UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Medicina

Programa de Pós-Graduação em Ciências da Saúde: Ginecologia e Obstetrícia

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“Que nada nos limite. Que nada nos defina. Que nada nos sujeite. Que a liberdade seja nossa própria substância.”

Simone de Beauvoir

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SUMÁRIO

LISTA DE ABREVIATURAS

ADP	Adenosine 5’-diphosphate (5’-difosfato de adenosina)
AMP	Adenosine monophosphate (adenosina monofosfato)
AMPc	Cyclic adenosine monophosphate (adenosina monofosfato cíclico)
ATP	Adenosine triphosphate (adenosina trifosfato)
AVC	Acidente vascular cerebral
COX 2	Ciclooxigenase 2
ERα	Estrogen receptor alpha (receptor de estrogênio tipo alfa)
ERβ	Estrogen receptor beta (receptor de estrogênio tipo beta)
FII	Fator de coagulação II
FSH	Follicle-stimulating hormone (hormônio folículo estimulante)
FX	Fator de coagulação X
FXa	Fator de coagulação X ativado
HDL	High-density lipoprotein (lipoproteína de alta densidade)
KKS	Kallikrein-kinin system (sistema calicreína-cinina)
LDL	low-density lipoprotein (lipoproteína de baixa densidade)
NF-kB	Nuclear factor kappa B (fator nuclear kappa B)
NO	Nitric oxide (óxido nítrico)

PAI-1 Plasminogen activator inhibitor -1 (Inibidor do ativador de plasminogênio -1)

PAR-1 Platelet protease activator-1 (ativador de protease plaquetária-1)

PDTC Pyrrolidine dithiocarbamate (ditiocarbamato de pirrolidina)

PI3K-AKT Phosphatidylinositol 3-kinase - protein kinase B (fosfatidilinositol 3 quinase - proteína quinase B)

SHR Spontaneously hypertensive rat (ratos espontaneamente hipertensos)

uPA Urokinase plasminogen activator (ativador de plasminogênio urocinase)

WKY Wistar Kyoto

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RESUMO

Mulheres na pós-menopausa têm um risco aumentado de desenvolver doenças cardiovasculares, como eventos pró-trombóticos associados à hipertensão. Embora o estrogênio pareça ter efeitos protetores em mulheres no período pré-menopausa, os mecanismos moleculares envolvidos nos eventos trombóticos após a queda dos níveis de estrogênio ainda não foram explicados. O objetivo deste trabalho foi investigar como plaquetas e a aorta contribuem para criar e manter um estado pró-trombótico em um modelo animal de hipertensão pós menopausa em ratas ovariectomizadas. Para tal, ratas espontaneamente hipertensas (SHR) e ratas Wistar Kyoto (WKY) normotensas, ambas com idade de 14 semanas, foram submetidas à ovariectomia bilateral e mantidas com dieta livre de fitoestrógenos. Então, diferentes parâmetros relacionados à função vascular, reatividade plaquetária e coagulação sanguínea foram analisados. A depleção dos níveis de estrogênio levou à hiper-reatividade plaquetária tanto em animais SHR quanto WKY. Acredita-se que o mecanismo envolvido esteja relacionado com a diminuição da expressão de COX2 na aorta e redução na velocidade de hidrólise de nucleotídeos como AMP, ADP e ATP presentes no soro e na superfície das plaquetas. O plasma das ratas ovariectomizadas apresentou um potencial pró-coagulante, o que foi confirmado através do aumento na geração de calicreína e FXa detectados na superficie de anéis de aorta. Ainda, observou-se que anéis de aorta obtidos dos animais SHR ovariectomizados tinham um potencial maior de gerar trombina do que anéis equivalentes de animais WKY. Nesse caso, o mecanismo envolvido pode estar relacionado ao aumento da expressão de fator tecidual e outros marcadores da via extrínseca da cascata de coagulação (FII, FX e PAR-1) e de fibrinólise (uPA e PAI-1) na aorta e plaquetas. Além disso, células de músculo liso de aorta pré-

tratadas em cultura com um *pool* de plasma obtido dos animais ovariectomizados desenvolveram um perfil pró-coagulante e um aumento da expressão de fator tecidual tempo-dependente. Esse perfil pró-coagulante induzido nas células de músculo liso é dependente de uma sinalização inflamatória, ja que o PDTC, um inibidor de NF-kB, atenuou a atividade pró-coagulante na superfície das células e reduziu a expressão do fator tecidual. Portanto, a diminuição dos níveis de estrogênio induz um fenótipo pró-trombótico tanto em animais normo quanto hipertensos, o que parece estar associado com uma hiperreatividade plaquetária e aumento da expressão do fator tecidual na aorta e nas plaquetas. O mecanismo envolve uma via de sinalização pró-inflamatória que oferece suporte a uma maior geração de trombina na aorta e nas células vasculares.

Palavras-chave: trombose, hipertensão, fator tecidual, plaquetas, estrogênio, ovariectomia, menopausa.

ABSTRACT

The risk of cardiovascular diseases such as the association of hypertension and prothrombotic events increase in postmenopausal women. Although estrogen seems to have some protective effects in premenopausal period, the molecular mechanisms underlying thrombotic events after estrogen depletion still remains obscure. The aim of the present study was to investigate how platelets and aorta contributes to create and maintain a prothrombotic state in an experimental model of postmenopausal hypertension in ovariectomized rats. For this purpose, a bilateral ovariectomy was performed in 14-week-old female spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats maintained with a phytoestrogen free diet. Then, vascular parameters as well as molecular mechanisms of platelet, coagulation and aortic prothrombotic functions were accessed. Estrogen depletion induced an exacerbated platelet aggregation response in both SHR and WKY animals. The mechanism involved seems to be related to a decrease in aortic COX2 expression and reduction in AMP, ADP and ATP hydrolysis by 5'-nucleotidases in serum and platelet surface. A procoagulant potential was observed in plasma from ovariectomized rats and this was confirmed by the increase of kallikrein and FXa generation in aortic rings. Interestingly, aortic rings derived from ovariectomized SHR presented a greater thrombin generation capacity compared to equivalent rings from WKY rats. In this case, the mechanism involved seems to be related to the increase in tissue factor expression and other markers of extrinsic coagulation (FII, FX and PAR-1) and fibrinolysis (uPA and PAI-1) activation pathways in aorta and platelets. Similarly, aortic smooth muscle cells pre-treated with a plasma pool derived from estrogen depleted animals developed a procoagulant profile with a time-dependent increase in tissue factor expression. This procoagulant profile induced in aortic

smooth muscle cells was dependent of an inflammatory signalling, since PDTC, a NFκB inhibitor, attenuated the procoagulant activity and tissue factor expression. In summary, estrogen deficiency induces a prothrombotic phenotype in both normo and hypertensive rats which was associated with platelet proaggregatory ability and an increase in tissue factor expression in aorta and platelets. The mechanism involves a proinflammatory signalling that support greater thrombin generation on aorta and vascular smooth muscle cells.

Keywords: Thrombosis, hypertension, tissue factor, platelets, estrogen, ovariectomy, menopause.

INTRODUÇÃO

As doenças cardiovasculares figuram dentre uma das principais causas de morte no mundo (Oliveira, 2020; WHO, 2021) e também no Brasil (Oliveira, 2020). Os fatores de risco para essas doenças são muitos, mas sabe-se que a idade é um dos principais (Heit, 2008; Oliveira, 2020). Quando se fala de doenças cardiovasculares na população feminina especificamente, a menopausa também ganha um papel importante.

A menopausa pode ser caracterizada pela diminuição dos níveis circulantes de estrogênio (Bacon, 2017), e as mudanças fisiológicas desse período contribuem para o aumento do risco de desenvolvimento de doenças cardiovasculares, como o tromboembolismo (El Khoudary, 2018; Melo, 2017). A prevalência de hipertensão arterial em mulheres no período pós-menopausa é um fatores de risco importantes para o desenvolvimento dessas doenças (Brahmbhatt, 2019).

Este trabalho foi realizado com o objetivo de avaliar algumas das consequências da diminuição de estrogênio no que diz respeito à ocorrência de eventos pró-trombóticos em um modelo animal.

REVISÃO DA LITERATURA

1 Estratégia para localizar e selecionar informações

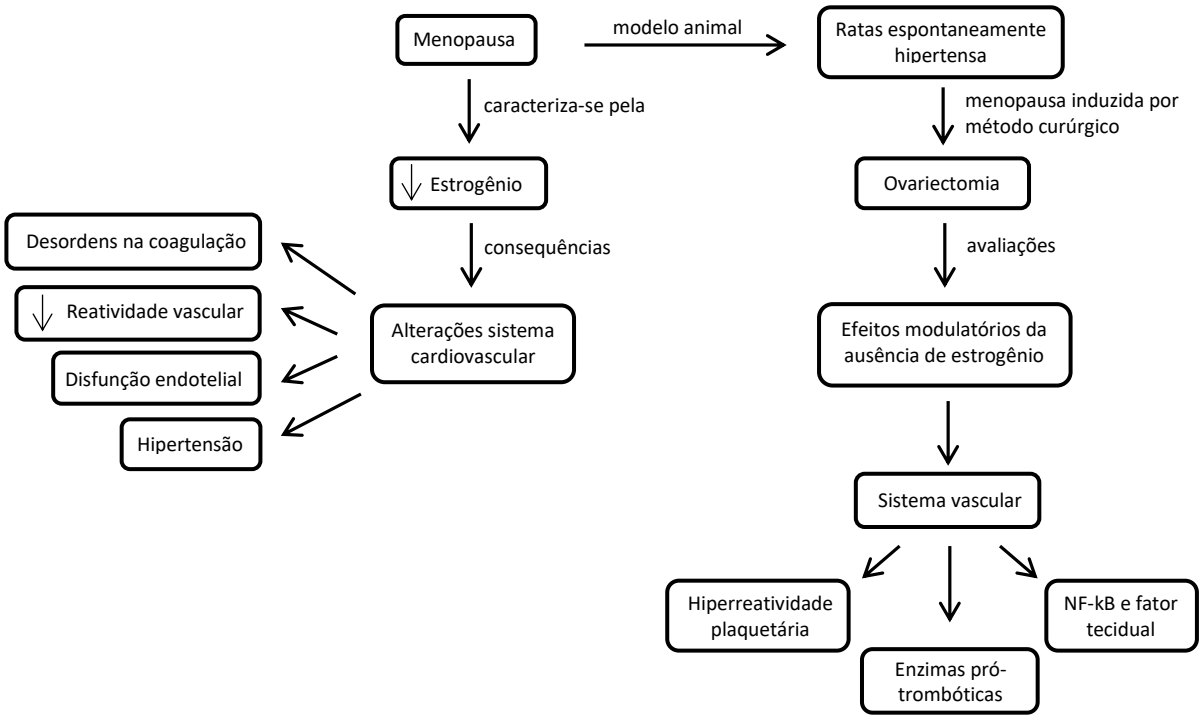
Para elaboração da revisão da literatura deste trabalho, foi realizada uma busca eletrônica por artigos em bases de dados no período entre fevereiro de 2021 e dezembro de 2021. As bases de dados utilizadas foram *Pubmed* e *Scielo* e os seguintes descritores foram cruzados entre si: *menopause and cardiovascular disease; menopause and hypertension; animal model and hypertension; animal model and menopause; hemostasis and blood coagulation and tissue fator; estrogen and thrombosis.*

Depois de concluída a busca, os artigos foram selecionados primeiramente a partir de uma leitura prévia dos resumos, excluindo-se os que não condiziam com o tema deste trabalho. Para os artigos restantes, priorizaram-se aqueles publicados nos últimos 15 anos e foi realizada uma leitura mais detalhada dos trabalhos. Também buscou-se informações em livros físicos e no site oficial da Organização Mundial da Saúde (em inglês, OMS). Ao final, foram utilizadas 27 referências, dentre artigos (23), livros (1), teses (1) e meios *online* (1).

Na tabela a seguir, descrevem-se resumidamente os resultados das buscas de artigos nas bases de dados:

Descritores	*PubMed*	*SciELO*	Artigos utilizados
Menopause and cardiovascular disease	11.374 artigos	49 artigos	9 artigos
Menopause and hypertension	3.479 artigos	60 artigos	3 artigos
Animal model and hypertension	22.226 artigos	18 artigos	3 artigos
Animal model and menopause	1.891 artigos	2 artigos	3 artigos
Hemostasis and blood coagulation and tissue factor	11.775 artigos	6 artigos	4 artigos
Estrogen and thrombosis	2.251 artigos	6 artigos	4 artigos

2 Mapa conceitual



3 Epidemiologia da doença cardiovascular e tromboembólica na menopausa

Doenças cardiovasculares, como infarto do miocárdio, acidente vascular cerebral (AVC) e tromboembolismo, são a principal causa de mortes no mundo (ESHRE, 2006). Segundo dados da Organização Mundial da Saúde, a estimativa é de que em 2019 cerca de 17,9 milhões de pessoas tenham morrido em decorrência dessa causa, o que representa 32% dos óbitos daquele ano (Oliveira, 2020; WHO, 2021).

No Brasil, cerca de 30% dos óbitos relacionados a doenças crônicas não transmissíveis referem-se a doenças cardiovasculares, especialmente as doenças coronariana e cerebrovascular. Segundo estudo de 2017, a prevalência de doença cardiovascular na população brasileira é de 6025 casos em 100.000 habitantes (Oliveira, 2020).

O tromboembolismo é considerado a terceira causa de morte de origem cardiovascular após a doença coronariana e acidente cérebro vascular (da Silva, 2021; Heit, 2008; Raymundo, 2019). O tromboembolismo é uma doença grave, com alto risco de mortalidade, especialmente o tromboembolismo pulmonar (da Silva, 2021; Heit, 2008). No Brasil, muitos estudos sobre a incidência desses eventos são realizados através de autópsias, nos levando a inferir que o número de casos possa estar subestimado (da Silva, 2021).

As doenças cardiovasculares são também responsáveis por uma sobrecarga financeira importante para os sistemas de saúde, de forma que o estudo sobre o tema ganha ainda mais relevância. Sabe-se que a idade é um fator de risco importante para o aparecimento dessas doenças (Heit, 2008; Oliveira, 2020), mas,

para as mulheres, as variações hormonais da menopausa parecem também influenciar sobre esse processo.

A menopausa é definida como o período final da fase reprodutiva da mulher, quando ocorre uma série de alterações hormonais. A menopausa natural é diagnosticada após um período de 12 meses de amenorreia, no qual há redução no número de folículos ovarianos e diminuição da secreção de hormônios ovarianos. O período anterior à menopausa é chamado de transição menopausal, caracterizado por ciclos menstruais irregulares, diminuição dos níveis de estrogênio e aumento dos níveis de hormônio folículo estimulante (em inglês *follicle-stimulating hormone*, FSH). O período pós-menopausa se estende até o final da vida da mulher (Bacon, 2017).

As modificações do período pós-menopausa incluem mudanças no perfil de distribuição do tecido adiposo e desenvolvimento de processos ateroscleróticos que contribuem para o risco de desenvolvimento de doenças cardiovasculares (Melo, 2017). Essas mudanças hormonais que ocorrem na menopausa estão ligadas ao aumento na incidência de doenças cardiovasculares. Alguns trabalhos, como Marko (2018) e Bacon (2017), sugerem um efeito cardioprotetor do estrogênio. Dentre os efeitos, está o aumento do HDL e diminuição do LDL e melhora das funções endoteliais das coronárias. Há também evidências de os baixos níveis de estrogênio nesse período contribuem para o aumento do risco de eventos cardiovasculares.

O trabalho de El Khoudary (2018) avaliou uma série de estudos que mostraram outros efeitos do período pós-menopausa na saúde da mulher. Há evidências de as mudanças hormonais aumentam o risco de desenvolver a chamada síndrome metabólica, que envolve hipertensão, dislipidemia, diabetes

melittus e aumento na gordura abdominal, aumento da proteína C reativa (estabelecendo um estado pró-inflamatório) e um estado pró-trombótico (ESHRE 2006; Khoudary, 2018). A ocorrência de eventos trombóticos pode estar relacionada com a idade em que ocorreu a menopausa – mulheres que tiveram menopausa precoce ou menopausa tardia aparentam ter maior risco de apresentar distúrbios trombóticos (Canonico, 2014). Há também aumento de depósitos de gordura próximo ao coração, risco de disfunções endoteliais e rigidez endotelial (Khoudary, 2018).

Uma das alternativas para diminuir os riscos das doenças cardiovasculares é o uso de fármacos, como estatinas e anticoagulantes, porém observa-se que os benefícios desse tratamento não são iguais para homens e mulheres, o que pode estar associado às mudanças hormonais da menopausa (Marko, 2018).

