UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

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Marcadores de hipóxia e sobrevivência em células endoteliais sob modelo de shear stress respondendo ao meio condicionado por ligas de Cobalto-Cromo

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Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre pelo Programa de Pós-Graduação em Biotecnologia.

Prof. Dr. Willian Fernando Zambuzzi

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“Nada na vida deve ser temido, somente compreendido. Agora é hora de compreender mais para temer menos.”

-Marie Curie

LISTA DE ABREVIAÇÕES

CoCr-Cobalto-Crômio
    Variações: w/CoCr- disco com duplo ataque ácido; wo/CoCr- disco sem ataque ácido
AA- acidentes automobilísticos
AEPS - Anuário Estatístico Da Previdência Social
AKT- Protein Kinase B
ANOVA- Análise de Variância
BSA: proteína albumina bovina
CFM -Conselho Federal de Medicina
Chrysin- composto químico comercial utilizado como antagonista de HIF-1α
col1A1- Colágeno
DAE- Duplo Ataque Ácido
DMOG- composto químico comercial utilizado como agonista de HIF-1α
ECM- Matriz Extracelular
FAK- Quinase De Adesão Focal
GAPDH- Gliceraldeído 3-Fosfato Desidrogenase
HIF- Fatores Induzíveis Por Hipóxia
    Variações: versões alfa: HIF-1α, HIF-2α e HIF-3α; versões beta HIF-1β
HRE- Elemento De Resposta À Hipóxia
HUVEC- células endoteliais de veia umbilical.
IBGE- Instituto Brasileiro De Geografia E Estatística
IL- Interleucina
INSS- Instituto Nacional do Seguro Social
JNK- c-Jun N-Terminal Cinase
    Variações: 1, 2
MAPK-P38 – Proteína Cinase Ativada Por Mitogênio
MG32- Inibidor Do Proteassoma
MMP-9 - Matriz Metaloproteinase
    Variações: -6, -8
NOS- Óxido Nítrico Sintase
    Variações: eNOS- Óxido Nítrico Sintase Endotelial; NOS2: Óxido Nítrico Sintase
ºC- Grau centígrado
OIT- Organização Internacional Do Trabalho
OMS- Organização Mundial Da Saúde
PCR- Proteína C Reativa
    Variações: RT e qPCR: Pcr em tempo real
PI3K-Quinase Inibidora De Phosphoinositol-3
RNA- Ácido Ribonucleico
SFB- Soro Fetal Bovino
SS- shear stress
SUS- Sistema Único de Saúde
TBST- (Tris-buffered-saline + Tween 20) Solução salina tamponada com Tris + Tween
TIMP- Inibidor de Metalopeptidase
Tris- tris(hidroximetil) aminometano
UV- Ultra Violeta
VEGF- Fator De Crescimento Endotelial Vascular
    Variações: VEGFR- receptor

RESUMO

O osso é um tecido conjuntivo especializado, altamente dinâmico e capaz de se restabelecer quando acometido em pequenas lesões. Por outro lado, em lesões maiores (tamanho crítico), é necessário intervenções terapêuticas para recuperar-se. Neste contexto, muito se tem discutido sobre aplicações conceituais de bioengenharia de tecidos na busca por novas alternativas, em detrimento dos procedimentos tradicionais e atualmente utilizados na clínica. Tais procedimentos envolvem, sobretudo, o uso de material autógeno para o preenchimento destas lesões. Apesar dos aspectos positivos, fatores como segundo sítio cirúrgico, quantidade e qualidade do material, período de convalescença do paciente e aumento de custo público, reforçam a necessidade de novas estratégias terapêuticas. Embora ligas de titânio venham sendo amplamente utilizadas com resultados clínicos já estabelecidos, novas ligas vêm sendo propostas no intuito de melhorar essa performance biológica; dentre elas a liga Cobalto-Crômio (CoCr) tem ganhado destaque. Há mais de 20 anos, nosso grupo de pesquisa investiga novos biomateriais osteo-substitutos e processos a serem aplicados em bioengenharia e mais recentemente, tem se intensificado a aplicação de recursos metodológicos de vanguarda para desvendar mecanismos de transdução de sinais que regem a adesão e diferenciação de osteoblastos, dois processos celulares fundamentais para estimular a regeneração do osso. No entanto, muito pouco tem sido reportado sobre o efeito desses materiais em células do endotélio, tampouco reunindo informações moleculares que envolvam mecanismos de hipóxia (mecanismo conhecido capaz de guiar processos de angiogênese). Desta forma, o objetivo deste será avaliar a resposta biológica de células endoteliais (HUVECs) a biomateriais a base de CoCr, sobretudo olhando para o comportamento celular frente a expressão do gene HIF e sua capacidade de reciclagem via proteassoma. Para tanto, o meio condicionado dos biomateriais (condicionado por 24 horas; ISO10993-5:2016) foi utilizado para o tratamento de células endoteliais por 72 horas, as amostras foram coletadas para as análises biológicas. Para validar a importância da via proteassômica, foi inibida com MG32. Conclui-se que ligas de CoCr promovem a viabilidade e atividade de células endoteliais, assim como a ação mimética do shear stress, ambos ativam o mecanismo de Hif-1α, que apresenta um potencial para ser utilizado em momentos futuros da medicina personalizada, por exemplo.

Palavras chave: Implantes; ligas metálicas; Angiogênese; CoCr; Células endoteliais; HIF; osteogênese.

ABSTRACT:

Bone is a specialized connective tissue, highly dynamic and capable of reestablishing itself when affected by minor injuries, but when in larger lesions (critical size), therapeutic interventions may be needed to recover. In this context, much has been discussed about conceptual applications of tissue bioengineering in the search for new alternatives, to the detriment of traditional procedures currently used in the clinic. Such procedures involve, above all, the use of autogenous material to fill these lesions. Despite the positive aspects, factors such as a second surgical site, quantity and quality of the material, the patient's recovery period and increased public costs, reinforce the need for new therapeutic strategies. Although titanium alloys have been widely used with established clinical results, new alloys have been proposed in order to improve this biological performance; among those, Cobalt-Chromium alloy (CoCr) has gained prominence. For more than 20 years, our research group has been investigating new osteo-substitute biomaterials and processes to be applied in bioengineering, and recently, the application of cutting-edge methodological resources has intensified to unravel signal transduction mechanisms that orchestrate adhesion and differentiation of osteoblasts, two fundamental cellular processes to stimulate bone regeneration. However, very little has been reported on the effect of these materials on endothelial cells, nor have they gathered molecular information involving hypoxia mechanisms (a known mechanism capable of guiding angiogenesis processes). Thus, the objective of this study will be to evaluate the biological response of endothelial cells (HUVECs) to CoCr-based biomaterials, especially when looking at the cellular behavior of HIF gene expression and its ability to recycle via proteasome. For this purpose, the conditioned medium of biomaterials (conditioned for 24 hours; ISO10993-5:2016) was used for the treatment of endothelial cells for 72 hours, samples were collected for biological analyses. To validate the importance of the proteasomal pathway, it was inhibited with MG32. It is concluded that CoCr alloys promote the viability and activity of endothelial cells, as well as the mimetic action of shear stress, both activating the Hif-1α mechanism, which has potential to be used in future moments of personalized medicine, for example.

Keywords: Implants; metal alloys; Angiogenesis; CoCr; Endothelial cells; HIF; osteogenesis.

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1 -INTRODUÇÃO

Não há dúvidas que a ciência vem ganhando protagonismo importante ao longo dos anos. Elucidações quanto à origem da vida, meio ambiente, relação homem-natureza, relação universal, homem-tecnologia, vida e longevidade, entre tantas outras ciências possíveis, levam o ser humano compreender melhor seu ambiente, prever e medicar-se contra doenças, aumentando sua expectativa de vida (Van Deursen, J.M. 2019).

Com isso, a longevidade no Brasil e no mundo cresceu (World Health Organization et al., 2019; Machado, E.V. 2021). Segundo site do IBGE, em 1980, a possibilidade de uma pessoa com 60 anos chegar aos 80 anos era de 34,4%, em 2019 essa porcentagem estava em 60,4%. Outro dado do IBGE, relativo à longevidade, é que, no intervalo de aproximadamente 50 anos (1960-2008), o número de idosos aumentou em 500%. Em 2016 o Brasil alcançou a 5ª maior população mundial de idosos, segundo a OMS. Estes números são fielmente guiados pelo aumento populacional, no ano de 2021 o Brasil ocupou a 6ª colocação mundial de países populosos, segundo o IBGE (IBGE, 2021).

A longevidade nos traz novos obstáculos e, assim como cresce a população idosa, o cuidado com ela torna-se fundamental, gerando a necessidade de políticas públicas capazes de garantir o envelhecimento com qualidade (Giordano et al., 2020). Nesta perspectiva, doenças relacionadas ao avanço da idade populacional, ocasionam a necessidade de desenvolvimento de produtos e processos, com novas formas de cuidado, mais eficientes, menos invasivas, mais rápidas, com menos efeitos colaterais, para oferecer a melhor qualidade de vida possível.

Além da longevidade, o aumento populacional também trouxe outros números significativos para o Brasil: como os acidentes de trabalho - o Brasil ocupa o 5º lugar no ranking internacional de acidentes de trabalho não letal, segundo o AEPS 2018. O levantamento feito pela OIT, no período de 2013 a 2020, indicou que os ferimentos mais frequentes dentro do espaço de trabalho estão dentre os 21% corte, laceração, ferida, contusão ou punctura, fratura representa 17% e contusão/esmagamento ocupam 15% deste total. O INSS registra que os afastamentos cedidos por motivo de fraturas chegam a 40%. Sendo que, osteomuscular e tecido conjuntivo representam 23% desse total (AEPS, 2018; OIT, 2020).

O Brasil, segundo a OMS, ocupou em 2020, a 4ª posição no ranking de acidentes automobilísticos (AA). Estima-se que a cada uma hora no Brasil em média cinco pessoas vão a óbito, sendo então a segunda maior causa de mortes externas. Os que mais tem probabilidades de se envolver em um acidente de trânsito, resultando em lesões graves e óbitos são os homens jovens, condutores de motocicletas, na faixa etária economicamente ativa, de 20 a 39 anos. Além dos fatores humanos, o Conselho Federal de Medicina (CFM), afirma em um levantamento publicado no ano de 2019, que nos últimos dez anos o Sistema Único de Saúde (SUS) teve um custo direto de quase 3 bilhões de reais entre custos de assistência/socorro, até as internações e procedimentos nos hospitais em decorrências de AA (CFM, 2019). As lesões ortopédicas constituíram a segunda causa de internação mais frequente dentre os pacientes, vítimas de Acidentes de Trânsito, concentrando-se a maioria (70,1% dos casos) dessas lesões na região dos membros inferiores, particularmente na coxa (IPEA, 2020).

Dentre as necessidades que a longevidade e ferimentos de naturezas diferentes nos trazem, existe uma importância significativa relacionado ao processo de cicatrização, as quais em outros momentos não seriam um problema. A cicatrização traz consigo questões tais como cuidados e cuidadores especializados, técnicas específicas de tratamentos e aplicações, justificativas e especificações para a utilização de um biomaterial. Logo, é necessário que se conheça os pontos positivos e negativos dos biomateriais disponíveis e sua compatibilidade com o tecido ósseo, que é um ponto comum quando se fala de acidentes automobilísticos graves, acidentes em espaço de trabalho e em lesões agravadas pela idade ou por consequência de doença.

A cicatrização, reparo e regeneração do tecido perdido são fatores importantes na reabilitação de pacientes enquadrados nos parâmetros a cima; ao se pensar na qualidade de vida nos tempos atuais é necessária uma constância no fluxo de renda, que fica impossibilitada que ocorre uma injuria ou um acidente do qual se necessita a intervenção, por tanto é importante que essa cicatrização rápida e efetiva por meio de biomateriais biologicamente ativos seja sempre ponderada. Características de biocompatibilidade com células indiferenciadas, de estímulos a fatores tróficos, tais como fatores de crescimento e proteínas morfogenéticas, os quais se encarregam de regenerar o tecido afetado, recuperando sua originalidade morfofuncional, ao se aliarem na busca por resultados melhores e mais adequado para o crescimento do tecido perdido aumentam a eficiência e benefícios de se usar um biomaterial em comparação a outro.

Assim, propor novos biomateriais, ou mesmo melhor conhecer a sequência biológica de respostas do hospedeiro, são relevantes neste cenário. Especificamente em relação ao tecido ósseo, esses biomateriais devem desempenhar diferentes funções: como osseointegração, no caso de implantes metálicos, como o titânio e ligas de Cobalto/Cromo; reabsorção, no caso de biomateriais cerâmicos com a capacidade de garantir o preenchimento de lesões extensas, garantindo que células utilizem sua estrutura como scaffold para preservar e garantir sua atividade (PIRES et al., 2015).

