



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

**ESTABILIDADE DE MEMBRANA E MICROSCOPIA DE FORÇA ATÔMICA EM
ERITRÓCITOS DE VOLUNTÁRIAS DE DIFERENTES IDADES**

Estudante: Mariana Vaini de Freitas

UBERLÂNDIA, MG

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Estudante: **Mariana Vaini de Freitas**
Orientador: **Professor Dr. Nilson Penha-Silva**

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ABREVIATURAS

A₁	Absorvância com valor mínimo de hemólise
A₂	Absorvância com valor máximo de hemólise
A₅₄₀	Absorvância a 540 nm
AFM	<i>Atomic force microscopy</i> (microscopia de força atômica)
ATP	Trifosfato de adenosina
Cat	Catalase
D₅₀	Concentração de agente SDS que causa 50% de hemólise
dD	Variação na concentração de agente surfactante necessária para promover hemólise
Glu	<i>Glucose</i> (glicose)
H₂O₂	<i>Hydrogen peroxide</i> (peróxido de hidrogênio)
Hb	<i>Hemoglobin</i> (hemoglobina)
HDL-C	<i>High-density lipoprotein cholesterol</i> (Lipoproteína de alta densidade)
HSA	<i>Human serum albumin</i> (albumina)
Ht	<i>Hematocrit</i> (Hematócrito)
LDL-C	<i>Low-density lipoprotein cholesterol</i> (lipoproteína de baixa densidade)
MCV	<i>Mean corpuscular volume</i> (volume corpuscular médio)
MCH	<i>Mean corpuscular hemoglobin</i> (hemoglobina corpuscular média)
MCHC	<i>Mean corpuscular hemoglobin concentration</i> (concentração de hemoglobina corpuscular média)
nHDL-C	<i>Non-high density lipoprotein cholesterol</i> (Colesterol de lipoproteína de não alta densidade)
PUFA	<i>Polyunsaturated fatty acid</i> (ácido graxo polinsaturado)
Ra	<i>Arithmetic mean roughness</i> (média aritmética da rugosidade)
RBC	<i>Red blood cell</i> (eritrócito)
RDW	<i>Red cell distribution width</i> (variação de tamanho dos eritrócitos)
RNS	<i>Reactive nitrogen species</i> (espécies reativas de nitrogênio)
ROS	<i>Reactive oxygen species</i> (espécies reativas do oxigênio)
Rp	<i>Average height</i> (altura média)
Rv	<i>Average depth</i> (profundidade média)
Rz	<i>Maximum height</i> (altura máxima)
SDS	<i>Sodium dodecyl sulfate</i> (dodecil sulfato de sódio)

SFA	<i>Saturated fatty acid</i> (ácido graxo saturado)
SOD	<i>Superoxide dismutase</i> (superóxido dismutase)
t-C	<i>Total cholesterol</i> (colesterol total)
TG	<i>Triglycerides</i> (triglicérides)
UFA	<i>Unsaturated fatty acid</i> (ácido graxo insaturado)
VLDL-C	<i>Very low density lipoprotein</i> (Lipoproteína de muito baixa densidade)

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APRESENTAÇÃO

O Laboratório de Biofísicoquímica (LABFIQ) do Instituto de Genética e Bioquímica da Universidade Federal de Uberlândia padronizou métodos de estudo do efeito de solutos osmoestabilizantes e caotrópicos na membrana de eritrócitos. Esses métodos têm sido aplicados para entender como diversas condições fisiológicas dos voluntários, a exemplo de envelhecimento, doenças degenerativas e atividades físicas, podem influenciar os parâmetros de estabilidade dessas células.

Dentre os métodos, podemos citar choque hipotônico e estabilidade da membrana em etanol e dodecil sulfato de sódio (SDS). A dinâmica de lise pela ação caotrópica do SDS é caracterizada por uma transição entre dois estados, o estado íntegro e o estado lisado dos eritrócitos. A linha de transição espectrofotométrica é então analisada por regressão sigmoideal, com a determinação do ponto de meia-transição, que representa a concentração de SDS (D_{50}) na qual 50% das células estão hemolisadas.

Esses dados de estabilidade têm sido posteriormente correlacionados com variáveis hematológicas e bioquímicas coletadas dos voluntários de diferentes tipos de populações. As análises bivariadas são feitas inicialmente para estabelecer correlações significantes positivas e negativas entre os parâmetros e servem para formar os grupos que irão embasar as análises multivariadas. A estatística multivariada, por sua vez, nos fornece informações sobre quais variáveis influenciaram mais fortemente os parâmetros de estabilidade dos eritrócitos.

Além disso, o LABFIQ fez parceria com o Laboratório de Novos Materiais Isolantes e Semicondutores (LNMIS) do Instituto de Física da UFU, sob responsabilidade do professor Dr. Noelio Oliveira Dantas, para realização de microscopia de força atômica (AFM) em amostras sanguíneas. Esse método fornece dados sobre a rugosidade e topografia da membrana eritrocitária e dimensões celulares.

Sendo assim, o presente trabalho correlaciona os dados de estabilidade da membrana do eritrócito em SDS com variáveis hematológicas e bioquímicas e os dados fornecidos pela AFM, em voluntárias com idade entre 20 e 90 anos.

Conforme as normas definidas pelo Programa de Pós-Graduação em Genética e Bioquímica da UFU, o Capítulo 1 desta tese traz a Fundamentação Teórica e o Capítulo 2 e 3 trazem a íntegra dos trabalhos experimentais realizados.

CAPÍTULO 1

FUNDAMENTAÇÃO TEÓRICA

Membranas biológicas

As membranas celulares proporcionam a definição do meio intra e extracelular e possibilitam que as reações bioquímicas se desencadeiem em cada meio, permitindo trocas seletivas de acordo com as necessidades do organismo.

As membranas biológicas são trilaminares, sendo a lâmina interna de natureza hidrofóbica e as lâminas externas de natureza hidrofílica. Embora a membrana seja composta por diversas classes de lipídios, os que predominam são os fosfolipídios, que podem representar até 95% dos lipídios de membrana (Cooper, 1977). O colesterol é um importante lipídio encontrado nas membranas de células eucarióticas, uma vez que confere às camadas lipídicas maior espessura e rigidez (Roduit, Van Der Goot *et al.*, 2008). A lâmina ou núcleo hidrofóbico da membrana é constituído pelas cadeias hidrocarbonadas dos fosfolipídios e pela porção hidrofóbica do colesterol livre. As proteínas imersas ou apenas contíguas à membrana estabelecem numerosas forças atrativas de van der Waals com os fosfolipídios ao seu redor (Cribier, Morrot *et al.*, 1993). A composição de uma membrana pode variar de acordo com a função que ela desempenha (Storry, 2004).

É através de características próprias das membranas, como fluidez e estabilidade, que a célula suporta estresses externos e garante sua integridade e funcionalidade. A deformabilidade da célula é regulada, dentre outras características, pelas propriedades viscoelásticas da membrana (Nash e Meiselman, 1983).

A fluidez é uma propriedade da matéria entre o estado sólido e o estado líquido, e está relacionada à dinâmica da estrutura da membrana (Shiga, Maeda *et al.*, 1990). A organização de uma membrana biológica exige um nível adequado de fluidez, que pode ser designado de fluidez crítica (**Figura 1.1**).

No ponto de fluidez crítica, a membrana congrega a estabilidade necessária para sua preservação com um nível de elasticidade necessário para que as células sofram as alterações conformacionais exigidas pelo exercício de funções complexas, como a transdução de sinais hormonais para moléculas no interior da célula, onde elas

vão regular funções celulares relacionadas com o metabolismo, a diferenciação e a proliferação (Sinensky, 1974).

A manutenção dessa fluidez de membrana depende de vários mecanismos homeostáticos. Alguns agentes e solventes podem modificar sistematicamente essas estruturas, como variação nas concentrações de sal, pH, colesterol, ácidos graxos saturados e insaturados dos fosfolipídios, além de certas doenças (Girasole, Pompeo *et al.*, 2010). Além disso, as propriedades mecânicas da membrana dependem da sua composição que sofre modificação nos processos que acompanham o envelhecimento. O acúmulo de produtos de oxidação lipídica nas membranas e o crescimento dos domínios com colesterol durante a idade levam a um aumento da rigidez média e da viscosidade da bicamada lipídica (Rizvi e Maurya, 2007; Starodubtseva, 2011).

A fluidez de membrana é aumentada pela elevação nos teores de ácidos graxos insaturados nos fosfolipídios e diminuída pela elevação no teor de ácidos graxos saturados e de colesterol (**Figura 1.1**). Um aumento excessivo no teor de UFA e/ou uma diminuição exagerada no teor de colesterol pode promover fusão da membrana, tornando-a sujeita à lise por fusibilidade. Por outro lado, um aumento excessivo no teor de SFA e/ou de colesterol vai promover rigidificação de membrana, tornando-a sujeita à lise por friabilidade. Assim, tanto aquém quanto além da fluidez crítica, a membrana perde estabilidade e funcionalidade (Caliskan, Caliskan *et al.*, 2000).

O calor também aumenta a fusibilidade de membrana (**Figura 1.1**). O impacto da temperatura sobre a estabilidade de membrana num organismo homeotérmico ocorre diferentemente de organismos pecilotérmicos. Micro-organismos que têm seu 'habitat' em regiões de altas temperaturas têm maior teor em ácidos graxos saturados em seus fosfolipídios de membrana do que organismos que vivem em baixas temperaturas (Yatvin, 1977).

A produção e concentração de osmólitos, que são solutos orgânicos de baixa massa molecular, é uma estratégia de manutenção da estabilidade celular amplamente preservada em diferentes tipos de organismos (Yancey, 1985; Santoro, Liu *et al.*, 1992). Além disso, a osmoestabilização é utilizada em processos biotecnológicos para criopreservação de eritrócitos e outras células por longos

períodos. Os osmoestabilizadores são comumente pequenos solutos, como glicerol, sorbitol, trealose e dextrana (Boutron e Arnaud, 1984; Santoro, Liu *et al.*, 1992; Scott, Lecak *et al.*, 2005).

Eritrócitos e parâmetros hematológicos

Os eritrócitos são um dos principais componentes do sangue. A estrutura do eritrócito inclui a membrana plasmática, citoesqueleto hexagonal ligado à membrana por proteínas integrais, e concentrado de hemoglobina. O eritrócito humano não tem núcleo nem mitocôndria. Assim, o conteúdo intracelular é frequentemente considerado como uma solução homogênea rica em proteína, a maior parte hemoglobina. Reticulócitos, presentes cerca de 1% no sangue, são precursores diretos de eritrócitos maduros e contêm várias quantidades de RNA e ribossomos (Mohandas e Gallagher, 2008). A contagem de reticulócitos fornece informações iniciais para a investigação de anemia e eritropoiese (Marks e Glader, 2009).

Os índices hematológicos são valores laboratoriais que funcionam como biomarcadores adicionais indicando a saúde, o estado nutricional e a resposta aos tratamentos médicos (Hausman, Fischer *et al.*, 2012). Dentre eles, a concentração de hemoglobina (Hb) dentro do eritrócito difere dependendo da idade das células e do indivíduo. Com o envelhecimento, são observadas modestas mudanças na quantidade de Hb em adultos saudáveis (Marks e Glader, 2009). Uma importante propriedade da hemoglobina é a sua contribuição com a viscosidade interna, o qual determina as propriedades reológicas dos eritrócitos. A viscosidade intracelular pode ser representada pela concentração de hemoglobina corpuscular média (CHCM), que é calculada a partir do conteúdo da hemoglobina e do volume da célula. Quando há uma alta concentração da hemoglobina intracelular, a deformação do eritrócito é diminuída e a passagem pelos capilares pode ser retardada (Shiga, Maeda *et al.*, 1990).

O volume corpuscular médio (VCM) é a medida do volume médio dos eritrócitos. Altos valores de VCM podem indicar anemia macrocítica associada à deficiência de vitamina B₉ (ácido fólico) e/ou B₁₂ (cobalamina). Quando VCM é

diminuído, os eritrócitos são menores do que o normal (microcitose), tal como é visto na anemia por deficiência de ferro ou talassemias (Batool, Wang *et al.*, 2013).

RDW é um índice quantitativo da variabilidade no tamanho dos eritrócitos e indica a anisocitose na corrente sanguínea. Os valores mais altos refletem uma grande heterogeneidade no tamanho das células na circulação. Este parâmetro é utilizado na área clínica como índice auxiliar para diagnóstico de anemia microcítica. Assim, valores elevados de RDW são associados à anemia por deficiência de ferro (Batool, Wang *et al.*, 2013) e, recentemente, têm sido relacionados com processos inflamatórios (Ozturk, Unal *et al.*, 2013), estresse oxidativo (Semba, Patel *et al.*, 2010), risco de doenças cardiovasculares, hipertensão e diabetes (Felker, Allen *et al.*, 2007; Cavusoglu, Chopra *et al.*, 2010; Patel, Semba *et al.*, 2010; Malandrino, Wu *et al.*, 2012; Nishizaki, Yamagami *et al.*, 2012; Tanindi, Topal *et al.*, 2012; Tziakas, Chalikias *et al.*, 2012; Keskin Kurt, Aras *et al.*, 2013). O mecanismo que define a associação entre os maiores valores de RDW e mortalidade ainda não foram definidos. Nesse contexto, é possível que este parâmetro seja um novo marcador para múltiplas deficiências fisiológicas associadas à idade (Cauthen, Tong *et al.*, 2012).

Membrana dos eritrócitos

O volume médio do eritrócito é cerca de 90 fL, com diâmetro médio de aproximadamente 7 µm, correspondendo a uma esfera de 2,8 µm de raio. Essa célula possui um excesso de área de superfície de 40%, o que propicia a forma de disco bicôncavo suportar certa elevação de água sem causar lise, além de garantir sua deformabilidade (**Figura 1.2**). O volume celular pode aumentar 1,5 a 1,8 vezes em ambiente hipotônico, sem destruição ou ruptura da membrana (Shiga, Maeda *et al.*, 1990; Mohandas e Gallagher, 2008).