4 Eventos pró-trombóticos e hipertensão arterial na menopausa

A hipertensão é um dos principais fatores de risco para o desenvolvimento de doenças cardiovasculares (Brahmbhatt, 2019). Vários estudos mostram que, na mulher, a prevalência de hipertensão é maior na pós-menopausa e indicam que o estrogênio tem um papel importante na regulação da pressão arterial (Abramson, 2014; Brahmbhatt, 2019; di Giosia, 2018). Os dados do *guideline* sobre hipertensão de 2018 mostram que há uma prevalência baixa de hipertensão em mulheres na fase reprodutiva, os seja, pré-menopausa. Porém, a partir dos 45 anos, há um aumento nessa prevalência (Brahmbhatt, 2019; di Giosia, 2018). A estimativa é que a prevalência de hipertensão em mulheres com mais de 65 anos seja de aproximadamente 60% (Abramson, 2014; di Giosia, 2018).

Foi observado em modelos animais que há no endotélio vascular e células de músculo liso receptores de estrogênio e que esse hormônio pode contribuir na modulação da pressão arterial através do aumento de óxido nítrico (NO), que tem ação vasodilatadora (Abramson, 2014; Brahmbhatt, 2019; di Giosia, 2018). O estrogênio também é capaz de aumentar a síntese de monofosfato de adenosina cíclico (AMPc), cuja ação aumenta a concentração de cálcio intracelular nas células endoteliais, contribuindo com a vasodilatação. Assim, a redução nos níveis de estrogênio na menopausa pode explicar o porquê mulheres nessa fase da vida tendem a desenvolver hipertensão (Brahmbhatt, 2019).

Há também estudos que indicam que o estrogênio possui influência sobre o sistema renina-angiotensina-aldosterona e sistema simpático (Abramson, 2014; di Giosia, 2018). Tem-se observado que o estrogênio é capaz de aumentar a síntese de angiotensina através da regulação de genes específicos. A revisão feita por di Giosia (2018) traz estudos que indicam que as mulheres, quando comparado a homens, possuem a expressão reduzida de receptores tipo I de angiotensina tipo II, enzima conversora de angiotensina e renina plasmática. Associado a isso, a redução dos níveis de NO e maior atividade de angiotensina II causa aumento da reabsorção de sódio nos rins e consequentemente aumento da pressão arterial.

Está descrito na literatura sobre o aumento na concentração de um peptídeo vasoconstritor chamado endotelina em mulheres pós-menopausa, isso porque o estrogênio tem um efeito inibidor sobre a síntese de endotelina. Esse peptídeo contribui para o aumento da pressão arterial principalmente ao se ligar no receptor de endotelina tipo A, localizado no músculo liso vascular, causando vasoconstrição. O aumento da proporção de hormônios andrógenos na mulher, após redução dos

níveis de estradiol na menopausa, também está relacionado ao aumento da pressão arterial, pois maiores níveis de testosterona promovem a produção de angiotensinogênio (Abramson, 2014).

A complacência arterial é uma característica que se refere à distensibilidade das grandes artérias. A complacência arterial é menor em mulheres do que em homens e uma maior rigidez das artérias pode estar associado a um maior risco de doenças cardiovasculares e arterioscleróticas (Brahmbhatt, 2019; di Giosia, 2018). Em mulheres de idade mais avançada, já na menopausa, observa-se que a complacência arterial é ainda menor, o que se relaciona ao fato de que mulheres possuem uma maior hipertensão sistólica nesse período comparado aos homens (di Giosia, 2018). Isso pode acarretar em um maior risco de desenvolver hipertrofia do ventrículo esquerdo – fator que é tido como causa de doenças coronarianas e AVC (Abramson, 2014).

5 Modelos experimentais de hipertensão e menopausa

O emprego de modelos animais na área da pesquisa é importante para o estudo da etiologia, fisiopatologia e complicações de doenças e para avaliar a eficácia e mecanismo de ação de tratamentos. Quando comparado a estudos com humanos, os modelos animais apresentam como vantagens o fato de ser possível o controle de fatores como dieta e o ambiente em que se encontram, além de ser possível a obtenção de amostras biológicas para estudos experimentais detalhados (Leong, 2015).

No que diz respeito ao estudo da hipertensão, há vários modelos animais, cada um deles relacionados a um fator etiológico que contribui para a causa da hipertensão nos seres humanos, uma vez que essa doença é multifatorial (Leong, 2015; Sarikonda, 2009).

Em 1963, Okamoto e Aoki apresentaram um novo modelo animal para estudos de hipertensão. São os chamados ratos espontaneamente hipertensos (SHR). Trata-se de uma cepa obtida através de cruzamento entre animais em que a hipertensão não é causada por intervenções fisiológicas, farmacológicas ou cirúrgicas (Leong, 2015). Nesses animais, a hipertensão é uma característica genética e a fisiopatologia da doença se assemelha bastante ao que é observado na hipertensão primária em seres humanos. Esses animais são um dos principais modelos animais para estudo da hipertensão (Leong, 2015; Sarikonda, 2009).

A hipertensão nos animais SHR é observada a partir da quarta semana de vida, atingindo geralmente de 180-200mmHg. Além da hipertensão em si, os animais podem apresentar outras disfunções consequentes dessa condição, como hipertrofia cardíaca, insuficiência cardíaca e disfunção renal (Leong, 2015). Há, dentre os animais SHR, várias linhagens que foram criadas ao longo dos anos, diferentes entre si por questões genéticas ou mecanismos neuro-humorais, mas com a característica central mantida: serem espontaneamente hipertensos (Sarikonda, 2009).

Outros modelos mimetizam diferentes aspectos da hipertensão. Em 1934, foi desenvolvido um modelo animal para hipertensão renovascular, onde há aumento da concentração de renina circulante e consequente aumento de angiotensina I, convertida a angiotensina II, um potente vasoconstritor. Nesse modelo, há o

clampeamento de pelo menos uma das artérias renais, realizado de maneira cirúrgica. Também é possível trabalhar com animais cujos fatores para o aumento da pressão arterial são a deficiência da enzima oxido nítrico sintase, responsável pela produção de óxido nítrico ou então animais submetidos à administração de angiotensina II, administração de dietas específicas ou de estressores ambientais (Leong, 2015, Sarikonda, 2009).

Da mesma forma, quando se fala em menopausa, modelos animais são importantes para o estudo dessa condição e das consequências dela na vida da mulher. Buscam-se modelos que se aproximem ao máximo do perfil endócrino e neuroendócrino observado em mulheres. Os modelos que utilizam ratos e camundongos são os mais usados, pois além das facilidades na manipulação, a função ovariana é bastante conhecida (Wu, 2015).

As fêmeas apresentam ciclos estrais rápidos e sucessivos, que duram cerca de quatro a cinco dias e que se assemelham ao ciclo menstrual das mulheres no que diz respeito às mudanças hormonais. Em um determinado período da vida, esses ciclos passam a ser irregulares, e há baixa nos níveis de hormônios sexuais. A menopausa pode ser provocada de maneira química, através do uso de 4-vinilciclohexano diepóxido, um composto capaz de causar depleção folicular acelerada em roedoras, levando à falência ovariana. Esse modelo químico é tido como um bom modelo para o estudo da menopausa, pois há atresia folicular e esses folículos são mantidos nos animais, tornando o perfil hormonal mais similar ao que ocorre no período pós-menopausa das mulheres (Koebele, 2016).

Um dos modelos mais usado para estudo da menopausa é a ovariectomia, que induz a menopausa de maneira cirúrgica. Esse modelo é tido como ideal

quando o objetivo do estudo é avaliar os efeitos da ausência de hormônios gonadais, além de ser uma boa alternativa quando o objetivo do estudo for avaliar o efeito de tratamentos hormonais na menopausa (Koebele, 2016). Além disso, conforme trazido pelo estudo de Medina-Contreras (2020), a ovariectomia é um bom modelo de estudo para avaliações referentes à síndrome metabólica, pois, após o procedimento, as fêmeas passam a apresentar aumento da gordura visceral e desenvolver distúrbios metabólicos como a resistência à insulina, dislipidemia e obesidade.

6 Hemostasia e a via do fator tecidual

Hemostasia é definida como uma resposta fisiológica normal do organismo que tem como objetivo interromper sangramentos provenientes de lesões vasculares. Ela é composta por uma série de eventos que envolvem os vasos sanguíneos, plaquetas, fatores de coagulação, fatores de anticoagulação, proteínas da fibrinólise e seus inibidores (Zago, 2013).

Sempre que a parede de um vaso sanguíneo é rompida, ocorre a ativação do sistema de coagulação. Há a formação de um tampão de plaquetas em um primeiro momento e após uma série de mecanismos são ativados de maneira coordenada para evitar a perda de sangue. É preciso haver um ajuste fino entre os fatores coagulantes e anticoagulantes – em condições normais, os mecanismos anticoagulantes estão mais ativos. Quaisquer distúrbios que interfiram nesse sistema podem resultar em hemorragias ou formação de trombos (Dahlbäck, 2000).

As células endoteliais que constituem a parede interna dos vasos sanguíneos e tem papel importante na manutenção da hemostasia (Figura 1). Elas são capazes de regular o tônus vascular e garantir uma superfície antitrombótica para o fluxo sanguíneo. São nas células endoteliais que estão armazenadas proteínas como o fator de von Willebrand e p-selectina. Além disso, o endotélio produz substâncias como: óxido nítrico e prostaciclina – importantes vasodilatadores que inibem a função plaquetária; glicosaminoglicanos, componentes da via da proteína C e inibidores da via do fator tecidual – que inibem fatores da cascata de coagulação. Porém, em casos de lesão direta, inflamação ou infecções, o endotélio passa a apresentar funções pro-trombóticas (Zago, 2013).

A exposição da matriz extracelular causa a atração de plaquetas para a lesão, que mudam seu formato, tornando-se mais esféricas e emitindo pseudópodos (Figura 1). Essa alteração de forma induz que outras plaquetas se mobilizem para o local da lesão, liguem-se ao Fator de von Willebrand e colágeno presentes na lesão vascular, promovendo a adesão plaquetária. Substâncias como colágeno, trombina e adrenalina, liberadas no momento da lesão do vaso, ajudam a promover a ativação e agregação plaquetária propriamente ditas, formando um tampão no local onde houve lesão vascular. A ativação plaquetária também é importante para a ligação de fatores de coagulação na superfície das plaquetas, pois promove um rearranjo nos fosfolipídeos de membrana, onde fatores de coagulação ativados poderão se ligar, dando sequência ao processo de coagulação (Dahlbäck 2000; Löwenberg 2010; Oliveira, 2009).

Simultaneamente, há o chamado processo de iniciação da cascata de coagulação, com a ação do fator tecidual, ativando o fator de coagulação VIIa que

induz a ativação de pequenas quantidades de fatores IX e X (Dahlbäck 2000) (Figura 1). O fator Xa liga-se ao fator Va (proveniente de grânulos das plaquetas) e converte uma pequena quantidade de protrombina em trombina. A trombina formada é o que inicia a próxima etapa do processo, chamada de amplificação.

A trombina formada na etapa anterior tem papel fundamental no processo de coagulação: atua na ativação de plaquetas – o que expõe receptores para fatores de coagulação ativados, ativa fatores de coagulação, como fator V e fator VIII. A dissociação entre fator VIII e fator de von Willebrand nesta etapa deixa mais fator de von Willebrand livre para mediar a adesão e agregação plaquetária. Já a ativação do FVIII contribui para a formação de FXa, As plaquetas ativadas ficam com fosfolipídeos carregados negativamente expostos, onde se ligam os fatores de coagulação. A trombina também promove a formação do fator XIa na superfície das plaqueta (Dahlbäck 2000; Oliveira, 2009) (Figura 1).

A próxima etapa ocorre na superfície das plaquetas e é chamada de propagação. O fator XIa liga-se ao fator VIIIa. O fator Xa se associa ao fator Va na superfície das plaquetas, formando o complexo protrombinase. Esse complexo promove a formação de grandes quantidades de trombina, responsável pela conversão de fibrinogênio em fibrina. A trombina formada ainda é responsável pra ativação do fator XIII que modifica a estrutura da fibrina, levando a estabilização dessa rede de fibrina e, consequentemente, do coágulo (Dahlbäck 2000; Oliveira, 2009) (Figura 1).

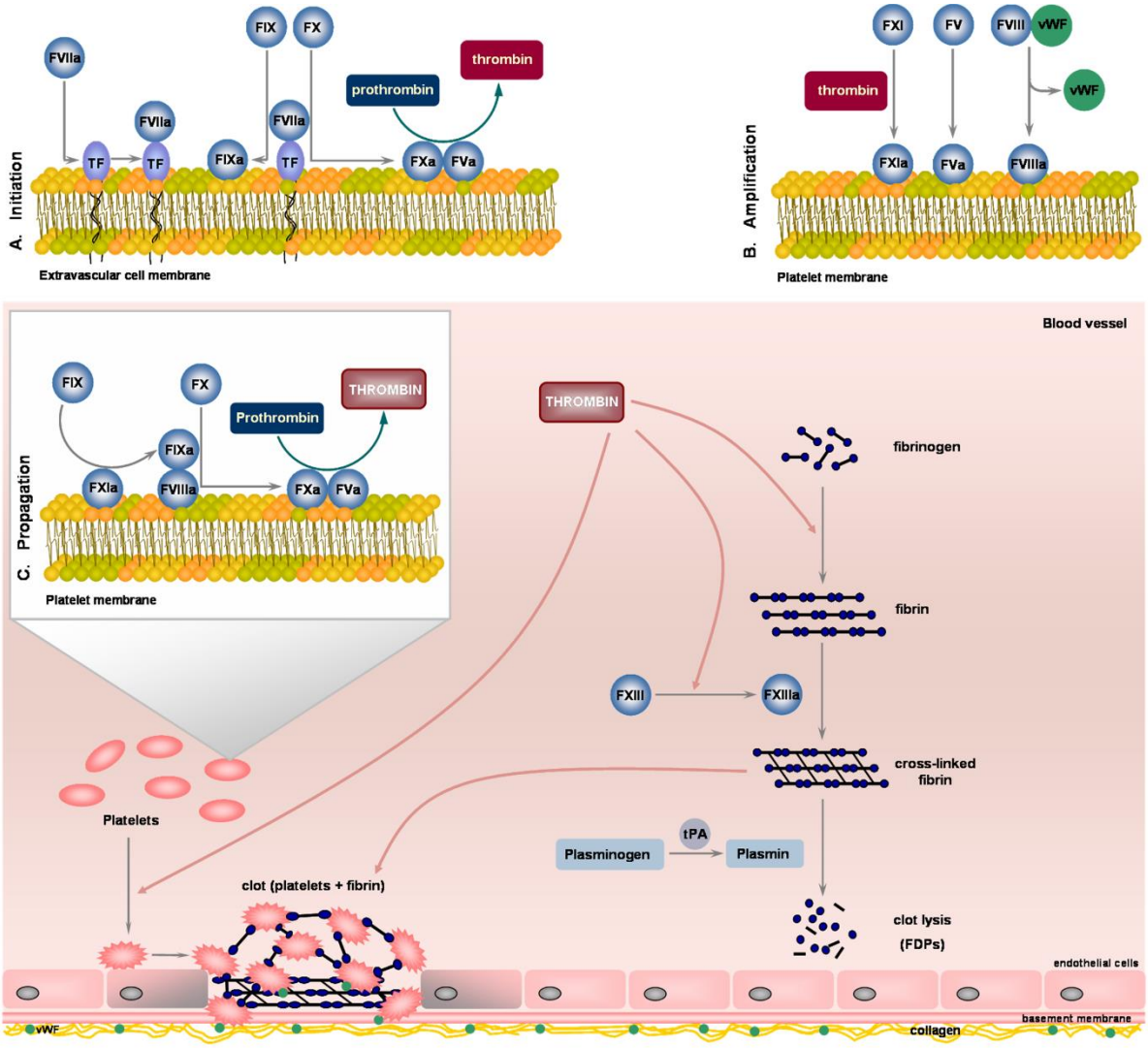


Figura 1. Modelo da coagulação sanguínea (Pinto, 2010).

7. Ações vasculares do estrogênio e sua relação com eventos pró-trombóticos

Apesar da contribuição para a manutenção da hemostasia, as plaquetas também podem estar envolvidas em desordens patológicas que podem culminar com a formação de trombos. Fatores como a taxa de cisalhamento sanguíneo, disfunção de células endoteliais e processos inflamatórios contribuem para que haja a formação de trombos. Em condições normais, as plaquetas não interagem com o endotélio, porém, em estados inflamatórios é comum que haja a ativação das

plaquetas, que ficam mais suscetíveis a aderir ao endotélio. Além disso, as próprias células do endotélio podem apresentar um estado de inflamação, permitindo a adesão das plaquetas. Também, uma maior taxa de cisalhamento pode levar a um aumento na concentração de fator de von Willebrand disponível para ligação às plaquetas, levando a formação de coágulos que podem causar um AVC ou infarto. Da mesma forma, as plaquetas podem contribuir para a formação de placas ateroscleróticas – o rompimento de uma placa arteroesclerótica expõe as estruturas do endotélio as quais as plaquetas aderem-se (Löwenberg, 2010).