Tecidos ósseos e seus processos regenerativos

O tecido ósseo por muito tempo foi tido como apenas uma parte inorgânica dos vertebrados, sem muita relevância aos processos fisiológicos desenvolvidos, ou com relação a características endócrinas destes organismos. Atualmente, sabe-se que o osso é um tecido de extrema importância na garantia de homeostase do organismo através de comunicação efetiva com demais tecidos e células, tais como: tecido endotelial, tecido adiposo, células do sistema imune, células da medula, além de células cancerígenas, e também interferem em eventos hormonais (Van Der Eerden, 2014). A comunicação intra-tecidual e manutenção deste sistema ocorre entre os agentes modeladores do tecido ósseo, desempenhados por células específicas, osteoblastos, osteoclastos, osteócitos, pré-osteoblastos e células mesenquimais (BAHNEY et al., 2019). Estas células mantêm uma sincronia importante durante os mecanismos de osteogênese, atuante em eventos sequenciais durante o desenvolvimento de vertebrados, como, por exemplo, partindo de um molde cartilaginoso que, durante o processo de amadurecimento, é substituído gradualmente por tecido ósseo e por vasos sanguíneos que irão irrigar este novo tecido (Sivaraj et al., 2016).

Os vasos sanguíneos também desencadeiam respostas importantes em eventos de ossificação intra-membranosa (Fig.1). Importante notar que durante esses eventos morfogenéticos, o osso recebe uma vascularização significativa e essas células endoteliais tem sido salientadas por desempenharem relevância na osteogênese através de sinais angiócrinos. Além de seu papel convencional como um sistema de condução de gases, nutrientes, metabólitos ou células. Os vasos sanguíneos no sistema esquelético desempenham papéis ativos no controle de vários aspectos da formação óssea e fornecem

nichos para células-tronco hematopoiéticas que residem na medula óssea. Além disso, estudos recentes destacaram os papéis dos vasos sanguíneos durante a cicatrização óssea e relevância crucial na osteogênese (SCHOTT et al., 2021; ZHAO et al, 2020).

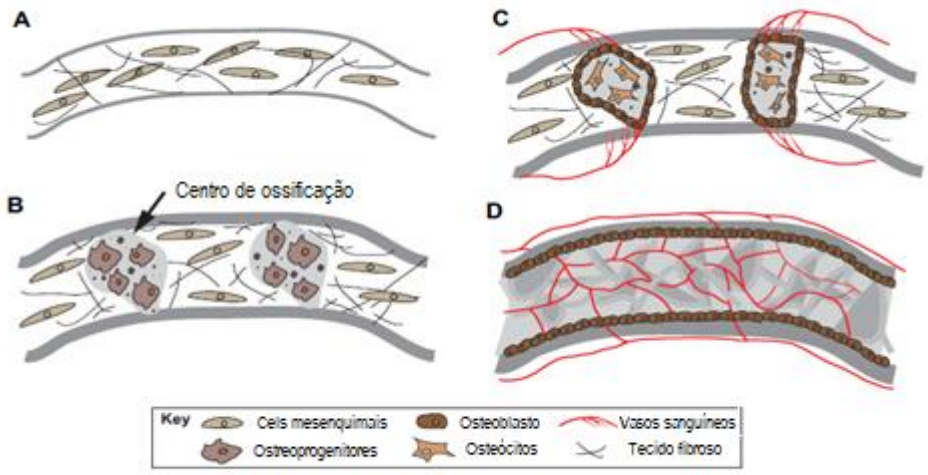

Fig.1. Angiogênese intramembranosa. As células mesenquimais se condensam para formar estruturas esponjosas (A) e se diferenciam em osteoprogenitores e osteoblastos, que secretam MEC e formam centros de ossificação e, por fim, osteócitos totalmente diferenciados (B). As proteínas da matriz e os fatores pró-angiogênicos gerados pelos centros de ossificação atraem os vasos sanguíneos (C). A vascularização subsequente do osso promove a osteogênese (D). Adaptado de Sivaraj at al, 2016.

Embora tenha propriedades importantes de recapitular eventos do desenvolvimento durante a regeneração de partes perdidas, o tecido ósseo não o faz em caso de grandes lesões, necessitando por tanto de intervenção cirúrgica e preenchimento de biomateriais (Chelsea, et al, 2019). Uma fratura óssea pode apresentar-se de diversas formas, desde fissuras no tecido, até uma separação total ou até fragmentação do osso, nos quais é necessária uma intervenção significativa para uma boa cicatrização (**Fig.2**).

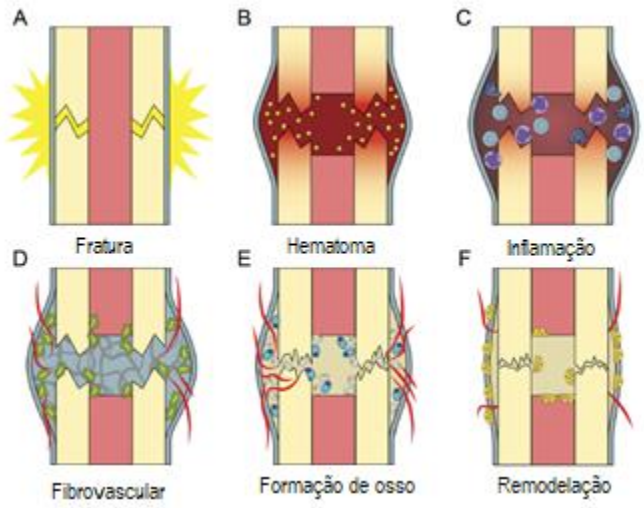

Fig.2. Regeneração de fraturas. Um coágulo se forma imediatamente fornecendo uma matriz provisória, aprisionando células e plaquetas, que desempenharão papel importante durante os mecanismos morfogenéticos subsequentes. A degranulação plaquetária libera quimiocinas e recrutam células mesenquimais e angiogênese. O osso é formado e passa por mecanismos de remodelação. Adaptado de Chiesa S. et al, 2019.

Em um caso de ferimento moderado, após a lesão, há o extravasamento do conteúdo dos vasos sanguíneos que foram rompidos e inicia-se sinalização inflamatória por meios de citocinas. Nestes mecanismos, as primeiras células que chegam na lesão são as de ação imunológica, os macrófagos, células T, células B, eosinófilos e neutrófilos em resposta à sinalização inicial, após esse período de instalação da inflamação outras células se apresentam para a recuperação do tecido, como os já citados osteoblastos, células mesenquimais, condrócitos e por fim osteoclastos (CHIESA et al.,2020). Nesta etapa também há uma maior sinalização angiogênica, o que resulta no recrutamento de vasos sanguíneos para este tecido em regeneração. A partir desta estrutura, o tecido ósseo começa a se regenerar graças à sincronia entre osteoblastos e células endoteliais, responsável pela estruturação e mineralização do tecido ósseo. Assim que o tecido é estabilizado e mineralizado, os osteoblastos atuam remodelando o osso para que este volte a ficar o mais parecido possível com o tecido original (Bahney, et al, 2019).

A vasculatura óssea desempenha um papel vital no desenvolvimento, remodelação e homeostase óssea. A formação de novos vasos sanguíneos é tão crucial durante o desenvolvimento ósseo primário quanto no reparo de fraturas em adultos. Tanto o reparo ósseo quanto a remodelação óssea envolvem a ativação e interação complexa entre as vias angiogênicas e osteogênicas (ZHAO et al., 2020). Curiosamente, estudos demonstraram que a angiogênese precede o início da osteogênese. De fato, o fluxo

sanguíneo reduzido ou inadequado tem sido associado à cicatrização prejudicada de fraturas e distúrbios de baixa massa óssea relacionados à velhice, como a osteoporose. Da mesma forma, a penetração lenta dos vasos sanguíneos do hospedeiro em grandes enxertos de tecido ósseo de engenharia tem sido citada como um dos principais obstáculos que ainda impedem as estratégias atuais de engenharia de construção óssea (Gemini-Piperni et. Al, 2014), como na figura 3. Assim, conhecer melhor o papel de células endoteliais em mecanismos de regeneração tecidual é de extrema importância no desenvolvimento de biomateriais cada vez mais biomiméticos e responsáveis pela melhor resposta biológica em mecanismos de regeneração e osseointegração.

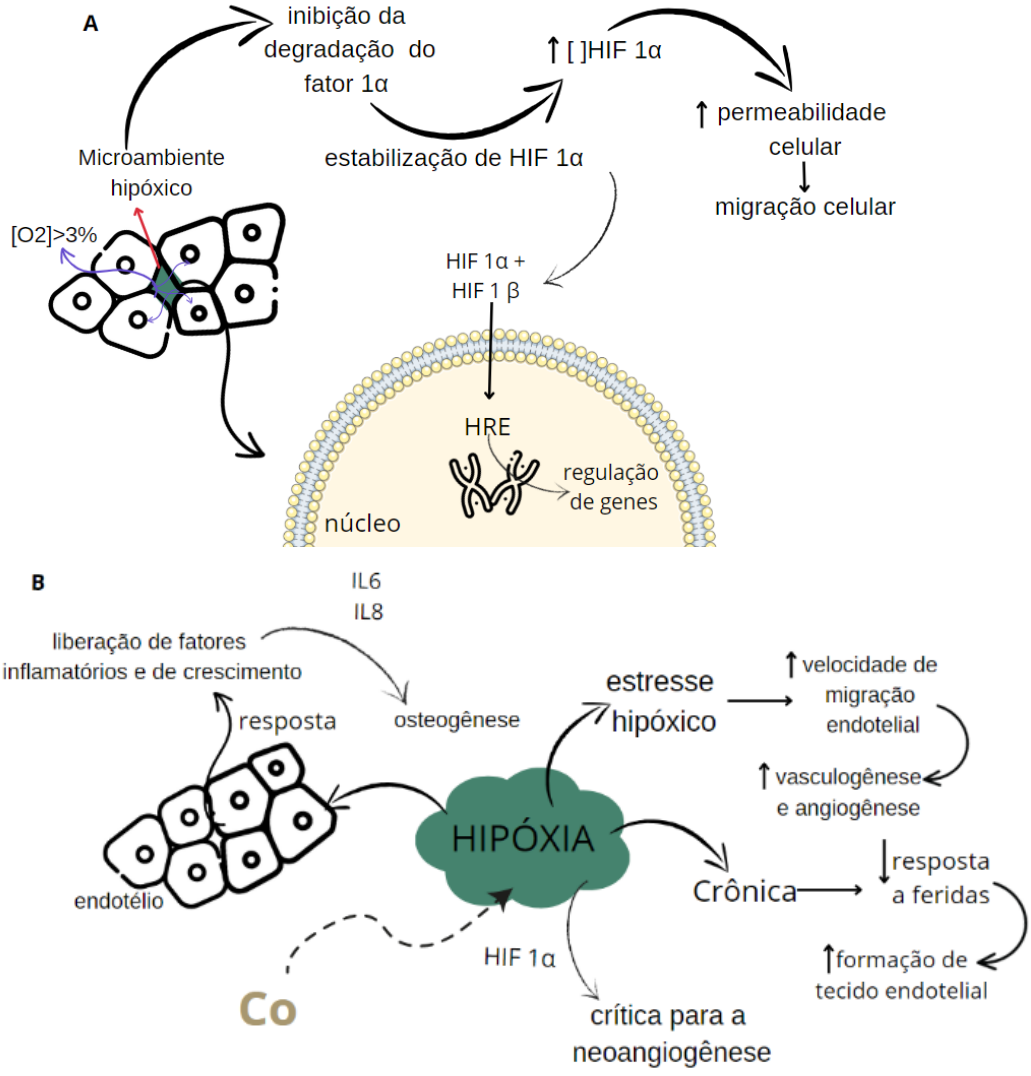

Figura 3. Esquema ilustrando as diferentes ações da hipóxia no tecido endotelial. A. micro ambiente hipóxico, e as respostas desencadeadas pelas células endoteliais. B. potencializações exercidas pela hipóxia.

Vascularização do osso, hipóxia e sua regeneração

Durante mecanismos biológicos de regeneração, a vascularização é primordial para guiar mecanismos celulares através de uma sinalização angiócrina e parácrina. Destaca-se neste contexto, a aceleração de mecanismos angiogênicos em resposta a hipóxia.

Certas características da superfície do biomaterial influenciam enormemente a atividade celular e a integração célula-material (Hamad et al., 2002) (p. ex, formação de componentes de adesão focal e organização do citoesqueleto com efeitos na maturação dos osteoblastos e subsequente mineralização). Especificamente, Zhao & Zhou (2016) observou que a incorporação do Cobalto induz uma expressão aumentada dos marcadores para angiogênese e osteogênese em células-tronco mesenquimais, e desta forma, conhecer os mecanismos moleculares envolvidos neste panorama faz-se necessário, visto que parâmetros adicionais agregam novas características durante o desenvolvimento de novos materiais ou superfícies, aumentando o desempenho do material, diminuição do tempo de reabilitação do paciente, etc.

