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Lady Katerine Serrano Mujica

ESTRESSE OXIDATIVO E METABOLISMO ÓSSEO EM MODELO ROEDOR DA SÍNDROME DOS OVÁRIOS POLICÍSTICOS (SOP)

Santa Maria, RS
2020

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Farmacologia, Área de Concentração em Toxicologia e Nutracêutica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção grau de **Doutor em Farmacologia.**

Orientador: Prof. Fabio Vasconcellos Comim
Co-orientador: Prof. Paulo Bayard Dias Gonçalves

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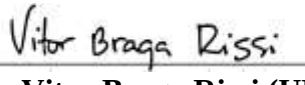

Prof. Dr Vitor Braga Rissi (UFSC)

Santa Maria, RS
2020.

Sempre acredite que o poder do absoluto é
a realização mais alta e mais complexa do ser
humano e que por isso resume ao mesmo tempo
toda sua grandeza e toda a sua miséria.

Gabriel Garcia Márquez

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As demais pessoas que contribuíram de forma direta ou indireta para a realização desse trabalho.

RESUMO

ESTRESSE OXIDATIVO E METABOLISMO ÓSSEO EM MODELOROEDOR DA SÍNDROME DOS OVÁRIOS POLICÍSTICOS (SOP)

AUTORA: Lady Katerine Serrano Mujica

ORIENTADOR: Fabio Vasconcellos Comim

A síndrome dos ovários policísticos (SOP) é considerada a causa mais comum de infertilidade endócrina em mulheres, acometendo cerca de 8% da população feminina em idade reprodutiva. É um distúrbio endócrino complexo, associado a resistência insulínica, aumento de marcadores inflamatórios e de estresse oxidativo, dislipidemia, obesidade visceral e aumento de risco de doenças cardiovasculares e de diabetes mellitus. Mais recentemente, tem sido levantado a possibilidade que a SOP possa, direta ou indiretamente, afetar também o metabolismo ósseo.

Em ratas, o excesso de androgênios ou estrogênios no período neonatal induz a alterações metabólicas e reprodutivas similares às observadas na SOP em humanos. Por este motivo, tem sido desenvolvidos diversos estudos com modelos animais que visam confirmar as origens da SOP. Entre os modelos mais empregados estão aqueles em que se administram doses de propionato de testosterona.

Desta forma, o presente trabalho tem como objetivo principal estudar o modelo animal de SOP induzido em roedores com propionato de testosterona, permitindo avaliar as características metabólicas, estresse oxidativo e metabolismo ósseo causada pela androgenização. Os resultados do estudo indicam que as mudanças no estresse oxidativo podem ser promovidas pela exposição ao propionato de testosterona após o nascimento, o que está provavelmente associado à anovulação e / ou desarranjo lipídico.

Palavras-Chave: SOP, Testosterona, Androgenização, Fetal, Metabolismo Ósseo

ABSTRACT

OXIDATIVE STRESS AND BONE METABOLISM IN A RODENT MODEL OF POLICYSTIC OVARY SYNDROME (PCOS)

AUTHOR: Lady Katerine Serrano Mujica

ADVISER: Fabio Vasconcellos Comim

Polycystic ovary syndrome (PCOS) is considered the commonest cause of endocrine infertility in women and affects around 8% of females at reproductive age. It is a complex disturbance, frequently associated with insulin resistance, increase of inflammatory markers, oxidative stress, dyslipidemia, and an increased risk to the development of cardiovascular disease and diabetes. Recently, it has been considered the possibility that PCOS may affect the bone metabolism directly or indirectly.

In rats, neonatal androgen or estrogen excess replicate similar metabolic and reproductive abnormalities seen in human PCOS. Indeed, studies with animal models have been carried out to confirm the origins of PCOS. Among the models employed are those based on the administration of testosterone propionate.

Therefore, the present work has the main aim to study a rodent animal model of PCOS induced by testosterone propionate and evaluating metabolic, oxidative stress, and its impact on the bone tissue. Study findings indicate that changes in oxidative stress could be promoted by testosterone propionate exposure after birth, which is likely associated with anovulation and/or lipid disarrangement.

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Key words: PCOS, testosterone, fetal androgenisation, bone metabolism

Lista de figuras

Artigo I

Artigo II

Artigo III

Lista de suplemento de figuras

Artigo I

Lista de tabelas

Lista de suplemento de tabelas

Artigo II

Anexos

Artigo I

Artigo II

Tabela de conteúdo

1. Revisão de Literatura

1.1. Síndrome dos Ovários Policísticos (SOP)

A síndrome dos ovários policísticos (SOP) foi descrita pela primeira vez de maneira sistemática por Irving Stein e Michael Leventhal, em 1935. A SOP é um distúrbio endócrino comum que afeta aproximadamente 5-15% das mulheres em idade reprodutiva (KNOCHENHAUER et al., 1998; ASUNCION et al., 2000; ELSENBRUCH et al., 2006; DIAMANTI-KANDARAKIS, 2008), sendo caracterizado por sintomas de anovulação (amenorréia, oligomenorréia e ciclos menstruais irregulares)(MORGANTE et al., 2018) e hiperandrogenismo (hirsutismo, acne e alopecia) e a principal causa de infertilidade por anovulação (HEIM et al., 1999; SLIM et al., 2007; REZVANFAR et al., 2012; RAMEZANI TEHRANI et al., 2014). Apesar de ser um distúrbio comum, a etiologia da SOP ainda é obscura e a origem desta é complexa e multifatorial.

A SOP foi descrita pela primeira vez de maneira sistemática por Irving Stein e Michael Leventhal, em 1935, seu conceito é muito amplo, por isso, observou-se a importância de normatizar alguns parâmetros para melhor definir essa síndrome(ANDREWS, 1952).

Em 1990, em reunião de consenso promovido pelo National Institute of Health decidiu-se incluir, entre as portadoras da síndrome, mulheres com hiperandrogenismo clínico ou laboratorial e ciclos espaniomenorréicos (menos que seis ciclos por ano), desde que afastadas outras alterações como síndrome de Cushing, hiperprolactinemia, deficiência enzimática da supra-renal e distúrbios da tireoide (BANI MOHAMMAD & MAJDI SEGHINSARA, 2017)

Em 2003, o Consenso de Rotterdam, na Holanda, propôs como critérios diagnósticos: ciclos espaniomenorréicos ou amenorréia (anovulação crônica), sinais clínicos ou bioquímicos de hiperandrogenismo, presença de ovários policísticos, desde que fossem excluídas a deficiência enzimática da supra-renal, neoplasias de ovário secretoras de androgênios e a síndrome de Cushing(ROTTERDAM, 2004).

Em 2006, a Sociedade para o Estudo do Excesso de Androgênios (Androgen Excess Society) estabeleceu os seguintes critérios: hiperandrogenismo (hirsutismo e/ou hiperandrogenismo), disfunção ovariana (anovulação e/ou ovários policísticos) e exclusão de outras endocrinopatias(AZZIZ et al., 2006).

Quadro 1. -Consensos para a Síndrome de Ovários Policísticos.

NIH (1990)	**The Rotterdam Consensus (2003)**	**AE-PCOS (2008)**
Presença de dois critérios	Presença de dois de três critérios	Presença de dois critérios
Disfunção menstrual **Hiperandrogenemia e / ou Hiperandrogenismo**	Anovulação crônica Hiperandrogenemia e / ou Hiperandrogenismo Ovários policísticos	Hiperandrogenemia e / ou Hiperandrogenismo Disfunção menstrual e /ou ovários policísticos

Uma série de evidências experimentais vem apontando para uma possível origem embrionária da SOP (FRANKS et al., 2008). A hipótese de que aspectos ambientais, incluindo a dieta e exposição à esteróides sexuais, associados a uma predisposição genética possa desencadear a síndrome, tem ganhado um grande destaque no meio científico (LEGRO et al., 1998; VINK et al., 2006). As ações de xenoestrogênios, em especial o bisfenol-A, no desenvolvimento da SOP também vem sendo exploradas pelos pesquisadores (PUTTABYATAPPA et al., 2017; PUTTABYATAPPA et al., 2019). Ainda assim, o estudo em mulheres com SOP apresenta muitas dificuldades que podem inviabilizar a evolução desses conhecimentos, por vários motivos:

- Tempo médio de vida muito elevado para estudos longitudinais.
- Dificuldade de acesso aos órgãos alvo (hipotálamo, hipófise, ovários),
- Heterogeneidade da síndrome.