Um ateroma é formado a partir de pequenas lesões nos vasos sanguíneos, onde ocorre o acúmulo de macrófagos e, posteriormente, de células de gordura, que são estabilizados por colágeno. Entretanto, pode haver ruptura dessa rede de proteção de colágeno, desestabilizando o ateroma ao ponto que parte dele pode se desprender, originando um trombo (ESHRE, 2006). O estrogênio tem um papel importante na etapa inicial da formação do ateroma, pois influencia no metabolismo de lipídeos de forma a evitar o depósito inicial das células de gordura. Entretanto, quando a placa de ateroma já está formada, o estrogênio aumenta a expressão de metaloproteinases da matriz presentes no ateroma – são essas enzimas as responsáveis pela degradação do colágeno que estabiliza a placa de gordura. Assim, embora o estrogênio tenha um efeito protetor do endotélio, pode também influenciar na formação de trombos (ESHRE 2006).

A revisão feita por Del Principe e colaboradores (2015) relata uma série de estudos que avaliaram a reatividade de plaquetas isoladas e, em sua maioria, as plaquetas isoladas de animais fêmeas apresentavam uma reatividade maior. A mesma situação foi observada em estudos sobre a atividade de plaquetas humanas.

Uma das razões para tal efeito é que as plaquetas isoladas de fêmeas teriam um maior número de sítios de ligação para fatores pró-trombóticos, como o fibrinogênio e trombina, por exemplo. Ainda, um dos estudos avaliados na revisão mostrou que esses sítios de ligação podem estar ainda mais aumentados em mulheres pós-menopausa quando comparado a mulheres pré-menopausa. Isso reforça a tese de que as mudanças hormonais ao longo da vida da mulher possam ter influência sobre eventos trombóticos, modificando de alguma forma os sistemas que controlam a hemostasia.

O principal hormônio sexual feminino, o estrogênio, tem mostrado um efeito cardioprotetor, conforme já abordado brevemente. Após a menopausa, ainda há síntese de estrogênio, em menor proporção, pelo tecido adiposo, cérebro e células do músculo liso do endotélio vascular. A ação do estrogênio sobre o sistema cardiovascular se dá através da ligação a diferentes tipos de receptores, sendo os mais estudados: receptor de estrogênio tipo alfa (ERα) e receptor de estrogênio tipo beta (ERβ) (Iorga, 2017). Diferentes ações ocorrem quando o estrogênio se liga aos diferentes receptores: em linhas gerais, tem-se que ao ligar-se aos ERα, há proliferação celular e inibição da apoptose, enquanto que a ligação aos ERβ induz diferenciação celular e apoptose.

Além disso, há receptores de estrogênio em diferentes estruturas celulares. Quando na membrana celular, os receptores ERα e ERβ estão localizados em *rafts* lipídicos e a ligação do estrogênio nesses receptores tem uma ação rápida, não genômica e não dependente de síntese de proteínas, promovendo, por exemplo, a vasodilatação através da ativação de proteínas quinases, que ativam a óxido nítrico sintase endotelial, promovendo o aumento de óxido nítrico. O estrogênio também é

capaz de ativar a via de sinalização PI3K-AKT, responsável por processos de proliferação celular e inibição de apoptose (Del Príncipe, 2015).

Esses receptores de estrogênio também fazem parte da membrana mitocondrial de células cardíacas e a ação do estrogênio nesses receptores contribui para a manutenção das funções dessa organela e tem ação cardioprotetora. A homeostase mitocondrial contribui para o controle da apoptose celular e da produção de espécies reativas de oxigênio, por exemplo (Del Principe, 2015; Iorga, 2017). Experimentos animais mostram que a presença de estrogênio diminuiu a área de infarto e aumentou a contratilidade cardíaca em ratas adultas, quando comparadas a animais ovariectomizadas. O estrogênio induziu a um aumento nos níveis da enzima aldeído desidrogenase 2, já conhecida por sua ação cardioprotetora (Iorga, 2017).

Estudos sobre a cardioproteção promovida pela ação sobre receptores ERα presente nas mitocôndrias mostraram que, camundongos *knockout* para ERα mitocondrial após isquemia cardíaca tiveram menor recuperação da área cardíaca, maior incidência de taquicardia e menor fluxo sanguíneo nas coronárias (Del Principe, 2015; Iorga, 2017).

JUSTIFICATIVA

A queda nos níveis circulantes de estrogênio está associada ao aumento do risco cardiovascular e pró-trombótico em mulheres na pós-menopausa. Mulheres hipertensas podem apresentar problemas de regulação da pressão arterial durante a fase de redução hormonal, incluindo aquelas já submetidas ao controle farmacológico tradicional para hipertensão (Giménez, 2006). Apesar do estrogênio ter um papel protetor no sistema vascular, o mecanismo celular e molecular pelo qual as plaquetas e células vasculares contribuem para o desenvolvimento de um perfil pro-trombótico durante a hipertensão na menopausa ainda não é totalmente conhecido. Portanto, acreditamos que este trabalho possa ser relevante para:

- Agregar conhecimento a cerca do mecanismo pelo qual o sistema de hemostasia funciona durante a menopausa e hipertensão e sobre qual é o papel das plaquetas, células vasculares e da via do fator tecidual nesse processo;
- Entender como a redução dos níveis de estrogênio altera o sistema de coagulação sanguínea e o balanço de enzimas pró-trombóticas nas plaquetas e na aorta;
- Identificar novos alvos terapêuticos potenciais que auxiliem no controle do risco cardiovascular durante a fase de redução hormonal e que possam ser testados futuramente em modelos pré-clínicos.

HIPÓTESES

Hipótese alternativa

A redução dos níveis de estrogênio modula a resposta de plaquetas e células vasculares induzindo um estado pro-trombótico em ratas hipertensas ovariectomizadas

Hipótese nula

A redução dos níveis de estrogênio não exerce nenhuma influência na resposta de plaquetas e células vasculares e, portanto, não altera o equilíbrio hemostático em ratas hipertensas ovariectomizadas.

OBJETIVOS

Objetivo principal

Investigar os efeitos modulatórios da ausência de estrogênio e sua associação com eventos pró-trombóticos em ratas ovariectomizadas espontaneamente hipertensas.

Objetivos secundários

- Estabelecer um modelo experimental de hipertensão na menopausa através do procedimento de ovariectomia em ratas espontaneamente hipertensas da linhagem SHR;
- Investigar os mecanismos de agregação plaquetária no plasma rico em plaquetas de ratas ovariectomizadas hipertensas;
- Investigar os mecanismos de coagulação sanguínea em ratas ovariectomizadas hipertensas;
- Investigar os mecanismos pró-coagulantes na superfície de anéis de aorta de ratas ovariectomizadas hipertensas;
- Investigar a expressão de diferentes fatores de coagulação, fibrinólise e inflamação em extratos de aorta de ratas ovariectomizadas hipertensas;
- Investigar o papel da via do fator tecidual em células de músculo liso de aorta tratadas com o plasma de ratas ovariectomizadas hipertensas;

- Investigar o papel da via do NF-kB na expressão de fator tecidual em células de músculo liso de aorta tratadas com o plasma de ratas ovariectomizadas hipertensas.

A seguir, os resultados obtidos nesta dissertação, que serão apresentados na forma de um artigo científico a ser submetido ao períodico Molecular and Cellular Endocrinology (IF 2020, 4.1).

REFERÊNCIAS

ABRAMSON, Beth L.; MELVIN, Rochelle G. Cardiovascular risk in women: focus on hypertension. **Canadian Journal of Cardiology**, v. 30, n. 5, p. 553-559, 2014.

BACON, Janice L. The menopausal transition. **Obstetrics and Gynecology Clinics**, v. 44, n. 2, p. 285-296, 2017.

BRAHMBHATT, Yasmin; GUPTA, Maitreyee; HAMRAHIAN, Seyed. Hypertension in premenopausal and postmenopausal women. **Current hypertension reports**, v. 21, n. 10, p. 1-10, 2019.

CANONICO, Marianne et al. Age at menopause, reproductive history and venous thromboembolism risk among postmenopausal women: The Women's Health Initiative Hormone Therapy clinical trials. **Menopause (New York, NY)**, v. 21, n. 3, p. 214, 2014.

DAHLBÄCK, Björn. Blood coagulation. **The Lancet**, v. 355, n. 9215, p. 1627-1632, 2000.

DA SILVA, Jaqueline Pinheiro et al. Perfil Epidemiológico do Tromboembolismo Pulmonar no Brasil de 2015 a 2019. **BEPA. Boletim Epidemiológico Paulista**, v. 18, n. 208, p. 1-10, 2021.

DEL PRINCIPE, Domenico et al. The relevance of estrogen/estrogen receptor system on the gender difference in cardiovascular risk. **International journal of cardiology**, v. 187, p. 291-298, 2015.

DI GIOSIA, Paolo et al. Gender differences in epidemiology, pathophysiology, and treatment of hypertension. **Current atherosclerosis reports**, v. 20, n. 3, p. 1-7, 2018.

EL KHOUDARY, Samar R.; THURSTON, Rebecca C. Cardiovascular implications of the menopause transition: endogenous sex hormones and vasomotor symptoms. **Obstetrics and Gynecology Clinics**, v. 45, n. 4, p. 641-661, 2018.

ESHRE CAPRI WORKSHOP GROUP. Hormones and cardiovascular health in women. **Human reproduction update**, v. 12, n. 5, p. 483-497, 2006.

GIMÉNEZ, José et al. 17β-Oestradiol enhances the acute hypotensive effect of captopril in female ovariectomized spontaneously hypertensive rats. Experimental Physiology, v. 91, n. 4, p. 715-722, 2006.

HEIT, John A. The epidemiology of venous thromboembolism in the community. **Arteriosclerosis, thrombosis, and vascular biology**, v. 28, n. 3, p. 370-372, 2008.

IORGA, Andrea et al. The protective role of estrogen and estrogen receptors in cardiovascular disease and the controversial use of estrogen therapy. **Biology of sex differences**, v. 8, n. 1, p. 1-16, 2017.

KOEBELE, Stephanie V.; BIMONTE-NELSON, Heather A. Modeling menopause: The utility of rodents in translational behavioral endocrinology research. **Maturitas**, v. 87, p. 5-17, 2016.

LEONG, Xin-Fang; NG, Chun-Yi; JAARIN, Kamsiah. Animal models in cardiovascular research: hypertension and atherosclerosis. **BioMed research international**, v. 2015, 2015.

LÖWENBERG, E. C.; MEIJERS, J. C.; LEVI, M. Platelet-vessel wall interaction in health and disease. **The Netherlands journal of medicine**, v. 68, n. 6, p. 242-251, 2010.

MARKO, Kathryn I.; SIMON, James A. Clinical trials in menopause. **Menopause**, v. 25, n. 2, p. 217-230, 2018.

MEDINA-CONTRERAS, J. M. L. et al. Ovariectomized rodents as a menopausal metabolic syndrome model. A minireview. **Molecular and Cellular Biochemistry**, v. 475, n. 1, p. 261-276, 2020.

MELO, Jorgileia Braga de et al. Fatores de risco cardiovasculares em mulheres climatéricas com doença arterial coronariana. **International Journal of Cardiovascular Sciences**, v. 31, p. 04-11, 2017.

OLIVEIRA, Gláucia Maria Moraes de et al. Estatística Cardiovascular–Brasil 2020. **Arquivos brasileiros de Cardiologia**, v. 115, p. 308-439, 2020.

OLIVEIRA, Markus Berger. Distúrbios de agregação plaquetária e coagulação sangüínea no envenenamento pela taturana Lonomia obliqua. 2009.

PINTO, Antônio FM et al. Lonomia obliqua venom: In vivo effects and molecular aspects associated with the hemorrhagic syndrome. Toxicon, v. 56, n. 7, p. 1103-1112, 2010.

RAYMUNDO, Selma Regina de Oliveira et al. O que mudou nas últimas décadas na profilaxia do tromboembolismo venoso em pacientes internados: artigo de revisão. **Jornal Vascular Brasileiro**, v. 18, 2019.

SARIKONDA, Kiran V. et al. Experimental animal models of hypertension. **Journal of the American Society of Hypertension**, v. 3, n. 3, p. 158-165, 2009.

WHO. **Cardiovascular diseases (CVDs)**, 2021. Página inicial. Disponível em <http:// https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)>. Acesso em 23 de nov. de 2021.

WU, Julie M. et al. Ovarian aging and menopause: current theories, hypotheses, and research models. **Experimental Biology and Medicine**, v. 230, n. 11, p. 818-828, 2005.

ZAGO, Marco A et al. Tratado de hematologia. São Paulo: Editora Atheneu, 2013.

ARTIGO EM INGLÊS

Estrogen depletion modulates aortic prothrombotic signalling in normotensive and spontaneously hypertensive female rats

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Abstract

The risk of cardiovascular diseases such as the association of hypertension and prothrombotic events increase in postmenopausal women. Although estrogen seems to have some protective effects in premenopausal period, the molecular mechanisms underlying thrombotic events after estrogen depletion still remains obscure. The aim of the present study was to investigate how platelets and aorta contributes to create and maintain a prothrombotic state in an experimental model of postmenopausal hypertension in ovariectomized rats. For this purpose, a bilateral ovariectomy was performed in 14-week-old female spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats maintained with a phytoestrogen free diet. Then, vascular parameters as well as molecular mechanisms of platelet, coagulation and aortic prothrombotic functions were accessed. Estrogen depletion induced an exacerbated platelet aggregation response in both SHR and WKY animals. The mechanism involved seems to be related to a decrease in aortic COX2 expression and reduction in nucleotide hydrolysis in serum and platelet surface. A procoagulant potential was observed in plasma from ovariectomized rats and this was confirmed by the increase of kallikrein and FXa generation in aortic rings. Interestingly, aortic rings derived from ovariectomized SHR presented a greater thrombin generation capacity compared to equivalent rings from WKY rats. In this case, the mechanism involved seems to be related to the increase in tissue factor expression and other markers of extrinsic coagulation (FII, FX and PAR-1) and fibrinolysis (uPA and PAI-1) activation pathways in aorta and platelets. Similarly, aortic smooth muscle cells pre-treated with a plasma pool derived from estrogen depleted animals developed a procoagulant profile with a time-dependent increase in tissue factor expression. This procoagulant profile induced in aortic smooth muscle cells was dependent of an inflammatory signalling, since PDTC, a NFκB inhibitor, attenuated the procoagulant activity and tissue factor expression. In summary, estrogen deficiency induces a prothrombotic phenotype in both normo and hypertensive rats which was associated with platelet hyperreactivity and an increase in tissue factor expression in aorta and platelets. The mechanism involves a proinflammatory signalling that support greater thrombin generation on aorta and vascular smooth muscle cells.

Keywords: Thrombosis, hypertension, tissue factor, platelets, estrogen, ovariectomy, menopause.

1. Introduction

The arterial vascular wall contributes to thrombotic complications in a variety of diseases, including hypertension, atherosclerosis, diabetes, stroke and sepsis (Esmon and Esmon, 2011; Lacolley et al., 2012). Besides vascular tone regulation, vascular wall cells (mainly endothelial and vascular smooth muscle cells) can generate a hypercoagulable state by supporting procoagulant enzyme formation (Pawlinski et al., 2004). Vascular wall also functionally modulates and interacts with tissue factor expressing cells that participates in thrombus growth such as platelets, neutrophils and monocytes (Grover and Mackman, 2018). In hypertension, experimental evidence points toward a hemostatic balance impairment in vasculature. Spontaneously hypertensive rats (SHR) exhibited accelerated FeCl3-induced thrombus formation in carotid arteries and its vascular smooth muscle cells (VSMCs) supported a greater thrombin generation capacity when compared to VSMCs from normotensive control rats (Ait-Aissa et al., 2015). Clinical studies also have reported higher plasma levels of D-dimers, fibrinogen, thrombin-antithrombin complex (TAT) and FVII in hypertensive patients than in normotensive individuals (Junker et al., 1998; Arikan and Sen, 2005) and plasma from hypertensive patients possess enhanced thrombin generation capacity compared healthy controls (Elias et al., 2019).

Concerning the prothrombotic events in postmenopausal women the contribution of cellular and molecular mechanisms involving vascular wall are still unclear. Gender differences in the risk of cardiovascular diseases are well known. Premenopausal women have lower risk than men at the same age, however, this apparent cardio protection disappear with the onset of menopause (Knowlton and Lee, 2012). Indeed, thromboembolic and coronary heart diseases are the leading cause of death in women after loss of ovarian function. This risk is even higher when

associated with hypertension, obesity, diabetes or coagulation disorders during menopause (Anagnostis et al., 2019).