Dentre esses processos envolvidos nas respostas celulares, a hipóxia parece ser uma importante via de sinalização intracelular, sobretudo por estar em destaque de modo bifásico, participando em processos ligados à osteogênese e angiogênese (Liu et al., 2017; Fercana et al., 2017), as células detectam microambientes hipóxicos através da inibição da degradação do fator 1α induzida por hipóxia (HIF-1α) e o subsequente aumento da sua concentração, conduzindo a alterações na sua morfologia e dinâmica. Tabata et al. (2019), demonstrou que a permeabilidade endotelial vascular aumentava quando a monocamada endotelial vascular era exposta a estresse hipóxico, ou seja, a migração endotelial e a angiogênese são influenciadas diretamente pela hipóxia cuja resposta principal é dada por Hypoxia-inducible factor-1 (HIF-1).

Quando em hipóxia a forma HIF-1α é estabilizado, levado ao núcleo, formando o heterodímero ativo de HIF-1 com HIF-1β. Posteriormente, o HIF-1 ativo se liga ao elemento de resposta à hipóxia (HRE) no DNA, regulando a expressão de genes-alvo, como o fator de crescimento endotelial vascular (VEGF) (Niu et al., 2018). Sob condições normóxicas, os membros da subunidade α, HIF-1α, HIF-2α e HIF-3α sofrem hidroxilação dependente de oxigênio, resultando em ubiquitinação e degradação pelo proteassoma (Saito et al.,2010).

A hipóxia é um importante sinal fisiológico que pode influenciar a diferenciação celular e garantir o aumento da oferta de oxigênio em locais privados de oxigênio. A hipóxia é o estado do sangue quando este está com uma baixíssima concentração de oxigênio (<3%), no tecido ou corrente sanguínea, que quando mantida desta forma por muito tempo gera estresse e morte celular, a hipóxia crônica diminui as respostas da cicatrização de feridas celulares, para evitar tal, a resposta tecidual é a tentativa de formar mais tecido epitelial o qual é induzido principalmente pelo HIF-1. O HIF-1 também é expresso durante a fase inflamatória inicial da cicatrização de feridas (Malda et al., 2007), chamada hipóxia local aguda (Saito et al.,2010; Laschke et al., 2006).

O estresse hipóxico aumenta a velocidade de migração das células endoteliais vasculares, sendo esta migração coletiva de células endoteliais vasculares fundamental para a vasculogênese e angiogênese, e para a manutenção da integridade da monocamada. A resposta da monocamada endotelial vascular exposta ao estresse hipóxico é a liberação de mediadores inflamatórios e fatores de crescimento (angiogênicas endoteliais vasculares) e por alterações na expressão gênica de acordo com o tempo de exposição (DETMAR, et Al., 1997). Segundo Niu et al. (2018) alguns dos resultados foram relatados de que a via do HIF-1α é um mediador crítico da neoangiogênese, que é necessária para a regeneração do esqueleto. Em outra pesquisa, de Deng et al. (2019) com biomateriais e hipóxia, o Cobalto foi utilizado como indutor da hipóxia para se explorar os mecanismos subjacentes na cicatrização e angiogênese no osso. A expressão de HIF-1a e VEGF aumentaram significativamente sob hipóxia (Tabata et al., 2019).

Niu et al. (2019) afirmam que seus resultados do estudo apoiam o conceito da regulação positiva de IL-6 e IL-8 mediada por hipóxia em células osteoblásticas humanas via HIF-1α, que pode promover a osteogênese, facilitando a angiogênese. Nos últimos anos, a manipulação terapêutica da via do HIF-1α para o tratamento de distúrbios metabólicos ósseos e cartilagens, como a osteoporose e artrites tem crescido frente as novas e velhas necessidades da ortopedia e implantologia (Lee et al.,2014; Gupta, N & Nizet, V., 2018). O uso de terapêuticas direcionadas a HIF indicam que esta não apenas facilita a neovascularização, mas também pode alterar o metabolismo dos tecidos-alvo para proteger contra a hipóxia.

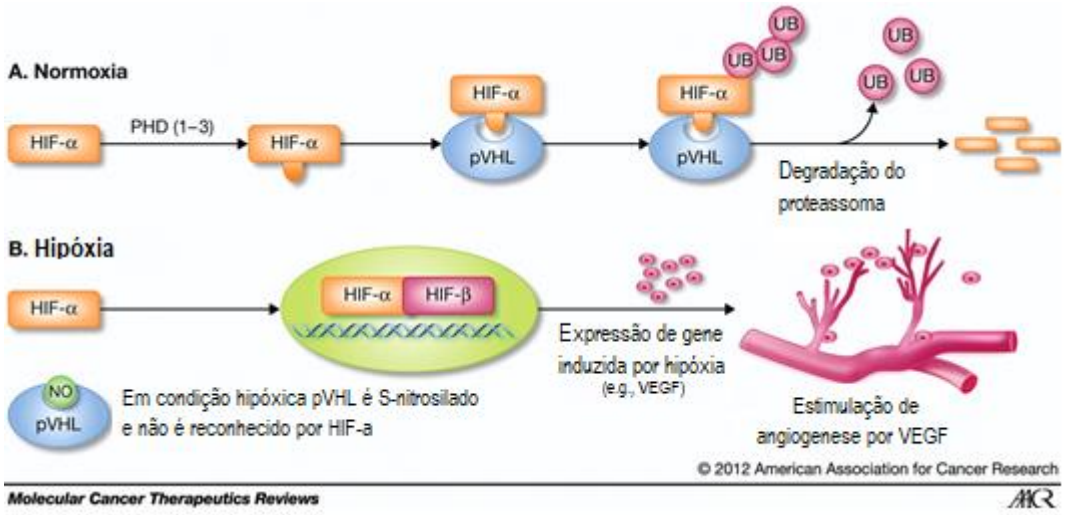

Figura 3. Mecanismos moleculares desencadeados pela normóxias (**A**) e hipóxia (**B**). Note a importância da via proteassômica envolvida na regulação da atividade de HIF. Adaptado de RAHIMI, 2012.

O mecanismo de reparo ósseo periimplante recapitula eventos de osteogênese, requerendo um repertório já conhecido de células e moléculas (RAHIMI, 2012). Dentre as células necessárias, as células endoteliais, regentes de processos angiogênicos, reveste-se de grande importância na regeneração do tecido, sobretudo pela sua capacidade de transportar moléculas tróficas e células. Além disso, nos últimos anos, trabalhos têm mostrado também relevância dos vasos sanguíneos na comunicação parácrina com células indiferenciadas, impactando seu processo de diferenciação osteoblástica, contribuindo com a formação do osso. Em outras palavras, as células endoteliais, embora ainda pouco estudadas neste cenário, contribuem com eventos de sucesso dos implantes e merecem maiores cuidados na investigação (SCHOTT, et al., 2021)

Assim, dada a importância do fenótipo de células endoteliais em processos de reparo cicatricial periimplante, o objetivo deste estudo foi melhor avaliar o envolvimento de vias de sobrevivência e proliferação celular, além de fatores indutores de hipóxia em resposta ao meio enriquecido com cobalto-cromo em células endoteliais dinamicamente respondendo ao shear-stress (mimetizando o fluxo sanguíneo).

2 - Especificamente, os objetivos foram:

1. Estabelecer curvas de viabilidade ao modelo proposto através de atividade mitocondrial;

2. Avaliar a expressão de genes envolvidos com o fenótipo das células endoteliais: VEGF, eNOS, VEGFR, VEGFR2;
3. Avaliar genes envolvidos com proliferação celular e remodelamento da Matriz extracelular;
4. Avaliar a expressão de HIF1;
5. Avaliar marcadores descritos nos itens anteriores após inibição químico de HIF1.

Desenho Experimental.

Como células endoteliais respondem indiretamente ao material implantado, decidimos avaliar este comportamento celular através do contato indireto, onde as células foram tratadas com o meio condicionado pelos materiais. Os discos (0,9 mm de diâmetro) foram incubados em meio de cultura adequados por 24 horas, a fim de estabelecermos o meio condicionado, como previsto pela ISO 10993-5:2016. Este meio condicionado contem moléculas/partículas potencialmente liberadas pelos materiais. O meio condicionado foi utilizado para tratar as células endoteliais, com o intuito de conhecermos sua citotoxicidade e capacidade de modular a expressão de genes envolvidos e seu fenótipo, sobretudo destacando mecanismos de hipóxia.

Obs. Os resultados obtidos estão apresentados em 2 manuscritos, dos quais 1 deles já se encontra publicado e outro em fase final de preparação para submissão (ver a seguir).

3 - **ARTIGO PUBLICADO**

COBALT-CHROMIUM-ENRICHED MEDIUM AMELIORATES SHEAR-STRESSED ENDOTHELIAL CELL PERFORMANCE

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Key words: Biomaterials; Implants; Cobalt; Chromium; Endothelial cell; Blood vessel.

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ABSTRACT

Angiogenesis is a relevant mechanism to be considered for the success of bone healing, even considering endosseous implantable devices, providing adequate delivery of substances necessaries for the cell viability and bone *de novo* deposition. Within of the repertory of metal-based implantable alloys, cobalt-chromium (CoCr) has emerged with very interesting properties for biomedical applications. Additionally, we have shown that released molecules from implants devices are able to modulate cells away and because that we hypothesized these released molecules might act on endothelial cells. In order to better address this issue, we investigated the effect of Co-Cr-enriched medium on endothelial cells (HUVECs), considering a biological model subjecting those cells to shear-stress to partially mimic the physiological environment and further allow investigating intracellular pathways responsible to drive cytoskeletal rearrangement, cell viability and extracellular matrix (ECM) remodeling processes. Considering the analysis of the metalloproteinases (MMPs) activities, our data indicates an intense ECM remodeling in response to CoCr-enriched medium suggesting some role on angiogenesis once ECM remodeling is prerequisite to cell growth. This was better addressed by revealing its involvement on modifying both mRNA expression and protein levels of members of the MAPK family. Additionally, the expression of CDK4 gene was modulated within the cell response to Co-Cr-enriched medium, while the modulation in the expression of P15 and P21 indicates an important regulatory mechanism required. Overall, our results demonstrate that trace of CoCr elements triggers decisive intracellular signaling in shear-stressed endothelial cells, suggesting influence on angiogenesis-related mechanism and they bring novel insights to explain the biological activity of CoCr as it has been emerged as interesting biomedical materials within the medical and dentistry field.

INTRODUCTION

As an alternative to implants within medicine and dentistry fields, titanium (Ti) alloys have been used for more than decades, but studies with other biomaterials such as the cobalt-chromium (Co-Cr) alloy have been performed due on their mechanical strength and also considering its biocompatibility when into human tissues and these both characteristics are of extreme importance for alternative biomaterials [1]. Taking these lessons into account, an ideal biomaterial should presents characteristics able to favor the adequate interaction with the host tissue and interfering on their osseointegration [2]. Thus, it sounds relevant to evaluate the cellular behavior in response to cobalt-chromium (Co-Cr), mainly considering the template of analysis widely used to define previously the biological properties of titanium alloys showing the cellular responses related to their growth and differentiation processes [3]. Additionally, it has been reported the cytotoxicity of cobalt on fibroblast and its ability to trigger inflammatory mediators [4], and because that the cicatrization peri-implant as well as the homeostasis from the host bone might be affected by the presence of these materials [5,6]. Thus, strategies are used to better assimilate the biomaterial to the host tissue, and e.g. nanotopography might facilitates the osseointegration process [3,7,8].

Tissue healing peri-implant requires hierarchical involvement of different types of cells, such as mesenchymal stem cells and immunological cells, which harmonically provides adequate environment to drive the osseointegration of those implantable devices. On this sense, it is already discussed that different cells equally respond differently to the biomaterial's surface [9,10]. In addition to the importance of the success of osseointegration, angiogenesis is relevant and needs to be better considered during bone healing surrounding the implanted devices providing prompt delivery of molecules necessaries for the bone *de novo* deposition [11,12]. Considering the endothelium tissue, the lumen of the blood vessels is constituted by a laminar monolayer of endothelial cells, which they are constantly exposed to blood flow forces [13], requiring responses to the mechanotension generated by the shear stress. On this context, endothelial cells paracrine respond for the tissue homeostasis and their relevance on osteogenesis has been addressed [14].

Over the last few years, we have shown an interesting repertory of intracellular molecules able to predict the viability and metabolism of bone cells [2,15–19], highlighting a metabolism map of specific signaling pathways cascade and reprogramming gene expression. Specifically to the cobalt-chromium alloys, we have previously shown that they

are able to release active molecules when in solution, which dynamically interacts with cells and triggers specific intracellular signals. Based on these results, we suggested cobalt-chromium able to affect and interfere on surrounding tissue [18], so it is probable that this cobalt-chromium-enriched medium might modulate endothelial cells as well and overall contribute with bone tissue healing at peri-implant region. However, there are few studies showing the effects of Co-Cr on the behavior of endothelial cells, even considering the importance of them during tissue healing process [10].

