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Universidade Estadual Paulista “Julio de Mesquita Filho”

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Efeitos do tratamento com iodeto de potássio sobre HUVEC’s incubadas com plasma de grávidas diagnosticadas com distúrbios hipertensivos da gravidez

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EFEITOS DO TRATAMENTO COM IODETO DE POTÁSSIO SOBRE HUVEC'S INCUBADAS COM PLASMA DE GRÁVIDAS DIAGNOSTICADAS COM DISTÚRBIOS HIPERTENSIVOS DA GRAVIDEZ

Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Estadual Paulista – UNESP como requisito para a obtenção do título de Mestre em Biotecnologia
Orientadora: Profª Dra. Valeria Cristina Sandrim

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The scientific man does not aim at an immediate result. He does not expect that his advanced ideas will be readily taken up. His work is like that of the planter — for the future. His duty is to lay the foundation for those who are to come, and point the way. He lives and labors and hopes."

— Nikola Tesla

PRÓLOGO

Esta dissertação será apresentada na forma de uma introdução geral sobre os distúrbios hipertensivos da gravidez focando no diagnóstico diferencial de pré-eclâmpsia, heme-oxigenase 1 e iodeto de potássio, seguida por um manuscrito referente ao projeto de dissertação conforme as normas da revista *American Journal of Obstetrics and Gynecology*, revista a qual foi submetido para publicação.

SUMÁRIO

INTRODUÇÃO

A hipertensão gestacional afeta entre 5% e 7% das grávidas ao redor do mundo [1] e se caracteriza como a maior causa de morte materna no Brasil, cerca de 37% das mortes obstétricas diretas [2]. Sua etiologia ainda é desconhecida, mas a falha na invasão das arteríolas espiraladas pelo citotrofoblasto bem como a isquemia destas placentárias são tidas como o ponto de partida para a síndrome. O critério para diagnóstico consiste em hipertensão após a 20ª semana de gravidez, com pressão sistólica maior ou igual a 140mmHg e/ou pressão diastólica maior ou igual a 90mmHg em duas tomadas de pressão com diferença de 4 horas entre elas e resolução em até 12 semanas pós-parto; quando acompanhada de proteinúria, edema e/ou sinais de danos a órgãos vitais como fígado, rins e sistema nervoso central o diagnóstico diferencial é de pré-eclâmpsia (PE), forma mais grave que pode levar mãe e feto a óbito. Fatores de risco para o desenvolvimento da PE incluem diabetes e/ou hipertensão pré-existentes e obesidade, além de fatores étnicos, imunológicos e genéticos, afetando entre 25% e 31% das filhas de mulheres que apresentaram tal quadro [3].

As consequências da PE para as mães e os nascituros variam com a intensidade da síndrome, mas é comum que haja a necessidade de interromper a gravidez prematuramente, e há um maior risco de haver morte intrauterina ou neonatal do que em gestações normais, sendo os países em desenvolvimento os que apresentam mais mortes maternas do que em países desenvolvidos [4]; os recém-nascidos costumam apresentar baixo peso ao nascer, e há estudos mostrando uma maior predisposição para distúrbios vasculares e metabólicos de filhos de mulheres que foram acometidas por PE, como pressão sanguínea elevada e maior índice de massa corporal, assim como taxas de colesterol e triglicérides aumentadas na adolescência e vida adulta [5,6].

Normalmente ao final do segundo trimestre de gravidez células do citotrofoblasto migram até o terço mais interno do miométrio, remodelando as arteríolas espiraladas tornando-as menos sensíveis ou até insensíveis a estímulos vasoconstritores. Alterações na estrutura destes vasos incluem a substituição da camada elástico-muscular e do endotélio por trofoblastos endovasculares tornando-as mais calibrosas e, portanto, aumentando a vazão de sangue materno para os espaços intervilosos da placenta e para o tecido uterino. Este remodelamento permite que o útero e a placenta recebam um grande volume sanguíneo à baixa pressão, propiciando um ambiente propício às trocas

que devem ocorrer entre o sangue materno e fetal, bem como a nutrição do tecido uterino que se encontra em intensa adaptação [7]. Ao final da gestação a volemia materna pode chegar a ser 50% maior do que o anterior à gravidez, e a maior complacência e menor responsividade dos vasos uterinos à vasoconstrição são as maiores responsáveis pela manutenção da normotensão materna juntamente a fatores hormonais, sendo comum ainda que as mulheres grávidas apresentem uma pressão arterial periférica mais baixa do que o normal em decorrência de uma queda expressiva na resistência vascular periférica.

Na PE, no entanto há um remodelamento insuficiente do tecido uterino, em que cerca de 30% a 50% das arteríolas espiraladas são modificadas, tendo, portanto, o restante permanecido com sua estrutura vascular preservada. A manutenção do endotélio e da musculatura lisa vascular prejudica a circulação feto-placentária, já que os vasos continuam tendo um menor calibre (cerca de 50% do diâmetro total vascular uterino e placentário em mulheres grávidas normais) e, portanto não cedem ao maior volume de sangue que naturalmente é produzido e direcionado ao tecido, além de serem capazes de responder aos inúmeros estímulos vasoconstritores provenientes da constante geração de substâncias que o desenvolvimento fetal propicia [8]. A má perfusão resultante deste remodelamento insuficiente da vasculatura acaba ocasionando vasoconstrição e uma diminuição no aporte sanguíneo da placenta, gerando hipóxia e eventos isquêmicos pontuais. A privação crônica de oxigênio do tecido placentário estimula a produção de radicais livres, mediadores lipídicos, citocinas e s-flt1 (do inglês *soluble fms-like tyrosine kinase -1*) [9]. Tais substâncias atuam sistemicamente produzindo um quadro de disfunção endotelial responsável pela sintomatologia e pelas complicações da PE. À medida que o feto a e placenta se desenvolvem, o útero se expande e o volume sanguíneo aumenta, há uma retroalimentação da condição já que este aumento da volemia ao não ser compensado pela vasodilatação uterina e sistêmica gera ainda mais tensão vascular e, portanto, mais fatores perturbadores que intensificam o quadro hipertensivo e de disfunção endotelial já instalado.

No estado fisiológico o endotélio se encontra em estado quiescente, expressando um fenótipo antitrombótico, antiproliferativo, anti-inflamatório e altamente responsivo às variações de pressão intravascular advindas das modificações normais de volemia e débito cardíaco, sendo a resposta ao aumento de pressão regulada principalmente através da produção de óxido nítrico (NO) através do estímulo do fluxo de sangue

laminar. A produção de NO se dá pela ativação da óxido nítrico sintase endotelial (do inglês, *endothelial nitric oxide synthase* ou eNOS) utilizando L-arginina na presença de cofatores como tetrahidrobiopterina; o NO então se difunde até o músculo liso vascular e ativa a guanilato ciclase gerando vasodilatação via monofosfato cíclico de guanosina. A eNOS também pode ser ativada por moléculas como bradicinina, adenosina, fator de crescimento endotelial (VEGF) em resposta à hipóxia e serotonina, esta liberada pela agregação plaquetária. A manutenção do estado normal do endotélio depende da ligação do NO a grupos cisteína de moléculas reguladoras da atividade transcricional como NFκB (p50/p65) e nas mitocôndrias pela inibição do complexo IV, onde inibem a ativação do tecido e a via intrínseca da apoptose, respectivamente. Quando este mecanismo é esgotado pelo desequilíbrio na geração e neutralização de espécies reativas do oxigênio (ERO's) como peróxido de hidrogênio (H_2O_2), ocorre a ativação do endotélio e instala-se a condição denominada disfunção endotelial (DE) [10].

