T.C.
AKDENİZ ÜNİVERSİTESİ
SAĞLIK BİLİMLERİ ENSTİTÜSÜ
Fizyoloji Anabilim Dalı

DUVAR KAYMA GERİLİMİ DEĞİŞİKLİKLERİNE ENDOTEL HÜCRELERİNİNİN YANITI: ERİTROSİT AGREGASYONUNUN ETKİSİ

Özlem YALÇIN

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Tez Danışmanı
Prof. Dr. Oğuz Kerim BAŞKURT

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1. Baskurt OK, Yalcin O, Gungor F, Meiselman HJ: Hemorheological parameters as determinants of myocardial tissue hematocrit values. *Clinical Hemorheology and Microcirculation 35:* 45-50, 2006.

2. Yalcin O, Aydin F, Ulker P, Uyuklu M, Gungor F, Armstrong JK, Meiselman HJ, Baskurt OK: Effects of red blood cell aggregation on myocardial hematocrit gradient using two approaches to increase aggregation. *American Journal of Physiology 290 (Heart Circ. Physiol.)* H765-H771, 2006.

3. Yalcin O, Meiselman HJ, Armstrong JK, Baskurt OK: Effect of enhanced red blood cell aggregation on blood flow resistance in isolated-perfused guinea pig heart preparation. *Biorheology 42*: 511-520, 2005.

4. Yalcin O, Uyuklu M, Armstrong JK, Meiselman HJ, Baskurt OK: Graded alterations of red blood cell aggregation influence in vivo blood flow resistance. *American Journal of Physiology 287 (Heart Circ Physiol)* H2644-H2650, 2004.

5. Baskurt OK, Yalcin O, Ozdem S, Armstrong JK, Meiselman HJ. Modulation of endothelial nitric oxide synthase expression by red blood cell aggregation. *American Journal of Physiology 286 (Heart Circ Physiol)* H222-229, 2004.

6. Baskurt OK, Yalcin O, Meiselman HJ: Hemorheology and vascular control mechanisms. *Clinical Hemorheology and Microcirculation 30:* 169-178, 2004.

7. Bor-Kucukkatay M, Yalcin O, Gokalp O, Kipmen-Korgun D, Yesilkaya A, Baykal A, Ispir M, Senturk UK, Kaputlu I, Baskurt OK: Red blood cell rheological alterations in hypertension induced by chronic inhibition of nitric oxide synthesis in rats. *Clinical Hemorheology and Microcirculation 22*: 267-275, 2000.

Akdeniz Üniversitesi Sağlık Bilimleri Enstitüsü Müdürlüğü'ne

Bu çalışma, jürimiz tarafından Fizyoloji Anabilim Dalı'nda Doktora tezi olarak kabul edilmiştir.

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Tıp Fakültesi Fizyoloji Anabilim Dalı

ONAY:

Bu tez, Sağlık Bilimleri Enstitüsü Yönetim Kurulunca belirlenen yukarıdaki jüri üyeleri tarafından uygun görülmüş ve Enstitü Yönetim Kurulu'nun ……../……../2007 tarih ve ……./……. sayılı kararıyla kabul edilmiştir.

Prof. Dr. Nurettin Oğuz
Enstitü Müdürü

ÖZET

Endotel hücreleri kan akımından kaynaklanan kayma gerilimine maruz kalırlar. Endotel hücrelerinde kayma geriliminin etkisiyle otokrin ve parakrin faktörlerin üretimi akut ve kronik olarak düzenlenir. Kayma geriliminin pek çok genin ekspresyonunu düzenlediği ve damar duvarının patofizyolojisinde önemli rolleri olduğu çok iyi bilinmektedir.

Endotel hücreler üzerinde in vivo etki gösteren mekanik kuvvetler, bu hücreler üzerindeki kan akımından kaynaklanır ve duvar kayma gerilimi olarak ifade edilirler: duvar kayma geriliminin büyüklüğü, damar duvarına komşu sıvının hızı (duvar kayma hızı) ile bu alandaki sıvının vizkositesinin çarpımı ile belirlenir. Çeşitli çalışmalarda, hemodinamik koşullarda ve duvar kayma gerilimindeki değişikliklerin damar endotelinde NO sentezleyen mekanizmaları etkileyebileceği gösterilmiştir. Kanın akışkanlık özellikleri duvar kayma gerilimini etkileyen en önemli faktörlerden biridir. Sıçanlarda artmış eritrosit agregasyonunun iskelet kası küçük arterlerinde endotelyal nitrik oksit sentaz (eNOS) ekspresyonunu ve fonksiyonunu azalttığı ve kan basıncını arttırdığı gösterilmiştir. Potansiyel kayma gerilimi reseptörü olan eNOS'un, endotel hücrelerinde kayma gerilimi değişikliklerinin algılanmasında rol oynadığı bildirilmiştir. Bu çalışmada; cam kapiller tüplerin içine yerleştirilen endotel hücrelerinde eritrosit agregasyonunun arttırılmasıyla azalan duvar kayma gerilimine cevaben NO bağımlı mekanizmalardaki değişiklikler incelenmiştir.

Çalışmada cam kapiller tüpler içine özel bir teknikle insan göbek kordonu kaynaklı venöz endotel hücreleri yerleştirilmiştir. Cam tüpler içinde kültür edilmiş endotel hücreleri sabit basınç koşullarında farklı karakterde kan örnekleriyle perfüze edilmiştir. Endotel hücreleriyle kaplanmış kapillerin perfüzyonu; normal insan kanı, plazma kapsamı değiştirilerek eritrosit agregasyonu arttırılmış kan (dextran grubu) ve eritrosit yüzey özellikleri değiştirilerek agregasyonu modifiye edilmiş (F98 grubu) eritrosit süspansiyonları kullanılarak yapılmıştır. Endotel hücreleriyle kaplı kapiller borular yukarıda belirtilen deney gruplarına uyumlu eritrosit süspansiyonlarıyla 30 dakika veya 6 saat boyunca perfüze edilmişlerdir. Perfüzyon sonrası endotel hücrelerinde eNOS protein ve mRNA ekspresyonları tayin edilmiştir. Ayrıca endotel hücrelerinde nitrit-nitrat düzeyleri ölçülmüştür.

Cam kapiller tüpler içinde kültür edilmiş endotel hücrelerinde akıma cevaben NO üretiminin ve eNOS serin 1177 ekpresyonunun arttığı bulunmuştur. Kapiller tüplerin, eritrosit agregasyonu arttırılmış kan örnekleriyle perfüzyonu NO üretimini ve eNOS serin 1177 ekspresyonunu azaltmıştır.

Bu deneysel veriler eritrosit agregasyonunun değiştirilmesiyle meydana gelen duvar kayma gerilimi değişikliklerinin, NO bağımlı mekanizmalarla izlenebileceğini göstermiştir.

Anahtar kelimeler: Duvar kayma gerilimi, endotelyal nitrik oksit sentaz, nitrik oksit

ABSTRACT

Vascular endothelial cells are the primary cell types exposed to shear stress originating from blood flow. They regulate the production of autocrine and paracrine vasoactive factors both acutely and chronically in relation with the effect of shear stress. It is well known that shear stress selectively regulates the expression of many different genes and contributes the pathophysiology of vascular wall.

Mechanical forces which have in vivo effects on endothelial cells are originated from blood flow and they are called wall shear stress: magnitude of wall shear stress is determined by multiplying the velocity of fluid adjacent to vessel wall (wall shear stress) with viscosity of the fluid. In several studies it was shown that alterations on hemodynamic conditions or wall shear stress may have effects on NO-synthesizing mechanisms of vessel endothelium. Fluidity of blood is a major determinant of wall shear stress. In rats, increased erythrocyte aggregation reduces the expression and function of endothelial nitric oxide synthase (eNOS) while it increases the blood pressure. It has been shown that eNOS which is a potential receptor of the l shear stress, plays a role in sensing of the alterations in shear stress by endothelial cells. In the present study we wanted to investigate the alterations of NO-dependent mechanisms in endothelial cells that are settled in capillary tubes, in response to a reduction in wall shear stress resulting from an increase in erythrocyte aggregation.

In this study, we have located human umbilical cord originated venous endothelial cells into glass capillary tubes with a special technique. Endothelial cells cultured in glass capillary tubes were perfused with blood samples of different characteristics in constant pressure. Perfusion of capillaries covered with endothelial cells is realized by use of following three samples; normal human blood, blood which has increased erythrocyte aggregation due to changed plasma content (dextran group), and blood which has modified aggregation due to changed erythrocyte surface attributes (F98 group). Capillary tubes of study groups filled with endothelial cells were perfused for 30 minutes or 6 hours with appropriate erythrocyte suspensions. After the perfusion, eNOS protein levels and mRNA expression were measured in endothelium cells. Nitrite-nitrate levels of endothelial cells were also measured.

It is observed that both NO production and eNOS serin 1177 expression increased in the cultured endothelial cells inside glass capillary tubes in response to flow. Perfusion of capillary tubes with blood samples whose erythrocyte aggregation was increased, decreased the NO production and eNOS serin 1177 expression.

This experimental data show that alterations in wall shear stress caused by modification in erythrocyte aggregation can be monitored with NO-dependent mechanisms.

Key words: Wall shear stres, endothelial nitric oxide synthase, nitric oxide

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İÇİNDEKİLER DİZİNİ

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SİMGELER ve KISALTMALAR DİZİNİ

Ca^{++}	:	Kalsiyum
cGMP	:	Siklik guanozin 3',5' monofosfat
cAMP	:	Siklik adenozin monofosfat
IP3	:	İnozitol trifosfat
cp	:	Centi poise
EDRF	:	Endotel kaynaklı gevşetici faktör
eNOS	:	Endotelyal nitrik oksit sentaz
S1177	:	Serin 1177
T495	:	Threonin 495
EDTA	:	Etilendiamin tetraasetik asit
Htc	:	Hematokrit
iNOS	:	Uyarılabilir nitrik oksit sentaz
K^{+}	:	Potasyum
Mg^{++}	:	Magnezyum
n	:	Viskozite
fl	:	Femtolitre
Na^{+}	:	Sodyum
NOS	:	Nitrik oksit sentaz
Pa	:	Pascal
PBS	:	İzotonik fosfat tamponu
PI3K	:	Fosfoinozitid 3 kinaz
PKA	:	Proten Kinaz A
PKC	:	Protain kinaz C
PLC	:	Fosfolipaz C
PKG	:	Protein kinaz G
AMPK	:	AMP-tarafından aktive edilen protein kinaz
PP1	:	Protein fosfataz 1
PP2A	:	Protein fosfataz 2A

VEGF	:	Vasküler endotelyal büyüme faktörü
Q	:	Akım
R	:	Yarıçap
SS	:	Kayma gerilimi
W	:	Duvar kayma gerilimi
V	:	Akım hızı
c-Src	:	Rous Sarcoma oncogene
Ras	:	Rat Sarcoma
mSOS	:	Mammalian son-of-Sevenless
Shc	:	SH2-containing collagen-related proteins
ERK1/2	:	Ekstraselüler regülated kinaz
Grb2	:	Growth factor reseptör bound protein
GTP	:	Guanozin trifosfat
Cl^-	:	Klor
ICAM-1	:	İntraselüler adezyon molekülü 1
TNF-α	:	Tümor nekrozis faktör-α
ET-1	:	Endotelin-1
NF-Kb	:	Nükleer faktör kappa B
FAK	:	Fokal adezyon kinaz
JNK	:	c-Jun Kinaz
LDL	:	Düşük dansiteli lipoprotein
TRE	:	Phorbol ester tissue 13-acetate responsive element
NADPH	:	Nikotinamid adenin dinükleotid fosfat
FAD	:	Flavin adenin dinükleotid
FMN	:	Flavin adenin mononükleotid
BH4	:	Tetrahidrobiopterin
Zn	:	Çinko
PMA	:	Phorbol mrystate acetat
MAPK	:	Mitojenle aktive olan protein kinaz

ŞEKİLLER DİZİNİ

ÇİZELGELER DİZİNİ

GİRİŞ

Dolaşım sisteminde, kan ve damar duvarı arasında lokalize olan endotel hücreleri, kan akımından kaynaklanan kayma gerilimine maruz kalırlar. Damarların içini kaplayan ve kan akımından etkilenen endotel hücrelerinin kültüre edilmesi, deneysel ortamlarda fizyolojik yanıtların araştırılmasını kolaylaştırmıştır. Endotel hücreleri, kayma geriliminin akut ve kronik etkisiyle otokrin ve parakrin vazoaktif faktörlerin üretimini regüle eder. Kayma kuvvetinin, endotele monosit bağlanması ve endotel hücrelerinin apoptozisini içeren [1, 2] pro-aterojenik olayların inhibe edilmesini içeren antiaterojenik etkileri olduğu çok iyi bilinmektedir. Kayma kuvvetinin aterosklerozu önleme mekanizması tam olarak bilinmemekle birlikte, bu etkisinde endotel hücrelerinin önemli rol oynadığı gösterilmiştir [1, 3, 4]. Kayma geriliminin, selektif olarak birçok farklı genin ekspresyonunu regüle ettiği ve damar duvarı patofizyolojisinde önemli etkileri olduğu bulunmuştur.

Kayma kuvveti (tanjansiyel kuvvet), endotel hücre yüzeyine paralel şekilde etki eden sürtünme kuvvetidir. Kayma gerilimi olarak da isimlendirilen bu kuvvetin büyüklüğü, damar sisteminde dolaşan sıvının akım hızı, viskozitesi ve kan damarlarının fiziksel boyutları tarafından belirlenir. Kan reolojik yönden, non-Newtonien bir sıvıdır. Yani, kan viskozitesi kayma hızına bağlı olarak değişir. Kayma hızı arttıkça kan viskozitesi azalır [5, 6]. Kayma hızının kanın büyük arterlerdeki değerine (100-400 sn^{-1}) ulaşmasından sonra ise kan Newtonien davranış gösterir [5]. Yani bu hızın üzerindeki hızlarda kan viskozitesi kayma hızına bağımlı değildir [7]. Damar sistemi içinde yer alan çok çeşitli boyuttaki damarlarda kan akımı birbirinden farklı karakterler gösterebilir. Kan akımının 0,5 mm'nin üzerinde çapa sahip damarlarda laminar olmaya eğilimli olmasına karşın, çapı daha küçük damarlarda bu davranışı değişir. Ek olarak, kayma gerilimi transmural basınç gradiyentlerinden etkilenir, çünkü damar distansiyonu, damar çapını arttırarak kayma gerilimini azaltma eğilimindedir. Kayma gerilimi üzerine damar distansiyonunun bu etkisi önemli olabilir. Çünkü kayma gerilimi damar yarıçapının 4. kuvvetiyle ters orantılı olduğundan, damar duvarı katmanları ve hücre yapısındaki varyasyonlardan dolayı aynı damarın farklı noktalarında kayma geriliminde farklılıklar oluşabilir [8].

Nitrik oksit (NO) dolaşımın kontrolünde anahtar bir rol oynar [9]. Vasküler düz kas tonusunun regülasyonunda ve vasküler direncin düzenlenmesinde NO'nun önemi çok iyi bilinmektedir [10-12]. Ayrıca, birçok fizyolojik fonksiyonda da (örneğin apoptozis) bu basit molekülün rolü olduğu gösterilmiştir [13, 14]. Nitrik oksit kaynaklarından biri de endotel hücreleridir.

Endotel hücrelerinde, L-arjinin'den NO sentazın endotelyal izoformu (eNOS) aracılığıyla nitrik oksit sentezlenir [15]. Endotel hücre kültürlerinde ve damarlarda, kayma geriliminin eNOS'dan NO üretimini stimüle ettiği çok iyi bilinmektedir, ancak kayma geriliminin NO üretimini regüle ettiği moleküler mekanizmalar hakkında farklı sonuçlar bulunmaktadır [16-20]. Kayma gerilimi, endotel hücrelerinden 2 farklı fazda NO üretimini stimüle eder: Birincisi akım başladıktan sonra saniyeler ile 30 dakika içerisinde görülen Ca^{+2}/kalmodulin (CaM) bağımlı NO üretim fazıdır. İkinci faz ise, saatlerce süren akım koşullarında gözlenen Ca^{+2}/CaM bağımsız, uyarılma fazıdır [21, 22]. Bu çalışmalardaki ortak bulgu, endotel hücrelerinde eNOS ekspresyonun maruz kalınan mekanik kuvvetlerin büyüklüğü ile modüle edildiğidir [23-25].

Endotel hücreleri, sıvı kayma kuvvetlerinin biyosensörleridir [15, 26]. Endotel hücrelerinde bulunan kaveola'nın, potansiyel kayma gerilimi sensörlerinden biri olduğu ileri sürülmektedir. Sinyal transdüksiyonunun başlatılmasından sorumlu olan mekanoreseptörlerin kimliğine ait çalışmalar kaveola'nın kayma gerilimine cevaben biyokimyasal sinyallere aracılık edebileceğini ortaya koymuştur. Endotel hücrelerinde bol bulunan caveola'nın membrandan iyon akışı ve sinyal transdüksiyonu gibi olaylarda birçok sinyal molekülüyle etkileşim içinde olduğu gösterilmiştir [27]. Kaveola'ya bağlı olduğu bilinen sinyal molekülleri arasında; G proteinleri, c-Src (Rous sarcoma oncogene) ailesi tirozin kinazları, ras (rat sarcoma), protein kinaz C, eNOS [28], shc (SH_2-containing collagen-related protein), Grb2 (growth factor receptor bound protein 2), mSOS (mammalian son-of-sevenless), raf-1 (v-raf-1 murine leukemia viral oncogene homolog 1) ve ERK1/2 (extracellular signal-regulated kinase 1/2) bulunmaktadır [29].

Bazal koşullarda, eNOS'un kaveolada caveolin-1'e bağlı şekilde olduğu görülür ve inaktif durumdadır. Kayma kuvvetinde kademeli artış, eNOS'un caveolin-1'den ayrılmasına, enzimin aktivitesine yol açan Ca^{+2}/CaM'e bağlanmasına neden olur [30, 31]. Ek olarak, GTP-bağlayan proteinlerin bu erken faz sırasında kritik bir rol oynadığı gösterilmiştir. Oysa pertussis toxin-duyarlı G proteinleri ($Gi\alpha$ ailesi) sığır aortik endotel hücrelerinde akım-bağımlı NO üretimini regüle ederken, insan umbilikal ven endotel hücrelerinde ise pertussis toksin duyarsız G proteinleri (küçük G proteini) işe karışmaktadır [32, 33]. Bu bulgulara göre, G proteinlerinin gerçek kimliği hücre tipine bağlıdır. Bununla beraber, ikinci faz sırasında akım bağımlı NO üretimi, G proteininden bağımsız olarak regüle edilmektedir [33].

Endotel hücreleri üzerinde in vivo etki gösteren mekanik kuvvetler, bu hücreler üzerindeki kan akımından kaynaklanır [1, 34, 35] ve duvar kayma gerilimi olarak tanımlanabilir. Duvar kayma geriliminin büyüklüğü, damar duvarına komşu sıvının hızı (duvar kayma hızı) ile bu alandaki sıvının vizkositesinin çarpımı ile belirlenir [2, 3]. Çeşitli çalışmalarda, hemodinamik koşullarda duvar kayma gerilimindeki değişikliklerin damar endotelinde NO sentezleyen mekanizmaları etkileyebileceği gösterilmiştir. Kanın akışkanlık özellikleri duvar kayma gerilimini etkileyen en önemli faktörlerden biridir [2, 3].

Kayma gerilimi, endotelyal yapının ve mekanosensitif genlerin ve nitrik oksit gibi (NO) vazoaktif faktörlerin üretimini de içeren hücresel fonksiyonların kontrolünde önemli bir role sahiptir [4, 36, 37].

Kan damarı içinde kanın bileşiminin her noktada birbirinin aynı olmadığı iyi bilinmektedir [38]. Eritrositlerin damarın merkezinde toplanma eğiliminden dolayı, damar çeperine yakın bölgede (marjinal zonda) plazmadan zengin ve daha düşük viskoziteye sahip, hücreden fakir bir bölge oluşur [38, 39]. Eritrositlerin agregasyon eğilimi, aksiyal migrasyon derecesi üzerinde güçlü bir etkiye sahiptir. Eritrosit agregasyon eğiliminin artması, aksiyal migrasyonu arttırır [39] ve bu koşullarda akım direnci azalır [39-41]. Bununla beraber, eritrosit agregasyonun akım dinamikleri üzerine etkilerinin oldukça karmaşık olduğu ve belirli bir damar segmentinden kanın geçiş süresi (yani akım süreleri) [42] ve damarın yerçekimine göre oriyantasyonu [38] gibi çeşitli faktörlerle module edildiği unutulmamalıdır.

Eritrosit agregasyonu modifikasyonunun, damar duvarına komşu marjinal zonda yerel kan bileşimini değiştirerek duvar kayma kuvvetini etkileyebileceği düşünülmektedir. Bu etki, özellikle plazma akışkanlığında önemli bir değişikliğin olmadığı, hücresel faktörlerdeki değişimlere bağlı eritrosit agregasyonu modifikasyonlarında önemli duruma gelebilir. Ancak, bu etkinin hangi boyutlarda olacağının hesaplanması veya doğrudan ölçümle bulunması mümkün değildir. Bu çalışmada, duvar kayma kuvvetlerinde eritrosit agregasyonunun modifikasyonuna bağlı olarak ortaya çıkacak değişikliklerin endotel NO sentez mekanizmalarına etkisi incelenmiştir. Bu kurgunun arkasında, endotel hücrelerindeki NO sentezine ilişkin mekanizmaların duvar kayma kuvvetlerindeki değişikliklerin tahmin edilmesinde bir araç olarak kullanılması düşüncesi vardır.

GENEL BİLGİLER

2.1. Kan Akışkanlığının Önemi

Kan dokusunun, organizmadaki homeostatik dengenin ve hücrelerin yaşamlarının sürdürebilmesi için sürekli hareket halinde olması gereklidir [43]. Kan hareketinin sürmesi kalp pompasının gücü ve damar sisteminin özellikleriyle yakından ilişkilidir. Kan dokusunun damar sistemi içindeki hareketi her şeyden önce kendi özelliklerine ve akışkanlığına bağlıdır. Kanın hücresel elemanlarının konsantrasyonları yanında, reolojik özellikleri de kanın akışkanlığını belirleyen önemli etkenlerden arasındadır [5, 7].

Bir damar yatağında kan akımı, basınç/direnç oranıyla belirlenir. Poiseuille yasasına göre, damar içinde akan kanın reolojik özellikleri ve sistemin geometrik yapısı damar yatağının akıma gösterdiği direnci belirler.

$$Q = \frac{\pi}{8} \frac{\Delta P \cdot r^4}{L \cdot \eta} \qquad (1)$$

Poiseuille eşitliğinde; akım direnci yarıçapın (r) dördüncü kuvveti ile ters, damarın uzunluğu (L) ve sıvının viskozitesi (η) ile doğru orantılıdır. Direncin yarıçapın dördüncü kuvveti ile ters orantılı olması nedeniyle, akım direncinin belirlenmesindeki en önemli faktör düşünülürken diğer faktörler neredeyse tamamen göz ardı edilmiştir. Hücresel patoloji teorisinin tıbbi pratik üzerindeki temel etkisi bu durumu daha da ileri götürmüş, (pekçok hastalığın teşhisinde veya anlaşılmasında ölü veya fikse edilmiş örneklerin mikroskobik incelenmesi temel alındığı için) bu sayede vasküler geometri tek önemli faktör durumuna gelmiştir [44]. Kan akışkanlığı gibi bir parametrenin önemsenmemesinin, Poiseuille eşitliğinde sabit faktörlerden biri olarak kabul edilmesinden kaynaklandığı düşünülebilir.

Doku metabolizması ve fonksiyonu yeterli kan akımı ile yakından ilişkilidir. Birçok doku, ihtiyacı olan kanı dokuya sağlamak üzere, direnci kontrol edilebilen ve bu sayede dokuda her zaman yeterli kan bulunmasını sağlayan vasküler yapı ile donatılmıştır [45]. Dolaşım sisteminde damar çapı oldukça geniş bir aralıkta değişir. Ek olarak, damar çapı yerel ve merkezi mekanizmalarla düzenlenir. Bu düzenleme sonucu kan akımı organizmanın bütün bölümlerinde gereken düzeyde tutulur.

Hemoreolojik parametrelerin bozulması durumunda doku perfüzyonunun olumsuz yönde etkilendiği ileri sürülmektedir. Ancak, kanın

reolojik özelliklerinin fizyopatolojik süreçlerdeki rolü üzerindeki tartışmalar halen sürmektedir [46]. Kanın akışkanlık faktörleri ve hemodinami arasındaki ilişki çok karmaşıktır. Normal koşullarda, kan akımı ve doku perfüzyonunda meydana gelen değişiklikler, vasküler kontrol mekanizmaları tarafından damar çapı değiştirilerek kompanse edilir. Ancak, damar yapısı belli hastalık süreçlerine bağlı olarak bozulmuşsa (aterosklerozis gibi), yeterli vazomotor rezerv bulunmadığından bu kompansasyon gerçekleşmeyebilir [47]. Kanın akışkanlık faktörleri (örneğin, plazma viskozitesi, eritrosit agregasyonu ve eritrosit deformabilitesi) perfüze olan dokunun metabolik durumuna duyarlıdır [47]. Kan komponentleriyle temas halinde bulunan iç ortam değişiklikleri bu elemanların reolojik özelliklerini, dolayısıyla da bütün kan dokusunu etkiler. Fizyopatolojik süreçler içinde reolojik değişikliklerin bu sürecin nedeni mi, sonucu mu olduğu ilk bakışta anlaşılmayabilir.

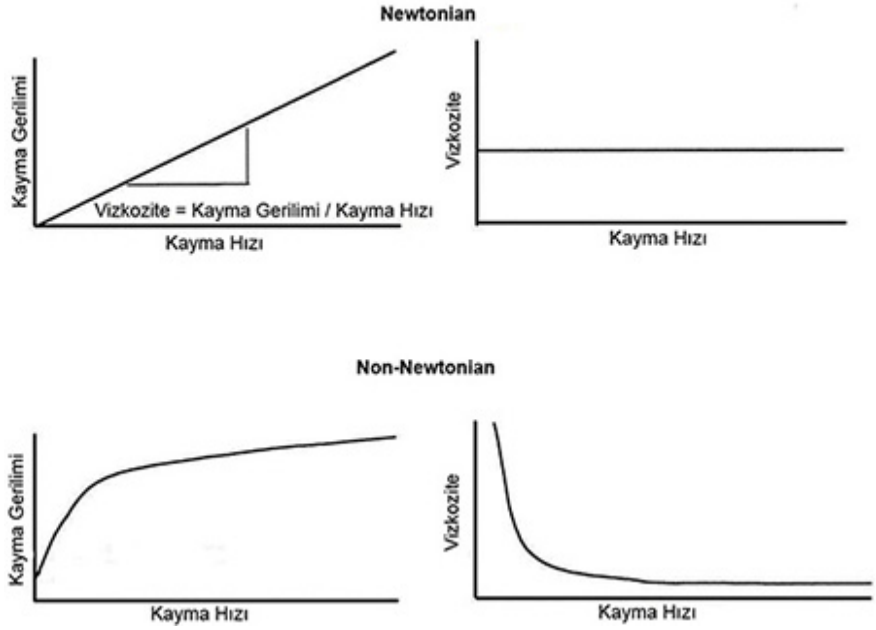
20. yüzyılın ilk yarısında Robin Fahraeus tarafından yapılan çalışmalar, humoral patoloji ve modern hemoreoloji kavramları arasında köprü olmuştur [38, 48]. Fahraeus'un kan süspansiyonunun stabilitesi üzerine yaptığı öncü çalışmalara ek olarak [49], kan damarlarının belirli bir kesitindeki kan akımı dağılımının aynı olmadığını gösterdiği çalışma da ilk in vivo hemoreolojik çalışmalardan biridir [50]. Bu çalışmanın sonuçları modern hemoreolojik kavramların oluşturulmasında çok önemli rol oynamış ve ayrıca patolojik koşulların çoğunda ölçülen hemoreolojik parametrelerin belirleyicisi olmuştur [3, 51, 52]. Diyabet, sepsis ve kardiyovasküler hastalıklar gibi patolojik durumların hemoreolojik profilleri çok iyi bilinmektedir [51, 52]. Tüketici egzersiz gibi fizyolojik durumlarda da önemli hemoreolojik değişikliklerin olduğu rapor edilmiştir [53]. Klinik çalışmalara ek olarak, patolojik süreçlerin hayvan modellerinde hemoreolojik değişiklikleri gösteren birçok çalışma bulunmaktadır [54-57].

Kanın in vivo reolojik davranışı ile ilgili en iyi bilinen çalışma 1933 yılında Whittaker ve Winton tarafından yapılmıştır [58]. Bu yayında, in vivo koşullarda belirlenen viskozitenin in vitro koşullarda ölçülen değerden daha düşük olduğu gösterilmiş [58] ve diğer yayınlarda da benzer sonuçlar bildirilmiştir [59-61]. Bu bulgulara göre hemoreolojik anormalliklerin derecesi ile doku perfüzyonundaki değişikliklerin gerçek derecesini tahmin etmede zorluklar ortaya çıkmıştır. İn vivo akım direncini sadece ex vivo koşullardaki reolojik verilere dayanarak tahmin etmek oldukça zordur. İn vitro ve in vivo çalışmalar arasındaki bu farklılıklar Robin Fahraeus tarafından geliştirilen kavramlarla kısmen açıklanabilir [38]: akım sırasında şekil değiştirebilen eritrositlerin aksiyal migrasyonu ve bunun Fahraeus etkisi (mikrovasküler düzeyde azalmış hematokrit) gibi sonuçları in vivo koşullarda belirlenen daha düşük viskoziteyi açıklayabilir [39, 41, 62].

2.2. Kanın Akışkanlık Özellikleri

Kan reolojik açıdan değerlendirildiğinde, non-Newtonien bir sıvı olarak tanımlanır. Non-Newtonien sıvılarda; kayma hızı ile kayma kuvveti arasındaki ilişki doğrusal olmayıp sıvı viskozitesi kayma hızına bağlı olarak

değişmektedir (Şekil 1). Kayma hızı arttıkça kan viskozitesinin azaldığı [5, 6], fakat kayma hızının büyük arterlerdeki değerine (100-400 sn^{-1}) ulaşmasından sonra ise kanın Newtonien davranış gösterdiği ifade edilmektedir [5]. Yani, kanın akışkanlığı kayma hızından bağımsız hale gelir [7] (Şekil 2.1). Damar sistemi içinde yer alan çeşitli boyuttaki damarlarda kan akımı birbirinden farklı karakterler gösterebilir.



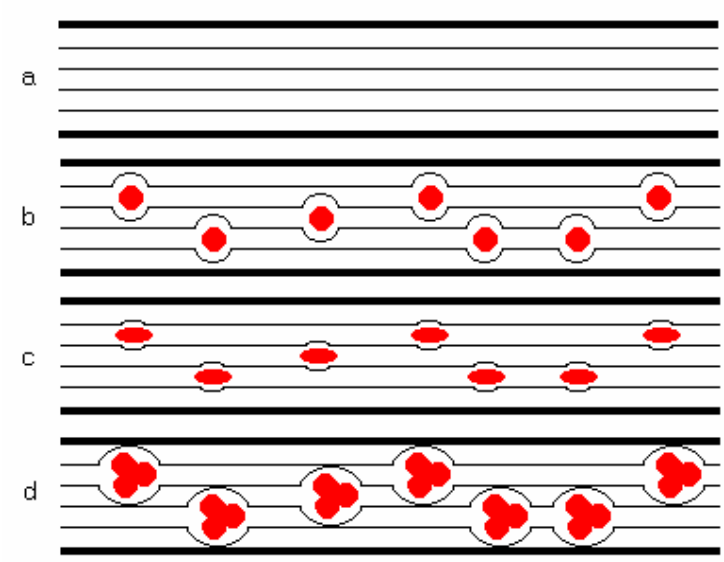
Şekil 2.1.: Newtonian ve non-Newtonian sıvılar için kayma gerilimi-kayma hızı ve viskozite-kayma hızı arasındaki ilişkiler (kaynak 44'den alınmıştır).

2.2.1. Büyük Damarlardaki Kan Akımı

Kan, büyük boyuttaki damarlarda iki fazlı bir süspansiyon özelliğindedir [5, 6]. Bu koşullarda, damar sisteminin geometrik özelliklerine, kanın fiziksel özelliklerine ve akım hızına bağımlı olarak laminer veya türbülan karakterde akım görülebilir. Laminer akım, sıvı tabakalarının birbiri üzerinde kayması şeklinde gerçekleşir ve buradaki hidrolik direnç oldukça düşüktür [6]. Fizyolojik koşullarda kan akımının karakteri laminerdir. Damar geometrisinde yerel değişikliklerle beraber kan akım hızında ani artışlar görülürse kan akımının karakteri türbülan hale dönüşebilir. Bu koşullarda akım direnci de artar.

Laminer akım koşullarında sıvının akışkanlığı, sıvı tabakaları (laminalar) arasındaki sürtünme kuvvetiyle belirlenir. Kan dokusu gibi iki fazlı sıvılarda, birinci faza (plazma) ait laminalar arasındaki sürtünme ikinci fazı oluşturan parçacıkların, bu laminaları ne ölçüde distorsiyona uğrattığı ile yakından ilişkilidir [52]. Kanın hücresel elemanlarından oluşan ikinci fazdaki parçacıkların kolay şekil değiştirebilen bir özellikte olmaları, onların laminer

akım çizgilerine oriyentasyonunu kolaylaştırarak, tabakalar arasındaki sürtünmeyi, dolayısıyla sıvının viskozitesini azaltır [5-7, 63]. Tersine, eğer laminalar arasında yer alan parçacıkların büyüklüğü artarsa, tabakalar arasındaki sürtünme ve viskozite artar [45, 64] (Şekil 2.2). Eritrositlerin tersinir kümelenme (agregasyon) eğilimi, özellikle düşük kayma kuvvetlerinin etkisinde parçacık büyüklüğünü arttırarak, viskoziteyi etkiler [65].



Şekil 2.2.: Plazmada içindeki eritrositlerin laminar akım çizgilerine etkileri. (a) eritrositlerin olmadığı durumda plazmanın oluşturduğu laminar akım çizgileri, (b) şekil değiştiremeyen (rijid) eritrositlerin varlığında akım çizgilerinin distorsiyonu, (c) şekil değiştirebilen eritrositlerin varlığında akım çizgilerinin azalmış distorsiyonu, (d) eritrosit agregasyonundan dolayı artmış distorsiyon (kaynak 44'den alınmıştır).

Kayma kuvvetleri yeterince büyükse, eritrositler plazma içinde bir sıvı damlası gibi davranırlar. Hidrodinamik kuvvetler küçüldükçe, eritrositler geniş diskoid yüzeylerinden birbirlerine yaklaşarak kümelenirler ve üç boyutlu agregatlar meydana getirirler [63]. Akım hızının yavaşlaması halinde böyle agregatlar oluşması kan akımı içinde sıvı tabakaları arasındaki sürtünme kuvvetini arttırır ve kanı daha visköz hale dönüştürürler [65].

2.2.2. Kapiller Kan Akımı (Mikrodolaşım)

İnsan dolaşım sisteminde kapiller damarlar 3–8 μm çaptadır. Bu koşullarda, kanın bütün olarak iki fazlı bir sıvı sistemi gibi düşünülmesi olanaksızdır. Bunun yerine, kanın hücresel elemanlarının ve plazmanın bu boyuttaki damarlardan geçişi ayrı ayrı değerlendirilmelidir. Yer yer kan hücrelerinin boyutlarından daha küçük bir çapa sahip olabilen bu damarlarda akım hızı, büyük ölçüde kan hücrelerinin şekil değiştirme yetenekleri (deformabilite) ile yakından ilişkilidir [43, 66, 67].

2.2.3. Kanın Akışkanlığını Belirleyen Faktörler

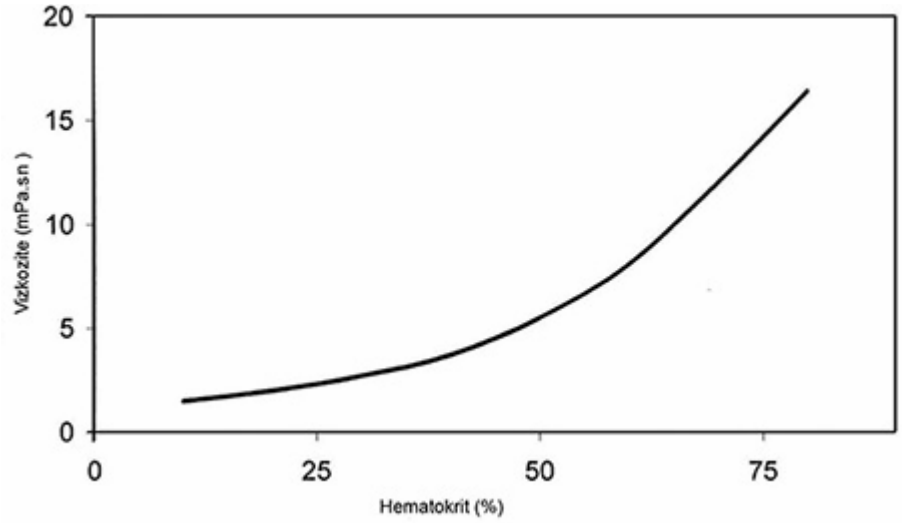
İki fazlı bir sıvı olan kanın akışkanlığı, tüm çok fazlı sıvılarda olduğu gibi her bir fazın reolojik özellikleri ve iki fazın birbirine oranı ile belirlenir. Bu iki fazı, kanın hücresel elemanları ve plazma oluşturur. Buna göre, kanın akışkanlığı; plazma viskozitesi, hematokrit değeri ve kan hücrelerinin reolojik davranışlarından etkilenir.

2.2.3.1. Plazma Viskozitesi

Plazma kandaki hücresel elemanlar için süspansiyon ortamı olarak görev yaptığından, akışkanlığındaki bir değişiklik, doğrudan kan viskozitesine yansır. Normal plazma viskozitesi 37 °C'de 1.10-1.35 centipoise arasında bir değere sahiptir [51, 52], ancak hastalık durumlarında daha yüksek değerler gözlenebilir. Genel olarak, plazma viskozitesi hastalık sürecinin nonspesifik bir belirtecidir ve akut faz reaksiyonları ile ilgili patofizyolojik durumlarda artar [68]. Bu artış, plazmanın protein içeriği ile yakın bir ilişki gösterir. Fibrinojen gibi akut faz reaktanları hastalık sırasında plazma viskozitesindeki artmaya önemli ölçüde katkıda bulunur [68].

2.2.3.2. Hematokrit Değeri

Laminar akım koşullarında, sıvı tabakalarının arasındaki direnci arttıran hücresel elemanların oransal miktarı, bu iki fazlı sıvının akışkanlığını belirleyen faktörlerin başında gelir. Hematokrit değeri ile kan viskozitesi arasında eksponansiyel bir ilişki vardır [5] (Şekil 2.3).



Şekil 2.3.: Kan viskozitesi üzerine hematokritin etkisi (kaynak 44'den alınmıştır).

2.2.3.3. Kanın Hücresel Elemanlarının Reolojik Davranışı

Kanın hücresel elemanlarının büyük çoğunluğunu oluşturan eritrositler, kitle halinde akım koşullarında dikkate alınması gereken tek hücre türüdür. Ancak, mikrodolaşım düzeyinde, hücrelerin bireysel hareketleri ön plana

çıktığından, her bir hücre türünün reolojik davranışının ayrı ayrı değerlendirilmesi gerekir. Eritrositlerin şekil değiştirme yetenekleri ve tersinir kümelenme eğilimleri, değişik koşullarda kanın akışkanlığının belirlenmesinde önemli rollere sahiptirler [69]. Akım hızının yüksek olduğu koşullarda eritrosit deformabilitesi kan viskozitesini belirleyen temel faktörler arasındadır [70]. Akımın yavaşlaması halinde, hücrelere etki eden kuvvetler küçülür ve kümelenme eğilimi ön plana çıkar. Eritrosit agregatlarının oluşması, bu koşullarda viskoziteyi yükseltir [67].

2.3. Eritrositler

Eritrositler solunum gazlarını taşımak üzere özelleşmiş hücrelerdir [71]. Olgun eritrositler 8 μm çapında, bikonkav disk şeklindedir. Bu diskin kenarlardaki maksimum kalınlığı 2.5 μm, ortadaki en düşük kalınlığı ise 0.8 μm'dir. Hücrenin yüzey alanı 140 μm^2, hacmi ise ortalama 90 femtolitre (fL) olarak belirlenmiştir [71]. Bu hücreler kemik iliğinde üretilir ve periferik dolaşıma katılmadan önce çekirdeklerini kaybederler. Olgun eritrositlerin, oksijen taşıyan pigment olan hemoglobinden oluşan amorf bir sitoplazmaları vardır. Bu özel yapı ve şekil hücrelere çok özel mekanik özellikler kazandırır. Eritrositlerin bikonkav-disk şeklinin korunması fonksiyonel yönden çok büyük öneme sahiptir. Bu özel şeklin korunmasında etkili beş faktör olduğu ileri sürülmüştür [71, 72]. Bunlar, membran içindeki elastik kuvvetler, yüzey gerilimi, membran yüzeyindeki elektriksel kuvvetler, osmotik ve hidrostatik basınçlardır. Bunların yanında, hücre şeklinin korunmasında eritrositlerin içinde bulundukları ortamın özellikleri de büyük önem taşımaktadır. Ayrıca, eritrosit membranı iç yüzeyinde yer alan ve membran iskeletini oluşturan proteinlerin bu düzenlemede rolü olduğu düşünülmektedir [73].

Eritrosit membranının akışkan yapısı, sahip olduğu lipid matriksten kaynaklanmaktadır. Bu tabakanın lipid kompozisyonu membran akışkanlığını değiştirmektedir [74]. Fakat bu değişimin membranın bütün olarak visko-elastik yapısı üzerine önemli bir etkisi olmadığı gösterilmiştir [66]. Eritrosit membranının viskoelastik özellikleri hemen bütünüyle eritrosit membran iskeletinin yapısı ve proteinler arasındaki ilişkiler ile belirlenir [75, 76].

2.3.1. Eritrosit Deformabilitesi

Eritrosit deformabilitesi, bu hücrenin belli bir kuvvetin etkisi altında şeklini tersinir olarak değiştirebilme yeteneğini ifade eder [66]. Eritrosit deformabilitesi; eritrositin geometrik özellikleri, sitoplazmik viskozitesi ve eritrosit membranının mekanik özellikleri tarafından belirlenir [66, 73, 77, 78].

Eritrositlerin normal bikonkav-disk şeklinin korunması deformabilite yeteneği açısından çok önemlidir. Bu özel geometrik şekil, hücreye yüzey alanını genişletmeksizin şekil değiştirme olanağı sağlar. Eritrosit şeklinde meydana gelen bozukluklar deformabilite yeteneğinde önemli azalmalara neden olur [73, 79].

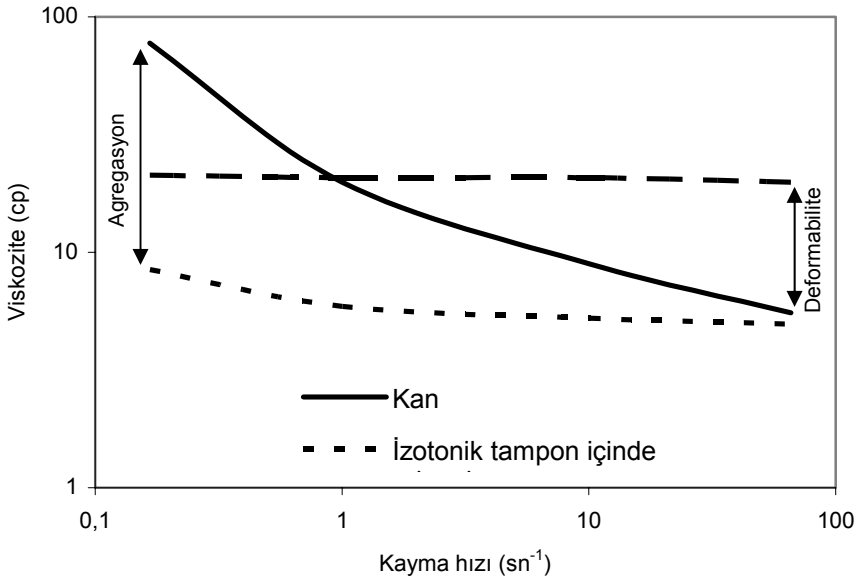
Eritrosit sitoplazmasının akışkanlığı da eritrositlerin mekanik özelliğini etkilemektedir. Sitoplazmanın önemli bir içeriği olan hemoglobin konsantrasyonu sitoplazma akışkanlığını belirler [73, 77]. Normal bireylerde eritrositlerin hemoglobin konsantrasyonu yaklaşık 27-37 g/dL arasındadır. Bu aralıkta sitoplazmik viskozite 5-15 centipoise kadardır [80]. Bu normal sınırlarda, sitoplazmik viskozitenin eritrosit deformabilitesi üzerinde etkisi ihmal edilebilir. Ancak, bu değerlerin üzerindeki konsantrasyonlarda sitoplazmik viskozitede büyük artışlar meydana getirir. Olgun eritrositlerde hemoglobin sentezi ve yıkımı olmadığından konsantrasyon değişimleri hücrenin su kapsamındaki değişimlere bağlıdır.

Eritrosit membranı esnek yapısından dolayı dış kuvvetlerin etkilerini sitoplazmaya aktararak eritrositlerin bütün içerikleriyle akıma katılmalarını sağlar. Eritrosit membranının şekil değiştirmeye izin vermesi yanında bir başka önemli özelliği de elastik yapıya sahip olmasıdır [66]. Eritrositlerin hidrodinamik kuvvetlerin etkisindeki şekil değiştirmeleri geri dönüşümlüdür. Şekil değişimine neden olan etkinin ortadan kalkması ile hücre diskoid şekline geri döner. Bu özelliğin hemen bütünüyle membran ve membran iskeletine bağlı olduğu açıktır [73, 79, 81].

2.3.2. Eritrosit Agregasyonu

Durgun haldeyken insan kanındaki eritrositler birbirine yapışmış, para dizileri gibi karakteristik morfolojileri ile gevşek agregatlar oluştururlar. Bu agregasyon için rulo formasyonu terimi de kullanılmaktadır. Plazmada, fibrinojen başta olmak üzere makromoleküllerin varlığı eritrosit agregasyonunun ortaya çıkmasında anahtar rol oynar [82]. Yüksek moleküler ağırlıklı, suda eriyebilen polimerler (dextran, polviynlpyrolidone) kullanılarak hazırlanan yapay süspansiyon ortamlarında da eritrosit agregatları oluşabilir. Eritrosit agregatları sıvı akımının başlaması veya agregatlara etki eden kayma kuvvetlerinin büyümesi ile parçalanırlar. Agregatları parçalamaya yetecek büyüklükte kuvvetleri 20-40 s^{-1} büyüklükteki kayma hızları oluşturabilir [83]. Bu özellik, agregasyon sırasında göreceli olarak zayıf kuvvetlerin ön planda olduğunu düşündürmektedir. Patolojik kanda veya büyük polimerlerle agrege olan eritrositlerde daha güçlü hücreler arası çekim kuvvetleri bulunmaktadır [83]. Hücreler yıkanıp protein ya da polimer içermeyen tamponlarda tekrar süspanse edilirlerse, eritrosit agregasyonu ortadan kalkar.

Eritrositlerin rulo formasyonu şeklindeki agregasyonu çok önemlidir. Çünkü bu özellik, normal insan kanının “shear thinning” (agregatların dağılmasına bağlı olarak artan kayma hızı ile kan viskozitesindeki azalma) davranışından sorumludur (Şekil 2.4). Bu durum in vivo akım dinamikleri üzerinde önemli etkilere sahiptir [42, 84-88].



Şekil 2.4.: Normal kan, izotonik tampon içerisinde süspanse edilmiş normal eritrositler ve plazmadaki rijid eritrositler için kayma hızı-viskozite eğrileri. Kayma hızı aralığının üst sınırlarında gözlenen viskozite farklılıkları eritrosit agregasyonunun etkileri ile ilişkiliyken düşük kayma hızlarında gözlenen viskozite farklılıkları eritrosit deformabilitesinin etkileri ile ortaya çıkmaktadır (kaynak 44'den alınmıştır).

Eritrosit agregasyonunun derecesi, eritrositleri bir arada tutan kuvvetlerle (agregan kuvvetler), bu kümeleri dağıtmaya çalışan kuvvetler (disagregan kuvvetler) arasındaki denge ile yakından ilişkilidir [82]. Disagregan kuvvetlerin başında ortamdaki hidrodinamik kuvvetler gelir. Hücre kümelerine etki eden kayma kuvvetleri büyüdükçe, kümelenme eğilimi azalır. Bunun yanında, eritrosit membranı yüzey yüküne bağlı olarak ortaya çıkan elektrostatik itim kuvvetleri ve eritrosit rijiditesi agregasyona karşı koyan kuvvetler arasındadır [82]. Eritrosit agregatlarını bir arada tutan agregan kuvvetler ile ilgili olarak ise iki hipotez öne sürülmüştür [82]

1- Köprüleme hipotezi: Bu hipoteze göre, birbirine yakın hücrelerin yüzeylerine adsorbe olan ve bu hücreler arasında köprüler oluşturan makromoleküller, agregatları bir arada tutarlar [89].
2- Deplesyon hipotezi: Makromoleküllerin eritrosit yüzeyinden fiziko-kimyasal mekanizmalarla uzak tutulması bir osmotik gradient ve hücrelerarası boşlukta bir sıvı hareketi oluşturur. Bu sıvı hareketinin yarattığı basınç farklılıkları komşu hücreleri birbirine doğru iter [90, 91].