Importantly, endothelial cells have emerged presenting important modulatory role within tissue, mainly taking into account their capacity to respond to chemical mediators (cytokines, hormones, etcetera), as well as to hemodynamic forces generated by the blood flow itself as shear stress, triggering intracellular processes called "mechanosignaling" [20,21], maintaining their viability trough biochemical signal [22]. The mechanosignaling process arises sequential and hierarchical steps - initially cells receive physical forces or stimulus (shear-stress); sensing occurs via cellular structures, triggering signal transduction via signaling molecules; then a transmission and propagation of the signal occurs, when this activate "cellular receptors" and finally a physiological response [23]. Basically, endothelial shear stress (SS) is a force present tangentially to the luminal surface of the blood vessel and is generated when the blood flow acts on the endothelial cells and modulates their biological activity [24], involving activation of several cascades of biochemical and genetic signaling [25].

Considering endothelial cells, these processes are associated with the dynamics of rearrangement of the cytoskeleton [26,27], cell-cell junctions [28], focal adhesion platforms (such as the proteins FAK and Src) [29] and requires transmembrane proteins activation as well [30], culminating on their alignment and functions [31]. In this aspect, we have shown previously that a circuit of mechanical tensional forces differentially requires FAK, Src, as well as PI3K and AKT [32]. We have also suggested this pathway upstream to important mechanism related with endothelial cell viability, able to activate NOS2 and modulate VEGF expression. In order to better address the biological performance of endothelial cells challenged with CoCr-enriched medium, it is necessary consider a biological model where endothelial cells are also experimentally respond to shear-stress, mimicking physiological issues.

Based on the above-mentioned, our study evaluates the behavior of shear-stressed human umbilical vein endothelial cells (HUVEC) responding to cobalt-chromium-enriched

medium, investigating intracellular parameters responsible to drive cell viability, such as cytoskeletal rearrangement and ECM remodeling processes.

MATERIAL AND METHODS

CoCr alloys and reagents

The metallic CoCr-based discs were gently donated by the S.I.N. (São Paulo, SP, Brazil). Ripa buffer (R0278), Phosphatase inhibitor cocktail 2 (P5726), bovine serum albumin (A7906), gelatin (48723), triton X100 (9284) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gotaq qPCR master mix (A6002) was purchased from PROMEGA (Madison, Wisconsin, EUA). The following antibodies were purchased from Cell Signaling (Danvers, MA, USA): Cofilin (D59) Antibody (#3318, 19 kDa); PhosphoCofilin (Ser3) Antibody (#3311, 19 kDa); Src (#2109, 60 kDa); Akt Antibody (#9272, 69 kDa); and JNK (46,54 kDa) (Phospho-MAPK Family Antibody Sampler Kit #9910); GAPDH (14C10) Rabbit mAb (#2118, 37 kDa).

Cell line and culture conditions

Human Umbilical Vein Endothelial Cells (HUVECs; ATCC, Manassas, Virginia, USA) immortalized were maintained in RPMI, containing antibiotics (100U/mL penicillin, 100mg/mL streptomycin) and 10% (v/v) Fetal Bovine Serum (Nutricell, Campinas, SP, Brazil). Cells were maintained in an incubator at 37°C, 5% CO_2, and 95% humidity.

CoCr-enriched medium

In order to prepare the CoCr-enriched medium [both dual acid-etching (DAE) treating surface (named w/DAE) and the machined surfaces (named wo/DAE], the experimental alloys (n = 6) were incubated in cell culture media (RPMI) without FBS up to 24 hours at 37°C, 5% CO_2, and 95% humidity [0.2g/mL (w/v); ISO 10993:2016]. CoCr-enriched medium contains molecules released from those metallic alloys and might affect the biology of endothelial cells. To test this hypothesis, CoCr-enriched medium was further used to treat the endothelial cells before they are subjected (or not) to the mechanical forces of the shear-stress, as mentioned previously [21,32,33].

Shear Stress

The shear stress was applied in HUVECs previously seeded in the periphery area of modified 100-mm of diameter, where previously 60-mm culture dishes were bonded

onto the centered bottom of 100-mm culture dishes using medical silicone and thereafter the this modified dishes were sterilized using UV light for 15 min, as described by dela Paz [34]. The adherent cells were maintained in RPMI up to semi-confluence was reached, at 37°C, in a humidified atmosphere containing 5% CO_2. The CoCr-enriched medium was used to challenge monolayers of endothelial cells, which they were further subjected to orbital shear-stress up to 72 hours at 37˚C in the presence of CO_2 using an SK-O180-Pro Digital Orbital Shaker (SCILOGEX, Rocky Hill, CT, EUA). Shear stress protocols respected a rotation frequency of 100 rpm in according to the formula: $\tau_{max}=a\sqrt{\rho\eta}(2\pi f)$ 3, where ρ = density and η = viscosity of the culture media and a = orbital rotation radius. Considering our experimental condition, Pa·s and a = 12 cm. Hence, our rotation frequency yields stress levels that correspond those observed when considering physiological arterial pressure (6-40 dynes/cm²). HUVECs from the same passage, which were not subjected to shear stress, were kept in the same CO2 incubator and were considered as static control.

Western blotting

After 72 hours of subjecting to shear stress and responding to CoCr-enriched medium, the challenged HUVECs were washed in ice-cold PBS and protein extracts were obtained using a RIPA lysis buffer (Sigma Aldrich, St. Louis, Missouri, USA), supplemented with a cocktail of anti-proteases and anti-phosphatases (Sigma Aldrich, St. Louis, Missouri, USA) up to 1 h on ice. Protein extracts were cleared by centrifugation 14,000 rpm for 15 min at 4°C. The precipitate was then resuspended in 100 µL of RIPA lysis buffer (Sigma Aldrich, St. Louis, Missouri, USA). The protein extracts were clarified and the protein concentration determined by the Lowry method [35]. Proteins extracts were resolved by SDS-PAGE and later transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). An equal volume of gel loading buffer [100 mmol L^{-1} Tris–HCl (pH 6.8), 200 mmol L^{-1} dithiothreitol (DTT), 4% SDS, 0.1% bromophenol blue, and 20% glycerol] was added to the samples and boiled for 5 min at 95°C. Aliquots of the samples (75-100 µg/lane) were resolved into SDS-PAGE (8, 10 or 12% gels) and later transferred to PVDF membranes (Millipore, USA), which were blocked with 5% nonfat dry milk dissolved in Tris-Buffered Saline (TBS)-Tween-20 (0.05%) and then incubated overnight with appropriate primary antibody (1:1,000) at 4°C. After 1x-washing in TBS–Tween-20 (0.05%) and 2x-washing in TBS, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse IgGs antibodies (1:2,000),

diluted in blocking buffer for 1h. Immunoreactive bands were detected using Enhance Chemiluminescence (ECL, Pierce, USA).

mRNA isolation and RT-qPCR analysis

Challenged HUVECs were harvested properly and total mRNA isolated using Ambion TRIzol Reagent (Life Sciences − Fisher Scientific Inc., Walthan, MA, USA) and thereafter treated with DNase I (Invitrogen, Carls-band, CA, USA). cDNA synthesis was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real Time PCR was carried out in a total of 10 μL, containing PowerUpTM SYBRTM Green Master Mix 2x (5μL) (Applied Biosystems, Foster City, CA, USA), 0,4 μmol L^{-1} of each primer, 50 ng of cDNA and nuclease free H2O. Results were expressed as relative amounts of the transcripts using 3 internal reference genes (ß-Actin, GADPH and 18S genes) to better normalize the data obtained in the qPCR analysis (housekeeping gene), using the cycle threshold (ct) method. Primers and details are described in **Table 1**.

Zymography analyses

The proteolytic activities of both MMP-2 and MMP-9 presented in challenged HUVECs-conditioned medium were assayed by gelatin-based zymography, as described by Lefebvre [36]. Both static (control) and shear-stressed cultures were used to harvest those conditioned media, which they were after clarified by centrifugation 14,000 rpm for 15 min at 4°C, and stored at -20°C. In the moment of the analysis, the samples were quantified using the Lowry protein assay [35] and diluted in non-reducing buffer [0.1 mol L^{-1} Tris–HCl, pH 6.8, 20% (v/v) glycerol, 1% (w/v) SDS and 0.001% (w/v) bromophenol blue]. Equal amounts of protein (150 μg) were loaded onto SDS–polyacrylamide gel [10% (w/v) and 4% (w/v) gelatin]. MMPs renaturation was performed in 2% (v/v) Triton X-100 overnight followed by the incubation in specific buffer [50 mmol L^{-1} Tris–HCl and 10 mmol L^{-1} CaCl2 (pH 7.4)] at 37°C overnight. Afterwards, gels were stained with 0.5% (w/v) Coomassie blue G 250 overnight, washed in a 30% (v/v) methanol and 10% (v/v) glacial acetic acid solution until the bands appear and then analyzed using software ImageJ.

Statistical analyses

Results were represented as mean ± standard deviation (SD). The samples assumed a normal distribution with $p < 0.05$ considered statistically significant and $p <$

0.001 considered highly significant. In the experiment where there were > 2 groups, we used two-way ANOVA with multiple comparisons, in order to compare all pairs of groups. In this case, the significance level was considered when alpha = 0.05 (95% confidence interval). The software used was GraphPad Prism 7 (GraphPad Software, USA).

RESULTS

In order to better analyze the effect of cobalt-chromium surfaces (with or without DAE) on the shear-stressed endothelial cells, RT-qPCR and Western Blotting technologies were performed.

Firstly, intracellular pathways related with cell viability and survival was evaluated. The AKT (mRNA level) pattern was increased when endothelial cells were subjected to shear-stress (**Fig. 1a**). It seems shear-stress triggers survival signaling requiring the overexpression of AKT, when in response to CoCr, as well as FAK (mRNA level; **Fig. 1b**). Additionally, SRC mRNA profile was also responsive to CoCr, with positive interference of shear-stress condition, and presented a very similar pattern of expression to AKT (**Fig. 1c**). However, this immediate response to CoCr by reprogramming AKT and SRC genes did not reflect to the protein amount (**Fig. 1d-g**), suggesting any post-transcriptional mechanism to drive this response. Both of AKT and the Src proteins were higher in response to wo/DAE than others groups, but this profile was reduced considering shear stress.

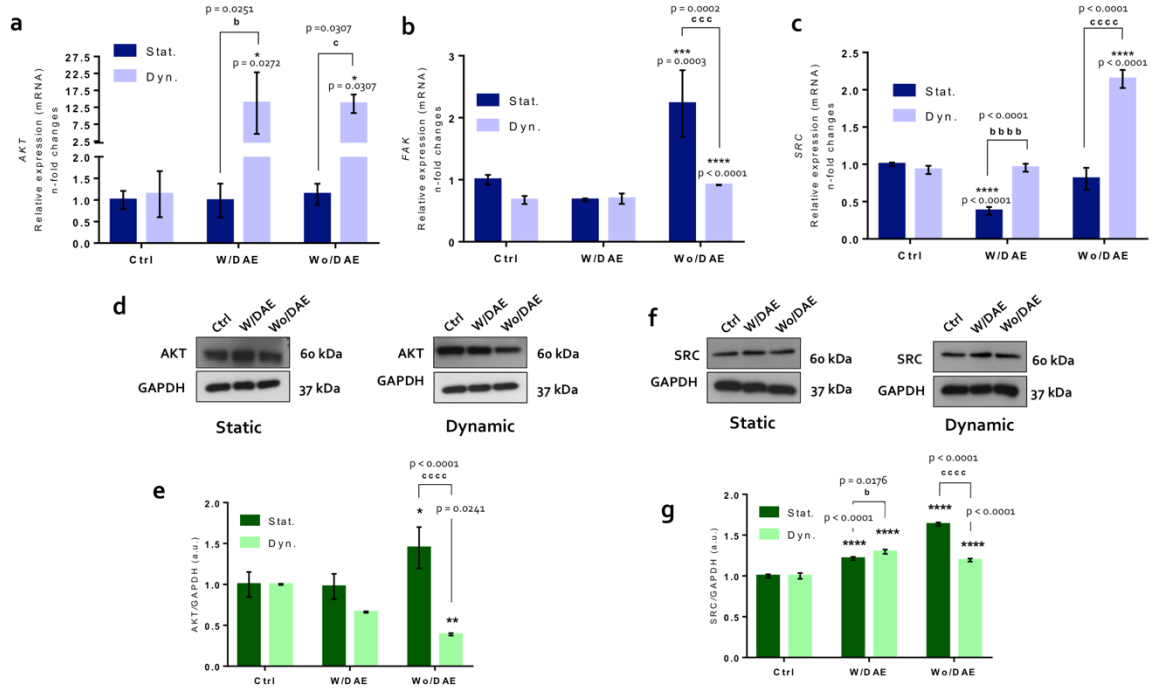

Fig. 1. Survival signaling. The survival panorama was evaluated firstly by qPCR technology, and the genes AKT (**a**), FAK (**b**) and SRC (**c**) were modulated by CoCr-enriched medium. The statistical analyses were performed using two-way ANOVA, with further Sidak's test for multiple comparisons. Significances were when $*p = 0.0272$ (W/DAE vs Dyn.), $*p = 0.0307$ (Wo/DAE vs Dyn.) for AKT; $***p = 0.0003$, $****p < 0.0001$ for FAK and $****p < 0.0001$ for SRC. Additionally, differences were also considered comparing the effects of different surfaces concomitant to shear-stress: $^{b}p=0.0251$ and $^{c}p=0.0307$ for AKT; $^{ccc}p = 0.0002$ for FAK and $^{bbbb}p < 0.0001$ and $^{cccc}p < 0.0001$ for SRC. Protein profile was also shown by using western blotting technology: AKT (**d,e**) and SRC (**f,g**). Representative blottings are displayed, and the graphs represent arbitrary values obtained by the densitometry of the bands, which they were normalized by the average values of the respective GADPH bands (loading control). $*p = 0.0241$; $**p = 0.0020$ for AKT and $****p < 0.0001$ for SRC. Moreover, significances were when $^{cccc}p<0.0001$ (AKT) and $^{b}p = 0.0176$, $^{cccc}p < 0.0001$ (SRC). To normalize the data obtained from qPCR technique, we have used 3 reference genes GAPDH, β-actin and 18S.