A DE se caracteriza pela perda da capacidade endotelial de manter o fenótipo regulado por NO e favorecimento da ativação do tecido por sinalização redox, causada por fatores como estresse oxidativo, citocinas inflamatórias, hipertensão, hiperglicemia entre outros. Na DE as ERO's produzem H_2O_2 em presença de superóxido dismutase que de maneira semelhante ao NO, se difunde pela célula e se liga aos mesmos resíduos de cisteína que o NO inibiria. A H_2O_2, entretanto, provoca a fosforilação de fatores de transcrição, indução de remodelamento da cromatina e ativação de proteases [10]. O endotélio ativado por antígenos patogênicos, por exemplo, é importante para permitir que haja a correta migração de leucócitos para o local da infecção já que tem a expressão de agentes quimiotáticos aumentada, bem como sua permeabilidade, retornando ao seu estado normal depois que o patógeno é eliminado. Na DE, no entanto, o estímulo não é patogênico e não há reversão para o estado normal, ocasionando muitas vezes uma resposta inflamatória descontrolada, mas principalmente lesões em órgãos intensamente vascularizados ocasionando falência renal, hepática, parada cardíaca e acidente vascular cerebral hemorrágico, decorrentes destas alterações [11].

Na PE, a produção de NO está deficiente [12] o que à medida que a gestação avança permite a progressão do quadro hipertensivo da mulher grávida. Não existem tratamentos para a PE além dos sintomáticos, com drogas hipotensivas como metildopa, e em casos de pico de pressão, hidralazina e nifedipina, havendo ainda a possibilidade de não-responsividade ao tratamento, provavelmente associada à polimorfismos de

haplótipos codificantes da eNOS [13]. A síndrome só cessa realmente com o nascimento da criança, sendo a placenta a origem da condição. Além dos riscos para a mãe durante a gestação, existem evidências de que mulheres que já foram acometidas pela PE tem maior predisposição para doença renal crônica [14] e cardiovascular [15], além de correrem maiores riscos de morrer por doenças cerebrovasculares [16,17] do que mulheres que não passaram por síndromes hipertensivas na gravidez. O tratamento para casos graves de PE inclui também a administração intravenosa de sulfato de magnésio com o intuito de evitar convulsões, devendo ser mantida até 24 horas pós-parto com o intuito de minimizar as chances de convulsões [9].

Em condições de desequilíbrio de fatores oxidantes e antioxidantes como a PE existem mecanismos celulares que são ativados pelo excesso de ERO's e agentes eletrofílicos xenobióticos, sendo um deles o fator nuclear relacionado a fator eritroide tipo 2 (do inglês, *nuclear factor (erythroid-derived 2)-like 2* ou Nrf2). O Nrf2 é um membro da subfamília de fatores de transcrição chamados cap'n'collar, sendo esta parte da família bZip, que em condições normais está associado à Keap1 (do inglês *Kelch-like ECH-associated protein 1*), proteína ancorada ao citoesqueleto celular (filamentos de actina) que promove a constante degradação proteasomal do Nrf2 através de poliubiquitinação dependente de Cullin 3, inibindo a translocação do Nrf2 para o núcleo. No estresse oxidativo há a dissociação do Nrf2 da Keap1 que ao acessar o núcleo celular, se liga às proteínas Maf G ou Maf K para formar um heterodímero [18], que então irá se ligar a sequências genéticas denominadas elementos da resposta antioxidante (ARE, do inglês, *antioxidant response elements*), regiões promotoras da transcrição de várias enzimas antioxidantes tais como a heme-oxigenase 1 (HO-1) [19]. O Nrf2 é um dos fatores de transcrição mais importantes na sinalização do estresse oxidativo e toxicidade celular, sendo responsável pela promoção da transcrição de outras inúmeras enzimas antioxidantes e desintoxicantes como proteínas da fase I e II do metabolismo de xenobióticos como quinona oxido-redutase 1 (NqO1) e glutatione S-transferases (GST) por exemplo.

A HO-1 é uma das enzimas mais bem estudadas produzidas pela estimulação do Nrf2, consistindo, portanto, de uma proteína importante na resposta celular ao estresse oxidativo. Ela converte heme livre, com atividade pró-oxidante, em ferro livre, monóxido de carbono (CO) e biliverdina, também um fator pró-oxidante, sendo esta última ainda convertida em bilirrubina pela ativação do Nrf2 e genes codificantes das

duas enzimas biliverdina redutase [20,21]. A inibição específica de HO-1 reduz os efeitos da ativação do Nrf2, o que explicita um possível efeito de feedback entre a enzima e o fator de transcrição; o CO ainda age como o NO, estimulando a produção de GMP cíclico e portanto de proteína quinase G, também um fator estimulante da via do Nrf2 [22]. Em camundongos-fêmea prenhes a deleção do gene *HMOX1* codificante para a enzima promove remodelamento das arteríolas espiraladas inadequado e placentação insuficiente, seguida de restrição do crescimento intrauterino da prole e letalidade fetal; a inibição da enzima em ratas prenhes gera hipertensão no animal [23], demonstrando, portanto, o alto grau de dependência da ação da HO-1 para uma gestação normal.

A ativação do Nrf2 por nutrientes encontrados nos mais diversos alimentos demonstra efeitos preventivos importantes [24], refletindo dados epidemiológicos mundiais que demonstram as diferenças de longevidade e incidência de doenças cardiovasculares entre países com culturas alimentares diferentes [25,26,27]. Considerando as afecções circulatórias frequentemente presentes em mulheres que tiveram PE, é de interesse científico que substâncias antioxidantes e anti-inflamatórias que possam contribuir para a prevenção e tratamento da PE e destas consequências sejam identificadas. Compostos orgânicos como antioxidantes fenólicos, carotenoides e ácidos graxos ômega-3 (DHA e EPA) de cadeia longa já foram relacionados a melhores indicadores de saúde vascular; os ácidos graxos ômega-3 ativam o fator de transcrição pelo seu produto oxidado 4-hidroxihexenal[28,29,30]; os carotenoides agem provavelmente em grande parte do mesmo modo, gerando produtos oxidados [31,32], e os antioxidantes fenólicos aparentam agir através de seus produtos oxidados via quinona [33,34].

Em se tratando de longevidade e saúde cardiovascular, os asiáticos, mas principalmente os japoneses constituem um povo extensivamente estudado, com uma expectativa de vida média de 83,7 anos, quase 10 anos maior do que a brasileira e quase 5 anos maior do que a norte americana, e uma expectativa de vida saudável de 74,9 anos em 2015 segundo a OMS [35]. Uma diferença notável no estilo de vida japonês é o elevado consumo de algas marinhas, uma das maiores fontes nutricionais de iodo disponíveis. Os japoneses consomem de 1200 a 5280 μg do mineral por dia frente a uma média de 166μg consumidos por norte-americanos [36], e seus índices de saúde são significativamente melhores, com menores taxas de câncer próstata, ovário e mama

[36,37], assim como mortes devido a doença cardiovascular [38] e mortalidade infantil [39].