A membrana dos eritrócitos é composta por vários componentes de proteína e lípidio. Cerca de 52% da massa da membrana consiste de proteínas, 40% de lípidos e 8% de carboidratos (Mohandas e Evans, 1994). Os eritrócitos são responsáveis por transportar o oxigênio pelo organismo. A fluidez de membrana dos eritrócitos garante

as trocas gasosas entre a célula e o tecido, e ainda permite a manutenção da integridade celular dentro do sistema vascular (Waugh, Narla *et al.*, 1992; Bransky, Korin *et al.*, 2007). Existem três fatores essenciais na regulação da habilidade de deformação do eritrócito: geometria do eritrócito (área de superfície/volume); viscosidade citoplasmática, determinada pela concentração de hemoglobina; e características mecânicas da membrana (Mohandas e Gallagher, 2008).

A bicamada lipídica da membrana é composta por proporções iguais em peso de colesterol e fosfolípidos. Embora o colesterol seja distribuído igualmente entre as bicamadas, os principais fosfolípidos estão dispostos de forma assimétrica (Mohandas e Gallagher, 2008). A composição dos lipídios em eritrócitos humanos depende parcialmente do hábito nutricional (Bhandaru, Srinivasan *et al.*, 1982).

A membrana dos eritrócitos contém grande quantidade de proteínas que interagem entre si e são responsáveis por propriedades antigênicas, função de transporte e propriedades mecânicas da célula. A organização estrutural ordenada e específica dos componentes da membrana eritrocitária é responsável pelas características da grande deformabilidade e estabilidade da membrana que são necessários para a célula desempenhar a sua função fisiológica (Da Costa, Galimand *et al.*, 2013).

O eritrócito é capaz de resistir a grandes forças de cisalhamento durante a passagem pela circulação em virtude da sua flexibilidade e das propriedades mecânicas e físicas únicas da sua membrana celular (Williams, 1973). Eritrócitos são amplamente utilizados como modelo para estudo de propriedades e funções celulares, como transporte, estabilidade mecânica e resposta a fatores externos. Uma característica distinta é a deformabilidade quando passam pelos capilares de pequenos diâmetros (Zinchuk, 2001). As principais características de seu comportamento celular são alta elasticidade, rápida resposta a ampla variedade de estressores e alta resistência estrutural (Mohandas e Gallagher, 2008).

Quando os capilares apresentam diâmetro menor que dos eritrócitos, as células sofrem deformação para garantir o fluxo sanguíneo. Nos vasos maiores a deformação é também importante para reduzir a resistência hidrodinâmica. Assim que as forças

aplicadas vão subitamente cessando, a forma da célula em disco bicôncavo pode ser restaurada (**Figura 1.2**) (Kim, Kim *et al.*, 2012).

Os fatores determinantes da deformabilidade celular são a razão da área de superfície para volume; a viscosidade intracelular ou concentração de hemoglobina; e rigidez ou flexibilidade da membrana. Estes fatores variam com a idade da célula. Então, em relação a toda população de eritrócitos há certa heterogeneidade em termos de deformabilidade (Kim, Kim *et al.*, 2012).

As propriedades mecânicas e reológicas da bicamada lipídica estão intimamente relacionadas à dinâmica da estrutura da membrana, representada pela facilidade de movimento molecular dentro dela (fluidez da membrana) (Shiga, Maeda *et al.*, 1990). Um menor teor de ácidos graxos insaturados em relação aos saturados em fosfolípidos é uma característica que reduz a fluidez da membrana do eritrócito. Por outro lado, um elevado teor de ácidos graxos insaturados em relação aos saturados em fosfolípidos é uma característica que aumenta a fluidez da membrana. Além disso, a fluidez diminui com o aumento do teor de colesterol na membrana (Sinensky, 1974; Mohandas e Gallagher, 2008), sendo o colesterol associado à manutenção da estabilidade da membrana do eritrócito (Coldman, Gent *et al.*, 1970).

Modificações na estrutura dos eritrócitos

Os eritrócitos apresentam tempo de vida de aproximadamente 120 dias. Durante esse tempo, sofrem vários estresses mecânicos e químicos, o que resulta em perda de área de membrana, de volume e de deformabilidade (Shiga, Maeda *et al.*, 1979; Mohandas e Gallagher, 2008). Com o envelhecimento da célula, a área da superfície, a concentração de hemoglobina e a densidade celular mudam. Nesse sentido, o próprio envelhecimento humano associado ao estresse oxidativo provoca alteração na estrutura molecular e nas funções dos eritrócitos (Rizvi e Maurya, 2007). Além disso, estudos sobre deformabilidade, integridade estrutural e forma do eritrócito têm avançado para descobrir os mecanismos moleculares que definem

certas doenças associadas à membrana celular (Gallagher e Jarolim, 2005; Da Costa, Galimand *et al.*, 2013).

Durante o envelhecimento *in vivo* e *in vitro*, os glóbulos vermelhos sofrem várias modificações bioquímicas. A alteração na forma do eritrócito pode ser induzida por substâncias químicas e depleção metabólica, ou por forças externas físicas. Podemos citar a depleção de ATP, a diminuição do pH interno, indução por agentes aniônicos, e a variação morfológica da forma normal de repouso, a partir de um disco achatado bicôncavo chamado discoide, indo a um corpo mais ou menos esférico coberto com afiadas protuberâncias chamadas espículas (equinocitose ou externalização) (Girasole, Pompeo *et al.*, 2010). Já os agentes catiônicos induzem no eritrócito a forma de um copo (estomatocitose ou internalização). Os produtos químicos aniônicos são preferencialmente incorporados na camada externa da membrana, enquanto os catiônicos são incorporados na metade interna (Mohandas e Gallagher, 2008). Estas conversões ocorrem através de processos que envolvem a modificação do esqueleto da membrana lipídica (Werre, Willekens *et al.*, 2004).

Os estudos indicam que as propriedades mecânicas e reológicas de membranas de eritrócitos podem ser alteradas de forma reversível pela presença ou ausência de determinados constituintes do sangue (por exemplo, glicose e algumas proteínas) (Williams, 1973). Concentrações elevadas de glicose podem causar compactação do eritrócito, devido ao aumento da pressão osmótica, impedindo a entrada de água dentro da célula. Este fato pode proteger eritrócitos humanos contra a lise hipotônica, o que não significa um aumento na funcionalidade das células (Lemos, Marquez-Bernardes *et al.*, 2011).

Além disso, o colesterol se caracteriza como um componente essencial e funcional das membranas celulares, que são extremamente sensíveis às alterações do perfil de lipídeos plasmáticos (Cooper, 1977; Dwight, Mendes Ribeiro *et al.*, 1996; Caliskan, Caliskan *et al.*, 2000; Tziakas, Chalikias *et al.*, 2009). O colesterol plasmático pode ser direcionado para a membrana do eritrócito (Martinez, Vaya *et al.*, 1996), diminuindo sua fluidez, ou seja aumentando sua rigidez (Cooper, 1977), o que altera as propriedades funcionais da célula bem como as características reológicas do sangue

(Chabanel, Flamm *et al.*, 1983; Koter, Broncel *et al.*, 2002). O excesso de colesterol é um fator de risco para o desenvolvimento de aterosclerose e doença coronariana (Bernstein, 1985) e, nesse sentido, a composição dos lipídios da membrana do eritrócito tem ganhado importância devido a possibilidade de conexão com doenças cardiovasculares (Tziakas, Chalikias *et al.*, 2010; Michel, Virmani *et al.*, 2011; Tziakas, Chalikias *et al.*, 2012).

O estresse oxidativo é um importante fator que causa modificação na estrutura e função dessa célula. No eritrócito são encontrados oxigênio (O_2) e ferro no estado ferroso (Fe^{2+}). O transporte de oxigênio depende do estado reduzido do ferro na hemoglobina. Todos esses elementos podem gerar sozinhos ou juntos espécies reativas que causam danos aos próprios eritrócitos e no endotélio vascular. Essas espécies reativas apresentam elétron desemparelhado no orbital externo e são capazes de extrair elétron de moléculas vizinhas para completar seu próprio orbital. Há dois grupos de espécies: espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS). Nos eritrócitos, ROS são produzidos tanto acidentalmente quanto fisiologicamente em diferentes reações catalisadas por enzimas (Cristiana, Nina *et al.*, 2012).

Quando formados em pequenas quantidades, essas espécies podem ser combatidas pelas defesas antioxidantes e mecanismos de reparo celular; no entanto, quando produzidas em excesso, elas geram um estado de desequilíbrio chamado estresse oxidativo, que promove dano em ácidos nucleicos, proteínas e lipídios (Gilgun-Sherki, Melamed *et al.*, 2004). O ataque oxidativo causa alterações na organização estrutural e funcional das membranas celulares, incluindo diminuição na fluidez da membrana, aumento na sua permeabilidade, inativação de enzimas e decréscimo no teor de ácidos graxos essenciais (Van Ginkel e Sevanian, 1994), além de estar relacionado com câncer e doenças cardiovasculares (Barreiros, David *et al.*, 2006). Os ácidos graxos polinsaturados (PUFA) de membranas constituem as estruturas mais vulneráveis a esses danos, sendo alvo do processo chamado lipoperoxidação (Begum e Terao, 2002).

Sendo assim, a perda de área de superfície devido a diminuída coesão da membrana, ou fragmentação celular como consequência da redução da estabilidade mecânica da membrana, bem como um aumento de volume da célula, devido a um defeito nos transportadores iônicos, irão comprometer a função e a capacidade de deformação do eritrócito, e levar a sua remoção prematura da circulação ([Mohandas e Gallagher, 2008](#)).

Sistemas de proteção ao eritrócito

Os organismos apresentam um sistema antioxidante que tem a função de inibir ou reduzir os danos causados pelas espécies oxidativas através de diversos mecanismos. Dentre as estratégias, existem as enzimas antioxidantes, como superóxido dismutase (SOD), catalase (CAT) e glutathione peroxidase (GPx); e as moléculas, que incluem albumina, ferritina, ácido ascórbico, β -caroteno, α -tocopherol, ácido úrico e bilirrubina ([Barreiros, David et al., 2006](#)). Se os sistemas antioxidantes sofrem disfunção, pode ocorrer oxidação da hemoglobina, de lipídios e de proteínas da membrana ([Bunn, 1972](#)).

A albumina é a proteína sérica mais abundante em mamíferos, com função em geral de transportar pelo sangue as substâncias pouco solúveis em água, como ácidos graxos, esteroides, bilirrubina e drogas, sendo que ainda apresenta possível papel antioxidante ([Bruschi, Candiano et al., 2013](#)). Essa proteína age de maneira protetora em eritrócitos submetidos a rápida hemólise osmótica ([Katchalsky, Kedem et al., 1960](#)) ou quando em meio com o surfactante dodecil sulfato de sódio (SDS) ([Fonseca, Arvelos et al., 2010](#)). Níveis baixos de albumina sérica podem ser preditores de mortalidade em pessoas mais velhas ([Hausman, Fischer et al., 2012](#)).

Através da enzima SOD, as células tentam manter baixas as concentrações do radical superóxido. A maior quantidade da enzima SOD é encontrada no fígado e nos eritrócitos. Existem três isoformas de SOD em tecido humano: SOD1 ou Cu/Zn SOD está presente no citosol e contém dois átomos de cobre e zinco, SOD2 ou MnSOD é uma molécula muito maior que contém quatro átomos de manganês e é encontrada

na mitocôndria, e SOD3 ou ECSOD, que contém cobre e zinco e possui um peptídeo sinalizador que direciona a enzima para o meio extracelular. Alterações significativas na concentração celular de cobre, manganês e zinco têm o potencial de alterar a atividade antioxidante da SOD (Zelko, Mariani *et al.*, 2002; Miao e St Clair, 2009; Cristiana, Nina *et al.*, 2012).

A catalase (Cat) atua na sinalização da proliferação celular, ativação plaquetária e apoptose. É uma hemoproteína que se encontra livre no citoplasma de eritrócitos, sendo dependente de NADPH. Sua principal função é proteger a hemoglobina, removendo o peróxido de hidrogênio (H_2O_2) gerado em eritrócitos humanos (Goyal e Basak, 2010).

O H_2O_2 não é um radical livre, mas pode ser classificado como ROS uma vez que gera o radical hidroxila ($OH\bullet$) na presença de metais de transição (Fe^{2+} , Cu^+). H_2O_2 é lipossolúvel e, portanto, pode se difundir entre os lipídios de membrana e danificar o eritrócito. Daí a função da catalase e da GPx em decompor o H_2O_2 , formando oxigênio e água (Cristiana, Nina *et al.*, 2012).

Além disso, o estado de hipercolesterolemia gera alta produção de ROS. Estes podem prejudicar a função endotelial, danificando a parede dos vasos sanguíneos. A maioria das terapias para diminuir o colesterol também apresentam efeitos antioxidantes, a exemplo das estatinas (Cristiana, Nina *et al.*, 2012).

Estabilidade da membrana eritrocitária

Os eritrócitos são modelos interessantes de estudo da estabilidade de membrana, em função da menor invasividade em seu processo de obtenção e da simplicidade de monitoração baseada na liberação de hemoglobina, que pode ser quantificada por espectrofotometria a 540 nm. Essa absorvância é proporcional à extensão da lise dos eritrócitos (Penha-Silva, Firmino *et al.*, 2007).