Since menopause is characterized by a natural decline in estrogen production, this hormone has been implicated in several pathophysiological aspects of vascular wall. Estrogen depletion is associated with changes in lipid metabolism, increased vascular and cardiac oxidative stress, reduced nitric oxide (NO) availability and reduced vascular reactivity response to vasodilators (Wassmann et al., 2001; Barp et al., 2012; Knowlton and Lee, 2012). In fact, estrogen receptor (ER) subtypes ERα and ERβ are expressed in endothelial and VSMCs and the activation of both receptors mediates NO production (Knowlton and Lee, 2012). Systemically, the decrease in estrogen production is associated with rise in arterial blood pressure in rat models of ovariectomy (Fang et al., 2001; Harrison-Bernard et al., 2003; Peng et al., 2003; Ito et al., 2006). Angiotensin system seems to be an important mechanism involved, since estrogen can regulate angiotensinogen, renin, angiotensin II (Ang II) receptors AT1 and AT2 expression, and aldosterone production (Hoshi-Fukushima et al., 2008; Ahmad et al., 2018). AT1 expression increased in blood vessels, kidney and brain stem in estrogen-depleted SHR (Ito et al., 2006) and, in the heart, estrogen loss caused diastolic dysfunction by altering cardiomyocytes relaxation properties (Zhao et al., 2014). Interestingly, Ahmad et al (2018) also found that chymase is the main Ang II generating enzyme in cardiomyocytes isolated from ovariectomized SHR and chymase activity positively correlates with cardiovascular dysfunction in this model.

Together with systemic blood pressure changes and Ang II involvement as a relevant player, platelet hyperreactivity has also been described in experimental models of menopausal hypertension. Platelets derived from salt-sensitive SHR ovariectomized rats showed an increased aggregatory response to classical agonists, which correlates with the rise in blood pressure levels (Otsuka et al., 1997; Sasaki et al., 2000). Once Ang II has well reported prothrombotic effects, this may be one of the mechanisms involved (Mogielnicki et al., 2005; Celi et al., 2010). However, it is not completely understood how platelets, aorta and VSMCs contribute to create or even maintain a prothrombotic state after estrogen level drops down. For this purpose, we designed a menopausal hypertension model in which bilateral

ovariectomy surgery was performed in 14-week-old female SHR and normotensive Wistar Kyoto (WKY) rats maintained in a phytoestrogen free diet. After 50 days of ovariectomy surgery, both WKY and SHR animals evolved to a vascular prothrombotic state associated with nucleotide metabolizing system down-regulation, platelet hyperreactivity and tissue factor expression up-regulation in both aorta and platelets. We found that aorta and vascular cells are important supporting procoagulant enzyme assembly and generation by a mechanism that was significantly exacerbated in hypertensive aorta and was dependent of tissue factor and NF-κB-mediated proinflammatory signalling.

2. Material and methods

2.1 Animals

A total number of 20 Wistar Kyoto (WKY) and 20 spontaneously hypertensive (SHR) female rats was used in this study. The animals (60-days old, weighting 170-200 g) were acquired from the Federal University of Rio Grande do Sul Animal House (Porto Alegre, RS, Brazil) and maintained throughout the experiment (90 days total period) in the animal facility of our institution at Hospital de Clínicas de Porto Alegre (Porto Alegre, RS, Brazil), following the Brazilian Law number 11.794/2008, which provide guidance and regulation for scientific research involving animals. They were kept at 20–24 oC in a 40-60 % relative air humidity environment with 12 h dark/light cycle and free access to water and food. During its first 60 days the animals received regular standard rodent chow and in the next 90 days they received a soy-free based diet (PRAGSOLUÇÕES, SP, Brazil) to avoid phytoestrogens interference. All the procedures involving animals were carried out accordingly to the Brazilian Guideline for the Care and Use of Animals for Scientific and Educational Purposes (DBCA, RN 30/2016) as stated by the CONCEA (National Council for Control of Animal Experimentation). Our experimental protocol was approved by the Institute's Animal Ethics Committee of the Experimental Research Center at Clinical Hospital, Porto Alegre, RS, Brazil (protocol number 19001/2019).

2.2 Experimental design

It was developed an experimental model of postmenopausal hypertension in ovariectomized rats. As detailed in Fig. 1A, the animals arrived at our institution 60

days after birth and this date was considered as the day zero of our protocol. At the day 40 (with approximately 14 weeks of age) the animals were randomly assigned to undergo either bilateral ovariectomy (OVX) or SHAM surgery (referred here as gonadal-intact animals). The experimental groups (n = 10/group) were as follows: (i) Wistar Kyoto rats – SHAM operated (WKY-SHAM); (ii) Wistar Kyoto rats – ovariectomized (WKY-OVX); (iii) Spontaneously Hypertensive rats – SHAM operated (SHR-SHAM); and (iv) Spontaneously Hypertensive rats – ovariectomized (SHR-OVX). At the day 90 (50 days after surgery), protocol was finished and animals were euthanised for sample collection. Five days before either surgery (at day 35) or euthanasia (at day 85) blood pressure was measured. At the same days of blood pressure measurements, a vaginal smear was also collected for estrous cycle phase evaluations. All animals submitted to OVX surgery were in diestrous phase to avoid metabolic variations. During the entire protocol rats were weighted once a week.

2.3 Surgical procedure

Surgery was conducted under inhaled general anaesthesia with isoflurane vaporized in oxygen (5 % for induction, 2 % for maintenance) and animal's body temperature was maintained at 37 ^{o}C throughout the procedures. The ovariectomized group of rats had both ovaries removed, while sham operated group were submitted to the identical surgical protocol, but does not have their ovaries removed. After anesthetic induction, animals were positioned in lateral decubitus, were trichotomized in both right and left flanks and an incision of approximately 0.5 cm caudally to the last rib was carried out to reach the abdominal cavity. Thus, after muscle and subcutaneous tissue dissection, the ovary was exposed and two ligation sutures were positioned between the falopian tube (close to the uterine horn), vessels and periovarian adipose tissue. The organ was then removed after a single incision between the two suture ligations. Same procedure was repeated contralaterally and muscle and skin layers were then sutured using polyglactin 910 (Vicryl®) 4-0 and mononaylon 5-0, respectively. Analgesic support was provided after the surgery by a single dipyrone dose (500 mg/kg, via i.m) and over the first three days post-surgery with tramadol (5 mg/kg, via i.p) twice a day, every 12 h. After 50 days (at day 90, Fig. 1A) of ovariectomy, animals were deeply anesthetized with

isoflurane for blood and organ collection. Euthanasia occurred by exsanguination until cardiorespiratory arrest.

2.4 Biological sample preparation

Blood (approximately 10 mL) was obtained by cardiac puncture in 1:10 (v/v) 3.8 % trisodium citrate. Then platelet rich plasma (PRP) was prepared immediately by centrifugation at 200 x g in three cycles of 5 min each. The PRP was used for all platelet aggregation experiments at the same day of its collection. Platelet poor plasma (PPP) was obtained by blood centrifugation at 1,500 × g for 10 min. PPP was aliquoted and stored at – 20 oC until use in the coagulation assays, cell treatments and nucleotidase activity determinations. Abdominal and the descending thoracic aorta and uterus were also collected. Abdominal and thoracic aorta were washed in cold phosphate buffered saline and kept on ice until use. The thoracic aorta was immediately sectioned in two-millimetre rings and processed as previously described (Ait-aissa et al., 2015) for the aortic ring procoagulant profile assays (see details below). The abdominal aorta was immediately frozen in liquid nitrogen and stored at –80ºC for tissue extract processing and western-blot experiments. Uterus was weighted, frozen and also kept at –80ºC.

2.5 Cardiovascular parameters

At the days 35 and 85 of the protocol (Fig. 1A), heart rate, systolic and diastolic blood pressure were measured in conscious rats by the tail-cuff method (Insight, Ribeirao Preto, SP, Brazil). All the animals were properly adapted to the equipment and measurement procedure before protocol starts. Three consecutive readings of blood pressure and heart rate were recorded per animal.

2.6 Platelet aggregation

The platelet aggregation agonists ADP (10 µM) or collagen (2.5 µg/mL) were incubated for 5 min at 37 oC in 96-well flat-bottomed plates containing Tyrode-albumin buffer, pH 7.4. Aggregation response was then triggered by the addition of PRP suspension (3-4 x 105 cells/µL) and changes in turbidity were monitored at 650 nm in intervals of 11 s for 30 min using a microplate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, CA, USA). The decrease in turbidity over time was

measured in absorbance units and results expressed as area under the aggregation curves, as previously described (Berger et al., 2010).

2.7 Nucleotide hydrolysis

The hydrolysis of extracellular nucleotides was determined in serum and platelets by an enzymatic assay as already described (Naasani et al., 2017). E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) and ecto-5'-nucleotidase activities were measured through the malachite green method using ATP, ADP and AMP as substrates and KH2PO4 as Pi standard. Nucleotide spontaneous hydrolysis during the incubation times were monitored by running simultaneously assay batches in the absence of serum or platelet extracts. All samples were run in triplicate and enzyme specific activity was expressed as nmol Pi released/min/mg of protein. Phosphodiesterase activity (E-NPP, nucleotide pyrophosphatase/phosphodiesterase) was assessed by using the synthetic chromogenic substrate thymidine 5'-monophosphate p-nitrophenyl ester, p-Nph-5'-TMP (Sigma-Aldrich, Saint Louis, MO, USA) at a final concentration of 0.5 mM. The kinetics of p-nitrophenol release was monitored at 405 nm during a total time of 30 min with 14 s intervals between reads. Enzyme activity was expressed as mOD of p-nitrophenol released per min per mg of protein.

2.8 Blood coagulation

The following coagulation parameters were measured in citrated-plasma: Activated partial thromboplastin time (aPTT), prothrombin time (PT), thrombin time (TT) and fibrinogen levels (FBG). These parameters were determined using commercially available kits following the general manufacturer's instructions (Sullab Diagnosticos, Porto Alegre, RS, Brazil).

2.9 Aortic ring assays

To measure the procoagulant profile acquired by normo and hypertensive ovariectomized rat aorta we design an aortic ring assay based on the procedure previously described by Ait-aissa et al (2005), with some modifications. Thoracic aortic rings (approximately 2 mm) were resuspended in Krebs-Ringer bicarbonate buffer containing 2 g/L bovine serum albumin and incubated at 37 oC for 5 min.

Then, a mixture of activated factor VII (FVIIa) (2 nM), factor X (FX) (1.2 nM), activated factor V (FVa) (2.5 nM) and prothrombin (10 nM) was added and thrombin generation monitored by changes in absorbance at 405 nm after the addition of 0.2 mM S2238 substrate (H-D-Phe-Pip-Arg-p-nitroanilide). Similarly, activated factor X (FXa) generation was also measured in the presence of aortic rings by adding a mixture containing FVIIa (2 nM), FX (8 nM) and 0.2 mM S2222 substrate (Bz-Ile-Glu-Gly-Arg-p-nitroanilide). For plasma kallikrein generation assay, the aortic rings were incubated in Krebs-Ringer with human plasma deficient in prothrombin (diluted 1:5) containing 100 nM corn trypsin inhibitor. Kallikrein formation was then followed at 405 nm by adding S2302 substrate (H-D-Pro-Phe-Arg-p-nitroanilide). In this case, both prothrombin deficient plasma and corn trypsin inhibitor were used to avoid thrombin and activated factor XII (FXIIa) interference, respectively. Kinetic readings were taken over 40 min (14 s intervals) for all the aortic ring assays using 96-well plates in a final volume of 150 µL.

2.10 Nitrite/nitrate measurements

Total nitrate and nitrite levels (NOx-) were determined as an indication of nitric oxide (NO) production. The measurements were performed in total plasma and aorta extracts according to the Griess method (Miranda et al., 2001).

2.11 Cell culture experiments

Vascular smooth muscle cells (VSMC) (A7r5 cell line, ATCC® CRL-1444), derived from rat thoracic aorta, were cultured in DMEM (high glucose) containing 10 % fetal bovine serum (FBS), 50 U/mL penicillin and 100 µg/mL streptomycin following the standards for cell culture maintenance. VSMCs were used to investigate if the plasma-derived from normo and hypertensive OVX rats could induce a procoagulant profile on cell surface. For this purpose, a similar experimental design as described by Berger et al (2019) was followed. Confluent VSMC monolayers seeded in 96-well plates were treated for 1 h with 5, 10 or 30 % of plasma obtained from OVX and SHAM-operated animals diluted in DMEM without FBS. Then, the medium was removed and cells were washed twice in PBS. The procoagulant profile triggered on VSMC surface was determined by the aPTT assay after addition (50 µL) of a normal rat plasma pool collected from healthy animals. To

verify if the procoagulant profile induced on VSMC was time-dependent, VSMC monolayers were treated with 10 % diluted plasma and cells were washed and processed for aPTT assay after different time-points of incubation (30 min, 1, 2, 4 and 24 h), using the same procedure described before. In another set of experiments, VSMC were pre-treated overnight (in the presence of 1 % FBS) with PBS or 0.1 µM PDTC to block the NF-κB pathway. Then, it was added 10 % diluted plasma from OVX and SHAM animals in absence of FBS. Incubation was maintained for additional 2 h and after a washing step, aPTT was measured as above using normal plasma pool. In all experimental settings clot formation kinetics was monitored (650 nm) at a time interval of 10 s during a total time of 20 min on a SpectraMAX 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.12 Western blotting

Protein expression of different biomarkers related to thrombosis was evaluated in tissue (aorta) and cell (platelets and VSMCs) extracts by immunoblot. Following standard procedures, proteins (30-50 µg) were separated by SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, incubated with primary and secondary-horseradish peroxidase conjugated antibody and revealed using the colorimetric kit Opti-4CN (Bio-Rad, Hercules, CA, USA). Protein expression levels was normalized against β-actin and quantified using Image J software (available at https://imagej.nih.gov/ij/). The following antibodies and dilutions were used: Cyclooxygenase 2 (COX-2) – 1:500 (D5H5 #12282 – Cell Signaling Technology, Danvers, MA, USA); inducible nitric oxide synthase (iNOS) – 1:200 (M-19 #sc-650 Santa Cruz Biotechnology, Dallas, TX, USA); tissue factor (TF) – 1:500 (I-20 #sc-23596 – Santa Cruz Biotechnology, Dallas, TX, USA); kallikrein-1 (KLK1) – 1:500 (13G11 #19901 – QED Bioscience, San Diego, CA, USA); factor X (FX) – 1:500 (C-20 #sc-16341 – Santa Cruz Biotechnology, Dallas, TX, USA); factor II (FII) – 1:500 (D-15 #sc-23355 – Santa Cruz Biotechnology, Dallas, TX, USA); protease activated receptor – 1 (PAR1) – 1:500 (ATAP2 #sc-13503 – Santa Cruz Biotechnology, Dallas, TX, USA); plasminogen activator inhibitor – 1 (PAI-1) – 1:1000 (M-20 #sc-6644 – Santa Cruz Biotechnology, Dallas, TX, USA); urokinase plasminogen activator (uPA) – 1:500 (#CSB-PA14319A0Rb, Cusabio Biotech Co.,

Wuhan, China); and β-actin – 1:1000 (#A-1978, Sigma-Aldrich, Saint Loius, MO, USA).

2.13 Data analysis

The data are presented as means ± SE, and significant differences were analysed by one-way ANOVA followed by an unpaired t-test with Bonferroni correction for multiple comparisons. Cardiovascular parameters were analysed by the method of Generalized Estimating Equations (GEE) followed by Bonferroni's test. Data from body and uterus weight were analysed using Kruskal-Wallis followed by Dunn's test. P-values of 0.05 were significant considered to be significant. Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) or SPSS 20.0 software.

3. Results

In this work we design an experimental model of post-menopausal hypertension in ovariectomized rats trying to focus our efforts on the understanding how are the thrombotic mechanisms in aorta after estrogen reduction. For this purpose, we submitted Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) female rats feeding a phytoestrogen free diet to a bilateral ovariectomy (OVX) surgery (Fig.1A). Rat body weight increased in groups that had their ovaries removed (Fig. 1B). The OVX surgery efficiency was also confirmed by uterine atrophy, as demonstrated by the significant reduction in the ratio between uterus and rat body weight observed in ovariectomized animals (Fig. 1C). As expected, normotensive WKY animals had lower systolic (SBP) and diastolic (DBP) blood pressure levels compared to SHR at the baseline pre-OVX measurements (Table 1). The difference between these parameters increased significantly 50 days post-OVX surgery. Estrogen depletion led to hypertension in WKY animals, since SBP increased from 132 ± 4.7 to 169 ± 3 mmHg while in SHR it led to an exacerbated hypertensive response with SBP increasing from 159 ± 3.5 to 176 ± 2.5 mmHg (Table 1).