Conhecido por sua participação determinante na síntese de hormônios tireoidianos tiroxina e triiodotironina assim como sua importante função no desenvolvimento neurológico fetal, o iodo tem funções fisiológicas que são atualmente pouco discutidas, mas que estão voltando ao cenário científico em diversas áreas, notadamente em se tratando de saúde da mulher [40,41]. O iodeto tem cerca de três vezes mais capacidade antioxidante do que o ácido ascórbico [42],é transportado principalmente pelo simportador sódio/iodeto (NIS, do inglês, *sodium/iodide symporter*) e armazenado em tecidos extratireoidianos que expressam tal receptor como tecido mamário (principalmente em lactação), mucosa gástrica, glândulas salivares e lacrimais [43] sendo crucial para regulação da proliferação folicular tireoidiana independente de TSH [44]; é naturalmente presente em mucosas e glândulas exócrinas e quando em presença de peroxidases (lactoperoxidase por exemplo, presente em glândulas salivares e mamária) e peróxido de hidrogênio, forma ácido hipoiodoso, poderoso agente antibiótico [43]; o iodo molecular tem efeito altamente antibiótico ao se ligar aos resíduos de cisteína e metionina, nucleotídeos e ácidos graxos de bactérias, desestabilizando-as e eventualmente levando-as a morte [45]; e pesquisas recentes tem estudado a função pró-apoptótica do iodo molecular, do iodeto e de mediadores lipídicos iodados como a 6-iodolactona sobre células tumorais em doses baixíssimas em relação às que levariam células normais à morte [46].

A deficiência grave de iodo na dieta acarreta principalmente em condições como o bócio, hipotireoidismo clássico e subclínico (níveis de hormônios tireoidianos normais com elevação do nível de hormônio tireoestimulante ou TSH) e quando acomete mulheres grávidas, há um elevado risco de prejuízo à saúde a da mãe, que corre maior risco de desenvolver PE, abortar espontaneamente [47], e da criança, que pode nascer com cretinismo (devido ao hipotireoidismo congênito, a criança sofre de malformações físicas e neurológicas debilitantes e irreversíveis) e/ou prematuramente [48,49,50]. Devido aos altos índices de bócio endêmico e malformações decorrentes da deficiência de iodo vários países, incluindo o Brasil, iniciativas governamentais determinaram a adição do micronutriente ao sal de cozinha, o que diminuiu sensivelmente a incidência destas condições. No entanto, de acordo com o Guia Alimentar para a População Brasileira, o consumo de sal não deveria exceder os 5 gramas diários, que fornece

125µg na forma de iodato de potássio, valor menor do que a dose diária recomendada de 150µg. Mulheres grávidas e lactantes devem aumentar seu consumo de iodo para 200µg ao dia, que caso dependa inteiramente do consumo de sal, acabaria por propiciar a um consumo excessivo e inadequado de sódio, já que a ingestão de algas marinhas ou peixes de água salgada do brasileiro é ínfima, ambos fontes naturais de iodo.

Um estudo de 2014 relacionou pela primeira vez um maior estresse oxidativo e uma menor atividade de enzimas antioxidantes como superóxido dismutase e catalase em mulheres grávidas com deficiência branda de iodo (iodo urinário entre 50 e 99 µg/L em comparação ao nível normal de 100 a 300 µg/L por dia) [51]; outro estudo de 2016 encontrou uma associação positiva entre níveis ligeiramente ou moderadamente baixos de iodo urinário detectados em crianças com um estresse oxidativo maior em comparação às crianças com níveis suficientes de iodo [52]. Amostras de mulheres com PE grave apresentaram níveis baixíssimos de iodo urinário (4,25 +/- 2,7µg/dL de urina) em comparação às de gestantes normais (20,89 +/- 6,4 µg /dL de urina) [53], sugerindo que o iodo urinário possa ser um marcador para a PE; o mesmo estudo ainda relata um aumento nos níveis de triiodotironina (T3), hormônio tireoidiano ativo, em mulheres com pré-eclâmpsia grave (1,86 +/- 0,4 µg/dL) em comparação aos de gestantes normais (1,45 +/- 0,3 µg/dL; $p < 0,001$), ainda que os níveis de TSH estivessem normais, sugerindo uma maior utilização do hormônio na PE. O iodeto também parece estimular a proliferação de células endoteliais *in vitro* independentemente de receptores de membrana ao estimular a fosforilação de VEGFR-2 (do inglês, *vascular endothelial growth fator receptor 2*) no resíduo Tyr1214, mas não no Tyr1175 [54], podendo, portanto, participar de processos angiogênicos que estão reduzidos na PE.

Em relação à ativação direta do Nrf2, um estudo reporta que o iodo é capaz de ativá-lo em culturas de queratinócitos humanos imortalizados e pele humana, demonstrando proteção contra danos causados por raios UVB e redução da inflamação cutânea [55], principalmente com o tratamento com iodeto de potássio. A possível ativação desta via em células endoteliais de cordão umbilical humano (HUVEC) incubadas com plasma de gestantes hipertensas ou pré-eclâmpticas poderia representar mais uma evidência do potencial antioxidante do iodo.

Considerando as evidências epidemiológicas acerca do consumo de alimentos ricos em iodo [56] bem como os estudos demonstrando uma menor excreção e possível

aumento de necessidade da ingestão do micronutriente por mulheres grávidas pré-eclâmpticas é objetivo do presente estudo avaliar se esta ação ocorre *in vitro* avaliando a resposta destas células ao potencial antioxidante dessas moléculas, bem como outras respostas que estas células possam desenvolver frente a halogênio.

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MANUSCRITO

RESUMO

GALVÃO, V.E. **Efeitos do tratamento com iodeto de potássio sobre HUVEC's incubadas com plasma de grávidas diagnosticadas com distúrbios hipertensivos da gravidez.** 2019. Dissertação (Mestrado), Instituto de Biociências de Botucatu – Universidade Estadual Paulista, Botucatu, 2019.

Distúrbios hipertensivos da gravidez (HPD) são caracterizados por aumento de pressão arterial após a 20ª semana gestacional; a presença de proteinuria e/ou sinais de danos a órgãos como fígado, rins e sistema nervoso central indicam pré-eclâmpsia (PE). Estes distúrbios afetam de 5 a 7% das gestações ao redor do mundo e ainda que uma causa única não tenha sido identificada, o processo de placentação insuficiente se mostra crucial para o desenvolvimento destas afecções. A placenta isquêmica e disfuncional secreta moléculas que irão afetar o endotélio, tecido que reveste os vasos internamente, e este responde de inúmeras maneiras com o intuito de manter a homeostasia. A enzima heme-oxigenase 1 tem um papel importante na manutenção da função endotelial ao converter grupos heme em moléculas antioxidantes, antiapoptóticas e vasoativas. Foi reportado que a produção de heme-oxigenase 1 pode ser estimulada pelo iodeto de potássio (KI) em queratinócitos, bem como fragmentos de pele humana. Neste estudo células endoteliais de veia umbilical humana (HUVEC's) foram incubadas com plasma de mulheres grávidas apresentando HPD com o objetivo de avaliar o potencial do KI de estimular a expressão da heme-oxigenase 1 nestas células. Os resultados mostram que o KI foi capaz de induzir a expressão da enzima no grupo saudável ($p=0,0065$) e de reduzi-la a níveis normais no grupo hipertenso ($p=0,0018$); o tratamento reduziu a citotoxicidade do plasma de grávida pré-eclâmpticas sobre as HUVEC's, mas não teve efeito algum sobre a expressão de heme-oxigenase 1. Estes achados sugerem que o KI afeta vias de produção da heme-oxigenase 1 e que o plasma de mulheres pré-eclâmpticas contém fatores que previnem este efeito.