Desta forma, a utilização de modelos animais pode trazer contribuições importantes para o entendimento dos mecanismos dessa síndrome (ainda que não possa definir com exatidão o problema primário causador da SOP em humanos) e possivelmente para estratégias terapêuticas e de prevenção.

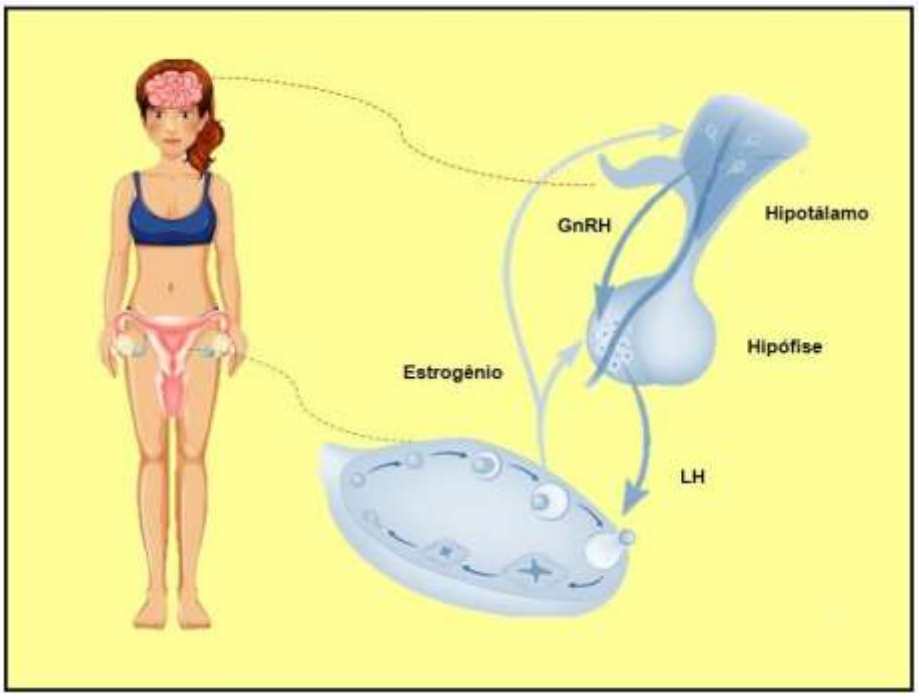
1.1.1 Aspectos Reprodutivos

O hiperandrogenismo, o principal fenótipo da SOP, é resultante principalmente de uma secreção exagerada de androgênios por parte dos ovários e das adrenais. O aumento da produção e esteroides de origem ovariana é causado por uma série de alterações: o hiperestímulo central da LH nas células da teca, a ação da insulina como co-gonadotrofina (EHRMANN et al., 1999; AZZIZ, 2016; AZZIZ et al., 2016, JAKIMIUK et al., 2001), bem como expressão aumentada de enzimas das células da teca envolvidas na síntese de androgênios (JAKIMIUK et al., 2001; GOODARZI et al., 2011). Especificamente, foi demonstrado que nas células da teca ovariana existe uma expressão aumentada do gene *CYP17A1* na SOP, sendo esse um gene chave na regulação da esteroidogênese (PUSALKAR et al., 2009).

Na SOP, o excesso de produção de androgênios (NESTLER & JAKUBOWICZ, 1997), cujos níveis também se encontram aumentados em 40-50% das mulheres com SOP (HOMBURG, 2009). A justificativa para esse aumento é uma alteração da função normal do eixo hipotálamo-hipófise gondal (HHG) e que pode ser resultante de alterações nos mecanismos de retroalimentação mediados por esteroides sexuais na secreção de GnRH a nível hipotalâmico (PASTOR et al., 1998). Quanto ao aspecto ovariano, evidências mostram que os androgênios podem estimular o crescimento dos folículos pré-antrais em seu desenvolvimento inicial, em momento que ainda são independentes do estímulo de gonadotrofinas. Este processo, denominado “stockpilling” resulta em um ovário com seis a oito vezes mais folículos pré-antrais e pequenos folículos antrais, na sua maioria atréticos (PASTOR et al., 1998; MACIEL et al., 2004).

Figura 1. -Eixo hipotalamo- hipófise- gonadal. O hipotálamo se conecta a adenohipófise.

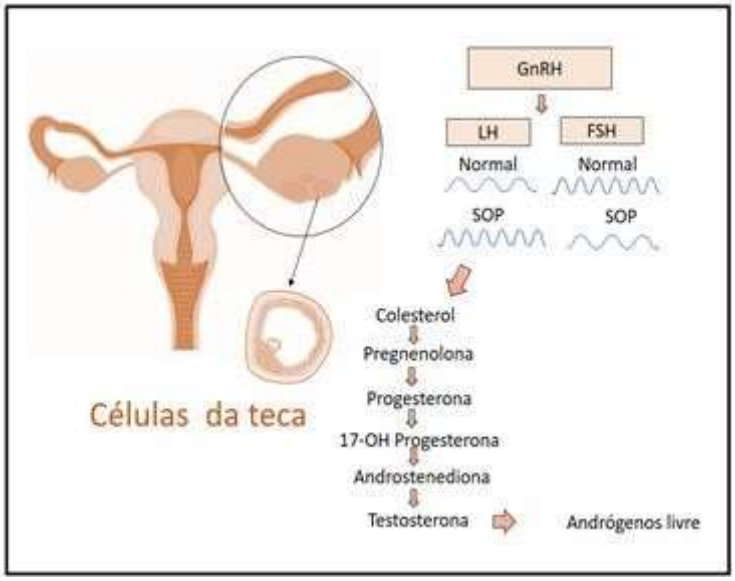
A adenohipófise é uma glândula que compõe a porção anterior da hipófise e possui cinco tipos de células com atividade de secreção hormonal que são responsáveis pela produção das gonadotrofinas sendo esses hormônios atuantes no ovário.



A hiperandrogenemia da SOP está diretamente ligada ao aumento de estimulação do LH nas células da teca ovariana onde são sintetizadas a androstenediona, a DHEA e a testosterona (GILLING-SMITH et al., 1994; GILLING-SMITH et al., 1997; BURGER, 2002; ABBOTT et al., 2005). A falta de ciclicidade e o aumento numérico de folículos antrais (com células da granulosa luteinizadas e com menor atividade da aromatase) acabam também favorecendo um maior acumulo de androgênios. Um interessante estudo *in vitro* mostrou que, mesmo após diversas passagens, as células da teca de mulheres com SOP persistiam com uma maior capacidade de produzir androgênios quando cultivadas nas mesmas condições de cultivo que o grupo controle(GILLING-SMITH et al., 1994). Além disso, os níveis de androgênios na SOP estão significativamente aumentados devido a hiperatividade enzimática da 5α-redutase e esta limita a ação da aromatase atuando como um inibidor competitivo impedindo a seleção folicular (SAM & EHRMANN, 2015).

Figura 2.- Função hormonal

Em uma parte de mulheres com SOP ocorre uma diminuição relativa no pico de FSH um aumento nos picos de LH, que sensibiliza as células tecais e amplifica o hiperandrogenismo ovariano funcional. Estas alterações são observadas geralmente em mulheres com peso normal, uma vez que a obesidade leva a redução da amplitude de secreção das gonadotrofinas.



Outras condições comumente presentes nestas pacientes favorecem um aumento de produção dos androgênios. A obesidade, observada em até 50% dos casos, pode estar associada a níveis elevados de insulina, que semelhantemente ao LH, é estimulador da secreção de androgênios pela teca (WATHES et al., 2007). Na presença de resistência à ação da insulina a produção hepática da globulina carreadora de hormônios sexuais (SHBG) pode estar reduzida gerando uma consequente elevação da testosterona livre, o que por sua vez, significa uma maior biodisponibilidade da testosterona nos tecidos-alvos e agravamento da hiperandrogenemia (MILLER et al., 2004).