A estabilidade osmótica do eritrócito sofre influência de fatores como volume, tamanho e forma celulares, tipo e quantidade de hemoglobina, viscoelasticidade e

composição química e estrutural da membrana (Perk, Frei *et al.*, 1964). Assim, como as propriedades das membranas de eritrócitos podem ser alteradas por determinados constituintes do sangue e pela própria saúde do indivíduo, estudos têm sido conduzidos a fim de analisar a influência dos parâmetros hematológicos e variáveis bioquímicas na estabilidade da membrana celular (De Freitas, De Oliveira *et al.*, 2010; Fonseca, Arvelos *et al.*, 2010; Lemos, Marquez-Bernardes *et al.*, 2011; Bernardino Neto, De Avelar Jr. *et al.*, 2013; De Arvelos, Rocha *et al.*, 2013; De Freitas, Marquez-Bernardes *et al.*, 2013).

O volume de água do eritrócito é osmoticamente regulado, particularmente pela concentração intracelular de íons potássio (K^+) e extracelular de íons sódio (Na^+). O controle da quantidade de íons é importante para manutenção do volume e forma da célula e, conseqüentemente, da viscosidade intracelular relacionada com a concentração da hemoglobina (Cristiana, Nina *et al.*, 2012). As condições do meio podem alterar essa dinâmica. Alguns solutos promovem a organização da membrana, como água e osmólitos, e ainda outros solutos podem gerar sua desorganização (Cunha, Arvelos *et al.*, 2007; Penha-Silva, Arvelos *et al.*, 2008; Fonseca, Arvelos *et al.*, 2010).

Assim, as substâncias estabilizantes e desestabilizantes são capazes de mudar o comportamento físicoquímico das membranas. O efeito desses solutos na estabilidade de membrana em eritrócitos de populações saudáveis ou em condições específicas tem sido estudado através de experimentos que incluem o choque hipotônico e a ação de solutos estabilizantes e caotrópicos, como o etanol, ureia e dodecil sulfato de sódio (SDS) (Cunha, Arvelos *et al.*, 2007; Penha-Silva, Firmino *et al.*, 2007; De Freitas, Netto Rde *et al.*, 2008; Penha-Silva, Arvelos *et al.*, 2008; De Freitas, De Oliveira *et al.*, 2010; Fonseca, Arvelos *et al.*, 2010; Lemos, Marquez-Bernardes *et al.*, 2011; Bernardino Neto, De Avelar Jr. *et al.*, 2013; De Arvelos, Rocha *et al.*, 2013; De Freitas, Marquez-Bernardes *et al.*, 2013; Mascarenhas Netto, Fabbri *et al.*, 2013).

A interação de surfactantes com as membranas pode ajudar a entender os mecanismos biológicos de manutenção da integridade celular, uma vez que essas substâncias são capazes de se ligar à célula e afetar as propriedades das membranas.

As mudanças induzidas pelos surfactantes vão desde uma súbita mudança de permeabilidade até lise e fusão da membrana (Shalel, Streichman *et al.*, 2002).

O SDS é um surfactante aniônico que apresenta uma cadeia de 12 átomos de carbono ligados a um grupamento sulfato, o que confere à molécula propriedades anfipáticas requeridas para um detergente. Em concentração alta, SDS solubiliza lipídios e desestabiliza proteínas de membrana (efeito de solubilização), e em baixa concentração o detergente se intercala na membrana, o que aumenta a permeabilidade e permite a entrada de água, causando ruptura celular (efeito osmótico). O mecanismo osmótico pode induzir uma taxa de hemólise lenta, enquanto o efeito da solubilização provoca rápida hemólise. Esses são os dois mecanismos sugeridos para o processo de lise do eritrócito (Shalel, Streichman *et al.*, 2002).

A extensão da hemólise pode ser analisada pelo gráfico da absorvância em 540 nm (A_{540}) em relação ao aumento da concentração de SDS (**Figura 1.3**). O ajuste por regressão linear sigmoidal através da equação de Boltzmann fornece os seguintes parâmetros:

- A_1 e A_2 , que representam o platô mínimo ($A_{\min}$) e máximo ($A_{\max}$) de A_{540} ;
- D_{50} , que é a concentração de SDS que promove lise de 50% dos eritrócitos;
- dD , que representa a variação da concentração de surfactante responsável pela transição de hemólise.

Microscopia de força atômica

Várias abordagens para a compreensão da nanoestrutura de membranas estão focadas na análise de parâmetros de ondulação da membrana. A busca por métodos de análise de tal superfície representa um avanço para a microscopia de varredura por sonda (Girasole, Pompeo *et al.*, 2010; Kozlova, Chernysh *et al.*, 2013).

A microscopia de força atômica (AFM) tornou-se uma ferramenta fundamental em vários campos de pesquisa, incluindo bioquímica e biologia celular, uma vez que permite estudos topográficos de alta resolução de materiais, bem como a obtenção de

informações mecânicas sobre sistemas biológicos em seu ambiente natural (Dulinska, Targosz *et al.*, 2006).

A AFM é realizada através de uma sonda afiada montada na extremidade de um braço de suporte flexível que interage com a superfície da amostra. A amostra, em geral, é verificada nas direções X e Y, por meio de um *scanner* piezoelétrico, e as alterações na altura (sentido Z), devido às interações da ponta com substrato, são captadas por detector sensível à posição. Um computador é utilizado para processar a informação e produzir imagens topográficas bi e tridimensionais da superfície da amostra (**Figura 1.4**) (Girasole, Pompeo *et al.*, 2007).

Essa técnica tem sido utilizada para estudar a topografia e as propriedades mecânicas das células vivas e fixadas (Nowakowski, Luckham *et al.*, 2001). Investigações por AFM de células vivas e estruturas celulares têm fornecido dados importantes sobre as propriedades viscoelásticas da membrana celular e da organização do citoesqueleto a partir da confecção de imagem em escala micro- ou nanométrica (Muller, 2008). Essas informações sobre as propriedades micromecânicas dos sistemas biológicos ajudam a compreender a arquitetura da célula e suas funções (Dulinska, Targosz *et al.*, 2006).

A membrana do eritrócito é uma superfície de múltiplos componentes que pode fornecer informações importantes (Mohandas e Gallagher, 2008). O comportamento e estrutura do eritrócito têm sido estudados por AFM, devido a sua estrutura relativamente simples e facilidade de isolamento (Kamruzzahan, Kienberger *et al.*, 2004), além da técnica permitir a operação não invasiva no estado líquido ou ar, e pela facilidade na preparação da amostra. As imagens das células podem ser obtidas à temperatura ambiente, sem necessidade de fixação, coloração ou marcação (Muller, 2008).

Vários estudos teóricos e experimentais de propriedades mecânicas de eritrócitos revelaram que essas células apresentam um comportamento mecânico único. As principais características são a resposta altamente elástica e rápida ao estresse mecânico e alta resistência estrutural. Alterações nas propriedades biofísicas

das células vermelhas podem ser indicadores básicos para a saúde humana (Chen, Cai *et al.*, 2002; Mohandas e Gallagher, 2008; Wu, Hu *et al.*, 2009).

Autores têm analisado a rugosidade da superfície celular pela AFM através dos parâmetros: média aritmética da rugosidade (*arithmetic mean roughness* - Ra), altura máxima (*maximum height* - Rz), altura média (*average height* - Rp) e profundidade média (*average depth* - Rv). Em dada amostra de uma espécie, é esperado que a superfície celular dos eritrócitos seja homogênea e que a distribuição de valores de rugosidade medidos em muitas áreas de uma única célula siga uma curva de Gauss. Assim, o valor médio da rugosidade pode ser considerado um marcador de célula (Girasole, Pompeo *et al.*, 2007).

Considerações finais

Diante desse contexto, os métodos de estabilidade da membrana do eritrócito em SDS e microscopia de força atômica foram utilizados para entender os fatores hematológicos e bioquímicos que se correlacionam com a integridade da membrana celular.

As análises estatísticas bivariada e multivariada foram aplicadas aos dados experimentais para entender essa complexidade de fatores fisiológicos que afetam a membrana em diferentes grupos etários.

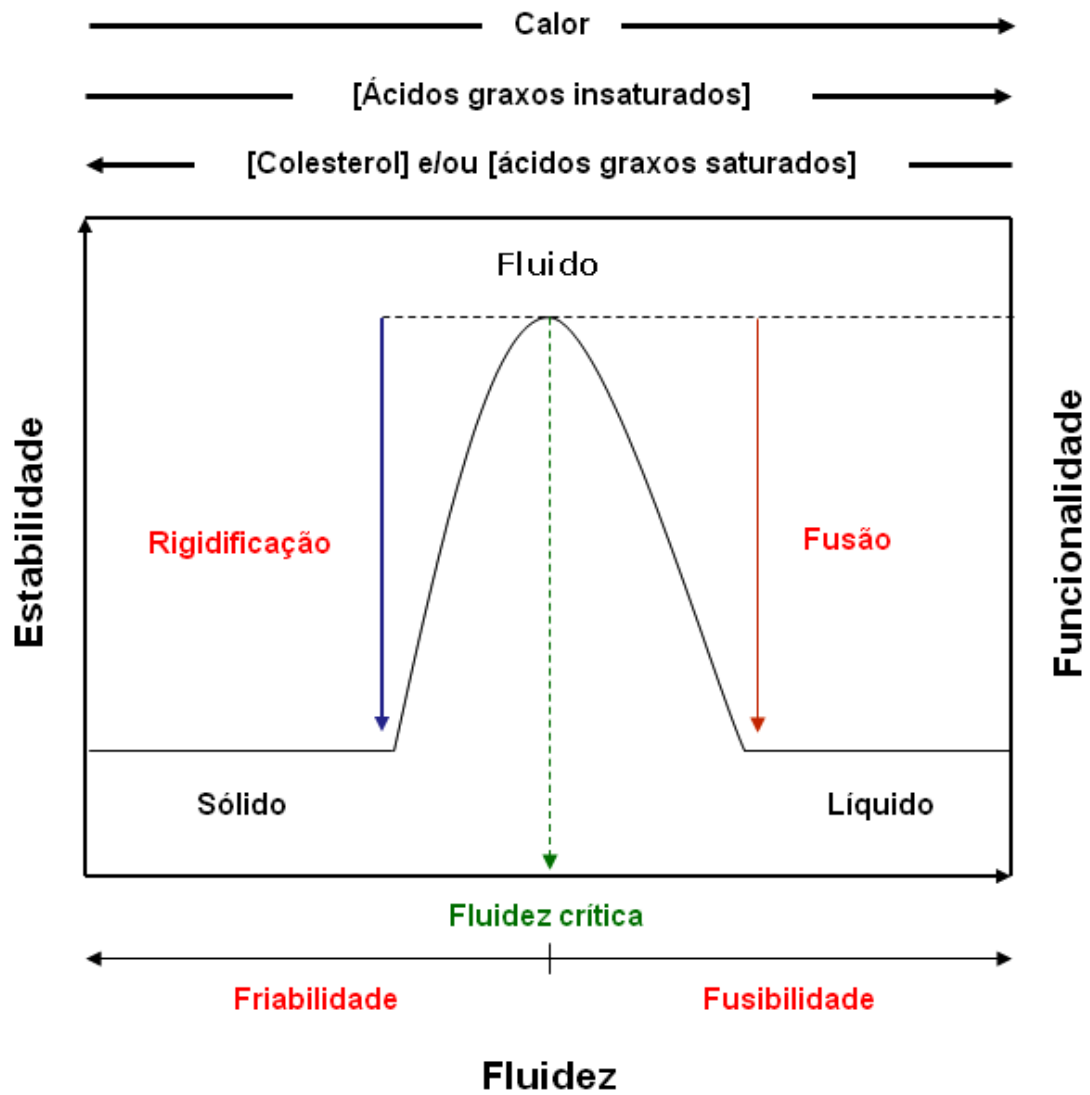


Figura 1.1 Dependência da estabilidade e da funcionalidade de uma membrana celular com sua fluidez. Essas duas propriedades da membrana não são necessariamente coincidentes.

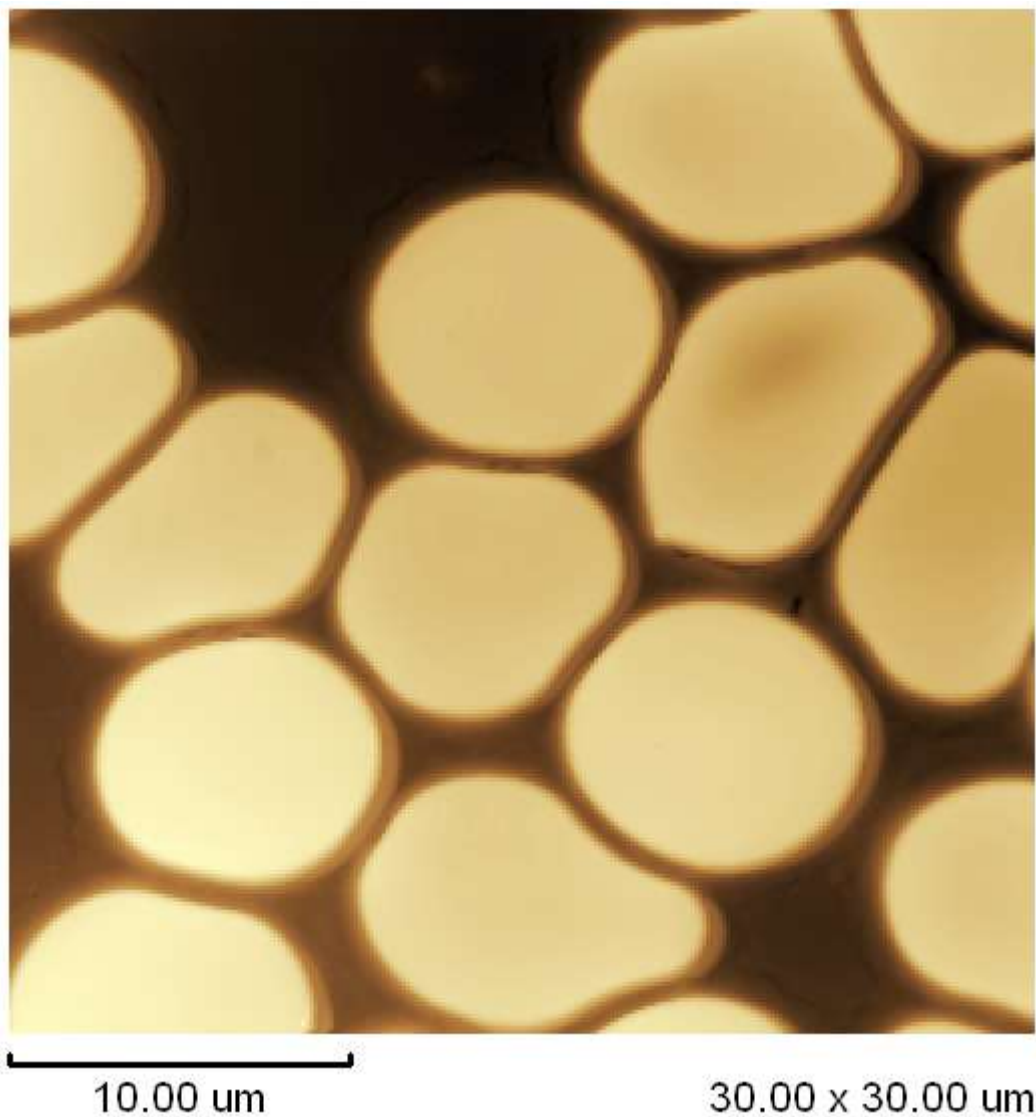


Figura 1.2 Deformabilidade do eritrócito. O excesso de área de volume e as características elásticas permitem que o eritrócito sofra alterações na sua conformação durante o contato com outras células e passagem pelos pequenos capilares. Quando as forças externas cessam, a célula volta ao típico formato bicôncavo (área de $30 \mu\text{m}^2$).