As cytoskeleton rearrangement seems to be involved with cell viability in response to biomaterials, we decided to evaluate the profile of cofilin phosphorylation at Ser03, and the ratio of Cofilin phosphorylation was assessed in endothelial cells responding to the different groups using Western Blotting technology. The phosphorylation of cofilin was very dynamic considering both parameters: surface of materials and shear stress. Once again, shear-stress compromises the response to the CoCr (**Fig. 2a-c**). Mechanistically, the **Fig.2d** depicts a probable intracellular pathway able to drive cofilin phosphorylation.

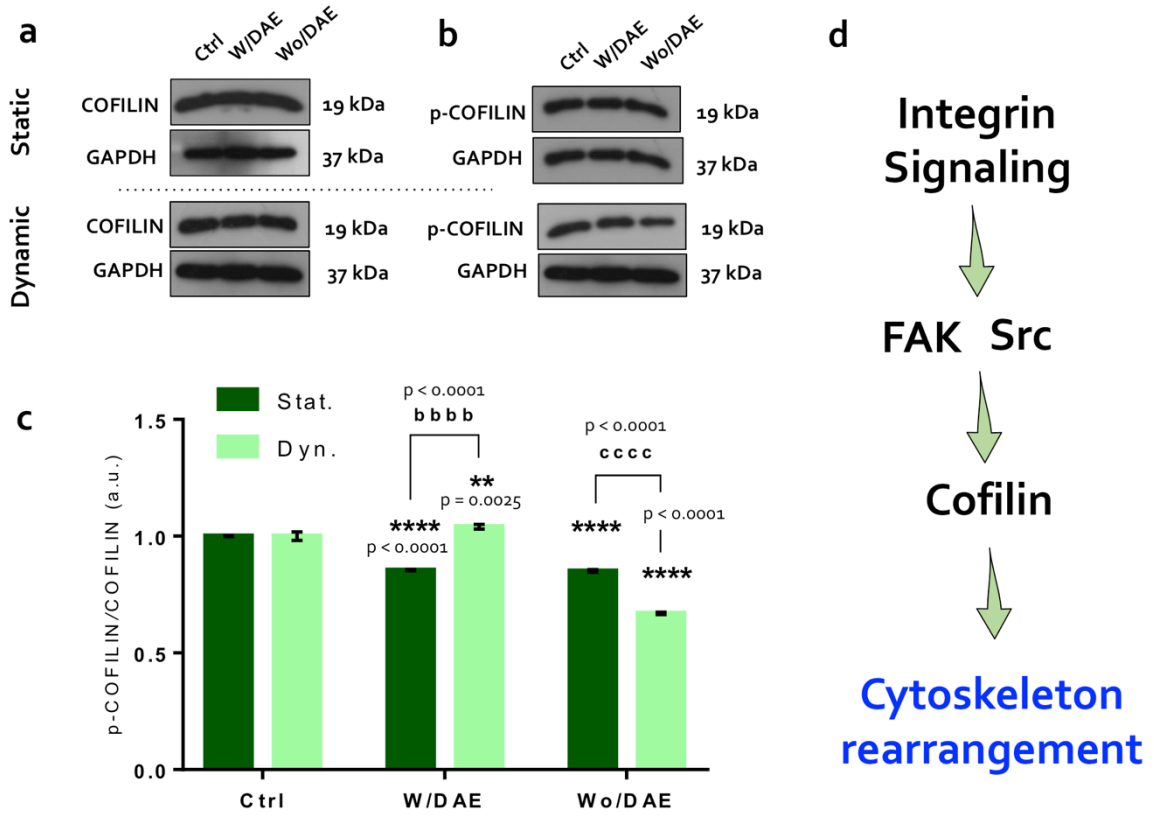

Fig. 2. Cytoskeleton dynamics. The phosphorylation of cofilin was evaluated in order to estimate cytoskeleton rearrangement dynamics: (**a**) cofilin, (**b**) p-cofilin and (**c**) representative blotting showing p-cofilin/cofilin ratio. Arbitrary values obtained by densitometry analyses of the bands are represented in the graphs. The letter "**d**" depicts on the signaling cascade required during cytoskeleton rearrangement. Significances were when: $^{**}p = 0.0025$; $^{****}p < 0.0001$. Results were represented as mean ± standard deviation of three independent experiments. The statistical analyses were performed by using two-way ANOVA, with further Sidak's test for multiple comparisons ($^{bbbb}p < 0.0001$ and $^{cccc}p < 0.0001$).

Thereafter, the MAPK reprogramming genes (P38, Erk1, JNK) were evaluated by RT-qPCR technology. Although our data shows significant changes on MAPK-P38 in response to the both w/DAE and wo/DAE (**Fig. 3a**), the presence of shear stress decreased this significance, suggesting an influence of autocrine mechanosignaling in response to the biomaterials. In addition, MAPK-ERK1 gene was significantly decreased in response to w/DAE and wo/DAE (**Fig. 3b**), here considering both static and dynamic conditions, and this pattern was also found for JNK (**Fig. 3c**). In relation to JNK, the amount of protein was also evaluated (**Fig. 3d**). Again, in both cases there are significances in the responses considering static and dynamic model, reinforcing the effect of shear-stress in this scenario. As it was evident the maintenance of survival signaling in response to the variations to

present CoCr materials, we decided to evaluate the reprogramming of genes related with cell cycle, looking for estimate mechanism involved with angiogenesis. Thus, the expressions of CDK4, P21 and P15 genes were also analyzed by RT-qPCR technology.

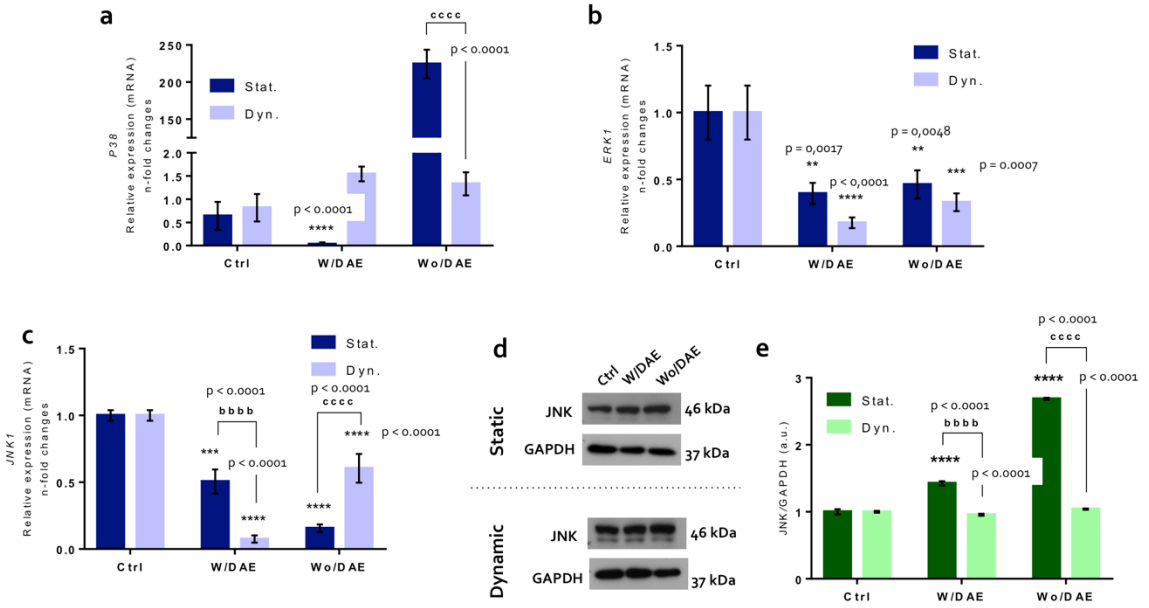

Fig. 3. MAPK member requirement in response to CoCr-enriched medium. Gene expression profile of MAPK signaling-related members [(**a**) P38, (**b**) ERK1 and (**c**) JNK1] was evaluated by RT-qPCR technology, and GAPDH, β-Actin and 18S were used as reference genes. Statistics were performed using two-way ANOVA, and Sidak's test for comparison the effects of different surfaces (w/DAE and wo/DAE) with shear-stress (p values are displayed in the graphs).

CDK4 mRNA pattern was modulated by the presenting surface modifications (**Fig. 4a**), as well as to the shear-stress condition. Regarding both genes related with the arrest of cell cycle, both p15 and p21 genes were reprogrammed in response to the CoCr, with also interference of shear-stress in this response (**Fig. 4b,c**; respectively).

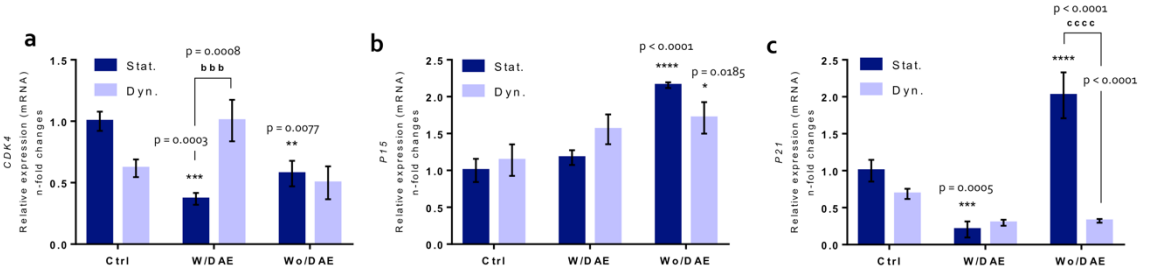

Fig. 4. Cell cycle members. RT-qPCR was performed to evaluate gene expression of cell cycle members: (**a**) CDK4, (**b**) P21 and (**c**) P15 genes were evaluated and the statistics

performed by two-way ANOVA with Sidak's test as a multiple comparison of effects of different surfaces with shear-stress. Significances were when compared to control group: "*", and when compared the effects of different surfaces (w/DAE and wo/DAE) with shear-stress (represented by the letters "b" or "c"). Technically, the data was normalized by 03 references genes: GAPDH, actin-β and 18S.

Considering the success of the endosseus implants ECM remodeling emerges as important pre-requisite to support bone *de novo* deposition and angiogenesis. In order to better address this issue, we evaluate the ECM remodeling in response to CoCr by investigating MMPs, at mRNA and activity levels, performing RT-qPCR and zymography respectively. Considering the transcriptional profile, MMP2 was significantly up-modulated in response to CoCr and sensible to the surfaces of the materials (**Fig. 5a**), as well as MMP9 (**Fig. 5b**). Complimentarily, we have also addressed the analysis of negative modulators of MMP's activity: TIMP1 (**Fig. 5c**), TIMP2 (**Fig. 5d**), and RECK (**Fig. 5e**). Important to report that all of them were differentially modulated respecting the surfaces of the materials, with a significant increase when they were compared with the control. Moreover, shear-stress exerts crucial relevance on the reprogramming of those genes activation (**Fig. 5**). Lastly, we have investigated whether collagen (col1A1) was modulated in response to CoCr and our qPCR data shows there is a significant up-expression of Col1A1 in response to wo/CoCr associated with shear-stress (**Fig. 5f**).