Na SOP as adrenais podem também ser responsáveis por uma produção excessiva de androgênios. Acredita-se que 20 ao 30% das pacientes com SOP apresentem hiperandrogenismo adrenal (MORAN et al., 1999; KUMAR et al., 2005) manifesto através de níveis elevados dos seguintes: sulfato de deidroepiandrosterona (DHEAS), 11β-hidroxiandrostenediona (11β-OHA), deidroepiandrosterona (DHEA), Androstenediol e Androstenediona (LOUGHLIN et al., 1986). A etiologia do hiperandrogenismo adrenal na SOP não é totalmente conhecida, tendo sido propostos como mecanismos desta alteração o maior catabolismo do cortisol, a resposta amplificada dos androgênios adrenais, a disregulação de enzimas esteroidogênicas chaves, e os níveis normais de ACTH (LOUGHLIN et al., 1986).

1.1.2. Aspectos Metabólicos

Mulheres com SOP apresentam comumente muitas alterações metabólicas. Entre esses fatores, destacam-se a resistência insulínica, o diabetes, a obesidade com aumento de acúmulo de gordura visceral, e a dislipidemia (KELLY et al., 2002). Resistência Insulínica, é característica marcante da Síndrome Metabólica, está presente em 50-80% de as mulheres com SOP (LEGRO et al., 1998; BARANOVA et al., 2011).

O conceito de síndrome metabólica é um estado de anormalidades clínicas e laboratoriais associado a maior risco de desenvolvimento de doenças cardiovasculares. O perfil lipídico clássico dessa síndrome se caracteriza por elevação dos triglicerídeos e dos níveis de LDL, bem como redução do HDL-colesterol. Essas condições somam-se aos demais componentes para determinar elevação do risco cardiovascular e morte prematura, que podem ocorrer nas mulheres com síndrome de ovários policísticos. (STOLAR, 2007).

Quadro 2. -Características metabólicas em mulheres com SOP.

Características Metabólicas		Referencias
Diabetes	Sim	(DUNAIF, 1997; PASQUALI et al., 2000)
Obesidade	Sim	(PASQUALI et al., 2000; GAMBINERI et al., 2002; PASQUALI et al., 2011)
Dislipidemia	Sim	(WILD et al., 2011; KIM & CHOI, 2013)

1.1.2.1 Obesidade

Obesidade e SOP parece estar em estreita relação, diversos estudos mostram associação direta entre SOP e Obesidade uma vez que mais de 50% das mulheres com SOP apresentam algum grau de obesidade (DUNAIF, 1997; MARCONDES et al., 2007; DIAMANTI-KANDARAKIS, 2008; NADERPOOR et al., 2015), além disso, a obesidade ajuda piorar os aspectos hormonais, dermatológicos, metabólicos e reprodutivos da SOP (RANDEVA et al., 2012). Em estudos familiares demonstraram que o aumento de peso promove as chances da síndrome (SINGH et al., 2010). Pesquisas demonstram que a exposição ao excesso de androgênios intra-útero, no período neonatal ou na vida adulta, aumenta a expressão de genes envolvidos na lipogênese predispondo ao acúmulo de massa gorda, na cavidade abdominal (DUNAIF et al., 1989; LIOU et al., 2009). Este depósito de gordura visceral, está relacionado,

com uma maior expressão de receptores androgênicos no tecido e redução da lipólise induzida pelos androgênios (DICKER et al., 2004; BLOUIN et al., 2009; DE ZEGHER et al., 2009).

O excesso de gordura abdominal produz grande quantidade de ácidos graxos livres ao sistema porta, que contribuem para o aumento da produção de glicose pelo fígado e de insulina pelo pâncreas (DICKER et al., 2004). Além disso, sabe-se que a gordura visceral altera as concentrações de adipocinas (leptina, adiponectina, resistina, interleucina-6, fator de necrose tumoral-alfa), peptídeos que ao estar alterados, interveem na ação da insulina (YILDIZ et al., 2010).

A obesidade nas pacientes com SOP agrava as manifestações de hiperandrogenismo, porque inibe a liberação da SHBG, aumentando a taxa de androgênios livres (DUNAIF, 1997; EHRMANN et al., 1999; NESTLER et al., 2002). Na SOP, a RI e o hiperandrogenismo também estão presente em mulheres magras, porém, nas obesas, os níveis de LH são maiores, há maior supressão da SHBG e da IGFBP-1, sendo mais severos a RI e o quadro androgênico (KIRCHENGAST & HUBER, 2004; AL-RUTHIA et al., 2017).

1.1.2.2. Dislipidemia

As dislipidemias são alterações do metabolismo das lipoproteínas (LP) também frequentemente associadas à SOP. As LP são macromoléculas compostas por lipídios e proteínas responsáveis pelo transporte de TG e colesterol no organismo, tanto os de origem exógena quanto os de endógena sendo alterações constantes na SOP, caracterizadas por alterações nas concentrações séricas de qualquer um dos tipos de lipoproteínas como por exemplo, a lipoproteína de baixa densidade (LDL- colesterol) e a lipoproteína de alta densidade (HDL- colesterol). Na SOP as anormalidades nas lipoproteínas apresentam padrões variados, com aumento dos triglicerídeos (TG), do colesterol total (CT) e das LDL- colesterol, associados com redução da HDL- colesterol, o que confere a essas mulheres uma dislipidemia aterogênica (KIM & CHOI, 2013), Dislipidemia comumente observada em pacientes com obesidade, síndrome metabólica, resistência à insulina e diabetes mellitus tipo 2, sendo um fator de risco para desenvolvimento de aterosclerose (KIRCHENGAST & HUBER, 2001; WILD et al., 2011).

1.1.2.3. Resistencia à insulina

A resistência à insulina (RI) refere-se a uma quantidade aumentada de insulina necessária para executar a ação metabólica. Além dos efeitos metabólicos, a insulina exerce ações mitogênicas e reprodutivas. RI é um dos principais mecanismos de desarranjo metabólico

na SOP, ocorre de 50 até 70% desta população, dependendo do estudo. Evidências *in vitro* e *in vivo* sugerem a associação entre os níveis de insulina e os andrógenos (NESTLER et al., 1998; TSILCHOROZIDOU & CONWAY, 2004). A hiperinsulinemia que é observada na SOP é principalmente resultado do aumento da secreção de insulina basal, juntamente com diminuição da depuração da insulina hepática (DUNAIF, 1997).

A insulina se liga ao receptor da superfície celular, que é estruturalmente homólogo ao receptor do fator de crescimento semelhante à insulina 1 (IGF-1) (SALTIEL & KAHN, 2001). A insulina estimula a captação de glicose aumentando a translocação do transportador de glicose responsivo à insulina 4 (GLUT4) das vesículas intracelulares para a superfície celular. Essa via é mediada pela ativação da fosfatidilinositol 3-quinase (PI3-K), enquanto o crescimento e a diferenciação celular são mediados pela via MAPK-ERK que estimula uma cascata de enzimas, incluindo serina / treonina, Raf, MAPK e MAPK-ERK1 / 2 (DIAMANTI-KANDARAKIS et al., 2012).

O aumento dos níveis de insulina circulantes decorrentes da resistência a ação deste hormônio, leva a alterações em diferentes sistemas no organismo. Por exemplo, a insulina estimula a androgênese ovariana pelo aumento da secreção de hormônio luteinizante e da proteína carregadora do fator de crescimento semelhante à insulina (IGFBP-1), que está suprime a globulina ligadora de hormônios sexuais (SHBG) (TSILCHOROZIDOU & CONWAY, 2004). A hiperinsulinemia pode contribuir também para o desenvolvimento de obesidade, dislipidemia e esteatose hepática nas mulheres com SOP. Além disso, é possível que concentrações mais baixas de adiponectina e acúmulo de gordura no fígado, músculo esquelético e tecido perimuscular possam desempenhar um papel único na patogênese da RI em mulheres com SOP (SHULMAN, 2014; MORRISON et al., 2017).