Bu iki hipotez, eritrosit yüzeyine yakın bölgedeki makromolekül konsantrasyonları için farklı tahminlerde bulunurlar. Köprüleme hipotezine göre, yüzeye yakın bölgede makromolekül konsantrasyonunun süspansiyonun diğer bölümlerine göre daha yüksek olması gerekirken, tersine deplesyon hipotezine göre daha düşük olmalıdır [82]. Eritrosit yüzeyine komşu bölgede makromolekül konsantrasyonlarının yerel olarak doğrudan ölçülmesine yönelik çalışmalar başarısızlıkla sonuçlanmıştır.

Ancak, hücre elektroforezi çalışmaları deplesyon hipotezini doğrulayan ipuçları sağlamıştır [91].

Eritrosit agregasyonu gerek plazmanın, gerekse eritrositlerin hücresel özelliklerindeki değişimlerden etkilenir. Plazma bileşenlerinden özellikle fibrinojen konsantrasyonu eritrosit agregasyonunu etkileyen en önemli faktörlerden biridir [82]. Fibrinojen yanında diğer akut faz reaktanları, plazma globulin fraksiyonlarındaki değişimler, osmolarite ve pH değişiklikleri, hematokrit değerindeki artış eritrosit agregasyonunu etkiler [43, 82].

Diğer taraftan, özellikle eritrosit hücresel özelliklerindeki değişikliklerin de eritrosit agregasyonunu etkilediği son yirmi yılda yapılan çalışmalarla ortaya konmuştur [82, 92, 93]. Eritrositlerin karakteristikleri, deformabilite, eritrosit morfolojisi ve yüzey yükü farklılıkları ve membrana IgG bağlanımı gibi hücresel özelliklerin, eritrositlerin intrinsik agregasyon eğilimlerini belirgin ölçülerde değiştirebileceklerine dair deneysel kanıtlar bulunmuştur [83]. Hücresel özelliklerin, eritrositlerin intrinsik agregasyon eğiliminde bireyler arası, hatta türler arasında da belirgin farklılıklar oluşturabilecek düzeylerde etkili olabildikleri, bazı durumlarda patolojik süreçlerde gözlenenden daha yüksek düzeylerde agregasyon değişikliklerinden sorumlu olabilecekleri anlaşılmaktadır [83].

2.3.2.1.Eritrosit Agregasyonunun Deneysel Amaçlı Olarak Değiştirilmesi

Eritrosit agregasyonu deneysel amaçla plazma (süspansiyon ortamı) kapsamı değiştirilerek modifiye edilebilir. Bu amaçla yüksek molekül ağırlıklı ve fibriler yapıdaki biyomoleküller (örneğin dekstran) birçok deneysel çalışmada kullanılmıştır [59, 94-96]. Genellikle, bu biyomoleküllerin çözeltilerinin deney sırasında dolaşıma enjekte edilmesiyle gerçekleştirilen eritrosit agregasyonu artışı, beraberinde plazma viskozitesi yükselmesini de getirir. Ayrıca, bu yolla sonuçta ortaya çıkacak eritrosit agregasyonu değişikliğinin ne büyüklükte olacağı kolaylıkla kontrol edilemez.

Buna karşılık, son birkaç yılda kullanılmaya başlanan yeni bir teknik eritrosit hücresel özelliklerinin değiştirilmesiyle, süspansiyon ortamında herhangi bir değişiklik yapılmaksızın eritrosit agregasyonunun modifiye edilmesine olanak vermektedir [97-99]. Bu yöntemle, eritrosit yüzeyine kovalan olarak bağlanabilen, uçlarında özel reaktif gruplara sahip polietilenglikol esaslı ko-polimerler kullanılarak, eritrosit yüzey özellikleri değiştirilmektedir. İyi kontrol edilen koşullarda eritrositlerle birlikte inkübe edilen reaktif ko-polimerin konsantrasyonu değiştirilerek, inkübasyon sonrası yıkanarak otolog plazmalarında süspansiyon haline getirilen hücrelerin agregasyon dereceleri ayarlanabilmektedir. Bu şekilde, eritrosit agregasyonunun kademeli olarak değiştirilebilmesi ve bu değişimlerin hemodinamik etkilerinin incelenebilmesi olanaklı hale gelmiştir.

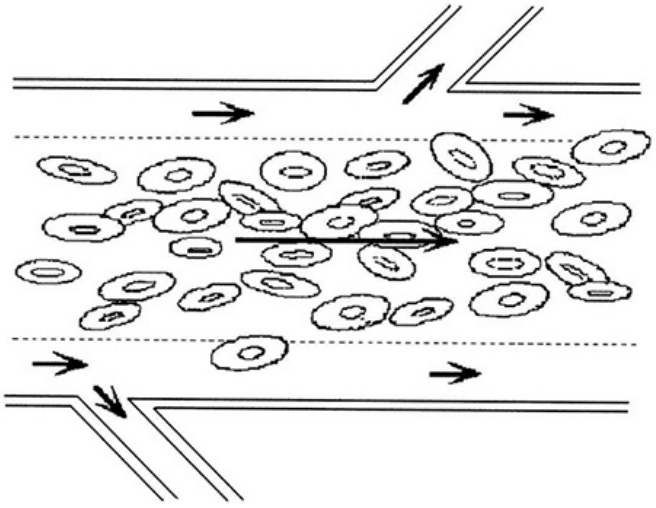
İki yöntem arasındaki en önemli farklılık, süspansiyon ortamı viskozitesiyle ilişkilidir. Biyomoleküllerin süspansiyon ortamına eklenmesi halinde ortaya çıkan eritrosit agregasyonu aksiyal migrasyonun daha etkin

olmasına yol açsa bile, süspansiyon ortamı viskozitesinin artmış olması nedeniyle damar duvarına komşu bölgede bulunan sıvının akışkanlığında önemli bir değişiklik yaratmayabilir. Oysa eritrosit agregasyonunun reaktif polimerlerin eritrosit yüzeyine bağlanması yoluyla değiştirilmesi halinde, süspansiyon ortamı akışkanlığında bir değişiklik olmayacağından, aksiyal migrasyon sonucunda damar duvarına komşu sıvı tabakalarında hematokrit değerinin düşmesi, bu bölümde viskozitenin efektif olarak azalmasıyla sonuçlanacaktır.

2.4. Kanın Akışkanlığında Yerel Değişimler

Özellikle 1000 mikrometrenin altında çapa sahip olan damarların belli bir kesitinde kanın bileşiminin her yerde aynı olmadığı ve aksiyal migrasyondan etkilendiği de iyi bilinmektedir [39]. Aksiyal migrasyon, eritrositlerin damar merkezinde toplanma eğilimi sonucu ortaya çıkar (Şekil 2.5). Aksiyal migrasyonun derecesi ile eritrosit agregasyon eğilimi arasında doğrusal ilişki vardır: eritrosit agregasyon eğiliminin artması, aksiyal migrasyonu arttırır [38]. Bu koşullarda akım direncinin azalması beklenir [41, 59].

Aksiyal migrasyon, damar duvarına yakın bölgede plazmadan zengin, daha düşük viskozite ve hematokrite sahip, hücreden fakir bir marjinal zon oluşturur [38, 39]. Duvar kayma gerilimi şiddetinin, damar duvarına komşu olan sıvının hareket hızı (duvar kayma hızı) ile marjinal alandaki sıvının viskozitesi tarafından belirlendiği göz önünde bulundurulursa, eritrosit agregasyonundaki artma ve bunun sonucu artan aksiyal migrasyonun duvar kayma kuvvetinde azalmaya neden olması beklenebilir [2, 3].



Şekil 2.5.: Akım sırasında damar merkezinde eritrositlerin birikmesi; yan dalların hücreden fakir sıvı tabakası ile beslenmesine ve dolayısıyla doku hematokritinin azalmasına neden olmaktadır (kaynak 44'den alınmıştır).

Eritrosit agregasyonunun derecesi damar sisteminin çeşitli bölümlerinde farklı büyüklüklerde olabilir. Bu büyüklüğü belirleyen en önemli faktör bu bölümlerde hüküm süren hemodinamik koşullarla ilgili olmak üzere, yerel kayma kuvvetleridir [99, 100]. Eritrosit agregatlarının düşük kayma kuvvetlerinin hakim olduğu venöz damarlarda ve çeşitli nedenlerle kan akımının yavaşladığı damar bölümlerinde yoğunluk kazanması ve bu bölümlerde kan viskozitesinin artışına neden olarak akım direncini yükseltmeleri beklenir [94-96].

2.5. Duvar Kayma Gerilimi

Silindirik borularda sıvının hareketi sırasında ortaya çıkan bu kuvvet dyne/cm^2 olarak, yani birim alana uygulanan kuvvet biçiminde ifade edilir. Dolaşım sisteminde, kan ve damar duvarı arasında lokalize olan endotel hücreleri, kan akımından kaynaklanan kayma geriliminin etkisinde kalırlar [1, 34, 35]. Endotel hücresine doğal akım koşullarında etki eden kuvvetler "duvar kayma gerilimi" olarak ifade edilebilirler.

2.5.1. Duvar Kayma Kuvvetini Belirleyen Faktörler

Duvar kayma geriliminin büyüklüğü, damar duvarına komşu olan sıvının hareket hızı (duvar kayma hızı) ile damar duvarına temas eden sıvının viskozitesi tarafından belirlenir [2, 3]. Kanın akışkanlık özellikleri duvar kayma gerilimini etkileyen en önemli faktörlerden biridir [2, 3]. Kayma gerilimi, endotel hücresi yapısının modifikasyonu, mekanosensitif genlerin aktivasyonu ve nitrik oksit gibi (NO) vazoaktif faktörlerin üretimini de içeren hücresel fonksiyonların kontrolünde önemli bir role sahiptir [4, 36, 37].

Duvar kayma kuvveti (**δ**); sıvı akım hızı (Q), damar yarıçapı (r), sıvının viskozitesi (η) değişiklikleri tarafından belirlenir ve aşağıdaki formüle göre hesaplanır.

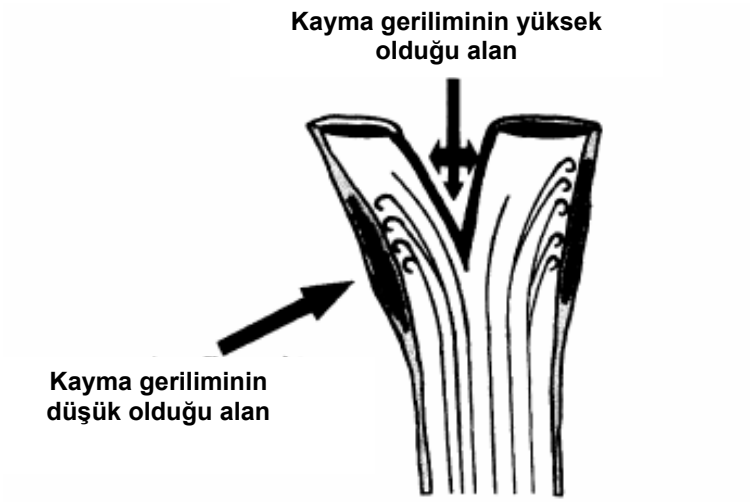
$$\delta = \frac{4 \cdot Q \cdot \eta}{\pi \cdot r^3} \qquad (2)$$

Dolaşım sisteminin belirli noktalarında damar yapısı ve dolaşımdaki hücresel elemanların davranışı gözönünde bulundurularak duvar kayma kuvveti hesaplanabilir. Ortalama kayma gerilimi büyük venlerde en düşük seviyede iken (<1 dyne/cm^2),. küçük arteriyollerde yüksek akım hızı ve küçük çap nedeniyle (60-80 dyne/cm^2) yüksek olmaya eğilimlidir.

Duvar kayma gerilimini tam olarak belirlemek kolay değildir. Duvara yakın bölgede akan kanın hızı ve hız gradiyenti belirlenmelidir, ancak bu değerleri belirlemek teknik olarak zordur. Gradiyent, yüksek oranda hız profilinin şekline ve duvardan olan uzaklığına bağlıdır. Ayrıca, eritrositlerin aksiyal migrasyonu, kan viskozitesini hücreden fakir olan damar duvarına yaklaştıkça azaltması nedeniyle, damar duvarına yakın bölgedeki viskozite

tam olarak bilinemez. Bu nedenle arteryal duvar kayma gerilimi ölçümleri, %20-50 arasında hatalı olabilmektedir [101].

Zhao ve ark.'nın yaptığı çalışmada, damar konfigürasyonunun kayma kuvvetlerini etkilediği gösterilmiştir [102]. Aynı damarın farklı bölgelerinde kayma kuvvetinin <1-60 dyne/cm^2 aralığında değiştiği ortaya konmuştur [102]. Arteryel sistemde dallanma bölgelerinde kan akımı sabit değildir (Şekil 2.6) ve bu koşullarda duvar kayma gerilimi değişiklikleri önemli olmaya başlamaktadır.



Şekil 2.6.: Damar dallanmalarının duvar kayma gerilimine etkisi (kaynak 103'den alınmıştır).

Duvar kayma gerilimi kardiyak döngü sırasında değişmektedir. Kan akım hızı ve duvar kayma gerilimi sistolde yüksek iken diyastolde daha düşüktür. Ancak diyastol fazı, kardiyak döngünün 2/3'ünü oluşturması nedeniyle diyastol fazındaki duvar kayma gerilimi seviyesi, ortalama duvar kayma geriliminin büyük kısmını oluşturmaktadır. Sistol fazı boyunca nabız basıncındaki artışın yanı sıra duvar kayma geriliminde görülen artış da arter çapında meydana gelen artışa bağlı olarak sınırlandırılmaktadır. Bu etki özellikle elastik arterlerde görülmektedir [103].

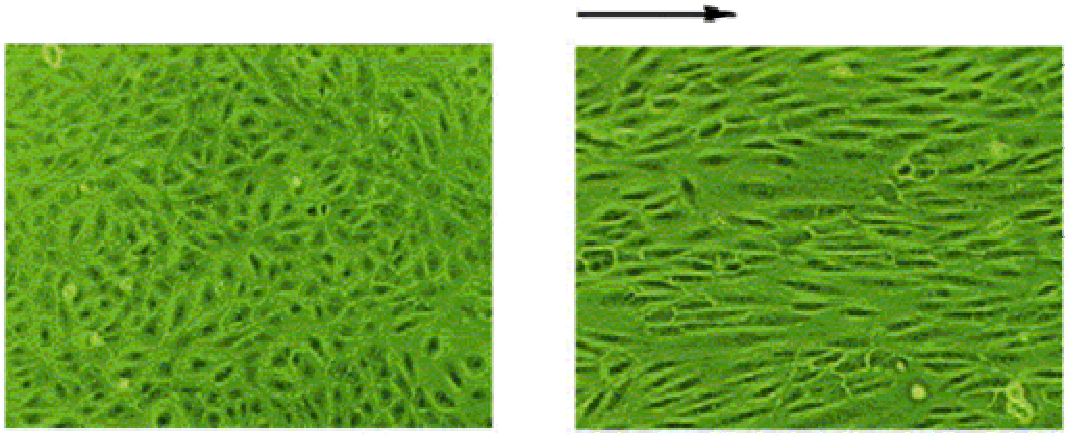
2.5.2. Kayma Gerilimi ve Endotel Hücreleri

Damar duvarının iç yüzeyinde bulunan endotel hücreleri, kan akımı tarafından ortaya çıkarılan kayma kuvveti ve sistolik, diyastolik kan basınçları arasındaki farklarla oluşan basınç pulsasyonlarına duyarlıdır. Basınç pulsasyonu, damar duvarında mekanik gerim meydana getirir [104]. Biyomekanik kuvvetlerin biyokimyasal aracılara ek olarak endotel hücre fonksiyonunun önemli belirleyicileri olduğu çok iyi bilinmektedir. Örneğin; duvar kayma gerilimi ve mekanik gerim, endotelyal gen ekspresyonunun belirleyicileri iken [105], duvar kayma gerilimi endotel hücreler tarafından

üretilen vazoaktif mediyatörlerin üretimini modifiye ederek arteryel çap değişimlerini regüle eder [106, 107]. Kayma gerilimi tarafından upregüle edilen endotelyal genlerin arasında transkripsiyon faktörleri, büyüme faktörleri, adezyon molekülleri ve çeşitli enzimlerin bulunduğu in vitro olarak gösterilmiştir [108].

Aterosklerotik lezyonlar düşük kayma hızı ile karakterize, anormal akım koşullarına sahip alanlarda ortaya çıkmaktadır [109, 110]. Son çalışmalarda normal ve anormal akım koşullarına sahip alanlarda gen ekspresyonlarının değiştiği gösterilmiştir [111]. İn vitro koşullarda, kayma gerilimin 0.4 Pa olduğu doku kültürlerinde aterojenik fenotipin ekspresyonu uyarılırken, 1.0-1.5 Pa düzeylerindeki kayma gerilimi etkisinde ateroprotektif endotelyal gen ekspresyonlarının indüklendiği bildirilmiştir [3, 112].

Biyomekanik kuvvetler, endotel hücre fonksiyonu yanında endotel yapısına da etki eder. Endotel hücreleri duvar kayma gerilimi doğrultusunda şekillerini değiştirirler: yüksek duvar kayma gerilimi noktalarında hücreler daha uzun bir yapı sergiler [113-115] (Şekil 2.7). Bu değişikler, stres fiberlerinin dağılımlarının, yayılımlarının değişmesine ve sayılarının artmasına sebep olur [114, 116]. Çalışmalarda uygulanan farklı karakterlerdeki kayma gerilimi de farklı morfolojik yapıların gelişmesine neden olur. Osilatuar kayma gerilimine maruz kalan sığır endotel hücreleri kültür koşullarında, poligonal biçimlerini korumuşlardır. Bu hücrelerde aktin stress fiberleri oluşumu da gözlenmemiştir [113].



Şekil 2.7.: Endotel hücrelerinin akım yönünde şekil değiştirmeleri (kaynak 3'den alınmıştır).

Biyomekanik kuvvetler ve endotel hücre fonksiyonu arasındaki ilişki, mekanotransdüksiyon olarak adlandırılan ve son yıllarda birçok araştırmaya konu olan ilgi çekici bir mekanizma olmuştur [36, 117-119]. Mekanotransdüksiyonda, endotel hücrelerinin luminal yüzeyinde etkili olan

mekanik kuvvetler, hücre iskeleti aracılığıyla diğer alanlara doğru iletilmektedir [120]. Bu kuvvetler, özellikle bazal adezyon noktalarında çok etkilidir ve bu noktalar ekstraselüler matriks ve çekirdek membranı ile bağlantılıdır. Dolayısıyla kuvvetin, tüm hücre boyunca dağılmasını sağlarlar. Hücre membranı kayma geriliminin yarattığı şekil değiştirmeye cevaben, gerim duyarlı iyon kanallarını ve integrinleri aktive eder [117, 118]. Çekirdeğe iletilen biyomekanik kuvvetler, trombosit-kaynaklı büyüme faktörleri, doku plazminojen aktivatörü, endotelin-1, endotelyal nitrik oksit sentaz (eNOS) ve intraselüler adezyon molekülü-1 (ICAM-1) gibi genlerin promoterlarında gerim-duyarlı sekanslar aracılığıyla duyarlılık meydana getirebilirler [105].

Kayma gerilimi endotel hücresinden NO üretimini bifazik şekilde etkiler: 1. akım başlamasını takiben saniyeler ile 30 dakika içerisinde NO üretimi Ca^{+2}/CaM bağımlı iken, 2. saatlerce süren akım koşullarında NO üretiminin Ca^{+2}/CaM bağımsız olduğu bildirilmiştir [22, 121]. Ayrıca, endotel hücrelere etki eden mekanik kuvvetlerin büyüklüğü de eNOS ekspresyonunu değiştirebilmektedir [23-25].

2.5.3. Kayma Gerilimi ve İyon Kanalları

Endotel hücrelerinin akıma verdiği erken yanıtlar, kayma kuvvetlerinin algılanmasının hemen ardından meydana gelir. Bu yanıtlardan biri, akıma duyarlı iyon kanallarının aktivasyonudur [122]. Olası akım algılayıcıları olduğu düşünülen bu kanalların aktivasyonu, hücrede iyon akımlarının başlamasına neden olur. Kayma gerilimi, endotel hücrelerinde "inward rectifying" K^+ kanallarını aktive eder. Bu kanallar hücre membranının hiperpolarize olmasına sebep olur. Bu olayla eş zamanlı olarak kayma gerilimi endotel hücrelerinde "outward rectifying" Cl^- kanallarını da aktive eder. Bu kanalların aktivasyonu ise K^+ kanallarına bağlı olarak gelişen hiperpolarizasyonu antagonize eder ve membran depolarizasyonuna neden olur [122-129]. Kan akımının başlamasıyla hızlı olarak aktive olan akıma duyarlı K^+ kanalları, 0,1 dyn/cm^2 kayma geriliminde iyon akımının başlamasına neden olmaktadır. Yarı maksimal aktivasyona 0,7 dyn/cm^2 de ulaşan ve 15 dyn/cm^2 de sature olan bu kanallar, 1-5 dyn/cm^2 kayma gerilimine yanıt olarak membran potansiyelini 2-6 mV arttırarak hücre membranını depolarize etmektedir [125, 129, 130]. Kayma gerilimine duyarlı Cl^- kanalları ise K^+ kanallarından bağımsız olarak aktive olmakta ve K^+ aracılı hiperpolarizasyonun başlamasını takiben hücre membranının depolarizasyonuna neden olmaktadır [122, 129, 130]. İyon kanallarının akıma bağlı olarak gerçekleşen aktivasyonlarının fizyolojik rolü tam olarak bilinmemektedir. Ancak, bu kanalların hücre hacmini düzenleyen yolaklarla ilişkili olduğu düşünülmektedir. İyon kanallarının aktivasyonu ile endotel hücrelerinin akıma verdiği birçok yanıtın düzenlendiği gösterilmiştir. Bu yanıtlar arasında nitrik oksit yapımı, cGMP salınımı ve Na-K-Cl kotransport protein ekspresyonu yer almaktadır [131]. İyon kanallarının aktivasyonu yolu ile belirtilen düzenleme mekanizmalarının gerçekleşmesinde, ikincil mesajcı olan Ca^{+2}'un hücre içine taşınmasının önemli bir rolü olduğu düşünülmektedir [132, 133]. Sonuçta ortaya çıkan sitozolik Ca^{+2} artışının bir dizi hücre içi

yolağın aktivasyonuna ve NO gibi biyoaktif ajanların yapımına yol açtığı bilinmektedir.

2.5.4. Kayma gerilimi ve gen ekspresyonunun uyarılması

Kayma gerilimi, hücrenin yazgısı da (hücre siklusunun ilerlemesi ve apoptozis) dahil olmak üzere vasküler fizyoloji ile ilişkili pekçok geni modüle eder, çok sayıda proteini (büyüme faktörleri, adezyon molekülleri, kemotaktik moleküller, koagülasyon faktörleri ve protoonkogenleri) kodlayan genlerin düzenlenmesinde önemli etkilere sahiptir. Laminer kayma gerilimi endotel hücrelerinde G1'den S fazına geçişi baskılar [134, 135]. Bu durum siklin bağımlı kinazların inhibitörü olan p21'in mRNA ve protein düzeylerinde artmayla ilişkilidir. Fizyolojik düzeylerde laminar kayma gerilimi endotel hücrelerinde TNF-α, okside LDL ve anjiotensin II gibi çeşitli uyaranlara yanıt olarak gözlenen apoptozisi önler [136, 137]. Kayma gerilimi ile oluşan antiapoptotik etkinin süperoksit dismutaz ve NOS upregülasyonu ile gerşekleştiğine dair kanıtlar bulunmaktadır [138]. Kayma gerilimi NOS'un mRNA düzeylerini, mRNA stabilitesini ve NO üretimini arttırır [23, 33]. Kayma gerilimine arteryel düzeyde maruz kalan endotel hücrelerinden salınan vazokonstriktör endotelin-1 (ET-1) miktarının ve mRNA'sının azaldığı [139-143], düşük kayma gerilimi (5 dyne/cm^2) durumunda ise ET-1 salınımının ve mRNA düzeylerinin arttığı bulunmuştur [143, 144].

Endotel hücreleri uzun süre hemodinamik kuvvetlere maruz kalırlarsa hücrelerde nükleer faktör kappa B'nin (NF-kB) (transkripsiyon faktörü) aracılık ettiği bir genomik aktivasyon gözlenebilir. Bu sistemin aktivasyonunun, akım ve kayma gerilimi değişikliklerinin gen aracılı yanıtlarına aracılık ettiği ileri sürülmüştür. NF-kB bağlanma bölgeleri pekçok genin promoter bölgesinde bulunduğu için, bu sistem kayma gerilimindeki değişikliklere cevaben gen ekspresyonunu kontrol etmede çok önemli olabilir [46]. Kayma gerilimine uzun süre maruziyet hem NO üretimi ve salınımında hem de eNOS ekspresyonunda artışa yol açar, bu artmış üretimin moleküler temelini oluşturmaktadır [24].

2.5.4.1. Endotel hücrelerinde sinyal transdüksiyonun kayma gerilimi tarafından aktivasyonu

Transkripsiyon faktörlerinin aktivasyonu ile gen ekspresyonun uyarılması için ilk olarak mekanik uyarıların kimyasal sinyallere dönüştürülmesi gerekir. Bu mekanokimyasal transdüksiyona membran yüzeyinde ve sitoplazma içinde sinyal yolaklarının aktivasyonu katılır. Kayma gerilimi, membrandaki K^+ kanalı [123], G proteinleri [145], intraselüler Ca^{+2} [146], cAMP [147], cGMP [32], inozitoltrifosfat [148], protein kinaz C [144], mitojenle aktive olan protein kinazlardan (MAPK) JNK'lar gibi [149, 150], küçük GTPaz'lardan Ras [149] ve protein tirozin kinazlardan fokal adezyon kinazlar (FAK) [151, 152] gibi pekçok sinyal molekülünü modüle eder. Bu sinyalizasyon moleküllerinin modülasyonu kayma gerilimine cevaben endotel hücrelerinde birçok genin ekspresyonunun regülasyonuyla bağlantılıdır. Örneğin, K^+ kanalı eNOS'un kayma gerilimi tarafından uyarılmasına katılır. Kayma gerilimi reseptörler ve iyon kanallarını içeren pekçok membran

proteinlerini eş zamanlı olarak aktive eder. Ligand bağlanmasıyla uyarılmış spesifik konformasyonel değişikliklerin yerine mekanik kuvvetler nonspesifik şekilde sinyal transdüksiyonun başlamasına yol açarak membran proteinlerinin kümelenmesini ve konformasyonel değişikliklerini uyarır. Hücre membranında lateral mobilite gibi moleküler dinamikler mekanokimyasal transdüksiyonun ilk basamağında önemli rol oynar. Lipid akışkanlığındaki değişiklikler, kayma gerilimi tarafından lipozomlarda G proteinlerinin aktivasyonunu değiştirir. Endotel hücrelerinin abluminal tarafında ve özellikle aktin yapısında hücre iskeletinin bütünlüğünde, fokal adezyon komplekslerinin reorganizasyonu sinyal kaskatlarının başlamasında önemli rol oynar. Kayma gerilimi, endotel hücrelerinde fokal adezyon ve mikrofilamentlerin tekrar düzenlenmesini indükleyebillir [152-154]. Aktin filamentlerinin dağılması, kayma gerilimi tarafından MAPK aktivasyonu ve phorbol ester tissue 13-acetate-reponsive elementin (TRE) transkripsiyonel aktivitesini zayıflatır fakat kayma gerilimi ET-1'in gen ekspresyonunu arttırır. Bu bulgulara göre, hücre iskeleti farklı genlerin ekspresyonlarında farklı rollere sahiptir. NOS geni membranda başlayan sinyalizasyon tarafından regüle edilir

2.6. Nitrik Oksit

Nitrik oksit, birçok fizyolojik olayda önemli rolü olan, çok kısa yarı ömürlü (3-5 sn) bir moleküldür. İnsan ve diğer memeli organizmasında NO üretilebildiğinin ortaya konulması ile 1987 yılına kadar vücutta bulunuş nedeni ve metabolizması hakkında çok az şey bilinen NO'nun fizyolojik ve patolojik olaylardaki rolü anlaşılmış ve 1992'de yılın molekülü seçilmiştir [155, 156]. 1980 yılında Dr. Robert Furchgott, endotelyal muskarinik reseptörlerin asetilkolin ile aktive edilmesi durumunda endotel-kaynaklı gevşetici bir faktör (EDRF) salıverildiğini göstermiştir. Ardından 1987'de Dr. Moncada ve Dr. Ignarro'nun EDRF'nin NO olabileceğine dair kanıtlar elde etmesiyle bilimde birçok sorunun yanıtının NO olduğu anlaşılmıştır. Dr. Furchgott, Dr. Ignarro ve Dr. Murad NO'in kardiyovasküler sistemde bir sinyal molekülü olarak rolü ile ilgili çalışmaları nedeniyle 1998 yılında Nobel Tıp ve Fizyoloji Ödülüne layık görülmüşlerdir [156, 157]. Bu çalışmalar, atmosferi kirlettiği, ozon tabakasını deldiği ve asit yağmurlarına neden olduğu bildirilen NO'un kaderini bir anda değiştirmiştir.

NO, nonpolar, renksiz bir gazdır [158]. Suda az çözünen, lipofilik bir molekül olan nitrik oksit hücre membranından kolaylıkla geçer. Nitrik oksitin yüksüz bir molekül olması, çiftleşmemiş elektron taşıması nedeniyle hücreden hücreye hiçbir bariyerle karşılaşmadan kolaylıkla geçmesini sağlamaktadır [158, 159]. Aynı zamanda NO, taşıdığı çifleşmemiş elektron nedeniyle bir radikal molekül olarak isimlendirilebilir. Diğer serbest radikaller genelde hücreler için zararlı iken NO, düşük konsantrasyonlarda çok önemli fizyolojik işlevlerde rol almaktadır. Ancak, aşırı ve kontrolsüz NO sentezi hücreler için zararlı olabilmektedir [160]. NO, bu özellikleri ile çok ideal bir fizyolojik haberci molekül özelliği kazanmaktadır. NO asidik, nötr ve bazik pH'larda farklı etkiler gösterir, küçük miktarlarda (nanomolar

konsantrasyonlarda) üretildiği zaman hücre koruyucudur, milimolar konsantrasyonlara arttığı zaman ise toksiktir. NO'in aşırı üretimi veya baskılanması zararlı etkilere neden olabilir [26].

Hemoglobin NO'yu bağlar ve inaktive eder. Özellikle oksihemoglobin NO için kuvvetli bir inhibitördür. NO, nitrite de okside olabilir ancak nitrit tekrar oksitlenerek kısa sürede nitrata dönüşür [26].

2.6.1. Nitrik Oksitin Fonksiyonları

2.6.1.1. NO'nun Kardiyovasküler Sistem Üzerine Etkileri

NO sentez inhibitörlerleri ile yapılan çalışmalarda NO yokluğunun vasküler dirençte artışa ve kan basıncında yükselmeye neden olduğu bulunmuştur [13, 156, 161]. Bu çalışmalar, damar direncinin dengelenmesinde NO'in büyük homeostatik rolü olduğunu göstermektedir [15, 26].

Endotel hücresinde oluşan nitrik oksitin bir kısmı damar düz kas hücresine difüze olurken, geri kalan kısmı kana geçerek dolaşımdaki komşu hücreler (lökosit, trombosit) üzerinde etkili olur [15, 162]. Ayrıca eritrositlerin içine de difüze olarak hemoglobine bağlanabilir [163]. NO, düz kas hücresine difüze olduktan sonra cGMP'yi arttırarak düz kas gevşemesine neden olur, cGMP artışı 6 farklı mekanizma ile gevşemeyi meydana getirebilir. Bu mekanizmalar şunlardır [158]:

1. Sarkoplazmik retikulumda Ca^{2+}-ATPaz'ın aktivasyonuyla hücre içi Ca^{2+} konsantrasyonunun azalması.
2. Miyozin hafif zincirinin defosforilasyonu.
3. Düz kas hücresi membranındaki reseptör aracılı Ca^{2+} kanallarının inhibe edilmesi.
4. Hücre içi Ca^{2+} düzeyinin düşmesini sağlayan Ca^{2+} taşıyıcılarının, G proteinlerinin, reseptörlerin ve kanal proteinlerinin fosforile edilmesi.
5. Membrandaki Ca^{2+}-ATPaz'ın uyarılması.
6. Potasyum kanallarından potasyum geçişinin arttırılmasıyla hiperpolarizasyon oluşması.

2.6.1.2. NO'nun Gastrointestinal Sistem Üzerine Etkileri

NO, gastrointestinal sistemde birçok fizyolojik etkiye sahiptir. Sekresyon ve motilite, kan akımı, elektrolit ve su absorbsiyonu, mukozal koruma ve inflamasyon gibi olaylarda rolü olduğu gösterilmiştir [164]. Peristaltizmin esas transmitteridir.

NO, mide kan akımını arttırır. NO dönorleri vagal uyarı veya histaminle indüklenen asit salgılanmasını azaltabilir [165]. Mide tonus ve motilitesini baskılar, NO dönorleri duodenal mukus sekresyonunu arttırarak gastrik asite karşı mukozal koruma sağlar.

2.6.1.3. NO ve Sinir sistemi

NO merkezi sinir sisteminde hafıza oluşumu, denge ve koku alma gibi birçok fonksiyonu destekleyen bir nörotransmitter olarak işlev görmektedir

[13, 158, 166, 167]. Periferik sinir sisteminde ise nonadrenerjik nonkolinerjik sinir sistemini etkileyerek vazodilatasyon, solunum, mide ve barsak fonksiyonlarının düzenlenmesine katkıda bulunmaktadır [168].

2.6.1.4. NO'nun Renal Sistem Üzerine Etkileri

Nitrik oksit renal kan akımı, renal otoregülasyon, glomerüler filtrasyon, renin salgılanması ve tuz itrahı gibi renal fonksiyonların kontrolünde en önemli parakrin modülatör ve aracıdır. Böbrekte glomerular ve medüller mikrodolaşım, endojen NO tarafından regüle edilir [169, 170]. Tubuloglomerular feedback, kısmen NO salınımı tarafından düzenlenir [170].

2.6.1.5. NO'nun İmmün ve İnflamatuvar Sistemler Üzerine Etkileri

Proinflamatuar sitokinler iNOS'u uyararak NO üretimini arttırırlar. NO'in, makrofajların intraselüler ve ekstraselüler patojenleri ortadan kaldırmada etkilerine aracılık ettiği, bununla beraber sitotoksik etkide NO formunun değil O_2^- ile etkileşimi ile meydana gelen peroksinitritin etkili olduğu bildirilmiştir [13]. Ayrıca, makrofaj kökenli NO'in inhibitör etkileri tümör hücreleri üzerine olduğu gibi lenfositlerde de görülür [171]. Aktive olmuş makrofajların lenfositlerin mitojen veya antijenlere verdiği proliferatif cevaptaki baskılayıcı etkisi kısmen NO'e atfedilmektedir [172-174].

2.6.2. Nitrik Oksit Üretimi

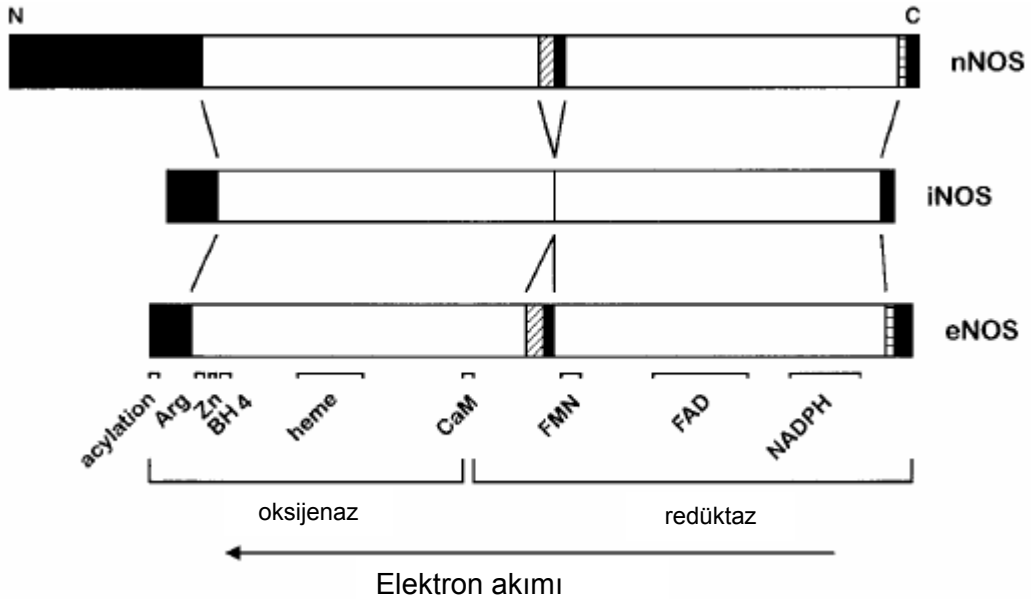
Nitrik oksit, nitrik oksit sentaz (NOS) enzimi tarafından sentezlenir. Fonksiyonel olarak 1990 yılında Bult ve arkadaşları tarafından tanımlanan NOS'un primer yapıları yüksek homolojiye sahip, üç farklı izoformu bulunmaktadır: nöronal NOS (NOS I veya nNOS); 2) indüklenebilir NOS (NOS II veya iNOS); 3) endotelyal NOS (NOS III veya eNOS) [13, 79, 175-178]. İki izoformu yapısal olarak eksprese edilirken (NOS 1, NOS 3) diğer izoformu yapısal değildir ve çeşitli sitokinler tarafından indüklenebilir (NOS 2). NO, NOS enzimlerinin katalizlemesiyle L-arjinin'in oksidasyonuyla oluşur. NOS enzimi, L-arginin amino asidinin terminal guanidino grubundaki nitrojenin oksidasyonunu sağlar [177, 179]. Bu reaksiyon, hem oksijen hem de nikotinamid adenin dinükleotid fosfat (NADPH) bağımlıdır ve NO yanısıra L-sitrullin oluşmasıyla sonuçlanmaktadır. NOS aracılı NO oluşumu için gerekli olan sübstratlar, L- arjinin aminoasiti, moleküler oksijen ve NADPH'dır. Kofaktör olarak ise tetrahidrobiopterin (BH_4), flavin adenin dinükleotid (FAD), flavin mononükleotid (FMN) ve sitokrom P450 kullanılmaktadır [158, 175, 177, 178]. Bundan başka; NOS, enzim aktivitesi için gerekli olan hem ve kalmodulin bağlama bölgesi içerir.

Bu üç NOS izoformunun subselüler lokalizasyonu da değişkenlik gösterir. nNOS ve iNOS çözünür sitozolik proteinlerdir. eNOS ise hücresel membranda partiküler subselüler fraksiyonda özellikle de plazmalemmal kaveola'da bulunur [180]. Endotel hücrelerinde eNOS'la bağlı halde bulunan kaveolin-1; eNOS aktivitesini baskılar. Agonist aktivasyonundan sonra hücre içi Ca^{+2} konsantrasyonundaki artış kalmodülinin eNOS'a bağlanmasına neden olur ve eNOS'un kaveolinden ayrılmasını sağlar. eNOS-kalmodülin kompleksi hücre içi Ca^{+2} konsantrasyonu azalana kadar NO sentezler. Ca^{+2}

konsantrasyonu azalmasıyla daha sonra yeniden inaktif eNOS-kaveolin kompleksi oluşur [180].

Belli koşullar altında; NOS, NO yerine serbest radikal ($^{-}O_2$) üretebilir [178]. NOS tarafından $^{-}O_2$ oluşumu, oksijenaz domaininin hem grubu aracılığıyla olur, sübstrat (L-arjinin) ve kofaktör (BH_4) varlığına bağımlıdır [181, 182]. NOS, her iki faktörün birinin konsantrasyonunun düşük olması durumda süperoksit oluştururken, sübstratı ve kofaktörü yeterli miktarda olduğu zaman NO üretir.

NOS enzimi, oksijenaz (1 - 491 aminoasit) ve redüktaz (492 - 1205 amino asit) olmak üzere 2 adet globüler protein motifine sahiptir [183]. Memeli NOS'ları sadece dimerik formda oldukları zaman fonksiyonel olarak aktiftirler. Redüktaz bölgesi, NO üretimi için gereken elektronları redükte olmuş NADPH'ı dehidrojenize ederek üretir [178]. Elektronlar daha sonra oksijenaz bölgesine geçerler. Kalsiyum bağlı kalmodulinin NOS'un COOH-terminal redüktaz ve NH_2- terminal oksijenaz domaininin arasına bağlanmasından sonra elektron transferi aktive olur. Kalmodulin, flavinlerden NOS'un hem bölgesine elektron transferinde görevli olup, NO biyosentezinde önemli bir basamak olan flavin indirgenmesinin hızını belirler [184-186]. Oksijenaz bölgesi, NO üretim yeridir ve hem, L-arjinin ve BH_4 bağlar. Ayrıca çinko (Zn) bağlama bölgesine sahiptir [187] (Şekil 2.8). Her NOS dimeri bir çinko iyonu içerir ve bu da dimerik molekülün stabilizasyonunu sağlar. Tüm NOS izoformları kalmodulin bağlanma bölgesi içerse de kalmodulinin NOS2'ye bağlanması onu kalıcı bir alt birim haline getirir. eNOS ve nNOS'a kalmodulin bağlanması ve aktivasyonu fizyolojik Ca^{+2} konsantrasyon değişikliklerine cevaben meydana gelirken, kendisine sıkıca bağlı kalmoduline sahip olan iNOS düşük Ca^{+2} konsantrasyonlarında bile maksimum flavin redüksiyonuna imkan sağlar [15, 26, 162, 188].



Şekil 2.8.: Nitrik oksit sentaz izoformlarının yapısı. Açık renkli kutucuklar homolog alanları gösterirken, taralı kutucuklar; nNOS ile eNOS arasındaki homolog bölgeleri göstermektedir. Arg, arjinin; BH4, tetrahidrobiyopterin; CaM, kalmodulin; FMN, flavin mononukleotid; FAD, flavin adenin nükleotid (kaynak 186'dan alınmıştır).

2.6.3. eNOS Aktivitesinin Kontrolü

eNOS, bazal koşullarda endotel hücresinde sürekli, ancak az miktarda NO sentezinden sorumludur. Endotel hücre kültürlerinde eNOS enzimi yapısal olarak eksprese olmasına rağmen bazı faktörlerin de enzim aktivitesini ve bazal ekspresyonunu etkilediği bilinmektedir. Kayma gerilimi, eNOS aktivitesini arttırmaktadır. Düzenli egzersiz yaptırılan köpeklerde de eNOS düzeyinin arttığı bildirilmiştir [189]. TNF-α, okside LDL artışı ve hipoksi gibi durumlarda ise eNOS aktivitesi azalmaktadır. Bu gibi durumlarda, mRNA'nın posttranskripsiyonel ömrünün kısalması eNOS sentezinin azalmasında önemli rol oynayabilir. Ayrıca kaveolanın transmembran proteini olan kaveolin de eNOS'u inhibe edebilir [180]. Bu inhibisyon Ca-kalmodulin kompleksi tarafından tamamen ortadan kaldırılabilir [180].

eNOS fosforilasyonu, eNOS aktivitesini kontrol eden kritik regülatör mekanizma olarak tanımlanır. eNOS üzerinde en az 5 spesifik fosforilasyon bölgesi tanımlanmıştır [35]. eNOS fonksiyonunda fosforilasyonun önemini destekleyen veriler bulunması ve giderek artmasına rağmen fosforilasyonun regülasyonunda rol alan protein kinazlar ve fosfatazlarla ilgili çelişkili bilgiler bulunmaktadır. Örneğin, eNOS'un serin 1177 aminoasitinin, in vivo ve in vitro koşullarda 5 farklı protein kinaz tarafından fosforillendiği gösterilmiştir [137, 190-193]. Serin 1177 bölgesi ile ilgili çok sayıda çalışma bulunmasına rağmen diğer bölgelerin fosforilasyonunun önemi hala tartışmalıdır [35]. eNOS enzimi fizyolojik uyarılara cevaben serin 1177, serin 633, serin 615, threonin 495 ve serin 114 bölgelerinden fosforillenmektedir.

eNOS-serin 1177 bölgesinin fosforillenmesi, enzim aktivitesinin artmasına neden olur, bu bölgenin fosforillenmesi ile bazal aktivitenin 2 kat arttığı bildirilmiştir [137, 194] eNOS-threonin 495 bölgesinin fosforillenmesi ise enzim aktivitesinin azaldığını göstermektedir [35, 180, 191, 195]. Literatürde diğer fosforilasyon bölgelerinin eNOS enzim aktivitesi üzerine etkilerine dair çelişkili bilgiler bulunmaktadır [35].

2.6.3.1. eNOS Fosforilasyonunda Protein Kinazlar ve Fosfatazların Etkisi

Tirozin kinazlar

Endotel hücrelerinde tirozin kinaz inhibitörleri kayma gerilimine yanıt olarak gözlenen NO üretimini inhibe etmektedir [16, 17]. Bu durum eNOS regülasyonunda tirozin kinazın rolü olabileceğini düşündürmektedir. Fizyolojik koşullar altında tirozin kinazların eNOS aktivitesini nasıl düzenlediği çok açık değildir. Bu konuyla ilgili iki olasılık vardır [35]:1- tirozin kinazlar eNOS'u tirozin rezidülerinden fosforiller; 2- tirozin kinazlar, enzim aktivitesini dolaylı olarak düzenleyen proteinleri fosforiller. Garcia-Cardena ve arkadaşları, sığır aortik endotel hücrelerinde tirozin fosfataz inhibitörü veya hidrojen peroksitin eNOS'un tirozin fosforilasyonunu stimüle ettiğini göstermişlerdir [196]. Tirozin fosforilasyonundaki artış, enzim aktivitesindeki azalmaya işaret eder. Bu inhibisyonun nedeninin de eNOS'la caveolin-1'in etkileşimi olabileceği ileri sürülmüştür [196]. Fleming ve arkadaşlarının çalışmasında ise; kayma

gerilimi veya tirozin fosfataz inhibitörü uygulanan endotel hücrelerinde Ca^{2+} bağımsız şekilde eNOS aktive olmuştur [197, 198].

Protein kinaz C (PKC): PKC bağımlı fosforilasyon eNOS enzim aktivitesini azaltır. Phorbol mristate acetate (PMA) ile protein kinaz C aktivasyonunun hücre kültüründe eNOS aktivitesini inhibe ettiği gösterilmiştir [199, 200]. PKC tarafından eNOS üzerinde fosforillenen bölgelerin threonin 495 ya da serin 114 olduğu gösterilmiştir [193, 201]. PMA uygulaması ile PKC'nin aktive edilmesi sonucu threonin 495 fosforile olmuştur [193, 202]. PKC inhibitörü kullanılarak bazal T495 fosforilasyonunda azalma tespit edilmiştir. Bu veriler eNOS-T495 fosforilasyonunda PKC'nin rolünü desteklemektedir.

Protein Kinaz B/Akt: eNOS regülasyonunda Akt'nin rolü eNOS-Serin 1177'nin fosforilasyonu şeklindedir [137, 203-205]. Fosfotidil inozitol 3-kinaz (PI3K) (Akt yolağında üst sıradaki düzenleyicilerden biri) spesifik inhibitörleriyle yapılan çalışmalarda kayma gerilimi, vasküler endotelyal büyüme faktörü (VEGF) ve insülin benzeri büyüme faktörü tarafından uyarılan eNOS-S1177 fosforilasyonu engellenmiştir [137, 203-205]. Michell ve arkadaşlarının çalışmasında Akt tarafından Serin 615'in de fosforillendiği bildirilmiştir [206].

Protein kinaz A (PKA) ve Protein kinaz G (PKG): PKA ve Akt'nin dahil olduğu birçok protein kinaz benzer fosforilasyon bölgelerini etkiler. Butt ve arkadaşlarının sonuçlarına göre de serin 1177'nin PKA ve PKG tarafından fosforillendiği bulunmuştur [190]. Serin 1177'a ek olarak PKA Serin 633'ü de fosforillemektedir. Serin 1177 ve S633'ün kayma gerilimi bağımlı fosforilasyonu PKA inhibitörleri tarafından engellenmiş [207], PKA aktivatörlerinin de fosforilasyonu indüklediği ortaya çıkarılmıştır [207]. Kayma gerilimi, VEGF ve bradikinin gibi uyaranların PKA bağımlı olarak Serin 633'ü fosforillediği gösterilmiştir [137, 203, 206]. Akt'nin ise serin 633'ü fosforilleyemediği bildirilmiştir [206].

AMP tarafından aktive edilen protein kinaz (AMPK): AMPK, Ca^{2+}/CaM varlığında serin 1177'u fosforiller. eNOS'la Ca^{2+}/CaM bağlanması AMPK tarafından oluşturulan serin 1177 fosforilasyonuna izin verecek konformasyonel değişikliği indükler [191]. Son zamanlarda peroksinitritin AMPK bağımlı mekanizma aracılığıyla Serin 1177 fosforilasyonunu stimule ettiği gösterilmiştir [208]. Ca^{2+}/CaM yokluğu ise threonin 495 fosforillenmesini sağlamıştır [191].

CaM bağımlı mekanizma: Bradikinin gibi bazı agonistlere cevaben PI3K bağımsız mekanizma aracılığıyla eNOS-S1177 fosforilasyonu meydana getirebilir [192, 193].

Fosfatazlar: Üç farklı sınıf serin/threonin protein fosfataz (protein fosfataz 1 (PP1), protein fosfataz 2A (PP2A), kalsinörin) eNOS defosforilasyonu ve aktivasyonun düzenlenmesinde görevlidir. eNOS-S1177'nin defosforilasyonu

PP2A tarafından regüle ediliyorken eNOS-T495'in defosforilasyonu PP1 tarafından yapılır [193, 202].