Palavras-chave: Pré-eclâmpsia, iodeto de potássio, heme-oxigenase 1, hipertensão, endotélio

TITLE PAGE

Potassium iodide effects in an *in vitro* model for hypertensive disorders of pregnancy

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Condensation: KI reduces cytotoxicity but does not affect heme oxygenase-1 in preeclampsia

Short title: Potassium iodide effect over HUVECs incubated with pregnant women plasma

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Competing Interest
Galvão declares she has no competing interests
Cavalli declares he has no competing interests
Sandrim declares she has no competing interests

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GALVÃO, V. E.; Sandrim, V.C.; Effects of potassium iodide treatment over HUVEC's in na in vitro model for preeclampsia. 21st National Biomedic Meeting, October 18th-20th, 2018, São Paulo State University, Botucatu, São Paulo, Brazil.

Ethical approval and consent of participants
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All participants provided written informed consent.

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A. Why was the study conducted?

- Iodine is most consumed in Japan and South Korea, two countries with the highest life expectancy and lowest incidence of hypertension and cardiovascular events in the world.
- Hypertensive disorders of pregnancy affect the endothelium.
- Potassium iodide was shown to induce heme oxygenase-1 in skin.
- This enzyme offers protective actions over endothelium, so KI could be therapeutic.

B. What are the key findings?

- Potassium iodide reduced cytotoxicity of preeclamptic plasma.
- KI induced heme oxygenase-1 expression in healthy group and normalized it in gestational hypertension group.
- Heme oxygenase-1 was not affected by KI in preeclamptic group.

C. What does this study add to what is already known?

- Plasma from preeclamptic pregnant women inhibits heme oxygenase-1 expression mediated by KI.

Keywords: Cytotoxicity, Preeclampsia

ABSTRACT

GALVÃO, V.E. **Effects of treatment with potassium iodide over HUVEC's incubated with plasma from women diagnosed with hypertensive disorders of pregnancy.** 2019. Thesis – (Master), Institute of Biosciences of Botucatu – São Paulo State University, Botucatu, 2019

Hypertensive disorders of pregnancy are characterized by increased blood pressure after the 20th gestational week; presence of proteinuria and/or signs of end-organ damage indicate pre-eclampsia. They affect 5%-7% of pregnancies worldwide and even though causes are still to be elucidated, placentation process failure is crucial for these diseases to develop. The ischemic, dysfunctional placenta secretes molecules that will affect the endothelium which responds in a number of ways attempting to maintain homeostasis. Heme-oxygenase 1 plays an important role in maintaining endothelial function as it catalyzes the conversion of heme groups into antioxidant, antiapoptotic and vasoactive products. Heme-oxygenase 1 production was shown to be stimulated by potassium iodide in skin cells lineage and in human skin explants. In this study, we used HUVECs incubated with pregnant women plasma to evaluate KI effect over this enzyme expression. Our results showed that KI was capable of inducing heme-oxygenase 1 expression in healthy pregnant group ($p=0.0065$) and reducing it in gestational hypertension group ($p=0.0018$); the treatment reduced plasma cytotoxicity but had no effect over pre-eclamptic group heme-oxygenase 1 expression. Our findings suggest that KI affects heme-oxygenase 1 pathways and that pre-eclamptic women plasma contains factors that prevent this effect. These preliminary results could guide future research on novel pre-eclampsia biomarkers and endothelial pathways to further elucidate its pathological mechanisms.

Keywords: Pre-eclampsia, potassium iodide, heme-oxygenase 1, hypertension, endothelium

INTRODUCTION

Gestational hypertension is characterized by either a systolic blood pressure level of 140 mmHg or higher, a diastolic blood pressure level of 90 mmHg or higher, or both, after the 20th gestational week which usually normalize up to 12 weeks post-partum[1]. If accompanied by proteinuria (higher than 0.3g/day) and/or signs of end-organ damage such as increased plasmatic liver enzymes, plasmatic and urinary creatinine as well as central nervous system alterations such as blurred vision and headaches, it is diagnosed as preeclampsia, the most dangerous presentation of hypertension in pregnancy [1]. Hypertensive disorders of pregnancy and mostly preeclampsia are a huge public health burden, with a worldwide incidence of 5-10% of all pregnancies [2, 3], being the major cause of maternal death in Latin America and Caribbean [4].

Hypertension induced by pregnancy etiology has not been fully elucidated yet, but there is a consensus that an inadequate trophoblastic implantation, as well as its consequences for the subsequent placentation process during pregnancy are crucial for the disorder development [5]. The presence of risk factors such as obesity, a family history of hypertension, and heart failure also increases chances of pregnancy complications [6, 7]. In hypertensive disorders of pregnancy this process is deficient, as only 30-50% of vessels are invaded and remodelled, causing the immature placenta to suffer ischemia-reperfusion lesions, as well as the secretion of a series of vasoactive molecules to the mother's bloodstream [5]. These molecules interact especially with the endothelium, where they provoke cellular reactions that will eventually lead to a dysfunctional tissue, which is responsible for most part of preeclampsia symptoms and signs [5, 8, 9]. Since the disease manifests as mostly an endothelial disorder [10] and it usually takes at least 34 weeks into pregnancy to be diagnosed [11], strategies seeking

the preeclampsia prediction and pathophysiology pathways evaluation models were designed.

Even though hypertension induced by pregnancy is an exclusively human disorder, animal models are used to study the condition such as angiotensin induced preeclampsia and reduced uterine perfuse pressure (RUPP) [12]; however, there are other models using *in vitro* and *in silico* strategies to better understand these disorder's risk factors, biomarkers and pathological mechanisms. These models can include cell culture and co-culture (endothelial, trophoblastic, immunological cell lines), tissue culture (placenta explants, etc) and even organ on a chip [12].

Incubation of endothelial cells with plasma or serum from preeclamptic patients was shown to provoke cellular responses compatible with endothelial activation by exhibiting upregulation of adhesion molecules such as vascular cell adhesion protein 1 [13] and platelet-derived growth factor [14]. When evaluating endothelin-1, a vasoconstrictor peptide also associated with preeclampsia pathophysiology [15, 16] our group showed that plasma from preeclamptic women contained more endothelin-1 than healthy pregnant samples, but provoked a regulatory effect over HUVECs by inducing the transcription of microRNA's that led to endothelin-1 levels in cell culture supernatant comparable to the ones from healthy pregnant group [17]. Therefore, the use of an *in vitro* approach provides insight into what different pathophysiological and compensatory mechanisms are activated by pregnant women's plasma over endothelial cells, directing the search for novel biomarkers, as well as novel cellular pathways.

Another protective molecule also associated with preeclampsia is heme oxygenase-1, an enzyme capable of converting free heme groups into free iron, carbon monoxide, and biliverdin, which is further converted into bilirubin by biliverdin

reductase [18]. Heme oxygenase-1 products have antioxidant, anti-inflammatory and antiapoptotic effects, which help to preserve the tissue integrity and function [19, 20]. Heme oxygenase-1 can be induced by excess heme groups as well as other stimuli such as radiation, hypoxia and cytokines[18], but it can also be produced through stimuli present in foods such as curcumin and caffeic acid [21] mediating even in part these substances antioxidant and anti-inflammatory effects. Iodine, which is obtained mostly from seaweed, dairy products and iodized salt, had modest anti-inflammatory effects in euthyroid individuals in mildly above the recommended average doses [22] and was negatively correlated with oxidant plasmatic profile in children [23]; in the form of potassium iodide, it was demonstrated that heme oxygenase-1 could be induced by iodine when using skin cells and human skin explants [24].