De forma previsível, a redução da hiperinsulinemia/ resistência insulínica através de medicamentos (metformina, liraglutida, glitazonas) e/ou mudanças do estilo de vida levando a redução da gordura visceral tem conseguido reduzir os níveis de androgênios e aumentar as taxas de fertilidade em mulheres com SOP (MARCONDES et al., 2007; DU et al., 2009; AZZIZ, 2016; AZZIZ et al., 2016; MACUT et al., 2017).

2. Estresse Oxidativo

2.1. Considerações gerais

O estresse oxidativo é um processo mediado pela formação de radicais livres diversos que resultam da degradação oxidativa dos lipídeos (lipoperoxidação) e de proteínas presentes

nos diversos sistemas de membrana da célula.

O estresse oxidativo é comumente referido como o desequilíbrio entre oxidantes e antioxidantes. Quando o desequilíbrio favorece oxidantes, geração de quantidades excessivas de espécies de oxigênio prejudicam nosso corpo de várias maneiras através a geração de quantidades excessivas de espécies reativas de oxigênio (ROSS, 1988; AGARWAL et al., 2005). Em outras palavras, células e tecidos reprodutivos permanecerão estável apenas quando o estado antioxidante e oxidante é equilibrado.

O desequilíbrio entre a formação e a defesa antioxidante resulta em vários processos deletérios para a célula e a longo da vida. Este desequilíbrio é denominado estresse oxidativo que são flutuações na concentração destes oxidantes exercem um papel na sinalização intracelular, enquanto aumentos descontrolados dessas espécies de oxigênio conduzem a reações em cadeia com proteínas, lipídeos, polissacarídeos e DNA (DROGE, 2002).

O balanço redox celular é regulado por uma quantidade relativa de substâncias oxidantes e redutoras. Ânion superóxido (O_2^-), radical hidroxila (OH), peróxido de hidrogênio (H_2O_2) e oxigênio singleto ($^1O_2$) constituem os principais componentes oxidantes endógenos (KAMATA & HIRATA, 1999; THANNICKAL & FANBURG, 2000). A primeira consequência desse processo é a profunda alteração das propriedades físicas e químicas das membranas, causando perda das suas funções especializadas (ROSS, 1988).

As células possuem diversas estratégias para neutralizar os radicais livres de oxigênio gerados durante as oxidações biológicas prevenindo assim a propagação dos danos. Trata-se de um sistema complexo que comprende:

- Inativação dos radicais livres de oxigênio por enzimas específicas.
- Neutralização dos radicais eventualmente formados pela ação de substâncias com propriedades sequestradoras de radicais livres.
- Reparo dos lipídeos oxidados. A geração de espécies reativas de oxigênio pela mitocôndria é um processo contínuo e fisiologicamente normal em condições aeróbicas(VERHAEGEN et al., 1995).

Em mulheres, a oxidação de ácidos graxos é importante para o eixo gonadotrófico e a gametogênese, ajudando na qualidade dos oócitos (WATHES et al., 2007; DUNNING & ROBKER, 2012; DUPONT et al., 2014). Eles também atuam como precursores para a síntese das prostaglandinas e esteroides, sendo essencial para a maturação dos oócitos durante os estágios do desenvolvimento embrionário (DUPONT et al., 2014)

2.2. Estresse Oxidativo na SOP

Alterações nos padrões de estresse oxidativo, são descritas nas mulheres com SOP, independentemente do IMC ou de apresentarem ou não anormalidades metabólicas (GUZICK et al., 1996; SABUNCU et al., 2001). Algumas consequências patológicas da SOP como a resistência à insulina, hiperandrogenismo e a obesidade, parecem estar aumentadas em mulheres que também apresentam quadro de estresse oxidativo.

Evidências sugerem uma forte correlação entre resistência à insulina e estresse oxidativo no tecido adiposo visceral em mulheres com SOP (CHEN et al., 2014; BANNIGIDA et al., 2020). Em estudos recentes com mulheres com SOP (MURRI et al., 2013; SESHADRI REDDY et al., 2018), os níveis de antioxidantes vem sendo descritos tanto como reduzidos (óxido nítrico e glutationa) (NACUL et al., 2007; MENG, 2019), ou elevados (paraoxonase, da atividade da superóxido dismutase, e níveis de albumina). A oxidação direta de lipídios tem sido reportada como aumentada (malondialdeido/MDA) assim como a oxidação de proteínas (xantina oxidase e albumina modificada pela isquemia (GATEVA & KAMENOV, 2012).

Estudos em ratas e em ovelhas com hiperandrogenismo, apresentaram maiores níveis de estresse oxidativo, quando comparadas com ratas controle (YANES et al., 2011), mostrando que existe uma relação da resistência à insulina e hiperandrogenismo com o quadro de estresse oxidativo, influenciando pela SOP.

3. Metabolismo Ósseo

3.1. Considerações gerais

Os ossos são um tecido metabolicamente ativo que continuamente são renovados durante toda a vida (LIESEGANG et al., 1998; CHANOIT et al., 1999; CORTET & MARCHANDISE, 2001; MARCH et al., 2010; YANES et al., 2011). Eles são constituídos por diversas células, matriz mineralizada e não mineralizada de tecido conjuntivo, e espaços que incluem a medula óssea, canais vasculares, canalículos e lacunas como resultado dos processos de remodelação óssea se desenvolve com base em dois processos a formação e a reabsorção óssea (ERIKSEN, 1986; TURNER, 1998). Os dois processos (formação e reabsorção) permitem a renovação e remodelação ósseas e é mantido a longo prazo junto um complexo sistema de controle que inclui hormônios, fatores físicos e fatores humorais locais (KUSHIDA et al., 1995; WOITGE et al., 1995). Condições como idade, doenças ósteo-metabólicas, mobilidade diminuída, ação de algumas drogas, podem alterar este equilíbrio entre formação e

reabsorção, levando ao predomínio de um sobre o outro (RAISZ, 1999)**.**

Dois tipos celulares, derivados de precursores originados na medula óssea, destacam-se, no processo de remodelamento óssea: os osteoblasto/osteócito (FIGURA 3). Os osteoclastos são células multinucleadas, exclusivas do tecido ósseo, e que uma vez recrutados, proliferam, aderem a superfície óssea e levam à sua degradação através da produção de vesículas compostas de ácidos e proteases. Os osteoblastos, por sua vez, ao tornar-se embebidos na matriz e sofrerem diferenciação, dão origem aos osteócitos. A sinalização molecular tanto para o desenvolvimento de osteoblastos quanto osteoclastos é complexa. Usualmente as cascatas de transdução necessárias para o processo de diferenciação dos osteoblastos envolvem os seguintes fatores: *Runx2* (efeito estimulador), *Bone Morphogenetic Proteins (BMPs)* (efeito estimulador), *transforming growth factor beta. (TGF-β)* (efeito estimulador e/ou inibidor), *Wnt/LRP5/β-catenin* (efeito estimulador), *insulin-like growth fator -I (IGF-I)* (efeito estimulador), *fibroblast growth fator (FGF)* (efeito estimulador), *Notch protein (*efeito inibidor) e *Hedgehog protein* (efeito estimulador)(LONG, 2011) Os principais fatores envolvidos na osteoclastogenese e reabsorção, por sua vez, são: osteoprotegerin (OPG), nuclear factor-kB (RANK) e seu ligante (RANKL) e o macrophage-colony stimulating fator (M-CSF), interleucin -1 (IL1)(BARON & KNEISSEL, 2013).