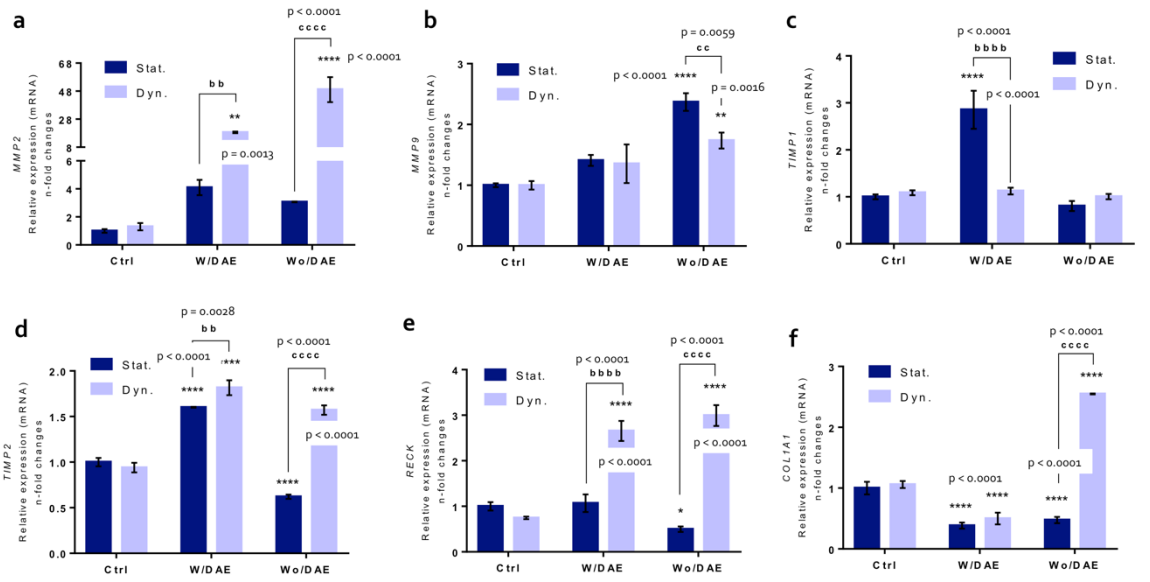

Fig. 5. Extracellular matrix remodeling. The extracellular remodeling was analyzed using RT-qPCR and zymography. (**a**) MMP2, (**b**) MMP9, (**c**) TIMP1, (**d**) TIMP2, (**e**) RECK and (**d**) COL1A1 mRNA profile expressions were evaluated and two-way ANOVA was performed to evaluate the significance of the data. Sidak's multiple comparison was

also performed. Statistics: "*" shows the significances when the test groups were compared with the control, and letters "b" and "c" mean significances between different surfaces (w/DAE and wo/DAE) and shear-stress.

To better conclude on ECM remodeling in this context, we further analyzed the activity of MMP2 and MMP9 by performing gelatin-based zymography and the **Fig. 6** brings an overall analysis, highlighting the activities of proMMPs and MMP at the active conformation (base on the molecular weight). Our data shows a differential activity of both MMPs2 (**Fig. 6a,b,e,f**) and 9 (**Fig. 6a-d**) comparing both surfaces investigated, it being increased on both cases when the static condition was considered; conversely, this activity was significantly down-modulated by shear-stressed endothelial cells. From our data it is clear that shear-stress signaling decrease the activities of MMPs in response to CoCr.

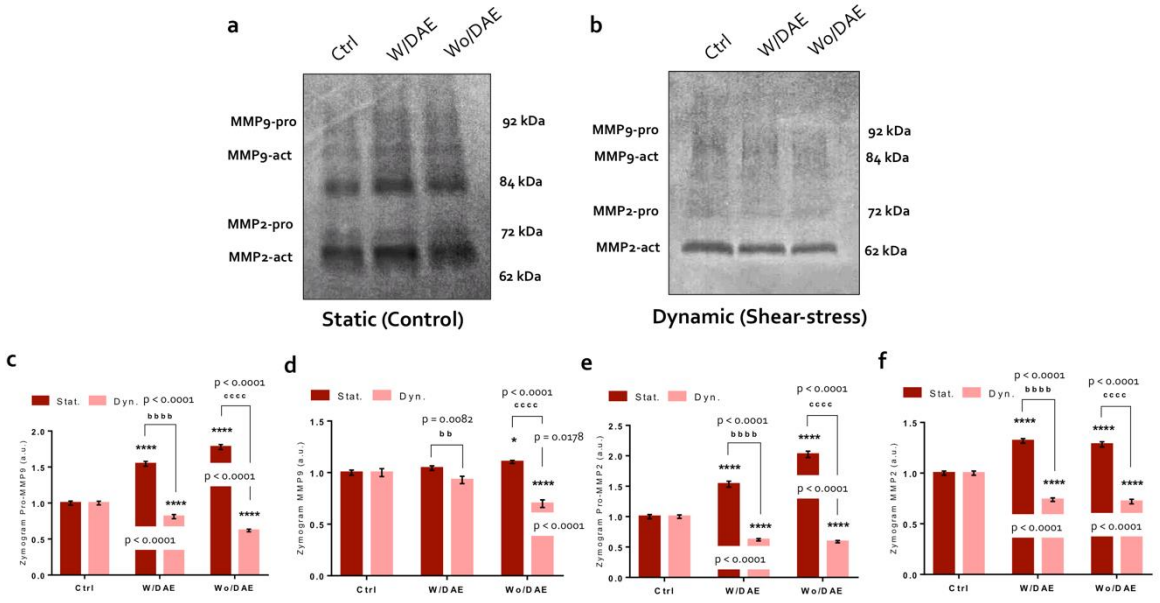

Fig. 6. Metalloproteinases activities were evaluated using Zymography. The activities of both MMP2 and MMP9 were analyzed using gelatin-containing gels. The zymogram depicting differences in (pro-)MMP-2 and (pro-)MMP-9 were shown (**a**, **b**; static and dynamic respectively). As detailed in material and methods, after activation step, the gel was stained with Coomassie blue by the methods outlined here. Two-way ANOVA with Sidak's multiple comparison test was used to compare the effects of different surfaces and shear-stress. The "*" shows the significance when compared to control group and the letters "b" and "c" show the statistical analyses when compared the effects of different surfaces (w/DAE and wo/DAE) with shear-stress.

The **Fig.7** summarizes the mechanism suggested by the data obtained in this study. It is possible to suggest that survival signaling drives proliferative phenotype, as well as the ECM remodeling, in response to CoCr.

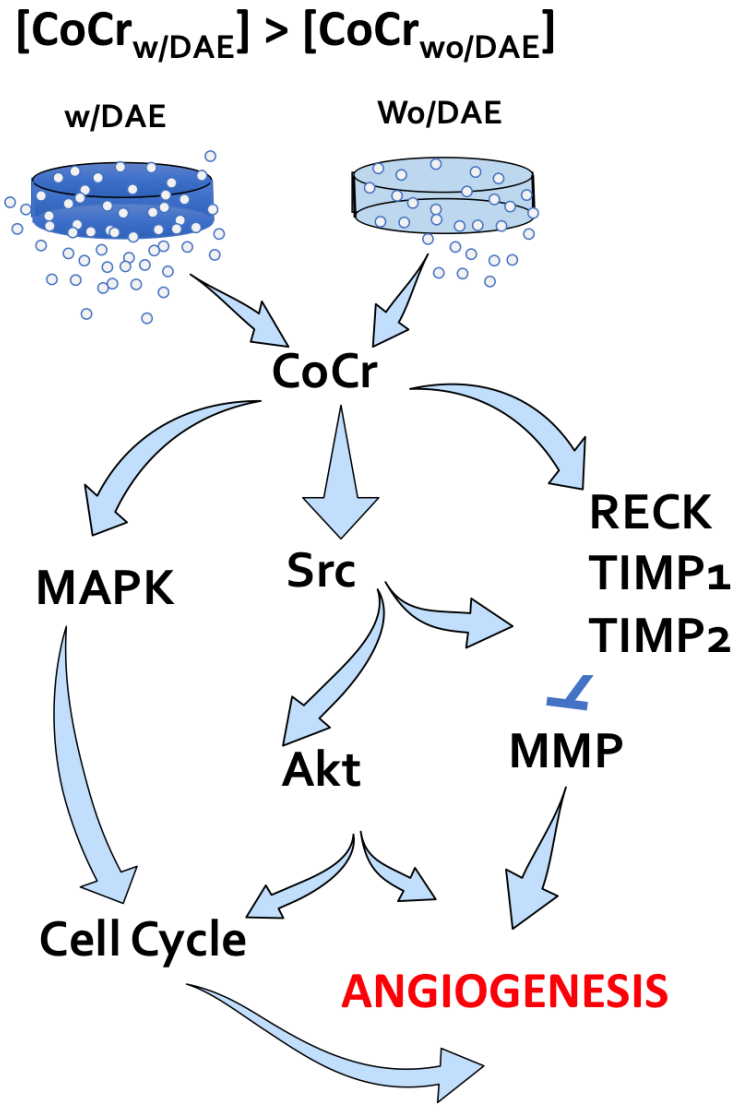

Fig.7. Schematization of the main data obtained in this study. The trace of released CoCr triggers intracellular mechanism in endothelial cell requiring activation of MAPK pathway, Src, AKT, and these upstream members drive the signaling involved with cell cycle and ECM remodeling. Taken these data into account, it is possible to suggest an involvement of angiogenesis in response to CoCr.

DISCUSSION

Biomedical materials development is placed in an interdisciplinary field, which brings together engineering, physical-chemical and biological aspects, looking for accelerating the recovery of patient. Historically, titanium materials are considered the gold standard

when looking for metal-based biomaterials focused on bone tissue repair in dentistry and medicine [3,15]. Despite the unquestionable biocompatibility of titanium, efforts have been worldwide focused on reducing the recovering time of the patients by enhancing the performance of biomaterial-related osseointegration [3]. In this sense, the proposal of novel biomaterials to be applied in biomedical field is very welcome, as well as modification of their surfaces are also relevant alternatives to enhance their performance when interacting with surrounding host tissue [3]. Based on this matter, other biomaterials than titanium have been proposed and CoCr and Zirconia have been emerged presenting important physicochemical and biological properties [16,37]. Although some progress is already reached in pre-clinical experimentation, there is a lack on understanding their effects on endothelial cell performance.

Recently, besides to the classical role of blood vessels in supporting cell metabolism away by supplying them with nutrients, there is now emerging evidences highlighting their cross-talk with bone cells [14]. Thus, considering the effect of biomaterials on endothelial cell metabolism and response seems being necessary, mainly when those biomaterials are proposed for bone injuries. In order to better address this issue, this study brings an experimental design considering the effect of CoCr-enriched medium on endothelial cell performance; these endothelial cells were also subjected to shear-stress to better mimic physiological condition of vascular biology.

Firstly, we have focused on evaluating the effect of CoCr on endothelial cell viability and survival pathway. Our data shows some effect on SRC and FAK, proteins involved in the integrin-based signaling pathway, which we have previously demonstrated being crucial to drive cell adhesion onto biomaterials surfaces [38]. Additionally, this involvement of Src seems to be important on participating with Akt pathway, a kinase responsible to the regulation of several central biological processes such as proliferation, survival, angiogenesis, among others [39]. Classically, changes in the levels of these proteins trigger signaling pathways related with cytoskeletal rearrangement, linking cytoskeleton proteins with cell survival processes [38,40,41]. Thus, to validate this hypothesis, we further evaluated the pattern of cofilin phosphorylation (at Serine 3). To date, cofilin is a cytoskeletal-related protein able to drive actin rearrangement by their capacity to interact with either actin monomers or F-actin according to the level of phosphorylation at the residue S3 [41,42]. Considering our experimental model, cofilin phosphorylation was sensible to the both surfaces evaluated here and this suggests an

adaptation of the endothelial cytoskeleton when in response to those modifications of CoCr surfaces.

Moreover, our data demonstrates that the interaction between surface modification and shear-stress are decisive external parameters to modulate gene expression and protein levels of Src, FAK and AKT, and of course driving endothelial cell viability. This mechanism also involves a dynamic participation of MAPKs in response to CoCr-enriched medium. To date, MAPK signaling is classically reported on driving intracellular signaling [43,44] and has been related with responses to different surfaces such as titanium, suggesting as a crucial step for cell survival and proliferation [45,46]. In conjunction, this set of results found corroborates with our previous published data, where we showed the effect of CoCr on osteoblasts and fibroblasts [16]. As experimental design, we have considered only an indirect effect of the surfaces on the cell metabolism, and the differential response of them to the variations on the surfaces of the materials (W/DAE and Wo/DAE) might be explained by the differential concentration of released elements to the extracellular compartment (here named CoCr-enriched medium); in fact, before treating the cells with, Fernandes et al (2018) measured the content of cobalt and chromium in the CoCr-enriched medium and the results surprisingly showed there is a significant increase on the amount of these released elements, quantified by graphite furnace technology [16].

Taken into account this panorama involving mechanism related with cell viability and survival, this prompted us to investigate the possibility of this CoCr-enriched medium in modulating endothelial cell growth. Considering the activation of CDK4 gene, our data shows that while CoCr-enriched medium seems decrease in a half the proliferative stimulus in the static condition, shear-stressed endothelial cells maintained it at a very similar profile of the control. In addition, this proliferating phenotype of endothelial cells in response to CoCr-enriched medium seems being modulated by p15 and p21 proteins [47,48], which they were found in this response. It is clear that our decision in considering shear-stressed cells was decisive to understand the real effect on endothelial performance, mainly because endothelial cells are considerate mechanosensitives and develop responses based on this phenotype. Importantly, the proliferating phenotype of endothelial cells is found in mechanism of angiogenesis and the modulation of this process is expected for the success of osseointegration of implants within host tissue.