2.6.4. Duvar Kayma gerilimi ve NO

Duvar kayma gerilimi değişikliklerinin NO bağımlı mekanizmaları etkilediği çok iyi bilinmektedir [16, 17, 20, 23, 24, 33]. NO, dolaşımın kontrolünde anahtar bir rol oynar [9, 10]. Vasküler düz kas tonusunun ve akım direncinin düzenlenmesinde NO'nun önemi iyi bilinmektedir [10-12]. Hipoksi ve kayma gerilimi gibi çeşitli faktörler eNOS aracılı NO sentezini etkiler [15, 25, 37, 209, 210]. Bu faktörler sadece mevcut enzimin aktivitesini değil endotel hücrelerinde eNOS proteinin ekspresyonunu da etkiler [15, 211]. Endotel hücreleri üzerinde etkili mekanik faktörler hücrelerdeki NO üretiminin ana belirleyicisi olarak bilinir [15, 211]. Duvar kayma geriliminin azalması NO üretimini azaltır ve düz kas tonusunun artmasına neden olur [212].

Endotel hücresinden NO üretimini belirlemede akım hızının rolünü teyit eden çok sayıda deneysel çalışma yayınlanmıştır [212-215]. Varin ve arkadaşlarının çalışmasında, sol koroner arter ligasyonu ile sıçanlarda kronik kalp yetmezliği oluşturulmuş ve bu hayvanlarda grasilis kası arterlerinde NO tarafından aracılık ettiği bilinen akım aracılı gevşeme yanıtlarının baskılandığı gösterilmiştir [212]. Aynı kas örneklerinde NOS ekspresyonun da azaldığı saptanmıştır [212]. İlginç olarak hem akım aracılı gevşeme hem de NOS'daki azalma kardiyak debi ve iskelet kas damarlarına kan akımını arttıran yüzme egzersizi ile önlenebilmektedir [212]. Miyauchi ve arkadaşları endotelyal NO aktivitesi üzerine sıvı kuvvetlerinin etkilerini gösteren sonuçlara ulaşmışlardır [215]. Çalışmalarında, sıçanlarda 45 dakika ağır egzersiz sonrası akciğer ve böbreklerde NOS aktivitesini araştırmışlardır. Egzersiz sırasında akciğerlerde kan akımı artışı gerçekleşirken, böbreklerde tam tersi olur. Egzersiz yapmayan sıçanlarla karşılaştırıldığında, egzersiz yapan sıçanların akciğerlerinde total NOS aktivitesinin arttığı, böbreklerde ise aktivitenin azaldığı saptanmıştır [215]. Yukarıda bahsedilen iki çalışmada kan akımındaki değişikliklere bağlı olarak duvar kayma geriliminin değişmesi durumunda NO sentezleyen mekanizmaların etkilendiği açık bir şekilde gösterilmiştir [212, 215].

2.6.5. Duvar Kayma gerilimi, Eritrosit agregasyonu ve NO

Artan eritrosit agregasyonu duvar kayma gerilimini azaltabilir, bu da NO sentez mekanizmalarında baskılanmaya neden olabilir. Bunun sonucunda vasküler direnç artışı gözlenebilir. Eritrosit agregasyonunun etkilerine dair bu hipotez Başkurt ve ark.'nın çalışmasında, poloxamer-kaplı eritrosit süspansiyonlarının izovolemik değişmeli transfüzyonu ile eritrosit agregasyonları kronik olarak arttırılan sıçanlarda deneysel olarak test edilmiştir [97]. Poloxamer kaplı eritrositler 0.4 L/L hematokritle otolog plazmalarında süspanse edilmiş ve sıçanların total kan hacminin %30'u değişecek şekilde değişmeli transfüzyon yapılmıştır. Bu işlem sonrasında, sıçanlarda agregasyon derecesini yaklaşık 4 kat arttırdığı ve transfüzyonu izleyen 4 gün boyunca kademeli olarak azaldığı bulunmuştur [97]. Buna

karşılık, deney hayvanlarının kan basıncı 4 gün boyunca kademeli bir artış göstermiştir. Eritrosit agregasyonunun arttırıldığı sıçanlara ait grasilis kasından elde edilen arteriyol segmentlerinin akım aracılı gevşeme cevaplarında gözlenen değişiklikler, bu hayvanlarda NO bağımlı endotel cevaplarının baskılandığını düşündürmüştür. Bu sonuç grasilis kas örneklerinden alınmış dokularda azalmış eNOS ekspresyonunu gösterilmesiyle doğrulanmıştır [97].

2.7. Hipotez

Eritrosit agregasyonu değişiklikleri kanın damar içindeki segmental dağılımını etkileyerek, damar duvarına komşu bölgede kan bileşimini etkileyebilir. Bu etki sonucu ortaya çıkacak olan yerel hematokrit azalması ve buna bağlı olarak kan viskozitesinin düşmesi damar duvarına etki eden kayma kuvvetlerinin de azalmasına neden olabilir. Eritrosit agregasyonu ile birlikte plazma viskozitesinin de değişmesi, agregasyonla ilişkili olarak ortaya çıkacak olan bu etkiyi önleyebilir. Bu hipoteze dayalı olarak kurgulanan çalışmada silindirik, cam kapiller tüpler içinde yetiştirilen insan göbek kordonu endotel hücrelerinin, agregasyon özellikleri iki farklı yöntemle değiştirilen eritrosit süspansiyonlarıyla perfüzyona verdikleri yanıt incelenmiştir. Eritrosit agregasyonun değiştirilmesinde kullanılan iki yöntemden birisi hücrelerin yüzey özelliklerinin değiştirilmesi, ikincisi ise süspansiyon ortamının değiştirilmesi esasına dayalıdır. Dolayısıyla, agregasyonun arttırılması yanında süspansiyon ortamının (plazma) viskozitesinin de değiştirilmesinin bu etkide oynayacağı rol de araştırılmıştır. Endotel hücrelerinin bu süspansiyonların akımıyla uygulanan ve 15 dyn/cm^2 düzeyinde olacağı hesaplanan duvar kayma kuvvetine yanıtları NO sentez mekanizmalarının incelenmesiyle araştırılmıştır. NO sentez mekanizmalarının, eritrosit agregasyonu değişiklikleriyle ortaya çıkması beklenen duvar kayma kuvveti değişikliklerini saptamak amacıyla kullanılması planlanmıştır.

GEREÇ ve YÖNTEMLER

Bu çalışmada, iç yüzeyleri insan umblikal ven endotel hücreleriyle kaplanmış cam kapiller boruların farklı agregasyon özelliğine sahip eritrosit süspansiyonlarıyla perfüze edilmesinin, endotel hücrelerinin nitrik oksit sentaz mekanizmalarına etkileri incelenmiştir.

Çalışma Akdeniz Üniversitesi Tıp Fakültesi Fizyoloji Anabilim Dalı ve Merkez Araştırma Laboratuvarında gerçekleştirilmiştir. Endotel hücre izolasyonu için; sağlıklı, gönüllü annelere ait göbek kordonları kullanılmıştır. Göbek kordonlarında fazlaca hematom, ödem olmamasına ve uzunluğunun en az 20 cm olmasına dikkat edilmiştir. Akdeniz Üniversitesi hastanesinde gerçekleşen doğumlarda plasentanın çıkarılmasını takiben göbek kordonu alınmış, steril plastik torbaya konularak ilk 2 saat içinde endotel hücre izolasyonu için kullanılmıştır.

Perfüzyonda kullanılacak eritrosit süspansiyonlarının hazırlanması için gereken kan örnekleri 20-40 yaş arası sağlıklı, gönüllü bireylerin ön kol venlerinden alınmış (35-40 mL) ve ethylendiaminetetraasetic acid (EDTA; ED2P, Sigma Chemical Co., St. Louis, MO, ABD 1.5 mg/mL) ile antikoagüle edilmiştir. Eritrosit agregasyonu, iki farklı yaklaşımla (plazma bileşimi değiştirilerek ve eritrosit yüzey özellikleri değiştirilerek) modifiye edilmiştir.

3.1. Endotel Hücre İzolasyonu

Endotel hücreleri, kollajenaz izolasyon tekniği kullanılarak insan göbek kordonundan elde edilmiştir [216]. Yaklaşık 20 cm uzunluktaki göbek kordonuna ait venin iki ucuna laminar akımlı kabinde özel cam kanüller yerleştirilmiştir. Ven öncelikle 40 mL steril izotonik fosfat tamponu (PBS; 14040-091, GIBCO, Invitrogen Corporation, Carlsbad, California, ABD) ile yıkanmış ve damar içindeki kan artıkları uzaklaştırılmıştır. Göbek bağının içindeki PBS boşaltıldıktan sonra 1 mg/mL konsantrasyonunda kollajenaz enzimi (C2674; Sigma Chemical Co., St. Louis, MO, ABD) içeren PBS ile doldurulmuş ve enzim çözeltisinin venin içinde kalmasını sağlamak üzere kordonun her iki ucundaki kanüllere geçirilmiş silikon borular kıskaçlarla sıkıştırılmıştır. Göbek kordonu steril naylon poşet içinde 37 °C'de, %5 CO_2 içeren inkübatörde 15 dakika bekletilmiştir. İnkübasyon süresinin bitiminde yeniden laminar akım kabinine alınan göbek kordonu veni boşaltılmış ve 20 mL PBS ile yıkanarak sıvılar steril bir tüpe aktarılmıştır. PBS, enzim ve hücre karışımından oluşan bu süspansiyon, 1500 rpm'de 5 dakika santrifüj edilmiş, süpernatan aspire edilmiştir. Hücre peletinin üzerine 8 mL besiyeri eklenerek bu süspansiyon, önceden PBS/jelatin (G1393; Sigma Chemical Co., St.

Louis, MO, ABD) (1/1) ile kaplanmış, 2 adet doku kültür flaskına eşit miktarda paylaştırılarak (25 cm^2, 353109; Becton-Dickinson Co., N. Jersey, ABD) 37 °C sıcaklıkta ve %5 CO_2 içeren inkübatörde bekletilmiştir. İnkübasyon süresince kültür ortamı birinci, ikinci ve yirmidördüncü saatlerde, sonrasında 3-4 gün aralıklarla değiştirilmiş ve hücrelerin çoğalarak tüm alanın %70-80'ine dağılması için (5-6 gün) beklenmiştir.

Endotel hücreleri izolasyonunda ve aşağıda tanımlanan işlemlerde kullanılan besiyeri, medium 199 (31150-022; GIBCO, Invitrogen Corporation, Carlsbad, California, ABD), fetal sığır serumu (%20 F7524; Sigma Chemical Co., St. Louis, MO, ABD), penisilin/streptomisin (P4333; Sigma Chemical Co., St. Louis, MO, ABD), hidrokortizon (1 mg/mL, H0135; Sigma Chemical Co., St. Louis, MO, ABD), epidermal büyüme faktörü (1 ng/mL, E9644; Sigma Chemical Co., St. Louis, MO, ABD) ve amphotericin (2,5 µg/mL, A2942; Sigma Chemical Co., St. Louis, MO, ABD) içermektedir. Hücrelerin flasklardaki büyümelerini gözlemlemek için, faz kontrast mikroskop (IX70; Olympus Optical Co. Ltd.; Tokyo, Japonya) kullanılmıştır.

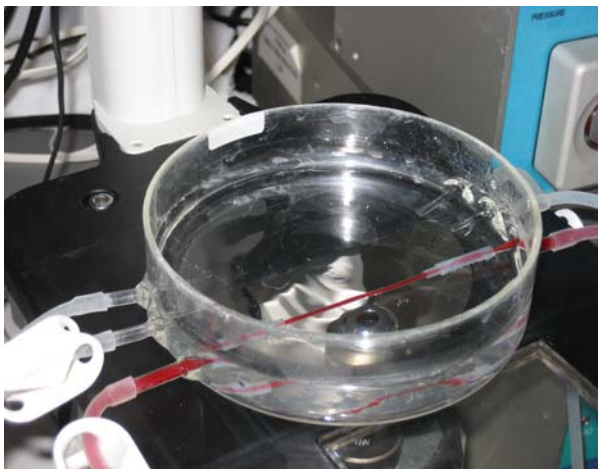
3.2. Silindirik, Cam Kapiller Boruların Hazırlanması

Endotel hücrelerinin cam yüzeye yapışmasını kuvvetlendirmek için kapiller tüpler 3-aminopropyltriethoxysilane (APES, A3648; Sigma Chemical Co., St. Louis, MO, ABD) ile kaplanmıştır. Bir mm çapa ve 7.5 cm uzunluğa sahip, cam silindirik kapiller borular 50 mL'lik steril falcon tüpe yerleştirilip, 1 gece derişik nitrik asitte (606K1888543; Merck KGaA Chemical Co., Darmstad, Almanya) bekletilmiştir. Ertesi gün ortamdan nitrik asit uzaklaştırılmış ve yerine aseton (13; Merck KGaA Chemical Co., Darmstad, Almanya) eklenerek 30 sn hafifçe çalkalanmış, bu işlem taze aseton ile iki kez tekrar edilmiştir. Bu işlemi takiben, %4 APES/Aseton karışımı hazırlanmış ve falcon tüpte bulunan cam kapillerin üzerine dökülüp 30 sn bekletilmiştir. APES'li karışımın yenilenmesinin ardından 2.5 saat süreyle çalkalanmıştır. Çalkalama süresinin sonunda APES'li karışım dökülüp yenisi eklenerek 1 gece karanlıkta bekletilmiştir. Ertesi gün kapiller tüplerin içindeki karışım dökülerek 2 kez daha asetonda 30 sn süresince hafifçe çalkalanmıştır. Kapiller tüpler, laminer akımlı kabine alınarak, distile su ile yıkanmış, 37°C'lik etüve konularak kurumaları sağlanmıştır. Kaplanan kapiller tüpler endotel hücre kaplama prosedürü öncesi etilen oksit ile steril hale getirilerek kullanılmıştır.

3.3. Silindirik, Cam Kapiller Boruların Kaplanması

Primer endotel hücrelerinin, doku kültür flasklarının %70-80'nini kaplayacak şekilde çoğalmalarının ardından kapiller boruların iç yüzeyine kaplanmaya uygun hale geldikleri kabul edilmiştir. Bu hücrelerin, flasklardan kaldırılması işlemlerinin tümü, laminer akımlı kabinde ve aşağıda belirtilen koşullarda gerçekleştirilmiştir. Kültür flaskına ortamdaki fazla kalsiyumun uzaklaşması için, 1 mL EDTA (E8008; Sigma Chemical Co., St. Louis, MO, ABD) solüsyonu eklenerek 1 dakika bekletilmiş, bu sürenin bitimiyle aspire edilerek uzaklaştırılmıştır. Daha sonra hücrelerin üzerine 3 mL tripsin (T4424; Sigma Chemical Co., St. Louis, MO, ABD)/EDTA (2/1) solüsyonu

eklenmiş ve 37 °C'lik inkübatörde 1 dk bekletilmiştir. İnkübasyon süresinin sonunda tekrar laminer akımlı kabine getirilen flaskların üzerine enzim aktivitesini sonlandırmak için 6 mL besiyeri eklenmiştir. Flask içindeki hücre süspansiyonu 15 mL'lik steril bir tüpe aktarılıp, oda ısısında 1500 rpm'de 5 dakika santrifüj edilmiştir. Hücrelerin çöktürülmesinin ardından üstteki sıvı aspire edilmiş, aynı tüpe 300 μl besiyeri eklenmiş ve süspansiyon pastör pipetiyle karıştırılarak homojen hale getirilmiştir. Bu işlemler sonucunda her bir flaskdan yaklaşık $2,5x10^6$/mL hücre elde edilmiştir. Cam kapiller tüplerin homojen şekilde kaplanması için laboratuvarımızda geliştirilen bir teknik uygulanmıştır. Her bir kapillerin kaplanması için aynı göbek bağına ait 2 adet flask kullanılmıştır. Kaplama sırasında silindirik borunun yarısı için bir flaskdan elde edilen hücre süspansiyonu (300 μL) kullanılmıştır. Hücre süspansiyonun 100 μL'si yatay durumda tutulan kapiller borunun içine doldurularak 15 dakika beklenmiştir. Bu sürenin sonunda borunun içindeki hücre süspansiyonu boşaltılarak, 100 μL yeni süspansiyonla doldurulmuş, boru 90 derece, saat yönünde döndürülürek 15 dakika daha beklenmiştir. İşlem, tekrarlanarak boru bu defa 180 derece saat yönünün tersine çevrilmiştir. 15 dakika sonra boru 90 derece saat yönünde döndürülerek başlangıç konumuna getirilmiş ve 15 dakika daha beklenmiştir. Bu işlemler 37 °C'de, %5 karbondioksit içeren etüvünde gerçekleştirilmiştir. Kapiller borunun ilk bölümde üstte kalan yüzü alta gelecek şekilde yerleştirilerek aynı işlemler tekrarlanmış ve endotel hücrelerinin boru içine yerleştirilmesi işlemi tamamlanmıştır. Hücrelerin yerleştirilme işleminden sonra kapiller tüpler özel cam petri kaplarına (35 mm çaplı) yerleştirilmiştir. Petri kabının içi 30-40 mL besiyeri ile doldurulmuş, kapiller tüpler petri kabının içindeki özel bağlantı borularına silikon tüplerle irtibatlanmıştır (Şekil 3.1). Hücrelerin kapiller tüplere homojen şekilde yayıldıkları mikroskobik olarak teyit edildikten sonra, kapiller tüp içindeki hücrelerin çoğalmaları için petri sistemi, %5 CO_2 içeren inkübatörde, 24 saat 37 °C'de inkübe edilmiştir. İnkübasyon süresi boyunca, kapiller boruların içinden, irtibatlandıkları bağlantı boruları kanalıyla, 60 dakikada bir, 30 saniye süresince 407 μL/dakika akım hızında besiyeri geçmesi sağlanmıştır.



Şekil 3.1: Cam kapiller tüp içerisine yerleştirilen endotel hücrelerin büyütülmesi ve perfüzyonu sırasında kullanılan 35 mm çaplı özel petri kabı.

3.4. Endotel Hücre Kimliğinin Tesbiti:

Endotel hücre yüzeyinde eksprese olan Von Willebrand faktör (vWF) immunohistokimyasal olarak gösterilmiştir. İmmunohistokimyasal boyama işlemi için 24 kuyucuklu petri kabı ve APES ile kaplı yuvarlak cam lamel (406/189/13; vWR International Ltd., Leicestershire, İngiltere) kullanılmıştır. Lameller kullanılmadan önce steril hale getirilip petrideki kuyucuklara yerleştirilmiştir. Eşit oranda hazırlanan jelatin/PBS karışımı (1 mL) lamellerin üzerine eklenip 1 saat laminar akımlı kabinde bekletilmiştir. %80 confluent olmuş bir flask seçilip tripsinize edilmiştir. Hücreler, santrifügasyonla çöktürülüp tripsin enzimi uzaklaştırılmış ve yerine 10 mL besiyeri eklenmiştir. Lamellerin üzerindeki fazla jelatin inkübasyonun sonunda uzaklaştırılmış ve 10 mL hücre süspansiyonundan 1 mL alınıp lamellere eklenmiştir. Kuyucuklu petrinin kapağı kapatıldıktan sonra CO_2 etüvünde 1 saat beklenmiştir. Bu süre sonunda besiyeri değiştirilmiş ve yenisi eklendikten sonra 24 saat confluent olmaları için beklenmiştir. Sürenin sonunda, lamellerdeki besiyeri uzaklaştırıldıktan sonra 2 kez PBS ile yıkanmıştır. Formaldehit (%2) (F8775; Sigma Chemical Co., St. Louis, MO, ABD) ile 10 dk fiksasyonun ardından üzerine %0.2 Triton-X (T9284; Sigma Chemical Co., St. Louis, MO, ABD) eklenip 10 dakika oda ısısında bekletilmiştir. Lamellerin üzerindeki Triton-X/Formaldehit atılıp 5 kez PBS ile yıkanmıştır. Hücrelere, bloklama için %2 keçi serumu (G9023; Sigma Chemical Co., St. Louis, MO, ABD) eklenmiş ve 20 dakika oda ısısında inkübe edilmiştir. Serumun uzaklaştırılmasıyla, vWF için üretilmiş primer antikor (SF3520; Sigma Chemical Co., St. Louis, MO, ABD), 1:500 oranında dilüe edilip hücrelere eklendikten sonra 1 saat +4 °C'de bekletilmiştir. Hücreler, 5 kere PBS ile yıkanıp 1:500 dilüsyonda sekonder antikorla (F9887; Sigma Chemical Co., St. Louis, MO, ABD) muamele edilmiştir. İnkübasyon süresi boyunca +4 °C'de 1 saat bekletildikten sonra 5 kez PBS ile yıkanmıştır. Petri kabı içerisine, floresans boyamalar için solmayı önleyici kapatma solüsyonu (M1289; Sigma Chemical Co., St. Louis, MO, ABD) damlatılmış ve üzerine hazırladığımız lamel konulup floresans mikroskobunda vWF görüntülenmiştir.

3.5. Hücre Sayımı ve Hücre Canlılığı Testi

Hücre canlılığı testi için, doku kültür flaskı içerisindeki hücreler tripsinize edilmiştir. Tripsinizasyonun ardından hücreler santrifüjle çöktürülmüş ve dipteki hücre peletine 500 µL PBS eklenmiştir. Bu karışımdan 48 µL alınıp 2 µL tripan mavisi (T8154; Sigma Chemical Co., St. Louis, MO, ABD) ile karıştırılmıştır. Hücreler thoma lamının sayım kamarasına yerleştirilip ışık mikroskobunda (BX50; Olympus Optical Co. Ltd., Tokyo, Japonya) 20X büyütmede incelenmiştir. Membranı mavi boyanan hücreler ölü, boyanmayan hücreler ise canlı kabul edilmiştir.

Hücre sayımı için, tripsinizasyon ve santrifüj işlemleri sonunda dipteki pelete 1 mL besiyeri eklenerek süspanse edilmiştir. Süspansiyonu thoma lamının sayım kamarasına yerleştirip 5 farklı alandan hücre sayımı

yapılmıştır. Toplam sayı 5'e bölünüp çıkan değer 10^6 ile çarpılıp 1 mL'deki hücre sayısı hesaplanmıştır.

3.6. Gruplandırma

Endotel hücreleriyle kaplanmış kapiller tüpler dört gruba ayrılarak aşağıda tanımlanan şekilde deneye alınmışlardır:

1. Kontrol (sıfır akım) grubu (Kontrol): Bu gruptaki kapiller borular, normal eritrositlerin otolog plazma içinde, hematokriti 0.3 L/L ye ayarlanmış süspansiyonu ile doldurulmuş ancak, sıvı akımı olmaksızın 30 dakika veya 6 saat süreyle Bölüm 3.6.'da tanımlanan koşullarda bekletilmişlerdir.

2. Normal eritrosit – otolog plazma grubu (Normal): Bu gruptaki kapiller borular, normal eritrositlerin otolog plazma içinde, hematokriti 0.3 L/L ye ayarlanmış süspansiyonu ile Bölüm 3.6.'da tanımlandığı şekilde 30 dakika veya 6 saat süreyle perfüze edilmişlerdir.

3. Normal eritrosit – Dextran 500 grubu (Dextran): Bu gruptaki kapiller borular, normal eritrositlerin %0.5 Dextran 500 (MW: 500 kD; D5251, Sigma Chemical, St. Louis, MO) içeren otolog plazma içinde, hematokriti 0.3 L/L ye ayarlanmış süspansiyonu ile Bölüm 3.6.'da tanımlandığı şekilde 30 dakika veya 6 saat süreyle perfüze edilmişlerdir.

4. Modifiye eritrosit – otolog plazma grubu (F98): Bu gruptaki kapiller borular, yüzeyleri Pluronic F98 ile kaplanarak agregabiliteleri arttırılmış eritrositlerin otolog plazma içinde, hematokriti 0.3 L/L ye ayarlanmış süspansiyonu ile Bölüm 3.6.'da tanımlandığı şekilde 30 dakika veya 6 saat süreyle perfüze edilmişlerdir.

3.7. Kan Örneklerinin Hazırlanması:

3.7.1. Kan Örneklerinin "Kontrol" ve "Normal" Grupları İçin Hazırlanması

Gönüllülerden elde edilen kan örnekleri, 2700 rpm'de 10 dakika santrifüj edilerek plazma ve eritrosit paketi birbirinden ayrılmıştır. Eritrosit paketi, 3 kez PBS ile yıkanmış ve otolog plazmada hematokrit 0.3 L/L olacak şekilde süspansiyon haline getirilmiştir.

3.7.2. Kan Örneklerinin "Normal Eritrosit – Dextran 500 Grubu (Dextran)" İçin Hazırlanması

Gönüllülerden elde edilen kan örnekleri, 2700 rpm'de 10 dakika santrifüj edilerek plazma ve eritrosit paketi birbirinden ayrılmıştır. Plazmaya %0.5 konsantrasyonda olacak şekilde dextran 500 (MW: 500 kD, D5251; Sigma Chemical Co., St. Louis, MO, ABD) eklendikten sonra, 3 kez PBS ile

yıkanmış olan eritrosit paketi, bu plazma içinde hematokrit 0.3 L/L olacak şekilde süspansiyon haline getirilmiştir.

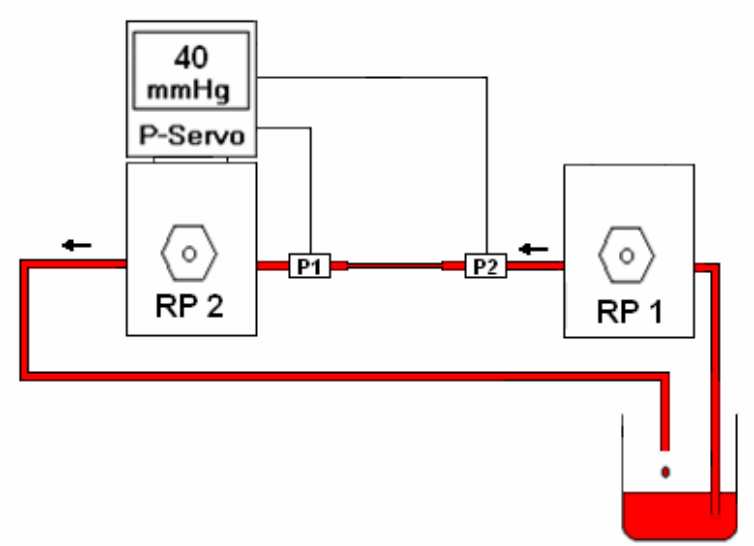
3.7.3. Kan Örneklerinin "Modifiye Eritrosit – Otolog Plazma" Grubu (F98) İçin Hazırlanması

Eritrosit agregasyonu, hücrelerin, polietilenglikol esaslı bir poloxamer (Pluronic F98-SC) moleküllerinin eritrosit yüzeyine kaplanması ile modifiye edilmiştir (46). Eritrositlerin kaplanmasında 0.025 mg/mL konsantrasyonunda reaktif polimerler (Pluronic F98-SC) kullanılmıştır. Pluronic F98-SC, University of Southern California, Department of Physiology and Biophysics (ABD) laboratuvarlarında Prof. Dr. Herbert J. Meiselman ve Dr. Jonathan K. Armstrong tarafından geliştirilmiş ve bu çalışmada kullanılmak üzere hediye edilmiştir. Gönüllülerden elde edilen kan örnekleri, 2700 rpm'de 10 dakika santrifüj edilerek plazma ve eritrosit paketi birbirinden ayrılmıştır. Eritrosit paketi, 3 kez PBS ile yıkanmıştır. Kan örneklerinden ayrılan eritrositler, 30 mM triethanolamine tamponu (290 mOsm/kg, pH 8,60) ile hematokrit ~0.1L/L olacak şekilde süspanse edilmiştir. HCL tamponunda (10 mM) +4 °C'de çözülen F98-SC polimeri (100 mg/mL) triethanolamine tamponu içindeki eritrosit süspansiyonuna 0.025 mg/mL konsantrasyonda olacak şekilde eklenip, 2 saat boyunca +4 °C'de bir tüp sallayıcı üzerinde sürekli karıştırılarak inkübe edilmiştir. Bu sürecin sonunda Pluronic F98 ile kaplanan hücreler PBS'le 3 kere yıkandıktan sonra hematokrit değeri 0.3 L/L olacak şekilde otolog plazmada resüspanse edilmiştir.

Perfüzyonda kullanılan eritrosit süspansiyonlarının viskozitesi koni-plak tipi ölçüm sistemine sahip bir rotasyonel viskometre (Model DV II, Brookfield Engineering Labs., Middleboro, MA, USA) kullanılarak, 37,5 ve 1,500 s^{-1} kayma hızlarında ölçülmüştür. Plazma viskozitesi ise 1,500 s^{-1} kayma hızında tayin edilmiştir. Hematokriti 0.1 L/L olan kan örneği Westergreen yöntemi ile sedimentasyon hızı ölçümü için kullanılmıştır. Sedimentasyon tayini sırasında 60 dakika total süre boyunca her 5 dakikada bir değerler kaydedilmiştir. Her bir örnek hazırlama işleminden sonra ışık mikroskobu (BX50; Olympus, Tokyo, Japonya) ile 20X objektifte görüntüleme yapılmıştır.

3.8. Endotel Hücresiyle Kaplanmış Kapillerlerin Perfüzyonu

Endotel hücreleriyle kaplanmış kapillerin perfüzyonu ayrı ayrı deneylerde, hematokriti 0.3 L/L'ye ayarlanmış normal insan kanı (Normal grubu), plazma kapsamı değiştirilerek eritrosit agregasyonu arttırılmış (Dextran grubu) ve eritrosit yüzey özellikleri değiştirilerek agregasyonu modifiye edilmiş (F98 grubu) eritrosit süspansiyonları kullanılarak yapılmıştır.



Şekil 3.2: Servo kontrollü perfüzyon sistemi. RP1: Kanın rezervuardan kapiller tüp içine infüzyonunu sağlayan sabit akım pompası. RP2: Sistem basıncının 40 mmHg'da sabit kalmasını sağlayan servo kontrollü pompa. P1, P2: Basınç tranduserları.

Perfüzyon işlemi basınç servo kontrollü bir pompa sistemi (Model: PS/200/Q; Living Systems Instrumentation, Burlington, Ohio, ABD) kullanılarak yapılmış, sıvı akım hızı her bir eritrosit süspansiyonu için kapiller boru içinde 15 dyn/ cm^2 duvar kayma kuvveti (δ) olacak şekilde, Q kapiller tüpün içinden geçen sıvının akım hızı, r kapiller borunun yarıçapı, η perfüzyonda kullanılan sıvının viskozitesi olmak üzere, aşağıdaki formülle hesaplanmıştır.

$$\delta = \frac{4 \cdot Q \cdot \eta}{\pi \cdot r^3}$$

Perfüzyon basıncı bütün deneyler için 40 mmHg'da olarak uygulanmıştır. Perfüzyon işleminde kullanılan servo kontrol sistemi, kapiller borunun iki ucuna bağlı iki ayrı döner pompanın (roller pump) hızlarını denetleyerek akım hızını ve perfüzyon basıncını birbirinden bağımsız olarak ayarlayabilmektedir. Perfüzyon sisteminin şematik görüntüsü Şekil 3.2'de gösterilmiştir.

Endotel hücreleriyle kaplı kapiller borular aşağıda belirtilen deney gruplarına uyumlu eritrosit süspansiyonlarıyla 30 Dakika veya 6 saat boyunca perfüze edilmişlerdir. Perfüzyon işlemi özel petri kutuları içinde, 37 °C'de gerçekleştirilmiştir (Şekil 3.1).

3.9. Perfüzyon Sonrasında İncelenen Parametreler

Perfüzyon işlemleri sonrasında kapiller tüplerin iç yüzeyine tutunmuş endotel hücreleri tripsin/EDTA solüsyonu (2,5 g/mL tripsin, 0,5 mM EDTA) kullanılarak cam yüzeyden ayrılmıştır. Tripsinizasyonun ardından hücreler,

PBS ile 2 kez yıkanmış ve hücreler Bölüm 3.5.'de belirtilen yöntemle sayılmış ve devamında yaklaşık $120x10^4$/mL endotel hücresine 100 μl lizis tamponu (100 mL içinde 100 mM NaF (S7920; Sigma Chemical Co., St. Louis, MO, ABD), 50 mM Hepes (H3375; Sigma Chemical Co., St. Louis, MO, ABD), 150 mM NaCl (S6753; Sigma Chemical Co., St. Louis, MO, ABD), 1 mM $MgCl_2$ (5832; Merck KGaA Chemical Co., Darmstad, Almanya), 1 mM EGTA (E4378; Sigma Chemical Co., St. Louis, MO, ABD), 1 mM PMSF (P7626; Sigma Chemical Co., St. Louis, MO, ABD), 1 mM Na-O-Vanadate (S6508; Sigma Chemical Co., St. Louis, MO, ABD), 10 mM Sodyum pyrophosphate (221368; Sigma Chemical Co., St. Louis, MO, ABD), 10 μg/mL Leupeptin (L2023; Sigma Chemical Co., St. Louis, MO, ABD), 10 μg/mL Aprotinin (A6191; Sigma Chemical Co., St. Louis, MO, ABD), 10 μg/mL Pepstatin (P5318; Sigma Chemical Co., St. Louis, MO, ABD), %10 Gliserol (G7757; Sigma Chemical Co., St. Louis, MO, ABD), %1.2 Triton X-100 (X100; Sigma Chemical Co., St. Louis, MO, ABD) eklenmiştir. Otomatik pipet yardımıyla hücre süspansiyonu iyice karıştırıldıktan sonra 5 sn düşük devirde sonike edilerek hücrelerin parçalanması sağlanmıştır. Perfüzyon sonrasında ölçülecek olan herbir parametre için kontrol, normal, dextran ve F98 grubuna ait 4 deney yapılmıştır.

3.10. Nitrit/Nitrat Ölçümü

Endotel hücre süspansiyonunda total nitrat-nitrit düzeyi, ticari kit (780001; Cayman Chemicals Co., Michigan, ABD) kullanılarak Griess yöntemiyle spektrofotometrik olarak tayin edilmiştir [217]. Bu yöntemde; ilk basamak, nitratın, nitrat redüktaz aracılığıyla nitrite dönüştürülmesidir. İkinci basamak ise, Griess reaktiflerinin eklenmesiyle nitritin koyu mor azo bileşiklerine çevrilmesidir. Sonuçta elde edilen değerler, total nitrat/nitrit konsantrasyonunu göstermektedir. Basınç servo-kontrollü sistemde, kapiller tüplerin içindeki endotel hücrelerinin 30 dk perfüzyonu sonrasında hücreler, tripsinize edilmiştir. Örneklere bölüm 3.9.'da bahsedilen işlemler uygulandıktan sonra +4 °C, 100.000 g'de 20 dk santrifüj edilmiştir. Santrifüj sonrası, süpernatanttan 80 μL örnek alınıp 36 kuyucuklu petri kabına konmuştur. Örneğin üzerine, 10 μL enzim kofaktörü karışımından ve 10 μL nitrat redüktaz karışımı eklendikten sonra 3 saat oda ısısında inkübe edilmiştir. İnkübasyondan sonra kuyucuklara 50 μL Griess R1 reaktifi ve aynı miktarda R2 reaktifi eklendikten sonra 10 dakika oda ısısında bekletilmiştir. Bu sürenin sonunda 540 nm dalga boyunda absorbanslar kaydedilmiştir. Absorbans değerleri nitrat standart eğrisinde yerine konarak nM cinsinden total nitrat+nitrit miktarları elde edilmiştir.

3.11. eNOS Protein Ekspresyonu

Endotel hücresinde eNOS ve fosforile-eNOS protein düzeyleri, immunoblot yöntemi ile saptanmıştır. Tespit edilen iki perfüzyon süresinin sonunda elde edilen hücrelere, 100 μL lizis tamponu eklenip otomatik pipetle iyice karıştırıldıktan sonra 5 sn sonike edilip hücrelerin parçalanması sağlanmıştır. Örnekler, 14.000 rpm'de 20 dakika +4 °C'de santrifüj edilip, hücre membranları çöktürülmüş ve total protein içeren supernatant kısmına 5

μL proteaz inhibitör kokteyli (P8340; Sigma Chemical Co., St. Louis, MO, ABD) eklenerek protein elektroforezi işlemine kadar -20 °C'de muhafaza edilmiştir.

3.11.1. Numunelerin Protein Miktar Tayini

Protein miktar tayini, üretici firmanın kullanım tarifine uygun olarak (Lowry yöntemi ile) ticari kit (500-0006; Bio-rad Laboratories, Washington DC, CA, ABD) kullanılarak gerçekleştirilmiştir. Standard eğri, sığır serum albumini (BSA), (K35187818; Merck KGaA Chemical Co., Darmstad, Almanya) ile oluşturulmuştur. Her bir örnekteki protein miktarı 50 μg/mL konsantrasyonda olacak şekilde ayarlanmış ve SDS-poliakrilamid jele yüklenerek elektroforez yapılmıştır (SDS-PAGE).

3.11.2. SDS-Poliakrilamid Jel Elektroforezi (SDS-PAGE) ve Immunoblot

Proteinlerin ayrıştırılması için %7.5'lik poliakrilamid jel kullanılmış ve moleküler ağırlık standardı (161-0318; Bio-rad Laboratories, Washington DC, CA, ABD) örneklerle birlikte kuyucuklara yüklenerek 80 V akım ile 2 saat süreyle elektroforez yapılmıştır. Elektroforez sonrası, jelde moleküler ağırlığına göre ayrılmış olan proteinler, nitroseluloz membrana (162-0115; Bio-rad Laboratories, Washington DC, CA, ABD) ıslak transfer yöntemi ile 90 dakika süresince 80 V akım uygulanarak aktarılmıştır. Transfer sonrası membran, %5 lik süt tozunda 1 saat bloke edilmiş ve daha sonra 1 saat boyunca 4 kez tazelenen TBS-T solüsyonu (%0,1 Tween 20 içeren TBS) kullanılarak yıkanmıştır. Primer antikor olarak 1:1000 dilüsyonda hazırlanmış eNOS (9572; Cell Signaling, Sigma-Aldrich Co., St. Louis, Mo, ABD), fosfo (Ser 1177)eNOS (9571S; Cell Signaling, Sigma-Aldrich Co., St. Louis, Mo, ABD) ve fosfo(Thr 495)eNOS (9574S; Cell Signaling, Sigma-Aldrich Co., St. Louis, Mo, ABD) kullanılmış ve antikorla membran, 16 saat, +4 °C'de çalkalanarak inkübe edilmiştir. Pozitif kontrol olarak, hücrelerde sabit ekspresyonu olan β-aktin (4967; Cell Signaling, Sigma-Aldrich Co., St. Louis, Mo, ABD) proteinine özgü antikor kullanılmıştır (1:1000 dilüsyonda). Primer antikor inkübasyonu sonrasında membranlar, yine 1 saat boyunca TBS-T ile devamlı çalkalanarak yıkanmıştır. İşaretlemek için HRP-konjuge IgG (7074; Cell Signaling, Sigma-Aldrich Co., St. Louis, Mo, ABD) kullanılmış ve 1:3000 oranında sulandırılarak (TBS-T solüsyonunda), membranlarla 1 saat oda ısısında çalkalanarak inkübe edilmiştir. Sekonder antikor uygulaması sonrasında membranlar tekrar 1 saat süresince çalkalanarak yıkanmış ve ECL (RPN2106; Amersham, GE Healthcare Co., Buckinghamshire, İngiltere) uygulanarak kemiluminisans oluşturulmuştur. Daha sonra membran, radyografik filme konularak görüntüleme gerçekleştirilmiştir. Dansitometrik analizler, filmlerin taranarak JPEG formatında kaydedilmesinden sonra Image J programı kullanılarak yapılmıştır. Sonuçlar; her grup için eNOS/β-aktin, fosfo (Ser1177)eNOS/β-aktin, fosfo(Thr495)eNOS/β-aktin şeklinde β-aktin proteininin miktarına oranlanarak ayrı ayrı hesaplanmıştır. Sonuçlar her grup için ünite cinsinden ifade edilmiştir.

3.12. RNA İzolasyonu

Literatürdeki veriler doğrultusunda belirlenen [218] 6 saatlik perfüzyon sonrası, kapiller tüplerden tripsinize edilen endotel hücreleri, RNA stabilizasyon sıvısına (RNA*later,* 76104; Qiagen Inc., Valencia, CA, ABD) alınmış ve deneyin başlangıcına kadar -20 °C'de bekletilmiştir. Deney günü, örnekler buzda eritildikten sonra RNAlater'ı uzaklaştırmak için 3000 rpm'de, 20 dakika +4 °C'de santrifüj edilmiş ve RNA izolasyonu için kullanılan ticari kitin (1828665; Roche, F. Hoffmann-La Roche Ltd., Basel, İsviçre) kullanım kılavuzunda önerildiği şekilde total RNA izolasyonu yapılmıştır. RNA miktarı spektrofotometrik olarak ölçüldükten sonra kullanım anına kadar –20 °C de saklanmıştır.

3.12.1. eNOS mRNA Düzeyi

İnsandaki eNOS ve β–aktin gen bölgelerine ait dizi analizi incelenerek amplifikasyon için uygun primer dizileri belirlenmiştir. eNOS ve β–aktin mRNA düzeyi, revers transkriptaz PCR yöntemi ile tayin edilmiştir. Bu amaçla öncelikle, vasküler endotel hücrelerinden elde edilen total RNA, revers transkriptaz ve random dokuzlu oligonükleotid (5' AGACTACAG) kullanılarak cDNA'ya dönüştürülmüştür. Daha sonra her bir reaksiyon için 10 μg cDNA kullanılarak, aşağıda dizinleri ve lokalizasyonları belirtilen özgül primerler ile (her birinin konsantrasyonu 0.2 μM olmak üzere) tablo 1' de belirtilen PCR koşulları kullanılarak çoğaltılmışlardır. Olası DNA kontaminasyonunu elimine etmek amacıyla, eNOS için forward ve reverse primerler, sırasıyla 4 ve 7. ekzonda bulunacak şekilde planlanmışlardır. Böylece, mRNA nın amplifikasyonuyla 519 bp'lik bir bölgenin çoğaltılması sağlanmıştır. Kontrol olarak insan aktin mRNA'sından elde edilen PCR ürünü kullanılmıştır. Tüm örnekler, bromofenol-formamid boyası (jel yürütme boyası) eklenerek %3' lük agaroz jele yüklenmiş ve 60 V da 1 saat süren elektroforez sonrası, jel etidyum bromür ile boyanarak Vilbert Lourmat (Vilber Lourmat Co., Paris, Fransa) görüntüleme cihazı ile analiz edilmiştir.

İnsan eNOS Primerleri	GenBank Acc.No	Lokalizasyon
Forward 5'-GGCCCACGAACAGCGGCTTC	AF519768	4916–4935
Reverse 5'-TGCTCCAGGGGCACCTCAAGG	AF519768	7220–7200
İnsan β-Aktin Primerleri		
Forward 5'- AGCACGGCATCGTCACCAACT	NM001101	287–308
Reverse 5'- TGGCTGGGGTGTTGAAGGTCT	NM001101	467–446

Tablo 1: PCR Koşulları

	Sıcaklık	Süre	Döngü Sayısı
Ön Denatürasyon	95°C	5 dakika	1x
Zincir ayrılması (Denaturation)	95°C	30 saniye	40x
Primer-Kalıp eşleşmesi (Annealing)	58°C	1 dakika	
Zincir Uzaması (Elongation)	72°C	1 dakika	
Son Basamak Uzaması	72°C	5 dakika	1x

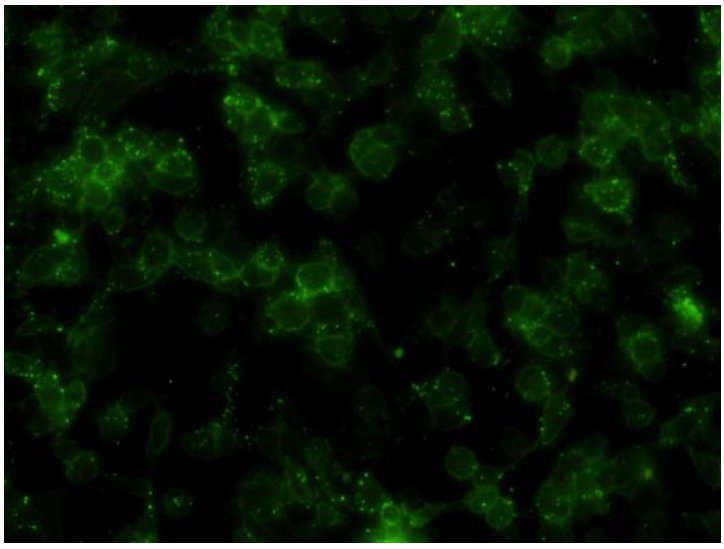
3.13. İstatistik

Değişkenler, median olarak ifade edilmiştir. Gruplar arasındaki farkların istatistiksel önemliliği için Kruskal-Wallis varyans analizi kullanılmıştır. Kruskal-Wallis sonucunda gruplar arasında istatistiksel olarak önemli fark tespit edilen değişkenler için ikili grup karşılaştırmaları amacıyla Dunn's Post-Hoc testi uygulanmıştır.

BULGULAR

4.1. Endotel Hücre Kimliğinin Tespiti

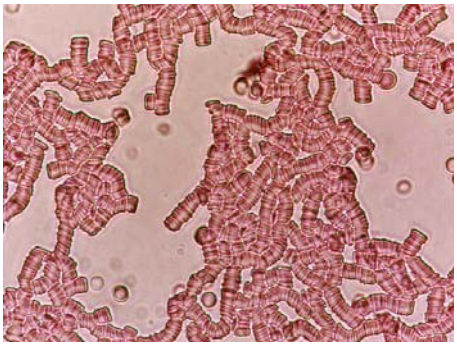
İnsan göbek kordonundan izole edilen venöz endotel hücrelerin yüzeyinde eksprese olan Von Willebrand faktörün (vWF) immunohistokimyasal görüntüleri şekil 4'de gösterilmiştir. İzole edilen hücrelerin endotel hücreye spesifik bir marker olan vWF ile görüntülenmesi endotel hücreleri olduklarına dair sağlam bir kanıt oluşturmuştur.



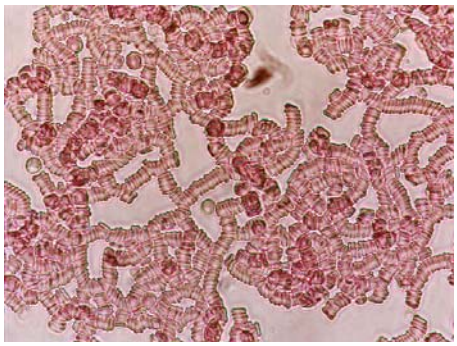
Şekil 4.1.: Endotel hücrelerindeki Von Willebrand faktörün immünohistokimyasal görüntüleri

4.2. Eritrosit Agregasyonu Değişiklikleri

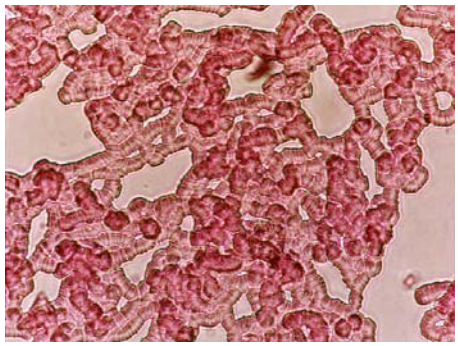
Kapiller cam tüpler içinde kültür edilmiş endotel hücrelerinin perfüzyonu sırasında kullanılan kan örneklerine ait morfolojik incelemeler şekil 4.2.1'de gösterilmiştir. Işık mikroskobu aracılığıyla elde edilen görüntülerde otolog plazma içerisinde hazırlanan Normal grubunda rulo formasyonu şeklinde eritrosit agregatları gözlenmiştir. Otolog plazmasında, hücre yüzey özelliklerinin değiştirilmesiyle eritrosit agregasyonu arttırılan *F98 grubunda* ve plazma kapsamı değiştirilerek eritrosit agregasyonu arttırılan *Dextran grubunda* ise eritrosit agregasyonu üç boyutlu bir şekle dönüşmüş ve büyük kitleler halinde kümelenme eğilimi göstermiştir (Şekil 4.2.1).



Normal grubu



F98 grubu

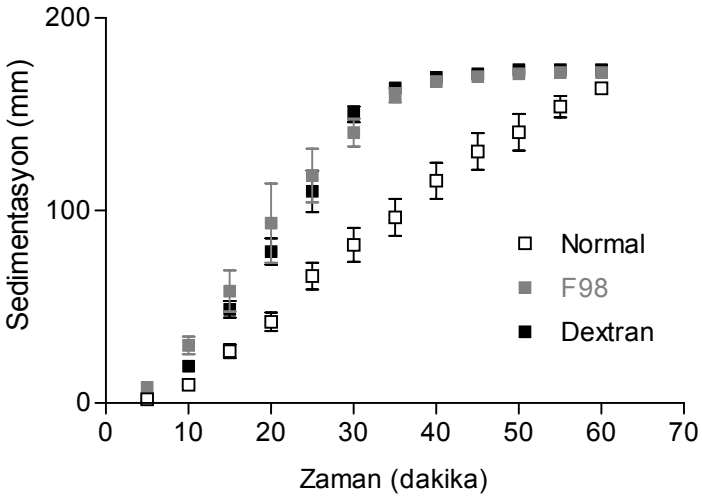


Dextran grubu

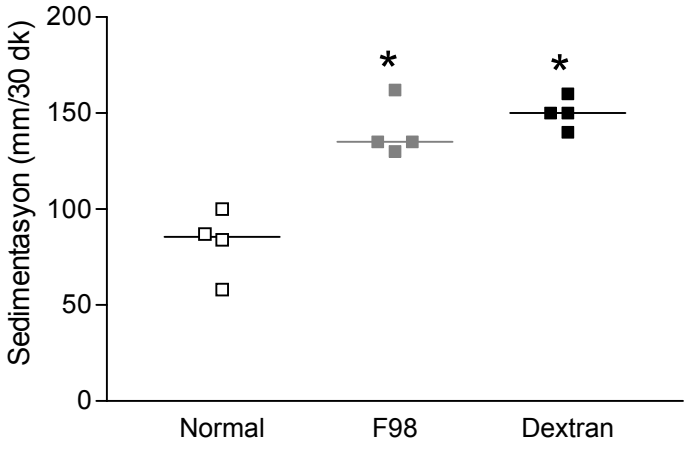
Şekil 4.2.1: *Normal, F98* ve *Dextran* gruplarında perfüzyon sırasında kullanılan kan örneklerinin fotomikroskobik görüntüleri.