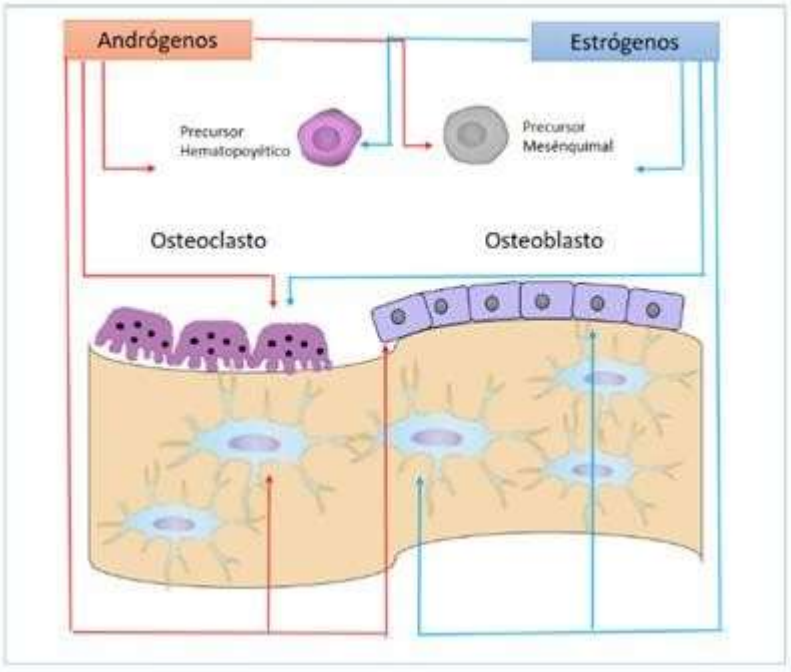
Os ossos são compostos essencialmente por dois tipos de tecidos: o tecido trabecular, uma estrutura de aspecto esponjoso; e o tecido cortical, de forma compacta ao redor do componente trabecular e que contribui para a manutenção da sua integridade estrutural (HESP et al., 1987). Além das diferenças estruturais, os dois tipos diferem também quanto a outros aspectos como a distribuição espacial das células, densidade da matriz mineralizada, distribuição dos vasos sangüíneos e área ocupada pela medula óssea (ALVAREZ et al., 1995). Em função de sua maior superfície em relação ao volume, o osso trabecular é metabolicamente mais ativo que o cortical (KUSHIDA et al., 1995).

Os marcadores da formação óssea são detectados somente no soro e os marcadores de reabsorção óssea podem ser detectados no soro ou na urina (ALLEN et al., 2000). Os marcadores da formação óssea são a osteocalcina (OC), fosfatase alcalina total (FAT), fosfatase alcalina óssea (FAO), peptídeo carboxiterminal do procolágeno tipo I (PINP) e peptídeo aminoterminal do procolágeno tipo I (PINP) enquanto os marcadores de reabsorção óssea são a fosfatase ácida tartarato resistente (TRAP), o telopeptídeo carboxiterminal do colágeno tipo I (ICTP), piridinolina (U-PYD), deoxipiridinolina (U-DPD), hidroxiprolina (U-HYP), porção aminoterminal do procolágeno I (U-NTX) e porção carboxiterminal do procolágeno I (U-CTX) (SEEMAN, 1999; ALLEN et al., 2000; DELMAS & COMMITTEE OF SCIENTIFIC

ADVISORS OF THE INTERNATIONAL OSTEOPOROSIS, 2001). Os marcadores do metabolismo ósseo são empregados, em humanos, no diagnóstico definitivo ou no monitoramento de doenças ósseas metabólicas (CHAVASSIEUX et al., 2001; MIKI et al., 2003; PRZEDLACKI et al., 2009).

Figura 3.-Metabolismo ósseo.

Os estrógenos e os andrógenos influenciam a geração e a vida útil dos osteoclastos e osteoblastos, bem como a vida útil dos osteócitos. Modificada: (ALMEIDA et al., 2017)



	Fatores estimuladores da formação óssea  Osteoblasto	Fatores estimuladores da reabsorção óssea  Osteoclasto	Inibidor da reabsorção óssea  Osteoclasto
Fatores de crescimento	BMP-2 BMP-4 BMP-6 BMP-7 IGF-I IGF-II TGF-β FGF PDGF	TNF EGF PDGF FGF M-CSF GM-CSF	
Citocinas		IL-1 IL-6 IL-8 IL-11 PGI_2 PGG_2 PGE_2 PGH_2 PGE_4	INF-γ IL-4

3.2. Metabolismo Ósseo na SOP

Oligo / amenorreia, hiperandrogenismo, obesidade e hiperinsulinemia, características

da SOP podem produzir efeitos de longo prazo sobre a densidade mineral óssea (BMD). Enquanto o estrogênio desempenha um papel importante no desenvolvimento e manutenção da massa óssea em mulheres principalmente na via RANK/RANKL/OPG, inibindo a formação de osteoclastos, a influência dos andrógenos na massa óssea de mulheres não foi totalmente elucidado. Embora a aromatização de andrógenos a estrogênios no ovário e tecido extra-glandular, com subsequente receptores de estrogênio em tecidos-alvo, seja o principal mecanismo de ação androgênica no metabolismo ósseo, as ações de androgênios não-aromatizáveis também tem sido descritas (SAITO & YANAIHARA, 1998; SEEMAN, 1999; CHAVASSIEUX et al., 2001; MIKI et al., 2003; PRZEDLACKI et al., 2009). Assim, o hiperandrogenismo em mulheres com SOP poderia afetar o osso.

Por outro lado, a massa óssea é alcançada desde o final da adolescência até os trinta anos e a disfunção na menstruação durante este período, pode possivelmente influenciar negativamente na densidade mineral óssea (RECKER et al., 1992) acredita-se que as irregularidades menstruais e amenorréia em mulheres mais jovens com SOP podem representar um risco para o desenvolvimento de osteoporose durante a vida adulta. Até à data, no entanto, os dados relativos A BMD em mulheres com SOP são conflitantes; foram reportados nenhuma diferença na DMO, aumento e diminuição da BMD, (DI CARLO et al., 1992; ADAMI et al., 1998; KIRCHENGAST & HUBER, 2001; YUKSEL et al., 2001; KASSANOS et al., 2010; PIOVEZAN et al., 2019). Uma meta-analise recente identificou redução de massa óssea em mulheres com SOP e IMC < 27 kg/m^2; todavia, até o momento, não é conhecido o impacto da SOP sobre a incidência de fraturas ósseas (PIOVEZAN et al., 2019).

4. Modelos animais de SOP

De acordo com a proposta do epidemiologista David Barker, a ocorrência de acontecimentos intrauterinos ou agravos no período neonatal, podem resultar em alterações estruturais, fisiológicas e metabólicas para o organismo e assim desencadear doenças endócrino- metabólicas na vida adulta (BARKER, 1990; BARKER, 1997).

Baseado nesta hipótese houve um considerável número de estudos mostrando que o excesso de androgênio em fases precoces da vida (no período fetal e neonatal), poderia resultar em manifestações com fenótipo similar à SOP em animais na vida adulta. Nesse sentido, o uso de modelos animais para a pesquisa da sindrome, e inúmeros modelos experimentais foram desenvolvidos com o propósito de mimetizar os eventos ocorridos em mulheres. Assinalam-se, entre eles, a exposição a androgênios (testosterona, a dihidrotestosterona, sulfato de dehidroepiandrosterona), estrogênios, inibidores de aromatase, antiprogestagênios, luz

contínua e manipulações genéticas (WALTERS et al., 2012). Os modelos mais utilizados para pesquisa são: camundongos, ratos, ovelhas e macacos Rhesus. Entre os animais, a ocorrência espontânea de achados semelhantes SOP como aumento de androgênios e LH só foi descrita isoladamente em macacas rhesus(ABBOTT et al., 2017).

Em 1961 foi descrito na literatura, que injeções de propionato de testosterona até o quinto dia de vida em ratas induziriam a infertilidade destes animais por anovulação na idade adulta (BARRACLOUGH, 1961). Após, diversos estudos observaram que utilizando o princípio que a exposição precoce ao excesso de androgênios reproduz na vida adulta características metabólicas, como hiperinsulinemia, aumento dos níveis de LH, e morfológicas, como os ovários policísticos, modelos animais com SOP foram sendo refinados e finalmente estabelecidos (ABBOTT et al., 2005; DUMESIC et al., 2005; ZHOU et al., 2005). O propionato de testosterona foi estudado em diferentes fases da vida de roedores, observando-se que ratas expostas durante os primeiros 5 dias de vida passaram a apresentar, durante a vida adulta, estro persistente, anovulação, presença de ovários policísticos e folículos atrésicos, um fenótipo semelhante a SOP (MCDONALD & DOUGHTY, 1972). Curiosamente, a exposição de ratas tardiamente, com 15-25 dias de idade não induziu um fenótipo semelhante a SOP com ovários morfologicamente normais, destacando o fato de que os efeitos do androgênio, podem ocorrer apenas durante períodos específicos (TYNDALL et al., 2012). Porém, foi observado que o modelo animal roedor exposto a testosterona durante a fase prenatal (nos dias 16, 17 e 18 de gestação) e pós-natal no dia (5 de nascido), apresentam alterações reprodutivas e metabólicas que mimetizam a SOP.