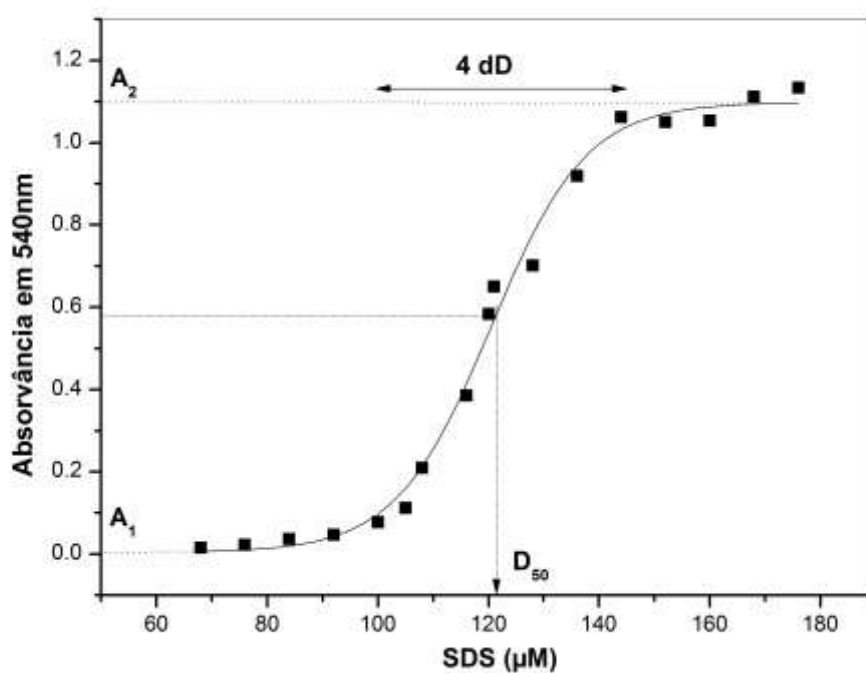


Figura 1.3 Curva típica de lise de eritrócitos pelo surfactante SDS ajustada por regressão sigmoideal. D_{50} é a concentração de SDS em que há lise da metade da população de eritrócitos da amostra, dD é a variação da concentração de surfactante que promove a transição de lise, A_1 ($A_{\min}$) e A_2 ($A_{\max}$) são respectivamente os valores mínimo e máximo de absorvância associados à sigmóide (adaptado de [De Freitas, Marquez-Bernardes et al., 2013](#)).

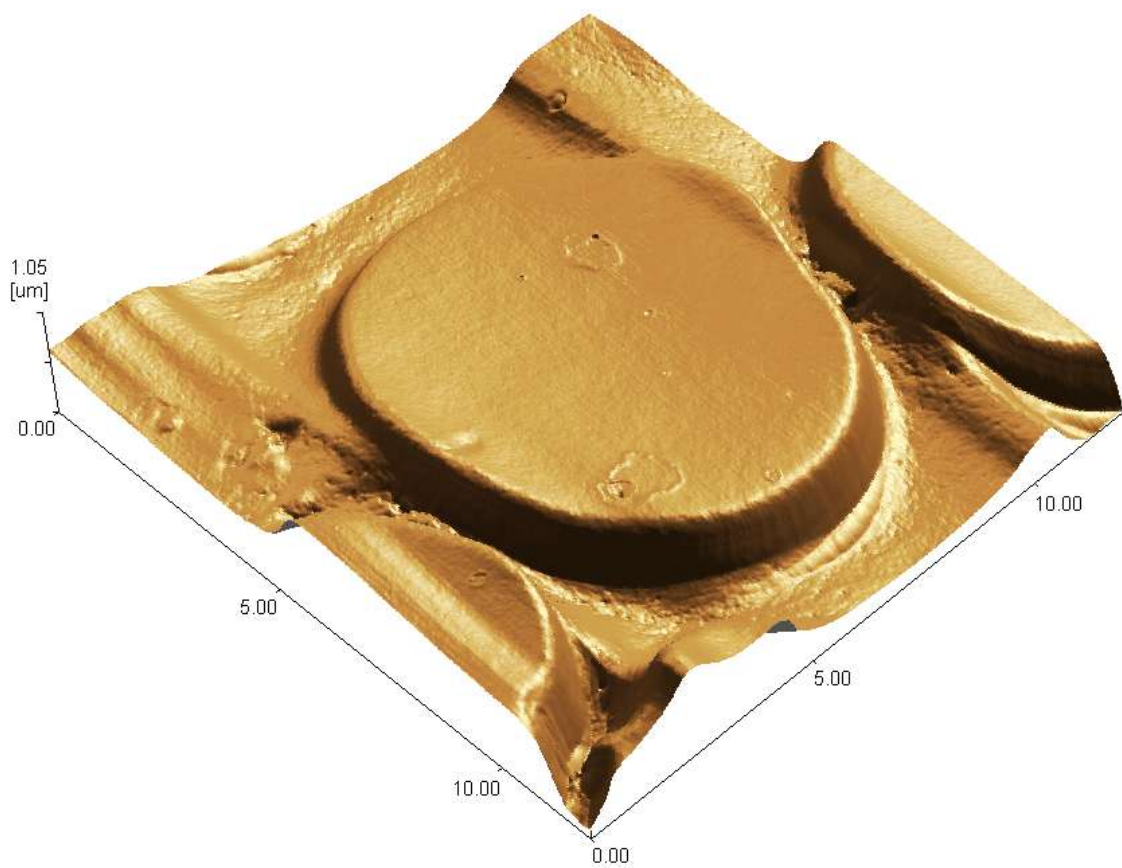


Figura 1.4 Imagem do eritrócito humano por microscopia de força atômica (AFM), em modo contato (área de $12 \mu\text{m}^2$).

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CAPÍTULO 2

INFLUENCE OF AGE ON THE CORRELATIONS OF HEMATOLOGICAL AND BIOCHEMICAL VARIABLES WITH THE STABILITY OF ERYTHROCYTE MEMBRANE IN RELATION TO SODIUM DODECYL SULFATE

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Influence of age on the correlations of hematological and biochemical variables with the stability of erythrocyte membrane in relation to sodium dodecyl sulfate

RBC stability and aging

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RESUMO

Objetivo: Avaliar a influência da idade sobre as variáveis bioquímicas, hematológicas e de estabilidade de membrana de eritrócitos em relação ao dodecil sulfato de sódio (SDS), em uma população de 105 voluntários do sexo feminino entre 20 e 90 anos.

Métodos: A estabilidade de membrana de eritrócitos foi determinada por regressão não linear da dependência da absorvância de hemoglobina liberada em função da concentração de SDS, tendo sido representada pelo ponto de meia transição da curva (D_{50}) e pela variação da concentração do detergente que promove a lise (dD).

Resultados: Houve um aumento dependente da idade na estabilidade da membrana em relação ao SDS. A análise por regressão linear múltipla mostrou que este aumento da estabilidade é significativamente relacionado com a variação percentual da distribuição de volume dos eritrócitos (RDW) e das variáveis bioquímicas albumina sérica e colesterol total.

Discussão: A associação positiva entre a estabilidade de eritrócitos e a RDW pode refletir um possível mecanismo envolvido no significado clínico deste índice hematológico.

Palavras-chave: Albumina, Envelhecimento, Eritrócito, Membrana, RDW

ABSTRACT

Objectives: To evaluate the influence of age on the relationships between biochemical and hematological variables and stability of erythrocyte membrane in relation to the sodium dodecyl sulfate (SDS) in population of 105 female volunteers between 20 and 90 years.

Methods: The stability of RBC membrane was determined by non-linear regression of the dependency of the absorbance of hemoglobin released as a function of SDS concentration, represented by the half-transition point of the curve (D_{50}) and the variation in the concentration of the detergent to promote lysis (dD).

Results: There was an age-dependent increase in the membrane stability in relation to SDS. Analyses by multiple linear regression showed that this stability increase is significantly related to the hematological variable red cell distribution width (RDW) and the biochemical variables blood albumin and cholesterol.

Discussion: The positive association between erythrocyte stability and RDW may reflect one possible mechanism involved in the clinical meaning of this hematological index.

Keywords: Ageing, Albumin, Erythrocyte, Membrane, RDW

INTRODUCTION

Stability and fluidity are membrane properties that are essential to allow the cells to resist the action of internal and external stressors and maintain the deformability necessary to carry out their complex functions.^{1,2}

The maintenance of the fluidity and functionality of a membrane depends on several factors, which include the relative content of phospholipids and cholesterol, the concentration of ions³ and the osmolarity of the medium.⁴

In erythrocytes, the membrane fluidity contributes to the deformability required for the passage of these cells through small diameter capillaries as well as for the exchange of gases between hemoglobin (Hb) and the external tissues. However, during their lifetime the red cells undergo various chemical and mechanical stresses, resulting in loss of membrane area and deformability.^{5,6}

The behavior of erythrocyte membrane is influenced by the supply conditions of the lipid constituents of the membrane,⁷ different kinds of erythrocytopathies and other pathological conditions,³ gender, aging,⁸ and many chemical agents.⁹

The in vitro evaluation of the stability of the erythrocyte membrane in relation to hypotonic stress and the chaotropic action of solutes such as ethanol and sodium dodecyl sulfate (SDS)^{8,10–15} is an essential task to investigate the myriad of conditions that affect the structural homeostasis of biological membranes.

These conditions surely comprise the concentration of many blood biochemical and some hematological variables, which also depend on the nutritional and health status and the use of many types of drugs.

It is within this context that this study evaluates the influence of hematological and biochemical variables on the stability of human erythrocyte membrane.

METHODS

Population

The research was approved by the Ethics Committee of the Federal University of Uberlândia (Protocol 127/11). The sample population consisted of 105 female volunteers in the age range of 20–90 years (11 (19–25), 24 (25–31), 5 (31–37), 7 (37–43), 5 (43–49), 5 (49–55), 8 (55–61), 14 (61–67), 8 (67–73), 11 (73–79), 3 (79–85), 4 (85–91)). Only women were recruited to avoid possible physiological variations caused by gender.^{16,17}

The participants of this study were recruited in a clinical laboratory (Labormed, Uberlândia, MG, Brazil) and selected after interview conducted before donation of blood samples. All subjects selected were non-smokers and non-chronic consumers of alcohol. We excluded subjects with known clinical history of chronic diseases (diabetes, coronary heart disease, and dementia) and those who were under chronic drug use.

Blood collection

After overnight (8–12 hours) fasting, blood was collected by intravenous puncture into evacuated tubes (4 ml) with (1) 1.8 mg/ml K₃EDTA, for collection of the whole blood, (2) 1.8 mg/ml K₃EDTA and 3 mg/ml NaF for determination of glucose, and (3) a spray dried clot activator, for serum separation.

Determination of the stability of human erythrocytes in relation to sodium dodecyl sulfate

Test tubes with 1 ml of 68–176 μ M SDS solutions in 0.9 g dL⁻¹ NaCl were pre-incubated at 37°C for 10 minutes. After addition of 10 μ l of whole blood, gently mixing, and incubation for 30 minutes at 37°C, the tubes were centrifuged at 1500 $\times$ g for 10 minutes. The supernatants were used for evaluation of the optical density at 540 nm

(A_{540}) in an UV-VIS spectrophotometer (Shimadzu™, model UV1650TC, Japan). The graphics of A_{540} in relation to the SDS concentration were fitted to sigmoidal regression lines, according to the Boltzmann equation:

$$A_{540} = \frac{A_1 - A_2}{1 + e^{(D - D_{50})/dD}} + A_2 \quad (1),$$

where A_1 and A_2 represent the minimum and maximum plateaus of A_{540} , D is the SDS concentration, D_{50} is the SDS concentration capable of promoting 50% of hemolysis, and dD represents the variation in the SDS concentration responsible for the transition of hemolysis.

Determination of hematological and biochemical variables

Hemogram was obtained using an automated system of analysis (Sysmex K4500; Sysmex Corporation™, Mundelein, IL, USA). The lipid profile was performed in an automatic analyzer (Hitachi 917, Roche Diagnostics™, Indianapolis, IN, USA). Serum albumin was colorimetrically assayed using a commercial kit (Labtest, Belo Horizonte, MG, Brazil) and an UV-VIS spectrophotometer (Shimadzu™, model UV1650TC, Japan).