One urinary iodine assessment performed in preeclamptic women showed a severe iodine deficiency [25] and another showed that blood from preeclamptic women contained significantly less iodine compared with healthy pregnant controls, and that umbilical cord blood of those women's fetus contained more iodine than control subjects [26], suggesting iodine could participate in preeclampsia pathophysiology. Hence, the objective of this study then was to assess potassium iodide effects over HUVECs incubated with plasma from pregnant women diagnosed with gestational hypertension or preeclampsia as well as its capability to induce heme oxygenase-1 system.

MATERIAL AND METHODS

Source of biological samples

The plasma samples used were obtained as described before [17]. A group of women was recruited from the ambulatory clinic at HCFMRP-USP. That study was approved by the Institutional Review Board at Ribeirão Preto Medical School, Brazil

(reference 4682/2006, approved date June 20, 2006) and was performed following the principles of the Declaration of Helsinki. All subjects gave written informed consent. Exclusion criteria included twin pregnancy, hemostatic abnormalities, chronic hypertension, diabetes mellitus, fetal abnormalities, cancer, and cardiovascular, autoimmune, renal, and hepatic diseases. Gestational hypertension and preeclampsia were diagnosed according to the American College of Obstetricians and Gynecologists [1]. All maternal venous blood samples were collected in tubes containing heparin. The tubes were then centrifuged (1000 g for 3 min) and plasma samples were stored at −80°C

Preparation of potassium iodide (KI) solution and redox titration

Potassium iodide was purchased from Sigma-Aldrich Brazil (Catalogue number 221945-100G). KI solution was prepared by solubilizing it in deionized water and for the redox titration, we used sodium thiosulfate, previously titrated with a potassium iodate solution as primary standard. The stock solution concentration was 58mM. For the experiments we diluted that solution in growth culture to achieve final concentration of 100µM and 1000µM.

Human umbilical vein endothelial cell culture, incubation with patients' samples *pools* and potassium iodide.

For this *in vitro* model of preeclampsia, we used human umbilical vein endothelial cells (HUVECs, ATCC® CRL-2873™). Cells were cultivated in sterile 25cm² flasks with growth medium (Gibco, CA, USA) supplemented with fetal bovine serum 10% v/v (Gibco), 50 µg/ml penicillin, 50 µg/ml streptomycin and 0,5 µg/ml amphotericin B (Gibco). For the experiments described ahead, cells were detached from culture flask (Corning, Costar, Netherlands) using tripsin solution (Trypsin/EDTA 0.5/0.2 mg/ml in phosphate buffered saline, PBS) centrifuged at 1200rpm for 10 minutes, resuspended in growth medium free from FBS containing antibiotic and

antifungal solution, and seeded on 96 well microplate (1.10^4 cells/well) overnight in an incubator at 37ºC, 5% CO_2 tension and 95% humidity to ensure cell adhesion. The next day, the *pools* of plasma were prepared to be used on our assays by mixing equal volumes of each sample from healthy pregnant patients (HP, n= 12), gestational hypertensive (GH, n=10) or preeclamptic patients (PE, n=11) at 10%v/v and diluting it in growth medium q.s. prior to incubation. The plasma *pools* were added to the plate, as well as the KI solution (100µM and 1000µM). After 24 hour incubation period the supernatant was collected for posterior cytotoxicity, total antioxidant capacity and heme oxygenase-1 quantification assays, while cells were promptly used for viability assay.

Cell Viability and Cytotoxicity

Cell viability was assessed using fluorescent probe Prestoblue™ (Thermofisher, catalogue number A-13261). The probe was added to the cells (10%v/v) and the microplate was once again incubated at 37ºC, 5% CO_2 tension and 95% humidity for an hour. After the incubation period, the fluorescence was read in a microplate reader with an excitation wavelength of 560nm and emission wavelength of 590nm. Control groups consisted in HUVECs with plasma *pools* only and those readings were used as 100% viability. Cytotoxicity was assessed using Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Catalogue number 88954). This colorimetric assay relies on two subsequent enzymatic reactions to measure lactate dehydrogenase (LDH) activity. LDH is present on cell culture supernatant when cell membrane integrity is lost indicating cytotoxicity; after an 24-hour incubation with patients *pool* 30µL of supernatant were collected and immediately transferred to another 96 well microplate, to which 30µL of the substrate mix was added. After 30 minutes of incubation at room temperature

protected from light, 30 µL of stop solution was added and the absorbance was read in 490nm wavelength, with additional 680nm reading for background signal elimination.

Total Antioxidant Capacity (TAC) and heme oxygenase-1 quantification

TAC of culture supernatant was assessed using Ferric Reducing Antioxidant Power (FRAP) assay [27]. FRAP reagent was prepared mixing 50mL of 23mM acetate buffer (pH= 3.6), 5mL of 10mM TPTZ solution and 5mL 20mM $FeCl_3.6H_2O$. 10µL of supernatant sample was added to a 96 well microplate, to which 290µL of FRAP reagent was mixed, and the microplate was incubated in 37ºC for 4 minutes and absorbance was read at a 593nm wavelength. The standard solution was prepared using $Fe_2SO_4.7H_2O$ and the unit used was mM equivalent Fe^{2+}.

For the heme oxygenase-1 (HO-1) quantification assay, we used the Human Total HO-1/HMOX1 DuoSet IC ELISA (Catalogue number DYC3776) and performed the assay by manufacturer's instructions. Readings were performed at 450nm wavelength.

Transmission electron microscopy (TEM)

Cells were seeded in tissue culture 6 well plate (500,000 cells/well) overnight and incubated with plasma from groups already defined as well as preeclamptic plasma plus 1000µM KI for 24 hours. Cells were detached from well surface using tripsin-EDTA solution for a minute; the enzyme was neutralized with BFS and the cells were transferred to microtubes for centrifugation (1200rpm, 10 minutes). The supernatant was then discarded and the cell pellet was resuspended in Karnovsky fixative for another centrifugation. The process was repeated one more time before the cells were resuspended in fixative and delivered at Electron Microscopy Center, UNESP, Botucatu *campus* for further processing.

Statistical Analysis

The software used was GraphPad Prism 6. For statistical analysis of clinical features from patients enrolled, we used ANOVA followed by Dunnet's test for multiple comparisons when variables obeyed normal distribution or Kruskal-Wallis followed by Dunn's test for multiple comparisons when at least one group followed a non-parametric distribution. P values are described in Table 1.

For the statistical analysis of results obtained from cell culture we compared the incubation with plasma and KI with control group consisting of cells incubated only with plasma from each studied group; we performed ANOVA followed by Dunnet's test for multiple comparisons, with statistically significant p values <0.05, and "*" mark was used. We also compared groups treated only with plasma or plasma and KI in each concentration; in this case we performed ANOVA followed by Bonferroni's test, with statistically significant p values <0.05 and "#" mark was used.