SOP experimental pode ser induzido por outros fármacos (como a dexametasona, estradiol, di-hidrotestosterona, letrazol) (Figura 4), ou através da manipulação genética em várias espécies de animais. A maioria dos experimentos é realizada em roedores, embora alguns estudos sejam realizados em animais maiores. O modelo murino é o mais usado em decorrência da disponibilidade de mais de 200 linhagens bem caracterizadas e da habilidade de deletar ou superexpressar genes específicos por meio da tecnologia dos nocautes e transgênicos (DUMESIC et al., 2005).

Embora existam limitações intrínsecas relacionadas a origem do fator desencadeante (etiologia), a utilização de modelos animais tem se mostrado valiosa estratégia para entender a interação entre fatores reprodutivos e metabólicos na SOP.

Figura 4.- Modelos animais para o estudo de SOP.

Modelos de estudo para SOP	Macaco 	Ovelha 	Rato 	Mulher 
Tipo de ovulação	Monovulatorios	Monovulatorios	Poliovulatorios	Monovulatorios
Puberdade	32 meses	24 meses	2 meses	144 meses
Dias de gestação	165 dias	152 dias	24 dias	290 dias
Protocolos de androgenização	Androgenização (Pré-natal) • Propionato de Testosterona • Letrozol • DHEA • Estradiol Androgenização (Pós-natal) • Propionato de Testosterona • Letrozol • DHEA • Estradiol	Androgenização (Pré-natal) • Propionato de Testosterona • Letrozol • DHEA • Estradiol • Dexametasona Androgenização (Pós-natal) • Propionato de Testosterona • Letrozol • DHEA • Estradiol	Androgenização (Pré-natal) • Propionato de Testosterona • Letrozol • DHEA • Estradiol • Dexametasona Androgenização (Pós-natal) • Propionato de Testosterona • Letrozol • DHEA • Estradiol • Dexametasona	

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5. Objetivos

5.1. Objetivo geral

Avaliar marcadores de metabolismo, estresse oxidativo e metabolismo ósseo em modelo roedor de Síndrome de ovário policístico.

5.2. Objetivos específicos

Comparar o perfil de estresse oxidativo em dois diferentes fenótipos obtidos pelos protocolos de androgenização pré-natal (PreN) e pós-natal (PostN) (ambos tratados com propionato de testosterona).

Reexaminar o papel da Síndrome de Ovário Policístico no metabolismo ósseo, investigando um modelo jovem roedor de SOP (que exibia ovários policísticos e menos ciclos estrais).

Estudar o impacto da ovariectomia em lipídios, glicose e marcadores de estresse oxidativo em conjunto em modelo androgenizado de SOP.

ARTIGO I

TRABALHO PUBLICADO:

Oxidative stress and metabolic markers in pre- and postnatal polycystic ovary syndrome rat protocols

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Abstract

Several studies have described an enhanced inflammatory status and oxidative stress balance disruption in women with polycystic ovary syndrome (PCOS). However, there is scarce information about redox markers in the blood of androgenised animal models. Here, we evaluated serum/plasma oxidative stress marker and metabolic parameter characteristics of prenatal (PreN) and postnatal (PostN) androgenized rat models of PCOS. For PreN androgenisation (n=8), 2.5 mg testosterone propionate was subcutaneously administered to dams at embryonic days 16, 17, and 18, whereas PostN androgenisation (n=7) was accomplished by subcutaneously injecting 1.25 mg testosterone propionate to animals at postnatal day 5. A unique control group (n=8) was constituted for comparison. Our results indicate that PostN group rats exhibited particular modifications in oxidative stress marker; an increased plasma ferric-reducing ability (FRAP) and increased antioxidant capacity reflected by higher albumin serum levels. PostN animals also presented increased total cholesterol and triglyceride-glucose (TyG) levels, suggesting severe metabolic disarrangement. Study findings indicate that changes in oxidative stress could be promoted by testosterone propionate exposure after birth, which is likely associated with anovulation and/or lipid disarrangement.

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder impacting 5%–10% of women at reproductive age (FRANKS, 1995; NORMAN et al., 2007; BALEN et al., 2009). Although PCOS has been identified mainly by reproductive features (oligo-amenorrhea, hyperandrogenism, polycystic ovary appearance), other metabolic and inflammatory conditions, including disruption in cholesterol and glucose levels and the oxidative stress balance, have also been reported (DIAMANTI-KANDARAKIS et al., 2008; DEEPIKA et al., 2014; AZZIZ et al., 2016a; VICTOR et al., 2016; ABRUZZESE et al., 2017; BANULS et al., 2017; BEHBOUDI-GANDEVANI et al., 2017). A recent publication has found that total oxidative stress and antioxidant capacity were increased in PCOS against controls (ZHANG et al., 2017). In this study, which also evaluated the four phenotypes of PCOS, a higher oxidative stress was related to increased androgens, plasma glucose, triglycerides and decreased $apoA_1$ concentrations (ZHANG et al., 2017).

Studies in rodent models replicate many of the abnormalities observed in PCOS women and, for this reason, have been used to explore the pathophysiological basis of the disorder.(SLOB et al., 1983; MANNERAS et al., 2007; MOTTA, 2010; MANNERAS-HOLM et al., 2011; AMALFI et al., 2012; HEBER et al., 2013). Currently, there is scarce information about the redox state in the blood of PCOS rats, once the majority of the studies have focused most of their attention to the ovaries, liver, fat and muscle tissues[17,29,35].

Therefore, the aim of this study was to compare the oxidative stress profile in two different phenotypes obtained by pre and post-natal androgenization protocols (both with testosterone propionate). This research worked with an anestrous rat model of PCOS (postnatal androgenized rat or PostN group), an estrous rat model (prenatal androgenized rat or PreN group), and an androgenized postnatal estrous rat model (PostN L group)(TYNDALL et al., 2012; SERRANO MUJICA et al., 2017).

Our results indicate that modifications in the oxidative stress markers in the blood occurred in the presence of severe reproductive and metabolic disarrangement observed in the female rats submitted to postnatal androgenisation with testosterone propionate.

Materials and Methods

Animals

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Santa Maria (UFSM), Brazil, under protocol number 100/14. The procedures with animals were in agreement with the guidelines of the Brazilian National Council of Control of Animal Experimentation that follows the “Principles of Laboratory Animal Care” established by the National Institutes of Health, USA.

Overall, 46 females Wistar rats *(Rattus norvegicus albinus*) were used in this study and housed at the Laboratory Animal Reproduction (BioRep) the Federal University of Santa Maria (UFSM). The animals were maintained at a temperature of 22 °C, 55–65% humidity under artificial illumination on a light–dark cycle of 12:12 h; with daylight from 7 a.m. to 7 p.m. Food and water were given *ad libitum*.

A total of 30 female rats were submitted to the protocol for synchronization of oestrus. They received an intraperitoneal injection of 10 IU of equine chorionic gonadotropin (eCG; Folligon™, Intervet, Sao Paolo, Brazil), followed 48 h later by 10 IU of human

chorionic gonadotropin (hCG; Pregnyl™, Organon, Cascavel, Brazil), and were placed with a male for 24 h. Matches were controlled; vaginal plug was checked every 12 h. Observation of the vaginal plug was considered as the first day of pregnancy. Female rat pups were divided into four groups for androgenisation by treatment with testosterone propionate or two control groups. Dams were maintained with their pups until weaning (21 days). Prenatal hormone exposure was accomplished by treatment of pregnant dams during embryonic days 16, 17, and 18 through subcutaneous injection of 2.5 mg testosterone propionate (Androgenol™, Hertape Calier, Juatuba, Brazil) (PreN group), whereas vehicle control exposures were accomplished by similar treatment of pregnant dams with 2.5 mg corn oil (Control PreN). Postnatal hormone exposures were performed by the treatment of 5-day-old animals through a subcutaneous injection of 1.25 mg testosterone propionate (PostN group), whereas vehicle control postnatal 5-day-old animals received a subcutaneous injection of 1.25 mg corn oil (Control PostN) (SERRANO MUJICA et al., 2017). The final groups were as follows: prenatal (PreN n = 8), postnatal (PostN n = 7), and control group (Control PreN plus Control PostN) (n=8). Another androgenized group, postnatal leuprolide (PostN L n=7), included the treatment with an intramuscular injection (i.m.) of 0.40 mg leuprolide acetate depot (Lectrum™, Sandoz, Brazil) in 2-day-old rats before postnatal androgenisation with testosterone propionate. Information of other groups of leuprolide treatment including the number of animals per group (e.g. prenatal androgenized plus leuprolide) is available in the supplemental material.