Normal, F98 ve *Dextran* gruplarında perfüzyon için kullanılan eritrosit süspansiyonlarında eritrosit sedimentasyonunun 60 dakika içindeki seyri Şekil 4.2.2'da gösterilmiştir. Sedimentasyon tayini sırasında 60 dakika total süre boyunca her 5 dakikada bir değerler kaydedilmiştir. Üç boyutlu agregatların gözlendiği *F98* ve *Dextran* grubunda eritrosit sedimantasyonun zaman içindeki seyrinin *Normal* grubundan farklı olduğu gözlenmiştir. Eritrosit agregatlarının *F98* ve *Dextran* grubunda *Normal* grubuna göre daha hızlı bir şekilde çöktüğü ve bu farkın özellikle 30. dakikaya kadar belirgin olduğu dikkati çekmektedir (Şekil 4.2.2). *F98* ve *Dextran* grubunda sedimentasyon hızlarının 30. dakida *Normal* grubuna göre istatistiksel olarak önemli düzeyde arttığı bulunmuştur ($p<0.05$). Bütün gruplara ait son ölçüm noktası olan 60. dakikada sedimentasyon hızlarının benzer olduğu tespit edilmiştir.

A.



B.



Şekil 4.2.2.: *Normal, F98* ve *Dextran* gruplarında kullanılan eritrosit süspansiyonlarının sedimentasyon değerleri, A. 5 dakika aralıklarla sedimentasyon değerini gösterirken B. 30. dakikadaki sedimentasyon değerlerini göstermektedir (Her grup için yatay çizgi median değerini göstermektedir. n=4, *: Normal grubundan fark, $p<0.05$).

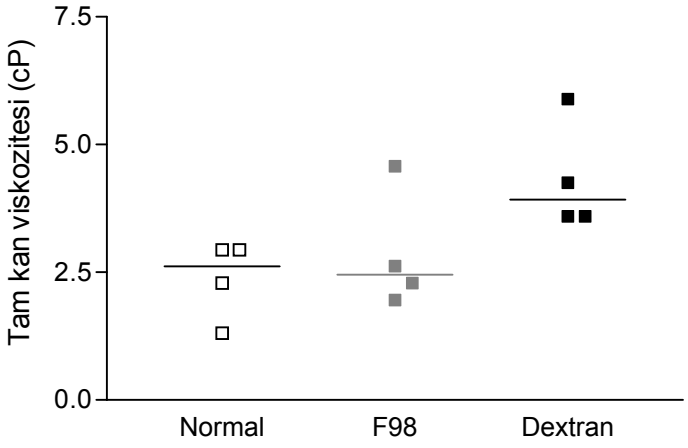
4.3. Plazma ve Eritrosit Süspansiyonu Viskoziteleri

Kapiller, cam boruların perfüzyonu sırasında kullanılan eritrosit süspansiyonlarının viskoziteleri 37,5 ve 1,500 sn^{-1} kayma hızlarında ölçülmüştür. Viskozite değerleri, 750 sn^{-1} kayma hızında *Dextran* grubunda,

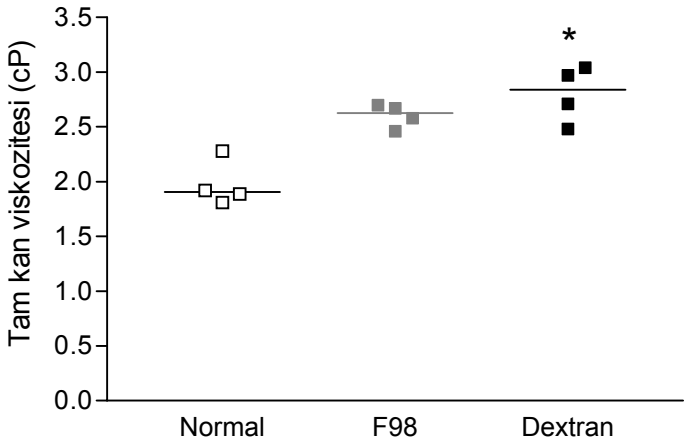
Normal grubuna göre istatistiksel olarak önemli düzeyde artmıştır ($p<0.05$). Tam kan viskozitesi aynı kayma hızında *F98* ve *Dextran* grupları arasında önemli düzeyde değişmemiştir. (Şekil 4.3.1).

Plazma viskozitesi *Dextran* grubunda *F98* ve *Normal* grubuna göre istatistiksel olarak önemli düzeyde yüksek ($p<0.01$) bulunmuştur (Şekil 4.3.2).

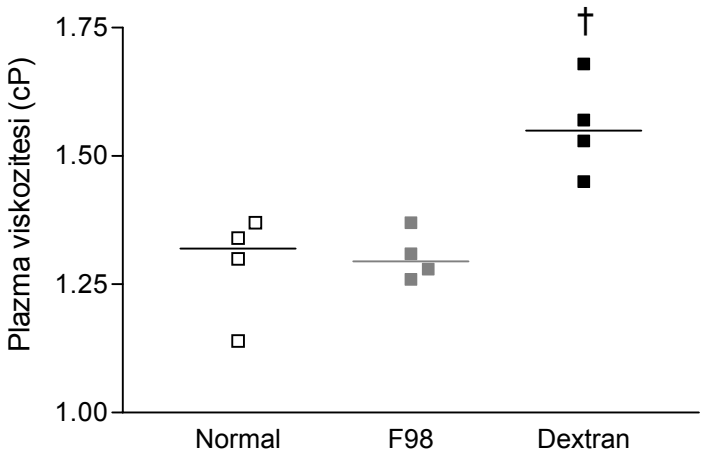
A.



B.



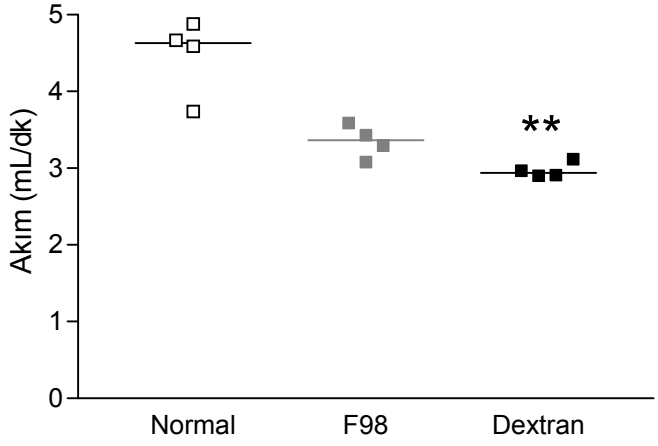
Şekil 4.3.1: *Normal, F98* ve *Dextran* gruplarında kullanılan eritrosit süspansiyonlarının A. 37 sn^{-1}, B. 750 sn^{-1} kayma hızlarında tam kan viskozite değerleri (Her grup için yatay çizgi median değerini göstermektedir. n=4, *: Normal grubundan fark, $p<0.05$).



Şekil 4.3.2.: *Normal, F98* ve *Dextran* gruplarında kullanılan eritrosit süspansiyonlarının 750 sn^{-1} kayma hızında plazma viskoziteleri 750 (Her grup için yatay çizgi median değerini göstermektedir.n=4, †: F98 grubundan fark, $p<0.05$).

4.4. Perfüzyon Sırasında Kaydedilen Akım Değerleri

Farklı eritrosit agregasyonu özelliklerine sahip kan örnekleriyle endotel hücrelerinin perfüzyonu sırasında uygulanan akım değerleri Şekil 4.4'de gösterilmiştir. Akım değerlerinin *Dextran* grubunda *Normal* grubuna kıyasla istatistiksel olarak önemli düzeyde düştüğü tespit edilmiştir. *F98* grubu ile *Normal* grubu ve *F98* ile *Dextran* grupları arasında uygulanan akım değerleri yönünden fark bulunamamıştır.



Şekil 4.4: *Normal, F98* ve *Dextran* gruplarında perfüzyon basıncını 40 mmHg değerinde tutmak için uygulanan akım değerleri (Her grup için yatay çizgi median değerini göstermektedir. n=4, **: Normal grubundan fark, $p<0.01$).

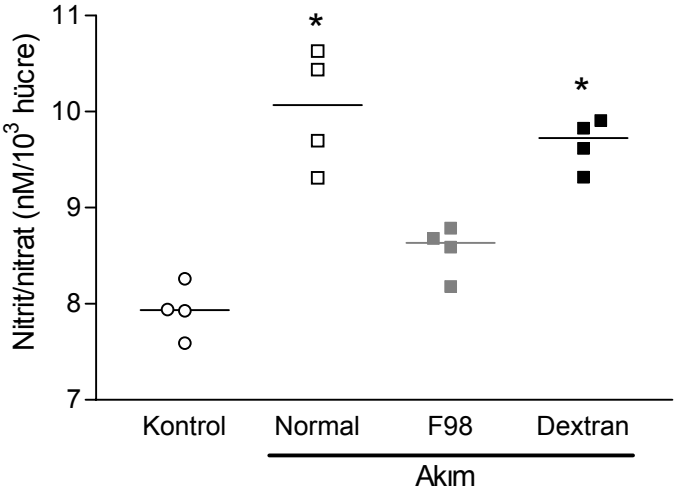
4.5. Hücre İçi Nitrit-Nitrat Değişimi

Materyal ve Metod bölümünde tanımlanan yöntemle duvar kayma kuvvetini 15 dyn/cm^2 değerinde sabit tutmak üzere hesaplanan akım değerleri ile farklı özellikteki eritrosit süspansiyonlarıyla perfüze edilen

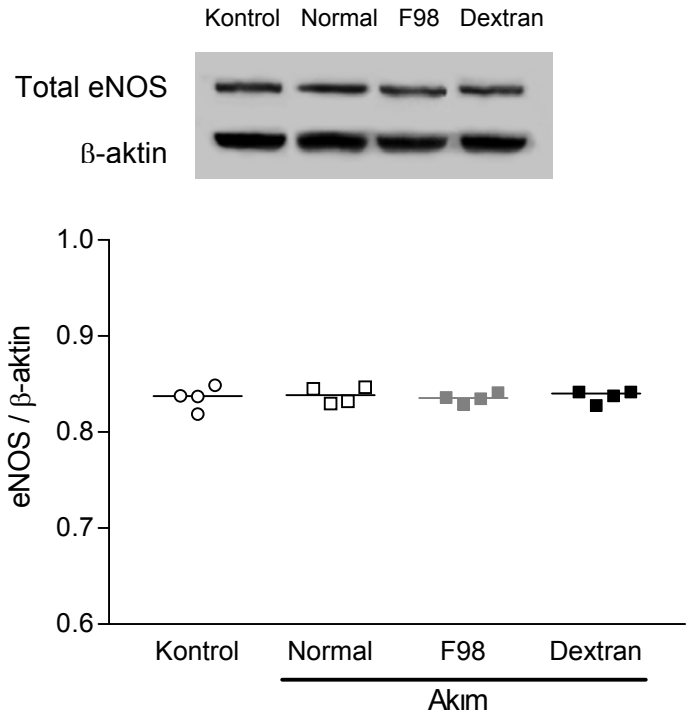
kapiller boruların iç yüzeyini düşeyen endotel hücrelerinde ölçülen nitrit-nitrat değerleri şekil 4.5.'de gösterilmiştir *Normal* grubunda, 30 dakika 15 dyn/cm^2 duvar kayma kuvveti etkisi altında bırakılan endotel hücrelerindeki nitrit/nitrat düzeylerinin, akım uygulanmayan *Kontrol* grubuna göre önemli ölçüde arttığı saptanmıştır ($p<0.001$). Aynı duvar kayma kuvvetini oluşturacağı hesaplanan akım hızıyla, eritrosit yüzey özellikleri değiştirilerek agregasyonu hızlandırılmış eritrosit süspansiyonu ile perfüze edilen kapillerlerdeki endotel hücrelerinde (*F98 grubu*) nitrat/nitrat düzeylerinde bu artış gözlenmemiştir. Diğer taraftan, F98 grubunda kullanılan eritrosit süspansiyonu ile aynı agregasyon özelliklerine sahip ancak plazma viskozitesi de yüksek olan *Dextran* grubunda ise *Normal* grubuna yakın nitrit/nitrat düzeyi saptanmıştır. *Normal* ve *Dextran* gruplarında nitrit/nitrat konsantrasyonu farklı bulunmamıştır.

4.6. eNOS Protein Ekspresyonları

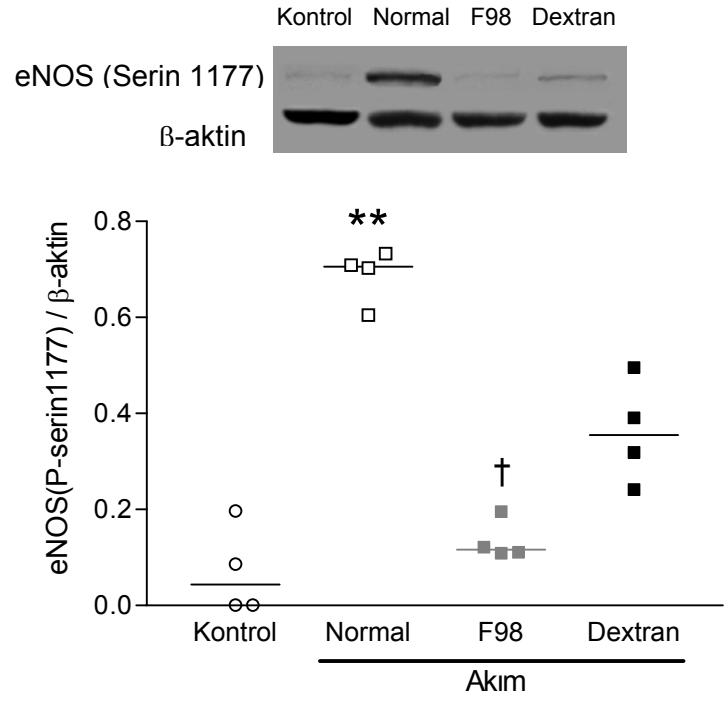
Kapiller cam tüplerin 30 dakika perfüzyonu sonrasında endotel hücrelerinin eNOS ekspresyonları şekil 4.6.1'de gösterilmiştir. Tüm gruplarda saptanan total eNOS protein ekspresyonlarının değişmediği bulunmuştur. Şekil 4.6.2 aynı hücrelerdeki serin1177 pozisyonunundan fosforile olan eNOS düzeylerini göstermektedir. Serin1177 fosforile eNOS düzeylerinin *Normal* grubunda *Kontrol* grubuna göre istatistiksel olarak önemli düzeyde arttığı bulunmuştur ($p<0.01$). *F98* grubuna ait serin1177 fosforile eNOS düzeyleri *Normal* grubuna göre azalmıştır ($p<0.05$). eNOS proteininin bir başka fosforilasyon bölgesi olan threonin 495 için elde edilen sonuçlar ise şekil 4.6.3'te gösterilmiştir. Bu sonuçlara göre eNOS threonin 495 fosforilasyonu tüm gruplarda önemli düzeyde değişmemiştir.



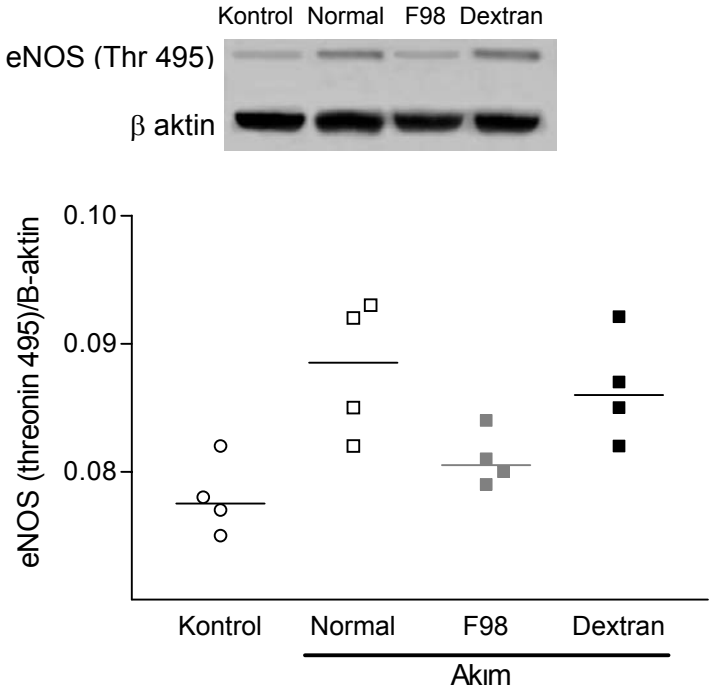
Şekil 4.5.: *Kontrol, Normal, F98* ve *Dextran* gruplarında kapillerlerin 30 dakika perfüzyonu sonrasında endotel hücrelerinin nitrit/nitrat konsantrasyonu (Her grup için yatay çizgi median değerini göstermektedir. n=4; *: Kontrol grubundan fark; $p<0.05$).



Şekil 4.6.1: *Kontrol, Normal, F98* ve *Dextran* gruplarında 30 dakika perfüzyon sonrası endotel hücrelerinde eNOS protein ekspresyonları (n=4).



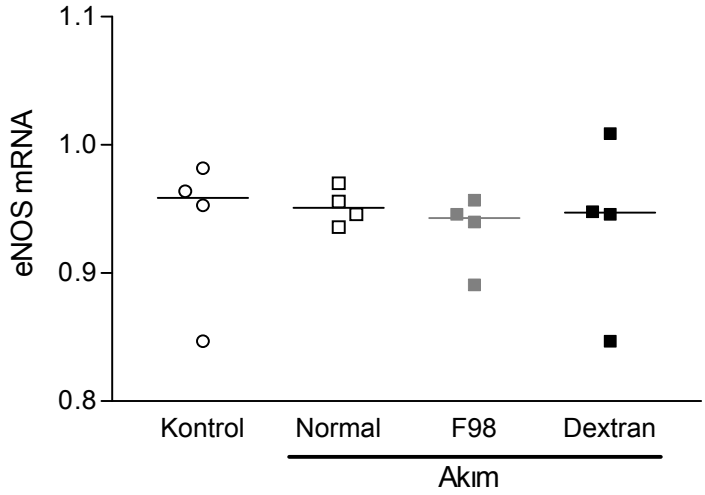
Şekil 4.6.2: *Kontrol, Normal, F98* ve *Dextran* gruplarında 30 dakika perfüzyon sonrası endotel hücrelerinde serin1777 fosforile eNOS protein düzeyleri (Her grup için yatay çizgi median değerini göstermektedir. n=4; **: Kontrol grubundan fark; p<0.01, †:Normal grubundan fark, p<0.05).



Şekil 4.6.3: *Kontrol, Normal, F98* ve *Dextran* gruplarında 30 dakika perfüzyon sonrası endotel hücrelerinde threonin 495 fosforile eNOS protein düzeyleri (Her grup için yatay çizgi median değerini göstermektedir. n=4).

4.7. eNOS mRNA Değişiklikleri

Endotel hücrelerinin 6 saat perfüzyonu sonrası eNOS mRNA düzeyleri şekil 4.7'de gösterilmiştir. Tüm gruplarda 6 saat 15 dyn/cm^2 duvar kayma kuvveti etkisi altında bırakılan endotel hücrelerindeki eNOS mRNA düzeylerinin benzer olduğu bulunmuştur.



Şekil 4.7: *Kontrol, Normal, F98* ve *Dextran* gruplarında 6 saat perfüzyon sonrası endotel hücrelerinde eNOS mRNA değikişiklikleri (Her grup için yatay çizgi median değerini göstermektedir. n=4).

TARTIŞMA

Bu çalışmanın bulguları, duvar kayma gerilimi değişikliklerinin endotel hücrelerini etkilediğini ve bu etkilerin de NO sentez mekanizmalarının incelenmesiyle saptanabileceğini göstermektedir. Duvar kayma gerilimi değişikliklerinin tahmin edilmesinde NO üretimi ve eNOS protein ekspresyonlarının birer prob olarak kullanılabileceği gösterilmiştir. Silindirik cam kapiller tüplerin içinde kültüre edilen endotel hücrelerinin farklı agregasyon özelliklerine sahip kan örnekleriyle perfüzyonu nitrik oksit üretimini, eNOS proteininin serin 1177 fosforilasyonunu etkilemektedir.

Vasküler sistemde duvar kayma gerilimi ve kan akımı endotel hücrelerinde NO sentezinin ana belirleyicilerindendir [17, 23-25, 219], küçük arterlerde kan akımının azalması ile akım aracılı dilatasyon cevaplarının ve eNOS ekspresyonun baskılanması bunun göstergesidir [212]. Eritrosit agregasyonu artışı damar duvarına yakın bölgede plazmadan zengin daha düşük viskoziteli bir bölgeye sahip kan damarlarında, eritrositlerin aksiyal birikmesini arttırma eğilimindedir [39]. Kan dağılımının her yerde aynı olmamasından dolayı azalmış duvar kayma gerilimi kan damarlarında NO ile ilgili mekanizmaları etkileyebileceği beklenebilir [3, 38, 39]. Bu iki önerme (kan akımının azalması ve agregasyonun etkisiyle damar duvarına yakın düşük viskoziteli bölge) birbirinden bağımsız değildir, hatta endotel hücrelerinde gözlenen mekanik kuvvetlerin azaltılmasında birlikte çalıştıkları söylenebilir [25, 42, 84].

Duvar kayma gerilimi değişiklikleri endotel hücresinde eNOS enzimi aracılı NO sentezini etkileyebilir. Kayma geriliminin endotel hücrelerinde eNOS aktivitesini arttırması yönündeki moleküler mekanizmalarla ilgili birkaç çalışma bulunmaktadır [17, 20, 33]. Duvar kayma geriliminin eNOS protein ekspresyonuna etkileri de daha önceki çalışmalarla gösterilmiştir [24, 218, 220]. Bununla birlikte, kayma geriliminin bu iki etkisi (NO üretimi ve eNOS protein ekspresyonu) zaman içinde değişiklik gösterebilir. eNOS aktivitesini gösteren NO üretimi hemodinamik değişimlerden hemen sonra görülürken eNOS protein ekspresyonunun değişimleri için daha uzun bir süreye ihtiyaç vardır [23, 213]. Örneğin, egzersiz sırasındaki vasküler adaptasyonlar üzerinde iskelet kasındaki NO üretimi ile ilişkili mekanizmalar hemen etkisini gösterirken [23, 213], düzenli fiziksel egzersizin eNOS protein ekspresyonlarının artışını uyarması için birkaç haftaya ihtiyaç vardır [214, 221, 222]. Kayma geriliminin NO üretimi üzerine bifazik etkili olduğu gösterilmiş; ilk 1 saate kadar NO üretiminin artış fazı, 4. saate kadar ekstra artışın gözlenmediği bir plato fazı ve 4.-8. saat arasında ise tekrar bir artış paterni izlediği bildirilmiştir [223]. İnsan göbek kordonu venöz endel hücrelerine farklı düzeyde kayma gerilimi (3, 6, 12, 25 dyne/cm^2) uygulanmış

ve farklı düzeylerdeki kayma gerilimin NO üretimine (nitrit-nitrat) etkileri incelenmiştir [217]. Kayma gerilimi uygulamasının başlamasıyla zaman içinde NO üretiminin arttığı bildirilmiştir. İlk yarım saatte 12 dyne/cm2 kayma geriliminde nitrit-nitrat miktarının 106 nmol/mg.saat iken 25 dyne/cm2 kayma geriliminde 113 nmol/mg.saat olduğu tespit edilmiştir [217]. Pulmoner arter kaynaklı endotel hücrelerinde yapılan çalışmada kayma geriliminin ilk yarım saat içinde nitrit-nitrat üretimini önemli düzeyde arttırdığı, saatlerce devam eden kayma gerilimi sırasında ise nitrit-nitrat üretimi artışının yükselerek devam ettiği bildirilmiştir [223]. Bizim çalışmamızda da 30 dakika kayma gerilimi uygulamasından sonra NO sentez mekanizmalarının değiştiği gösterilmiştir (şekil 4.5.1). Normal grubunda nitrit-nitrat düzeyinin kontrol grubuna göre istatistiksel olarak önemli düzeyde arttığı bulunmuştur. Kayma geriliminin etkisiyle 30 dakika perfüzyondan sonra Normal grubunda (otolog plazmalarında normal eritrositlerin agregasyonu) saptanan nitrit-nitrat artışı yukarıda bahsedilen çalışmalarla benzer sonuçları işaret etmektedir. Yüzeyleri Pluronic F98 ile kaplanarak agregabiliteleri arttırılmış eritrositlerin otolog plazma içinde süspanse edildiği F98 grubunda ise, nitrit-nitrat miktarının önemli düzeyde değişmediği bulunmuştur. Plazma kapsamı değiştirilerek eritrosit agregasyonu arttırılan Dextran 500 grubunda, nitrit-nitrat miktarının kontrol grubuna göre istatistiksel olarak önemli düzeyde arttığı gösterilmiştir. Dextran grubunda tespit edilen nitrit-nitrat artışının Normal grubundan daha düşük olduğu fakat iki grup arasındaki farkın istatistiksel olarak önemli olmadığı bulunmuştur. Bu sonuçlara göre; eritrosit agregasyonu artmış gruplarda NO sentez mekanizmalarının baskılandığı, ancak plazma viskozitesi de artmış ise bunun bir ölçüde önlenebildiği söylenebilir. Başkurt ve ark. yaptıkları çalışma da bu düşünceyi destekler [97]. Çalışmada, duvar kayma gerilimi değişikliklerinin endotel hücrelerini etkileyebildiği ve bu etkilerin de NO sentezleyen mekanizmaları etkileyebileceği gösterilmiştir [97]. Poloxamer-kaplı eritrosit süspansiyonlarının izovolemik değişmeli transfüzyon ile eritrosit agregasyonları kronik olarak arttırılan sıçanlarda, eritrosit agregasyonu artışı ile NO sentez mekanizmaların baskılandığı ve kan basıncı artışına bağlı olarak vazomotor tonusun değiştiği bulunmuştur. Ortalama arteryel basınç, non-invazif bir metod kullanılarak transfüzyon sonrası günlük olarak ölçülmüş, basıncın sadece agregasyonu arttırılmış grupta kademeli olarak arttığı bulunmuştur. Eritrosit agregasyonunun arttırıldığı sıçanlara ait grasilis kasından elde edilen arteriyol segmentlerinin NO bağımlı relaksasyon cevaplarının da değiştirilebildiği gösterilmiştir. Akım aracılı dilatasyon cevaplarının NO'ya bağımlı olduğu bilinmektedir. Agregasyonu arttırılmış grupta yüksek akım hızlarında maksimum dilatasyon cevabı gözlenmiş ve maksimum dilatasyon yüzdesinin sadece kontrol grubunun %50'si kadar olduğu saptanmıştır. Bu veriler, asetilkolinle indüklenmiş dilatasyon cevaplarının baskılandığını gösteren sonuçlarla desteklenmektedir, NO aracılı mekanizma eritrosit agregasyonu arttırılmış sıçanlarda önemli düzeyde baskılanmıştır [97]. Bu sonuç grasilis kas örneklerinden alınmış dokularda azalmış eNOS ekspresyonunu gösterilmesiyle doğrulanmaktadır [97]. Burada incelenen mekanizmalar büyük olasılıkla azalan kan akımı ve/veya artan eritrosit aksiyal birikimi ile ortaya çıkan azalmış duvar kayma

gerilimi ile ilgilidir; eritrosit agregasyonu artışı, değişmiş kan basıncı ve artmış vasküler direnç sonucu NO-aracılı vasküler kontrol mekanizmalarını downregüle eder ve diğer bir deyişle hemoreolojik parametreler vazomotor kontrol mekanizmalarını etkiler.

Bu çalışmada, eritrositlerin Pluronic F98 ile kaplanması sırasında kullanılan 0.025 mg/mL dozu ile Dextran grubunda kullanılan %0.5 konsantrasyonunun eritrosit agregasyonunu arttırma özelliklerinin aynı düzeyde olmasına dikkat edilmiştir. Bahsedilen iki grupta kullanılan konsantrasyonlar Başkurt ve ark.'larının çalışmasındaki veriler doğrultusunda seçilmiştir [40]. Eritrosit agregasyonu değişikliklerinin göstergesi olan sedimentasyon hızları incelendiği zaman; F98 grubunda ve Dextran 500 grubunda Normal grubuna göre önemli düzeyde arttığı bulunmuştur. Sedimentasyonun artması eritrosit agregasyonu artışını göstermektedir. Eritrosit agregasyonunun fotomikroskobik görüntülerinde de agregasyonun F98 ve Dextran grubunda arttırıldığını desteklemektedir Eritrosit agregasyon özelliklerini gösteren ölçümler sonucu eritrosit agregasyonunun F98 ve Dextran grubunda önemli düzeyde arttırıldığı ifade edilebilir. Kan örneklerinde perfüzyon öncesi tam kan viskozitesi ölçümleri ve plazma viskozitesi ölçümlerinin sonuçları da planlanan eritrosit agregasyonu artışını doğrulayan sonuçlar göstermiştir. Plazma viskozitesi ölçümleri sonucu, eritrositlerin Pluronic F98 ile kaplanması sırasında plazma kapsamının değiştirilmeden agregasyon artışı sağlanabildiğini göstermiştir. Eritrosit agregasyonu plazma kapsamı %0.5 dextran ile değiştirilen kan örneklerinin plazma viskozitesi Normal grubuna ve F98 grubuna göre önemli düzeyde artmıştır. Eritrosit agregasyonu arttırılmış kan örneklerine sahip Dextran grubunda tam kan viskozitesinin Normal grubuna göre istatistiksel olarak önemli düzeyde arttığı bulunmuştur. Agregasyonu arttırılmış F98 grubu ve Dextran grubu arasındaki nitrit-nitrat üretimindeki farklar hipotezimizi açıklayabilir. Eritrosit agregasyonu artışı duvar kayma gerilimini etkileyerek endotel hücrelerde NO sentezleyen mekanizmaları etkilemiştir. Bu etkisini de özellikle damar duvarına komşu bölgede kan bileşimini değiştirerek göstermekte; aksiyal migrasyondan dolayı yerel hematokrit azalması ve buna bağlı olarak kan viskozitesinin düşmesi damar duvarına etki eden kayma kuvvetlerinin azalmasına neden olmaktadır. Bu etkinin plazma akışkanlığında önemli bir değişikliğin olmadığı, hücresel faktörlerdeki değişimlere bağlı eritrosit agregasyonu artışı yapılan F98 grubunda önemli olduğu gösterilmiştir. Eritrosit agregasyonu ile birlikte plazma viskozitesinin de değişmesi, agregasyonla ilişkili olarak ortaya çıkacak olan bu etkiyi önleyebilir.

Bu çalışmada, eritrosit agregasyonunu arttırmak için iki farklı yöntem kullanılmıştır: Birinci yöntemle; son birkaç yıldır kullanılmaya başlanan yeni bir teknikle eritrosit hücresel özellikleri değiştirilmekte ve süspansiyon ortamında herhangi bir değişiklik yapılmaksızın eritrosit agregasyonu modifiye edilebilmektedir [98]. Bu yöntemle, eritrosit yüzeyine kovalan olarak bağlanabilen, uçlarında özel reaktif gruplara sahip polietilenglikol esaslı kopolimerler (13 kDa, Poloxamer) kullanılarak, eritrosit yüzey özellikleri

değiştirilmektedir. İkinci yöntemde ise eritrosit agregasyonu artışı yüksek molekül ağırlıklı ve fibriler yapıdaki biyomolekül olan dextran 500 ile plazma kapsamı değiştirilerek gerçekleştirilmiştir. Eritrosit agregasyonunu arttırmak için kullanılan poloxamerle kaplama yöntemi, plazmaya yabancı polimerlerin eklenmemesi özelliği bakımından benzersizdir. Eritrositler otolog plazmaları içerisinde süspanse edilirler. Eritrosit agregasyonunun hemodinamik etkilerini değerlendiren önceki çalışmalarda eritrosit agregasyonunu arttırabilmek için 500 kDa dextran gibi yüksek molekül ağırlıklı polimerler kullanılmıştır [40, 42, 49, 95, 224, 225]. Bu büyük polimerlerin eritrosit agregasyonunu düzenlemede etkin oldukları kanıtlanmış olsa da bazı potansiyel dezavantajları vardır: 1. Plazma viskozitesi artar (örn. 1.5 g/dl'lik Dextran 500 ortam viskozitesini %80 oranında arttırmaktadır [225] ve dolayısıyla vasküler duvara yakın sıvı viskozitesi de artmış olur. 2. Plazma proteinleri polimer eklenmesi ile dilue edilir. 3. Bazı deney hayvanlarında (örn. sıçan) dextran ve benzeri polimerler anafilaktik reaksiyonlara yol açarak patofizyolojik durumu uyarırlar [84]. 4. Çözelti içerisindeki makromoleküllerin neden olduğu artmış kolloid osmotik basınç intra-ekstraselüler sıvı hacimlerini değiştirebilir.

Aksiyal migrasyondan en çok etkilenen damar sistemi olan mikrodolaşımdaki damarların çapı ve perfüzyon basıncı dikkate alınarak, cam kapiller çapı 1 mm olarak belirlenmiş ve sistem basıncı 40 mmHg'ya ayarlanmıştır [226]. Endotel hücrelerine 3 farklı özellikteki kan süspansiyonlarının akımıyla uygulanan ve 15 dyn/cm^2 düzeyinde olacağı hesaplanan duvar kayma kuvveti uygulanmıştır. Hesaplanan duvar kayma kuvvetinin düzeyi literatürde verilen ortalama duvar kayma gerilimi değeri gözönünde bulundurularak seçilmiştir [3].

Bizim çalışmamızda farklı tam kan viskozitelerine sahip kan örneklerinin perfüzyonu sırasında uygulanan akımların değiştiği bulunmuştur. Dextran grubunda uygulanan akımın Normal grubuna göre önemli düzeyde düşük olduğu gösterilmiştir (Şekil 4.4). Agregasyonu arttırılmış grupları oluşturan F98 ve Dextran grubu için uygulanan akım değerleri arasında istatistiksel düzeyde önemli fark tespit edilememiştir. Dextran grubunda eritrosit agregasyonunun artması ve dolayısıyla hemodinamik direncin artması akımdaki azalmayı açıklayabilir. Kan örneklerinde eritrosit agregasyonu artışı duvar kayma gerilimini etkileyerek akımın değişmesini sağlayabildiği düşünülmektedir.

eNOS fosforilasyonu, eNOS aktivitesi için kritik regülatör mekanizmadır. eNOS enziminin serin, threonin ve tirozin rezidülerinden fosforillendiği gösterilmiştir [17, 196, 198, 227]. eNOS serin 1177 fosforilasyonu enzim aktivitesinin artmasına neden olur [137, 203]. eNOS threonin 495 fosforilasyonu ise enzim inaktivasyonunu gösterir [191]. Koyun plasenta arterlerinden izole edilen endotel hücrelerinde 15 dyne/cm2 kayma gerilimi uygulamasının ardından eNOS serin 1177 fosforilasyonunun 20. dakikada arttığı, bununla birlikte total eNOS protein ekspresyonunun ve eNOS threonin 495 fosforilasyon düzeyinin ise ilk yarım saat içinde değişmediği tespit edilmiştir [228]. Fetal pulmoner arter kaynaklı endotel hücrelerinde 20

dyne/cm^2 kayma gerilimine cevaben 2., 4. ve 8. saatlerde eNOS protein ekspresyonları ölçülmüştür [223]. eNOS protein ekspresyonlarının 2. saatte değişmemesine rağmen 4. saatte eNOS protein ekspresyon düzeyinin önemli düzeyde arttığı bulunmuştur. Boo ve arkadaşlarına ait çalışmada, kayma geriliminin protein kinaz A ve fosfoinozitid-3-kinaz aracılığıyla eNOS enziminde serin 1179 (insan S1177) fosforilasyonunu yaparak NO üretimini arttırdığı gösterilmiştir [207]. Bu bölümde eNOS fosforilasyon bölgeleri için verilen aminoasit numaraları sığır sekansı temel alınarak ifade edilmiştir Ayrıca çalışmada diğer potansiyel fosforilasyon alanlarının kayma geriliminden nasıl etkilendiği ve bu değişikliklerin 1 saat süre boyunca nasıl değiştiği belirlenmiştir. Sığır kaynaklı aortik endotel hücrelerine 15 dyne/cm^2 kayma gerilimi uygulanmış ve bu hücrelerde 2.-5.-15.-30.-60. dakikalarda total eNOS proteini ekspresyonu, serin 1179 fosforilasyonu ve threonin 497 (insan T495) fosforilasyonu incelenmiştir [207]. Total eNOS protein ekspresyonlarının tüm zaman dilimlerinde önemli düzeyde değişmediği bulunmuştur. Kayma geriliminin etkisiyle eNOS serin 1179 fosforilasyonu 2. dakikada artmaya başlamış, 30. dakikada maksimum fosforilasyon gözlenmiş ve 1 saat sonunda maksimum fosforilasyon düzeyi değişmemiştir. eNOS threonin 495 fosforilasyonu kayma geriliminden belirtilen zaman aralıklarında önemli düzeyde değişmediği bulunmuştur. Bu çalışmada eNOS protein ve fosforilasyon düzeyleri için kayma geriliminin uygulanma süresi olarak maksimum fosforilasyonun gözlendiği 30. dakika tercih edilmiştir. Kapiller tüplerin 30 dakika perfüzyonundan sonra total eNOS protein ekspresyonları 4 grupta değişmemiştir (Şekil 4.6.). Total eNOS protein düzeylerinin 30 dakika perfüzyon sonunda aynı düzeyde kalması yukarda bahsedilen çalışmaların sonuçlarıyla uyumludur [207, 223, 228]. eNOS serin 1177 fosforilasyonun, Normal grubunda önemli düzeyde arttığı gösterilmiştir. Normal grubunda eNOS serin 1177 fosforilasyonunun kontrol grubuna göre 7 kat arttığı bulunmuştur. eNOS enziminin serin fosforilasyonu F98 grubunda normal grubuna göre önemli düzeyde azaldığı saptanmıştır. Dextran grubunda ise eNOS serin 1177 fosforilasyonunun F98 grubuna ve Kontrol grubuna göre daha yüksek olduğu fakat aradaki farkın istatistiksel olarak önemli olmadığı tespit edilmiştir. eNOS serin 1177 fosforilasyonu enzim aktivitesi artışını gösterirken nitrit-nitrat üretiminin de aktiviteyi yansıtması iki parametrenin beraber değerlendirilmesi gerekliliğini ortaya çıkarır. Dextran grubunda tespit edilen nitrit-nitrat üretimi artışları enzim aktivitesinin arttığını göstermektedir, bu sonuçlar enzim fosforilasyonu sonuçlarıyla benzerlik göstermektedir. Agregasyonu arttırılmış iki grup arasında enzim aktivitesi bakımından iki farklı yanıt elde edilmiştir. Bunun 2 nedeni olabilir: 1. Dextran grubuna ait kan örnekleriyle perfüzyon sırasında kapiller duvarına yakın bölgede düşük hematokritli yüksek viskoziteli sıvının bulunduğu ve bu sıvının eNOS aktivitesi üzerinde aksiyal migrasyon artışı nedeniyle ortaya çıkacak azalmayı önleyici bir etkisi olabilir. Dextran grubunda eNOS serin 1177 fosforilasyonu ve nitrit-nitrat düzeylerinin F98 grubuna göre yüksek olması bu fikri desteklemektedir. 2. Eritrosit agregasyon eğiliminin artışı aksiyal migrasyonun artmasını sağlar, damar duvarına etki eden kuvvetin büyüklüğünün azalmasına neden olur. Duvar kayma kuvvetinin azalması eNOS fosforilasyonun F98 grubunda artmamasına neden olabilir.

eNOS threonin 495 fosforilasyonu tüm gruplarda önemli düzeyde değişmemiştir. Threonin 495 fosforilasyonunun tüm gruplarda değişmemesi Boo ve arkadaşlarının çalışmasıyla uyumludur [207].

Kayma gerilimine cevaben eNOS mRNA düzeylerinin nasıl etkilendiği Davis ve arkadaşları tarafından gösterilmiştir [218]. Sığır aortik endotel hücrelerine 15 dyne/cm^2 düzeyde kayma gerilimi 6 saat uygulandığı zaman statik koşullara göre eNOS mRNA miktarının 4-5 kat arttığı bulunmuş ve bu artış tirozin kinaz c-Src'ye bağlı yolak aracılığıyla meydana gelmiştir. Davis ME ve ark. çalışmasında eNOS mRNA yarı ömrünün (48 saat) 6 saat süreyle 15 dyne/cm^2 kayma gerilimi uygulaması sonucu 5 saat daha uzatılabildiği gösterilmiştir [218]. Tirozin kinazlardan c-Src'nin kayma gerilimine cevaben 2 ayrı yolla eNOS ekspresyonunu arttırdığını ileri sürmüşlerdir; 1. Kısa sürede eNOS transkripsiyonunda artışa yol açar 2. eNOS mRNA stabilizasyonunun uzatılmasına neden olur. Kayma gerilimine cevaben eNOS mRNA stabilitesinin uzatılması eNOS'un devamlı ekspresyonuna izin veren kritik bir olaydır. Xiao Z ve arkadaşları, sığır aortik endotel hücrelerinde arteryel düzeyde (25 dyne/cm^2) 6 saat kayma geriliminin uygulanması ile eNOS gen ekspresyonunu arttırdığını bulmuşlardır [229]. Bu artışın da akımsız gruba göre 7.95 kat olduğunu tespit etmişlerdir.

. Bizim çalışmamızda kullanılan 6 saatlik perfüzyon süresi yukarıda bahsedilen çalışmaların verileri doğrultusunda belirlenmiştir. Perfüzyondan sonra gruplar arasında reverse transkriptaz PCR yöntemiyle 40 döngüden oluşan PCR şartlarında oluşan ürünlerin birbirinden farklı olmadığı bulunmuştur.

Bu çalışmanın sonuçlarına göre; eritrosit agregasyonu modifikasyonunun yerel kan bileşimini değiştirerek duvar kayma kuvvetini etkilediğini, bu etkinin, özellikle plazma akışkanlığında önemli bir değişikliğin olmadığı, hücresel faktörlerdeki değişimlere bağlı eritrosit agregasyonu modifikasyonlarında önemli duruma geldiği gösterilmiştir. Eritrosit agregasyonunun arttırılması yanında plazma viskozitesinin değiştirilmesinin de bu etkide rol oynayabileceği bulunmuştur. NO sentez mekanizmalarının, eritrosit agregasyonu değişiklikleriyle ortaya çıkan duvar kayma kuvveti değişikliklerini saptamak amacıyla kullanılabileceğini gösterilmiştir.

SONUÇLAR

1- Işık mikroskobu aracılığıyla elde edilen görüntülerde otolog plazma içerisinde hazırlanan Normal grubunda rulo formasyonu şeklinde eritrosit agregatları gözlenmiştir. Otolog plazmasında, hücre yüzey özelliklerinin değiştirilmesiyle eritrosit agregasyonu arttırılan *F98 grubunda* ve plazma kapsamı değiştirilerek eritrosit agregasyonu arttırılan *Dextran grubunda* ise eritrosit agregasyonu üç boyutlu bir şekle dönüşmüş ve büyük kitleler halinde kümlenme eğilimi göstermiştir.

2- Üç boyutlu agregatların gözlendiği *F98* ve *Dextran* grubunda eritrosit sedimantasyonun zaman içindeki seyrinin *Normal* grubundan farklı olduğu gözlenmiştir. Eritrosit agregatlarının *F98* ve *Dextran* grubunda *Normal* grubuna göre daha hızlı bir şekilde çöktüğü ve bu farkın özellikle 30. dakikaya kadar belirgin olduğu dikkati çekmektedir (Şekil 4.2.2). *F98* ve *Dextran* grubunda sedimentasyon hızlarının 30. dakida *Normal* grubuna göre istatistiksel olarak önemli düzeyde arttığı bulunmuştur ($p<0.05$). Bütün gruplara ait son ölçüm noktası olan 60. dakikada sedimentasyon hızlarının benzer olduğu tespit edilmiştir.

3- Viskozite değerleri, 750 s^{-1} kayma hızında *Dextran* grubunda, *Normal* grubuna göre istatistiksel olarak önemli düzeyde artmıştır.

4- Uygulanan akım değerlerinin *Dextran* grubunda *Normal* grubuna kıyasla düşük olduğu tespit edilmiştir. *F98* ile *Dextran* grupları ve *Normal* ile *Dextran* grupları arasında akım değerlerinin istatistiksel olarak önemli düzeyde farklı olmadığı bulunmuştur.

5- *Normal* grubunda, 30 dakika 15 dyn/cm^2 duvar kayma kuvveti etkisi altında bırakılan endotel hücrelerindeki nitrit/nitrat düzeylerinin, akım uygulanmayan *Kontrol* grubuna göre önemli ölçüde arttığı saptanmıştır. Aynı duvar kayma kuvvetini oluşturacağı hesaplanan akım hızıyla, eritrosit yüzey özellikleri değiştirilerek agregasyonu hızlandırılmış eritrosit süspansiyonu ile perfüze edilen kapillerlerdeki endotel hücrelerinde (*F98 grubu*) nitrat/nitrat düzeylerinde bu artış gözlenmemiştir. Diğer taraftan, F98 grubunda kullanılan eritrosit süspansiyonu ile aynı agregasyon özelliklerine sahip ancak plazma viskozitesi de yüksek olan *Dextran* grubunda ise *Normal* grubuna yakın nitrit/nitrat düzeyi saptanmıştır. *Normal* ve *Dextran* gruplarında nitrit/nitrat konsantrasyonu farklı bulunmamıştır.

6- Tüm gruplarda saptanan total eNOS protein ekspresyonlarının değişmediği bulunmuştur.

7- Serin1177 fosforile eNOS düzeylerinin *Normal* grubunda *Kontrol* grubuna göre istatistiksel olarak önemli düzeyde arttığı bulunmuştur ($p<0.01$). *F98* grubuna ait serin1177 fosforile eNOS düzeyleri *Normal* grubuna göre azalmıştır ($p<0.05$). eNOS proteininin bir başka fosforilasyon bölgesi olan threonin 495 için elde edilen sonuçlar ise şekil 4.6.3'te gösterilmiştir. Bu sonuçlara göre eNOS threonin 495 fosforilasyonu tüm gruplarda önemli düzeyde değişmemiştir.

8- Endotel hücrelerinin 6 saat perfüzyonu sonrası eNOS mRNA düzeylerinin tüm gruplarda aynı olduğu bulunmuştur.

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ÖZGEÇMİŞ

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Effects of red blood cell aggregation on myocardial hematocrit gradient using two approaches to increase aggregation

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Yalcin, Ozlem, Funda Aydin, Pinar Ulker, Mehmet Uyuklu, Firat Gungor, Jonathan K. Armstrong, Herbert J. Meiselman, and Oguz K. Baskurt. Effects of red blood cell aggregation on myocardial hematocrit gradient using two approaches to increase aggregation. *Am J Physiol Heart Circ Physiol* 290: H765–H771, 2006. First published September 19, 2005; doi:10.1152/ajpheart.00756.2005.—The normal transmyocardial tissue hematocrit distribution (i.e., subepicardial greater than subendocardial) is known to be affected by red blood cell (RBC) aggregation. Prior studies employing the use of infused large macromolecules to increase erythrocyte aggregation are complicated by both increased plasma viscosity and dilution of plasma. Using a new technique to specifically alter the aggregation behavior by covalent attachment of Pluronic F-98 to the surface of the RBC, we have determined the effects of only enhanced aggregation (i.e., Pluronic F-98-coated RBCs) versus enhanced aggregation with increased plasma viscosity (i.e., an addition of 500 kDa dextran) on myocardial tissue hematocrit in rapidly frozen guinea pig hearts. Although both approaches equally increased aggregation, tissue hematocrit profiles differed markedly: *1*) when Pluronic F-98-coated cells were used, the normal transmyocardial gradient was abolished, and *2*) when dextran was added, the hematocrit remained at subepicardial levels for about one-half the thickness of the myocardium and then rapidly decreased to the control level in the subendocardial layer. Our results indicate that myocardial hematocrit profiles are sensitive to both RBC aggregation and to changes of plasma viscosity associated with increased RBC aggregation. Furthermore, they suggest the need for additional studies to explore the mechanisms affecting RBC distribution in three-dimensional vascular beds.

myocardium; plasma viscosity; Pluronic F-98; dextran

THE DIFFERENCE BETWEEN large blood vessels and microvascular hematocrit values was first described by Fahraeus (12) and later confirmed by other experimental studies (17, 18, 21, 33). The mean hematocrit value for blood in the vasculature of a given tissue, usually referred to as tissue hematocrit, tends to be significantly lower than the hematocrit value in arterial or venous blood (28). Therefore, the venous hematocrit value that is routinely measured for clinical purposes is actually an incorrect estimate of the mean hematocrit value of the total circulating blood volume. Furthermore, the reduced tissue hematocrit contributes to the reduced apparent viscosity of blood under in vivo flow conditions (34) and affects oxygen transfer to the tissues being perfused (17).