The first evidence that ovariectomized animals evolve to a prothrombotic state was obtained from ex-vivo platelet aggregation assays (Fig. 2). Platelets from both WKY or SHR rats presented an exacerbated aggregatory response to ADP (Fig. 2A-

C) and collagen (Fig. 2D-F) after OVX. Hypertensive animals from SHR group did not have a higher aggregatory response compared to WKY-SHAM group, but this effect slightly increased after OVX surgery compared to WKY-OVX group (Fig. 2B-C and E-F). To investigate the mechanisms behind the platelet pro-aggregatory response our primary hypothesis was the nitric oxide system. Interestingly, nitrate/nitrite levels in plasma and aorta and inducible nitric oxide synthase (iNOS) expression in aorta did not change significantly between groups, despite a little increase observed in nitrite/nitrate levels in plasma of spontaneously hypertensive ovariectomized rats (SHR-OVX) (Fig. 3A-C). On the other hand, when the aorta cyclooxygenase-2 expression was analysed, a reduction around of 50 % was observed both in WKY and SHR groups after OVX surgery (Fig. 3C). The second hypothesis was to investigate nucleotide metabolism involved in platelet aggregation, since ADP is one of the most important aggregation agonist and adenosine is a physiological inhibitor which control this process. As showed in Figure 4, E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) and ecto-5'- nucleotidase activities reduced almost at a same extent in serum and platelets of normo (WKY) or hypertensive (SHR) animals after ovarian removal. The presence of basal hypertension just increases ADP hydrolysis on platelets, but estrogen depletion strongly downregulates this parameter (Fig. 4F). Looking to phosphodiesterase activity (E-NPP), SHR had highest activity levels compared to normotensive rats in both serum and platelets. After OVX, an opposite effect was observed, E-NPP increase in serum from WKY group and decrease in platelets from spontaneously hypertensive animals (Fig. 4D and H).

Similar to platelet aggregation, plasma coagulation parameters also changed significantly after ovariectomy (Fig. 5). Extrinsic, intrinsic and common pathways of blood coagulation were evaluated in citrated plasma by the classical tests aPTT, PT, TT and fibrinogen levels measurements. In general, plasma from OVX animals coagulated faster than those from SHAM-operated animals independent of the basal hypertension levels pre-surgery. This event was particularly evident in intrinsic pathway measurements (aPTT was around of 25 s in the SHAM group, while the coagulation time was 15 s in OVX) but was also observed in extrinsic and common pathway measurements, such as in PT and TT, respectively (Fig. 5A-C and E). In

agreement with these alterations, fibrinogen levels drop down in estrogen depleted groups, being more evident in WKY-OVX group (Fig. 5F).

Aiming to understand how aorta can contribute to generate and maintain a prothrombotic state, we design some experiments to measure the complex assembly and activity of the main procoagulant enzymes on aortic ring surface. As showed in Figure 6, aortic rings can support the complex assembly responsible for kallikrein, FXa and thrombin generation. However, the procoagulant enzyme generation was higher in aortic rings from estrogen deficient rats (Fig. 6A-C). Interestingly, hypertension exacerbated thrombin generation in aortic rings from SHR-OVX group compared to those from WKY-OVX group which were originally normotensive pre-ovarian removal surgery (Fig. 6C). Corroborating these findings, tissue factor expression (the main molecule known as an initiator of extrinsic coagulation pathway), increase in both aorta (Fig. 7A) and platelet (Fig. 7B) surface from ovariectomized animals. In a similar way, OVX increase the aortic expression of other prothrombotic (FII and FX) (Fig. 8A and C) and fibrinolytic (PAI-1 and uPA) (Fig. 8 B and D) proteins, being uPA markedly up-regulated by the presence of basal hypertension such as in SHR-OVX animals (Fig. 8D). As a consequence of procoagulant enzyme activation, the protease activated receptor 1 (PAR-1) (main receptor cleaved by thrombin and FXa-induced intracellular signalling) also appear up-regulated in WKY-OVX and SHR-OVX aorta (Fig. 8C).

Next, we move forward to investigate if the plasma-derived from estrogen deficient animals could also activate and induce a prothrombotic profile in naive vascular smooth muscle cells (VSMC) in vitro. For this purpose, we choose a VSMC cell line (A7r5) derived from rat thoracic aorta, which was treated for 1h with increasing concentrations of plasma from normo and hypertensive ovariectomized rats. After this pre-treatment period, cells were washed and then a poll of plasma derived from normal healthy rats was added and coagulation time was immediately measured on VSMC surface. As showed in Figure 9, normal healthy plasma coagulated faster on VSMC surface pre-treated with OVX-derived plasma from both normo (WKY-OVX) and hypertensive (SHR-OVX) animals. The procoagulant profile triggered by OVX plasma was dose dependent (Fig. 9A) and more pronounced in VSMC treated up to 2 h, being similar to the control levels in cells treated by 24 h

(Fig. 9B). We also observed that OVX-derived plasma increases significantly the tissue factor expression on VSMC mainly between 30 min and 2 h after the treatments (Fig. 9C and D). In the last experiments (Fig. 10), we raise the hypothesis that this tissue factor up-regulation and the consequent procoagulant profile triggered on VSMC could be linked to an inflammatory intracellular signalling. To properly test this hypothesis, previously to the addition of OVX-derived plasma, cells were treated with PDTC, a NF-κB pathway inhibitor. As showed in Figure 10A, blocking NF-κB mediated pathway reduced significantly the procoagulant effect triggered by WKY-OVX and SHR-OVX plasma on VSMC surface. The reduction in VSMC-induced procoagulant activity was accompanied by a down-regulation in tissue factor expression on cells previously treated with PDTC (Fig. 10B).

4. Discussion

In the present study we develop an experimental model of postmenopausal hypertension induced by bilateral ovariectomy in 14-week-old Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) female rats. Our main finding shows that acute endogenous estrogen deprivation after ovariectomy promptly increases the blood pressure independent of a previous basal hypertensive condition, leading to a systemic vascular prothrombotic state. The mechanism behind involves a platelet hyperreactivity which was directly related to downregulation of nucleotide degradation system and tissue factor up-regulation in both platelets and aorta. Aorta participates in the mechanism being able to support procoagulant enzyme generation, including thrombin, and this event is significantly exacerbated in hypertensive aorta. Interestingly, we found that plasma derived from ovariectomized animals can trigger a prothrombotic profile in cultured vascular cells by increasing tissue factor expression through NF-κB-dependent pathway.

Since hypertension is an important risk factor for vascular thromboembolic events in the postmenopausal period, we decide to use SHR animals, which are a known model of preestablished and sustained hypertension. Previous reports showed that ovariectomized SHR feeding a high-salt diet develops an exacerbated level of hypertension, which was not observed in normotensive animals (Fang et al., 2001; Harrison-Bernard et al., 2003; Peng et al., 2003). In contrast, Ito et al (2006) measuring blood pressure by radiotelemetry, detected a significant SBP increase in

both ovariectomized normotensive (WKY) and SHR feeding a phytoestrogen free diet with standard levels of NaCl. Another important point to be considered in this case is the animal age at the moment of ovary removal surgery. Rats ovariectomized at young age only develops hypertension when fed a high-salt diet (Harrison-Bernard et al., 2003; Fang et al., 2001), whereas adult rats ovariectomized at ages from 12- to 14-week-old have increased arterial pressure feeding a phytoestrogen-devoid diet containing both standard or high-NaCl amounts (Peng et al., 2003; Ito et al., 2006). In the present work, we ovariectomized 14-week-old animals previously feeding a phytoestrogen free diet with standard NaCl and we follow them by a longer period of 50 days. In a similar way to that observed by Ito et al (2006), we found that both normotensive WKY and SHR animals had increased SBP, DBP and HR compared to the basal measurements before bilateral ovariectomy surgery, which indicated for us that it would be a good model to study thromboembolic alteration during postmenopausal hypertension. Regarding the mechanism involved in estrogen depletion-induced hypertension, Ahmad et al (2018) found an up-regulation of chymase-derived angiotensin II (Ang II) in cardiomyocytes isolated from ovariectomized SHR. Once Ang II has a prothrombotic effect causing platelet hyperreactivity (Fang et al., 2013; Mogielnicki et al., 2005), it is possible that chymase-derived Ang II has also a role in the prothrombotic events observed by us here. However, this hypothesis needs to be better explored in future experiments.

The first evidence of a systemic prothrombotic state was the procoagulant activity observed in plasma coagulation tests and platelet hyperreactivity. Platelets from ovariectomized animals had an enhanced response when stimulated with ADP and collagen. Accordingly, platelets from ovariectomized Dahl-salt-sensitive rats also showed an exacerbated aggregatory response to thrombin by a mechanism dependent of intracellular calcium and PKC activation (Sasaki et al., 2000). Here, we found that probably other mechanisms are also involved. Nitrite/nitrate levels in plasma and iNOS expression in aorta remains unchanged, while COX-2 decrease in aorta. Both nitric oxide and COX-2 are important platelet regulators, being able to cause vasodilation and reduce the aggregatory response through cGMP and PGI2 generation, respectively (Shah, 2005). Thus, COX-2 downregulation can contribute for a prothrombotic state by maintaining a vasoconstrictive response and platelet hyperreactivity. Other mechanism we found was related to the inhibition of nucleotide

degradation system in estrogen depleted groups. The same nucleotides used in energy metabolism also participate as platelet aggregation regulators. ATP and ADP are stored in platelet dense granules and are secreted during platelet activation. Extracellular ATP is rapidly metabolized in a cascade hydrolysis into ADP, AMP and finally adenosine by the ecto-enzymes E-NTPDase (CD39), ecto-5'- nucleotidase (CD73) and E-NPP (Koupenova and Ravid, 2018). Secreted ATP and ADP, as well as ADP deriving from ATP degradation, activate P2Y1, P2Y12, and P2X1 receptors. Through the P2Y12 receptor ADP strongly modulates the growth and stability of thrombus by potentiating platelet dense granule release, platelet aggregation and procoagulant activity (Ballerini et al., 2018). On the other hand, adenosine through the P1 receptors acts as a counterregulatory molecule increasing cAMP intracellular levels and inhibiting platelet aggregation (Koupenova and Ravid, 2018). Thus, nucleotide hydrolysis can control the balance between pro and anti-aggregatory profiles. Interestingly, ATP, ADP and AMP hydrolysis markedly reduce almost at a same extent in serum and platelets of normo or hypertensive ovariectomized animals. This means that estrogen deprivation changes nucleotide metabolism shifting the balance, favouring ADP/ATP accumulation and, thus, contributing to platelet hyperreactivity.

Besides its clear role in thrombogenesis through the aggregatory response, platelets, and also endothelial and VSMC participate in the initial and amplification phases of clot formation. They offer a proper surface of negatively charged phospholipids for coagulation factor complex assembly (Esmon and Esmon, 2011). Exposure of phosphatidylserine on the outer membrane of platelets and vascular cells regulated by flip-flop mechanism is thought to account for the procoagulant profile of these cells. A previous report showed that aortic rings and VSMC from SHR animals had increased amounts of negatively charged phospholipid procoagulant activity, which was correlated to a greater thrombin generation at the surface of SHR-derived aortic rings in comparison to WKY control rings (Ait-aissa et al., 2015). The authors found that hypertension-induced intracellular calcium elevation in SHR cells promoted the flip-flop process causing exposure of negatively charged phospholipids (Ait-aissa et al., 2015). While changes in membrane phospholipid rearrangement allows coagulation factor binding, the protein effectively responsible for triggering FXa and thrombin generation on vascular cell surface is

the tissue factor (TF) (Grover and Mackman, 2018). In fact, it has been shown that SHR has higher levels of TF associated with VSMC, but the present work is the first to demonstrate that estrogen depletion exacerbates TF expression in platelets and aorta. TF was up-regulated at similar extend in both normo and hypertensive ovariectomized animals. TF is the major cellular initiator of blood coagulation (Pawlinski et al., 2004). It is a type I transmembrane glycoprotein that orchestrates the initiating cascade by binding to FVII and facilitating its activation to VIIa. FVIIa that is not bound to TF has little activity (Riewald and Ruf, 2002). Once activated, the complex TF-VIIa binds to its substrate FX, generating FXa. The TF-VIIa-Xa complex efficiently cleaves prothrombin (FII) producing the main procoagulant enzyme, thrombin. TF-VIIa-Xa and thrombin also intimately links coagulation to inflammation, since these proteases are PAR-1 and PAR-2 activators triggering proinflammatory cytokines through NF-κB pathway (Riewald and Ruf, 2002). In agreement to TF up-regulation in aorta, we observed that aortic rings derived from ovariectomized rats supported an increased generation activity of kallikrein, FXa and thrombin on their surface. In this case, ovariectomized SHR presented a greater thrombin generation capacity compared to equivalent rings from normotensive rats, suggesting that estrogen depletion shifted vascular hemostatic balance toward a prothrombotic phenotype.

It is known that high blood pressure can induce microlesions on vascular wall (Wassmann et al., 2001; Mogielnicki et al., 2005). This fact associated with higher radial hydraulic conductance in hypertensive vessels can facilitate blood clotting factors not only to bind to directly exposed endothelial cells, but also effectively crossing through the lumen reaching medial VSMCs (Lacolley et al., 2012). Thus, we rise the hypothesis that procoagulant markers could be detected directly on vascular tissue extracts. In fact, aorta derived from estrogen depleted rats had increased levels of procoagulant (FX and FII) and fibrinolytic (PAI-1 and uPA) protein biomarkers, which corroborates with higher TF expression and procoagulant enzyme generation on aortic rings. To further explore VSMCs contribution to the hypercoagulable state we design some additional experiments in which A7r5 VSMC line was treated in culture with plasma-derived from ovariectomized animals. The rationale behind these experiments was based on previous observations showing the capacity of activated platelets and even VSMCs to secrete soluble TF-enriched

microparticles that circulates free in the blood stream (Pawlinski et al., 2004). Then we further wondering if the plasma derived from hypercoagulable estrogen-depleted animals could also induce a procoagulant activity on naïve VSMCs. Interestingly, VSMCs treated with estrogen deficient plasma had TF expression induced in the first 2 h. In accordance, when normal plasma was added to these cells previously treated with estrogen deficient plasma, it coagulates faster in comparison to that was added to untreated cells. It seems that estrogen deficient plasma from WKY and SHR activates and trigger procoagulant profile on VSMC independent of basal hypertension levels before ovariectomy. We also observed that the mechanism behind these effects in VSMCs involves an inflammatory pathway mediated by NF-κB, since PDTC (a NF-κB blocker) reduces significantly both TF expression and procoagulant activity on VSMC surface. As mentioned before, thrombin and TF complex (TF-VIIa-Xa) generated during hypercoagulable states are linked to proinflammatory signalling through the activation of PAR-1 and PAR-2 receptors (Riewald and Ruf, 2002). For instance, in endothelial cells thrombin participates in a positive feedback loop, activating PAR-1 and inducing TF expression up-regulation which will in turn contribute to increase FXa and thrombin formation (Minami et al., 2004). Once activated, PAR-1 regulates TF expression triggering IκBα proteolytic degradation and inducing nuclear translocation of NF-κB and c-Rel/p65 complexes (Pendurthi et al., 1997). In a similar way, PAR-1 also regulates the expression of other proinflammatory molecules such as ICAM, VCAM and IL-6 (Minami et al., 2004; Grover and Mackman, 2018). Since PAR-1 is up-regulated in aorta from ovariectomized animals it is reasonable to suppose that such mechanism strongly contributes to the prothrombotic profile described here in our model.

5. Conclusions

Overall, the results of this study aid to understand the cellular and molecular mechanisms behind the highest risk of prothrombotic events and its association with hypertension in postmenopausal women. We found that surgically induced estrogen deprivation in rats leads to a vascular prothrombotic state associated with a decrease in nucleotide metabolizing system, platelet hyperreactivity and tissue factor expression up-regulation in both aorta and platelets. Aorta and vascular cells are important players being able to support procoagulant enzyme generation by a

mechanism that was significantly exacerbated in hypertensive aorta. Interestingly, plasma derived from hypercoagulable estrogen-depleted rats can also trigger a prothrombotic profile in cultured naive vascular cells by increasing tissue factor expression through NF-κB-dependent pathway. Thus, our data suggest that targeting tissue factor mediated events could be a therapeutic option for vascular thromboembolic complications in the postmenopausal period.

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7. References

Ahmad S, Sun X, Lin M, Varagic J, Zapata-Sudo G, Ferrario CM, Groban L, Wang H. Blunting of estrogen modulation of cardiac cellular chymase/RAS activity and function in SHR. J Cell Physiol. 2018 Apr;233(4):3330-3342.

Ait Aissa K, Lagrange J, Mohamadi A, Louis H, Houppert B, Challande P, Wahl D, Lacolley P, Regnault V. Vascular smooth muscle cells are responsible for a prothrombotic phenotype of spontaneously hypertensive rat arteries. Arterioscler Thromb Vasc Biol. 2015 Apr;35(4):930-7.

Anagnostis P, Paschou SA, Katsiki N, Krikidis D, Lambrinoudaki I, Goulis DG. Menopausal Hormone Therapy and Cardiovascular Risk: Where are we Now? Curr Vasc Pharmacol. 2019;17(6):564-572.

Arikan E, Sen S. Endothelial damage and hemostatic markers in patients with uncomplicated mild-to-moderate hypertension and relationship with risk factors. Clin Appl Thromb Hemost. 2005 Apr;11(2):147-59.

Ballerini P, Dovizio M, Bruno A, Tacconelli S, Patrignani P. P2Y12 Receptors in Tumorigenesis and Metastasis. Front Pharmacol. 2018 Feb 2;9:66.

Barp J, Sartório CL, Campos C, Llesuy SF, Araujo AS, Belló-Klein A. Influence of ovariectomy on cardiac oxidative stress in a renovascular hypertension model. Can J Physiol Pharmacol. 2012 Sep;90(9):1229-34.