Euthanasia and sample collection

At 110 days of age, the animals were transferred and then anaesthetized with isoflurane plus administration of tramadol chloride (Tramadol™, Pfizer, Sao Paolo, Brazil) intramuscularly (20–40 mg/kg). Between 9:00 a.m. and 10:00 a.m., blood samples were collected before the animals were finally sacrificed using cardiac puncture under deep anaesthesia in the absence of pedal and corneal reflexes. Blood samples were centrifuged at 4 °C and 5000 rpm/4696 *g* (Sorvall-Thermo Scientific, Asheville, NC, USA) for 15 min to separate the blood solid components from the serum and plasma (EDTA), and stored at -80 °C.

Laboratory measurements

Total blood cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) triglyceride, albumin, glucose, were measured enzymatically in serum using a commercial assay kit (Labtest Diagnostics®, Lagoa Santa, Brazil).

Ferric-reducing ability of plasma (FRAP)

FRAP was assessed as previously described(BENZIE & STRAIN, 1996). In brief, the FRAP reagent was freshly prepared and warmed at 37 °C by mixing the following solutions: (1) 0.3 M sodium acetate buffer solution (pH 3.6); (2) 10 mM 2,4,6-tripyridyl-1-5-triazine in 40 mM HCl solution; and (3) 20 mM FeCl3 solution at the ratio of 10:1:1 (v/v/v), respectively. Plasma (10 µL) was incubated with 90 µL of FRAP reagent in a microplate for 30 min at room temperature in the dark. Subsequently, the level of absorbance of the mixture was measured at the wavelength of 595 nm using a spectrophotometer. The FRAP values were calculated by using a calibration standard curve of $FeSO_4$ (0–2000 µM). All measurements were performed at the same ocasion. The intra-assay coefficient of variation was between 1-2%.

Measurements of advanced oxidation protein product (AOPP) levels in serum

Samples were prepared as follows: in a tube, 20 µL of serum from each rat was diluted into 100 µL in phosphate buffered saline, followed by addition of 10 µL of 1.16 M KI, and 20 µL absolute acetic acid. The absorbance of the reaction mixture was immediately read using a SpectraMax 1601 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 340 nm against a blank containing 100 µL phosphate buffered saline, 20 µL acetic acid, and 10 µL KI solution(MEDEIROS et al., 2016). As the linear range of chloramine-T absorbance at 340 nm is between 0 and 100 µM, AOPP concentrations were expressed in µM chloramine-T equivalents. All measurements were performed at the same ocasion. The intra-assay coefficient of variation was 4%.

Total oxidation status (TOS)

TOS of serum was measured using a colorimetric measurement method(EREL, 2005). Briefly, 225 μL Reagent 1 (xylenol orange 150 μM, NaCl 140 mM, and glycerol 1.35 M in 25 mM H_2SO_4 solution, pH 1.75) was mixed with 35 μL of serum sample and the

absorbance of each sample was read spectrophotometrically at 560 nm as a sample blank. Subsequently, 11 μL Reagent 2 (ferrous ion [5 mM] and *o*-dianisidine [10 mM] in 25 mM H_2SO_4 solution) was added to the mixture, and approximately 3–4 min. After mixing, the last absorbance was read at 560 nm. The analytical sensitivity of the method was found to be 0.0076 absorbance/amount [AX ($\mu M^{)-1}$]. The assay was calibrated with H_2O_2 and the results are expressed in terms of micromolar H_2O_2 equivalent per litre (μmol H_2O_2 Equiv/L). The detection limit of the method was determined by evaluating the zero calibrator 10 times. All measurements were performed at the same ocasion. The intra-assay coefficient of variation was 6.5%.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Comparisons among the groups were performed by ANOVA followed by post hoc comparisons by the Tukey test. In the absence of a normal distribution, verified by Shapiro–Wilk test, the data were analysed by a Kruskal-Wallis test, followed by Dunn's post hoc test. Proportion among groups was compared by the Fisher´s test. Differences between independent variables of two groups were accessed by the Student T-test or Mann-Whitney test according to the presence or absence of a normal distribution. Significance was assumed at $P < 0.05$.

Results

Weight of the animals

PreN androgenised rats showed a lower weight at first postnatal day (mean ± SD; 5.16 ± 0.21 g) in comparison to PostN androgenised (6.56 ± 0.4 g) and Control (6.35 ± 0.44 g) groups ($P < 0.001$) (Figure 1A). At day 60, PreN rats continued to be lighter than PostN and Control rats: (mean ± SD) of weight was respectively 197.3 ± 8.0 g for PreN, 219.5 ± 1.5 g for PostN, and 210.5 ± 13.3 g for Control rats ($P = 0.01$) (Figure 1B). Finally, at 110 days, all groups displayed similar weights: (means ± SD) of 308.8 ± 15.2 g, 314 ±15.0 g, and 316.5 ± 10.67 g, respectively (Figure 1C). Groups subjected to leuprolide acetate treatment did not exhibit any modifications in the total weight (data not shown).

Oxidative stress markers and antioxidant capacity (albumin) in serum and plasma

PostN group rats displayed a significant increase in FRAP, a marker of direct oxidation, compared to PreN group rats. The (mean ± SD) of FRAP in PreN was 369.2 ± 103 μmol/L g protein^{-1}, whereas that in the PostN group was 962 ± 210.8 μmol/L g protein^{-1} (P = 0.03) (Figure 2A). The (mean ± SD) of FRAP in Controls was 644.3 ± 258.6 μmol/L g protein^{-1}. The difference between PostN and Controls did not reach statistical significance.

AOPP, another marker of direct oxidative stress, was comparable among the three groups (Figure 2B). The PreN, PostN, and Control values were similar, with (mean ± SD) of 36.46 ± 20.1, 49.75 ± 23.32, and 45.85 ± 21.3 mmol/g protein^{-1}, respectively.

Values for TOS, which estimates the final oxidant status, were superimposed among the three groups. As shown in Figure 2C, similar features were observed in the PreN (mean ± SD of 63.78 ± 14.68 μmol/L g protein^{-1}), PostN (mean ± SD of 74.77 ± 24.9 μmol/L g protein^{-1}, or Control (mean ± SD of 67.5 ± 20.9 μmol/L g protein^{-1}) groups. Notably, neonatal treatment with leuprolide acetate showed no effect on FRAP, AOPP, or TOS in all groups (Supplemental Figure 1A-I). Levels of serum albumin, a surrogate marker of antioxidant capacity, were elevated in the PostN group, showing (mean ± SD) 5.28 ± 0.18 mg/dL versus the Control group (mean ± SD of 4.53 ± 0.12 mg/dL) (P = 0.01). Intermediate values (mean ± SD) of 4.95 ± 0.4 were exhibited in the PreN group (Figure 2D). The treatment with leuprolide in the PostN L group was associated with a significant reduction in albumin levels in comparison to PostN rats (Supplemental Figure 1K). Those changes did not occur with controls (Supplemental Figure 1J) neither with PreN rats (Supplemental Figure 1L)