The lower mean hematocrit value for a given tissue generally reflects the significant reduction of hematocrit as the blood approaches the microcirculation. The mechanisms that underlie this reduction are multiple and interrelated and are related to the nonuniform distribution of red blood cells (RBCs) across the vessel diameter. This nonuniform distribution is due to two phenomena. First, because RBCs have a finite size, their local concentration near the wall is reduced, i.e., a wall exclusion effect, resulting in a statistical cell-poor layer having a width of about one-half of the cell diameter or less. Second, during flow, there is a tendency for RBCs to accumulate in the central region of vessels, a process known as axial migration (19, 28). The nonuniform distribution of cells leads to two important phenomena: plasma skimming and the Fahraeus effect (19, 28). Plasma skimming, i.e., a greater plasma flow in the branches, is due to the feeding of side branches from the cell-poor plasma layer adjacent to the vessel wall. The Fahraeus effect is the lowered hematocrit for blood within a given vessel segment compared with the hematocrit of blood that is discharged from that vessel segment and is due to the higher flow velocity of RBCs in the central flow zone relative to the plasma-rich fluid in the marginal zone (19, 28). In addition to these mechanisms, there is flow partitioning at vascular bifurcations, resulting in a preferential accumulation of RBCs in the branch with the higher flow rate. This phenomenon, termed "network Fahraeus effect," is effective in generating a Fahraeus effect at the circulatory network level rather than for individual vessels (16, 25).

The factors that determine the difference between the mean hematocrit value for a given tissue and systemic hematocrit are not well documented. Axial migration of RBCs during flow can be affected by various factors (10, 19), including the rheological properties of the cells and RBC aggregation. Fahraeus (12) was the first to show that greater aggregation enhanced RBC axial migration in experiments using horse blood versus human blood. Employing vertical glass tubes, Cokelet and Goldsmith (10) demonstrated that enhanced RBC aggregation may lead to a thicker cell-poor layer at the wall. These findings suggest that the reduction of tissue hematocrit might be dependent on the degree of RBC aggregation. Detailed studies of tissue hematocrit in myocardium have demonstrated spatial differences and the effects of cell rigidity and aggregation. First, Vicaut and Levy (33) demonstrated that tissue hematocrit values differ between subepicardial and subendocardial layers of the left ventricular myocardium and that there is a linear gradient between the two layers in hearts arrested during diastole by rapid freezing. Second, enhancing RBC

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aggregation by infusion of fibrinogen results in altered tissue hematocrit values in rat myocardium (2). Finally, reduced RBC deformability alters the tissue hematocrit gradient that exists in normal rat myocardium (3). Although variation of blood vessel morphometric parameters within the myocardium could lead to the observed gradients, differences in blood vessel sizes required to explain the gradients do not exist (33). Rather, it has been suggested that variations of discharge hematocrit at different myocardial layers are responsible for the gradient (33).

Although the effects of RBC aggregation on myocardial hematocrit gradient have been reported, increases of aggregation were achieved by adding fibrinogen to the plasma used to suspend the RBCs (2). The addition of fibrinogen alters the physicochemical characteristics of the plasma and increases plasma viscosity. Such changes may interfere with vascular control mechanisms, vessel size, and the distribution of RBCs. However, a new technique employing covalent attachment of Pluronic F-98 to the RBC surface has been developed, which allows the enhancing of RBC aggregation without the need for the infusion of a macromolecular solution and, hence, without an alteration of the suspending phase properties, e.g., plasma protein concentration and plasma viscosity. The present study was thus designed to compare the effects on myocardial hematocrit gradient of enhanced RBC aggregation, as induced either by the addition of a high-molecular-weight polymer to plasma or by Pluronic F-98 attachment to the RBC surface.

MATERIALS AND METHODS

Animals and groups. Adult guinea pigs of both sexes weighing 400–500 g were used in the experiments. The animals were randomly divided into control, *aggregation I*, and *aggregation II* groups. Another group of animals was used as blood donors for the preparation of RBC suspensions to be used in exchange transfusions. Blood from donor animals was obtained by cardiac puncture under light ether anesthesia, anticoagulated with EDTA (1.5 mg/ml), and handled as described below. The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University and was in accordance with the Declaration of Helsinki and the International Association for the Study of Pain guidelines.

Preparation. Guinea pigs were anesthetized with urethane (1 g/kg ip), tracheotomies were performed, and animals were ventilated with ambient air at a rate of 70 breaths/min with a tidal volume of 1 ml. The left carotid artery was catheterized using a polyethylene catheter (20 gauge). The catheter was positioned at the level of the ascending aorta and connected to a pressure transducer. Arterial blood pressure was recorded throughout the experiment and during the freezing of the heart. The left jugular vein was also catheterized and used for exchange transfusions together with the carotid artery. These catheters were also used for injection of labeled blood samples and blood sampling.

Exchange transfusions. During the exchange transfusions in all groups, ~40% of the total blood volume of animals was exchanged. The total blood volume of animals was estimated as 8% of their body weight. The control group received an exchange transfusion with normal hematocrit-matched guinea pig blood before the determination of myocardial hematocrit gradient. In the *aggregation I* group, RBCs coated with Pluronic F-98 to enhance aggregation were suspended in unaltered plasma and used for exchange transfusion. In the *aggregation II* group, the exchange transfusion was performed by using normal guinea pig RBCs suspended in plasma containing 1 g/dl Dextran 500 (500 kDa, Sigma Chemical, St. Louis, MO). Although RBC aggregation was enhanced in both aggregation groups, the means used to increase RBC aggregation, and hence the final plasma composition and viscosity, were different (1). The procedure (see *Determination of myocardial hematocrit gradient*) was started 5 min after the completion of the exchange transfusion.

Determination of myocardial hematocrit gradient. Tissue hematocrit gradient in the left ventricular myocardium was estimated by determining the activity of two different radionuclides, which labeled plasma and RBCs, as described by Vicaut and Levy (33). RBCs were labeled in vitro with ^{99m}Tc, and ^{125}I-labeled albumin (Amersham) was used to trace plasma. ^{125}I-labeled albumin was purified by using an ultrafilter with a molecular-mass cutoff of 50 kDa (Microsep) to eliminate any unbound tracer. ^{99m}Tc-labeled RBCs (0.2 ml packed cells) and 0.15 ml ^{125}I-labeled albumin solution (20 mg/ml albumin, 1 μCi) were suspended in 0.65 ml of saline and injected through the femoral vein. After 5 min, 1 ml of blood was sampled for the determination of ^{99m}Tc and ^{125}I activities, systemic hematocrit, and hemorheological parameters (see *Microscopic and rheological studies*).

After blood sampling was completed, a rapid midsternal thoracotomy was performed, and a plastic cylinder was immediately positioned around the heart. This cylinder was rapidly filled with liquid nitrogen, thus quickly freezing the heart. The frozen heart was then excised and kept at −20°C. A 2- × 3- × 5-mm tissue block with the long axis perpendicular to the left ventricular wall was cut and mounted on a cryostat disk, with the disk surface parallel to the left ventricular wall. With the use of a cryotome (Shandon Cryotome AS620, Cheshire, UK), each tissue block was sectioned through the thickness of the left ventricular wall, from epicardium to endocardium, to obtain 100-μm-thick myocardial slices. Sectioning was continued until the left ventricular cavity, clearly distinguished by the color of blood, was approached. Any myocardial tissue block with an unusual geometry observed during slicing (e.g., slices partially covered with blood) was excluded from the study. Each slice was numbered according to its depth in the tissue, with the epicardial layer corresponding to 0 and the layer closest to endocardium corresponding to 1. Slices between these two were represented by values between 0 and 1, depending on the depth and the number of 100-μm-thick slices. A transverse section of the heart was also used to measure the thickness of the left ventricular wall and to determine whether the heart was arrested during systole or diastole. Only the hearts arrested during diastole were used for data analysis.

Radionuclide (^{99m}Tc and ^{125}I) activities in the systemic blood specimens and the myocardial tissue slices were measured by a gamma counter (Atomlab 950, Biodex Medical Systems, New York, NY). Systemic hematocrit was determined by the microcapillary method (12,000 *g*, 5 min). Tissue hematocrit was estimated in each myocardial slice according to Vicaut and Levy (33). Briefly, for each tracer and each blood sample, a constant *K* between the radionuclide activity and the volume of the labeled compartment was calculated by using the measurements obtained for the systemic blood specimens: $K_{^{99m}Tc} = V_{blood} \times Hct/^{99m}Tc$ counts/min; $K_{^{125}I} = [V_{blood} \times (1 - Hct)]/^{125}I$ counts/min, where V_{blood} is the volume of blood used for gamma counting and Hct is the hematocrit determined by the microcapillary method. The resulting constants were then multiplied by the ^{99m}Tc and ^{125}I activity in each 100-μm-thick tissue slice to find the total volume of RBCs or plasma (i.e., $V_{RBC} = K_{^{99m}Tc} \times {}^{99m}$Tc counts/min, and $V_{plasma} = K_{^{125}I} \times {}^{125}$I counts/min). Tissue hematocrit in the slice was then calculated using these values [i.e., $Hct = V_{RBC}/(V_{RBC} + V_{plasma})$].

Aggregation I: Pluronic F-98 coating to increase RBC aggregation. In the RBC suspensions used for exchange in the *aggregation I* group, RBC aggregation was increased by employing a recently developed technique whereby poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) copolymers (Pluronics) that possess the ability to self-associate above a specific temperature are covalently attached to the RBC surface. The details of the method are described elsewhere (6). Briefly, RBCs and plasma were separated from anticoagulated (EDTA, 1.5 mg/ml) donor guinea pig blood by centrifugation at 1,400 *g* for 6 min. RBCs were washed three times with

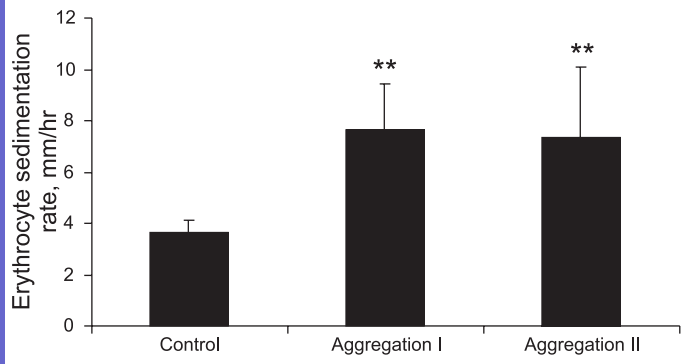

Fig. 1. Erythrocyte sedimentation rate measured for blood samples obtained after exchange transfusions in 3 groups. Pluronic F-98-coated cells were used in *aggregation I* group, and 500 kDa dextran were used in *aggregation II* group. Values are means (SD). $^{**}P < 0.01$, difference from control.

isotonic PBS (pH 7.4), resuspended in 30 mM triethanolamine buffer (290 mosmol/kg, pH 8.60) at a hematocrit of ~0.1 l/l, and the suspension cooled to 4°C. The low value of hematocrit (i.e., ~0.1 l/l) was necessary to obtain an even coating of RBCs with Pluronic and to avoid the formation of cross-linked aggregates of coated RBCs. Immediately before the use of RBCs, a reactive succinimidyl carbonate derivative of Pluronic F-98 [13 kDa, 80% poly(ethylene glycol)] was dissolved in 4°C hypotonic phosphate buffer (50 mM NaH_2PO_4 and 60 mM NaCl, pH 5) at a concentration of 10 mg/ml and added to the RBC suspension to obtain a final concentration of 0.025 mg/ml. The suspension was incubated for 2 h at 4°C with continuous gentle mixing. Pluronic-coated RBC were then separated and washed three times with isotonic PBS by gentle centrifugation (400 *g*, 5 min) and then resuspended in native plasma at 0.4 l/l hematocrit.

All Pluronic-coating procedures were performed at 4°C, i.e., below the critical micellization temperature (CMT), at which a phase transition occurs from predominantly single, fully hydrated copolymer chains to micelle-like structures. At a temperature above the CMT, RBC aggregation occurs due to the formation of micelle-like structures between adjacent Pluronic-coated RBC, with the strength of aggregation dependent on both the Pluronic concentration at the RBC surface and on the physicochemical characteristics (e.g., molecular weight and CMT) of the selected Pluronic (1). Although Pluronic-coated RBCs do not have an enhanced tendency to adhere to non-coated blood cells, they can form mixed aggregates, i.e., coated plus noncoated RBC, when lightly coated with Pluronic F-98 and suspended in an aggregating medium (1). Pluronic F-98 RBCs do not exhibit an enhanced tendency to adhere to cultured vascular endothelial cells (unpublished observations).

Aggregation II: addition of dextran to increase RBC aggregation. RBC aggregation in the suspensions used for exchange in the *aggregation II* group was increased by suspending washed cells at a hematocrit of 0.40 l/l in plasma containing 1 g/dl of Dextran 500.

Microscopic and rheological studies. Evaluation of hemorheological parameters subsequent to the exchange transfusions was carried out by using blood samples obtained before the freezing of hearts. Wet-mount preparations of dilute RBC suspensions in plasma were examined and photographed by using bright-field light microscopy. Erythrocyte sedimentation rates (ESR) were measured by using Westergren sedimentation tubes, and RBC-plasma suspensions were adjusted to a hematocrit of 0.1 l/l. Plasma viscosity was measured at a 1,500 s^{-1} shear rate using a Wells-Brookfield cone-plate viscometer (Brookfield, Middleboro, MA). Whole blood viscosity was also measured by using the same device at shear rates between 37.5 and 1,500 s^{-1}. All viscosity measurements were performed at 37°C.

Statistics. Results are means (SD), except where stated otherwise (e.g., Fig. 5). Comparisons between two means were made by Student's *t*-test, and the relationships between the depth of tissue and tissue hematocrit were tested by linear and nonlinear regression analysis.

RESULTS

Hemorheological alterations after exchange transfusions. In both *aggregation I* and *II* groups, RBC aggregation was found to be enhanced as determined by microscopic examination. In contrast to the control group where minimal aggregation was observed, RBCs in the aggregation groups formed aggregates with relatively few cells not associated with an aggregate; qualitative estimates via microscopy suggested essentially the same level of aggregation in the two groups. However, the appearance of the RBC aggregates differed somewhat between the two groups: those in the *aggregation II* group consisted of linear arrays of cells (i.e., "classic" rouleaux formation) with some secondary branching, whereas those in the *aggregation I* group tended to be slightly less regular with cells exhibiting linear and branched face-to-face aggregation (i.e., rouleaux) and some face-to-side structures. Such differences were anticipated due to the differing nature of the aggregation process between the two groups (1, 27). The aggregation strength and aggregate morphology of RBCs in the *aggregation I* group precluded measurements of aggregation with the use of an available photometric aggregometer (5), and hence ESR tests were employed. RBC sedimentation rates provided quantitative evidence for the enhanced aggregation (Fig. 1). Both aggregation groups had significant, approximately twofold, increases in aggregation versus control with no significant difference in ESR among these groups.

Whole blood viscosity values over the entire shear-rate range were higher in the *aggregation II* group than in the control and *aggregation I* groups (Fig. 2), reflecting the higher plasma viscosity in the *aggregation II* group (Fig. 3). In contrast, whole blood viscosity was only higher in the *aggregation I* group compared with the control group at the lowest shear rate. Although the lowest shear rate used in this study (i.e., 37.5 sec^{-1}) cannot be considered as representing low-shear blood viscometry (e.g., 1 s^{-1} or less), the significantly enhanced blood viscosity at this shear rate, combined with the unaltered plasma viscosity (Fig. 3), does reflect a higher disaggregation shear rate for cells in the *aggregation I* group (4, 23).

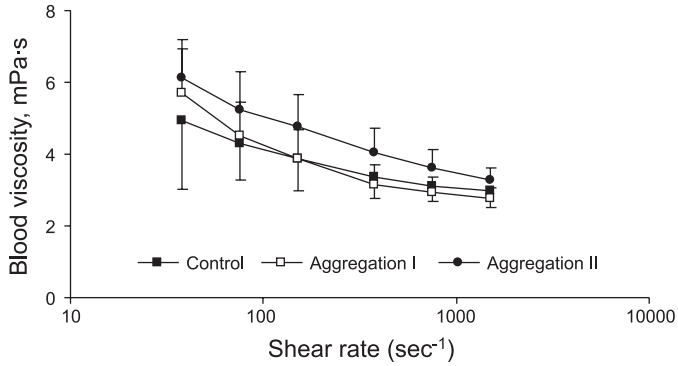

Fig. 2. Whole blood viscosities measured at shear rates between 37.5 and 1,500 s^{-1} for blood samples obtained after exchange transfusions in 3 groups. Pluronic F-98-coated cells were used in *aggregation I* group, and 500 kDa dextran were used in *aggregation II* group. Values are means (SD); 3 curves are significantly different from each other by 2-way ANOVA. Variation between groups is $F = 10.6$; variation between shear rates, $F = 24.4$.

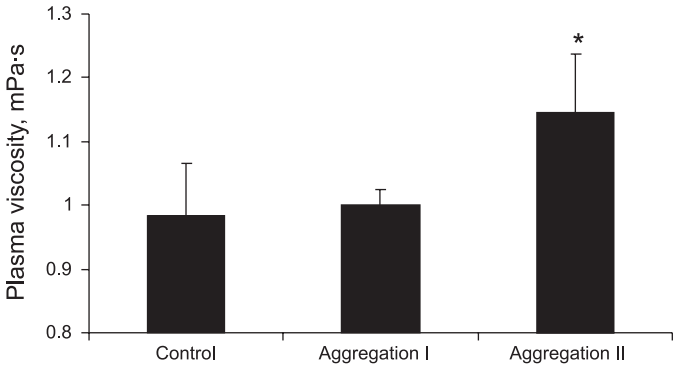

Fig. 3. Plasma viscosity measured for blood samples obtained after exchange transfusions in 3 groups. Pluronic F-98-coated cells were used in *aggregation I* group, and 500 kDa dextran were used in *aggregation II* group. Values are means (SD). *$P < 0.05$, difference from control.

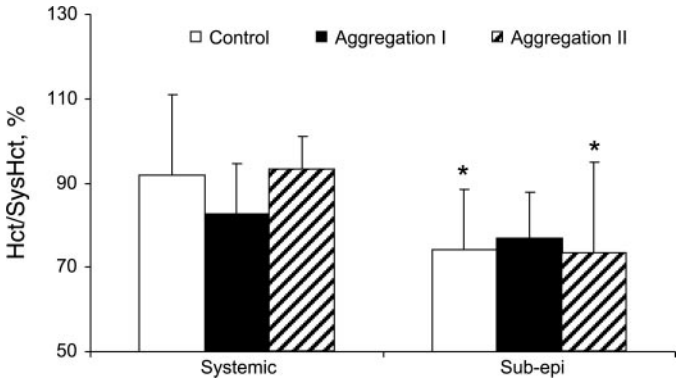

Fig. 4. Subepicardial (Subepi) and subendocardial layer hematocrits as a percentage of systemic hematocrit (SysHct). Pluronic F-98-coated cells were used in *aggregation I* group, and 500 kDa dextran were used in *aggregation II* group. Values are means (SD). *$P < 0.05$, difference from corresponding Sub-epi values.

Myocardial hematocrit values. Systemic hematocrit values together with tissue hematocrit values in the subepicardial and subendocardial layers are presented in Table 1, and subepicardial and subendocardial myocardial tissue hematocrit values expressed as a percentage of systemic hematocrit are shown in Fig. 4. In both aggregation groups, the subepicardial hematocrit was statistically less than the systemic hematocrit ($P < 0.05$ or better), with the largest decrease (i.e., 17%) noted for the *aggregation I* group. The ratios of subepicardial to systemic hematocrit did not differ among groups (Fig. 4). The mean subepicardial hematocrit value for the *aggregation II* group was slightly higher than the corresponding values for the control and *aggregation I* groups (Table 1), but the differences were not statistically significant by one-way ANOVA. In all groups the subendocardial hematocrit was significantly less than the systemic hematocrit ($P < 0.05$), with a 22% decrease for the *aggregation I* group and a 26% decreases for the control and *aggregation II* groups. Neither the absolute subendocardial hematocrit values nor the ratio of subendocardial to systemic hematocrit differed among groups.

Although the absolute values of subepicardial and subendocardial hematocrit did not differ among groups, there were marked differences in the ratio of subendocardial to subepicardial hematocrit (Table 1): *1*) Control group ratio was 0.82 ($P < 0.01$); *2*) *aggregation I* group ratio was 0.94 ($P > 0.10$); and *3*) *aggregation II* group ratio was 0.79 ($P < 0.01$). Thus the decrease in tissue hematocrit from epicardium to endocardium was significantly less than unity only in the control and *aggregation II* groups, whereas the small difference between the two hematocrits was not significant for the *aggregation I* group.

Figure 5 presents myocardial tissue hematocrit values for each group, expressed as a percentage of the subepicardial hematocrit ($\%Hct_{epi}$), for various tissue slices between the subepicardial and subendocardial layers. The percentage was calculated for each animal and then averaged for the group to obtain the mean ± SE. In Fig. 5, the subepicardial layer corresponds to a normalized depth of 0 and the subendocardial layer closest to the endocardium corresponds to a normalized depth of 1. Inspection and analysis of these results lead to the following observations. First, two-way ANOVA analysis indicated a significant difference among the three curves. Second, the control group data demonstrate the anticipated existence of a tissue hematocrit gradient, with the hematocrit value in the slice closest to endocardium being significantly lower ($P < 0.01$) than that in the subepicardial layer. The gradient was not linear but appears to initially decrease rapidly and then reach a stable level at ~80% of $\%Hct_{epi}$ at a normalized depth of ~0.5. Third, the *aggregation I* group data, i.e., RBCs coated with Pluronic F-98, appear to be essentially independent of position within the myocardium, with a slight tendency for decreased relative hematocrit in the two slices nearest the subendocardial layer. The average value of relative hematocrit for the 10 slices (99.1 ± 2.1, mean ± SE) did not differ from 100% (i.e., unchanged from subepicardial hematocrit). Fourth, the *aggregation II* group data demonstrated behavior intermediate between the control and *aggregation I* groups; i.e., over the first half of normalized depth, the relative hematocrit values were close to 100% and thus did not differ meaningfully from those for the *aggregation I* group. However, beyond a depth of ~0.5, the relative hematocrit decreased sharply and was coincident with the control group at the subendocardial position.

Table 1. *Systemic Hct, subepicardial, and subendocardial tissue Hct values in control, aggregation I, and aggregation II groups*

Group	*n*	Systemic Hct, l/l	Subepicardial Hct, l/l	Subendocardial Hct, l/l	Hct_{endo}/Hct_{epi}, %
Control	7	0.38 (SD 0.03)	0.34 (SD 0.05)	0.28 (SD 0.05)†‡	81.7 (SD 0.09)
Aggregation I	6	0.40 (SD 0.03)	0.33 (SD 0.07)†	0.31 (SD 0.05)*	94.8 (SD 0.19)
Aggregation II	6	0.41 (SD 0.03)	0.38 (SD 0.05)*	0.30 (SD 0.10)*‡	78.8 (SD 0.21)

Values are means (SD); *n,* number of hearts. Hct_{endo}/Hct_{epi}, subendocardial-to-subepicardial Hct ratio. *$P < 0.05$ and †$P < 0.01$, difference from systemic Hct, respectively; ‡$P < 0.01$, difference from subepicardial Hct in the same group.

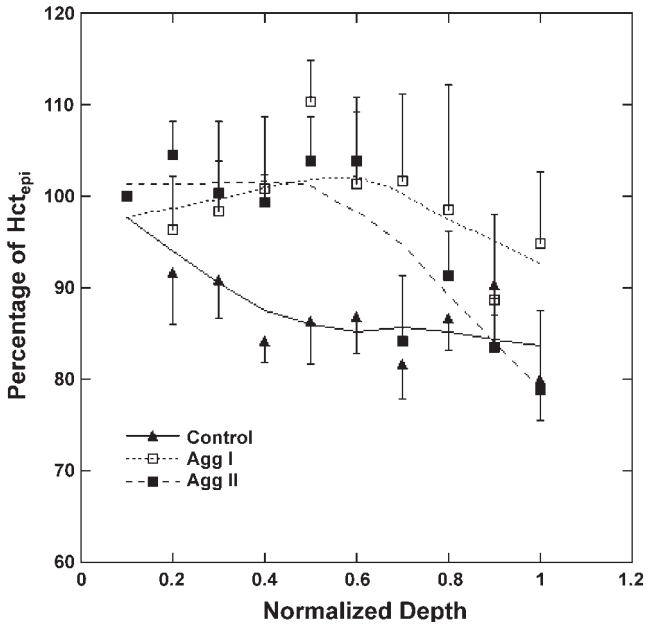

Fig. 5. Tissue hematocrit values as percentage of Sub-epi layer value (Hct_{epi}) in myocardial tissue slices after exchange transfusions in 3 groups. Sub-epi layer corresponds to normalized depth of 0, and subendocardial layer closest to endocardium corresponds to normalized depth of 1. Pluronic F-98-coated cells were used in *aggregation I* group (Agg I), and 500 kDa dextran were used in *aggregation II* group (Agg II). Values are means ± SE. Curves were obtained by computerized weighted smoothing method; 3 curves are significantly different from each other by two-way ANOVA. Variation between groups is $F = 9.7$; variation according to depth, $F = 2.5$.

DISCUSSION

The reversible aggregation of RBCs into multicell structures is a normal physiological process that occurs at low shear rates or at stasis and only in the presence of sufficiently large macromolecules in the suspending phase. Therefore, plasma composition is an important determinant of the degree of RBC aggregation (26). Additionally, it has been demonstrated that RBC surface properties play a significant role in RBC aggregation (27). Experimental and clinical data suggest that alterations of plasma composition, especially elevated levels of high-molecular-weight proteins (e.g., fibrinogen and macroglobulins), can lead to enhanced RBC aggregation (23, 27), and thus the circulatory effects of RBC aggregation are of clinical and physiological interest. The current study addressed the effects of enhanced RBC aggregation on myocardial hematocrit distribution, with the specific aim of examining possible differences between enhanced aggregation induced by two different experimental approaches. Recently developed methods for Pluronic coating of RBCs enabled the use of these two approaches (1).

In this study, the aggregating tendency of plasma was increased by the addition of Dextran 500, and thus the *aggregation II* group represents the use of altered plasma as the suspending media. Because the experimental protocol involved a 40% exchange transfusion of RBCs suspended in plasma containing 1 g/dl Dextran 500, this exchange should have resulted in a final plasma dextran concentration of ~0.4 g/dl. As anticipated, the addition of this high-molecular-weight macromolecule resulted in an increase of plasma viscosity (16%, Fig. 3) and markedly enhanced RBC aggregation (i.e., 101% increment in ESR, Fig. 1). Neither plasma viscosity nor plasma protein concentration was altered in the *aggregation I* group that received exchange transfusions with Pluronic F-98-coated RBC suspensions, yet RBC aggregation was enhanced to a similar degree (110%). Therefore, the use of these two methods for altering RBC aggregation represents a novel approach for comparing the effects on tissue hematocrit of enhanced aggregation induced by modified plasma composition or by modified RBC surface properties. The left ventricular myocardium is a useful tissue for such studies because it is possible to define tissue hematocrit values relative to the anatomic position of the tissue (2, 33).

The major finding of the current study is clearly shown in Fig. 5. The normal, control group hematocrit profile, with a significant decrease of hematocrit when traversing from subepicardial to subendocardial layers, is markedly altered by enhanced RBC aggregation. First, when aggregation is increased on using Pluronic F-98-coated RBC suspended in native plasma (*aggregation I* group), hematocrit differences across the myocardium are essentially abolished. Second, when RBC aggregation is increased on using a high-molecular-weight polymer (*aggregation II* group), hematocrit remains at subepicardial levels for ~60% of the myocardial thickness and then rapidly decreases to the control level in the subendocardial layer. Our results thus indicate that myocardial hematocrit profiles are sensitive to both RBC aggregation and changes of plasma viscosity associated with increased RBC aggregation. Tsai and coworkers (31, 32) have examined the microcirculatory effects of elevated plasma viscosity in extreme hemodilution produced by exchange transfusion with dextran solutions. Using a hamster skinfold model that allowed direct microscopic observation, they observed that functional capillary density was significantly higher when hemodilution was done using a high-viscosity Dextran 500 than when using a low-viscosity 70-kDa dextran. Our results (Fig. 5) partially support their observations: when using Dextran 500 and thus increasing plasma viscosity, hematocrit was maintained at subepicardial levels for ~60% of myocardial thickness, whereas the control group values were markedly lower over the same region. However, enhanced aggregation without increased plasma viscosity (*aggregation I* group) was more effective in maintaining hematocrit at subepicardial levels.

In the control group, the tissue hematocrit value at the subepicardial layer of the left ventricular wall was 92% of the systemic hematocrit (Fig. 4). This value is reasonably close to the average value previously reported for epicardial layers of rat left ventricular myocardium (2, 3, 33). A lower value (82%, Fig. 4) was found in the corresponding tissue samples obtained from the group with enhanced RBC aggregation but unaltered plasma composition (*aggregation I*). Based on previous observations of Fahraeus (12), Cokelet and Goldsmith (10), and others (11, 17, 21, 22), enhanced aggregation could result in lower tissue hematocrit values by promoting RBC axial migration and plasma skimming in smaller vessels. However, it has also been argued that the effects of RBC aggregation might be influenced by the relations between transit times of blood through the circulatory network and time constants of RBC aggregation (7, 8), thereby limiting the influence of aggregation on hemodynamic mechanisms favoring lower tissue hematocrit.

Although the main difference between the two methods to increase aggregation is assumed to be related to the plasma viscosity increase in the *aggregation II* group (Fig. 3), there does not appear to be experimental evidence suggesting that the alterations in plasma viscosity can affect the radial distribution of RBCs in vessels and hence tissue hematocrit. However, it can be speculated that increased plasma viscosity may lead to altered blood vessel diameters via its interference with endothelial cell vasomotor mechanisms that are dependent on wall shear forces (6, 13). Therefore, it would be interesting to test the effects of enhanced plasma viscosity without increasing RBC aggregation by introducing low-molecular-weight dextrans (e.g., 40 kDa) into the circulation. This approach was not included in the present study but should be considered in future experiments. Another important difference between the two approaches for increasing RBC aggregation is related to the strength of the aggregates. Pluronic F-98 coating of RBCs is known to produce irregular aggregates that resemble those found in various pathological conditions. The strength of aggregation and aggregate morphology are dose dependent and, when using high concentrations for cell coating, can result in aggregates that require significantly greater shear forces to cause disaggregation (1, 35). Although the in vivo behavior of Pluronic F-98-coated RBCs has not been studied in detail, it can be speculated that aggregates of these RBCs may persist in various portions of the circulatory system to a greater extent then those induced by 500 kDa dextran. Such behavior might be expected to enhance phase separation and related mechanisms that lead to lower microvascular hematocrit values. The essentially identical ESR values for the Pluronic F-98-coated and dextran groups (Fig. 1) only indicate that, at stasis, RBC aggregate sizes are similar and do not address the strength of the aggregates.

Unlike tissue hematocrit in the control group, tissue hematocrit in both aggregation groups remained at subepicardial levels for ~50 to 60% of the wall thickness and then either continued to remain at about this level (*aggregation I*) or decreased rapidly to the control level (*aggregation II*). The functional consequences of these changes are, at least, twofold. First, the increase of tissue hematocrit versus that in the control group (Fig. 5) should be beneficial, inasmuch as the oxygen-carrying capacity of the blood is increased. However, in the *aggregation II* group, this beneficial effect no longer exists for myocardial layers closest to the subendocardium. Subendocardial layers are prone to the highest extravascular pressures that tend to limit blood flow (20), making this region the most prone to ischemia. Second, blood viscosity increases in an exponential manner with increasing hematocrit, and thus the increase of hematocrit versus that in the control group should result in higher blood viscosity (23). In turn, it seems logical to assume that flow resistance in these layers is increased, thereby adversely affecting blood flow downstream in layers closer to the subendocardium. Thus, although it is possible to conjecture that the higher overall hematocrits in the *aggregation I* group are of physiological value, the actual flux of oxygen-carrying RBCs may be reduced due to decreased blood flow caused by higher blood viscosity.

The observations and conclusions herein are most likely limited to the specific experimental parameters employed (e.g., type of polymer coating and of dextran) and should be extended with caution to other vascular beds. However, the method used to determine myocardial tissue hematocrit values should be applicable in other studies. The radionuclide method for tissue hematocrit was originally developed by using rats as the experimental animal (33). However, preliminary attempts to utilize the Pluronic-coating technique with rat RBCs were not successful due to the tendency of these cells to undergo a discocyte-echinocyte shape change during in vitro processing. This shape transformation was observed with control (uncoated) and Pluronic-coated RBCs and is thus not related to the Pluronic-modification of the RBC surface. Conversely, guinea pig RBCs are more resistant to in vitro processing and preserve their normal discocytic geometry in the suspensions used for exchange transfusions (35). Unfortunately, there do not appear to be any data in the literature for tissue hematocrit in guinea pigs, thus precluding comparisons to prior studies.

It is interesting to note that although all RBC in the *aggregation II* group were exposed to plasma containing Dextran 500, only ~40% of the cells in the *aggregation I* group were coated with Pluronic F-98, and hence only these cells exhibited an intrinsic tendency for increased aggregation. Such a situation seems relevant to a model for altered aggregability of RBCs due to local disturbances of the microvascular environment (15). Under such pathophysiological conditions, circulating blood contains a mixture of modified RBC draining from damaged tissue and normal RBCs. Although it is possible that RBC subpopulations with different rheological properties preferentially aggregate with RBCs having similar properties (29) and although it is likely that Pluronic F-98-coated RBCs preferentially aggregate with each other, the Pluronic F-98-coating conditions used herein do not preclude the possibility of mixed RBC aggregates (1). Thus, although it is possible that the injected, uncoated ^{99m}Tc-labeled cells do not equally participate in aggregation in the *aggregation I* group, these cells would be affected by local microcirculatory flow dynamics and hence should reflect the spatial distribution of all RBCs.

The data obtained in this study represent the relaxed diastolic state of the myocardium, because only hearts arrested in diastole were used for the measurements. Although it was possible to detect that some hearts were arrested in systole, the radionuclide activity in the tissue slices obtained from these organs was very low, mostly as background activity, reflecting the low blood volume in the intramyocardial vessels (20). Therefore, it was not possible to use such hearts for determining tissue hematocrit. However, because perfusion of the left ventricular myocardium occurs mostly during diastole (20), information relevant only to the diastolic period should be of high physiological relevance. The dynamics of the rapid freezing process also suggest the physiological relevance of our results. Given that the rapid cooling of the heart within the rigid plastic chamber filled with liquid nitrogen starts at the same time on all external surfaces, Vicaut and Levy (33) have convincingly argued that the freezing process should not have a significant effect on hematocrit distribution. Most large blood vessels (arteries and veins) lie on or near the external cardiac surface, and these vessels are rapidly frozen or are at least cooled to near 0°C. Blood viscosity in these vessels thus increases very rapidly, thereby trapping blood in place before the total freezing of the tissue.

Finally, it should be noted that the exact mechanisms underlying the myocardial tissue hematocrit gradient are not clearly understood, although hemodynamic conditions through

the thickness of myocardium have been the subject of several detailed studies (14, 24, 30). The most important factor that changes with myocardial depth, especially in left ventricular myocardium, is the intramyocardial, extravascular pressure, i.e., extravascular compression, equivalent to the intraventricular pressure at the subendocardial layer and lowest at the subepicardial layer. These pressures also determine perfusion pressures and shear forces at various depths (20). Differences in hemodynamic conditions are strongly expected to influence RBC distribution and phase separation in the vascular system, although the complexity of hemodynamic conditions in the myocardium and the mechanisms related to RBC distribution currently make it inappropriate to offer extensive speculations regarding the influence of enhanced aggregation on myocardial hematocrit gradient (9). Further experimental and theoretical studies in this area are therefore warranted.

GRANTS

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Hemorheological parameters as determinants of myocardial tissue hematocrit values

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Abstract. It is well known that the hematocrit in microvessels with diameters smaller than 1000 μm is lower than either venous or arterial hematocrit, thereby resulting in significantly lower mean hematocrit values for vessels perfusing a given tissue (i.e., lower tissue hematocrit). The mechanisms that underlie this reduction of microvascular hematocrit include axial migration, plasma skimming and the Fahraeus Effect. It has been previously demonstrated in rats that a linear hematocrit gradient normally exists through the thickness of the left ventricular myocardium, and that this gradient is sensitive to alterations of the rheological properties of the circulating blood. The gradient is abolished if the RBC in the perfusate are rigid; fibrinogen infusions, and thus increases of both plasma viscosity and RBC aggregation, also affect this gradient. In a new series of studies, it has been observed that enhanced RBC aggregation affects the myocardial hematocrit gradient *regardless* of alterations of plasma viscosity. Although the exact mechanisms responsible for the myocardial hematocrit gradient, as well as its physiological significance, are not yet clearly known, it is possible to speculate that alterations in local hematocrit could adversely affect myocardial perfusion and function.

Keywords: Tissue hematocrit, microvascular hematocrit, erythrocyte deformability, erythrocyte aggregation

1. Microvascular hematocrit and tissue hematocrit

Hematocrit is one of the most widely measured parameters in medical practice. In addition to its hematological implications, hematocrit is functionally important since it is the major determinant of blood viscosity [3]. Hematocrit is usually measured either in venous blood samples or in samples obtained by finger prick, with such values assumed to represent blood in all parts of the circulatory system. However, hematocrit is not constant throughout the circulatory system, but becomes lower as blood approaches the microcirculation [30]; the instantaneous volume fraction of erythrocytes in blood within a given microcirculatory bed is defined as the *microvascular hematocrit* [8]. Optical methods and image analysis techniques have been used to calculate the hematocrit in microvessels, with results indicating that it may be significantly lower than that of blood in large vessels [26,29]: microvascular hematocrit values can be as low as $\sim$20% of large vessel hematocrit (Table 1).

The mean hematocrit of blood circulating in all sizes of blood vessels in a given tissue is termed *tissue hematocrit* [31,32], and can be estimated by using tracers that label red cells and plasma and then determining their ratio in a given tissue [11,32]. Values of tissue hematocrit have been reported to be

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Table 1
Microvascular hematocrit values measured in various tissue and species

Species	Organ/tissue	Microvascular hematocrit (tissue/systemic)	Method	Reference
Cat	Mesentery	0.08/0.33	Micro-occlusion	[25]
Hamster	Cremaster muscle	0.14/0.49	Optical	[21]
Rat	Cremaster muscle	0.18/0.37	Optical	[20]
Dog	Gastrocnemius-plantaris muscle	0.38/0.50	Isotope washout	[13]

Table 2
Tissue hematocrit values measured in various tissue and species

Species	Organ/tissue	Tissue hematocrit (tissue/systemic)	Method	Reference
Rat	Heart (subepicardium)	0.32/0.39	Double isotope labeling	[32]
Guinea pig	Heart (subepicardium)	0.34/0.38	Double isotope labeling	[33]
Human	Brain	0.38/0.43	PET	[27]
Rat	Brain	0.33/0.43	Double isotope labeling	[31]

significantly higher than microvascular hematocrit (Table 2), most likely due to the combined effects of lower hematocrit values in microvessels and higher hematocrit values in larger blood vessels. While microvascular hematocrit values can be as low as 20% of systemic hematocrit, tissue hematocrit values are about 75–89% of systemic hematocrit (Tables 1 and 2).

2. Mechanisms of hematocrit reduction in microvasculature

The difference between hematocrit values in vessels with different diameters reflects the reduction of hematocrit as blood approaches the microcirculation. The hemodynamic mechanisms that underlie this reduction are mainly related to the movement of RBC towards the central flow region in tube flow and thus towards a region of lower shear [16]. This movement is termed axial migration and results in phase separation [16,30], with a cell-poor layer formed at the peripheral region of a blood vessel and higher concentrations of RBC and hematocrit nearer the central flow zone.

One of the obvious outcomes of the abovementioned phase separation is plasma skimming, in which side branches of vessels receive blood flow mainly from the cell poor, low hematocrit peripheral flow zone. Furthermore, since the velocity of the central flow zone is higher than that of the marginal layers, RBC that accumulate in the central flow zone move faster relative to plasma thereby causing the "Fahraeus Effect" [16,30]. The Fahraeus Effect predicts lower hematocrit values within a small diameter tube compared to the hematocrit of the blood entering or discharged from that tube [16,30]. In addition to these mechanisms, flow partitioning at vascular bifurcations results in preferential accumulation of red cells in the faster branch [11,28], with this phenomenon termed the network Fahraeus Effect. Klitzman and Duling have proposed that shunting of RBC flow from arterial to venous vessels may also play role in hematocrit reduction [21].

3. Factors affecting microvascular/tissue hematocrit

Despite the availability of theoretical analyses [14] and experimental/clinical data regarding lower hematocrit values in the microvasculature, the factors responsible for these values have not yet been

fully elucidated. There are published reports of alterations of tissue/microvascular hematocrit under various conditions that, in general, usually reflect alterations of "normal" hemodynamics in various tissues. However, hematocrit reduction in a microvascular bed depends on the flow rate in that portion of the circulation [15]. Goncalez et al. demonstrated that acute pancreatitis results in decreased blood flow in most visceral organs and an increase of tissue hematocrit [17]. In contrast, Brizel et al. compared mammary tumor and normal tissue RBC flow velocity and hematocrit values in rats and concluded that tumor tissue is characterized by higher flow velocities and also by higher tissue hematocrit values [5]. Blood flow and tissue hematocrit measurements in myocutenous island flaps in pigs by Hjortdal et al. support the latter study, in that they reported decreased blood flow and decreased tissue hematocrit in the flap tissue [18]. Klitzman and Johnson also reported a positive correlation between blood flow and microvascular hematocrit in hamster cremaster muscle [22].

Klitzman and Duling indicate that muscle contraction induces a significant increment in microvascular hematocrit [21]. Frisbee et al. investigated microvascular hematocrit values in resting and contracting striated muscle tissue [12,13], and reported that microvascular hematocrit increased significantly with muscle contraction and was correlated with increased muscle blood flow due to the contractions [13]. They also reported a difference in the sustained effects of isometric and isotonic muscle contractions: sustained isometric contraction was characterized by a secondary drop of microvascular hematocrit from the peak value, whereas with isotonic contraction no decline in this value was observed [12]. They concluded that this difference may reflect the different character of microvascular blood flow, distribution patterns at bifurcations, and the number and spatial arrangement of open microvessel networks during isometric versus isotonic muscle contractions [12]. These studies thus underline the importance of hemodynamic factors as determinants of the difference between microvascular and systemic hematocrit values.

The dependence of microvascular tissue hematocrit values on local hemodynamic factors have also been demonstrated in myocardial tissue. Vicaut and Levy report a linear tissue hematocrit gradient in rat left ventricular myocardium, with the hematocrit being highest at the subepicardial layer [32]. There are no known morphological differences to explain this variation in tissue hematocrit between subepicardial and subendocardial layers of the left ventricular myocardium. While the left ventricular myocardium is a unique tissue in which local hemodynamic factors can be defined relative to their location within the thickness of the ventricular wall, hemodynamic conditions in intra-myocardial blood vessels are extremely complex [19]. Thus, it may not be possible to simply relate decreased tissue hematocrit to any given hemodynamic variable. Rather, it may only be possible to speculate that microvascular hematocrit values are strongly influenced by hemodynamic variables, with such variables being specific to the position within the myocardium.

4. Effect of hemorheological parameters on tissue hematocrit

It has been shown that hemodilution alters the magnitude of the difference between microvascular and large vessel hematocrit. Todd et al. reported that cerebral tissue hematocrit was reduced from 78% to 46% of arterial hematocrit when the arterial hematocrit was reduced from 0.43 l/l to 0.20 l/l [31]. In contrast, Lipowsky and Firrell demonstrated that the ratio of microvascular hematocrit to systemic hematocrit increased with hemodilution [24], and suggested that this alteration may indicate a more efficient utilization of the remaining volume of RBC [24].

RBC rheological properties have also been reported to be determinants of tissue hematocrit. Such findings are not unexpected since it is well known that RBC deformability and aggregation affect ax-

ial migration, phase separation, and related mechanisms. Lipowsky et al. observed that microvascular hematocrit in rat cremaster muscle increased during perfusion with RBC having impaired deformability [23]. Baskurt et al. studied myocardial hematocrit gradients in rat hearts using an approach described by Vicaut and Levy [32], and observed that the gradient seen in control animals was abolished following exchange transfusion with RBC having reduced deformability [2].

The effects of enhanced RBC aggregation on axial migration and phase separation were first demonstrated by Robin Fahraeus by comparing human and horse blood flowing in glass capillaries [9]. Cokelet and Goldsmith demonstrated that enhanced RBC aggregation promotes phase separation and reduces flow resistance in vertically oriented glass tubes [6]. Myocardial hematocrit gradient in rats was found to be influenced by increased RBC aggregation produced by fibrinogen infusions [1]; pre-existing linear hematocrit gradients were significantly altered such that tissue hematocrit at the subepicardial layer approached control levels with a steep change at the innermost layers of myocardium. However, it should be noted that fibrinogen infusion has two hemorheological consequences (i.e., increased plasma viscosity and increased RBC aggregation), and that both alterations may be expected to affect the mechanisms that determine microvascular hematocrit. More recently, the effects of enhanced RBC aggregation on myocardial hematocrit gradient were studied without altering plasma properties; RBC aggregation was increased by covalently attaching polymers to the RBC surface [33]. In these later studies, the myocardial hematocrit gradient was abolished and there was no difference between subepicardial and subendocardial myocardial layers [33].

5. Pathophysiological implications of tissue hematocrit alterations

The pathophysiological significance of tissue hematocrit alterations requires considering both oxygen carrying capacity and flow resistance as affected by blood fluidity. Obviously, there is a divergence between these two effects as hematocrit is altered: hematocrit reduction as blood approaches the microcirculation decreases blood viscosity and flow resistance, but also decreases the oxygen carrying capacity of blood. This situation is thus similar to that encountered when dealing with the concept of an "optimal hematocrit": both oxygen carrying capacity and blood viscosity increase with hematocrit, but since the rate of increase for viscosity is greater, oxygen delivery effectiveness (i.e., the ratio of hematocrit to viscosity) decreases above a certain critical hematocrit level [4,10]. Decreasing hematocrit below this critical level also decreases the hematocrit to viscosity ratio, again providing less than optimum effectiveness. Note that although several reports have generated experimental data regarding the optimal hematocrit concept and have attempted to identify the exact hematocrit value, these reports employed blood viscosity data obtained in large geometry viscometers: the applicability of these data to the microvasculature remains uncertain.

It is notable that Desjardins and Duling have presented strong arguments that oxygen transfer to tissue is not determined by microvascular hematocrit, but rather by the discharge hematocrit from microvessels [7]. Therefore, it is possible to suggest that alterations of microvascular hematocrit and tissue hematocrit may have different implications for tissue and organ function. Experimental determination of tissue hematocrit yields an average value that is influenced by both microvascular and discharge hematocrits (i.e., hematocrit in various sizes of venous vessels), and thus this averaging effect should be considered when attempting to evaluate the functional meaning of reduced or enhanced tissue hematocrit. Clearly, further studies are needed to understand the pathophysiological significance of microvascular/tissue hematocrit alterations that are the consequence of hemorheological changes.

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Effect of enhanced red blood cell aggregation on blood flow resistance in an isolated-perfused guinea pig heart preparation

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Abstract. The role of red blood cell (RBC) aggregation as a determinant of *in vivo* blood flow is still unclear. This study was designed to investigate the influence of a well-controlled enhancement of RBC aggregation on blood flow resistance in an isolated-perfused heart preparation. Guinea pig hearts were perfused through a catheter inserted into the root of the aorta using a pressure servo-controlled pump system that maintained perfusion pressures of 30 to 100 mmHg. The hearts were beating at their intrinsic rates and pumping against the perfusion pressure. RBC aggregation was increased by Pluronic (F98) coating of RBC at a concentration 0.025 mg/ml, corresponding to about a 100% increment in RBC aggregation as measured by erythrocyte sedimentation rate. Isolated heart preparations were perfused with 0.40 l/l hematocrit unmodified guinea pig blood and with Pluronic-coated RBC suspensions in autologous plasma. At high perfusion pressures there were no significant differences between the flow resistance values for the two perfusates, with differences in flow resistance only becoming significant at lower perfusion pressures. These results can be interpreted to reflect the shear dependence of RBC aggregation: higher shear forces associated with higher perfusion pressures should have dispersed RBC aggregates resulting in blood flow resistances similar to control values. Experiments repeated in preparations in which the smooth muscle tone was inhibited by pre-treatment with papaverine indicated that significant effects of enhanced RBC aggregation could be detected at higher perfusion pressures, underlining the compensatory role of vasomotor control mechanisms.

1. Introduction

The role of red blood cell (RBC) aggregation in determining *in vivo* blood flow is still not fully determined and hence is the subject of continuing investigation. On the one hand RBC aggregation is the major determinant of low shear blood viscosity (i.e., increased aggregation results in increased low shear viscosity) [4], and is therefore expected to be one of the major factors that affect blood flow, especially in low-shear zones of the circulatory system [10]. On the other hand, red blood cell aggregation is expected to promote axial accumulation of RBC during flow in the vasculature, thereby resulting in

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reduced frictional resistance at the vessel wall and more efficient RBC–plasma phase separation [11,13]. The latter effect may, in turn, influence the mechanisms that determine microvascular hematocrit, namely plasma skimming and the Fahraeus effect [13,17]. Obviously, these two phenomena are in conflict for predicting the overall effect of RBC aggregation on *in vivo* hemodynamic resistance, and thus it seems reasonable that the net effect should be related to their relative importance.