Berger M, Reck J Jr, Terra RM, Beys da Silva WO, Santi L, Pinto AF, Vainstein MH, Termignoni C, Guimarães JA. Lonomia obliqua venomous secretion induces human platelet adhesion and aggregation. J Thromb Thrombolysis. 2010 Oct;30(3):300-10.

Berger M, de Moraes JA, Beys-da-Silva WO, Santi L, Terraciano PB, Driemeier D, Cirne-Lima EO, Passos EP, Vieira MAR, Barja-Fidalgo TC, Guimarães JA. Renal and vascular effects of kallikrein inhibition in a model of Lonomia obliqua venom-induced acute kidney injury. PLoS Negl Trop Dis. 2019 Feb 14;13(2):e0007197.

Celi A, Cianchetti S, Dell'Omo G, Pedrinelli R. Angiotensin II, tissue factor and the thrombotic paradox of hypertension. Expert Rev Cardiovasc Ther. 2010 Dec;8(12):1723-9.

Elias A, Rock W, Odetalla A, Ron G, Schwartz N, Saliba W, Elias M. Enhanced thrombin generation in patients with arterial hypertension. Thromb Res. 2019 Feb;174:121-128.

Esmon CT, Esmon NL. The link between vascular features and thrombosis. Annu Rev Physiol. 2011;73:503-14.

Fang C, Stavrou E, Schmaier AA, Grobe N, Morris M, Chen A, Nieman MT, Adams GN, LaRusch G, Zhou Y, Bilodeau ML, Mahdi F, Warnock M, Schmaier AH. Angiotensin 1-7 and Mas decrease thrombosis in Bdkrb2-/- mice by increasing NO and prostacyclin to reduce platelet spreading and glycoprotein VI activation. Blood. 2013 Apr 11;121(15):3023-32.

Fang Z, Carlson SH, Chen YF, Oparil S, Wyss JM. Estrogen depletion induces NaCl-sensitive hypertension in female spontaneously hypertensive rats. Am J Physiol Regul Integr Comp Physiol. 2001 Dec;281(6):R1934-9.

Grover SP, Mackman N. Tissue Factor: An Essential Mediator of Hemostasis and Trigger of Thrombosis. Arterioscler Thromb Vasc Biol. 2018 Apr;38(4):709-725.

Harrison-Bernard LM, Schulman IH, Raij L. Postovariectomy hypertension is linked to increased renal AT1 receptor and salt sensitivity. Hypertension. 2003 Dec;42(6):1157-63.

Hoshi-Fukushima R, Nakamoto H, Imai H, Kanno Y, Ishida Y, Yamanouchi Y, Suzuki H. Estrogen and angiotensin II interactions determine cardio-renal damage in Dahl salt-sensitive rats with heart failure. Am J Nephrol. 2008;28(3):413-23.

Ito K, Hirooka Y, Kimura Y, Sagara Y, Sunagawa K. Ovariectomy augments hypertension through rho-kinase activation in the brain stem in female spontaneously hypertensive rats. Hypertension. 2006 Oct;48(4):651-7.

Junker R, Heinrich J, Schulte H, Erren M, Assmann G. Hemostasis in normotensive and hypertensive men: results of the PROCAM study. The prospective cardiovascular Münster study. J Hypertens. 1998 Jul;16(7):917-23.

Koupenova M, Ravid K. Biology of Platelet Purinergic Receptors and Implications for Platelet Heterogeneity. Front Pharmacol. 2018 Jan 30;9:37.

Knowlton AA, Lee AR. Estrogen and the cardiovascular system. Pharmacol Ther. 2012 Jul;135(1):54-70.

Lacolley P, Regnault V, Nicoletti A, Li Z, Michel JB. The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. Cardiovasc Res. 2012 Jul 15;95(2):194-204.

Minami T, Sugiyama A, Wu SQ, Abid R, Kodama T, Aird WC. Thrombin and phenotypic modulation of the endothelium. Arterioscler Thromb Vasc Biol. 2004 Jan;24(1):41-53.

Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide. 2001 Feb;5(1):62-71.

Mogielnicki A, Chabielska E, Pawlak R, Szemraj J, Buczko W. Angiotensin II enhances thrombosis development in renovascular hypertensive rats. Thromb Haemost. 2005 Jun;93(6):1069-76.

Naasani LIS, Rodrigues C, de Campos RP, Beckenkamp LR, Iser IC, Bertoni APS, Wink MR. Extracellular Nucleotide Hydrolysis in Dermal and Limbal Mesenchymal Stem Cells: A Source of Adenosine Production. J Cell Biochem. 2017 Aug;118(8):2430-2442.

Otsuka K, Ohno Y, Sasaki T, Yamakawa H, Hayashida T, Suzawa T, Suzuki H, Saruta T. Ovariectomy aggravated sodium induced hypertension associated with altered platelet intracellular Ca2+ in Dahl rats. Am J Hypertens. 1997 Dec;10(12 Pt 1):1396-403.

Pawlinski R, Pedersen B, Erlich J, Mackman N. Role of tissue factor in haemostasis, thrombosis, angiogenesis and inflammation: lessons from low tissue factor mice. Thromb Haemost. 2004 Sep;92(3):444-50.

Pendurthi UR, Williams JT, Rao LV. Inhibition of tissue factor gene activation in cultured endothelial cells by curcumin. Suppression of activation of transcription factors Egr-1, AP-1, and NF-kappa B. Arterioscler Thromb Vasc Biol. 1997 Dec;17(12):3406-13.

Peng N, Clark JT, Wei CC, Wyss JM. Estrogen depletion increases blood pressure and hypothalamic norepinephrine in middle-aged spontaneously hypertensive rats. Hypertension. 2003 May;41(5):1164-7.

Riewald M, Ruf W. Orchestration of coagulation protease signaling by tissue factor. Trends Cardiovasc Med. 2002 May;12(4):149-54.

Shah BH. Estrogen stimulation of COX-2-derived PGI2 confers atheroprotection. Trends Endocrinol Metab. 2005 Jul;16(5):199-201.

Sasaki T, Ohno Y, Otsuka K, Suzawa T, Suzuki H, Saruta T. Oestrogen attenuates the increases in blood pressure and platelet aggregation in ovariectomized and salt-loaded Dahl salt-sensitive rats. J Hypertens. 2000 Jul;18(7):911-7.

Wassmann S, Bäumer AT, Strehlow K, van Eickels M, Grohé C, Ahlbory K, Rösen R, Böhm M, Nickenig G. Endothelial dysfunction and oxidative stress during estrogen deficiency in spontaneously hypertensive rats. Circulation. 2001 Jan 23;103(3):435-41.

Zhao Z, Wang H, Jessup JA, Lindsey SH, Chappell MC, Groban L. Role of estrogen in diastolic dysfunction. Am J Physiol Heart Circ Physiol. 2014 Mar 1;306(5):H628-40.

8. Tables

Table 1. Cardiovascular parameters in WKY and SHR ovariectomized rats.

Parameter	Groups	Pre-OVX Baseline (Day 35)		Post-OVX (Day 85)		p-value
HR (beats/min)	WKY-SHAM	331 ± 15	(a)	346 ± 8.6	(a)	1.000
	WKY-OVX	332 ± 8.6	(a)	363 ± 7.8	(ab)	1.000
	SHR-SHAM	376 ± 14	(a)	331 ± 8	(a)	0.001
	SHR-OVX	358 ± 7	(a)	385 ± 8	(b)	0.091
SBP (mmHg)	WKY-SHAM	129 ± 3	(a)	126 ± 0.8	(a)	1.000
	WKY-OVX	132 ± 4.7	(a)	169 ± 3	(b)	<0.001
	SHR-SHAM	152 ± 4	(b)	143 ± 2.8	(c)	0.259
	SHR-OVX	159 ± 3.5	(b)	176 ± 2.5	(b)	<0.001
DBP (mmHg)	WKY-SHAM	82 ± 2.7	(a)	80 ± 2.4	(a)	1.000
	WKY-OVX	81 ± 2	(a)	102 ± 1.7	(b)	<0.001
	SHR-SHAM	95 ± 3	(b)	90 ± 3.2	(ac)	1.000
	SHR-OVX	100 ± 3.7	(b)	107 ± 3	(b)	0.737

Spontaneously hypertensive (SHR) and Wistar Kyoto rats (WKY) were ovariectomized (OVX)- or sham-operated (n = 10/group). Then, the cardiovascular parameters such as, heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured before (at the baseline, pre-OVX) and after the surgery (post-OVX). Details about the experimental design can be found on Fig. 1A. The results are presented as mean ± standard error. For data analysis the method of Generalized Estimating Equations (GEE) followed by Bonferroni's test was used. The p-value column indicates statistical differences between time (pre-OVX versus post-OVX). The letters indicate statistical differences between experimental groups. Same letter means no difference ($p>0.05$), while different letters means that there is statistical difference between groups ($p<0.05$). In all cases p values less than 0.05 were considered significant.

9. Figures

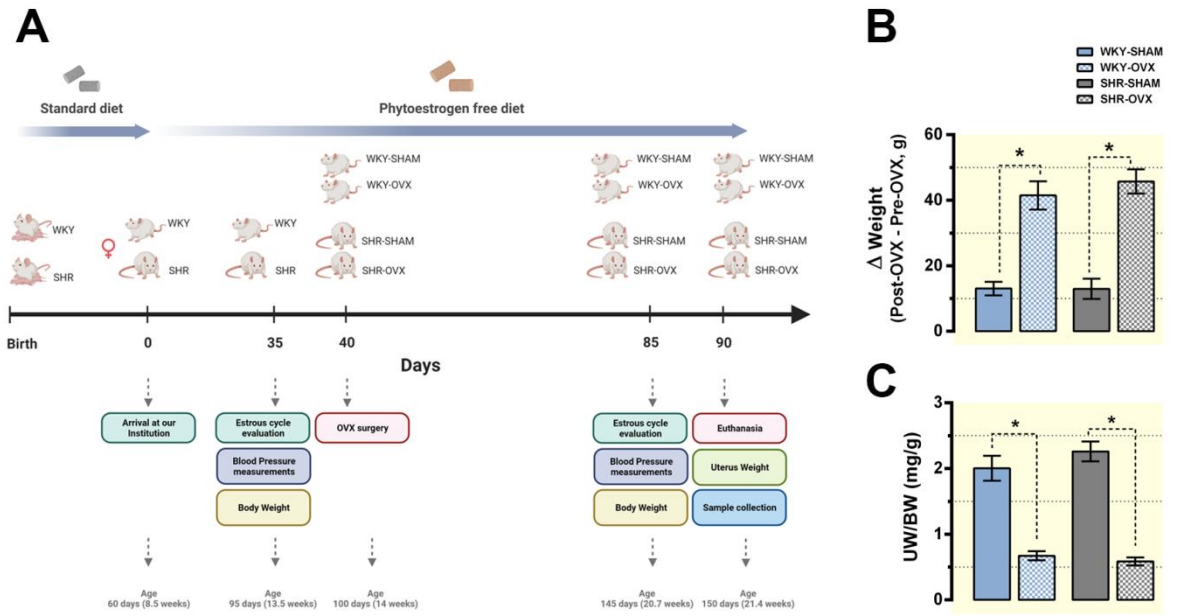

Figure 1. Experimental model of menopausal hypertension. (A) A total number of 20 female spontaneously hypertensive (SHR) and 20 normotensive Wistar Kyoto (WKY) rats arrived at our institution 60 days after birth and this date was considered as the day zero of our protocol. Before the day zero animals received a standard diet. During the entire protocol after the day zero they received a phytoestrogen free diet. At the day 40 (with approximately 14 weeks of age) the animals were submitted to a bilateral ovariectomy (OVX) or SHAM surgery (referred here as gonadal-intact animals). The experimental groups (n = 10/group) were WKY-SHAM, WKY-OVX, SHR-SHAM and SHR-OVX. Five days before either surgery (at day 35) or euthanasia (at day 85) blood pressure was measured, body weight was determined and estrous cycle phase was evaluated. At the day 90 (50 days after surgery), all animals were euthanised for sample collection. **(B)** Rat body weight variation between animals post-OVX surgery (at day 85) and pre-OVX (at day 35). **(C)** Ratio between uterus weight and body weight was determined as an index of OVX surgery efficiency.

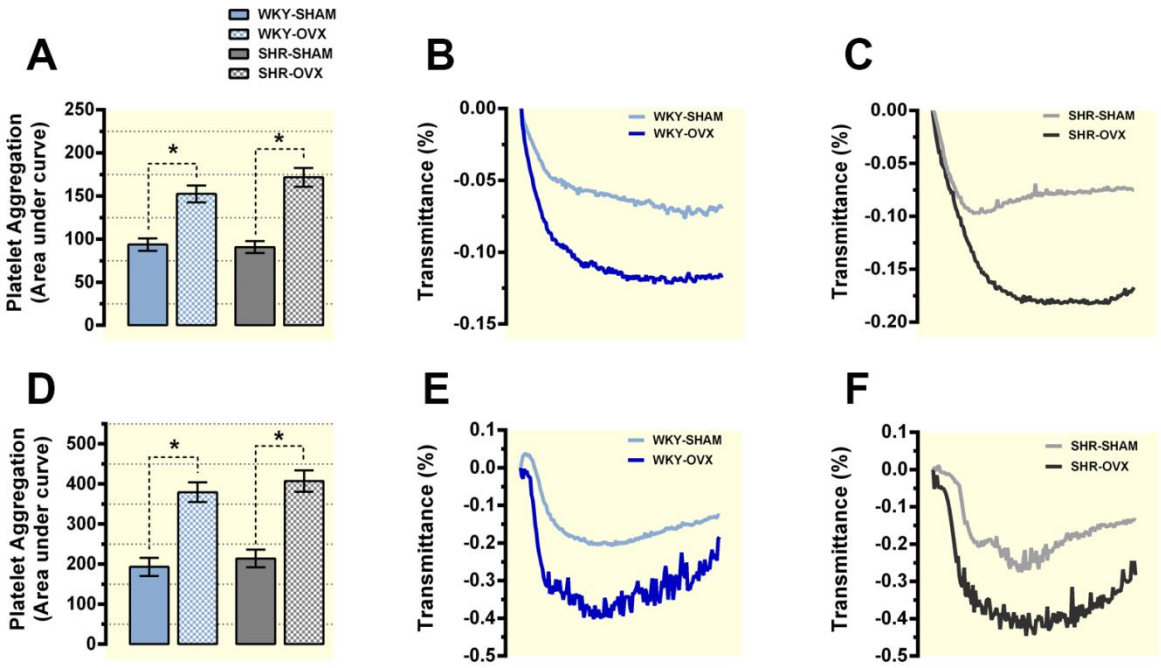

Figure 2. Platelet aggregation function in estrogen-depleted normo and hypertensive rats. A bilateral ovariectomy (OVX) or SHAM surgical procedure was performed in 14-week-old female spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. After 50 days, animals were euthanized and blood was collected for *ex-vivo* platelet aggregation functional tests. **(A)** ADP (10 µM)-induced platelet aggregation in platelet rich plasma (PRP). **(B)** Representative profile of ADP-induced aggregation curves for normotensive WKY rats. **(C)** Representative profile of ADP-induced aggregation curves for SHR rats. **(D)** Collagen (3 µg/mL)-induced platelet aggregation in PRP. **(E)** Representative profile of collagen-induced aggregation curves for normotensive WKY rats. **(F)** Representative profile of collagen-induced aggregation curves for SHR rats. Data are presented as mean ± SE and (*) represents significantly statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