The reference values (women over 16 years of age) were erythrocytes (RBC), 3.9–5.9 millions/mm³; hemoglobin (Hb), 12.0–16.0 g.dL⁻¹; hematocrit (Ht) 35.6–48.6%; mean corpuscular volume (MCV), 82–98 fl; mean corpuscular hemoglobin (MCH), 27–31 pg; mean corpuscular hemoglobin concentration (MCHC), 32.9–36%; red cell distribution width (RDW), 12–15%; glucose (Glu), 60–99 mg.dL⁻¹; albumin (HSA), 3.5–5.2 g.dL⁻¹; total cholesterol (t-C), <200 (optimum) and ≥240 mg.dL⁻¹ (high); high-density lipoprotein cholesterol (HDL-C), <45 (low) and >65 mg.dL⁻¹ (ideal), low-density lipoprotein cholesterol (LDL-C), <100 (good) and >160 mg.dL⁻¹ (high); very low density lipoprotein (VLDL-C), up to 40 mg.dL⁻¹; and triglycerides (TG), <150 (optimum) and >201 mg.dL⁻¹ (high).

Statistical analysis of experimental data

Data were tested for normality using the D'Agostino-Pearson test. In an initial analysis, the study population was stratified by age into two groups (20–50 and 51–90 years), using a cutoff age at which the average population of the region of study enters menopause.¹⁸ The two groups were compared with respect to all the variables considered in the study, using Student's t-test for normally distributed data and Mann–Whitney test for non-normally distributed data.

The study also used bivariate and multivariate statistics to search for the existence of relations between the erythrocyte stability parameters and the hematological and biochemical variables.

The existence of bivariate linear correlations between the stability parameters (D_{50} and dD) and the hematological and biochemical variables used the significance level of 0.05. These statistical analyses were performed with the use of the software package OriginPro 9 (MicroCal™, Northampton, MA, USA).

The multivariate analyses were performed by multiple linear regression (MLR), with the help of the software BioEstat 5.0 (Mamirauá™, Belém, PA, Brazil). In the MLR analyses, D_{50} or dD were considered the dependent variable and the hematological or biochemical parameters constituted the groups of independent variables. In this kind of analysis, the partial regression coefficient measures the strength of the relation between the dependent variable (D_{50} or dD) and a single independent variable of the group (hematological or biochemical), while the determination coefficient (R^2) measures the proportion of variance of the dependent variable (around its mean) that is explained by the whole set of independent variables.

RESULTS

Fig. 1 shows a typical curve of erythrocytes lysis induced by increasing concentrations of SDS. Nonlinear regression analysis (sigmoidal fitting) was always used to provide the parameters D_{50} , dD , A_1 and A_2 , also showed in the figure.

The preliminary analysis of the results obtained for the entire study population using the D'Agostino-Pearson test has shown the occurrence of normal distribution in the data of all the parameters considered in this study. But after stratification of the population by age, some results did not show normal distribution (**Table 1**).

The comparison of the studied variables between the two age groups (20–50 and 51–90 years) showed that the mean values of the stability variable D_{50} , the biochemical variables Glu, VLDL-C, LDL-C, t-C, and TG, and the hematological variables MCV and RDW were significantly higher in the 51–90 years group in relation to the other group. But the average of the MCHC values was significantly lower in 51–90 years group.

Table 2 shows the matrix of all the possible bivariate correlations between stability parameters (D_{50} and dD), age and hematological and biochemical variables. Within the limits of statistical significance considered in this study ($P < 0.05$), age was positively correlated with the values of D_{50} , MCV, RDW, Glu, TG, t-C, and VLDL-C, but negatively associated with the values of MCHC. Regarding the variables of stability, D_{50} was positively correlated with dD , age, HSA, t-C, LDL-C and HDL-C, while dD was positively correlated with MCH, MCHC, TG, t-C, LDL-C and HDL-C, but negatively correlated with A_{min} .

The influence of hematological and biochemical variables on the stability parameters (D_{50} and dD) was analyzed by MLR (**Table 3**). The power of MLR was 80% for an alpha error of 5% ($P < 0.05$) and an expected variance in the values of the stability variables of 15%. Both the set of biochemical variables and the set of hematological variables have been significantly correlated with the stability variable dD , but the stability variable D_{50} was significantly correlated only with the set of biochemical variables. The dependent variable dD was significantly correlated with the

hematological variables RDW and MCHC and the biochemical variables TG, LDL-C, and HDL-C. The dependent variable D_{50} was significantly correlated with the biochemical variables HSA and HDL-C, as well as with the age of the participants.

DISCUSSION

Although SDS, as detergent, is capable of solubilizing lipid components of biological membranes, at lower concentrations and under the experimental conditions of this study (30-minute incubation time) it has no major impact on the stability of the membrane. The plateau in the absorbance of free Hb designated as A_1 in **Fig. 1** is a proof of this statement. However, a progressive increase in the concentration of this detergent is associated with an (exponential) rise in the release of Hb up to a (SDS) concentration that defines the midpoint of the hemolysis curve (D_{50}), beyond which an increase in its concentration is associated with a hyperbolic rise in the Hb release (**Fig. 1**). That is why both D_{50} and dD are constants that present direct dependencies with the chemical stability of the erythrocyte membrane. The hemolysis by SDS is a complex process that can involve intercalation of this detergent in the cell membrane, leading to water penetration and membrane rupture (osmotic mechanism), but it is believed that hemolysis occurs by a process which is essentially based in the solubilization of lipid constituents of the cell membrane (chemical mechanism).^{14,15,19,20}

This study found a positive correlation between D_{50} and age (**Table 2**), indicating that the older female volunteers have erythrocytes that are more stable against the hemolytic action of SDS. Indeed, the group of older volunteers (51–90 years) presented higher values of the stability variables D_{50} and dD in relation to the younger participants (20–50 years) (**Table 1**). Since the stratification by age of these groups was based on the presumed age of entry in menopause, it is possible that hormonal and/or menstrual changes are associated with the erythrocyte stabilization with aging. The fact that menopause is a landmark in the transition of behavior of the erythrocytes was presented by Raval et al.,¹⁷ which reported that red cells of stored blood samples from postmenopausal women showed higher mechanical fragility (or lower mechanical resistance) than those from premenopausal women. This study agree

with the existence of this landmark, although mechanical resistance and chemical stability are conceptually different properties that cannot be compared in a single basis. Moreover, it is well known that blood storage modifies the structure of the erythrocytes,^{3,21} which may no longer represent the clinical conditions of their donors. That is why this study used only freshly collected blood samples to perform all tests.

Anyhow, an increased osmotic stability had already been associated to aging in female volunteers aged 20–90 years.⁸ The causes of such stabilizing effects must involve hematological and biochemical factors that may be better understood by examining the results of this study.

Concerning the hematological variables, the bivariate correlations presented in **Table 2** show that aging was associated with increased MCV and RDW and decreased MCHC in the studied population.

Multivariate statistical analysis was conducted in a tentative to clarify the relationships between the hematological changes associated with aging and changes in the variables of stability. The group set out to explain the variable D_{50} presented higher predictive ability (R^2 adj = 0.3453) and higher multiple correlation coefficient (0.6138) than the group set out to explain the variable dD (**Table 3**).

Although the stability variable that was related to age had been D_{50} and not dD, it is important to understand the inter-relations of this parameter with the hematological variables considered in this study. The variable dD exhibited positive bivariate correlations with MCH and MCHC (**Table 2**). The MLR showed that RDW was the predictor hematological variable that most strongly correlated with the stability variable dD (**Table 3**).

The MCV is a measure of the average volume of erythrocytes and increases with nutritional deficiencies of folate and/or cobalamin, which are very common among older individuals.²² The RDW is a measure of the variability of erythrocyte volume and their values increase with increasing heterogeneity in the volume of these cells.²³ Recent studies have shown that high RDW is strongly associated with increased risk of death and cardiovascular disease^{24–26} in older adults.²⁷ But the mechanisms

underlying the increase in RDW with increasing age and associated with mortality are not yet well defined. The positive correlation of the stability variable dD with RDW suggests that the mechanism by which that hematological variable is exerting its influence on the health prognosis of the patient is an excessive elevation in the membrane stability of RBC.

The stability variable D_{50} showed positive bivariate correlations with t-C, LDL-C, HDL-C and also HSA (**Table 2**). In MLR, albumin appeared as the biochemical variable with the strongest and significant association with D_{50} (**Table 3**). Certainly, the protective effect of albumin on the stability of erythrocyte membrane in relation to SDS is due to the capacity of this protein of binding to this denaturant,^{28,29} reducing its hemolytic action.¹³ On the other hand, the stability variable dD showed significant bivariate and multivariate correlations with TG, LDL-C, and HDL-C (**Tables 2 and 3**).

Besides the hematological causes of the stability increase, there are causes associated to the offer conditions of membrane constituents, such as cholesterol. High levels of cholesterol and triglycerides are common in older adults;³⁰ indeed, these trends were observed in the comparison between the age groups 20–50 and 51–90 years old (**Table 1**). The inclusion of free cholesterol into a biological membrane is an important mechanism of control of its fluidity. As shown in the classic studies done by Cooper,^{31–34} cholesterol present in the LDL can diffuse into the membrane of blood cells, contributing to regulation of its fluidity. The membranes of these blood cells, consisting predominantly of erythrocytes, can receive the excess of cholesterol from the blood and this, of course, has implications on its physicochemical properties. To the extent that the insertion of cholesterol contributes to membrane reach its critical fluidity, this process leads to the stabilization of these cells and certainly to increase in their time of permanence in blood, with consequent increase in blood populations of RBC. These statements can be supported by the positive correlation observed between the levels of total and LDL-C and the stability of erythrocyte membrane in a population of obese patients submitted to bariatric surgery.³⁵

In fact, patients with familial hypercholesterolemia presented increased content of cholesterol in the erythrocyte membrane³⁶ and mammals erythrocytes

showed decreased osmotic fragility with increase of membrane cholesterol.³⁷ Furthermore, a decrease in the blood levels of cholesterol is associated to a lower ratio between cholesterol and phospholipids in the membrane, with physicochemical changes as increased membrane fluidity.³⁸ These reports from the literature are consistent with the correlations found in this study between the stability variables and the blood concentrations of LDL-C in the bivariate and multivariate correlation analyses.

On the other hand, in experimental animals with considerably higher levels of cholesterol, the excessive diffusion of cholesterol to the cell membrane will manifest as hemolytic anemia.^{33,39}

This means that there must be a threshold of non-HDL-cholesterol associated with the inversion in the dependence of the stability of erythrocyte membrane and non-HDL-cholesterol levels, since the levels of non-HDL-C are correlated to the membrane properties.⁴⁰

As the increase in RDW, recently characterized as an important prognostic biomarker of cardiovascular disease^{26,41–44} was associated with an increase in cholesterol content of membrane,²⁶ it is quite possible that the predictive ability of RDW is associated with changes in the stability and function of the erythrocyte membrane. Indeed, the negative bivariate correlation of RDW with HDL-C and the positive bivariate correlations of RDW with VLDL-C and TG (**Table 2**), observed in this study, agrees with the association between high RDW values and the etiopathology of the cardiovascular disease.

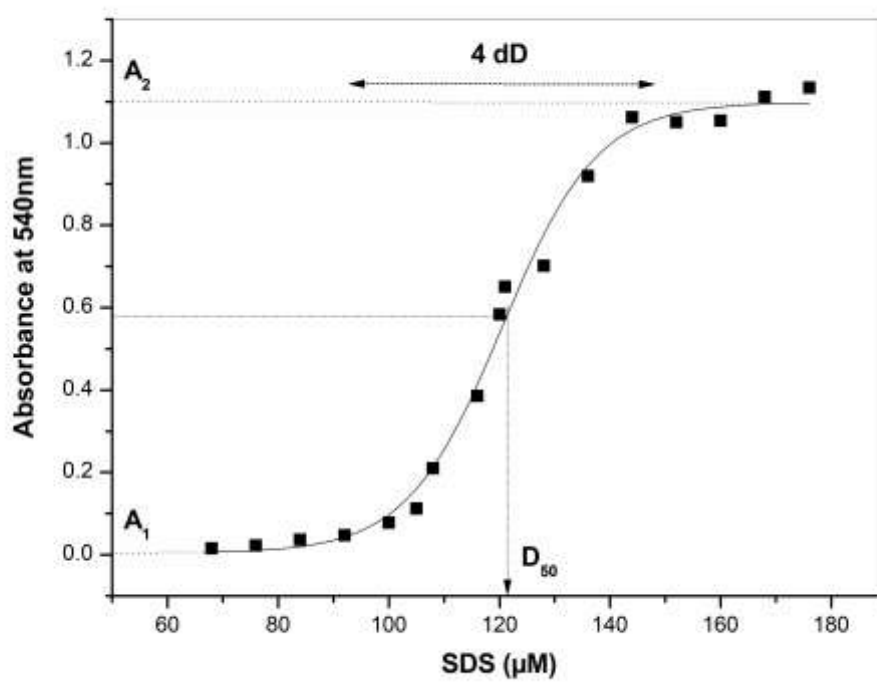


Figure 1. Sigmoidal fitting of a typical curve of hemolysis promoted by SDS. D_{50} is the concentration of SDS capable of promoting 50% of hemolysis. dD represents the change in SDS concentration responsible for the hemolysis transition. A_1 and A_2 represent the minimum and maximum average values of absorbance, respectively

Table 1. Comparison of stability, biochemical and hematological variables between age ranges

Variables	20-50 years		51-90 years		P
	(n = 53)		(n = 52)		
	Median	IQR	Median	IQR	
D ₅₀	120.6	9.933	123.7	10.02	0.0004 **
dD*	5.785	2.521	6.404	4.178	0.3189
A ₁ *	0.018	0.031	0.016	0.028	0.5792
A ₂	1.158	0.117	1.174	0.155	0.3780
Glu (mg.dL ⁻¹)	91.60	11.2	91.60	10.75	0.0131 **
HSA (g.dL ⁻¹)	4.160	0.51	4.100	0.47	0.5715
VLDL-C* (mg/dL)	15.50	10.6	24.10	12.8	<0.0001 **
LDL-C* (mg/dL)	100.4	37.2	105.5	40.5	0.0473 **
HDL-C (mg/dL)	53.60	15.4	49.90	15.55	0.2740
t-C (mg/dL)	165.7	42.7	184.5	52.45	0.0055 **
TG* (mg/dL)	82.20	52	117.6	60.6	0.0004 **
Hb* (g%)	13.40	1	13.30	1.5	0.4169
Ht (%)*	40.20	2.7	40.25	4.35	0.5934
RBC (milion/mm ³)	4.550	0.42	4.460	0.54	0.3144
MCV (fL)	88.30	4.2	90.55	5.15	0.0051 **
MCH* (pg)	29.60	2.2	29.90	1.95	0.3592
MCHC (%)	33.50	0.9	32.75	1.65	0.0008 **
RDW (%)	13.10	0.6	13.40	0.9	0.0085 **

*Non-normally distributed data (D'Agostine-Pearson test).