Evaluation of RBC aggregation versus *in vivo* flow resistance relationship is further complicated by a variety of specific factors: (1) The impact of RBC aggregation on network hemodynamic resistance depends on the relation between the residence time of a given population of RBC within a specific vascular segment and the time constants of the aggregation process [1,7,9]. If the residence time is too short for the development of RBC aggregates, alterations in RBC aggregation properties may not interfere with flow resistance in that segment; (2) The effects of RBC aggregation are also determined by the orientation of blood vessels. It has been previously demonstrated that RBC aggregation reduces flow resistance in vertically oriented tubes due to enhanced RBC–suspending medium phase separation, but leads to increased flow resistance in horizontally oriented tubes due to increased sedimentation at low flow rates [11]; (3) Alterations in RBC aggregation are usually associated with modified plasma composition, both under pathophysiological and experimental conditions [3,8,10,15,19,20]. For example, increased plasma fibrinogen concentration leads to enhanced RBC aggregation and also to increased plasma viscosity [15], with these two hemorheological changes possibly having different effects on flow resistance; (4) The net effect of a hemorheological alteration, including altered RBC aggregation properties, strongly depends on the status of vasomotor control mechanisms [5,21] and, if there is sufficient vasodilatory reserve capacity, it is possible to compensate for the additional hemorheological "load"; (5) Hemodynamic factors such as perfusion pressure and local shear forces are important determinants of the magnitude of effects due to altered hemorheological properties (e.g., the degree of RBC aggregation is a function of local shear forces).

The complexity and interactions between the factors indicated above may explain the discrepancy between published data, with the differences probably related to one or more factor. Therefore, experimental data obtained under various and controlled conditions may help to improve our understanding of the *in vivo* role of RBC aggregation. This study was thus designed to utilize a novel technique to alter RBC aggregation without changing plasma composition or properties [2]. The effects of increased RBC aggregation on flow resistance were investigated in an isolated-perfused heart preparation, with special emphasis on perfusion pressure and vasomotor mechanisms.

2. Materials and methods

2.1. Animals

Adult guinea pigs of either sex weighing 400–450 grams were used for the isolated heart preparation, and as blood donors for the preparation of RBC suspensions used for perfusion. Blood from the donor animals was obtained by cardiac puncture under light ether anesthesia, anticoagulated with EDTA (1.5 mg/ml) and handled as described below. The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University and was in accordance with the Declaration of Helsinki and IASP guidelines.

2.2. Preparation of isolated-perfused heart

Guinea pigs were anaesthetized by urethane (1 g/kg) and the trachea cannulated. The right carotid artery was also cannulated using a 20 G cannula that was advanced to the root of the aorta. A mid-sternal thoracotomy was performed and the animal was ventilated with ambient air at a rate of 70/min with a tidal volume of 1 ml. The pericardium was opened, the root of the aorta carefully dissected, and a 3-0 silk suture positioned around the root where the 20 G cannula was positioned. The heart was then rapidly excised by cutting all blood vessels, following which the excised heart was briefly cooled by immersion in 4°C phosphate buffered saline (PBS; pH = 7.4). The silk suture around the cannula in the aorta was then tied and perfusion of coronary vessels initiated, at 100 mmHg perfusion pressure, with oxygenated PBS warmed to 37°C; perfusion was started within two minutes following excision of the heart. The heart was placed in a water-jacketed organ bath kept at 37°C; the bath was kept covered with a plastic film in order to keep the heart in a humid environment. The heart continued to beat during the entire perfusion period with the rate determined by intrinsic pacemakers within the heart's normal conduction system, and was always pumping against the perfusion pressure in the aorta. This preparation thus allowed inflow of various RBC suspensions into the aorta with outflow drained from the organ bath chamber.

2.3. Perfusion of isolated heart preparation

Perfusion of isolated hearts at selected and constant pressures was performed using a pressure servo-controlled pump system (Living Systems Inc., Burlington, VT). The system (Fig. 1) consisted of two roller pumps having constant flow rates of 3.73 and 3.36 ml/min and a variable speed roller pump with its speed controlled by the pressure servo-control device (PS/200/Q, Living Systems, Inc.). This device monitors the pressure at the entrance of the catheter in the aortic root and controls the variable speed pump to maintain the perfusion pressure between 30–100 mmHg. The two constant flow rate pumps

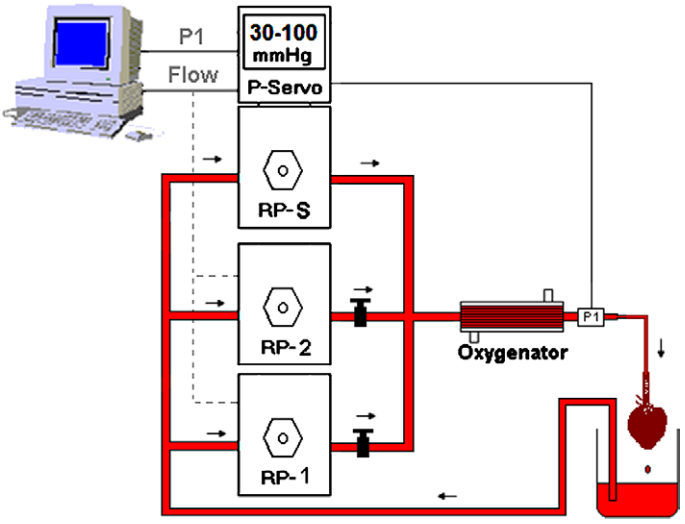


Fig. 1. Perfusion system for isolated heart preparation. RP-1 and RP-2 are constant flow rate roller pumps that can be turned on or off manually in order to achieve the flow rate necessary to obtain the desired perfusion pressure. RP-S is the servo-controlled pump whose flow rate is regulated by the feed back signal from the pressure transducer P1 (see text for details).

were activated manually as needed, either singly or together, in order to maintain the desired pressure. These pumps were not required at the lower pressures but were needed at higher pressures where the flow to maintain the pressure exceeded the capacity of the variable speed pump. Aortic perfusion pressure, together with volumetric flow rate obtained from the calibrated roller pumps, were recorded on a laboratory computer throughout the experiment. Flow resistance was calculated by dividing flow rate by perfusion pressure at each perfusion pressure.

The perfusion system employed complete re-use of the selected perfusate: the perfusate returning from the isolated heart was collected in a reservoir and then recirculated. An inline microfiber array oxygenator (Model OX, Living Systems, Inc.) was used to oxygenate the perfusion fluids (PBS or RBC suspensions) entering the aorta; the oxygenator employed room air, with the oxygen partial pressure of the RBC suspensions being 128 ± 15 mmHg (mean $\pm$ SE). RBC sedimentation problems in the perfusion system were minimized via a magnetic stirrer in the suspension reservoir and the use of small-bore tubing oriented primarily in a vertical position. The temperature of the entire system was controlled at 37°C via placing all relevant components in a closed, temperature-controlled plastic enclosure. As judged by the appearance of cell-free plasma, there was no detectable hemolysis in the perfusate throughout the entire perfusion period.

The protocol for the perfusion studies involved the following sequence: (1) using the desired perfusate, a series measurements were done with the isolated heart preparation having intact vascular tone; (2) the preparation was then perfused with PBS containing 10^{-4} M papaverine for 20 minutes in order to inhibit vascular smooth muscle tone; (3) the same series of measurements were repeated.

2.4. Modification of RBC aggregation

RBC aggregation was increased by employing a recently developed technique whereby poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) copolymers (Pluronics) that possess the ability to self-associate above a specific temperature are covalently attached to the RBC surface. The details of the method are described elsewhere [6]. Briefly, RBC and plasma were separated from anticoagulated (EDTA, 1.5 mg/ml) donor guinea pig blood by centrifugation at $1400 \times g$ for 6 minutes. The cells were washed three times with isotonic PBS, re-suspended in 30 mM triethanolamine buffer (290 mOsm/kg, pH = 8.60) at a hematocrit of ~0.1 l/l, and the suspension cooled to 4°C. The low value of hematocrit (i.e., ~0.1 l/l) was necessary in order to obtain an even coating of RBC with Pluronic and to avoid the formation of cross-linked aggregates of coated RBC. Immediately prior to use, a reactive succinimidyl carbonate derivative of Pluronic F-98 (13 kDa, 80% poly(ethylene glycol)) was dissolved in 4°C phosphate buffer (pH = 5) at a concentration of 10 mg/ml and added to the RBC suspension to obtain a final concentration of 0.025 mg/ml. The suspension was incubated for 2 hr at 4°C with continuous gentle mixing. All Pluronic-coating procedures were performed at 4°C (i.e., below the critical micellization temperature at which a phase transition occurs from predominantly single, fully hydrated copolymer chains to micelle-like structures). The Pluronic-coated RBC were washed 3 times with isotonic PBS by gentle centrifugation ($400 \times g$, five min), then re-suspended in native plasma at 0.4 l/l hematocrit.

2.5. Microscopic and rheologic studies

Wet mount preparations of dilute RBC suspensions used for the perfusion suspensions were examined and photographed using bright field light microscopy. Sedimentation rates were measured using Westergren sedimentation tubes and RBC–plasma suspensions adjusted to a hematocrit of 0.1 l/l.

2.6. Statistics

The data are presented as mean ± standard error. Comparisons were made using one-way and two-way ANOVA where appropriate. Pressure–flow and pressure–resistance curves were analyzed by non-linear regression analysis. Statistical significance was accepted at p values less than 0.05.

3. Results

3.1. Characteristics of RBC cell aggregation

Normal guinea pig RBC only slightly aggregate in autologous plasma, whereas Pluronic F98 coated RBC aggregates are larger (i.e., contain more RBC per aggregate) and form three-dimensional structures (Fig. 2). Erythrocyte sedimentation rates measured using 0.1 l/l hematocrit suspensions confirmed this enhanced aggregation, with about a hundred percent increase for the Pluronic coated RBC suspensions (Fig. 3).

3.2. Blood flow rate and flow resistance

In isolated heart preparations with intact vascular smooth muscle tone, blood flow increased with perfusion pressure for both control cells and those coated with Pluronic F98 (Fig. 4a). Flow resistance,

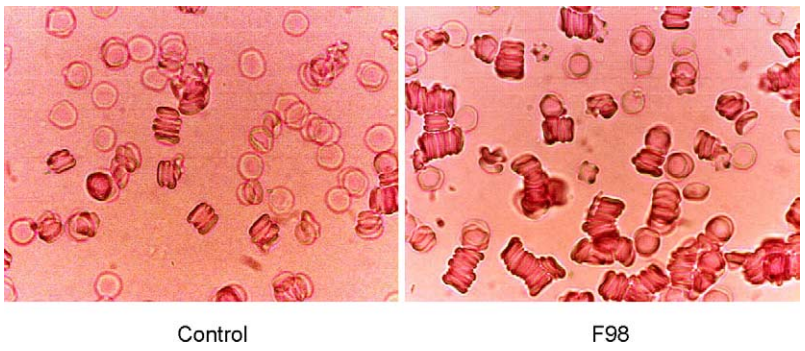


Fig. 2. Photomicrographs of RBC aggregates for control and Pluronic F98 coated cells suspended in plasma.

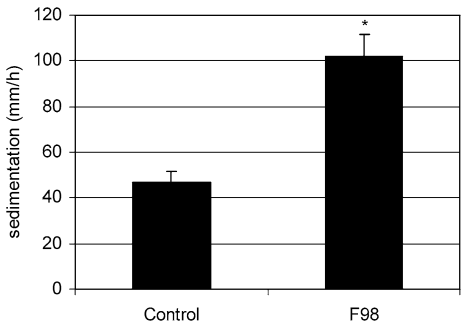


Fig. 3. Erythrocyte sedimentation rates of 0.1 l/l hematocrit suspensions for control and Pluronic F98 coated RBC in autologous plasma. Data presented as mean ± standard error. Difference from control; $^{*}p < 0.001$.

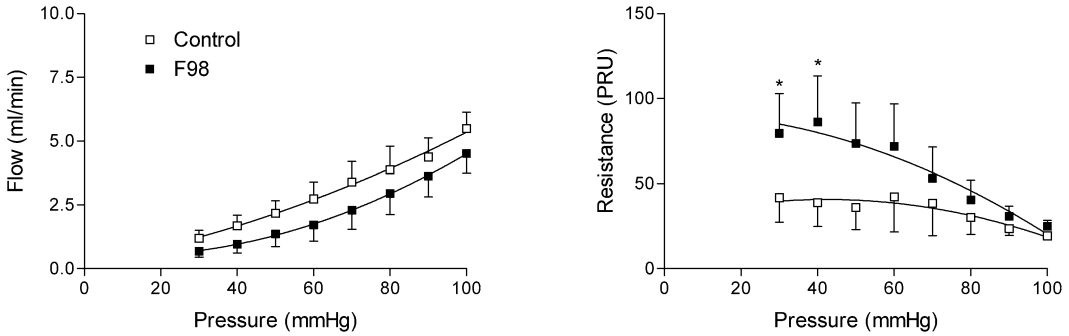


Fig. 4. Flow (a) and resistance (b) values plotted against perfusion pressure in isolated heart preparations with intact vascular smooth muscle tone. Data are mean ± standard error; $n = 6$ in each group. Difference from control; $^{*}p < 0.05$.

Table 1

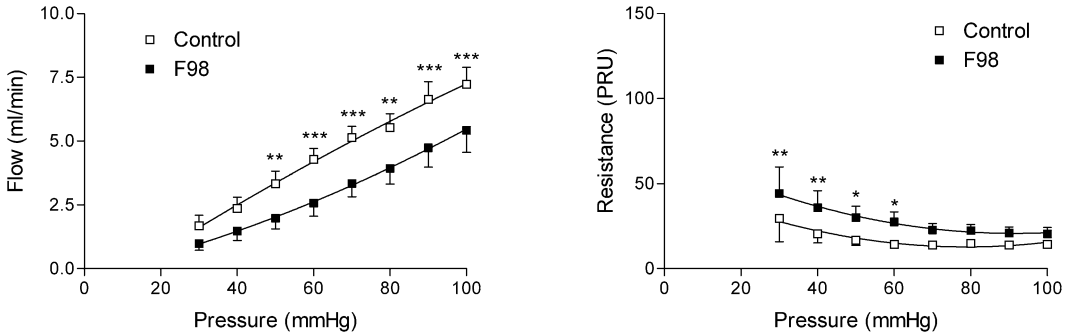
Linear regression equations and correlation coefficients for pressure–flow and pressure–resistance results obtained in intact and in papaverine-treated (Papa) preparations*

	Control		F98	
	Linear reg. equation	r	Linear reg. equation	r
Intact				
Flow	$y = -0.70 + 0.059x$	0.99	$y = -1.26 + 0.054x$	0.98
Resistance	$y = 53.45 - 0.30x$	−0.87	$y = 117.7 - 0.92x$	−0.97
Papa				
Flow	$y = -0.701 + 0.080x$	0.99	$y = -1.12 + 0.064x$	0.99
Resistance	$y = 28.63 - 0.17x$	−0.78	$y = 48.86 - 0.32x$	−0.93

*Equations were of the form $y = b + mx$, where y is either flow or resistance and x is pressure.

calculated as the ratio of perfusion pressure to blood flow, decreased for both suspensions with increasing perfusion pressure (Fig. 4b). However, it is clear from Fig. 4b that the dependence of flow resistance on perfusion pressure is more prominent for the coated RBC: for control cells resistance approximately doubled from 100 to 30 mmHg whereas there was about a 4-fold increase for coated cells over the same pressure range. Note that although the curves in Fig. 4 were drawn using second-order polynomial, similar "goodness of fit" results (i.e., correlation coefficients) were obtained using simple linear regression and the means at each pressure. Table 1 presents the linear regression results, where it can be seen that the regression line slopes for resistance were markedly higher for the coated RBC suspensions.

Figure 5 presents pressure–flow and pressure–resistance results for isolated heart preparations pretreated with 10^{-4} M papaverine. As anticipated, inhibition of vascular tone with papaverine greatly increased flow for both types of RBC suspensions at all pressures, and thus markedly reduced flow resistance (see Fig. 4). Flow measured during perfusion of papaverine-treated preparations with F98 coated RBC suspensions was decreased compared to normal guinea pig blood, with significant differences at perfusion pressures of 50 mmHg and higher (Fig. 5a). In addition, the differences in flow resistance between control and coated cells were significant over a wider range of perfusion pressures compared to the experiments in intact preparations (Fig. 5b). Linear regression results for resistance-pressure again indicated a steeper relation for perfusion studies using F98-treated RBC suspensions (Table 1).



(a) (b)

Fig. 5. Flow (a) and resistance (b) values plotted against perfusion pressure in isolated heart preparations treated with 10^{-4} M papaverine prior to the perfusion studies. Data are mean $\pm$ standard error; $n = 5$ in each group. Difference from control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

The results of this study clearly demonstrate that enhanced RBC aggregation leads to increased blood flow resistance in the myocardial vasculature, with the magnitude of this effect strongly influenced by perfusion pressure and vasomotor tone. However, it should be noted that these results may be specific to the experimental model used herein: (a) The isolated heart preparation has unique properties in terms of circulatory dynamics. The hydrodynamic conditions in the myocardial circulation are extremely complex [14] and results obtained in this environment may not be directly applicable to other vascular networks (e.g., resting skeletal muscle). Nevertheless, since a beating heart was employed, our experimental conditions may closely mimic the circulatory conditions in hearts with intact vascular connections; (b) The experimental model used in this study differs from previous studies due to the novel technique for increasing RBC aggregation. This new technique allows well-controlled modifications of aggregation intensity without altering plasma properties. Most previous *in vivo* and *in vitro* studies dealing with the hemodynamic consequences of RBC aggregation have involved the addition of polymers or proteins and hence alterations of plasma composition and viscosity [3,8,10,19,20]. Altered plasma viscosity may have significant effects on blood flow dynamics, especially at the microcirculatory level [18], and therefore it is important to differentiate between the effects of altered plasma viscosity versus altered RBC aggregation. The results of the current study reflect solely the influence of RBC aggregation and are not confounded by plasma viscosity alterations.

The dependence of flow resistance on perfusion pressure is an expected feature of hydraulic systems with elastic conduits, and relates, in part, to the effect of altered transmural pressure on vessel diameter. In systems perfused with blood, the non-Newtonian, shear-thinning behavior of blood also contributes to this dependence. The relationship between flow resistance and perfusion pressure is clearly seen in both Figs 4 and 5, with the dependence being more prominent when the perfusion is done with RBC suspensions having enhanced aggregation (Fig. 4, F98 group). This observation was also supported by regression analysis (Table 1); the negative regression coefficient in the F98 group is about 300% higher than control. Obviously, the stronger dependence of flow resistance on perfusion pressure in the F98 group led to significant differences from control at 30 and 40 mmHg perfusion pressures.

The two curves in Fig. 4b approach each other at higher perfusion pressures, with these higher pressures similar to those existing under physiological conditions. The degree of RBC aggregation is deter-

mined by the properties of blood itself (i.e., RBC intrinsic properties and plasma composition), but is also strongly influenced by the effective shear forces in the flow environment [16]. RBC aggregates may be dispersed during perfusion at higher pressures and hence higher flow, with the rheological behavior of the two suspensions (F98 and control) becoming similar. These findings suggest that the impact of intensified RBC aggregation may not be obvious at physiological perfusion conditions but would become evident when systemic or local circulatory functions are impaired. However, it should be noted that flow resistance at the highest perfusion pressure was, on average, about 30% higher for suspensions of F98 coated RBC compared to control cells (Fig. 4). While this difference at the highest perfusion pressure was *not* significant ($p > 0.1$), the apparent difference may reflect the influence of some aggregated RBC, even under higher shear stresses, due to the greater energy required to disperse F98-induced RBC aggregates [2]. Such aggregates may, to some degree, model the nature of RBC aggregation in pathological conditions where greatly enhanced aggregation is observed [16].

Experiments using preparations pre-treated with papaverine to inhibit smooth muscle tone were designed to provide insight to the role of vascular control mechanisms in the overall effect of altered RBC aggregation. Figure 5 indicates that flow resistance decreased significantly with papaverine treatment regardless of the aggregation intensity of the RBC suspension used for perfusion: this finding is the result of maximal vasodilation due to paralysis of vascular smooth muscles. There were also smaller flow resistance differences between control and F98 perfusions (e.g., the difference at 30 mmHg perfusion pressure was 88% in preparations with intact vascular control versus 51% in preparations with maximal vasodilation).

It is notable that differences between control and F98 resistance values were statistically significant at pressures of 60 mmHg or less (Fig. 5b), while the differences were only significant at pressures of 40 mmHg or less in hearts with intact vascular control (Fig. 4b). Careful examination of the results reveals that this apparent anomaly relates to differences in the variation of the data (Figs 4 and 5): the flow resistance data are characterized by smaller variations when vascular smooth muscle is paralyzed, with coefficients of variation in the papaverine treated preparations from 22% to 47% at pressures in the lower range, while the coefficients of variation were 78% to 119% for preparations with intact vascular tone over the same pressure range. Obviously, the greater variations for the intact preparations reflect intrinsic alterations of vascular tone which may not be equally effective in all preparations, whereas the autoregulatory response is abolished by papaverine. Therefore, based on these considerations, it appears that the impact of intensified RBC aggregation can be compensated for by vascular control mechanisms until the perfusion pressure decreases to very low levels. However, comparison of the control data presented in Figs 4 and 5 indicates that papaverine treatment resulted in a 26% to 40% decrement in flow resistance at physiological perfusion pressures. This decrement seems smaller than might be expected based upon the effective autoregulatory response of the coronary vasculature to reactive hyperemia [12]. This limited vasodilatory reserve observed in the isolated heart preparations used herein could be due to technical factors, and thus should be considered in applying these results to intact hearts.

It is important to underline the complexity of factors contributing to the overall *in vivo* hemodynamic effects of altered RBC aggregation. This complexity makes it extremely difficult to directly compare literature findings obtained under various conditions. For example, it has been previously demonstrated that flow resistance in an isolated guinea pig hind limb preparation was lower when perfused with aggregating RBC suspension (i.e., human RBC suspended in 3% dextran 70) compared to a non-aggregating RBC suspensions, with the difference only evident during perfusion at low flow rates [3]. These prior findings seem to contradict the results of the present study: flow resistances at lower flow rates were higher during perfusion with F98-coated RBC suspensions (Fig. 6). However, the following points

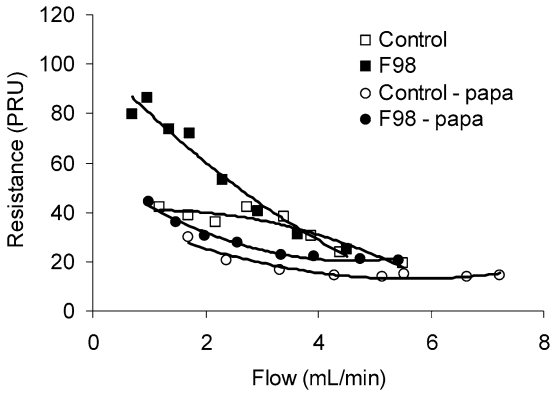


Fig. 6. Resistance values plotted against flow in isolated heart preparations with intact vascular tone (control) or treated with 10^{-4} M papaverine (papa) prior to the perfusion studies in order to abolish vascular tone.

should be considered in order to properly compare the two sets of data: (1) F98 coating of RBC results in a well-controlled, concentration dependent increment in aggregation. It has recently been shown that the hemodynamic effects of enhanced aggregation are determined by the intensity of aggregation and hence the concentration of F98 during the coating process [21]. Perfusion of guinea pig hindlimbs with suspensions of RBC coated with F98 at 0.025 mg/ml resulted in elevated flow resistance, whereas flow resistance was unaltered from control during perfusion with suspensions of RBC having a greater degree of aggregation due to the use of higher F98 levels [21]. Thus, the relationship between aggregation intensity and flow resistance may not be linear, but rather characterized by a multi-phasic response. It is therefore important to characterize the degree of enhanced aggregation when comparing experimental results, especially when aggregation and plasma characteristics are both altered (e.g., adding dextrans to suspending medium); (2) F98 coating of RBC can result in stronger aggregates (i.e., aggregates requiring higher shear forces for dispersion) compared to fibrinogen- or dextran-induced aggregates. RBC aggregates may thus exist in a greater portion of a circulatory network during perfusion with F98-coated RBC suspensions, thereby resulting in a more pronounced enhancement of flow resistance; (3) The preparation used herein for heart perfusion studies differed from that employed in previous studies [3,21]. The isolated, beating-heart preparation has unique hemodynamic characteristics that are not comparable with an isolated hind-limb preparation. Flow in the coronary vasculature varies throughout the cardiac cycle due to extravascular compression during systole [14], and this variability may have affected the formation of cell-poor layers adjacent to vessel walls and hence related mechanisms that lead to decreased vascular resistance. Experiments repeated on non-beating heart preparations may eliminate some of the factors that are specific to the preparation used in this study, and further aid understanding of the specific role of RBC aggregation in coronary circulation However, it is extremely difficult to control all relevant factors that play a role in this complex relationship, especially under clinical conditions; future work in this area will need to acknowledge this problem and develop suitable experimental protocols to either selectively examine or minimize their effects.

Acknowledgement

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Graded alterations of RBC aggregation influence in vivo blood flow resistance

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Yalcin, Ozlem, Murat Uyuklu, Jonathan K. Armstrong, Herbert J. Meiselman, and Oguz K. Baskurt. Graded alterations of RBC aggregation influence in vivo blood flow resistance. *Am J Physiol Heart Circ Physiol* 287: H2644–H2650, 2004. First published July 29, 2004; doi:10.1152/ajpheart.00534.2004.—Although the effects of red blood cell (RBC) aggregation on low-shear rate blood viscosity are well known, the effects on in vivo flow resistance are still not fully resolved. The present study was designed to explore the in vivo effects of RBC aggregation on flow resistance using a novel technique to enhance aggregation: cells are covalently coated with a block copolymer (Pluronic F-98) and then suspended in unaltered plasma. RBC aggregation was increased in graded steps by varying the Pluronic concentration during cell coating and was verified by microscopy and erythrocyte sedimentation rate (ESR), which increased by 200% at the highest Pluronic level. RBC suspensions were perfused through an isolated in situ guinea pig hindlimb preparation while the arterial perfusion pressure was held constant at 100 mmHg via a pressure servo-controlled pump. No significant effects of enhanced RBC aggregation were observed when studies were conducted in preparations with intact vascular control mechanisms. However, after inhibition of smooth muscle tone (using 10^{-4} M papaverin), a significant change in flow resistance was observed in a RBC suspension with a 97% increase of ESR. Additional enhancements of RBC aggregation (i.e., 136 and 162% increases of ESR) decreased flow resistance almost to control values. This was followed by another significant increase in flow resistance during perfusion with RBC suspensions with a 200% increase of ESR. This triphasic effect of graded increases of RBC aggregation is most likely explained by an interplay of several hemodynamic mechanisms that are triggered by enhanced RBC aggregation.

red blood cell; Pluronic coating; poloxamer; hemodynamics; sedimentation rate

ALTHOUGH RED BLOOD CELL (RBC) aggregation is generally accepted as a major determinant of low-shear whole blood viscosity (11), the effects of RBC aggregation on in vivo flow resistance are less conclusive. Numerous in vitro studies have been conducted to explore the effects of RBC aggregation on tube flow. The earliest study was by Fahreaus, who indicated that increased RBC aggregation resulted in thickening of a marginal zone of cell-poor plasma and decreased hydraulic resistance (28). More recent studies (2–4, 15, 22, 35, 38, 39) have confirmed these observations and described the effects of shear rate and tube diameter and the time dependency of the phenomenon. The effects of RBC aggregation also depend on the orientation of the flow system, as follows: *1*) in straight, horizontal tubes at low flow rates, aggregation-induced sedimentation of RBCs results in increased viscosity due to an elevation of tube hematocrit (Hct; Refs. 2, 4, 34); and *2*) in straight, vertical tubes at low flow rates, RBC aggregation leads to the formation of a cell-poor layer at the tube wall and an irregular core of RBCs at the tube center (3, 22, 37, 38, 42), which yields decreased flow resistance and lower apparent viscosity (3).

Given the geometric complexity of the microcirculation (36), observations in straight tubes may not be directly applicable to in vivo flow conditions. Frequent branching is characteristic of microvessels, and thus the residence time of blood in an individual vessel may not be sufficient to allow phase separation (3, 15). Furthermore, the orientation of individual microvessels may affect the development of a cell-poor marginal zone (22), and vascular control mechanisms may additionally complicate predictions based on in vitro findings (12). In fact, classical isolated limb and muscle studies by Whittaker and Winton (47) and later by Djojosugito et al. (23) have shown that the apparent viscosity of blood in vivo is lower than that measured in vitro. Factors such as the Fahraeus-Lindqvist effect (47), inertial energy loses, altered vessel geometry (27), and phase separation related to RBC aggregation have thus been suggested to play roles in the differences between in vivo and in vitro measurements of blood viscosity.

Several microvascular studies aimed at determining the specific effects of RBC aggregation on in vivo blood flow resistance have been reported; however, the results are not always in concordance. Intravital microscopy studies indicate that intensified RBC aggregation increases microvascular flow resistance (16, 17, 25, 32, 46). The effects of RBC aggregation on venous blood flow dynamics have been extensively studied (15–20), and results indicate that shear forces are low enough to allow significant aggregation and hence a predicted reduction in flow resistance. However, the expected reduction of flow resistance after increasing RBC aggregation by infusing 500-kDa dextran was not observed, which leads to the suggestion that RBC transit times through the nonbranched lengths of venules are too short to allow development of sufficient axial migration (15, 17–19). Conversely, using rat mesentery and cremaster preparations, Drussel et al. (25) indicated that greatly enhanced RBC aggregation achieved via 500-kDa dextran infusion markedly increased microvascular flow resistance. These authors report that a 5-fold increase of aggregate strength resulted in a 13-fold increase of mesentery resistance and a 3-fold increase in cremaster resistance. The difference between the vascular beds was suggested to be due to the effectiveness of blood flow regulation mechanisms (25).

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Studies of RBC aggregation effects in whole organ preparations are also not concordant and indicate that enhanced aggregation decreases, increases, or has no effect on flow resistance. Using a perfused isolated rat heart and 70-kDa dextran, Charansonney et al. (21) indicated that mild aggregation reduced resistance, whereas greatly enhanced aggregation elevated flow resistance. However, increased RBC aggregation caused by high-molecular-mass dextrans either increased blood flow resistance in the liver (39) or had no effect on uteroplacental blood flow (44). In a cat muscle preparation, Cabel et al. (20) observed an inverse relation between venous conductance and blood flow for normal blood, and this effect was reduced or absent for either nonaggregating or highly aggregating RBC suspensions; they thus concluded that RBC aggregation significantly contributes to venous vascular resistance in resting muscle and plays an important role in vascular system homeostasis (45). Using an isolated perfused rat hindlimb preparation and viscosity-matched suspending media, Baskurt et al. (7) observed that flow resistance was lower for aggregating vs. nonaggregating RBC suspensions.

As noted above, the results of microcirculatory studies do not agree with the findings of whole organ perfusion studies. Vicaut (45) mentioned that this controversy could be explained by the opposite effects of RBC aggregation on blood flow resistance at two different circulatory levels. The apparent viscosity reduction discussed above might be counteracted by the higher energy cost at the entrance of capillaries for disaggregation of RBCs in aggregating blood samples. Obviously, this higher energy cost should be reflected by microcirculation studies, whereas the whole organ studies should reflect a balance between the two opposing effects (45). Another explanation for the contradictory findings of microcirculation and whole organ studies might be the orientation of blood vessels under investigation. Most microcirculation studies that employ intravital microscopy technique are performed using tissues spread on a microscope stage, and blood flow orientation is usually horizontal. RBC aggregation has been demonstrated to increase flow resistance in tubes with such an orientation due to the sedimentation of aggregates (22). However, it has been suggested that in the intact vascular system, the residence times of blood in the vascular segments are insufficient for meaningful development of this sedimentation effect (15).

It should be noted that essentially all of the above-mentioned studies regarding the in vivo effects of RBC aggregation have utilized either large proteins (e.g., fibrinogen) or high-molecular-mass polymers (e.g., 70- or 500-kDa dextran) infused into the circulation to enhance aggregation. The present study was designed to employ polymer-coated RBCs (5) in native plasma to study the effects of graded alterations of RBC aggregation on vascular resistance in a guinea pig isolated and perfused hindlimb preparation.

MATERIALS AND METHODS

Animals. Adult guinea pigs of either sex (400–500 g body wt) were used in this study. Blood for perfusion of the hindlimbs of these animals was obtained from donor animals under light ether anesthesia by cardiac puncture and was anticoagulated with EDTA (1.5 mg/ml). The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University and was in accordance with the Declaration of Helsinki and International Association for the Study of Pain guidelines.

Modification of RBC aggregation. RBC aggregation was modified by employing a novel technique whereby copolymers that possess the ability to self-associate above a specific temperature are covalently attached to the RBC surface. These triblock copolymers, which are commercially known as Pluronics and commonly known as poloxamers, are composed of a central hydrophobic block of polypropylene glycol (PPG) flanked by two identical hydrophilic polyethylene glycol chains (40). Pluronics exhibit a critical micellization temperature (CMT) at which a phase transition occurs from predominantly single, fully hydrated copolymer chains to micellelike structures that are characterized by association of the hydrophobic PPG blocks (1, 6, 14). The CMT decreases with increasing mass of the PPG block, and by the appropriate selection of a Pluronic, RBC aggregation can be controllably and predictably enhanced or inhibited following covalent attachment of the Pluronic to the RBC surface (5). That is, at temperatures above the CMT, Pluronics attached to the RBC surface micellize with Pluronics on adjacent RBCs and thereby enhance RBC aggregation; the strength of the association is dependent on the temperature and the concentration of Pluronic used during the coating process. Conversely, below the CMT, Pluronics attached to the RBC surface inhibit RBC aggregation due to polymer-polymer repulsion (5).

Copolymer derivatization. Pluronic copolymers were a gift from BASF Performance Chemicals (Parsippany, NJ). These poloxamers contained 80% polyethylene glycol by mass and were designated as Pluronic F-68 (8.4 kDa) and Pluronic F-98 (13.0 kDa). To facilitate covalent attachment of the Pluronics to the RBC surface, a reactive derivative of the copolymers was first prepared. This was achieved by converting the terminal hydroxyl groups of the copolymer chain to succinimidyl carbonate (SC) groups as previously described (13) using a modification of the method described by Miron and Wilchek (33).

RBC coating. The SC derivatives of Pluronic F-68 (F68-SC) and Pluronic F-98 (F98-SC) covalently attach to primary amines, principally lysine residues, on the RBC surface when incubated at an elevated pH ($>$7.50) for a short time period (ca. $<$1 h). Any unreacted SC derivative hydrolyzes during the incubation period to a nonreactive form that is simply removed during subsequent RBC washing steps. To achieve graded increases of RBC aggregation, cells were incubated with various concentrations of the reactive F98-SC. At 37°C, Pluronic F-68-coated RBCs in autologous plasma exhibit aggregation and low-shear viscosity essentially identical to uncoated RBCs (14), and thus Pluronic F-68 was selected as an appropriate control for the presence of covalently attached copolymer without enhanced RBC aggregation.

Blood was centrifuged at 1,400 *g* for 6 min, and the plasma was separated and saved. RBCs were washed three times with isotonic phosphate-buffered saline (PBS, pH 7.4) and resuspended in 30 mM triethanolamine buffer (290 mosM/kg, pH 8.60) at a Hct of ~0.1 l/l, and the suspension was cooled to 4°C. All Pluronic-coating procedures were performed at 4°C (i.e., below the CMT) to obtain even coating of Pluronic on RBCs and to avoid the formation of cross-linked aggregates of coated RBCs. Immediately before use, the Pluronic-SC was dissolved in a 4°C hypotonic phosphate buffer (50 mM NaH_2PO_4 and 60 mM NaCl, pH 5) at a concentration of 10 mg/ml. The Pluronic-SC solution was promptly added to RBCs suspended in triethanolamine buffer to obtain six final concentrations between 0.00125 and 0.5 mg/ml, and the suspension was incubated for 2 h at 4°C with continuous gentle mixing on a tube rocker. Coated RBCs were separated, washed three times with PBS by gentle centrifugation (400 *g* for 5 min), and then resuspended in plasma at 0.4 l/l Hct.

Microscopic and rheologic studies. Wet mount preparations of dilute RBC suspensions in plasma were examined and photographed using bright-field light microscopy. Sedimentation rates were measured using Westergren sedimentation tubes, and RBC-plasma suspensions were adjusted to a Hct of 0.1 l/l. Owing to blood volume

demands of the perfusion protocol, these sedimentation rates were determined with a separate group of samples ($n = 4$) prepared as described above.

Isolated hindlimb preparation. Guinea pigs were anesthetized with urethane (20% solution in isotonic saline, 1 g/kg). The lower abdomen was opened by a midline incision, and a 24-gauge cannula was inserted into the left iliac artery and advanced to the femoral artery. The ipsilateral iliac vein was also catheterized using the same size of cannula. Visible branches throughout the dissected tract of the femoral artery and vein (i.e., collateral and cutaneous vessels) were cauterized to minimize flow through such routes, but the hindlimb was not surgically isolated from the rest of the body. The left leg was cooled during the cannulation procedure by externally applied ice. After cannulation was complete, the animal was euthanized via an overdose of pentobarbital sodium. Just before we started the perfusion studies, the lower body of the animal was submerged into a 37°C water bath.

Perfusion of the hindlimb preparation. The arterial cannula was connected to a pressure servo-control system composed of a roller pump, a pressure transducer monitoring the inflow pressure, and a control unit (PS/200/Q, Living Systems Instrumentation). A microfiber array oxygenator (model OX, Living Systems Instrumentation) was used in the arterial line to oxygenate the perfusion fluids (PBS or RBC suspensions) with room air. The Po_2 of the RBC suspensions used for perfusion was 130 ± 17 mmHg. RBC sedimentation problems in the perfusion system were minimized via a magnetic stirrer in the suspension reservoir and the use of vertically oriented small-bore tubing. The arterial perfusion pressure was set to 100 mmHg for all experiments; venous pressure was also recorded for the perfused limb. Arterial and venous pressures together with the volumetric flow rate obtained from the calibrated speed of the roller pump were recorded on a laboratory computer throughout the experiment. RBC suspensions were either uncoated or Pluronic-coated RBCs in plasma at a Hct of 0.4 l/l.

The protocol for the studies involved perfusing the hindlimb using the following sequence: *1*) cell-free PBS for 30 min, *2*) 5 ml of uncoated RBC suspension (control), *3*) 10 ml of PBS, *4*) 5 ml of coated RBC suspension, *5*) 10 ml of PBS before perfusion with the next RBC suspension, and *6*) repeat of *steps 4* and *5* for each test RBC suspension. Typical washout volumes ranged from 0.5 to 0.8 ml, and thus pressure and volumetric flow data used for calculation of flow resistance for PBS and RBC suspensions were obtained during the last 60 s of stable perfusion. For cells coated with Pluronic, the order in which the six different coating concentrations (0.00125–0.5 mg/ml) were used was varied between different preparations (i.e., low to high and high to low). Vascular resistance was calculated from the arterial-venous pressure difference and the volumetric flow rate. Flow resistance values were corrected for differences in vascular hindrance between preparations by dividing the calculated values by the flow resistance obtained during perfusion with cell-free PBS.

Blockage of smooth muscle tone. In one series of experiments, the PBS used for the initial 30-min perfusion contained 10^{-4} M papaverin to inhibit smooth muscle tone. This level of papaverin was also added to the RBC suspensions and the PBS perfused following each RBC suspension.

Statistics. Data are presented as means ± SE. Multiple comparisons were done by one-way ANOVA followed by Newman-Keuls post test or two-way ANOVA followed by a Bonferroni post test. Statistical significance was accepted at P values <0.05.

RESULTS

Graded alteration of RBC aggregation. Microscopic observations of dilute wet mounts of cells in plasma indicated that RBC aggregation was enhanced in a graded manner after incubation with F98-SC at concentrations between 0.00125 and 0.5 mg/ml (Fig. 1). It should be noted that the degree of aggregation (i.e., number of RBCs per aggregate) increased with F98-SC concentration and that three-dimensional structures dominate at and above F98-SC levels of 0.05 mg/ml. RBC aggregation quantified by measuring the sedimentation rate of dilute suspensions (i.e., 0.1 l/l Hct) was consistent with the microscopic observations, with the increases of RBC sedimentation statistically significant at and above F98-SC levels of 0.025 mg/ml (Fig. 2). Treatment of RBCs with F68-SC at concentrations between 0.00125 and 0.5 mg/ml had no effect on RBC aggregation as judged by both microscopic observations and sedimentation rates (data not shown).

Flow resistance in hindlimb preparations. Hydraulic resistance as determined during cell-free PBS perfusion of hindlimbs from seven animals averaged 24.8 ± 5.6 peripheral resistance units (PRU); this value decreased by 35% ($P < 0.05$) in six hindlimbs in which smooth muscle tone was inhibited with papaverin (Fig. 3).

At the 100-mmHg constant perfusion pressure employed for all studies, average blood flow for uncoated RBCs in seven hindlimbs with intact local vascular control mechanisms was 559 ± 129 μl/min [coefficient of variation (CV) = 61%]. These blood flow data were characterized by a substantial variation of individual flow rates ranging between 183 and 1,171 μl/min; the calculated mean blood flow resistance in this group was 251.3 ± 60.5 PRU (Fig. 3). In a separate group of six hindlimbs perfused with uncoated RBCs, blockage of vascular smooth muscle tone by papaverin resulted in a 130% increase of average blood flow rate to 1,279 ± 79 μl/min (CV = 15%) and a lower variation between animals (range, 999–1,456 μl/min). The calculated mean blood flow resistance in the papaverin-treated group was 83.1 ± 5.7 PRU and was thus significantly lower ($P < 0.001$) than the group with intact vascular control (Fig. 3).

Altered flow resistance by increased RBC aggregation. Flow resistance values measured during perfusion with RBC suspensions treated with various concentrations of F98-SC are presented in Fig. 4. The values shown are corrected for differences in vascular hindrance in each hindlimb (i.e., flow resistance with cell-free PBS) and are expressed as the percentage of the value determined during perfusion with uncoated RBCs. In the experiments with intact vascular control mechanisms, flow resistance decreased slightly for RBCs treated with F98-SC at a concentration of 0.0125 mg/ml; this concentration also caused a significant 60% increase in sedimentation rate (see Fig. 2). With increasing F98-SC levels, flow resistance tended to increase and then decrease, although none of the alterations at any F98-SC level were significantly different from uncoated cells. However, in the papaverin-treated hindlimb preparations, there was an increment of flow resistance during perfusion with RBC suspensions treated with 0.0125 mg/ml F98-SC. At 0.025 mg/ml F98-SC, the increase was more profound and significantly greater than for uncoated RBCs (see Fig. 4); this F98-SC concentration resulted in a 97% increase in sedimentation rate (see Fig. 2). Additional increases in F98-SC concentration to 0.05 and 0.25 mg/ml, which correspond to 136 and 162% increments of RBC sedimentation rates, resulted in a decrease in flow resistance almost to uncoated cell values. At the highest F98-SC concentration (0.5 mg/ml, a 200% increase of sedimentation rate), flow resistance was again significantly increased (see Figs. 2 and 4). Note that in papaverin-treated hindlimb preparations perfused with F68-SC-treated RBCs, no significant differences in flow resistance vs. uncoated cells

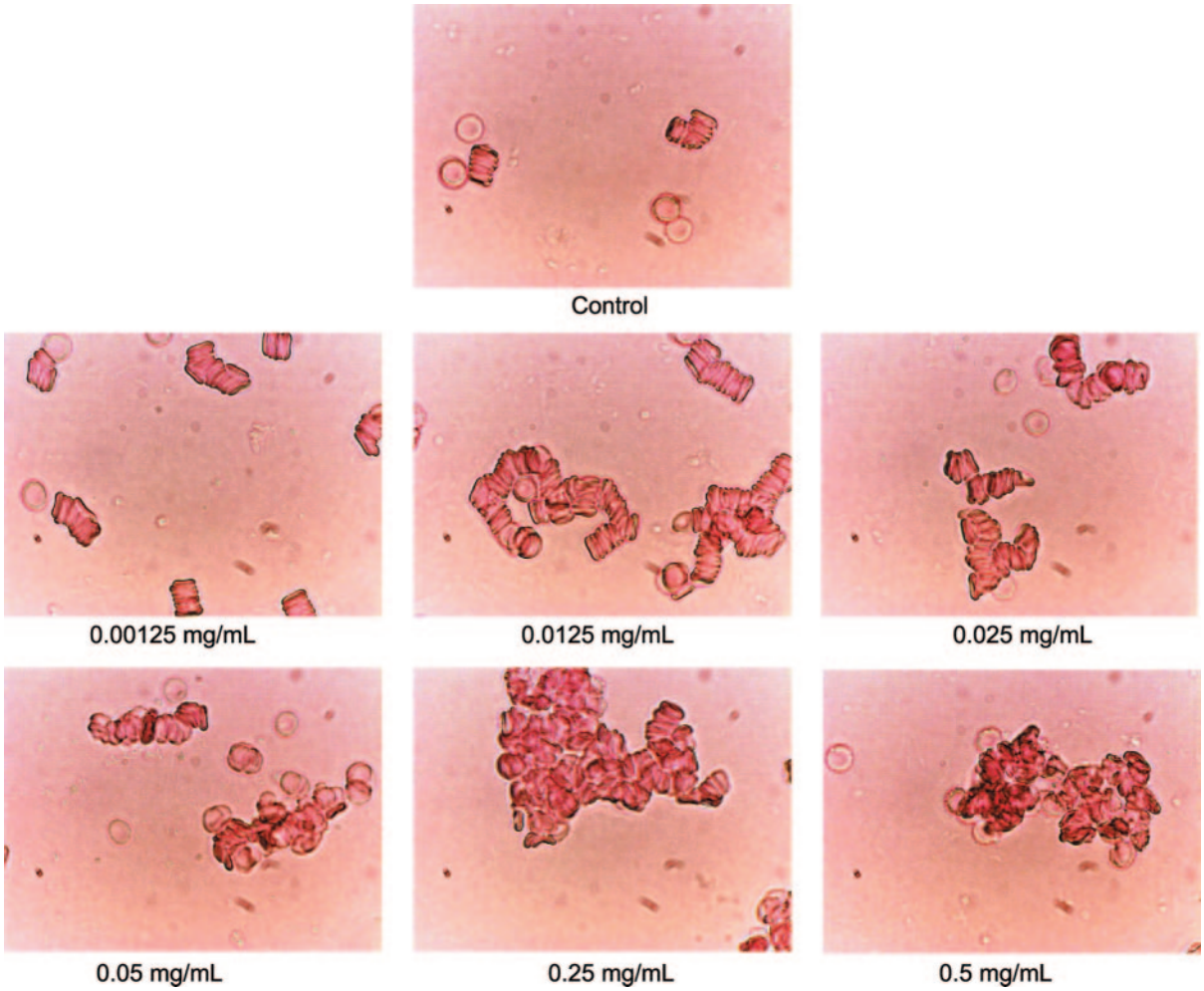

Fig. 1. Photomicrographs of red blood cells (RBCs) in plasma for uncoated (control) cells and cells coated with Pluronic F-98 at concentrations between 0.00125 and 0.5 mg/ml.

were observed at any concentration between 0.00125 and 0.5 mg/ml (Fig. 5).

DISCUSSION

The unique aspect of the present study is the novel method used to modify the aggregation behavior of RBCs when suspended in anticoagulated but otherwise unaltered and undiluted plasma. The method is based on the technique developed by Armstrong and co-workers in which nonionic Pluronic copolymers that possess the ability to self-associate (micellize) are covalently attached to the RBC membrane (5). This technique allows graded changes of aggregation by varying the concentration of the copolymer during the RBC coating process. Note

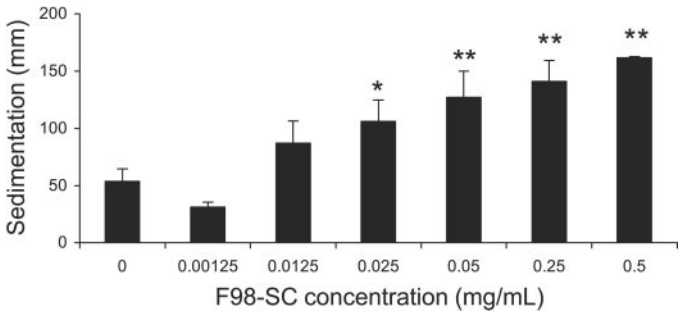

Fig. 2. RBC sedimentation, measured as (millimeter) drop in interface after 30 min for 0.1 l/l hematocrit RBC-plasma suspensions before and after RBCs were coated with Pluronic F-98 at concentrations of 0.00125–0.5 mg/ml. *$P <$ 0.05; **$P <$ 0.01, differences from uncoated RBC suspensions (i.e., zero concentration).

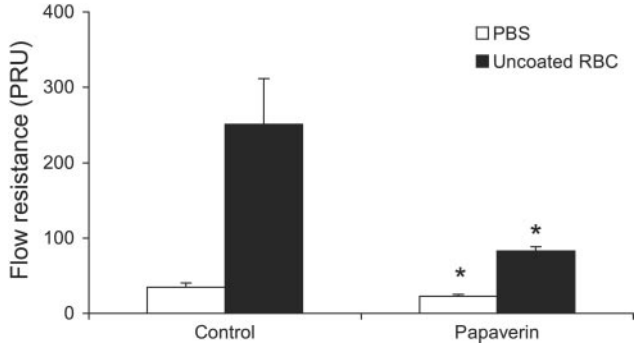

Fig. 3. Flow resistance [in peripheral resistance units (PRU)] calculated using flow rates during perfusion with PBS and with uncoated RBCs in plasma for hindlimb preparations with intact vascular control mechanisms (control) and for papaverin-treated preparations. Values are means ± SE. *$P <$ 0.05, difference from corresponding control value.