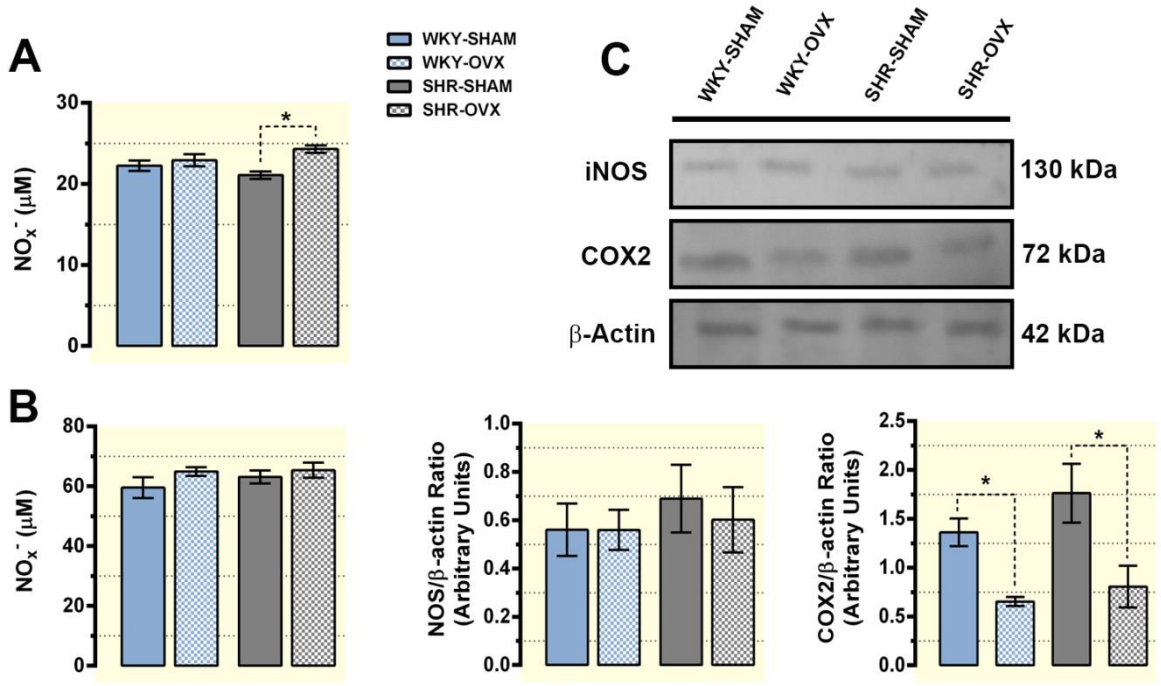

Figure 3. Nitrate/nitrite, nitric oxide synthase and cyclooxygenase 2 levels in normo and hypertensive ovariectomized rats. A bilateral ovariectomy (OVX) or SHAM surgical procedure was performed in 14-week-old female spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. After 50 days, animals were euthanized for plasma and aorta collection. **(A)** Plasma and **(B)** Aorta nitrite/nitrate (NO^-_x) levels as determined by the Griess method. **(C)** Aorta protein expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) as determined by western-blot (upper panel showing the representative blots from three independent experiments and lower panel showing its respective quantitative analysis normalized by the β-actin expression). Data are presented as mean ± SE and (*) represents significantly statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

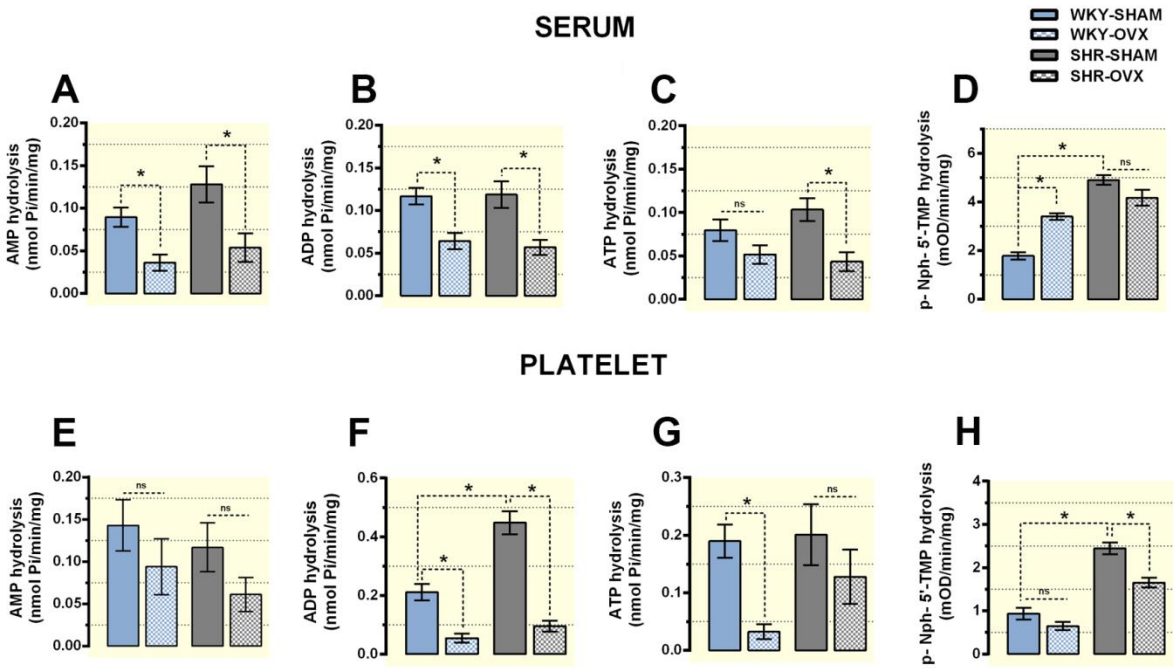

Figure 4. Hydrolysis of the main nucleotides involved in platelet aggregation in estrogen-depleted normo and hypertensive rats. A bilateral ovariectomy (OVX) or SHAM surgical procedure was performed in 14-week-old female spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. After 50 days, animals were euthanized and serum and platelets were collected to determine the enzymatic activity of ecto-nucleotidases involved in nucleotide metabolism that are relevant for platelet aggregation function. The activities of E-NTPDases (ectonucleoside triphosphate diphosphohydrolase), ecto-5'- nucleotidase and E-NPP (nucleotide pyrophosphatase/phosphodiesterase) were estimated in serum and platelets by the hydrolysis rate of (**A and E)** AMP, **(B and F)** ADP, **(C and G)** ATP and **(D and H)** 5'-TMP. Data are presented as mean ± SE and (*) represents significantly statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

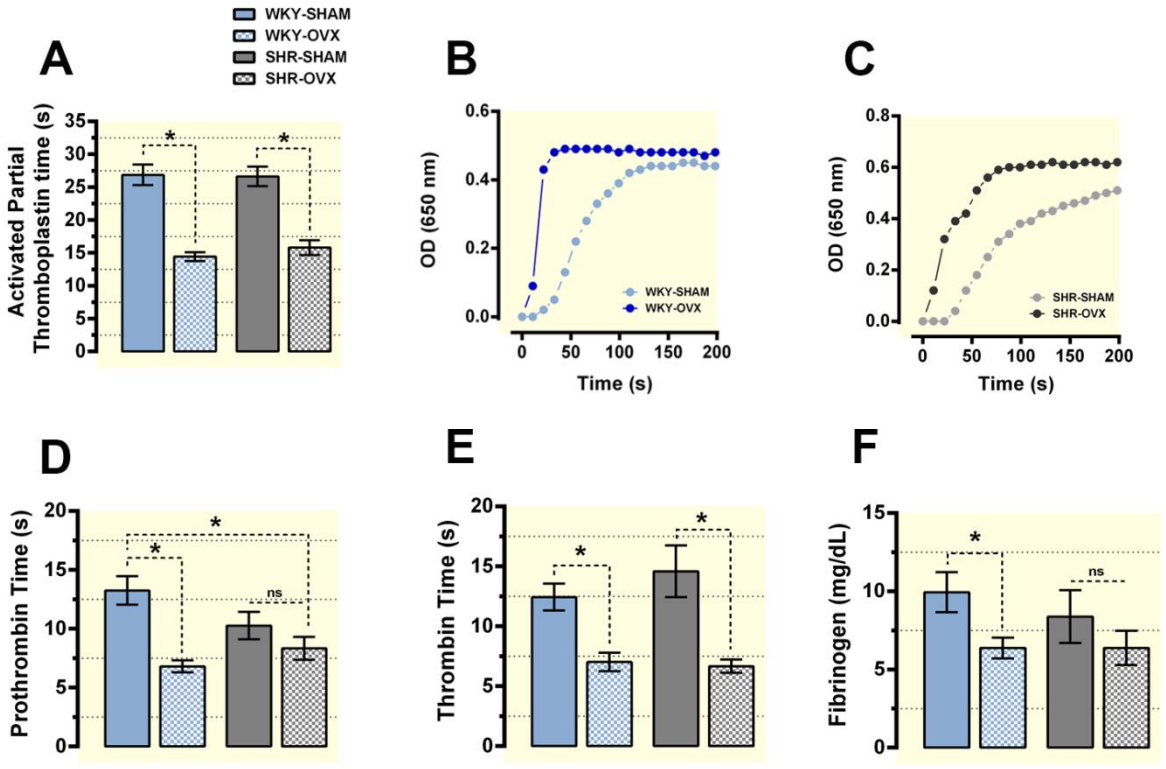

Figure 5. Blood coagulation parameters in estrogen-depleted normo and hypertensive rats. Citrated blood was collected through intracardiac puncture from ovariectomized (OVX) and SHAM-operated spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. The following coagulation parameters were determined in plasma: **(A)** activated partial thromboplastin time (aPTT); **(B)** kinetics of WKY rats aPTT; **(C)** kinetics of SHR rats aPTT; **(D)** prothrombin time (PT); **(E)** thrombin time (TT) and **(F)** fibrinogen levels. Data are presented as mean ± SE and (*) represents significantly statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

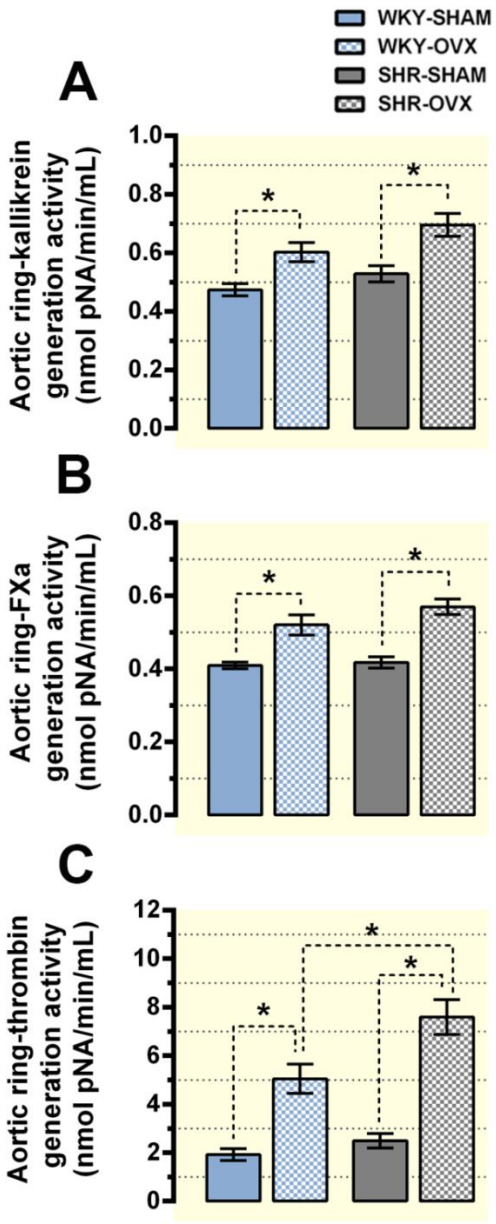

Figure 6. Procoagulant phenotype of aortic rings derived from normo and hypertensive ovariectomized rats. The descending thoracic aorta was collected from ovariectomized (OVX) and SHAM-operated spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. Then, two mm ring segments were prepared to measure its potential increasing rate of kallikrein, factor Xa (FXa) and thrombin generation. **(A)** Kallikrein generation was determined incubating the aortic rings with diluted prothrombin-deficient human plasma and adding the specific synthetic substrate S2302 to measured formed kallikrein. **(B)** FXa generation was determined incubating the aortic rings with purified human FX, FVIIa and calcium ions and adding the specific synthetic substrate S2222 to measured formed FXa activity. **(C)** Thrombin generation was determined incubating the aortic rings in the presence of purified human prothrombin, FVa, FVIIa, FX and calcium ions and adding the specific synthetic substrate S2238 to measured formed thrombin. Data are presented as mean ± SE and (*) represents significantly statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

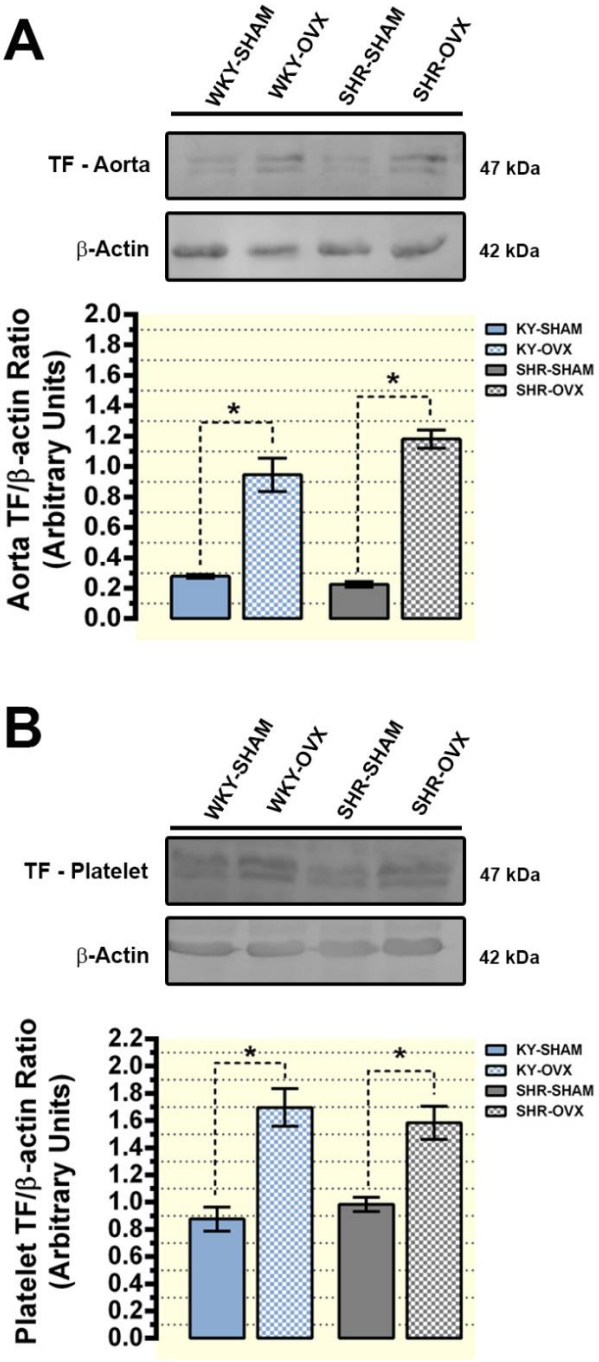

Figure 7. Tissue factor (TF) protein expression in aorta and platelets from normo and hypertensive ovariectomized rats. Abdominal aorta and platelets were collected from ovariectomized (OVX) and SHAM-operated spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. Aorta **(A)** and platelet **(B)** extracts were prepared, and TF protein expression were analysed by western blot. The upper panel shows representative images from three independent analysis, while the lower panel presents the quantitative data normalized by the β-actin expression. Data are presented as mean ± SE and (*) represents significantly statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

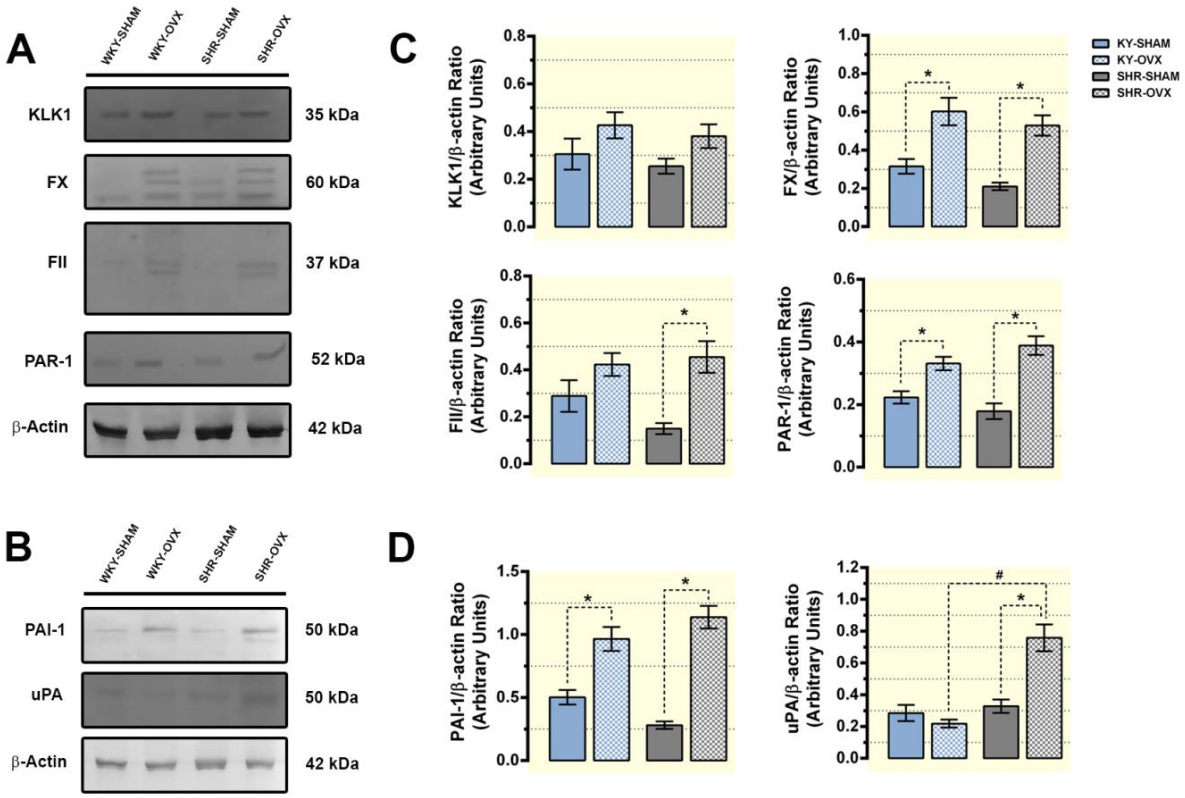

Figure 8. Prothrombotic and fibrinolytic molecular markers in aorta from estrogen-depleted normo and hypertensive rats. Abdominal aorta was collected from ovariectomized (OVX) and SHAM-operated spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. The tissue extracts were prepared and protein expression of prothrombotic markers – kallikrein (KLK1), factor X (FX), factor II (FII) and protease activated receptor – 1 (PAR-1) - **(A and C)** and fibrinolytic markers – plasminogen activator inhibitor – 1 (PAI-1) and urokinase plasminogen activator (uPA) - **(B and D)** were determined by western blot. The left panel shows representative images from three independent analysis, while the right panel presents the quantitative data normalized by the β-actin expression. Data are presented as mean ± SE and the symbols (* and #) represents significantly statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