**Statistically significant differences (*P* < 0.05) between age ranges (Student's t-test for normally distributed data and Mann-Whitney test for non-normally distributed data).

Table 2. Matrix of correlations (r) between pairs of biochemical, hematological and membrane stability variables

	Age	Amin	Amax	D50	dD	Hb	Ht	RBC	MCV	RDW	MCH	MCHC	Glu	HSA	TG	VLDL-C	LDL-C	nHDL-C	HDL-C	t-C
Age	1.0000																			
Amin	-0.0500	1.0000																		
Amax	-0.0167	0.0918	1.0000																	
D50	0.2557*	-0.0885	-0.0542	1.0000																
dD	0.0407	-0.6856*	0.0365	0.4672*	1.0000															
Hb	-0.0989	0.0575	0.4319*	0.0420	0.1415	1.0000														
Ht	0.0506	0.1234	0.4018*	0.0486	0.0350	0.9147*	1.0000													
RBC	-0.1165	0.1500	0.4054*	-0.0025	-0.0264	0.7362*	0.7929*	1.0000												
MCV	0.3018*	-0.0556	-0.1146	0.0796	0.0505	0.0263	0.0706	-0.5437*	1.0000											
RDW	0.3724*	-0.1395	-0.1327	0.0576	0.1379	-0.0298	0.0929	0.1658 [§]	-0.1199	1.0000										
MCH	0.0759	-0.1314	-0.0572	0.0534	0.1861 [§]	0.1458	-0.0333	-0.5556*	0.8477*	-0.2670*	1.0000									
MCHC	-0.3658*	-0.1472	0.0997	-0.0295	0.2495*	0.2332*	-0.1786 [§]	-0.1094	-0.1149	-0.2993*	0.4273*	1.0000								
Glu	0.1905 [§]	0.0351	0.1618 [§]	0.0973	0.0853	0.1555	0.1475	0.0833	0.0576	0.1465	0.0611	0.0154	1.0000							
HSA	-0.0257	0.0899	-0.0569	0.2414*	0.1071	0.1870 [§]	0.1804 [§]	0.1874 [§]	-0.0526	0.0889	-0.0390	0.0068	0.3068*	1.0000						
TG	0.2840*	-0.1800 [§]	0.0979	0.1160	0.2228*	0.1356	0.1334	0.0566	0.0831	0.2526*	0.0773	-0.0042	0.1781 [§]	0.2271*	1.0000					
VLDL-C	0.4268*	-0.1172	0.1022	0.0923	0.0770	0.0866	0.1269	0.0057	0.2020*	0.3008*	0.1207	-0.1043	0.1849 [§]	0.1137	0.7490*	1.0000				
LDL-C	0.1378	-0.1425	-0.1160	0.3961*	0.2555*	0.0092	-0.0520	-0.0328	-0.0325	-0.0135	0.0397	0.1340	0.1026	0.2125*	0.3974*	0.3048*	1.0000			
nHDL-C	0.2653*	-0.1602	-0.0609	0.3632*	0.2404*	0.0382	0.0013	-0.0253	0.0440	0.0946	0.0757	0.0751	0.1507	0.2175*	0.5955*	0.6065*	0.9421*	1.0000		
HDL-C	-0.0957	-0.1130	-0.1368	0.3390*	0.2039*	0.0358	-0.0221	-0.0895	0.1003	-0.2473*	0.1685 [§]	0.1409	0.0044	-0.0538	-0.2809*	-0.2363*	-0.0320	-0.1099	1.0000	
t-C	0.2322*	-0.2032*	-0.1125	0.4703*	0.3034*	0.0385	-0.0201	-0.0665	0.0762	0.0059	0.1342	0.1291	0.1506	0.1728 [§]	0.4730*	0.5054*	0.9083*	0.9362*	0.2397*	1.0000

* Statistically significant correlations ($P < 0.05$). [§] Borderline correlations ($0.05 < p < 0.10$).

Table 3. Multiple linear regression for D₅₀ and dD in relation to groups of independent variables

Dependent variable	Groups of independent variables	Partial Regression Coefficient	t	p	R ² adj	Multiple Correlation Coefficient	p
D ₅₀	a	77.8434	9.6448	<0.0001*			
	b1 (Age)	0.098	3.1247	0.0023*			
	b2 (HSA)	4.2583	2.4328	0.0167*	0.3453	0.6138	<0.0001
	b3 (LDL-C)	0.148	1.9301	0.0564**			
	b4 (HDL-C)	0.2998	3.9445	0.0001*			
	b5 (t-C)	-0.0446	-0.6812	0.4973			
dD	a	-44.5108	-3.6158	0.0005*			
	b1 (MCH)	0.2277	1.2834	0.2023			
	b2 (MCHC)	0.5976	2.0068	0.0475*			
	b3 (RDW)	1.3305	2.9349	0.0041*	0.2134	0.5161	0.0002
	b4 (TG)	0.0195	2.2710	0.0253*			
	b5 (LDL-C)	0.0819	2.1630	0.0329*			
	b6 (HDL-C)	0.1301	3.1047	0.0025*			
	b7 (t-C)	-0.0603	-1.7197	0.0886**			

MLR equation: $Y' = a + b_1X_1 + b_2X_2 + \dots + b_iX_i$

* Statistically significant correlations ($p < 0.05$). [§] Borderline correlations ($0.05 < p < 0.10$).

**Borderline correlations ($0.05 < p < 0.10$).

CONCLUSIONS

The values of D_{50} but not dD enhanced with increasing age of the volunteers in this study, which means that the erythrocyte stability in relation to SDS increased with the volunteers' age. Analyses of MLR showed that the blood levels of HSA are the main factor that influences the stability variable D_{50} and that the stability variable dD is associated with increased values of RDW.

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CAPÍTULO 3

INTER-RELATIONS BETWEEN HEMATOLOGICAL PARAMETERS AND MORPHOLOGICAL FEATURES OF ERYTHROCYTE MEMBRANE DETECTED BY ATOMIC FORCE MICROSCOPY IN DIFFERENT AGES

Este Capítulo foi redigido de acordo com os padrões da revista *Biochimica et Biophysica Acta*.

Inter-relations between hematological parameters and morphological features of erythrocyte membrane detected by atomic force microscopy in different ages

RBC stability and AFM

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Keywords: atomic force microscopy, antioxidant enzymes, arithmetic mean roughness, membrane stability, erythrocyte

RESUMO

Objetivo: Associar as características morfológicas dos eritrócitos obtidas por microscopia de força atômica (AFM) com a estabilidade da membrana e parâmetros bioquímicos e hematológicos em mulheres voluntárias entre 20 e 90 anos.

Métodos: A AFM em modo contato forneceu a média aritmética de rugosidade (Ra), a altura máxima (Rz), altura média (Rp) e profundidade média (Rv) da membrana celular, bem como largura, altura e profundidade da imagem do eritrócito. A curva sigmoidal de hemólise por dodecil sulfato de sódio (SDS) foi utilizada para obter as variáveis de estabilidade representadas pelo ponto de meia transição (D_{50}) e a variação da concentração de SDS (dD) necessária para promover a hemólise completa da amostra de sangue. Os dados de microscopia e estabilidade foram correlacionados com variáveis hematológicas, bioquímicas e com as enzimas antioxidantes superóxido dismutase (SOD) e catalase (Cat) de cada voluntário.

Resultados: Os valores de Ra foram menores no grupo de voluntários mais velhos e apresentaram correlação inversa com D_{50} , n-HDL e colesterol total. A atividade da Cat apresentou uma correlação inversa com A_{max} , triglicerídeos e VLDL-C, enquanto que a atividade da SOD apresentou uma correlação direta com o tamanho do eritrócito.

Discussão: Os menores valores de Ra encontrados em eritrócitos dos voluntários mais velhos podem estar relacionados com as alterações de membrana causadas por valores elevados de colesterol no sangue e diminuição da atividade das enzimas antioxidantes.

Palavras-chave: Microscopia de força atômica, enzimas antioxidantes, média aritmética da rugosidade, estabilidade da membrana, eritrócito

ABSTRACT

Objective: To associate the morphological characteristics of erythrocytes obtained by atomic force microscopy (AFM) with its membrane stability and biochemical and hematological parameters in volunteers between 20 and 90 years.

Methods: Contact-mode AFM provided arithmetic mean roughness (Ra), maximum height (Rz), average height (Rp) and average depth (Rv) of the cell membrane, as well as width, height and depth of the erythrocyte image. The sigmoidal curve of hemolysis by sodium dodecyl sulfate (SDS) was used to obtain the stability variables given by the half-transition point (D_{50}) and the variation in the SDS (dD) concentration necessary to promote complete hemolysis of the blood samples. The microscopy and stability data were correlated with hematological and biochemical variables and antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) of each volunteer.

Results: The values of Ra were lower in the group of older volunteers and presented inverse correlations with D_{50} and non-HDL- and total cholesterol. The Cat activity presented an inverse correlation with A_{max} , triglycerides and VLDL-cholesterol, while the SOD activity exhibited a direct correlation with the erythrocyte width.

Discussion: The smaller values of Ra found in the older volunteers' erythrocytes may be related to the membrane changes caused by high values of blood cholesterol and decreased activities of antioxidant enzymes.

Keywords: atomic force microscopy, antioxidant enzymes, arithmetic mean roughness, membrane stability, RBC

INTRODUCTION

The relations between composition, structures, shapes, properties and functions of cells constitute one of the central concerns of the cellular biology [1, 2].

The mammalian erythrocytes have a typical biconcave discoidal shape, which is determined by cytoskeletal membrane proteins and the architecture of the lipid bilayer. They can suffer reversible changes, but they are under the influence of internal or external factors [3] that can affect their biomechanical properties.

These factors surely comprise the hematological properties and the great diversity of nature and concentrations of all solutes contained in the blood, which are under great influence of lifestyle and nutrition. Besides heredity, lifestyle and diet are the most important factors related to health and different pathological processes.

That is why the study of the composition [4], morphology and biomechanical properties such as the viscoelasticity of the erythrocytes can provide information about the health status and allowing the diagnosis and treatment of diseases [3, 5-10]. Indeed, changes in composition and stiffness of membranes have been associated with diabetes, hypertension, anemia and cardiovascular disease [4, 11-13].

The ability of a membrane to preserve its structure before agents or stress conditions defines a property called stability. The mechanical stability of the erythrocyte membrane depends on its elasticity and rapid response to agents such as the shear tension and resistance to the passage through small diameter capillaries. On the other hand, the osmotic stability of the red cells depends on its ability in resisting the membrane rupture in hypotonic medium [14-16], while the chemical stability depends on the ability of these cells to resist the membrane rupture caused by chemical agents such as ethanol [17-19] and sodium dodecyl sulfate [20, 21].

Since the osmotic or chemical stability of erythrocytes have been associated to changes in hematological indices such as RDW [19, 21, 22], and blood analytes, such as albumin [20], glucose [23] and cholesterol [19, 21, 22], this means that membrane stability must be present in the study of the relations between composition,

structures, shapes, properties and functions of cells both in many conditions. Indeed, changes in erythrocyte membrane stability have been associated with aging [15, 21], changes in the energy intake [19], neurodegenerative [18] and infectious diseases [24]

The complex relations between composition, structure, shape, properties and functions of erythrocytes should also aggregate morphological features such as roughness and topographic cell dimensions, which can be obtained by atomic force microscope (AFM) [25,26]. Unlike other microscopy techniques, AFM provides three-dimensional visualization and analysis of the erythrocyte surface. This technique has a probing tip that scans the sample in order to form an image of its surface, typically at micro or nanoscale dimensions [27]. The image obtained can be understood in many aspects as a consequence of interactions between the sample surface and the probing tip [27-29]. There exists a great variety of causes for these interactions, such as: polarization forces, electrostatic forces, mechanical contact forces, or van der Waals forces [27]. In several studies, the AFM technique is widely used to measure the average size of nanostructures embedded in the glass [30], deposited onto a substrate [31] or electrodeposited in LbL film [32]. Some of these nanostructures have average sizes around 1.4 nm, being clearly observed in the AFM images [33] Thus, the AFM appears as a powerful technique to evaluate the surface characteristics of the sample with high accuracy [27]. Therefore, the combination of high resolution image and functionality in a physiological environment has provided investigate cells *in vivo* and *in vitro* by AFM [10, 34]. The roughness is an important parameter in the characterization and comparison of surfaces, which has received impulse from the application of AFM [35]. The creation of software for image processing of biological specimens has stimulated a great number of investigations in this field, especially in RBC [36].