RESULTS

Clinical features of patients from which plasma samples were obtained are shown in Table 1. Non-parametric distribution was found when analysing systolic and diastolic blood pressure. All affected patients were pregnant for the first time. No statistical differences were found between groups in terms of age or placental weight, but samples were obtained on earlier gestational week and delivery was also earlier in the preeclamptic group when compared with healthy pregnant group. Body mass index at sampling was significantly higher in hypertensive group, and systolic blood pressure were significantly higher in hypertensive and preeclamptic groups; both groups were already on methyldopa therapy at time of sampling, which can explain the lack of statistical difference between hypertensive and healthy pregnant groups diastolic blood pressure values; in preeclamptic group however, antihypertensive therapy did not

normalize blood pressure indexes and both systolic and diastolic measurements presented higher values.

Cellular viability was not affected by KI; plasma from gestational hypertensive group induced similar cytotoxic effects as seen in healthy pregnant group and KI did not affect those values, but plasma from the preeclamptic group was significantly more cytotoxic than the other two groups' (PE vs HP p=0.0378; PE vs GH p=0.0145, Figure 1B), even with 100μM treatment (PE vs HP p= 0.0031; PE vs GH p =0.0002). However, when associated with the highest KI concentration used, preeclamptic group cytotoxicity was significantly reduced when compared with preeclamptic control group (p=0.0017) and became similar to what was seen in healthy pregnant and gestational hypertension groups.

Given the cytotoxicity results, we analyzed the cellular ultrastructure. It is noticeable that cells treated with preeclamptic plasma and 1000μM KI showed different visual aspect in comparison with cells incubated only with gestational hypertension or preeclamptic plasma (Figure 2). There is a mild vacuolization in cytoplasm in these groups, but the overall images shown in D1 to D3 resemble the healthy pregnant group images supporting our cytotoxicity results.

Healthy pregnant group and gestational hypertension group had increased antioxidant capacity when 1000μM of KI was used compared with their respective control groups (p=0.0006 for HP; p=0.0025 for GH) (Figure 3A); antioxidant capacity of preeclamptic control group was significantly higher than seen in healthy and hypertensive control groups (p= 0.0035 HP vs PE; p=0.0066 GH vs PE), which was sustained when KI was added; neither healthy nor hypertensive groups' levels equated

to preeclamptic group results after KI treatment ($p < 0.0001$ PE vs HP; $p = 0.0004$ PE vs GH)

The results of heme oxygenase-1 dosages from hypertensive group (Figure 3B) showed increased expression of the enzyme compared with healthy and preeclamptic groups. In healthy pregnant group, the 100µM treatment worked as an optimal concentration for heme oxygenase-1 induction ($p=0.0065$). The 1000µM treatment reduced the enzyme production in the hypertensive group ($p=0.0018$), but no KI concentrations used changed preeclamptic heme oxygenase-1 production whatsoever; however, the most concentrated treatment brought the healthy pregnant group levels back to seen in control group, as well as hypertensive group levels. There was no difference in heme oxygenase-1 production between groups when 1000µM was used.

COMMENT

Principal Findings

Our findings show that KI was capable of inducing HUVECs heme oxygenase-1 expression when incubated with plasma from healthy pregnant women, reducing its expression in gestational hypertension group but did not affect preeclamptic group dosages. We also found that KI was well tolerated by HUVECs and even preserved these cells when the highest concentration was added to preeclamptic patients' plasma. This was the first time KI was used on an *in vitro* model for hypertensive disorders of pregnancy. Heme oxygenase-1 expression seems to be associated with a diminished cytotoxicity *in vitro,* and KI was capable of ameliorating this parameter in preeclamptic group even though it did not affect heme oxygenase-1 expression in high concentrations.

Results

To our knowledge this was the first time HUVECs were incubated with KI, even though another endothelial cell lineage was already used to assess KI effects over proliferation and vascular endothelial growth factor receptor activation [28] coming to interesting results. No other study to this date has assessed KI effects over endothelial cells when incubated with plasma from pregnant women, and this was the first time its effect over heme oxygenase-1 pathway was showed on aforementioned model.

Our transmission electron microscopy images of endothelial cells showed slightly altered patterns in morphological structures of diseased groups, with a noticeable increase in cytoplasmatic vacuoles in preeclamptic group; endothelial cells subjected to longer treatments than 24 hours used in this study with low doses of antineoplastic agent 5-Fluorouracil showed a similar pattern, associated with cellular distress [29]. In preeclamptic group, cells showed a fusiform morphology and abundant membrane projections resembling a process-bearing cell appearance were they fixated while adhered to the plate surface, which is mostly seen in distressed glioma cells [30]; since healthy pregnant group showed a flatted pattern with less cell projections, we may assume that what is seen in preeclamptic group display an abnormal morphology which is reversed by KI.

Antioxidant capacity of preeclamptic group was higher in all conditions studied, which did not reflect beneficial outcomes when taken with cytotoxicity results and microscopy images; in plasma, increased antioxidant levels were found in the same groups studied here, and attributed to a compensatory mechanism against oxidative stress in hypertensive and preeclamptic women [31]. We also found that KI has the potential to induce heme oxygenase-1 expression (possibly through Nrf-2 pathway

activation as was described before [24]) in healthy group and normalize expression in hypertensive pregnant group since it was demonstrated that KI has antioxidant and anti-inflammatory effects [22, 32]

Clinical Implications

Our study shows that KI is not harmful to endothelial cells *in vitro* even in high concentrations. We also showed that plasma from women diagnosed with gestational hypertension or preeclampsia affected endothelial cells in a different way, as well as KI treatment. Further studies should be carried on using different animal models for gestational hypertension and preeclampsia to assess the antioxidant and cytoprotective actions of KI over endothelial cells. Nevertheless, due to iodine importance for the fetus in pregnancy, urinary iodine should be evaluated alongside thyroid hormones to ensure sufficient intake even in later stages of pregnancy.

Research Implications

Our group has shown previously that plasma from pregnant women is capable of inducing different effects over HUVEC's through inhibition of gene expression by post-transcriptional regulatory molecules (microRNA's) [17] and these mechanisms should also be further studied particularly when KI is added to the preeclamptic samples. Heme oxygenase-1 expression in endothelial cells was shown to be induced by the activation of some transcriptional factors beyond Nrf-2 such as heat shot factor 1 [33] and also by peroxisome proliferator-activated receptors α and γ [34] and therefore, these proteins and their pathways as well as possible inhibitors should be assessed for a better understanding of plasma effects over HUVEC's seen in this study.

Strengths and Limitations

As a study limitation, we highlight the choice for healthy endothelial cell lineage, which could not necessarily reflect accurately the endothelial responses in terms of pharmacological mechanisms, since endothelium challenged by increased blood pressure and placental molecules in hypertensive disorders of pregnancy show altered function [35] and therefore, could respond differently when subjected to KI. However, as a biotechnological approach to isolate the plasma as only variable, our model is useful so as to enlighten specific pathways affected by different samples, therefore enabling a guided search of novel molecules present in plasma of hypertensive pregnant women. We also showed differences in endothelial responses to plasma from two different hypertensive disorders of pregnancy, which contribute to biomedical understanding of clinically similar but not equal disorders.