Finally, we analyzed the remodeling of the extracellular matrix by shear-stressed endothelial cell in response to CoCr-enriched medium, once ECM remodeling is reported as a crucial mechanism by favoring cell adhesion and viability during interaction with

different biomaterials [19,37,49,50]. Importantly, our data report a significant and dynamic reprogramming of ECM remodeling-related gene and it seems there is an adequate balance of MMPs and their inhibitors genes. Again, the shear-stressed endothelial cells respond to the CoCr at differential manner. It is known that shear-stress is reported as a factor provoking ECM remodeling in endothelial cells [51]. Conversely, the CoCr promoted an increase on the MMP activity by static endothelial cells, while this pattern was significantly decreased in concomitant response to the shear-stress and this finding strongly suggest that CoCr does not exerts a direct effect on the MMP's activity. Overall in this case, it is expected that shear-stressed endothelial cells responding to CoCr present a reprogram of MMP's inhibitors responsible to this balance of their activities, as it can be observed in the gene expression of RECK, TIMP1, and TIMP2.

Altogether, these results show that there is a fine control of ECM remodeling in response to CoCr, and the tensional forces of shear-stress drive this mechanism and this condition might reflects on angiogenesis. However, this mechanism requires novel strategies of analysis, maybe changing the intensity of these tensional forces, as it is found in the whole body.

Overall, our results demonstrate that CoCr-enriched medium affects shear-stressed endothelial cells favoring cellular mechanism required to angiogenesis and they bring the molecular basis to explain the biological relevance of CoCr as a promising biomedical device (**Fig.7**). It is important to note that despite the wide knowledge about the influence of implants surface on bone cells, very little advance has been achieved on their effects considering endothelial cells. Additionally, the biological model used in this study is also something new in the field of biomaterials development and the consideration of shear-stressed cells guarantees a better mimicking of physiological responses and should be considered in further studies focusing on better understand the biological behavior in response to biomaterials.

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4 - Manuscrito Submetido

Hypoxia modulates the phenotype of mechanically stressed endothelial cells responding to CoCr-enriched medium

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Key words: Biomaterials; Implants; Cobalt; Chromium; Endothelial cell; Blood vessel; Hypoxia.

ABSTRACT

Given the importance of the endothelial cell phenotype in dental peri-implant healing processes, the aim of this study was to better assess the involvement of endothelial cells responding to cobalt-chromium (CoCr)-enriched medium. Biologically, cobalt is widely used molecule to induce chemical experimental hypoxia because it stabilizes hypoxia inducible factors (HIF1α). The aplication of hypoxia models provides better experimental condition to allow its impact on cellular metabolism, by looking for biochemical and molecular issues. Thus, this study looks for understaing whether CoCr-based materials are able to modulate endothelial cells considering the hypoxic effect prmoted by cobalt. Firstly, our data shows there is a siginificant effect on endothelial phenotype by modulating the expression of VEGF and eNOS genes, whith low requirement of genes related with proteasome intracellular complex. Importantly, the data were validated using classical chemical modulators of hypoxia signaling [chrysin (*5,7*-dihydroxyflavone) and Dimethyloxalylglycine (DMOG)] in functional assays. Altogether, these data validate the hypothesis that hipoxya is important to maintain the phenotype of endothelial cells, and it is properly interesting during the tissue regeneration surrounding implants and so compromising osseointegration process. Finally, it is important to mention that the cobalt released from CoCr devices might contribute with an sufficient microenvironment surrounding implanted devices and it paviments new roads looking for more bioactive surfaces of implantable materials in human health.

Key words: Biomaterials; Implants; Cobalt; Chromium; Endothelial cell; Blood vessel; Hypoxia.

INTRODUCTION

Over the last few years bone has been known as endocrine tissue (Karsenty and Kousteni 2019; Zofkova 2015) by modulating cells and tissues away coordinated by bioactive molecules and it is extremely important in guaranteeing homeostasis of the organism through effective communication among tissues and organs in vertebrates; mostly of those evets comes from mineralized tissue remodeling performed by osteoblasts, osteoclasts, osteocytes, pre-osteoblasts and mesenchymal stem cells (Fu et al. 2007). These cells maintain an important synchrony each other since organogenesis until adult tissue remodeling where blood vessels perform important and coupled role (Kusumbe, Ramasamy, and Adams 2014).

Considering the bone tissue biology as well as its mechanism of regeneration, cells composing blood vessels trigger important responses in intramembranous ossification events and it is important to note that bone receives significant vascularization and it is the motivation on endothelial cells have been proposed being an important link to osteogenesis by releasing angiocrine signals (Collin-Osdoby 1994; Yang et al. 2012; Hu and Olsen 2017). Although playing well-known properties as serving a system for gases, nutrients, metabolites, and cells, blood vessels also plays active roles in controlling various aspects of bone formation and provide niches for hematopoietic stem cells (Collin-Osdoby 1994). Furthermore, recent studies have described the role of blood vessels during bone healing by evidencing their crucial function in osteogenesis (Zhao and Xie 2020; Schott, Friend, and Stegemann 2021).

Specifically, during wound healing as well as osseointegration of dental implants, endothelial cells are important players mainly because they release trophic molecules and contribute with the migration of osteogenic cells (Machado et al. 2019; Martins et al. 2021; da Costa Fernandes et al. 2021; Pinto et al. 2022). In addition, in recent years, studies have also shown the relevance of blood vessels in paracrine communication with undifferentiated cells, impacting their process of osteoblastic differentiation, contributing to bone formation and healing (da Costa Fernandes et al. 2021). However, the ability of endothelial cells to recapitulate already known bone formation steps is barely comprehended.

Thus, given the importance of the endothelial cell phenotype in dental peri-implant healing processes, the aim of this study was to better assess the involvement of endothelial cells responding to cobalt-chromium (CoCr)-enriched medium, an alloy presenting high strength, temperature endurance and wear resistance (Mavrogenis, Papagelopoulos, and Babis 2011; Vaicelyte et al. 2020). Biologically, cobalt is one of the most commonly used molecule to induce chemical experimental hypoxia because it stabilizes hypoxia inducible

factors 1α and 2α (HIF1α and HIF 2α) (Muñoz-Sánchez and Chánez-Cárdenas 2019). The aplication of hypoxia models provides an experimental condition to allow the characterization of the hypoxia-induced cellular responses. Summarizing, our data validate the hypothesis that hipoxya is important to maintain the phenotype of endothelial cells, and it is properly interesting during the tissue regenration surrounding CoCr-based dental implants and so compromising osseointegration.

MATERIAL & METHODS

CoCr alloys and reagents

CoCr-based discs were gently donated by the S.I.N. (São Paulo, SP, Brazil). Gotaq qPCR master mix (A6002) was purchased from PROMEGA (Madison, Wisconsin, EUA).

Cell line and culture conditions

Human Umbilical Vein Endothelial Cells (HUVECs; ATCC, Manassas, Virginia, USA) immortalized were maintained in RPMI, containing antibiotics (100U/mL penicillin, 100 mg/mL streptomycin) and 10% (v/v) Fetal Bovine Serum (Nutricell, Campinas, SP, Brazil). Cells were maintained in an incubator at 37 °C, 5% CO_2, and 95% humidity.

CoCr-enriched medium and treatments

In order to prepare the CoCr-enriched medium [both dual acid etching (DAE) treating surface (named w/DAE) and the machined surfaces (named wo/DAE], the experimental alloys (n=6) were incubated in cell culture media (RPMI) without FBS up to 24 h at 37 °C, 5% CO2, and 95% humidity [0.2 g/mL (w/v); ISO 10993:2016]. Co-Cr enriched medium contains molecules released from those metallic alloys and might affect the biology of endothelial cells.

For the treatment of HIF-1α agonist (DMOG) and antagonist (Chrysin), the concentration of 50 μM of each was used. After semiconfluence of the dishes, the medium used by the cells was replaced by the medium with the Hif-1α agonist or antagonist, depending on the group, for 72 hours. After this period, the cells were collected for analysis.

Shear stress mimicking blood flow

The shear stress was applied in HUVECs which were seeded on a modified culture dishes [(the periphery area of the modified 100-mm of diameter dish, where previously 60- mm culture dishes were bonded onto the centered bottom of 100-mm culture dishes using medical silicone and thereafter the modified dishes were sterilized using UV light for 15 min

(La Paz, et al.)]. The adherent cells were maintained in RPMI up to semiconfluence was reached, at 37 °C, in a humidified atmosphere containing 5% CO2. The CoCr-enriched medium and the treatment mediums were used to challenge monolayers of endothelial cells, which they were further subjected to orbital shear-stress up to 72 h at 37 °C in the presence of CO_2 using an SK-O180-Pro Digital Orbital Shaker (SCILOGEX, Rocky Hill, CT, EUA). Shear stress protocols respected a rotation frequency of 100 rpm in according to the formula: $\tau max = a\sqrt{\rho\eta(2\pi f)}$ 3, where ρ = density and η = viscosity of the culture media and a=orbital rotation radius. Considering our experimental condition, Pa·s and a=12 cm. Hence, our rotation frequency yields stress levels that correspond to those observed when considering physiological arterial pressure (6-40 dynes/cm²). HUVECs from the same passage, which were not subjected to shear stress, were kept in the same CO_2 incubator and were considered as static control.

mRNA isolation and qPCR technology

Mechanical stressed HUVECs were harvested properly and total mRNA isolated using Ambion TRIzol Reagent (Life Sciences–Fisher Scientific Inc., Walthan, MA, USA) and thereafter treated with DNase I (Invitrogen, Carls-band, CA, USA). cDNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real Time PCR was carried out in a total of 10 µL, containing PowerUpTM SYBRTM Green Master Mix 2x (5 µL) (Applied Biosystems, Foster City, CA, USA), 0,4 µmol L−1 of each primer, 200 ng of cDNA and nuclease free H_2O. Results were expressed as relative amounts of the transcripts using 3 internal reference genes (ß-Actin, GADPH and 18S genes) to better normalize the data obtained in the qPCR analysis (housekeeping gene), using the cycle threshold (ct) method. Primers and details are described in **Table 1**.

TABLE 1. Expression primers sequences and polymerase chain reaction cycle conditions

Gene	Primer	5'-3' Sequence	Reaction's Conditions
HiF1a	Forward	CATAAAGTCTGCAAXATGGAAGGT	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	ATTTGATGGGTGAGGAATGGGTT	
HSP70	Forward	CATTGTGCTCTAAAGCCGCC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	AATCCTGTAGGCCACTGCAC	
HSP40	Forward	GAAACCAAGGTAAGCGACGG	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGTCATGTGGATGCTGCCTT	
PSMB2	Forward	AGTACCTCATCGATCATGACAAG	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TTCGAACACTGAAGGTTGGC	
PSMB1	Forward	GAGACTTGGGGATGGAACCG	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GTCTCCATGAAAACCGCTGC	
PSMA2	Forward	CCCCGTCCGTGGGAATTAAA	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	ATGGACGAACACCACCTGAC	
UBX1	Forward	CCTCCTTCTCGTCACACACC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GGCAGCAGAACCAGATCCTT	
VEGF	Forward	TGCAGATTATGCGGATCAAACC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGCATTCACATTTGTTGTGCTGTAG	
VEGFR1	Forward	CAGGCCCAGTTTCTGCCATT	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TTCCAGCTCAGCGTGGTCGTA	
VEGFR2	Forward	CCAGCAAAAGCAGGGAGTCTGT	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGTCTGTGTCATCGGAGTGATATCC	
eNOS (NOS3)	Forward	TATTTGATGCTCGGGACTGC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	AAGATTGCCTCGGTTTGTTG	
β-ACTIN	Forward	ACAGAGCCTCGCCTTTGC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	GCGGCGATATCATCATCC	
GAPDH	Forward	AGGCCGGTGCTGAGTATGTC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGCCTGCTTC ACCACCTTCT	
18S	Forward	CGGACAGGATTGACAGATTGATAGC	95°C - 15s; 60°C - 30s; 72°C - 30s
	Reverse	TGCCAGAGTCTCGTTCGTTATCG	

Statistical analyses

Results were represented as mean ± standard deviation (SD). The samples assumed a normal distribution with $p < 0.05$ considered statistically significant and $p < 0.001$ considered highly significant. In the experiment where there were>2 groups, we used two-way ANOVA with multiple comparisons, in order to compare all pairs of groups. In this case, the significance level was considered when alpha=0.05 (95% confidence interval). The software used was GraphPad Prism 7 (GraphPad Software, USA).

RESULTS & DISCUSSION

The **Fig.1** brings CoCr-enriched medium significantly increases the involvement of genes related with endothelial cell phenotype such as eNOS (**a;** ~6-fold changes – w/DAE) and VEGF (**b;** ~3-fold changes - w/DAE) in shear-stressed endothelial cells, while ubiquitin gene was down-regulated (**c**), suggesting a low involvement of proteasome in those cells and it corroborates with the **Fig.2** which show a low involvemnet of genes related with the expresison of proteins involved with the assembling of proetasome complexes, except PSMA2 gene which seems be involved (**Fig.2a**), maybe in response to the hypoxia condition imposed by the cobalt.