It is within this context that this study investigates the relations of the membrane stability of human erythrocytes with hematological indices, several biochemical analytes, antioxidant enzymes and some morphological characteristics obtained by AFM technique.

MATERIAL AND METHODS

Population

The study was approved by the Ethics Committee of the Federal University of Uberlândia (Protocol 127/11). The population consisted of 29 participants from 20 to 90 years (14, 20-30 y and 15, 60-90 y).

The recruitment of the population occurred in a clinical laboratory (Labormed, Uberlândia, MG, Brazil). Only women were recruited to avoid possible physiological variations caused by gender [37, 38]. We excluded subjects with history of chronic diseases (diabetes, heart disease and dementia), chronic smokers and chronic consumers of alcohol and other drugs of abuse.

Blood collections

Blood was collected by intravenous puncture, after fasting 8 to 12 hours, in evacuated tubes (4 ml) K₃EDTA as anticoagulant.

Determination of hematologic and biochemical variables

Hemogram was done using an automated system of analysis (Sysmex K4500, Sysmex Corporation, Mundelein, IL, USA). The lipid profile was performed in an automatic analyzer (Hitachi 917, Roche Diagnostics, Indianapolis, IN, USA). Serum albumin was colorimetrically assayed using a commercial kit (Labtest) in a UV-VIS spectrophotometer (Shimadzu, model UV 1650 TC, Japan).

The reference values (women over 16 years) were: erythrocytes (RBC), 3.9 to 5.9 millions.mm³; hemoglobin (Hb), 12.0 to 16.0 g.dL⁻¹; hematocrit (Ht), 35.6 to 48.6%; mean corpuscular volume (MCV), 82-98 fL; mean corpuscular hemoglobin (MCH), 27-31 pg; mean corpuscular hemoglobin concentration (MCHC), 32.9 to 36%; red cell distribution width (RDW), 12 to 15%; glucose (Glu), 60 to 99 mg.dL⁻¹; albumin (HSA), 3.5

to 5.2 g.dL⁻¹; total cholesterol (t-C), <200 (optimum) and ≥240 mg.dL⁻¹ (high); high-density lipoprotein cholesterol (HDL-C), <45 (low) and >65 mg.dL⁻¹ (ideal), low-density lipoprotein cholesterol (LDL-C), <100 (good) and >160 mg.dL⁻¹ (high); very low density lipoprotein (VLDL-C), up to 40 mg.dL⁻¹; and triglycerides (TG), <150 (optimum) and >201 mg.dL⁻¹ (high).

Catalase (Cat)

The Cat activity was assayed by measuring the rate of disappearance of hydrogen peroxide [39]. Blood samples collected in evacuated tubes containing K₃EDTA were centrifuged at 1,300 x g for 5 min to remove plasma and leukocytes. The suspension of erythrocytes was then washed with phosphate-buffered saline pH 7.4 and submitted to centrifugation at 1,300 x g for 5 min, by three times, always discarding the supernatant. The erythrocytes were then lysed by mixing ultrapure water (MilliQ) in the 1:2 ratio. The hemolysate was dissolved in 50 mM phosphate buffer, pH 7.0, in the 1:500 ratio.

The control solution used in the spectrophotometric reading consisted of 3.5 mL of 50 mM phosphate buffer, pH 7.0, and 0.5 mL of the previous sample (final dilution ratio of 1:2000). To perform the test, 0.5 mL of the diluted sample in 2.5 mL of 50 mM phosphate buffer, pH 7.0, were placed in a cuvette for spectrophotometric reading at 240 nm (Shimadzu, UV 1650 model TC, Japan). The enzyme action was triggered by adding 1 mL of 30 mM H₂O₂. The absorbance readings were taken between 0 and 15 s after obtaining a positive absorbance value.

The erythrocyte catalase activity, expressed in k units per g hemoglobin per min, was obtained by using the following formula:

$$\left(\frac{2.3}{\Delta t}\right) \left(\frac{a}{b}\right) \log \left(\frac{A_1}{A_2}\right) \quad (1),$$

where Δt is the time variation (0 to 15 s), a is the final dilution factor of the test ($a = 2000$), b is the hemoglobin concentration of the sample in g/dL, and A_1 and A_2 represent the absorbance values at time 0 and 15 s, respectively.

Superoxide dismutase (SOD)

Whole blood samples collected in evacuated tubes containing K₃EDTA were used to detect the SOD activity using a Ransod kit (Randox, United Kingdom). The method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction. Absorbance readings were taken at 505 nm in a UV-VIS spectrophotometer (Shimadzu™, UV 1650 model TC, Japan).

Determination the erythrocytes stability in relation to sodium dodecyl sulfate (SDS)

Duplicate sets of test tubes (Eppendorf, Hamburg, Germany) containing 1 mL of 68-176 µM SDS solutions in 0.9 g.dL⁻¹ NaCl were pre-incubated at 37 °C for 10 min in a thermostated water bath (Marconi, MA 184 model, Piracicaba, SP, Brazil). After adding 10 µL of whole blood, the tubes were gently homogenized, incubated for 30 min at 37 °C, and then centrifuged at 1500 *xg* for 10 min. The supernatants were used for evaluation of the optical density at 540 nm (A_{540}) in a UV-VIS spectrophotometer (Shimadzu™, model UV 1650 TC, Japan). The graphics of A_{540} in relation to the SDS concentration were adjusted to sigmoidal regression lines, according to the Boltzmann equation:

$$A_{540} = \frac{A_1 - A_2}{1 + e^{(D - D_{50})/dD}} + A_2 \quad (2),$$

where A_1 and A_2 represent the minimum ($A_{\min}$) and maximum ($A_{\max}$) plateaus of the sigmoid, D is the SDS concentration, D_{50} is the SDS concentration capable of promoting 50% of hemolysis, and dD represents the variation in the SDS concentration responsible for full hemolysis of the blood sample.

Atomic force microscopy (AFM)

A whole blood sample containing K₃EDTA was spread on a mica sheet and dried at room temperature (22 °C) approximately 5 min prior to scanning by AFM. The blood smear was not fixed and no chemicals were added to immobilize the cells. The AFM images of the erythrocytes were recorded at room temperature with scanning probe microscope using contact mode (SPM – 9600, Shimadzu, Japan).

The software Vector Scan (SPM-Manager, Shimadzu, Japan) allowed the analysis of the images using two techniques: profile analysis and analysis of surface roughness. The profile analysis of the cell provided the width, height and depth of the erythrocyte image. A line was drawn on the image and profile display showed the width, height difference and angle in the specified section. The analysis of surface roughness provided the roughness parameters of the cell membrane extended in two dimensions: arithmetic mean roughness (Ra), maximum height (Rz), average height (Rp) and average depth (Rv).

Arithmetic mean roughness (Ra) represents a section of the reference length that is cut out from the roughness curve in the direction of the mean line. The X-axis is set in the direction of the mean line of this cut-out section, and the Y-axis in the direction of the longitudinal magnification. [Ra] value is given by the equation below when the roughness curve is expressed in $y=f(x)$.

$$Ra = \frac{1}{L} \int_0^L |f(x)| dx \quad (3),$$

where L represent the reference length.

Maximum height (Rz) is a section of the reference length that is cut out from the roughness curve in the direction of the mean line. Rz value is obtained by measuring the distance between the peak line and trough line of the cut-out section in the longitudinal magnification direction of the roughness curve.

Average height (Rp) represents the distance between the average of the section curve and the highest peak.

Average depth (Rv) is distance between the average of the section curve and the lowest trough.

Statistical analyses

D'Agostino-Pearson test was used to check the normality of the data. Comparison of experimental data between age groups was done using Student's t-test for normally distributed data and Mann-Whitney test for non-normally distributed data.

The existence of Spearman correlations between the stability parameters, the AFM morphological features, and the hematological and biochemical variables used the significance level of 0.05. Analyses were done with help of the software package OriginPro9 (MicroCal™, Northampton, MA, USA).

RESULTS

Table 1 presents the comparison between the age ranges for the antioxidant enzymes, erythrocyte stability parameters and hematological and biochemical variables of the volunteers (n=29). The age group of 60-90 y presented significantly higher values of MCV, RDW, Glu and VLDL-C, and values of D₅₀ and TG that had a borderline increase in relation to the younger volunteers group. On the other hand, the age group of 20-30 y showed the higher activities of SOD when compared with the older age group.

Table 2 presents the comparison between the age groups of morphological features of erythrocyte obtained by AFM. The values of the arithmetic mean roughness (Ra) were lower in participants of the older age group.

Table 3 shows the matrix of Spearman correlations between membrane stability, AFM topographic features, and hematological and biochemical variables. The erythrocyte width presented a positive correlation with SOD, while the cell height correlated positively with cell depth and TG. Cell depth was also positively correlated

with Hb, VLDL-C and TG. Regarding the roughness parameters, Ra had positive correlations with Rz, Rp and Rv, and negative correlations with D_{50} , MCV, MCH, nHDL-C and t-C, while Rz showed positive correlations with Rp and Rv. Regarding the antioxidant enzymes, CAT had inverse correlations with A_{max} , Hb, Ht, RBC, TG and VLDL-C, while SOD presented a direct correlation with the erythrocyte width.

Figure 1 shows typical AFM topographic images of erythrocytes from volunteers of the groups of 20-30 (A, B and C) and 60-90 years (D, E and F). A and D are erythrocytes in a $30\ \mu\text{m}^2$ scanning area. B and E are single erythrocytes in a $12\ \mu\text{m}^2$ scanning area used to obtain the cell profile (width, height and depth), as described in the figure legend. C and F are images obtained in the roughness analysis of the erythrocyte membrane (scanning area of $1\ \mu\text{m}^2$) using the arithmetic mean roughness (Ra) and the histogram of particle size distribution, in which Rz is maximum height. The values of Ra presented significantly lower values in the older volunteers group (Table 2).

DISCUSSION

The ability of AFM to detect cells and molecules in physiological conditions and native state has allowed the nanoscale study of the mechanical and structural heterogeneity of the erythrocyte [2, 40].

Only freshly-collected blood samples were used in the AFM analysis, as storage even at low temperatures produces gradual decrease in erythrocyte membrane roughness [26, 41].

Analysis by AFM requires the choice of a fixed area to allow for an adequate comparison among of roughness values between different samples [25]. That is why the values of Ra among different age groups were obtained with use of a fixed size area of $1\ \mu\text{m}^2$ (**Figure 1**, C and F).

Many changes may occur in the erythrocyte structure during its lifetime due to physiological changes associated with increasing age or presence of diseases. **Figure 1**

(C and F) illustrates the difference observed in the average roughness (Ra) between the age groups and the absence of significant differences for the other AFM features (**Table 2**). Ra has been used in the literature to characterize the cell membrane of erythrocytes submitted to various conditions, since it is directly or indirectly responsive to the integrity of cytoskeletal membrane [1, 26, 42-44]. The lowest average value of Ra found for the erythrocyte membrane of the older group can be certainly explained by the occurrence of physicochemical changes in the erythrocyte topography.

The absence of significant correlations between Ra and depth, width, and height (**Table 3**) confirms the lack of connection between cell morphology and membrane roughness. This means that the Ra values are derived from cell surface features, which allows its measurement in different size cells of a same sample [25].

Smaller values of average roughness were associated with defective membrane skeleton, treatment with destabilizing chemicals, and presence of spherocytes [44]. In addition, the morphological parameters width, length, valley, peak and average surface fluctuation of Ra undergo alterations in patients with anemia and thalassemia, which indicates the deformation in cell architecture induced by defects in membrane and cytoskeletal proteins. These alterations comprise increased average values of Ra in the iron deficiency anemia and decreased values of this variable in thalassemia when compared with healthy individuals [42]. Moreover, when the mechanical support exerted by the membrane skeleton on the lipid bilayer decreases, as occurs in certain blood diseases or after treatment with cytoskeletal-depolymerizing agents, the amount of cell surface roughness drops significantly [26].

The inverse correlation observed in this study between Ra and t-C, nHDL-C and D₅₀ (**Table 3**) indicate that the average roughness of the erythrocyte membrane decrease with increased blood levels of cholesterol, which certainly has an impact in the membrane cholesterol content and in the erythrocyte membrane stability in relation to SDS [21]. This makes sense, since the lipid environment is capable of modulating the cellular functionality. The lipid bilayer can undergo mechanical changes that will affect its conformational transitions [45], since high levels of LDL-cholesterol

will increase its cholesterol content [46, 47] and enhance its thickness and stiffness [48].

Moreover, the inverse relation observed between Cat and Amax, on one hand, and the direct correlation observed between SOD and the erythrocyte width, on the other (**Table 3**), are surely manifestations of the protective action of these antioxidant enzymes on the stability and integrity of the red cell membrane. Since increased oxidative stress will limit the erythrocyte lifespan, therefore, SOD and Cat play an important role in this process [49]. Indeed, mice with transplanted stem cells from knockout mice for SOD showed decreased survival of erythrocytes [50] and mutant mice with 50% higher expression of catalase had an increased longevity when compared with wild mice [51].

Erythrocytes are promoters of the oxidative processes and, at the same time, they are extremely susceptible to their damage, due to the high content of polyunsaturated fatty acids in their membranes and their high concentration of oxygen and hemoglobin [52, 53]. That's why a reduction in the content of antioxidant enzymes will increase the lipid peroxidation of the erythrocyte membrane, with consequent hemolysis and anemia [54].

A decrease in the content of antioxidant defenses has been detected in older populations [55]. The lesser content of SOD in the older volunteers of the present study (**Table 1**) may have contributed to the roughness changes observed in the erythrocyte surface. Indeed, the release of superoxide in the blood was associated with reduced erythrocytes roughness and loss in the ability of the red cells to maintain their typical discoid shape in smokers when compared to non-smokers healthy volunteers, probably due to a rearrangement in the macromolecular complexes of the membrane surface [43].