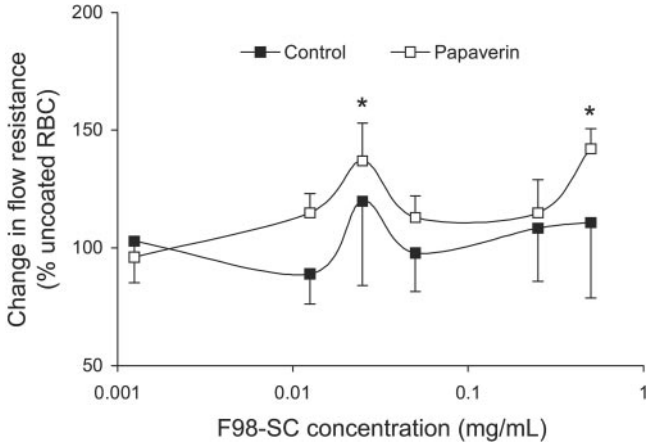

Fig. 4. Flow resistance during perfusion with RBC suspensions treated with SC derivatives of Pluronic F-98 (F98-SC). Results are expressed as the percentage of flow resistance during perfusion with uncoated cells, and thus a value of 100 indicates equality with uncoated RBCs. Data are for seven hindlimb preparations with intact vascular control mechanisms (control) and for six papaverin-treated preparations. *$P < 0.05$, difference from flow resistance measured during perfusion with uncoated cells.

that because F68-coated cells behaved like uncoated cells (Fig. 5), it is possible to exclude rheological artifacts (e.g., decreased RBC deformability) due to copolymer coating.

In contrast with the present study, almost all previous reports designed to investigate hemodynamic consequences of RBC aggregation have employed infusion of high-molecular-weight macromolecules such as dextrans or starches (7, 17, 20, 25, 32, 39, 44–46). However, these polymer infusion approaches have several disadvantages. First, it is not always possible to finely vary the degree of RBC aggregation, and thus most studies have employed two levels of aggregation (i.e., "normal" and "enhanced") instead of a range of aggregation tendencies. Second, high-molecular-weight molecules introduced into the circulation not only modify RBC aggregation but also influence the chemical and physical properties of plasma (e.g., increased viscosity and oncotic pressure). For example, 500-kDa dextran plasma levels of 0.6 to 1.4 g/dl as used in perfusion studies (16–19) result in 40–100% calculated increases of plasma viscosity (43). In turn, elevated medium viscosity may have significant effects on in vivo hydrodynamic resistance by altering local shear forces and hence vascular geometry (36). Furthermore, high-molecular-mass dextrans have very nonlinear van't Hoff's relations, and thus the resulting plasma oncotic pressure would be much greater than predicted simply by polymer concentration. Third, infusion of any macromolecular solution that is retained in the circulation dilutes plasma proteins and reduces RBC, white blood cell, and platelet counts. Finally, appropriate control experiments using lower-molecular-mass polymers (e.g., 40-kDa dextran) can be difficult to design and undertake due to differences in plasma viscosity at comparable polymer concentrations, in polymer concentration at a constant plasma viscosity, in clearance route, and in clearance rate over the time course of the experiment.

In addition to overcoming the abovementioned disadvantages, the Pluronic-coating method may permit exploration of the effects of abnormally elevated "pathological" aggregation of cells in native plasma. Aggregation at stasis for cells in normal plasma is characterized by linear arrays of RBCs (i.e., RBC rouleaux) with some degree of side and end branching of the rouleaux (26, 31, 41); such aggregation is observed for uncoated guinea pig RBCs and, to a certain extent, for RBCs treated with the lower concentrations of F98-SC (see Fig. 1). However, RBC aggregation in pathophysiological states (e.g., poorly controlled diabetes, nephrotic syndrome, myocardial infarction, and severe acute phase reaction) is much more intense, and the structures consist of somewhat shorter rouleaux formed into irregular three-dimensional clumps (31) that are more difficult to disperse by fluid shear forces (41). Although F98-coated RBCs show nonrouleaux formation at stasis (see Fig. 1), the aggregation is reversible with increasing shear (5), and the aggregability is controllable with F98-SC concentration. Intense, reversible aggregation can readily be induced at elevated F98-SC levels (see Fig. 1), which leads to greatly elevated low-shear blood viscosity (5) and may facilitate the development of in vivo models that are relevant to those conditions associated with greatly abnormal RBC aggregation.

The experimental results presented in Fig. 4 indicate that alterations of RBC aggregation tendency can influence blood flow resistance in the isolated hindlimb of guinea pigs. Two important aspects of these results are that the changes of flow resistance resulting from altered RBC aggregation intensity are modulated by local vascular control mechanisms, and the nature of the alterations in flow resistance does not vary monotonically with RBC aggregation intensity. With regard to the resistance results, note that although all visible branches of the femoral artery and vein (i.e., collateral and cutaneous vessels) were cauterized, the hindlimb was not surgically isolated, and thus arterial blood flow could include contributions from noncauterized vessels. The proportion of this type of flow was not determined due to technical limitations.

Dealing first with the influence of local vascular control mechanisms, it is notable that in hindlimbs treated with papaverin, enhanced RBC aggregation resulted in significantly increased flow resistance at two levels of aggregation intensity (i.e., for cells treated with 0.025 and 0.5 mg/ml F98-SC). Conversely, in control preparations with intact local vascular control, the lower concentration yielded only a nonsignificant increase, and no meaningful trend vs. uncoated cells was seen at the higher level. The local physiological mechanisms (i.e.,

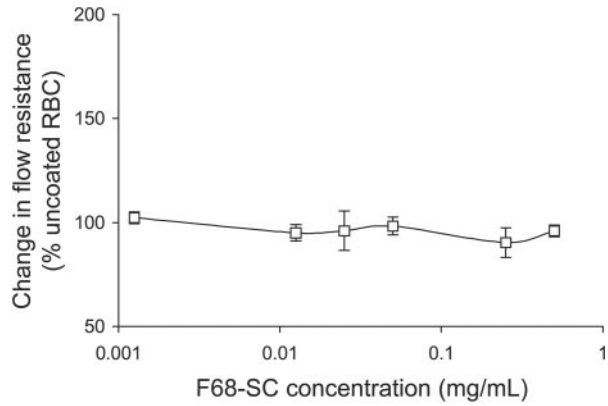

Fig. 5. Flow resistance during perfusion with RBC suspensions treated with SC derivatives of Pluronic F-68 (F68-SC). Results are expressed as the percentage of flow resistance during perfusion with uncoated cells, and thus a value of 100 indicates equality with uncoated RBCs. Data are for six papaverin-treated preparations.

metabolic and myogenic responses) that regulate vascular geometry are very effective in maintaining adequate blood flow in a given vascular bed (12), and the metabolic demand of the tissue plays an important role. Any acute alteration that affects the balance between this demand vs. supply provided by the blood flow is usually compensated for by the alterations of vascular hindrance (24). Intravascular pressure changes due to altered perfusate viscosity could also affect vascular hindrance via the myogenic response, and it has been shown that compensation for alterations of hemorheological properties (e.g., increased Hct and decreased RBC deformability) can be achieved if the tissue has sufficient vasodilatory reserve (10, 12). The differences between the responses of the two types of preparations (see Fig. 4) thus most likely have their basis in the degree of vasodilatory reserve available in the control hindlimbs. Note that the vascular tone in the control group (i.e., the nonpapaverin-treated group) might be lower than for normal animals inasmuch as central nervous influences were not present in the isolated limb preparation. Therefore, the ability to compensate for hemorheological alterations should be even greater in normal animals with intact central nervous control of vascular tone. Conversely, the somewhat elevated P_{O_2} value of the RBC suspensions used for perfusion and the resulting vasoconstriction could have augmented vasodilatory reserve in the control group.

Differences in the degree of vasodilatory reserve are also probably responsible for the variations (i.e., standard error) observed in the control data. Preparation to preparation differences of flow resistance for the control hindlimbs were large, especially at 0.025 and 0.5 mg/ml F98-SC, and were greater than for the corresponding concentrations in the papaverin series (see Fig. 4); flow resistance variations were also large when uncoated cells were used in the intact control preparations (see Fig. 3). These considerable variations most likely reflect the wide range of basal vascular tone in the intact preparations and could thus explain the large variations of flow resistance with enhanced RBC aggregation (see Fig. 4). That is, because the degree of vasodilatory reserve strongly depends on the level of vascular tone (12, 24), an initially high tone allows a large degree of compensation (i.e., vasodilation) for hemorheological abnormalities, whereas an initially low tone would have a smaller vasodilatory reserve available for compensation.

An interesting finding of this study is the nonmonotonic dependence of flow resistance alterations on RBC aggregation intensity: increasing intensity resulted in two regions of increased flow resistance separated by a region of essentially unaltered resistance (see Figs. 1, 2, and 4). Previous reports have primarily employed infusions of high-molecular-mass dextrans to alter aggregation with comparisons made between normal and hyperaggregating conditions (20, 44) or between nonaggregating and aggregating RBC suspensions (7, 39). However, Charansonney et al. (21) utilized two plasma concentrations of 500-kDa dextran in an isolated perfused rat heart preparation; rat RBCs in native plasma exhibit essentially no aggregation (8), and thus comparisons could be made with two levels of enhanced aggregation. They reported decreased flow resistance with the lower level of induced aggregation but increased flow resistance with the higher level (21). Guinea pig RBCs also exhibit low to moderate aggregation in native plasma (see Fig. 1), and increased aggregation first resulted in a significant increase in blood flow resistance in the papaverin series (see Fig. 4). Additional increases in aggregation then resulted in decreased flow resistance followed by another increase of flow resistance at the highest F98-SC level. Our results are thus conceptually consistent with the results of Charansonney et al. (21), although this study revealed three phases associated with graded increases of aggregation whereas their study revealed two.

The mechanisms underlying the observed effects of RBC aggregation on blood flow resistance (see Fig. 4) are most certainly complex and probably involve interactions between several theoretical and experimental considerations. In particular, RBC aggregation may affect in vivo flow resistance via the following mechanisms: *1*) RBC aggregation is known to be the main cause of increased blood viscosity under low-shear conditions, and this effect is explained by the increased disturbance of flow-stream lines with increased particle size (11); *2*) increased aggregation is expected to increase the energy cost for the breakdown of aggregates as the blood approaches the microcirculation and thereby increase flow resistance (45); *3*) increased aggregation is expected to increase the axial accumulation of RBCs and thereby increase plasma skimming and lower tissue Hct (9, 29, 30); the extent of this effect is dependent on the orientation of the blood vessels vs. gravity and the shear rates within the vessels (22, 29), however, this effect might be minimized by the insufficient residence times in the vasculature (15); *4*) increased axial accumulation of RBCs may result in decreased local viscosity at the marginal zone of blood vessels and, as has been demonstrated repeatedly in glass tubes (3, 22, 37, 42), decreased frictional resistance with the vessel wall; and *5*) decreased local viscosity of the marginal layers in blood vessels might be associated with decreased pressure gradients and hence lower wall-shear stresses for some vessels, thereby affecting vascular control mechanisms that are modulated by shear stress [for example, we have demonstrated that increased RBC aggregation results in diminished nitric oxide (NO)-dependent vascular control and decreased endothelial NO synthase expression (13)]; based on these considerations, increased RBC aggregation would result in increased vascular resistance due to inhibition of NO generation by the endothelium.

As is evident from the preceding paragraph, more than one process may affect the relations between RBC aggregation and vascular flow resistance. However, based on the results shown in Figs. 1, 2, and 4, one possible sequence with increasing RBC aggregation would involve an initial increase in flow resistance due to increased aggregate size and disturbed fluid stream lines followed by a decrease in flow resistance due to RBC axial accumulation, and then increased flow resistance related to the additional increment in aggregate size and strength, and, hence, increased energy costs for dispersion of RBC aggregates. Of course, other explanations are also possible, and thus a more detailed analysis of the origins of the complex relations between aggregation and vascular resistance is warranted.

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Modulation of endothelial nitric oxide synthase expression by red blood cell aggregation

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Baskurt, Oguz K., Ozlem Yalcin, Sadi Ozdem, Jonathan K. Armstrong, and Herbert J. Meiselman. Modulation of endothelial nitric oxide synthase expression by red blood cell aggregation. *Am J Physiol Heart Circ Physiol* 286: H222–H229, 2004. First published September 25, 2003; 10.1152/ajpheart.00532.2003.—The effects of enhanced red blood cell (RBC) aggregation on nitric oxide (NO)-dependent vascular control mechanisms have been investigated in a rat exchange transfusion model. RBC aggregation for cells in native plasma was increased via a novel method using RBCs covalently coated with a 13-kDa poloxamer copolymer (Pluronic F-98); control experiments used RBCs coated with a nonaggregating 8.4-kDa poloxamer (Pluronic F-68). Rats exchange transfused with aggregating RBC suspensions demonstrated significantly enhanced RBC aggregation throughout the 5-day follow-up period, with mean arterial blood pressure increasing gradually over this period. Arterial segments (≈300 μm in diameter) were isolated from gracilis muscle on the fifth day and mounted between two glass micropipettes in a special chamber equipped with pressure servo-control system. Dose-dependent dilation by ACh and flow-mediated dilation of arterial segments pressurized to 30 mmHg and preconstricted to 45–55% of the original diameter by phenylephrine were significantly blunted in rats with enhanced RBC aggregation. Both responses were totally abolished by nonspecific NO synthase (NOS) inhibitor (N^{ω}-nitro-L-arginine methyl ester) treatment of arterial segments, indicating that the responses were NO related. Additionally, expression of endothelial NOS protein was found to be decreased in muscle samples obtained from rats exchanged with aggregating cell suspensions. These results imply that enhanced RBC aggregation results in suppressed expression of NO synthesizing mechanisms, thereby leading to altered vasomotor tonus; the mechanisms involved most likely relate to decreased wall shear stresses due to decreased blood flow and/or increased axial accumulation of RBCs.

flow-mediated dilation; poloxamer coating

NITRIC OXIDE (NO) plays a key role in the control of circulatory function (21), and the importance of NO in the regulation of vascular smooth muscle tone and the maintenance of vascular resistance has been well established (13, 38, 44). In addition, several other physiological functions (e.g., apoptosis) have been attributed to this simple molecule (9, 25). Endothelial cells are the source of NO that modulates vascular resistance: NO diffuses to the underlying vascular smooth muscle where it activates guanylate cyclase to increase cGMP, thus resulting in smooth muscle relaxation (26) and decreased vascular resistance.

Endothelial NO is synthesized by the endothelial isoform of NO synthase (eNOS) using L-arginine as substrate (31). While eNOS is continuously expressed at a basal level in endothelial cells, its activation is dependent on Ca^{2+}/calmodulin complex levels, and thus NO generation by endothelial cells is dependent on intracellular calcium concentration (41). It has been shown that this calcium-dependent regulation of NO synthesis in endothelial cells is closely related to the hemodynamic conditions to which they are exposed (39): mechanical forces acting on endothelial cells trigger calcium entry and activate eNOS (54). Mechanical forces acting on endothelial cells can also activate eNOS by phosphorylation through calcium-independent mechanisms (15, 29, 54). Additionally, eNOS expression in endothelial cells can be modulated by the magnitude of the mechanical forces to which they are exposed (46, 53, 58).

Mechanical forces acting on endothelial cells in vivo are due to blood flow over these cells (i.e., tangential forces) and can be quantitated as wall shear stress: the magnitude of the wall shear stress is determined by the product of the fluid velocity profile adjacent to the vessel wall (i.e., wall shear rate) and the apparent viscosity of the fluid in this marginal area (33, 42). It has been demonstrated in a number of studies that alterations in hemodynamic conditions and wall shear stress may affect NO-synthesizing mechanisms in the vascular endothelium. Cardiac failure induced by coronary artery ligation, and hence decreased blood flow rates, leads to significantly diminished eNOS expression in skeletal muscle samples (55). This alteration can be reversed by regular exercise, indicating the importance of blood flow rate in maintaining physiological eNOS expression levels (55). These observations are supported by studies demonstrating enhanced eNOS expression in various regions of the circulatory system by regular exercise (24, 28, 30) and by increased blood flow due to arteriovenous shunts (23, 39).

The above-mentioned definition of wall shear stress suggests a significant role for the physical (i.e., rheological) properties of the blood in close contact with the vessel wall (33, 42). It is well known that the composition of blood across the diameter of a blood vessel is not uniform (20). Rather, red blood cells (RBCs) tend to migrate toward the axis, resulting in a plasma-rich, cell-poor marginal layer with relatively lower viscosity (16, 20). The extent of RBC axial migration is strongly affected by their tendency for aggregation, such that an enhanced tendency for RBC aggregation promotes axial migration (16) and, under the appropriate conditions, decreases flow resis-

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tance (6, 16, 47). Soutani et al. (50) clearly demonstrated the existence of this cell-poor layer and the influence of RBC aggregation on its thickness in an in vivo study using rabbit mesentery. They suggested that the widening of the cell-poor layer tends to reduce the flow resistance in the microvascular network (50). However, it should be noted that the effects of RBC aggregation on flow dynamics are highly complex and are modulated by a number of factors such as the orientation of blood vessels versus gravity (20) and transit times (i.e., flow times) through a given vessel segment (11).

The present study was designed to test the hypothesis that enhanced RBC aggregation would yield diminished eNOS expression as a result of decreased wall shear stress due to decreased blood flow rates and/or more effective RBC axial migration. In contrast with previous reports that employed the addition of high-molecular-weight water-soluble polymer solutions to circulating blood (e.g., Refs. 6, 11, 19, 35, and 48), enhanced RBC aggregation was achieved in the present study by covalent attachment of a 13-kDa poloxamer (Pluronic F-98) to the RBC surface (3). This technique allows controllable augmentation of RBC aggregation without diluting plasma proteins and does not involve chemical or viscosity changes of the plasma. Attachment of a 8.4-kDa poloxamer (Pluronic F-68) to the RBC does not alter aggregation for cells in plasma, thereby allowing control experiments that are closely matched to the group with enhanced aggregation.

MATERIALS AND METHODS

Animals

Adult rats of either sex weighing 250–300 g were used in the present study and were randomly assigned to each arm of the exchange transfusion protocol (see *Modification of RBC Aggregation*) or served as blood donors. Blood from donor animals was obtained from their abdominal aorta under light ether anesthesia, anticoagulated with EDTA (1.5 mg/ml), and used for preparing polymer-coated RBC suspensions. The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University and was in accordance with the Declaration of Helsinki and International Association for the Study of Pain guidelines.

Modification of RBC Aggregation

Enhanced RBC aggregation was achieved by employing a novel technique whereby copolymers that possess the ability to self-associate above a specific temperature are covalently attached to the RBC surface. These copolymers, known as poloxamers, are triblock macromolecules composed of a central hydrophobic block of polypropylene glycol (PPG) flanked by two identical hydrophilic polyethylene glycol (PEG) chains (49). Poloxamers exhibit a critical micellization temperature (CMT) at which a phase transition occurs from predominantly single, fully hydrated copolymer chains to micelle-like structures characterized by association of the hydrophobic PPG blocks (2, 5, 8). The CMT decreases with increasing mass of the PPG block, and, by proper selection of the poloxamer (8), RBC aggregation can be controllably and predictably enhanced or inhibited after covalent attachment to the RBC surface (3). That is, at temperatures above the CMT, poloxamers attached to the RBC surface micellize with poloxamers on adjacent RBCs, thereby enhancing RBC aggregation–the strength of the association being dependent on the poloxamer type, concentration, and temperature (3). Conversely, below the CMT, poloxamers attached to the RBC surface inhibit RBC aggregation due to polymer-polymer repulsion.

Derivatization of poloxamers. Pluronic copolymers were a gift from BASF Performance Chemicals (Parsippany, NJ). These poloxamers contained 80% PEG by mass and had the following designations and molecular weights: Pluronic F-68, 8.4 kDa; and Pluronic F-98, 13.0 kDa. To facilitate covalent attachment of the poloxamers to the RBC surface, a reactive derivative of the copolymers was first prepared. This was achieved by converting the terminal hydroxyl groups of the copolymer chain to succinimidyl carbonate groups using a modification of the method described by Miron and Wilchek (36). In brief, 1 mmol of poloxamer was dissolved in 100 ml of dioxane (warmed slightly to aid dissolution) and then cooled to room temperature; the clear solution was stirred using a magnetic stirrer and purged with nitrogen. A threefold molar excess (6 mmol) of *N*,*N*′-disuccinimidyl carbonate was then slurried in 10 ml of dry acetone and added to the solution. 4-(Dimethylamino)pyridine (6 mmol) was dissolved in 20 ml of dry acetone and added slowly to the reaction mixture, and the reaction was allowed to proceed for 24 h at room temperature under nitrogen. The reaction mixture was filtered to remove any solid precipitate, and the filtrate was poured slowly under high shear into 300 ml of diethyl ether to precipitate the derivatized poloxamer and remove the 4-(dimethylamino)pyridine. The precipitate was filtered and washed with 200 ml of diethyl ether, resuspended in 200 ml of isopropanol, filtered, and washed with 100 ml of isopropanol to remove any unreacted *N*,*N*′-disuccinimidyl carbonate. The precipitate was then resuspended in 200 ml of cyclolhexane, filtered, and washed with 100 ml of cyclohexane. Finally, the poloxamer succinimidyl carbonate derivatives (Pluronic F-68-SC and Pluronic F-98-SC) were dried under a stream of dry nitrogen for 12 h.

RBC coating. To achieve an even coating of poloxamer, and to avoid the formation of cross-linked aggregates of coated RBC, all poloxamer-coating procedures were performed at 4°C (i.e., below the poloxamer CMT). Blood was centrifuged at 1,400 *g* for 6 min, and the plasma was separated and saved. RBCs were washed three times with isotonic PBS (pH 7.4) and resuspended in 30 mM triethanolamine buffer (290 mosm/kg, pH 8.60) at a hematocrit of ~0.1 l/l, and the suspension was cooled to 4°C. Immediately before use, the reactive poloxamer to be used for coating of RBCs (either Pluronic F-68-SC or Pluronic F-98-SC) was dissolved in a 4°C hypotonic phosphate buffer (50 mM NaH_2PO_4 and 60 mM NaCl; pH 5) at a concentration of 10 mg/ml. The poloxamer solution was then promptly added to the RBCs suspended in triethanolamine buffer to obtain a final concentration of 0.25 mg/ml, and the suspension was incubated for 2 h at 4°C with continuous gentle mixing on a tube rocker. Coated RBCs were separated, washed three times with PBS by gentle centrifugation (400 *g*, 5 min), and then resuspended in their autologous plasma at 0.4 l/l hematocrit. The viscosity of the RBC suspensions at 37°C over a range of shear rates (0.1–94.5 s^{-1}) was measured using a Contraves LS-30 Couette viscometer (Contraves; Zürich, Switzerland).

Exchange Transfusions and Follow-up Procedures

Rats were anesthetized by xylazine-ketamine solution (10 mg/ml xylazine plus 50 mg/ml ketamine), 0.1 ml per 100 g body wt, and the left carotid artery and left jugular vein were catheterized using 20-gauge cannulas. Approximately 30% of total blood volume of each rat was exchanged with a poloxamer-coated RBC suspension by infusing the suspension slowly (0.28 ml/min) into the cannulated jugular vein using an infusion pump (model 975, Harvard Instruments; Holliston, MA) and withdrawing the same amount of blood from the carotid artery. Both the carotid artery and jugular vein were tied off at the end of the exchange transfusion procedure, and the surgical incision was closed by 3-0 silk sutures.

Blood pressure after the exchange transfusion was measured daily over a 4-day follow-up period using a tail-cuff method (BP HR200 Module plus MP100 system, Biopac Systems; Goleta, CA). Additionally, blood samples were obtained from tail tips of the rats every day during this period and used for microscopic examinations. Wet mount preparations of dilute RBC suspensions were examined and photographed using bright-field light microscopy. These photographs were

used to assess RBC aggregation via the microscopic aggregation index (MAI) technique (45). In the MAI method, the total number of RBCs in a field is divided by the number of RBC units (i.e., sum of the number of individual RBC and aggregates) in the same field; the MAI has a value of unity in the absence of aggregation and increases with enhanced RBC aggregation (45).

In a separate series of experiments, 2 mg of reactive Pluronic F-68 or F-98 in 0.1 ml isotonic saline were infused into the tail vein of rats ($n = 4$ for each poloxamer) daily over a 4-day period. Arterial blood pressure and RBC aggregation were monitored during this period using the methods described above to test the effect of free polymers on these parameters. These experiments were repeated using inactivated polymers (i.e., polymers incubated overnight at pH 8 at room temperature) for daily injections.

In Vitro Vascular Studies

On the fourth day after the exchange transfusion, rats were anesthetized by urethane (1 g/kg, 20% in isotonic saline).The gracilis muscle from the left leg of the animal was excised and transferred to a dissecting dish containing oxygenated Krebs solution, and an arterial segment of ~300 μm in diameter was isolated from the muscle under a dissection microscope. The arterial segment was transferred to a vessel chamber (CH/1, Living Systems; Burlington, VT), mounted between two glass micropipettes, and superfused with oxygenated Krebs solution [containing (in mmol/l) 119 NaCl, 24 $NaHCO_3$, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4 \cdot 7H_2O$, 1.6 $CaCl_2$, and 5.5 glucose] at 37°C. The micropipettes were connected to a pressure servo-control system composed of two roller pumps, two pressure transducers that monitored the inflow and outflow pressures, and a control unit (PS/200/Q, Living Systems), and the vessel segment was perfused from a separate source of the oxygenated Krebs solution described above. The servo-control system allowed us to set the flow rate through the arterial segment from 0 to 370 μl/min. The arterial segment preparation was placed on an inverted microscope (TS100, Nikon) equipped with a charge-coupled device camera (XC73CE Sony). The camera was connected to a video dimension-analysis system (model V94, Living Systems), which allowed continuous recording of the vessel diameter; intraluminal and perfusion pressures, fluid flow rate through the arterial segment, and the vessel diameter were continuously recorded via a digital computer.

Before being studied, the length of the arterial segment was adjusted to its in vivo length and the arterial segment was equilibrated at 30-mmHg intraluminal pressure and zero flow rate for 30 min. The following three types of studies were carried out: ACh dilation, flow-mediated dilation (FMD), and FMD plus N^{ω}-nitro-L-arginine methyl ester (L-NAME).

Acetylcholine dilation. At the end of the equilibration period, the segment was constricted to 45–55% of its original diameter by adding 10^{-7}–10^{-5} M phenylephrine to the superfusion solution. When the phenylephrine-induced contraction reached the desired 45–55% plateau level, increasing concentrations of ACh (10^{-8}–10^{-5} M) were added to the superfusion solution and the vessel diameter at each concentration was recorded.

Flow-mediated dilation. The arterial segment was washed with oxygenated Krebs solution and again constricted by phenylephrine to 45–55% of the basal diameter. FMD was assessed at a series of fluid flow rates between 8 and 35 μl/min while the intraluminal pressure was kept constant at 30 mmHg by the pressure servo-control system. Each flow rate was maintained for 2 min to obtain a steady vessel diameter.

Flow-mediated dilation with L-NAME. The arterial segment was washed again, and 10^{-3} M L-NAME was added to the vessel chamber. The procedure to assess FMD (i.e., phenylephrine to achieve 45–55% constriction, 8–35 μl/min flow rate) was repeated after 30 min of incubation with L-NAME.

Determination of eNOS Expression

Samples obtained from gracilis muscles were used to determine eNOS expression by Western blotting techniques; muscle samples from normal rats that did not receive exchange transfusions were also analyzed together with the samples from the Pluronic F-68 and F-98 groups. Tissue samples weighing ~300 mg were homogenized in 200 μl of 10% Triton X-100-HEPES buffer. The protein content of the homogenate was determined by Bradford reagent, and samples containing 100 μg protein were loaded onto 10% polyacrylamide gels; SDS-PAGE was carried out using a potential difference of 120 V. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes by incubating them in Tris-glycine-methanol buffer at 4°C overnight. The membranes were blocked in 1% fat-free milk powder solution prepared in 0.1% Tween 20 containing phosphate buffer (pH 7.4) for 2 h at room temperature. The membranes were then incubated in the presence of eNOS primer antibody (sc654, Santa Cruz Biotechnology; Santa Cruz, CA) at a dilution of 1/2,000 for 1 h at room temperature, followed by 1 h of washing in Tween 20-phosphate buffer to remove nonspecific binding. The membrane was then exposed to horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG-HRP, sc-2030, Santa Cruz Biotechnology) at a dilution of 1/200 for 1 h at room temperature. After being washed for 1 h, the membrane was treated by ECL detection reagent (RPN2106, Amersham Biosciences; Buckinghamshire, UK) and exposed to ECL-sensitive films. PVDF membranes were also used for the determination GAPDH expression, as the loading standard, as described above using GAPDH primer antibody (ab8245, Abcam; Cambridge, UK). The intensities of the bands were quantified by densitometric analysis using a gel scanner (Cobrascan CX 312 T, Radiographic Digital Imaging; Torrance, CA), with the results expressed as the ratio of densities of eNOS-specific bands to GAPDH bands.

Statistics

The results obtained from the Pluronic F-68 and F-98 groups were compared by Student's *t*-test where appropriate. Multiple comparisons were done by one-way ANOVA, followed by the Newman-Keuls post hoc test or two-way ANOVA, followed by the Bonferroni post hoc test. EC_{50} values (i.e., concentrations that caused 50% of maximal responses) were calculated by linear regression using statistical analysis software (GraphPad Software; San Diego, CA). Statistical significance was accepted at P values of <0.05.

RESULTS

RBC Aggregation

Examination of dilute RBC suspensions in autologous plasma indicated significantly enhanced RBC aggregation in the group that underwent exchange transfusions with Pluronic F-98-coated RBC suspensions. Typical examples for both Pluronic F-68 and F-98 groups are presented in Fig. 1, where it can be seen that multicell aggregates and RBC clumping exist in blood from the Pluronic F-98 animals, whereas RBC aggregation is negligible in the Pluronic F-68 group. It should be noted that due to strong associations between PPG blocks on the surfaces of adjacent Pluronic F-98-coated RBCs, the appearance of RBC aggregates in the Pluronic F-98 group was not similar to the regular linear rouleaux and the branched arrays of linear aggregates observed in normal human blood under physiological condi-

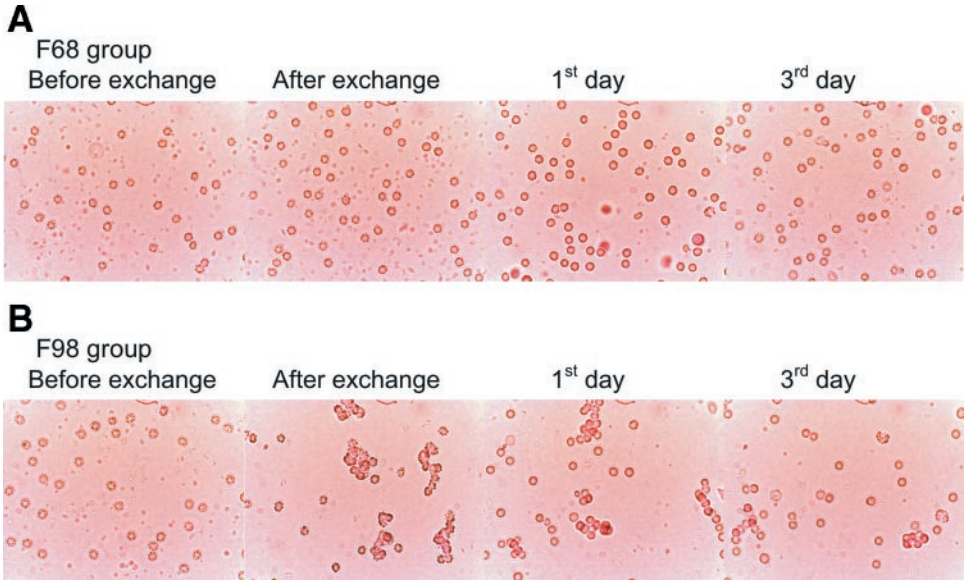

Fig. 1. Photomicrographs of red blood cells (RBCs) obtained from rats before and after exchange transfusions with Pluronic F-68-coated (*A*) or F-98-coated (*B*) RBC suspensions. RBCs are suspended in autologous plasma at low hematocrit (≈0.01 l/l).

tions (45). Rather, the RBC aggregation observed in Pluronic F-98-infused animals is characterized by stronger associations between individual RBCs as indicated by greater forces needed to break the mostly irregular clumps (i.e., RBC aggregates are more resistant to dispersion due to movement of the coverslip over the microscope slide) as previously demonstrated in vitro using low shear viscometry (3). However, this clumping was clearly reversible when sufficiently large shearing forces were applied to the suspension between the microscope slide and coverslip. The observed degree of clumping was reduced in the days after exchange transfusions in the Pluronic F-98 group (Fig. 1).

MAIs calculated using microphotographs of blood samples taken daily after the exchange transfusions are shown in Fig. 2. RBC aggregation was found to be ~3.5 times higher immediately after exchange transfusions with Pluronic F-98-coated RBC suspensions compared with either samples obtained before exchange transfusion or to samples from animals receiving Pluronic F-68-coated RBCs. RBC aggregation in the Pluronic F-98 group decreased gradually over the 4-day period after the exchange transfusions, yet the aggregation index was still 2.4 times higher than preexchange or Pluronic F-68 values. Aggregation indexes did not change significantly at any time point after exchange transfusions with Pluronic F-68-coated RBC suspensions (Fig. 2).

Blood viscosity measurements of poloxamer-coated RBCs suspended in autologous plasma were performed at 37°C. Pluronic F-98-coated RBCs showed a 4.9-fold increase and Pluronic F-68-coated RBCs showed a 0.3-fold decrease in low shear blood viscosity (shear rate = 0.1 s^{-1}) relative to control uncoated RBCs. No significant difference in high shear (94.5 s^{-1}) viscosity was observed.

Blood Pressure

Mean arterial pressure values measured using a tail-cuff method are presented in Fig. 3, where it can be seen that there was no alteration in arterial pressure in the Pluronic F-68 group during the days after the exchange transfusions. Conversely, in the group that received exchange transfusions with Pluronic F-98-coated RBC suspensions, there was a gradual increase in mean arterial pressure. Signifi-

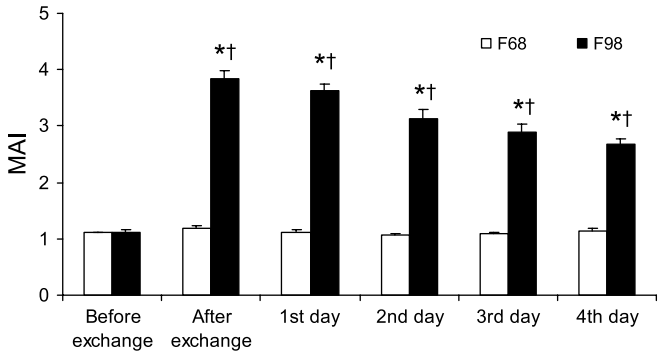

Fig. 2. Microscopic aggregation indexes (MAIs) of RBCs suspended in autologous plasma for the Pluronic F-68 and F-98 groups. Values are means ± SE; n = 8 rats/group. *Significant difference from the Pluronic F-68 group by two-way ANOVA, $P < 0.001$; †significant difference from before exchange by one-way ANOVA, $P < 0.001$.

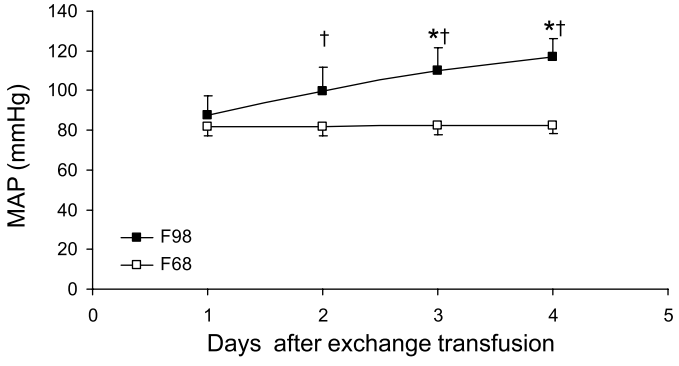

Fig. 3. Mean arterial blood pressure (MAP) of rats after exchange transfusions with Pluronic F-68 or F-98-coated RBC suspensions. Values are means ± SE; n = 8 rats/group. *Significant difference from the Pluronic F-68 group by two-way ANOVA, $P < 0.01$; †signficant difference from *day 1* by one-way ANOVA, $P < 0.001$.

cantly higher values were recorded in this group on and after the second day after exchange transfusions: mean arterial pressure was significantly higher than both the baseline values (i.e., the value before the exchange transfusion) and those for the Pluronic F-68 group (Fig. 3).

Effects of Free Poloxamers on Arterial Blood Pressure and RBC Aggregation

Daily injections of 2 mg of reactive Pluronic F-68 or F-98 in 0.1 ml of isotonic saline over a 4-day period did not lead to an increase of arterial blood pressure (data not shown). Strong RBC aggregation was observed in peripheral blood samples within 15 min of injection of reactive poloxamers, with return to normal aggregation by 4 h. Infusion of inactivated poloxamers according to the same protocol did not affect either RBC aggregation or arterial blood pressure.

ACh-Mediated Dilation

ACh-induced dilations of arterial segments pressurized to 30 mmHg at zero flow and preconstricted to 45–55% of the original diameter are shown in Fig. 4 for the Pluronic F-68 and F-98 groups. The shape of the ACh concentration-dilation curve obtained in the Pluronic F-68 group was similar to that obtained using the arterial segments obtained from normal animals that had not received exchange transfusions (data not shown). However, there was a rightward shift of the concentration-response curve in the Pluronic F-98 group versus the Pluronic F-68 group such that the concentration-response curve of the Pluronic F-98 group indicated a significantly blunted response (Fig. 4) with the dilation at 10^{-6} M ACh being significantly lower (72.1 ± 7.5 for Pluronic F-68 vs. 46.5 ± 9.5 for Pluronic F-98). Accordingly, the EC_{50} value for ACh was found to be 2.07×10^{-7} M in the Pluronic F-68 group, whereas in the Pluronic F-98 group the EC_{50} value was ~17 times higher (3.48×10^{-6} M).

Flow-Mediated Dilation

Responses of pressurized and preconstricted arterial segments to alterations in fluid flow rate through the segments are presented in Fig. 5. FMD at flow rates of 8 and 17 μl/min were significantly greater in the Pluronic F-68 group compared with the Pluronic F-98 group. FMD reached its maximum at 17 μl/min in the Pluronic F-68 group, whereas in the Pluronic F-98 group the maximum response to flow was observed at 26 μl/min (Fig. 4). The flow rate-dilation curve in the Pluronic F-68 group was similar to that obtained using arterial segments isolated from normal, nonoperated rats (data not shown). Prior L-NAME incubation of the arterial segments for 30 min abolished the FMD responses in both groups.

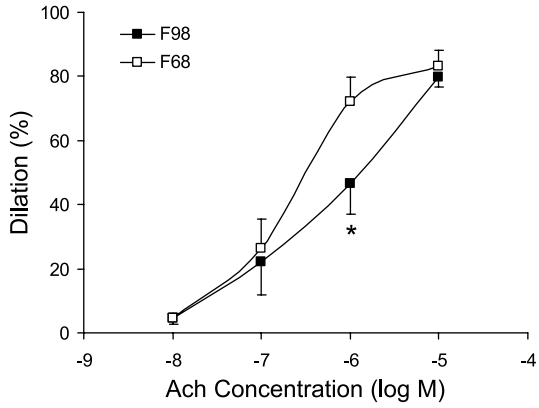

Fig. 4. Percent dilation induced by 10^{-8}–10^{-4} M ACh in ~300-μm-diameter arterial segments preconstricted to 45–55% of the original diameter with phenylephrine. Values are means ± SE; $n = 8$ segments/group. *Significant difference from the Pluronic F-68 group by two-way ANOVA, $P < 0.05$.

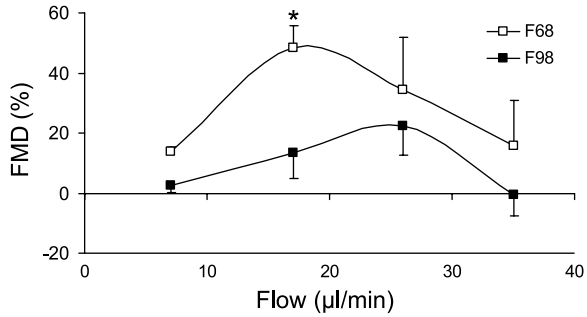

Fig. 5. Flow-mediated dilation (FMD; in %) induced by flow rates between 8 and 35 μl/min in ~300-μm-diameter arterial segments preconstricted to 40–50% of the original diameter with phenylephrine. Values are means ± SE; $n = 8$ segments/group. *Significant difference from the Pluronic F-68 group by two-way ANOVA, $P < 0.05$.

eNOS Expression

Western blotting results revealed that eNOS expression was markedly reduced in the Pluronic F-98 group 4 days after the exchange transfusion (Fig. 6): the ratio of eNOS band density to GAPDH band density was 3.2 times higher in the Pluronic F-68 group compared with the Pluronic F-98 group. There was

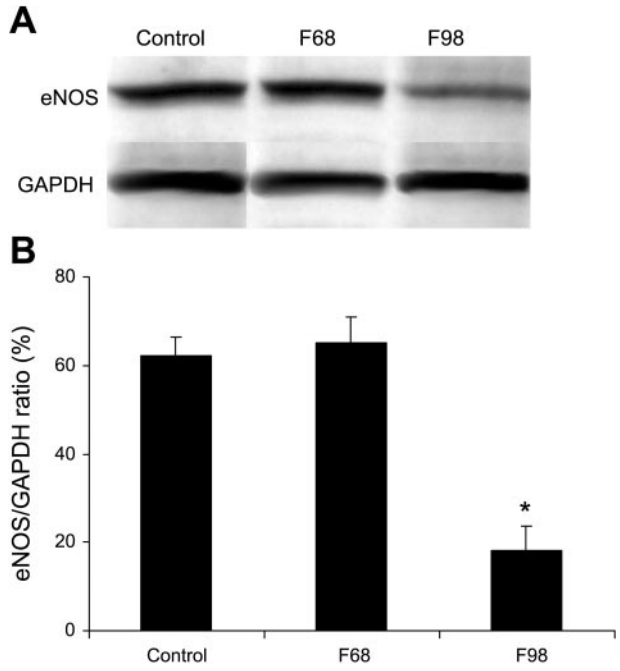

Fig. 6. Expression of endothelial nitric oxide synthase (eNOS) protein. *A*: typical pattern of Western blot results. *B*: eNOS-to-GAPDH ratio for control (nonoperated) animals and Pluronic F-68 and F-98 groups. Results are presented as means ± SE. *Significant difference from the Pluronic F-68 group, $P < 0.001$.

no significant difference between the values of the Pluronic F-68 group and nonoperated control animals that did not receive exchange transfusions.

DISCUSSION

With the use of a rat exchange transfusion protocol involving RBCs coated with a proaggregating poloxamer (i.e., Pluronic F-98), the results of the present study demonstrate that *1*) increased RBC aggregation leads to a gradual increment of arterial blood pressure; and *2*) the increment of blood pressure is accompanied by impaired NO-mediated vasodilatory responses together with a diminished expression of eNOS in vessels of skeletal muscle. Control experiments using RBCs coated with a nonaggregating poloxamer (i.e., Pluronic F-68) indicated no changes of arterial blood pressure or eNOS expression. The relationship between NO synthesis and blood pressure is well known: interventions to suppress NO synthesis by endothelial cells have been used as hypertension models (12, 58), and increased arterial pressure is known to alter NO-related vascular control mechanisms (40). The relationship between RBC aggregation and arterial blood pressure is also well known, at least from clinical observations (1, 32). It is thus suggested that the present study provides some insight to these complex relationships that are based on different types of observations.

Arterial blood pressure is directly proportional to the product of cardiac output and peripheral vascular resistance, with peripheral vascular resistance determined by vascular geometry, primarily blood vessel diameter, and the rheological properties of blood (14). RBC aggregation is one of the main determinants of blood's rheological behavior, with increased aggregation resulting in elevated blood viscosity at low to medium rates of shear (7). Thus, based on in vitro viscometric data, enhanced RBC aggregation would increase blood viscosity and flow resistance in large blood vessels. RBC aggregates should be dispersed (i.e., disaggregated) as they approach the microcirculation, where the size of blood vessels approaches that of individual RBCs; the energy cost of such disaggregation would increase if the aggregation tendency of RBC is enhanced (56). Therefore, increased hemodynamic resistance might be expected to be coincident with the stepwise increase of RBC aggregation resulting from the exchange transfusion with Pluronic F-98-coated RBCs: enhanced RBC aggregation would thus be the direct cause of the observed increase of arterial blood pressure (Fig. 3). Given such a mechanism, arterial blood pressure should have reached a maximum immediately after the rapid increase of RBC aggregation and then gradually returned toward control as RBC aggregation tended to decrease (Fig. 2). However, our experimental data do not support this prediction: mean arterial blood pressure rose gradually over the 4-day follow-up period, whereas RBC aggregation decreased ~30% (Figs. 2 and 3).

The lack of a close temporal linkage between increases of RBC aggregation and arterial blood pressure suggests the need to consider other mechanisms. For example, it could be suggested that the gradual increase of arterial blood pressure might be independent of RBC aggregation and rather related to delayed release of poloxamer from the RBC surface. If true, then an infused poloxamer should be expected to have an effect on arterial blood pressure. However, a separate series of studies in rats indicated that daily injections of reactive Pluronic F-68 or F-98 over a 4-day period did not lead to an increase of arterial blood pressure, although strong RBC aggregation was observed in peripheral blood samples during the first 4 h after infusions. This short-term increase in RBC aggregation can be explained by covalent attachment of poloxamers to plasma proteins and cross-linking of plasma proteins by the reactive poloxamer (both Pluronic F-68 and F-98), thereby inducing strong aggregation by markedly increasing the effective macromolecular size of plasma proteins. Note, however, that these short-term increases of aggregation had no meaningful effects on arterial pressure. Furthermore, in the event that the poloxamers did detach from the RBC surface in vivo, these "free" copolymers would be inactive and would not covalently attach to plasma proteins. Inactivated poloxamers were shown to be ineffective on both RBC aggregation and arterial blood pressure. These observations thus exclude the possibility of an effect of poloxamers (Pluronic F-68 and F-98) on blood pressure that is independent of long-term enhancement of RBC aggregation.

Although the in vitro viscometric data suggest increased vascular resistance to blood flow due to increased RBC aggregation, the effects of RBC aggregation on blood flow in vivo are controversial. Dispersion of strongly aggregated RBC at the microvascular level requires additional energy, yet widening of the cell-poor marginal layer near the vessel wall due to aggregation tends to decrease in vivo flow resistance (52). Therefore, the net effect of increased RBC aggregation at a given time point would reflect the interplay between these two opposite influences. Furthermore, it is now well established that the flow conditions in the vascular system (i.e., fluid shear stress) also affect vascular geometry, thereby modulating the vascular component of flow resistance. The possible effects of enhanced RBC aggregation on NO-related vascular control most likely relate to altered blood flow and blood composition near vessel walls. Given increased RBC aggregation and hence increased hemodynamic resistance, blood flow in the peripheral circulation would be diminished. It has been previously demonstrated that blood flow and wall shear stress in the vascular system are important determinants of NO synthesis by endothelial cells (15, 39, 46, 53, 59), with decreased blood flow shown to suppress eNOS expression and FMD responses in small arteries (55). Enhanced RBC aggregation would also tend to promote axial accumulation of RBC in blood vessels, resulting in a less-viscous, plasma-rich region near vessel walls (16). Diminished wall shear stress resulting from this nonuniform radial composition of blood should be expected to influence the NO-related mechanisms in blood vessels (16, 20, 33). Note that these two suggestions (i.e., decreased blood flow and a less-viscous region near vessel walls) are not mutually exclusive and most likely are synergistic in decreasing mechanical forces acting on endothelial cells (10, 11, 59).

Regardless of the specific mechanism(s) responsible for reduced fluid forces at vessel walls, such reductions should result in shifted vasomotor balance and increased peripheral resistance due to diminished NO generation by endothelial cells. Altered wall shear stress may affect NO synthesis by constitutive NOS existing in endothelial cells, and the molecular mechanisms of eNOS activation by mechanical shear stress acting on endothelial cells have been the subject of several studies (15, 29, 52). It has also been demonstrated that

wall shear stress modulates the expression of eNOS protein (17, 18, 53). However, the time scales for these two effects of shear stress differ. That is, while the modulation of existing eNOS activity follows rapidly after hemodynamic alterations, alteration of eNOS protein expression is known to occur over a longer span of time (23, 46). For example, while NO-related mechanisms in skeletal muscle are known to play a significant role in vascular adjustments during exercise (27, 34), regular physical exercise requires several weeks to induce increments in eNOS expression (24, 28, 30). It thus seems likely that the gradual increase of arterial blood pressure seen in the present study (Fig. 3) reflects the gradual downregulation of eNOS protein expression due to decreased shear forces on endothelial cells. This suggestion is supported by the suppressed NO-mediated dilation (both ACh and flow induced) responses (Figs. 4 and 5) and the markedly decreased eNOS expression in skeletal muscle samples on the day when arterial pressure was significantly higher than the control group (Fig. 6).