Importantly, the **Fig.1d** shows there is a significant stimulus of the CoCr-enriched medium in providing a hypoxia microenvironment (~5-fold changes - w/DAE). It is widely discussed that cobalt chloride ($CoCl_2$) imitates hypoxia *in vitro* by stabilizing hypoxia-inducible factor-alpha (HIF-1α), which is the master regulator in the cellular adaptive response to hypoxia and it opens new perspectives to better investigate the effect of the hypoxia condition on driving endothelial cell pehneotype in response to CoCr.

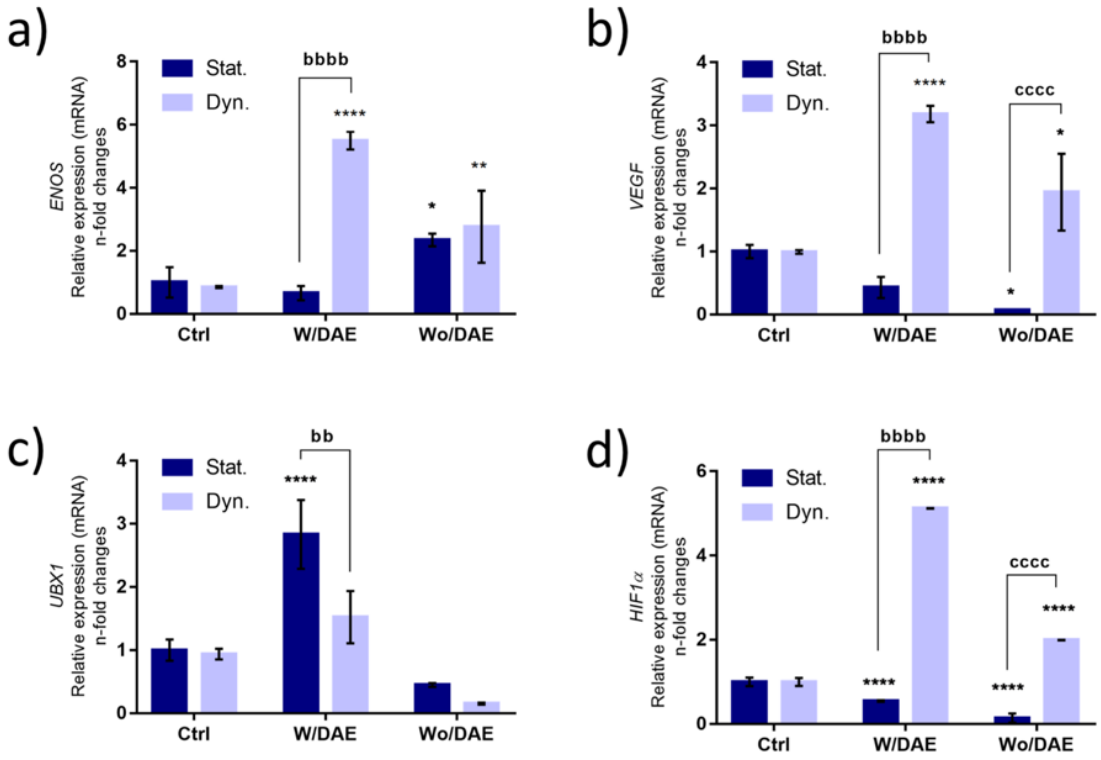

Fig.1. ***CoCr-enriched medium significantly promotes the activation of genes related to the endothelial cell phenotype and opens up the perspective of the involvement of the hypoxic condition.*** Endothelial cells were challenged with CoCr-enriched medium from discs subjected or not to surface modification by double acid etching (DAE). The results show the activation of eNOS (a) and VEGF (b) genes. Furthermore, the activation of the Ubiquitin gene (UBX1; c) and HIF1alpha (d) was investigated.

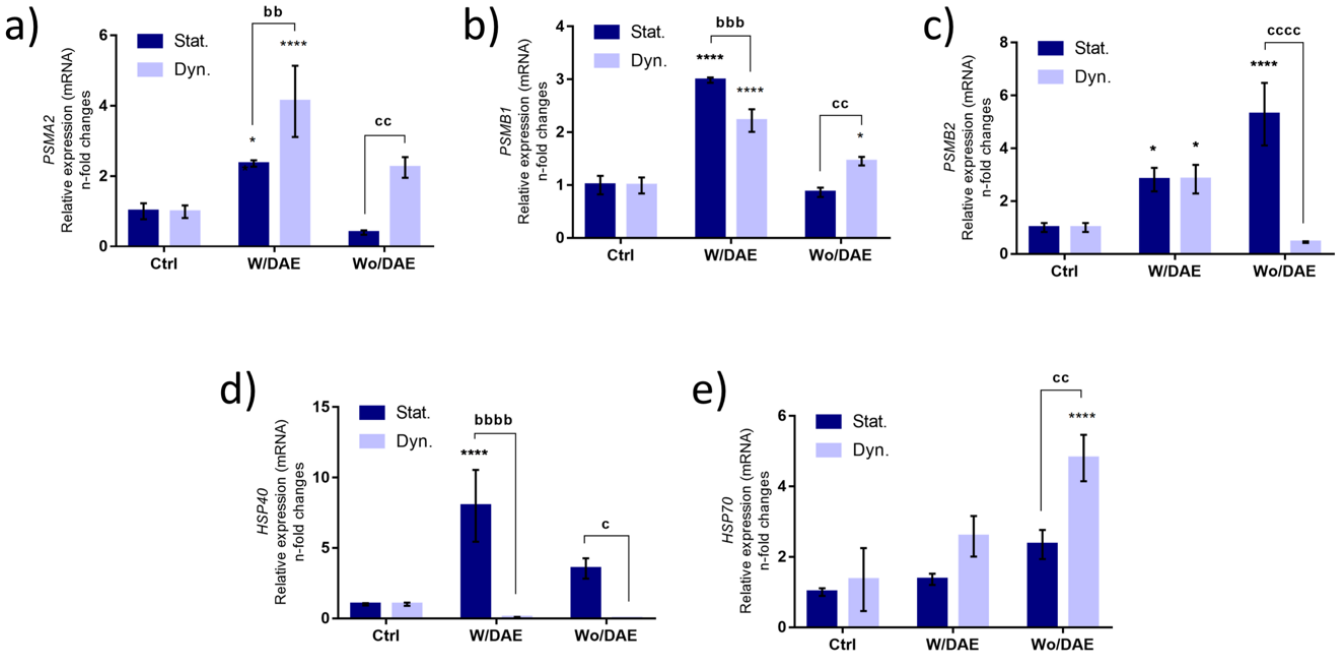

Fig.2. ***Genes related with proteasome complex were down regulated in response to CoCr-enriched medium.*** Except considering PSMA2 gene (**a**) that was upmdulated, the genes PSMB1 (**b**) and PSMB2 (**c**) were down-regulated, as well as HSP40 (**d**). HSP70 gene was significantly up-modulated in response to CoCr (e).

Thereafter in order to validate the relevance or influence of Hypoxia in modulating the expresison of genes related with endothelial phenotype, we have previoulsy pre-treated endothelial cells with chrysin (*5,7*-dihydroxyflavone) and Dimethyloxalylglycine (DMOG). While chrysin is a potent inhibitor of HIF-1α (Fu et al. 2007), DMOG is a competitive inhibitor of hypoxia-inducible factor (HIF)-hydroxylated prolyl hydroxylase and has been shown to play an important role against ischemia-reperfusion myocardial injury (Zhang et al. 2016). Importantly, chrysin was able to down-regulated the expression of eNOS (**Fig.3a**), VEGF (**Fig.3c**) and VEGFR2 (**Fig.3g**) genes, while DMOG contributes with the down-regulation of both VEGFR1 (**Fig.3f**) and VEGFR2 (**Fig.3h**) genes. Also, it is relevant to mention that VEGFR1 gene was significantly up-modulated in endothelial cell responding to chrysin (**Fig.3e**). Altogether, these data shows that hypoxia is an important prerequisite related with the remodeling tissue surrounding the CoCr devices.

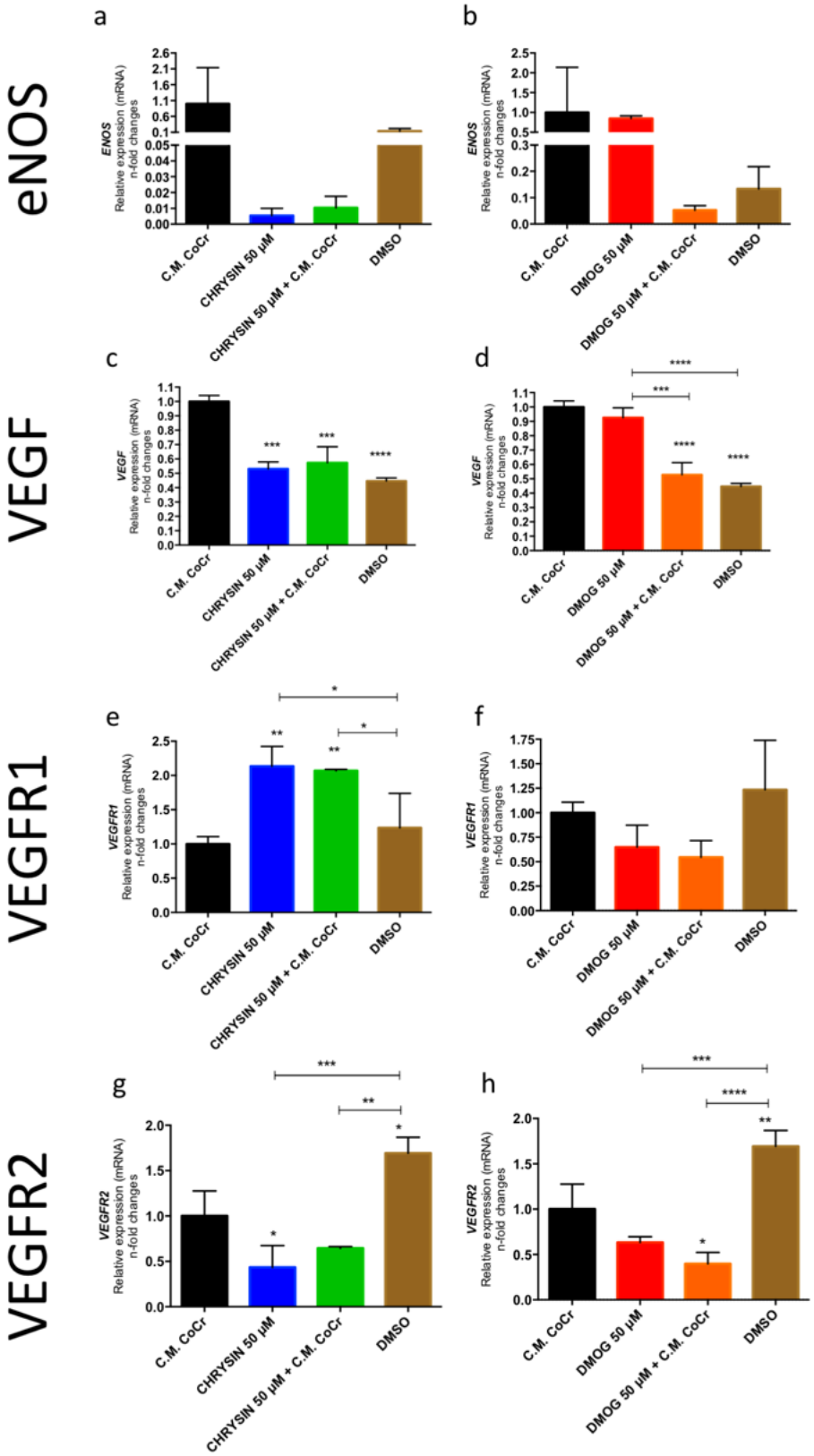

Fig.3. ***Functional assay shows there is decisive involvement of hypoxia in modulating endothelial cell phenotype related genes.***

Altogether, these data validate the hypothesis that hipoxya is important to maintain the phenotype of endothelial cells, and it is properly interesting during the tissue regeneration surrounding implants and so compromising osseointegration. Finally, it is important to mention that the cobalt released from CoCr devices might contribute with an ideal microenvironment surrounding implanted devices and it paviments new roads looking for more bioactive surfaces of implantable devices in human health.

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5 - CONSTATAÇÕES

1. Vias de sobrevivência celular são impactadas pelo meio enriquecido com CoCr;
2. Sinalização celular envolvida com rearranjo do citoesqueleto é requerida em resposta ao CoCr;
3. CoCr interfere em CDKs e, assim, contribui para a proliferação celular;
4. Células endoteliais tem seu fenótipo mantido pela hipóxia promovida pelo Cobalto liberado da liga.

6 - CONCLUSÃO

Este estudo mostrou que a liga de CoCr é um biomaterial metálico ativo capaz de modificar a atividades de células endoteliais através da expressão de genes envolvidos com adesão, proliferação, sobrevivência e função destas células, de maneira consequente ao papel hipoxiante do cobalto nesse microambiente.

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