Conclusions

We conclude that hypertensive disorders of pregnancy do not affect endothelial cells in the same way and that KI might offer benefits especially in gestational hypertension therapy. Our results also suggest that heme oxygenase-1 can be induced by KI in endothelial cells and that it is an active biochemical pathway in gestational hypertension. KI possibly attenuates the need for this pathway to be activated in this group, showing that this is a biochemical pathway that should be explored when handling the disorder. The lack of effect of KI over enzyme induction seen in preeclampsia suggests the existence of specific inhibitors of antioxidant and detoxifying enzymes expression in preeclamptic women plasma that might contribute to its severity and difficulty in handling symptoms and disorder progression using antihypertensive and antioxidant therapy.

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Table 1

Parameters	HP	GH	PE	*p* value
n	12	10	11	
Age (years)	22.3(2.27)	21.3(2.83)	21.6(4.14)	ns
GW sampling	37.3(1.33)	38.7(1.94)	31.5(3.62)*	* p<0.0001
BMI sampling (kg/m²)	28.3(2.28)	37.4(7.78)*	28.9(4.53)	* p=0.0006
SBP sampling (mmHg)	113.8(10.69)	126(10.50)*	136.2(16.14)**	*p=0.0310; ** p=0.0011
DBP sampling (mmHg)	71.83(8.96)	80.40(9.96)	90(10.00)*	*p=0.0001
Methyldopa (%)	0	100	90(10/11)	-
Nulliparous (%)	41.6 (5/12)	100(10/10)	100(11/11)	-
GW delivery	39.5(1.43)	40(1.00)	34.5(4.33)*	*p=0.0005
Placental weight (g)	536.8(106.28)	613(146.29)	408(160.88)	ns

Table 1 – Clinical characteristics of pregnant women and delivery conditions. Data shown as mean ± (SD) or median (underlined) ± (IQR). HP, healthy pregnant; GH, hypertensive pregnant; PE, preeclamptic; GW, gestational weeks; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. *p* values in comparison with HP group.

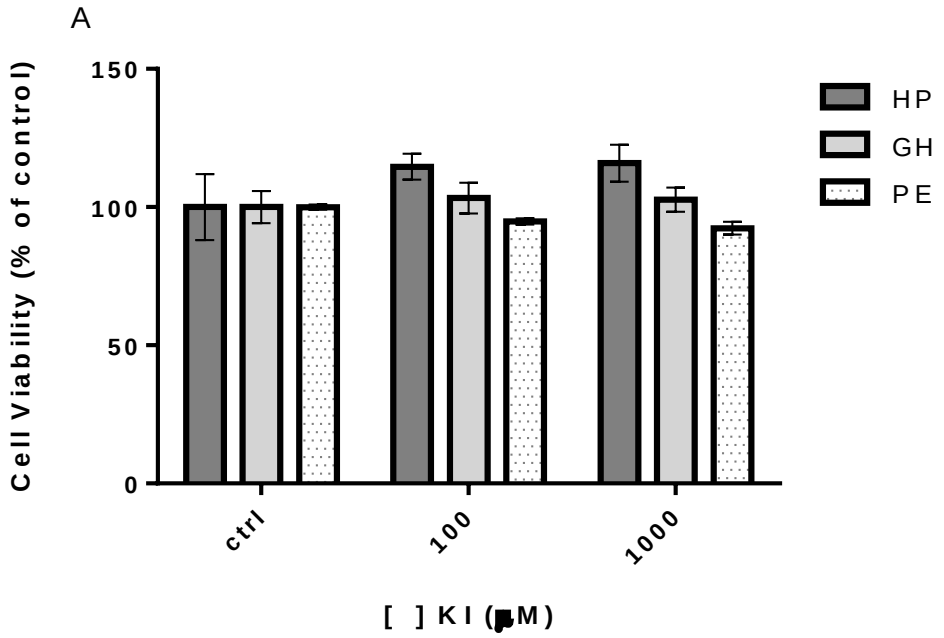
Figure 1- Cell viability (A) and cytotoxicity (B) of human umbilical vein endothelial cells incubated with pregnant women plasma and potassium iodide. HUVECs were incubated with healthy pregnant (HP), hypertensive pregnant (GH) or preeclamptic pregnant (PE) women's plasma pool and treated with 100µM or 1000µM of KI for 24 hours prior assays. Results are shown as mean ± SEM of quadruplicate wells per assay. (*) p<0.05 intragroup; (#) p<0.05 intergroup.

Figure 2 – Transmission electron microscopy images of endothelial cells incubated with healthy pregnant (A1-A3), gestational hypertension (B1-B3) and preeclamptic plasma (C1-C3). Magnification of 5µm, except for C3 (2 µm). D1 to D3 show cells incubated with preeclamptic plasma and 1000µM KI. Images are representative of one assay and were captured throughout the entirety of microscope field.

Figure 3 – Total antioxidant capacity (A) and heme oxygenase-1 (B). Total antioxidant capacity (A) and heme-oxygenase-1 quantification (B). Data shown as mean ± SEM of quadruplicate wells per assay.

LIST OF FIGURES

1)



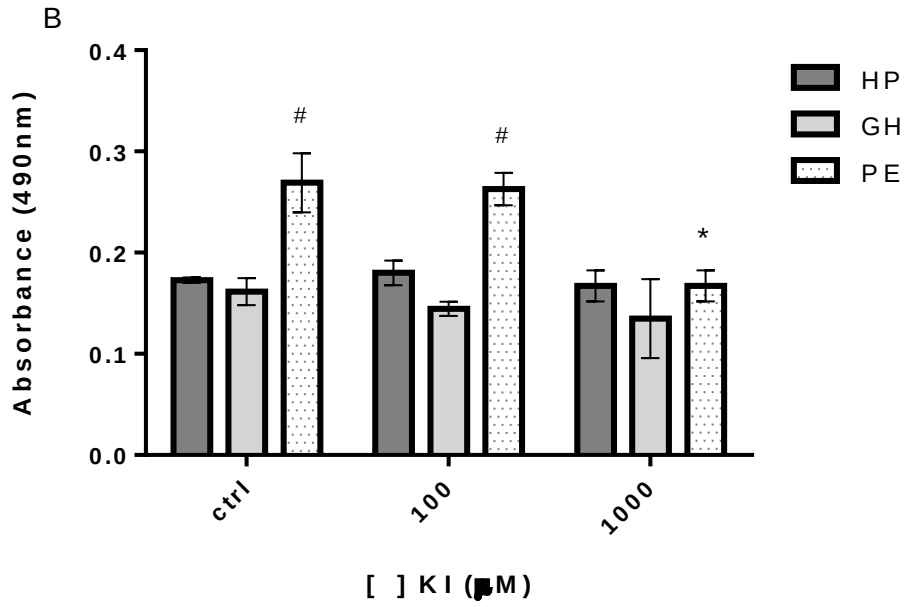


Figure 2 – Transmission electron microscopy images of endothelial cells incubated with pregnant women plasma.