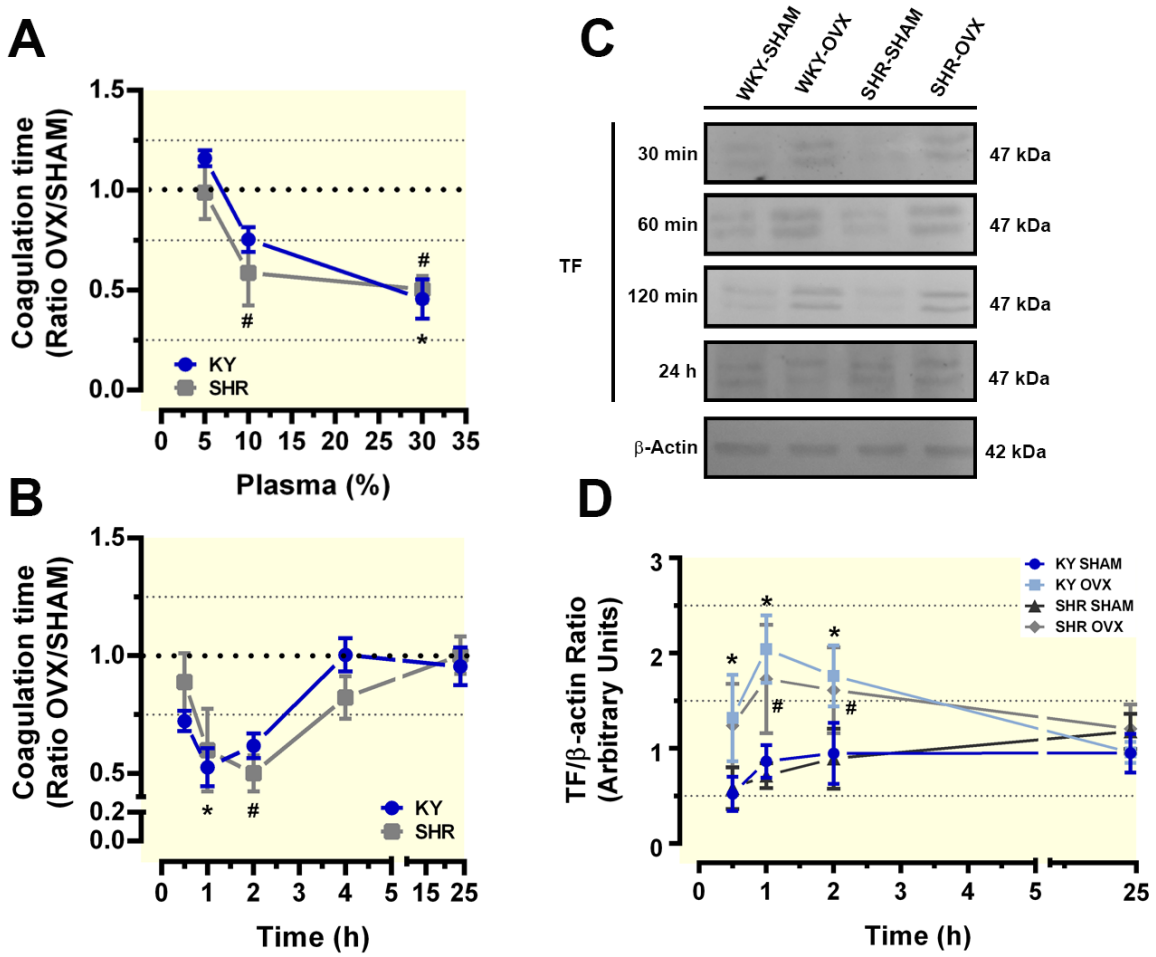

Figure 9. Plasma-derived from normo and hypertensive ovariectomized rats induced a procoagulant phenotype in vascular smooth muscle cells in culture. Plasma obtained from ovariectomized (OVX) and SHAM-operated spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats was used to treat a cell line (A7r5) of vascular smooth muscle cells derived from rat thoracic aorta. **(A)** A7r5 cells were treated with different concentrations of SHR or WKY-derived plasma for 1 h, then cells were washed, and coagulation time was determined through the aPTT assay by the addition of a rat plasma pool collected from healthy animals. **(B)** A7r5 cells were treated with diluted plasma (10 %) from SHR or WKY animals, at different time-point cells were washed and aPTT was determined as described above. **(C)** A7r5 cells treated with 10 % plasma from SHR or WKY animals were collected in each time-point, and tissue factor (TF) protein expression was determined by western blot. The panel shows representative images from three independent analysis. **(D)** Data from TF blots in each time-point were quantified and normalized by the β-actin expression. All data showed on graphs are mean ± SE analysed by one-way ANOVA followed by Bonferroni's-post hoc test. Coagulation data are presented as a ratio between coagulation time from ovariectomized versus SHAM-operated animals. The dotted line on graphs A and B indicates the normal coagulation pattern of A7r5 cells incubated with rat plasma pool collected from healthy animals. The symbols (*) and (#) in panels A and B indicate respectively the statistical difference of WKY and SHR versus the normal coagulation time from cells treated with plasma pool of healthy animals. The same symbols (* and #) in panel D indicate respectively the statistical difference between KY SHAM versus KY OVX and SHR SHAM versus SHR OVX groups.

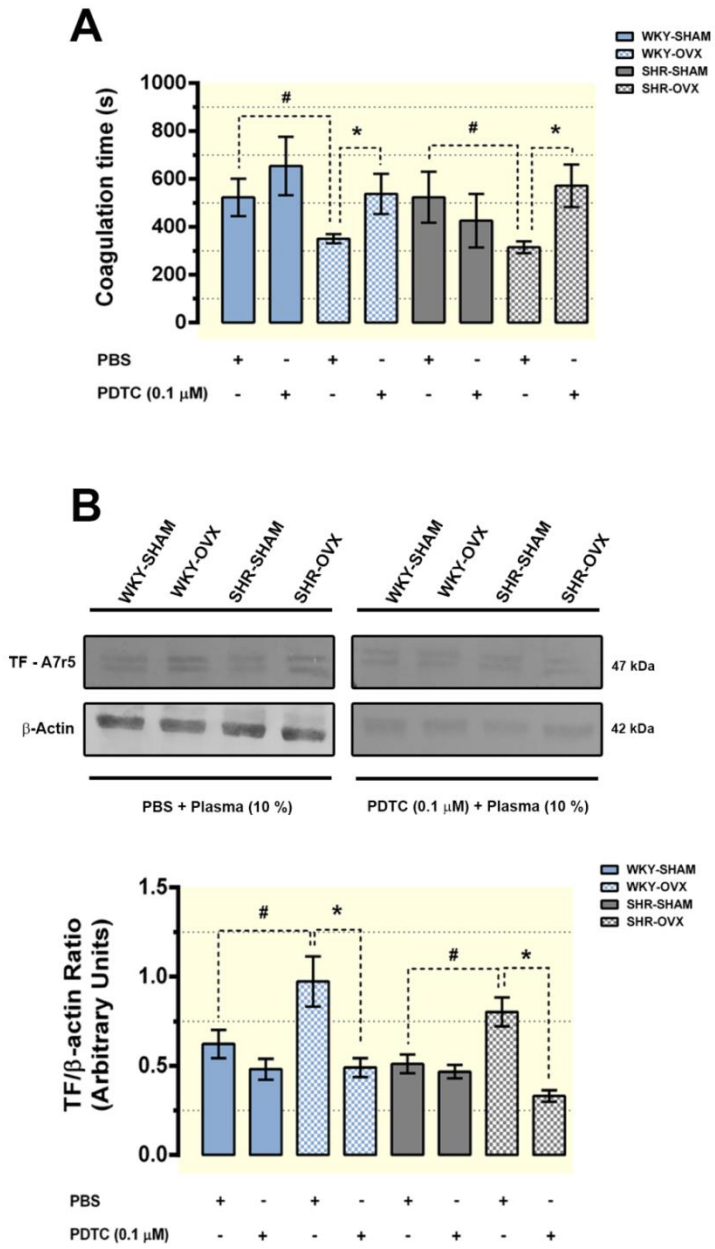

Figure 10. The procoagulant phenotype induced by plasma-derived from normo and hypertensive ovariectomized rats in vascular cells is dependent of NF-κB pathway. Vascular smooth muscle cells (A7r5) were pre-treated overnight with PBS or 0.1 μM PDTC to block the NF-κB pathway. Then, the cells were incubated for 2 h with 10 % plasma obtained from ovariectomized (OVX) and SHAM-operated spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. After a washing step, coagulation time was determined through the aPTT assay by the addition of a rat plasma pool obtained from healthy animals **(A)**. Similarly, in another set of experiments vascular cells were treated as above and collected for tissue factor (TF) protein expression analysis by western blot **(B)**. The upper panel in B shows representative images from three independent experiments, while the lower panel presents the quantitative data normalized by the β-actin expression. All data are presented as mean ± SE and symbols (* and #) represents significant statistical difference between indicated groups (one-way ANOVA followed by Bonferroni's-post hoc test).

CONSIDERAÇÕES FINAIS

De uma maneira geral, os resultados deste estudo são úteis para entender os mecanismos celulares e moleculares responsáveis pelo risco aumentado de eventos pró-trombóticos e sua associação com hipertensão em mulheres na pós-menopausa. Um resumo dos principais achados está ilustrado abaixo na Figura 2.

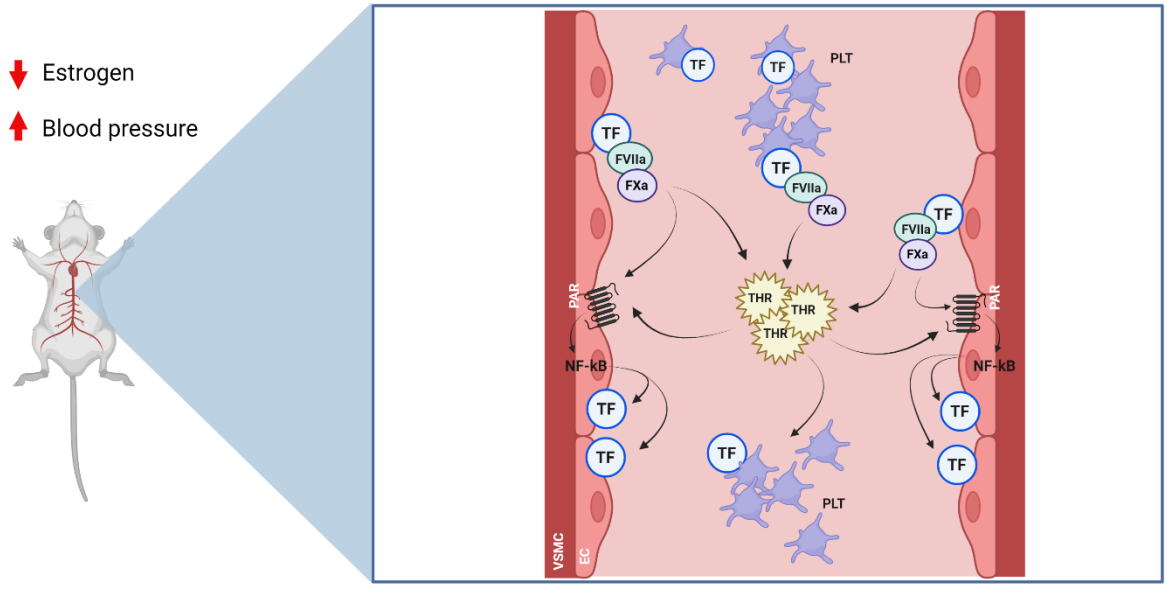

Figura 2. Mecanismo dos eventos pro-trombóticos em modelo experimental de hipertensão na menopausa. A ovariectomia bilateral leva a um aumento da pressão arterial. Esse evento está associado com hiper-reatividade plaquetária e aumento de expressão do fator tecidual nas plaquetas, aorta e células vasculares. A aorta e células vasculares de animais ovariectomizados hipertensos apresentam uma maior capacidade de geração de enzimas prócoagulantes como trombina e fator Xa que são formadas nas superfícies das células pela ligação ao fator tecidual. Trombina e fator Xa são os principais ativadores dos receptores do tipo PAR. Esses receptores regulam, através do NF-kB, a expressão do próprio fator tecidual que é encontrado em células endoteliais, células musculares lisas e plaquetas. Todo o processo parece fazer parte de um mecanismo de feedback positivo que alimenta o estado basal protrombótico. Siglas: TF, fator tecidual; FVIIa, fator VIIa, FXa, fator Xa, THR, trombina, PLT, plaquetas; EC, célula endotelial; VSMC, célula de músculo liso vascular; PAR, receptor ativado por protease; NF-kB, fator nuclear kappa B. Figura criada no BioRender (www.biorender.com)

Descobrimos que a depleção de estrogênio induzida cirurgicamente por ovariectomia bilateral em ratas leva a um estado pró-trombótico vascular associado

a uma diminuição no sistema de metabolização de nucleotídeos, hiperreatividade plaquetária e aumento da expressão proteica do fator tecidual tanto na aorta quanto nas plaquetas. A aorta e as células vasculares têm um papel fundamental em todo o processo, sendo capazes de suportar a geração de enzimas pró-coagulantes por um mecanismo que foi significativamente exacerbado na aorta dos animais espontaneamente hipertensos ovariectomizados. De forma interessante, observamos que o plasma hipercoagulável derivado das ratas com depleção de estrogênio também pode desencadear um perfil pró-trombótico em células vasculares saudáveis mantidas em cultura. O mecanismo nesse caso parece envolver o aumento da expressão do fator tecidual através de uma via dependente da ativação de NF-κB. De fato, o NF-kB pode regular a expressão de fator tecidual principalmente pelos receptores ativados por protease (PAR). Esses receptores são acoplados à proteína G e podem ser clivados e ativados por proteases como trombina, fator VIIa ou fator Xa. Como nossos resultados demonstram que a expressão e a geração de fator Xa e trombina aumentam após a ovariectomia é razoável supor que esses receptores estejam ativados e contribuam para o aumento do fator tecidual via NF-kB. Dessa forma, nossos dados sugerem que o fator tecidual é um alvo interessante a ser considerado para o desenvolvimento de novas estratégias terapêuticas direcionadas às complicações tromboembólicas vasculares no período pós-menopausa.

ANEXOS

1 Documento de aprovação do projeto de pesquisa na Comissão de Ética no Uso de Animais do Hospital de Clínicas de Porto Alegre

HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

Grupo de Pesquisa e Pós Graduação

Carta de Aprovação

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 2019/0001

Título: Efeitos modulatórios da ausência de estrogênio sobre a atividade de calicreína, enzimas pró-trombóticas e a produção de cininas no sistema vascular e renal de ratas ovariectomizadas hipertensas

Pesquisador Responsável: PAULA BARROS TERRACIANO

Equipe de Pesquisa:

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FERNANDA DOS SANTOS DE OLIVEIRA	JORGE ALMEIDA GUIMARÃES
CRISTIANA PALMA KUHL	ISABEL CIRNE LIMA DE OLIVEIRA DURLI
ELIZABETH OBINO CIRNE LIMA	TUANE NERISSA ALVES GARCEZ

Data de Aprovação: 28/04/2021

Data de Término: 28/02/2022

Espécie/Linhagem	Sexo/Idade	Quantidade	Data Reunião	Documento
RATO ISOGÊNICO	F/60 Dia(s)	18	12/02/2019	Projeto
RATO ISOGÊNICO	F/60 Dia(s)	18	12/02/2019	Projeto
RATO HETEROGÊNICO	F/60 Dia(s)	4	12/02/2019	Projeto

- Os membros da CEUA/HCPA não participaram do processo de avaliação onde constam como pesquisadores.

- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.

- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Impresso do sistema AGHUse-Pesquisa por CRISTIAN FIDALGO CABRAL em 28/04/2021 15:44:02

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Grupo de Pesquisa e Pós-graduação
29/04/2021 16:36:39