It should be noted that the vascular endothelium might not be the only source of eNOS in the muscle tissue samples used herein for Western blot analysis. It has been demonstrated that eNOS is expressed by rat skeletal muscle cells and is most probably localized to mitochondria (51). Although the level of eNOS expression in skeletal muscle cells was reported to be low (51), the relative contribution of eNOS from endothelial cells to the total eNOS determined in this study is somewhat uncertain. Unfortunately, it was technically impossible to isolate enough resistance blood vessel tissue from rat skeletal muscles and total total muscle tissue samples were employed. However, it seems highly unlikely that the skeletal muscle cell eNOS expression would be modified by the altered hemodynamic conditions: the existing literature clearly indicates a vascular origin for altered eNOS expression due to altered hemodynamics (30, 37, 55). Furthermore, decreased eNOS expression in the Pluronic F-98 group was associated by blunted eNOS-mediated vasomotor responses (Figs. 4 and 5) in resistance vessels isolated from the same muscles. Accordingly, altered eNOS protein content in the skeletal muscle tissue samples were accepted as a strong evidence for the effect of altered shear stresses on endothelial eNOS expression.

The poloxamer-coating method used herein to enhance RBC aggregation in vivo is unique in that it does not involve the addition of foreign polymers to circulating blood: RBCs are suspended in unaltered native plasma. In previous studies designed to assess the hemodynamic effects of RBC aggregation, high-mass-weight polymers such as 500-kDa dextran have been used to increase RBC aggregation (6, 11, 19, 35, 48). The use of these large polymers has been proven to be effective for modifying RBC aggregation, yet has potential disadvantages: *1*) plasma or suspending phase viscosity is increased [e.g., for 500-kDa dextran, 1.5 g/dl yields a 80% increase of suspending medium viscosity (43)] and hence the fluid viscosity near the vascular wall is modified; *2*) plasma proteins are diluted via the added polymer solution; *3*) in some experimental animals (e.g., the rat), dextran and similar polymers can lead to anaphylactic-type reactions and thus induce a pathophysiological state (10); *4*) the elevated colloid osmotic pressure caused by macromolecules in solution could alter intra- versus extravascular fluid volumes; and *5*) due to metabolism/excretion, polymer concentration decreases with the time after infusion (22), thus precluding long-term temporal studies of RBC aggregation effects. The latter point is also somewhat relevant to the coated cells used herein, although it is notable that the degree of aggregation for Pluronic F-98-coated cells decreased by <30% over the 4-day period after exchange transfusion (Figs. 1 and 2). The poloxamers used herein are not toxic at the concentrations that the experimental animals were exposed to but can be nephrotoxic at very high concentrations (57). The use of poloxamer-coated RBCs (3, 4) thus appears to represent an improved approach for investigating the in vivo effects of RBC aggregation.

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Hemorheology and vascular control mechanisms

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Abstract. Blood rheology is a well-known determinant of tissue perfusion and, according to the Poiseuille relation, hemodynamic resistance in a constant-geometry vascular network is directly proportional to blood viscosity. However, this direct relationship cannot be observed in all *in vivo* studies. Further, there are several reports indicating marked differences between the *in vivo* and *ex vivo* flow properties of blood. These differences can be explained, in large part, by considering special hemorheological mechanisms (e.g., Fahraeus–Lindqvist effect, axial migration) that are of importance in the microcirculation. Additionally, the influence of altered rheological properties of blood and its components on vascular control mechanisms requires consideration: (1) There is an indirect relation between blood rheology and microvascular tone that is mediated by tissue oxygenation, with a compensatory vasodilation occurring if tissue perfusion is impaired due to hemorheological deterioration; (2) Blood rheology may influence vascular tone through alterations of wall shear stress, which in turn determines endothelial generation of vasoactive substances (e.g., nitric oxide). This latter point is of particular relevance to the field of clinical hemorheology, since enhanced red blood cell aggregation has been shown to affect nitric oxide synthesis and thus control of vascular smooth muscle tone. Such multiple pathways by which hemorheological changes can affect vascular resistance help to explain the continuing difficulty of predicting correlations between *in vivo* and *ex vivo* hemorheological behavior; they also suggest the need for continued experimental studies in this area.

Keywords: Vascular resistance, viscosity, red blood cell, rheology

1. Introduction

The resistance to flow of simple fluids through rigid cylindrical tubes is known to be determined by geometrical and rheological factors, with the Poiseuille equation indicating that flow resistance is inversely proportional to the fourth power of radius and directly proportional to the length of the tube and the viscosity of the fluid. Although not always strictly correct, the Poiseuille equation has generally been applied to blood flow in vessels. Since the radius appears as the fourth power, vessel size was considered to be the most important determinant of flow resistance, with the other factors almost totally ignored. The major influence of cellular pathology theory on medical practice furthered this position: vascular geometry became the only important factor, with the diagnosis and understanding of most diseases based on the microscopic examination of dead and fixed tissue samples [4]. It is thus understandable why a functional parameter, such as the fluidity of blood, was previously not recognized as an important factor in medical practice but rather was considered to be no more than another "constant" in the Poiseuille equation.

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In the first half of the twentieth century, the work of Robin Fahraeus established a bridge between the very long medical tradition of humoral pathology and modern hemorheological concepts [18,27]. In addition to his pioneering work on the suspension stability of blood [23], he was the first to observe the uneven distribution of blood flow in the cross-section of blood vessels [22], and thus made one of the first *in vivo* hemorheology studies. The results of his studies played a very important role in the establishment of modern hemorheological concepts, and lead to hemorheological parameters being measured in a vast number of pathophysiological conditions [16,33,44]. Cardiovascular diseases, diabetes, and sepsis are among the disease processes with well-established hemorheological profiles [16,33]; significant hemorheological alterations have also been reported in extreme physiological states such as exhaustive exercise [51]. In addition to clinical studies, there are reports indicating hemorheological changes in animal models of pathologic states (e.g., [3,7,38,49]).

2. The importance of hemorheological alterations

While there now exists a very large collection of clinical and experimental studies reporting "*statistically significant*" alterations of hemorheological parameters in a wide variety of conditions, the importance of these alterations from a pathophysiological point of view has not yet been fully explored. Hemorheological alterations might have two distinct implications: (1) Hemorheological parameters might be altered due to pathophysiological processes, since it is well known that hemorheological parameters are sensitive to body homeostasis. For example, maintenance of normal red blood cell deformability depends on intact metabolic pathways [36], the proper microenvironment and "good cellular neighbors". Activated white blood cells have been demonstrated to induce impaired deformability in neighboring red cells [5]; (2) Altered hemorheology may play a role in the development of pathophysiological processes. A classic example of this type of relationship is sickle cell disease in which clinical signs and symptoms can be related to abnormal RBC rigidity. There are also genetic models of hypertension with red cell abnormalities [10,39], and it is possible that such alterations may play a role in the elevation of blood pressure by affecting peripheral vascular resistance [32].

The two abovementioned approaches to hemorheology-disease relationships may reflect a vicious cycle or, perhaps more correctly, a "chicken versus egg" dilemma. However, the two aspects of the cycle are not equally well established. That is, while there is a close relationship between homeostasis and hemorheology, and it is well known that general or local homeostasis can affect hemorheological factors, the role of hemorheology in maintaining homeostasis is not equally well defined. One factor contributing to this inequality are experimental studies indicating that the fluidity of blood *in vivo* might be quite different than that predicted based on measurements made outside of the vascular system.

3. Behavior of blood *in vivo*

Probably the most well-known study of the *in vivo* rheological behavior of blood is the Whittaker and Winton paper of 1933 [50]. In this publication they reported that the apparent viscosity of blood *in vivo* was lower than that measured via viscometry *in vitro* [50]; several other publications have reported similar results [6,14,20]. Such findings thus suggest difficulties in predicting the exact degree of alteration of tissue perfusion due to a given degree of hemorheological abnormality: flow resistance *in vivo* is more

complex than predicted based solely on *ex vivo* rheological data. Note that the apparent discrepancy between *in vitro* and *in vivo* results [50] can be explained, in part, using the concepts developed by Robin Fahraeus [27]: axial migration of deformable red blood cells during flow and its consequences such as the Fahraeus effect (i.e., reduced hematocrit at the microvascular level) may explain the lower apparent viscosity *in vivo* [17,40,43].

4. The role of vasomotor control in determining blood flow resistance

In addition to factors related to the rheological behavior of blood, factors related to blood vessels should also be considered when attempting to explain the difference between *in vivo* and *ex vivo* flow resistance. That is, blood vessel geometry and orientation are important determinants of the effects of hemorheological alterations on flow resistance [1,11,17,41,43,46]. Additionally, the role of vasomotion should also be considered, since vasomotion alters vessel radius and hence has a direct influence on vascular hindrance. Therefore vascular hindrance should be considered as a variable when exploring pressure–flow relationships of the vascular system, and not merely as an important but constant determinant of flow resistance.

Vascular hindrance is primarily affected by vessel radius, and hence is regulated by factors that affect vascular smooth muscle tone. While a variety of factors play role in this regulation, the most important is the metabolic demands of the perfused tissues [21,45]. Additionally, mechanical forces acting on the endothelium also influence smooth muscle tone and vascular hindrance [13,15].

4.1. Metabolic autoregulation

In general, autoregulation refers to the intrinsic ability of a vascular bed or organ to maintain blood flow at control levels in spite of changes of perfusion pressure; a logical extension of this definition would include challenges due to altered hemorheological factors. Autoregulation due to metabolic factors is a very effective mechanism for maintaining blood flow: a decrement in blood flow to a given tissue and related hypoxia can be normalized by decreased vascular hindrance even if the main cause of the blood flow decrement still continues to exist. The key element for this regulation is decreased tissue oxygen partial pressure due to insufficient blood flow [21,24,45], with the decrease being the result of decreased arterial pressure or decreased blood fluidity; the latter challenge can be considered to be a hemorheological “extra load”.

The role of vasomotion in determining flow resistance alterations induced by a hemorheological extra-load can be demonstrated experimentally. In a yet unpublished study, an isolated-perfused rat hind-limb preparation was used to investigate the role of vasomotor control in the response to hemorheological changes. The left femoral artery and femoral vein of rats were cannulated and the isolated hind limb preparation perfused using a pressure servo-control system (Fig. 1): hydrostatic pressure was monitored at the entrance of the arterial catheter and the speed of the roller pump (i.e., flow rate) adjusted to maintain arterial pressure at the set point (i.e., 100 mmHg), with flow resistance calculated based upon the measured pressure and flow rate. RBC-autologous plasma suspensions, at 0.4 l/l hematocrit, were prepared using either normal or chemically-rigidified rat red blood cells. The deformability of rat red cells was reduced, in a graded fashion, by incubating them with glutaraldehyde at concentrations between 0.0005–0.002%. At these low concentrations the decrease of deformability was subtle: RBC deformability measured at a low shear stress (1.58 Pa) was decreased by only about 16% at the highest glutaraldehyde concentration (Fig. 2A).

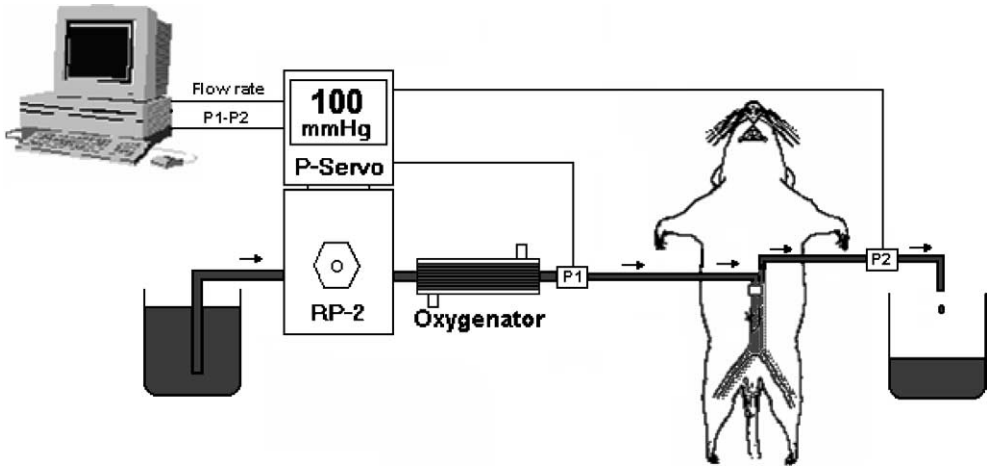

Fig. 1. Schematic representation of the experimental set up for determining blood flow resistance in isolated-perfused rat hind limb. P1 and P2 are pressure transducers, and P-Servo is the pressure servo controller device that maintains the pressure at P1 at 100 mmHg by automatically adjusting the flow rate generated by the roller pump (RP-2). Normal or glutaraldehyde treated rat RBC suspensions in autologous plasma were used for perfusion.

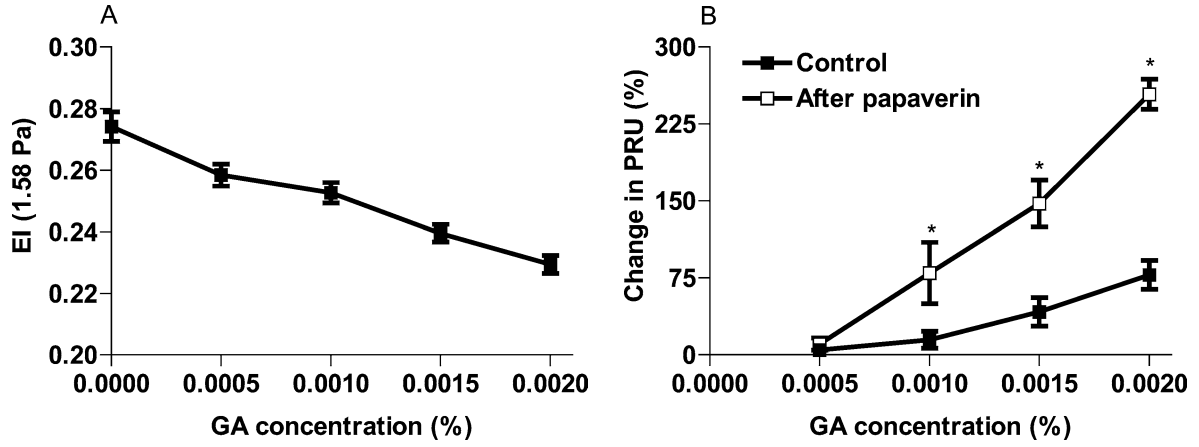

Fig. 2. Effect of glutaraldehyde concentration on RBC elongation index (i.e., deformability) as measured by ektacytometry at a shear stress of 1.58 Pa (panel A); Increase of blood flow resistance, relative to control (i.e., normal, untreated RBC), during perfusion with 0.4 l/l hematocrit glutaraldehyde-treated RBC suspensions (panel B). Data are mean $\pm$ standard deviation; $n = 4$ in both groups. *: Difference from control, $p < 0.001$.

In hind limb preparations with intact vasomotor control, infusions of red cell suspensions of decreasing deformability resulted in gradual increments of flow resistance (Fig. 2B). The increment in flow resistance during perfusion with the suspension of RBC treated with the highest glutaraldehyde concentration was about 78 percent. When vascular smooth muscle tone was inhibited by adding 10^{-4} M papaverin to the RBC suspensions being used, seminal observations included: (1) the absolute values of flow resistance markedly decreased (data not shown); (2) the change in flow resistance due to altered RBC deformability was significantly greater compared to preparations with intact vascular control mechanisms (Fig. 2B).

The experiment results shown in Fig. 2 clearly demonstrate the importance of vasodilatory reserve that can be used to compensate for alteration in tissue perfusion due to abnormal rheological behavior of blood (i.e., due to red cell deformability impairment in the example presented above). Nature also does such "experiments" by changing the geometry of blood vessels by pathological processes and thereby exhausting the vasodilatory reserve. In this case, any alteration that causes a decrease in blood flow (e.g., blood viscosity increment) cannot be compensated in the affected vascular network, whereas compensation can occur in vascular networks with sufficient autoregulatory reserve.

In another experiment series designed to demonstrate the importance of autoregulatory reserve in compensating for a hemorheological extra load, dogs were prepared by cannulating major blood vessels and the coronary sinus, and an electromagnetic flow probe was placed on the left coronary artery [8]. The dogs were then randomly assigned to one of two groups and a critical stenosis was applied to the left coronary artery of one group. The critical stenosis was applied using an adjustable mechanical occluder positioned proximally to the flow probe: (1) the artery was first constricted in small steps until a drop in coronary flow rate was just detected by the flow probe; (2) the occluder was than opened one step in order to achieve the flow rate that existed just before the flow rate reduction occurred – at this point the degree of narrowing of the artery varied between animals but was always greater than 70 percent constriction. The hematocrit of the dogs was increased about 50 percent by isovolemic exchange transfusion of packed dog red cells, resulting in about 100% increment in blood viscosity. Arterial and right atrial pressures and coronary artery blood flow were recorded before and after the hematocrit change.

As shown in Fig. 3, coronary artery resistance only slightly and non-significantly increased after the hematocrit was increased in the group with normal left coronary arteries (Fig. 3, left portion). However, in animals with stenosed left coronary arteries, flow resistance was significantly elevated after the same increase of hematocrit (Fig. 3, right portion). Additionally, erythrocyte deformability measured in blood samples obtained from coronary sinus (i.e., the blood returning from the coronary microvasculature) was more impaired if the left coronary arteries were critically stenosed (Fig. 4). Thus, changes of both flow resistance and RBC deformability in response to a hemorheological over load depend critically on the degree to which the coronary arteries are geometrically challenged [8].

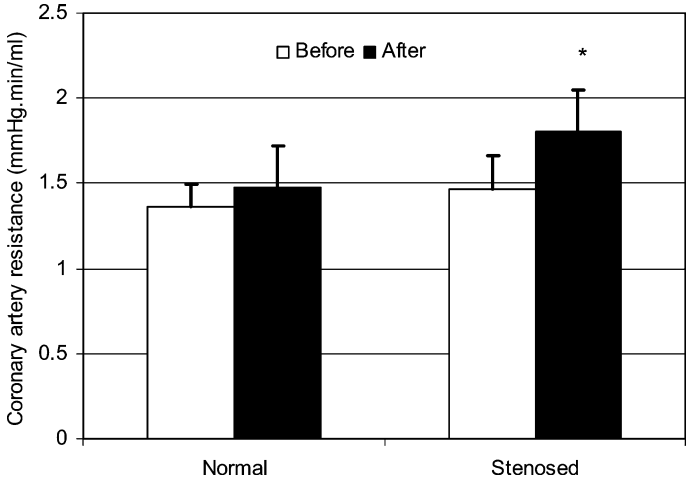

Fig. 3. Coronary artery resistance of dogs with normal or stenosed left coronary arteries before and after a 50% increment of hematocrit. Data are mean $\pm$ standard error; $n = 10$ for each group; *: Difference from the value obtained before the exchange transfusion, $p < 0.05$. (Redrawn from [8].)

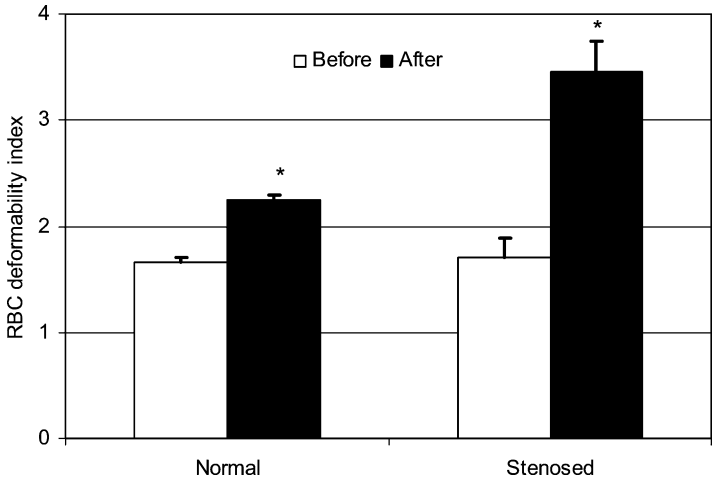

Fig. 4. RBC deformability indexes measured in blood samples obtained from the coronary sinus of dogs with normal or stenosed left coronary arteries before and after a 50% increment of hematocrit. Data are mean ± standard error; $n = 10$ for each group; *: Difference from value obtained before the exchange transfusion, $p < 0.05$. (Redrawn from [8].)

4.2. The effect of shear forces on vascular control mechanisms

Vascular tone is not only controlled by local metabolic factors, but also by specific vasoactive substances [12,42], with the most notable of these being nitric oxide [28]. Nitric oxide (NO) is a simple molecule that is synthesized in endothelial cells and diffuses to underlying smooth muscle cells where it activates guanylate cyclase [19,28,42]; the resulting increment in cyclic GMP results in smooth muscle relaxation [19,28]. NO is synthesized by NO synthases (NOS) using L-arginine as the substrate [31]. Endothelial NOS, which plays a major role in the regulation of vascular tone, is control by a variety of factors including hypoxia and shear stress [31]. These factors not only determine the activity of the existing enzyme, but also affect the expression of eNOS protein in endothelial cells [26,31]. Mechanical forces acting on endothelial cells are known to be an important determinant of the cell's NO output [25,47], with tangential wall shear stress affecting the NO synthesis mechanisms of endothelial cells [34,37]. Wall shear stress is, in turn, determined by the fluid velocity near the vessel wall and the viscosity of the fluid in this region [34,37]. Note that decreased wall shear stress down regulates NO output and may result in increased smooth muscle tone and hence increased vascular hindrance [48].

There are several experimental studies confirming the role of fluid velocity (i.e., local fluid shear forces) in determining NO output from vascular endothelium and hence flow-induced vasodilation mediated by NO (e.g., [29,30,35,48]). Using a rat chronic heart failure model induced by left coronary artery ligation, Varin et al. [48] indicate that flow-induced dilation in gracilis muscle arteries in response to various levels of fluid flow was totally abolished in vessels from rats with chronic heart failure; this response is well known to be mediated by NO. Varin et al. also demonstrated that NOS expression in skeletal muscle was suppressed in chronic heart failure. Interestingly, both the lack of flow-induced dilation and the suppression of NOS in rats with chronic heart failure could be prevented by a swimming exercise protocol that intermittently increased cardiac output and blood flow in skeletal muscle vessels [48].

A recent paper by Miyauchi et al. [35] also presents evidence supporting the effects of fluid forces on endothelial NO activity. In their study, rats were subjected to heavy exercise for 45 minutes, following which NOS activity was determined in the lungs and kidneys of these animals. Note that lung represents an organ with increased blood flow during exercise, while kidney represents the opposite (i.e., an organ with reduced blood flow during exercise). Their results indicated that compared to non-exercising animals, rats subjected to exercise had increased total NOS activity in their lungs but decreased total NOS activity in their kidneys, with the changes related to constitutive NOS activity [35]. The two examples cited above [35,48] thus clearly show that if wall shear stress is altered due to altered blood flow then NO synthesizing mechanisms are affected.

In addition to the blood flow rate near the vessel wall, the viscosity of the fluid in this region also affects wall shear stress [34,37]. It is well known that the composition, and hence the fluidity, of blood flowing in a cylindrical blood vessel is not uniform throughout the cross-section [17,27]. Rather, the distribution of RBC is not uniform and is influenced by axial migration [17]; increased RBC aggregation results in enhanced axial accumulation of red cells [11,22]. In turn, the enhanced accumulation of red cells in the central flow zone leaves a lower hematocrit, less viscous fluid in the marginal zone near the vessel wall [17]. Thus it can be expected that enhanced red cell aggregation may reduce wall shear stress and result in down-regulation of NO synthesis mechanisms and a resulting increase of vascular resistance.

The abovementioned hypothesis regarding the effects of RBC aggregation was tested in an experimental study performed on rats in which RBC aggregation was chronically enhanced by isovolumic exchange transfusions of poloxamer-coated red blood cell suspensions [9]. Poloxamer-coated red cells were suspended in autologous plasma at 0.4 l/l hematocrit and exchange transfused to rats to achieve a 30 percent exchange of the total blood volume. Note that in this approach, poloxamer copolymers are covalently attached to the RBC surface, and by appropriate selection of the poloxamer's molecular weight, RBC aggregation in autologous plasma can be significantly enhanced [2]. Conversely, use of a lower molecular weight poloxamer results in unaltered aggregation and thus provides a suitable control for the strongly aggregating suspensions.

In rats exchange transfused with RBC coated with a poloxamer known to enhance aggregation (F98), the extent of aggregation was found to be increased about 4-fold immediately after the exchange transfusion and to decrease gradually over a 4 day follow-up period [9]. Conversely, aggregation was not altered at any time point in rats exchanged with cells coated with the lower molecular weight poloxamer (F68). Mean arterial blood pressure, measured daily during the follow-up period using a non-invasive method, was found to increase gradually only in the group with enhanced aggregation. Moreover, NO-dependent relaxation responses of resistance artery segments obtained from the gracilis muscle of animals with enhanced RBC aggregation were found to be altered. Figure 5 demonstrates these findings and presents the flow-induced dilation response that is known to be NO mediated: in the group with enhanced red cell aggregation, the maximum dilation response was observed at a higher flow rate and the percentage of maximum dilation was only 50 percent of the control group. These data, supported by results indicating suppressed acetylcholine-induced dilation responses [9], suggest that NO related mechanisms were significantly depressed in the rats with enhanced red cell aggregation; this suggestion was confirmed by data indicating decreased eNOS expression in tissue samples obtained from gracilis muscle [9]. Such studies thus indicate that enhanced red cell aggregation may down-regulate NO-related vascular control mechanism yielding increased vascular resistance and altered blood pressure; thereby suggesting another means by which hemorheological parameters can influence vasomotor control.

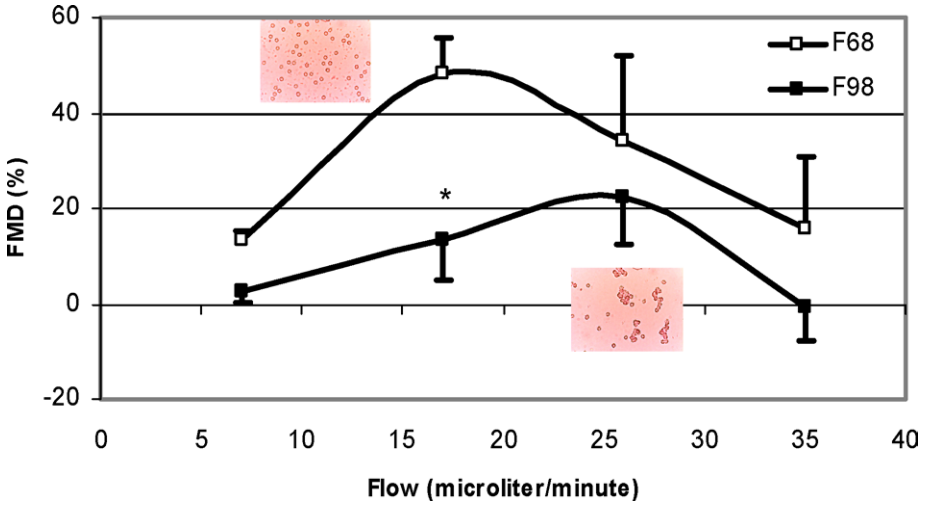

Fig. 5. Flow mediated dilation (FMD) in resistance arteries isolated from the gracilis muscles of rats exchanged with poloxamer-coated RBC. Cells coated with poloxamer F98 exhibit greatly enhanced aggregation while those coated with poloxamer F68 exhibit aggregation unaltered from control. Photographic insets indicate extent of RBC aggregation in blood samples from exchanged animals. Data are mean $\pm$ standard error; $n = 8$ for each group. (Redrawn from [9].)

5. Conclusion

The rheological behavior of blood is not merely a factor that, when combined with vascular hindrance (i.e., vessel geometry), determines hemodynamic resistance. Rather, hemorheological properties can also determine the magnitude of vascular hindrance via processes leading to altered vessel geometry. The effects of rheologic changes are probably mediated by a variety of vascular control mechanisms, and most likely include such phenomena as metabolic autoregulation and shear stress induced NO synthesis by vascular endothelium. Such multiple pathways by which hemorheological changes can affect vascular resistance may thus explain, in part, the continuing difficulty of predicting correlations between *in vivo* and *ex vivo* hemorheological behavior; they also suggest the need for continued experimental studies in this area.

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Red blood cell rheological alterations in hypertension induced by chronic inhibition of nitric oxide synthesis in rats

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Abstract. Nitric oxide (NO) plays a major role in vascular regulation. Modulation of NO synthesis is known to influence blood pressure. Inhibition of NO synthesis by NG-nitro-L-arginine methyl ester (L-NAME; 72 mg/kg/day, p.o., 21 days) resulted in 60% increase in blood pressure in rats. Red blood cell (RBC) transit time measured by the cell transit analyzer increased significantly in the L-NAME treated group, in comparison to normotensive rats. RBC aggregation measured in autologous plasma, by a photometric rheoscope also increased significantly in the hypertensive rats. RBC cytosolic free calcium concentration was also significantly higher in the hypertensive animals. Incubation of RBC from hypertensive and control animals with NO donor, sodium nitroprussid (SNP; 10-1000 µM) for 60 minutes resulted in a dose-dependant decrease in RBC aggregation, however aggregation index was significantly higher in hypertensive group at each SNP concentration. Incubation with SNP had no effect on RBC deformability in the control group, while a slight decrease in RBC transit time was observed only at 10 µM SNP in the hypertensive group. There results imply that NO may play a role in the regulation of rheological properties of RBC and the alterations in these properties may at least in part be involved in the development of L-NAME induced hypertension.

Key Words: Hypertension; erythrocyte aggregation; erythrocyte deformability; nitric oxide; L-NAME

1. Introduction

Hypertension is a pathophysiological condition with well-documented consequences related to many organ systems and tissues [18]. Blood tissue also seems to be affected in hypertensive individuals; hematocrit, plasma composition, coagulation system, platelet functions and white blood cells were reported to be influenced as well as red blood cells (RBC) [1,26]. Altered cholesterol/phospholipid ratio, transmembrane ion transport, mean cell volume and mechanical properties of RBC were reported in hypertension [1]. If hypertension is considered as a generalized vascular disease, these RBC alterations can be accepted as being secondary to the circulatory disorders, especially at the microcirculatory level [30,31]. Alternatively, altered RBC properties may play role in the development of hypertension [20], determining the fluidity of blood together with hematocrit value and plasma composition [12].

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Decreased RBC deformability and increased RBC aggregation are reported hemorheological alterations in patients with various clinical manifestations of hypertension [1]. Additionally, studies on spontaneous hypertension of rats revealed abnormalities in RBC structure and physiology, including alterations in cation homeostasis and mechanical properties [8,11,19,23]. There is considerable evidence that supports the existence of genetically determined alterations in these mechanisms and at least part of these alterations does not seem to be the result of non-specific effects of hypertension. Mchedlishvili et al. proposed that alterations in RBC mechanical properties lead to increased blood pressure and such changes (e.g., increased RBC aggregation) may play role in the increased peripheral vascular resistance [21,22].

Chronic inhibition of nitric oxide (NO) synthesis is a relatively new method to induce experimental hypertension [35]. Chronic administration of N-omega-nitro-L-arginine methyl ester (L-NAME), which is an orally active inhibitor of NO synthesis, induces persistent hypertension and renal damage [35]. The inhibition of NO synthesis results in diminished tonic vasodilatation that leads to increased peripheral resistance. The roles of renin-angiotensin system, calcium metabolism, salt retention and arachidonic acid derivatives in the development of L-NAME induced hypertension were also discussed [35].

Korbut and Gryglewski reported that NO generated by polymorphonuclear leukocytes or NO-donating agents (e.g., Sodium nitroprussid, SIN-1) preserves or enhances RBC deformability within a certain concentration range [16], although the mechanism of this beneficial effect on RBC mechanics is not known. Therefore, inhibition of NO-synthesis might be expected to have deleterious effects on RBC deformability. Such an alteration may contribute to the elevation of blood pressure by increasing peripheral vascular resistance [22].

This study aimed at investigating the RBC mechanical alterations in rats with chronic NO-synthesis inhibition by oral administration of L-NAME. In vitro experiments with a NO donor (i.e., sodium nitroprussid) had also been performed to obtain more direct evidence on the effects of NO on RBC mechanical properties in control and hypertensive rats.

2. Materials and methods

2.1. L-NAME hypertension model

Ten weeks old, male albino rats were used in the experiments. Rats were randomly divided into two to form Control and hypertensive (HT) groups. Standard rat chow and tap water was provided ad libitum. To induce hypertension in the HT group, L-NAME was added to drinking water at a concentration of 600 mg/l, and each rat received 72 mg/kg/day L-NAME on average, for 21 days. Control animals were kept under the same conditions, but L-NAME was not added to their drinking water. Blood pressure of these animals was monitored using a tail cuff blood pressure measuring system (Harvard Apparatus Ltd, Edenbridge, Kent, England).

2.2. Blood samples and measurements

Blood samples were obtained from the abdominal aorta of the rats, under light ether anesthesia and anticoagulated with Sodium Heparin (15 U/ml). These samples were used for the measurements of RBC aggregation, deformability, cytosolic free calcium and lipid peroxidation. Blood samples from a group of animals were used in *in vitro* experiments, as described below.

2.3. Incubation of RBC with sodium nitroprussid (SNP)

The effect of NO production in vitro was tested by incubating RBC with SNP at varying concentrations. RBC were washed with isotonic phosphate-buffered saline (PBS; pH: 7.4) twice and suspended in the same buffer at a hematocrit of 0.4 l/l. The suspension was divided into five aliquots and SNP was added to each aliquot to obtain the concentrations of 0, 10, 100, 300 and 1000 μM. The suspensions were incubated at room temperature for 60 min. The RBC were then separated by centrifugation, washed two times in PBS and resuspended in autologous plasma at a hematocrit of 0.4 l/l, to be used in RBC aggregation and deformability measurements as well as the determination of lipid peroxidation. Blood samples from both control and L-NAME hypertension groups were used for in vitro experiments.

2.4. Determination of aggregation parameters

RBC aggregation was assessed using a custom-built photometric aggregometer interfaced to a digital computer [6]. The shearing portion of the system consists of two parallel glass plates with a gap of 300 μm between them; a stepper motor, controlled by the computer, rotates one of these plates. An infrared light emitting diode and a phototransistor, combined with an amplifier and an A/D converter, were used to record light transmission-time data. The blood sample under study is placed between the glass plates, and is first sheared at 500 sec^{-1} for 10 sec to disperse RBC aggregates. After a sudden stop of motor, the infrared light transmission through the blood sample is monitored for 10 sec and recorded by the computer. The computer then calculates the area under the light transmission curve and reports a dimensionless index which reflects the extent of aggregation. Measurements were done in triplicate for each sample and the mean of these three measurements were used as the result. Measurements were done at room temperature (20±2 °C).

2.5. RBC deformability measurements

RBC deformability was estimated by measuring transit times through pores with 5 μm diameter and 15 μm length using a cell transit analyzer (CTA) [2]. CTA consists of an oligopore filter with 30 cylindrical pores, mounted between two reservoirs and an AC conductimeter. The conductimeter operates at 100 KHz and measures the electrical resistance between the electrodes placed in each reservoir. By adjusting the level of fluid in the reservoirs, a pressure gradient is created which forces the dilute RBC suspension (0.04% in PBS) in one of the reservoirs, to flow through the oligopore filter. The passage of a RBC through one of the 30 pores results in a resistance change between two reservoirs. A resistive pulse is generated at the output of the conductimeter circuit, which carries the information about the passage of that RBC through a 5 μm pore. This signal is then digitized and passed on a digital computer for analysis. Computer determines the width of each valid pulse that corresponds to the transit time (TT) of a RBC through a pore. Transit times of 1000 RBC for each specimen were determined and mean of them used to represent the deformability of that RBC population. The pressure gradient used in this study was 3 cmH_2O. All measurements were conducted at room temperature (20±2 °C).

2.6. Cytosolic calcium determinations

Cytosolic calcium was determined in intact RBC using the method modified from David-Dufilho *et al.* [14]. RBCs were separated using a density gradient (Histopaque-1071, Sigma Chemical Company, St. Louis, MO, USA) to eliminate contaminating WBC. After two washing steps with isotonic PBS, RBC suspension of 0.1% was prepared in isotonic PBS. RBC suspension was incubated at 37 °C, in the presence of Fura-2AM (Sigma Chemical Company, St.

Louis, MO, USA), at a concentration of 10 nM, for 25 min. The suspension was then centrifuged at 350 g for 5 min. to eliminate extracellular Fura-2AM and RBC were resuspended with PBS to give a suspension of 10^8 cells per ml. The fluorescence spectrum was measured at an excitation range of 335-385 nm, with an emission wavelength of 510 nm using a spectrofluorophotometer (Shimadzu RF-5000, Tokyo, Japan). The ratio between the fluorescence intensities of Fura 2-Ca^{2+} complex and the unchelated Fura 2 (F335/F385) reflects the cytosolic calcium concentrations [10].

2.7. Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS, as an indicator of lipid peroxidation were determined spectrophotometrically according to Stocks and Dormandy [29].

2.8. Statistics

Results are expressed as mean ± SE. Statistical comparisons between groups were done by "Student's t test" for two groups and "Repeated Measures ANOVA" followed by "Newman-Keuls post test" and p values <0.05 were accepted as statistically significant.

3. Results

3.1. L-NAME hypertension and RBC mechanical properties

L-NAME treatment resulted in a progressive increment in blood pressure (Figure-1). Systolic blood pressure of the rats that received L-NAME was 110 ± 10 mmHg at the start of the experiment, and increased to 176 ± 20 mmHg after three weeks of L-NAME treatment. Blood pressure in the control group was not significantly different from the starting value of the HT group and no significant alteration was observed throughout the experimental period. RBC aggregation was found to be significantly increased ($p<0.05$) in the hypertensive (HT) group in comparison with the control group (Figure 2). RBC TT was also significantly higher, indicating a decreased deformability in the HT group ($p<0.05$) (Figure 3). RBC cytosolic free calcium levels measured in a group of hypertensive animals were more then two times higher then the control value ($p<0.05$) (Figure 4) while RBC TBARS levels were not significantly different in control and HT groups (data not shown).

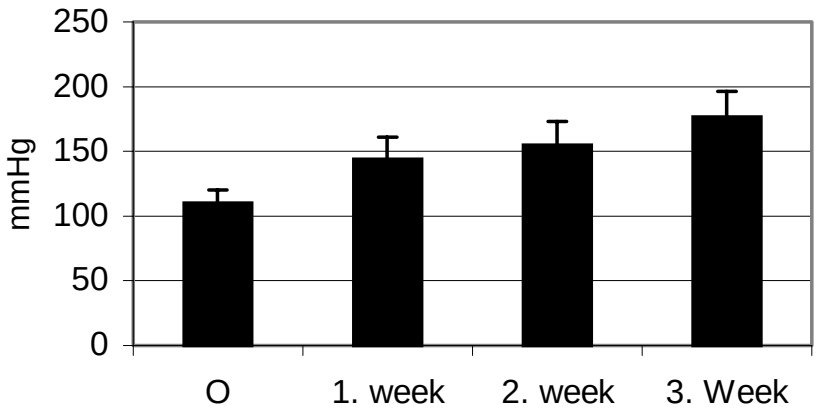

Fig. 1. Time course of systolic blood pressure in L-NAME treated group (n=12)

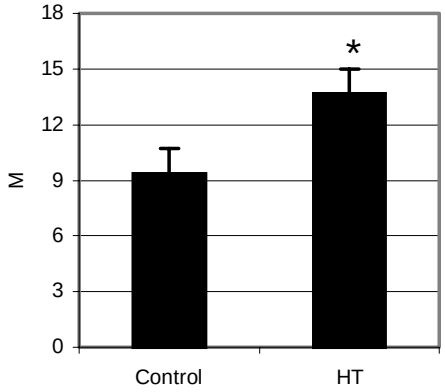

Fig. 2. RBC aggregation indexes (M) in the Control and L-NAME treated (HT) groups (n=12 in both groups; *: Difference from Control; $p<0.05$)

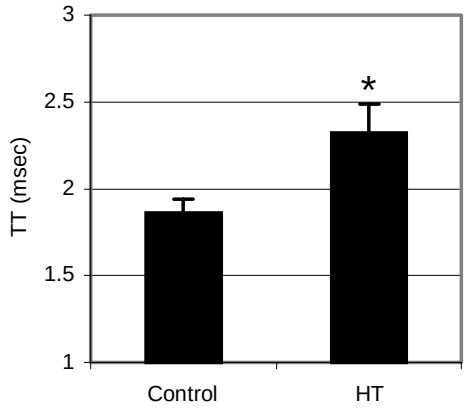

Fig. 3. RBC transit time (TT) in the Control and L-NAME treated (HT) groups (n=12 in both groups; *: Difference from Control; $p<0.05$)

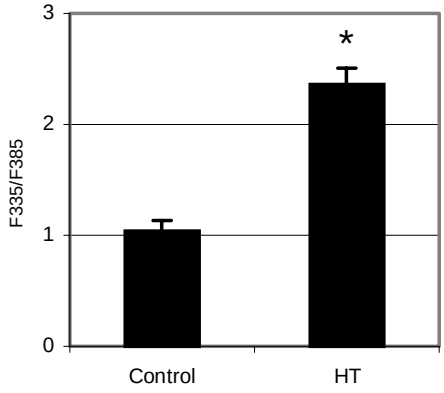

Figure 4: Cytosolic calcium levels of RBC in the Control and L-NAME treated (HT) groups (n=5 in both groups; *: Difference from Control; $p<0.05$)

3.2. Effects of SNP on RBC mechanical properties

SNP inhibited RBC aggregation in a dose dependent manner in the blood samples obtained from both Control and HT groups (Figure 5). However, at each concentration of SNP, the aggregation index of HT RBC remained higher than the control RBC (Figure 5). SNP had no effect on RBC deformability in the control samples, but a significant decrement in TT was

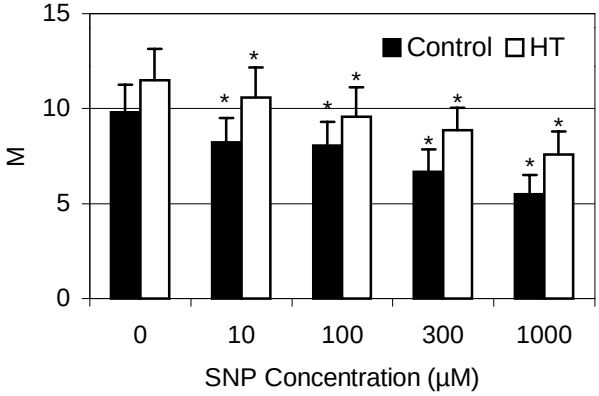

Fig. 5. Effect of SNP on RBC aggregation indexes (M) in the Control and L-NAME treated (HT) groups (n=10 in both groups; *: Difference from "0"; $p<0.05$)

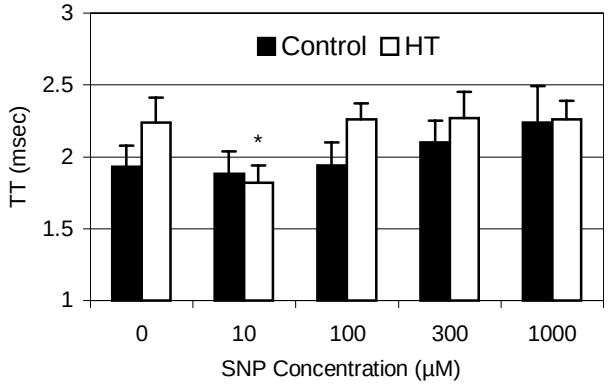

Fig. 6. Effect of SNP on RBC transit time (TT) in the Control and L-NAME treated (HT) groups (n=10 in both groups; *: Difference from "0"; $p<0.05$)

observed at 10 mM SNP concentration in the HT samples ($p<0.05$) (Figure 6). At higher concentrations the TT were not significantly altered. There was no significant alteration in TBARS levels at any concentration of SNP (data not shown).

4. Discussion

Chronic inhibition of NO synthesis by orally administered L-NAME is a well-documented model of hypertension and discussed in detail elsewhere [35]. In this study, the mean arterial blood pressure of the rats received L-NAME in their drinking water increased significantly starting in the first week and reached to 176 mmHg in three weeks. Both RBC aggregation and deformability were also found to be altered significantly in hypertensive animals in comparison with the normotensive, control animals. RBC mechanical properties (i.e., deformability and aggregation) were previously demonstrated to be altered in clinical hypertension [1] and in spontaneous hypertension of rats [19] and this study confirmed the existence of similar alterations in L-NAME induced hypertension.

Mechanical properties of RBC are dependent on the proper metabolic conditions and a normal homeostasis in their microenvironment [12,27]. This is especially true for the microcirculatory area and RBC can be affected while passing through this area if the microcirculatory function is impaired. Increased blood pressure for prolonged periods may seriously affect the microvascular function [33]. Released metabolites from damaged cells/tissues, altered pH, increased free radical generation and inflammatory reaction due to tissue damage may affect RBC properties [1]. Oxidative stress directly in the microvasculature of hypertensive rats has been clearly demonstrated [30,31]. It has been demonstrated that the number and activity of leukocytes were increased in hypertensive rats [25,26]. The enhanced leukocyte activity might also contribute to

tissue damage, especially the damage of oxidant nature. Activated leukocytes affect both deformability and aggregability of neighboring RBC [4].

Both of the above mentioned mechanisms of RBC damage in hypertension are not specific to the underlying cause of hypertension, but related to the non-specific tissue damage/inflammation caused by increased blood pressure. Besides these two possible mechanisms in which the effects on RBC are mediated by oxygen free radicals it had been proposed that inhibition of NO synthesis may also result in increased oxidant stress in microvasculature [30]. This suggestion is based on the observation of increased microvascular oxidant stress assessed by hydroethidine microfluorography after L-NAME superfusion on mesenteric arterioles and venules of normotensive rats [30].

The observed alterations of RBC deformability in hypertensive rats in this study can well be explained by oxidant damage. It has been previously shown by in vitro studies that oxygen free radicals alter RBC deformability possibly by affecting the membrane properties [3,7,32]. Oxygen free radicals may also affect RBC aggregability, possibly by altering their surface properties [7]. The effect of oxygen free radicals on RBC aggregability might be biphasic, depending on the magnitude of oxidant stress; at lower levels of oxidants the result is enhanced aggregability, while at higher levels the aggregation might be inhibited [5]. TBARS levels of RBC in L-NAME treated rats were not different from control animals. TBARS measurements are widely used as a measure of oxidant damage in RBC. However it has been previously observed that TBARS levels may not be altered in RBC under oxidant stress, depending on the experimental conditions, although other indicators of oxidant damage was present [3,7].

Altered RBC mechanical properties may also be the consequence of increased cytosolic free calcium concentration [15]. A significant increment in RBC cytosolic free calcium was observed in L-NAME treated animals. Increased RBC cytosolic free calcium concentrations were previously reported in hypertension and can be explained by altered permeability of the membrane and/or altered cation pump activities [9,11,23,24]. These alterations in calcium metabolism might be the consequence of alterations in the microenvironment of RBC that are discussed above. Alternatively, NO may play role in the regulation of cytosolic calcium concentration [13] and calcium homeostasis might be altered if its synthesis is inhibited.

Korbut and Gryglewski proposed that NO has a modulator effect on RBC deformability [16]. This effect seems to be dose dependent and NO might have opposite effects under certain circumstances. The same group of researchers reported the protective effect of NO synthase inhibitors on RBC deformability in endotoxaemia and septic shock [17,28]. Based on these observations specific/direct effects of NO on RBC mechanical properties could also be suggested [30,13]. In the samples obtained from control rats, incubation with SNP had no effect on RBC deformability. However, there was a significant decrement in RBC transit times in hypertensive samples incubated with 10 μM SNP, implying increased deformability. This effect that was observed only in hypertensive rat samples can be regarded as the correction of deterioration at 10 μM SNP concentration; the RBC TT just "returned" to control level, while at other concentrations RBC from hypertensive animals had longer TT. Incubation with SNP at low concentration seems to provide a "missing factor" that keeps deformability at normal.

SNP, used as NO donor in this study was reported to have significant effects on RBC shape and blood viscosity at 10 mM concentration (34). However, the doses of SNP used in this study were significantly below this concentration; the highest concentration used was 1 mM, and the concentration found to be effective in correcting the deteriorated RBC deformability was 10 μM.

The in vitro effect of SNP on RBC aggregability was an inhibition in the samples obtained both from L-NAME treated and control animals. This inhibition was dose dependant. A similar dose dependent inhibition of aggregation can be seen in oxidant treated RBC [7]. In our samples incubated with SNP TBARS levels were not altered, but this does not rule out a possible oxidant damage of RBC membranes, as discussed above. However, oxidant damage is also known to induce RBC deformability impairment [7,32]. The increased deformability at 10 μM SNP

concentration suggests that an oxidative damage is unlikely in the samples incubated with the NO donor, SNP.

Although RBC aggregation was found to be decreased with increased SNP concentrations, the aggregation was higher in hypertensive samples at all concentrations, implying that the increased RBC aggregation may not be directly related to NO insufficiency, as suggested for the deformability alteration. This difference between control and hypertensive samples might be due to plasma alterations in hypertension. Such alterations were reported in hypertension [1] and could be due to the inflammatory response and related acute phase reaction.

The in vitro effects of NO on RBC deformability observed in this study might be expected to improve blood fluidity and decrease flow resistance with a given vessel geometry. Therefore, NO deficiency may lead to RBC mechanical alterations that may contribute to the increased peripheral resistance [20,21,22], in addition to the well-documented effect related to the lack of tonic vasodilatation. Certainly, our findings do not rule out the RBC mechanical alterations resulting from impaired microvascular function in hypertension. However, the decreased deformability might at least in part be the direct result of NO deficiency in L-NAME treated rats.

Our results also support the hypothesis of Korbut and Gryglewski who suggested a regulatory role for NO on RBC mechanics [16]. The exact role of this regulatory function and the underlying mechanisms of the effects of NO on RBC mechanics need further investigation.

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