Further, the inverse correlations observed between Cat and TG and VLDL-C (**Table 3**) indicates that antioxidant defenses can increase under reduced blood fat levels. This agrees with the higher levels of VLDL-C observed in the group of older volunteer, although SOD and not Cat has been the anti-oxidant activity that declined in this group (**Table 1**).

Certainly, the induction of oxidative damage associated with increased caloric intake exerts deleterious effects on health [56, 57] involve increase in the blood fat levels. Indeed, hypercholesterolemia has been associated with high production of reactive oxygen species (ROS), blood vessels damage, endothelial dysfunction and increased oxidation of lipids and proteins in the erythrocyte membrane [58].

An inverse relation between those anti-oxidant enzymes and blood fat levels has also been seen in the hypocholesterolemic action of drugs such as the statins [59-62].

In summary, the lower values of Ra found in the erythrocytes of older people may be related to membrane changes caused by high values of blood cholesterol and decrease in the antioxidant activity of these cells.

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Table 1. Comparison of erythrocyte stability, antioxidant enzymes and hematological and biochemical variables between the two age ranges considered in this study

Variable	20-30 y (N = 14)		60-90 y (N = 15)		p
	Median	IQR	Median	IQR	
Age (y)	25.50	4.000	72.00	13.00	
A _{min} (ΔOD)	0.011	0.029	0.013	0.026	0.386
A _{max} (ΔOD) [‡]	1.215	0.096	1.195	0.111	0.743
D ₅₀ (μM SDS)	120.0	8.024	122.6	12.54	0.073 [§]
dD (μM SDS)	6.383	2.768	6.788	2.964	0.726
Hb (g/dL)	13.00	0.900	13.20	2.300	0.594
Ht (%)	39.50	2.900	40.00	6.600	0.316
RBC (millions/μL) [‡]	4.520	0.340	4.420	0.840	0.727
MCV (fL)	87.10	3.800	91.00	5.900	0.014*
RDW (%)	12.80	0.800	13.30	0.500	0.013*
MCH (pg) [‡]	29.15	2.200	30.00	1.300	0.205
MCHC (%)	33.10	1.100	32.80	1.300	0.134
Glu (mg/dL)	85.45	13.50	91.20	8.700	0.048*
HSA (g/dL)	3.900	0.480	4.100	0.400	0.707
TG (mg/dL) [‡]	63.40	69.10	102.6	59.80	0.058 [§]
VLDL-C (mg/dL)	11.80	10.50	20.50	11.90	0.011*
LDL-C (mg/dL)	79.30	39.60	96.90	32.20	0.367
nHDL-C (mg/dL)	90.45	38.50	118.0	26.40	0.156
HDL-C (mg/dL)	54.70	11.90	52.50	14.60	0.838
t-C (mg/dL)	145.4	51.10	172.1	38.00	0.199
Cat (k/g Hb/min)	7.148	2.047	6.870	2.427	0.807
SOD (U/g Hb)	115.9	117.9	85.26	46.86	0.015*

*Statistically significant ($p < 0.05$) and [§]borderline differences ($0.05 < p < 0.10$) between age ranges (Student's t-test, for normally distributed data, and Mann-Whitney's test, for non-normally distributed data)

[‡]Non-normally distributed parameters (D'Agostine-Pearson test)

Table 2. Comparison of morphological features detected by atomic force microscopy between the age groups

Parameters	20-30 y (N = 14)		60-90 y (N = 15)		p
	Median	IQR	Median	IQR	
Cell Profile					
width (µm)	7.890	0.650	7.780	1.020	0.540
height (nm)	393.0	145.7	394.6	147.4	0.892
depth (nm)	138.6	118.5	140.3	121.7	0.750
Membrane Surface (nm)					
arithmetic mean roughness (Ra)	1.598	2.554	0.809	0.579	0.002*
maximum height (Rz) [£]	13.01	12.10	14.77	9.971	0.913
average height (Rp)	5.104	5.821	5.241	4.481	0.659
average depth (Rv) [£]	7.675	5.437	8.469	5.096	0.982

*Statistically significant differences ($p < 0.05$) between age ranges (Student's t-test, for normally distributed data, and Mann-Whitney's test, for non-normally distributed data)

[‡]Non-normally distributed parameters (D'Agostine-Pearson test)

Table 3. Matrix of Spearman correlation coefficients (ρ) between pairs of variables

	Width	Height	Depth	Ra	Rz	Rp	Rv	Amin	Amax	D50	dD	Hb	Ht	RBC	MCV	RDW	MCH	MCHC	Glu	HSA	TG	VLDL-C	LDL-C	nHDL-C	HDL-C	t-C	Cat	SOD
Width	1																											
Height	0.140	1																										
Depth	-0.079	0.400*	1																									
Ra	-0.048	-0.173	-0.068	1																								
Rz	-0.081	-0.039	0.296	0.421*	1																							
Rp	-0.105	-0.034	0.293	0.466*	0.944*	1																						
Rv	-0.076	-0.079	0.153	0.410*	0.927*	0.788*	1																					
Amin	0.017	0.114	0.061	0.095	0.167	0.069	0.199	1																				
Amax	-0.325	0.234	0.312 [§]	0.144	-0.158	-0.067	0.178	-0.117	1																			
D50	-0.080	-0.190	-0.224	-0.392*	-0.266	-0.244	0.264	-0.180	-0.382*	1																		
dD	-0.221	-0.249	-0.286	-0.171	-0.160	-0.044	0.197	-0.773*	-0.022	0.530*	1																	
Hb	-0.206	0.214	0.393*	-0.246	-0.046	0.001	-0.067	-0.019	0.580*	-0.129	-0.081	1																
Ht	-0.084	0.087	0.248	-0.201	0.048	0.082	0.034	-0.071	0.553*	-0.172	-0.024	0.913*	1															
RBC	-0.135	0.100	0.186	0.113	0.033	0.070	0.047	0.007	0.649*	-0.356	-0.155	0.769*	0.826*	1														
MCV	0.006	0.155	0.181	-0.526*	-0.168	-0.106	0.248	-0.087	0.039	0.423*	0.212	0.319 [§]	0.330 [§]	-0.167	1													
RDW	0.047	-0.008	-0.167	-0.143	0.149	0.188	0.041	-0.048	0.001	0.192	0.288	0.093	0.294	0.134	0.327 [§]	1												
MCH	-0.171	0.338 [§]	0.318 [§]	-0.545*	-0.250	-0.203	0.313	-0.041	0.051	0.332	0.107	0.320 [§]	0.137	-0.297	0.830*	-0.016	1											
MCHC	-0.259	0.198	0.353 [§]	-0.063	-0.202	-0.199	0.231	0.022	0.022	0.018	-0.127	0.013	-0.350 [§]	-0.276	-0.144	-0.624*	0.358*	1										
Glu	-0.070	0.260	0.166	-0.080	-0.136	-0.109	0.115	0.134	0.240	-0.010	-0.069	0.144	0.128	0.070	0.235	-0.002	0.271	0.099	1									
HSA	0.240	-0.256	0.067	-0.089	-0.146	-0.067	0.158	-0.070	-0.005	0.307	0.109	0.250	0.250	0.024	0.397*	0.053	0.269	0.043	0.314 [§]	1								
TG	-0.217	0.389*	0.460*	-0.335 [§]	0.067	-0.002	0.090	-0.064	0.313 [§]	0.110	-0.044	0.444*	0.435*	0.352 [§]	0.196	0.023	0.204	-0.040	0.285	0.021	1							
VLDL-C	-0.210	0.336 [§]	0.509*	-0.329 [§]	-0.072	-0.132	-0.067	-0.106	0.390*	0.169	0.026	0.404*	0.383*	0.249	0.355 [§]	0.085	0.327 [§]	0.025	0.485*	0.180	0.888*	1						
LDL-C	-0.160	0.021	0.204	-0.367 [§]	-0.177	-0.179	0.214	-0.377*	0.071	0.368*	0.222	0.178	0.128	0.071	0.158	-0.187	0.150	0.259	0.166	0.059	0.400*	0.445*	1					
nHDL-C	-0.174	0.130	0.336 [§]	-0.429*	-0.180	-0.205	0.213	-0.350 [§]	0.146	0.354	0.199	0.263	0.223	0.121	0.240	-0.122	0.232	0.221	0.269	0.090	0.590*	0.651*	0.960*	1				
HDL-C	-0.013	-0.258	0.103	-0.087	0.002	-0.072	0.117	-0.216	-0.268	0.044	0.206	-0.059	-0.111	-0.145	-0.110	-0.376*	0.005	0.233	-0.065	0.123	-0.055	-0.103	0.024	0.018	1			
t-C	-0.144	-0.019	0.287	-0.471*	-0.183	-0.251	-0.166	-0.382*	-0.034	0.402*	0.254	0.154	0.101	-0.017	0.188	-0.223	0.217	0.300	0.226	0.143	0.507*	0.564*	0.910*	0.934*	0.329 [§]	1		
Cat	0.242	-0.168	-0.281	0.088	-0.049	-0.047	-0.101	0.173	-0.403*	0.124	0.049	-0.410*	-0.382*	-0.400*	0.033	-0.073	-0.036	-0.005	-0.197	-0.128	-0.596*	-0.494*	-0.175	-0.277	0.115	-0.179	1	
SOD	0.539*	0.001	-0.288	0.237	-0.100	-0.181	0.040	0.036	-0.335 [§]	-0.075	-0.241	-0.219	-0.091	0.012	-0.250	-0.185	-0.326 [§]	-0.162	0.085	0.109	-0.158	-0.242	-0.064	-0.094	0.169	-0.017	0.128	1

* Statistically significant ($p < 0.05$) and [§]borderline correlations ($0.05 < p < 0.10$)

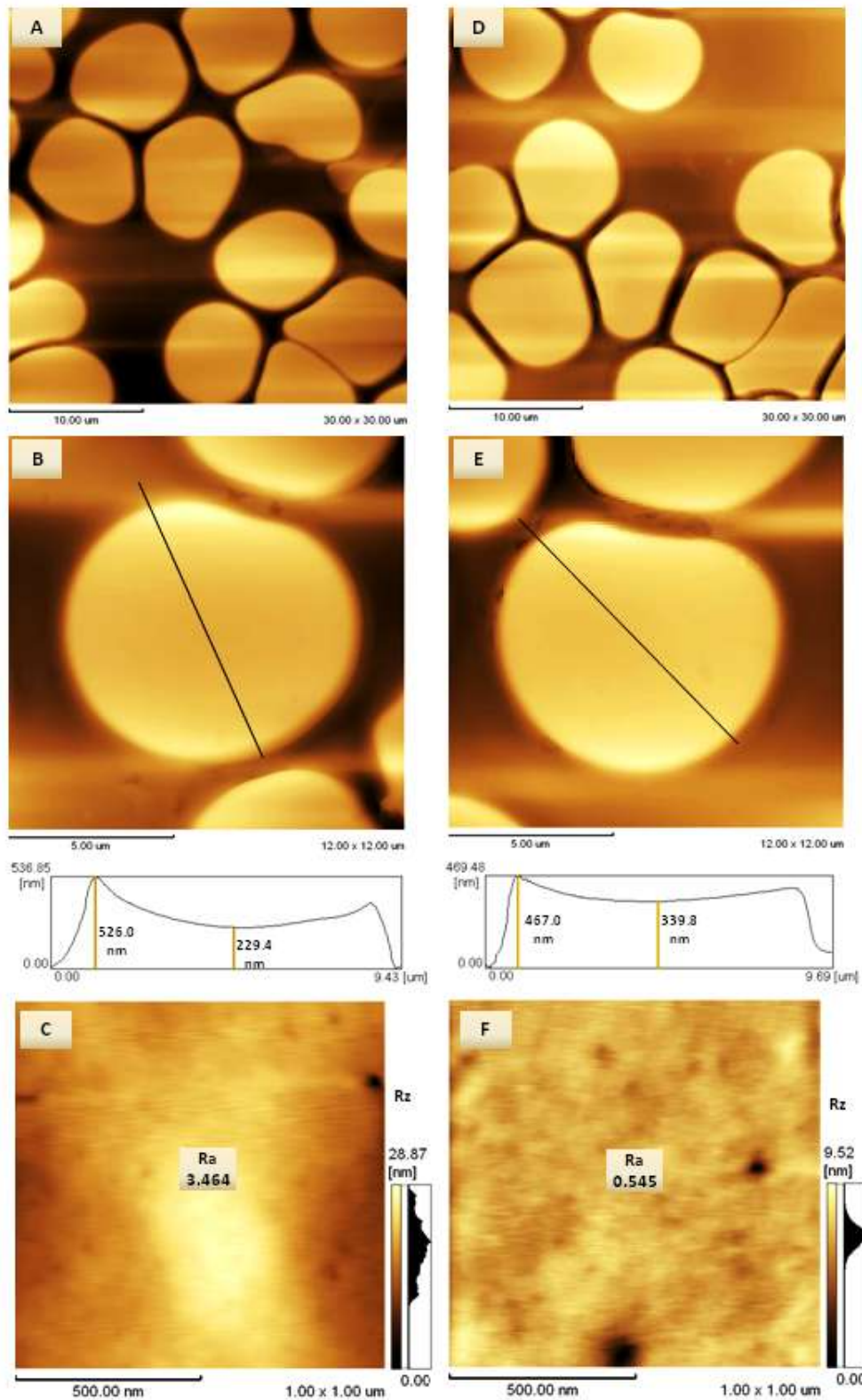


Figure 1. AFM images of erythrocytes of individuals between 20 - 30 (A, B and C) and 60 - 90 years (D, E and F). A and D show erythrocytes in a scanning area of 30 μm^2 . The erythrocyte is then focused on an area of 12 μm^2 (B and E) for analysis of its cellular profile, which provides width, height and depth of the cell. Finally, the erythrocyte membrane is analyzed over an area of 1 μm^2 (C and F) for obtaining the roughness parameters: arithmetic average roughness (Ra) and the histogram of particle size distribution in the membrane, where Rz is the maximum height.

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