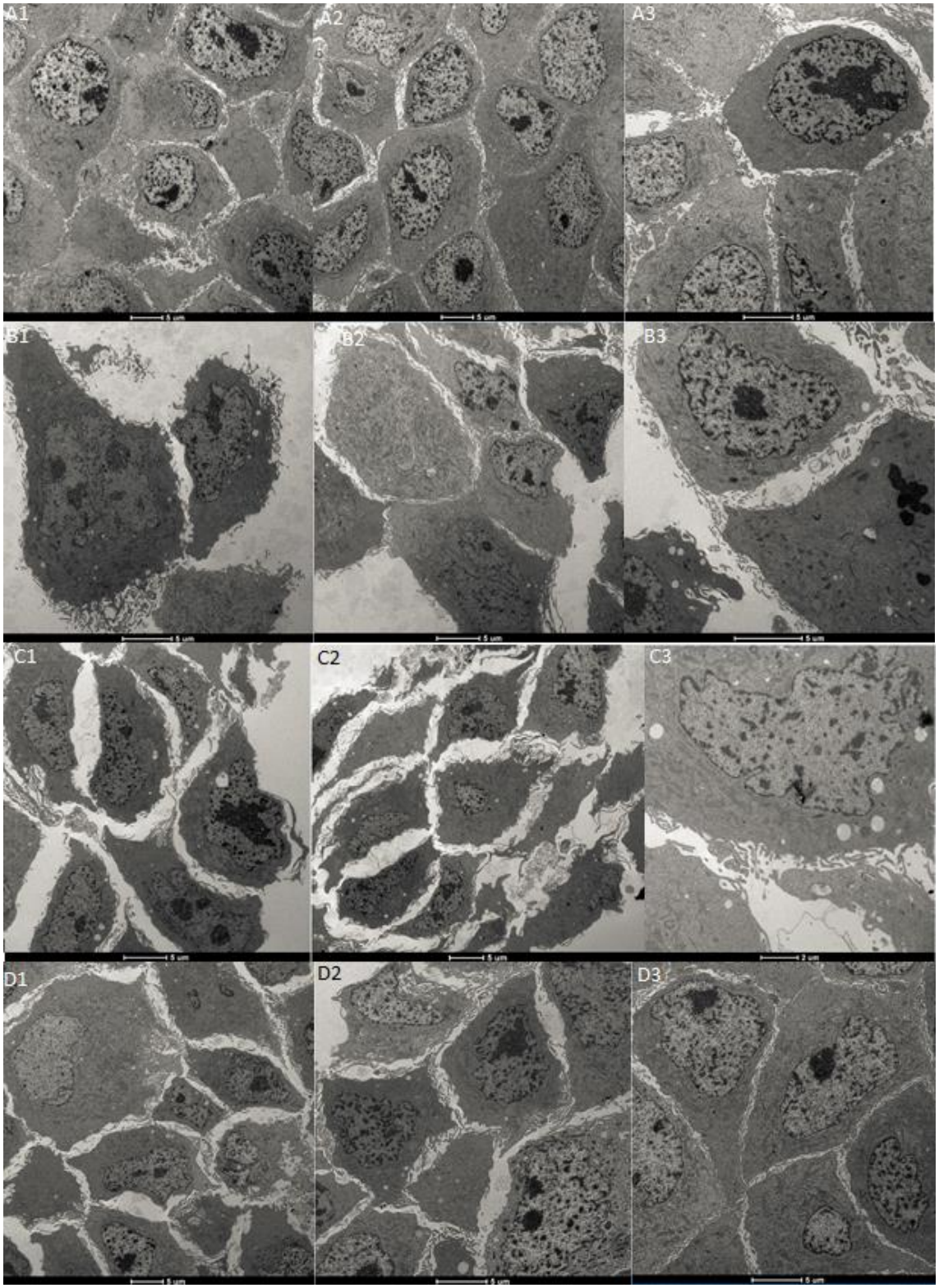


Figure 3 – Total antioxidant capacity (A) and heme-oxygenase 1 (B).

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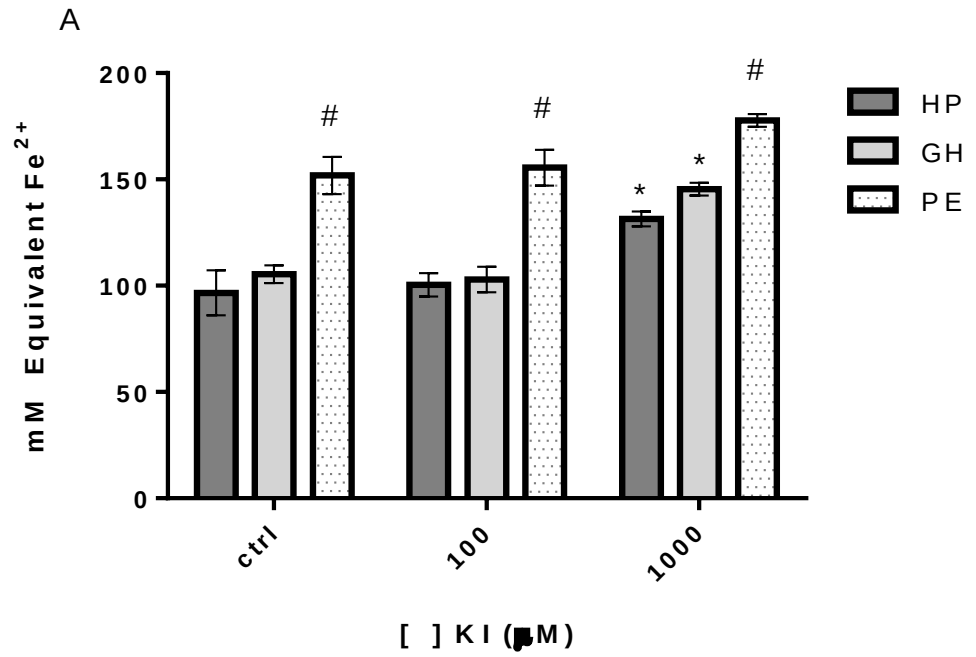
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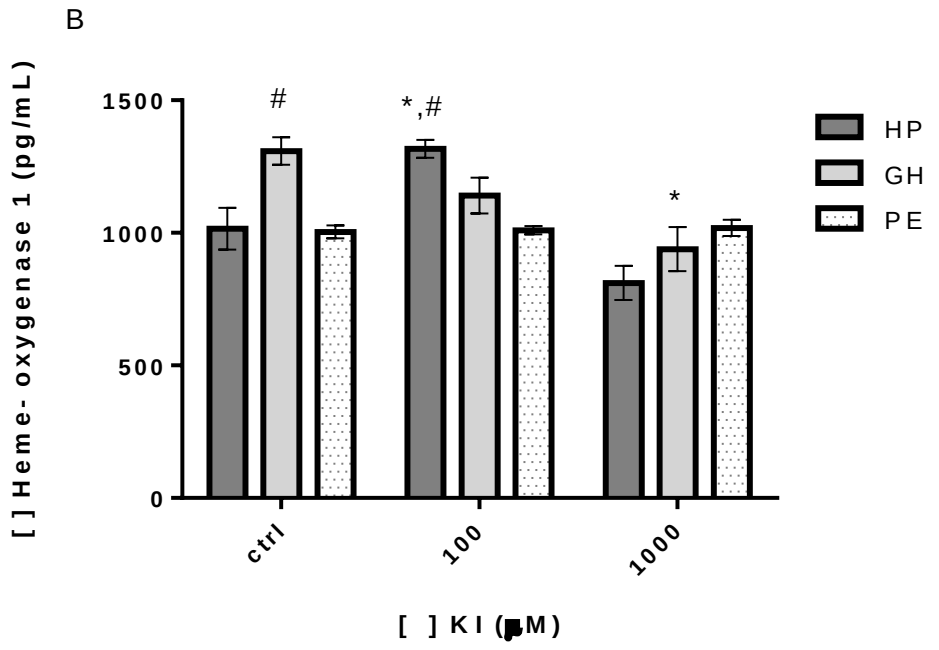
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