Biochemical variables

Total cholesterol levels were significantly reduced in PreN rats (mean ± SD 77.25 ± 11.4 mg/dL) in comparison with those in PostN rats (mean ± SD 100.7 ± 11.71 mg/dL) (P = 0.01) (Figure 3A). Differences between the total cholesterol levels in the Control group (mean ± SD 86.43 ± 5.25) versus PostN almost reached significance (P = 0.052). HDL cholesterol and LDL cholesterol level were similar between the three groups (Figure 3B and 3C). Notably, the HDL levels decreased in the PreN group after neonatal leuprolide treatment (Supplemental Figure 2G); other variables in PreN rats, such as glucose (Supplemental Figure 2L), total cholesterol (Supplemental Figure 2C), triglycerides (Supplemental Figure 2K) and TyG index (Supplemental Figure 2O) did not modify after

198 leuprolide treatment. PostN rats, in turn, did not show any metabolic modification after
199 administration of leuprolide acetate (Supplemental Figures 2B, 2F,2H, 2J, and 2N).
200 Triglyceride levels were increased in the PostN group (mean ± SD of 88.8 ± 9.3)
201 compared to those in the PreN group (mean ± SD of 63.5 ± 7.2) (Student t test, $P = 0.04$).
202 Overall, no changes in the triglyceride levels were identified in the three different groups
203 (Figure 3D), although a significant higher glucose was observed in both androgenized
204 rodent models (p=0.001) (Figure 3F). The product of triglycerides plus glucose (TyG)
205 was increased in PostN rats in comparison with that in the two other groups ($P = 0.02$)
206 (Figure 3E).
207 As shown in (Supplemental Figure 2M), Control rats treated with leuprolide acetate
208 (Control L) exhibited an increase in the TyG index, suggesting a worsening of metabolic
209 control (Supplemental Figure 2D). No other changes regarding total cholesterol, HDL
210 cholesterol or triglycerides were reported in control rats (Supplemental Figures 2A, 2E
211 and 2I).
212 Figure 4 summarizes the main dissimilarities between PreN and PostN androgenised rat
213 protocols in our study.
214
215 **Discussion**
216
217 Women with PCOS most frequently exhibit dyslipidaemia, glucose intolerance/diabetes
218 mellitus, and increased oxidative stress marker levels. Our study evaluated whether
219 different protocols of androgenisation leading respectively to a normal and abnormal
220 reproductive and metabolic rat phenotypes could be associated to a particular lipid status
221 and redox balance in the blood. We show that a combination of changes in oxidative stress
222 (increased direct oxidation and increased antioxidative profile) was observed in PostN
223 rats exhibiting anovulation/anestrous and increased TyG, whereas this phenomenon was
224 not observed in ovulatory/estrous PreN rats.
225 All groups of rats achieved similar final weight at the end of the study. As a result, it was
226 possible to avoid possible bias related to higher adipose accumulation, which is a
227 predominant characteristic of rodents following use of implants with dihydrotestosterone
228 (DHT)(MANNERAS et al., 2007; HOANG et al., 2015), letrozole (MALIQUEO et al.,
229 2013; HOANG et al., 2015; PANDEY et al., 2016; ULLAH et al., 2017), or other
230 miscellaneous protocols(WALTERS et al., 2012; WU et al., 2014; MARCONDES et al.,

2015). We also observed an earlier weight reduction in PreN group rats at birth and day 60. This finding was consistent with previous reports of rats androgenised with testosterone propionate prenatally.

An increase in direct oxidation, FRAP, was identified in the plasma of androgenized animals in our study. This result contrasts with the work of Dåneasa et al. 2016, where the serum levels of Malondialdehyde (MDA), a lipid peroxidation marker, were similar between letrozole-treated rats and its controls(DANEASA et al., 2016). No modifications in MDA where also reported in the ovary tissue of Sprague-Dawley rats submitted to free testosterone (2 or 5 mg) administration (AMALFI et al., 2012), although an increase in lipid peroxidation products of the ovary was found in letrozole rats by another author(PANDEY et al., 2016).

In the present study, antioxidant capacity was estimated in the serum of androgenized animals and controls through the levels of albumin. Albumin is capable of scavenging hydroxyl radicals with its reduced (-SH) cysteine residue (Cys34) and, therefore, considered one of the major antioxidant elements in the blood of humans and rats (SITAR et al., 2013; CIAPETTI et al., 2017; KINOSHITA et al., 2017; MANDIC et al., 2017; MASUDO et al., 2017; PRIETO et al., 2017).We identified increased serum albumin levels in PostN rats compared with Controls and the PreN group. In a previous study, no differences in glutathione peroxidase (GPx), another anti-oxidant marker was described between letrozole rats and controls (DANEASA et al., 2016). However, an increment of the antioxidant capacity measured by catalase activity and superoxide dismutase or glutathione peroxidase has been identified in the ovary of PCOS rats (AMALFI et al., 2012),(PANDEY et al., 2016),(DANEASA et al., 2016).

One reason for the divergences among experimental studies may be based on the existence of several approaches for the development of animal models of PCOS. Because of the diversity and limitation of rodent models, it has been claimed that there is no ‘gold standard’ reproducing all abnormalities seen in PCOS (MALIQUEO et al., 2014). For this reason, caution is necessary to avoid an indiscriminate generalization of the meaning of prenatal and postnatal models regarding the presented data.

Our results suggested a dual augmentation in oxidative and anti-oxidative status that agreed with some findings in the blood of women with PCOS. In a previous meta-analysis, the mean of MDA, a direct oxidant marker, was approximately 40% higher in PCOS than that in controls (MURRI et al., 2013).Other direct oxidants such as

dimethylarginine or homocysteine and nitric acid were also increased. However, in the same study, antioxidants markers were reduced (glutathione), increased (superoxide dismutase activity), or equal (total antioxidant capacity) to controls (MURRI et al., 2013). A recent study (544 PCOS and 468 control women) showed that all four typical phenotypes of PCOS based on the Rotterdam criteria were associated with higher TOS and oxidative stress index (OSI) in comparison to control women(ZHANG et al., 2017). Increased oxidative stress in PCOS was related to higher plasma glucose and triglycerides (ZHANG et al., 2017). Remarkably, all oligo- anovulatory PCOS women show an increased total antioxidant capacity in the serum, excepting PCOS women with presumed regular cycles (ZHANG et al., 2017). In our study, anovulatory rats (PostN) presented an increased triglyceride-glucose index (TyG). TyG, the product of triglycerides plus glucose, has been considered in humans and in rodents as a surrogate marker of insulin resistance and metabolic syndrome (SIMENTAL-MENDIA et al., 2008; GONZALEZ-TORRES et al., 2015; PINTO et al., 2016; QU et al., 2016). We showed that only PostN rats exhibited statistically significant higher TyG indices than controls, which may represent an additional link towards the disruption of the oxidative stress markers (SZCZUKO et al., 2016).

Although the comparison of two models (Pre and postnatal) plus an extra ovulatory control (PostN L rats) consisted in one strength of the present study, weakness should be considered as well. Limitations of our research from our point of view were related to sample size, the absence of subgroups (lean; obese/ young; aged) and the lack of inclusion of more antioxidant stress markers to the study.

To conclude, the results presented suggest that an increased direct oxidation and an increased anti-oxidative capacity could be associated with postnatal treatment with testosterone propionate (PostN), which is usually linked with anovulatory cycles and insulin resistance estimated by higher TyG. Androgenized rats treated with leuprolide acetate (PostN L) and presenting estrous cycles did not exhibit modifications in biochemical status or increased direct oxidation (FRAP) in plasma, but show a reduced antioxidant capacity estimated by albumin serum levels. Prenatal androgenisation, in turn, was related to a lower weight at birth but a less harmful phenotype. Altogether, these findings continue to support the central role of androgen excess, anovulation, and insulin resistance as the key factors to trigger redox abnormalities in PCOS. Additional studies of the impact of weight gain, high-glucose/ high fat diet (SZCZUKO et al., 2016),

or simply the long-term follow up will improve the comprehension of the intricacy mechanisms of oxidative stress in PCOS.

Author Contribution:

Study conception and design (FVC, LSM); acquisition of data (LSM, RNM, NG, AB, EDM); analysis and interpretation of data (FVC, MOP, LSM, VBR); drafting of manuscript (LSM, FVC); critical revision for important intellectual content of the draft (AQA, PBDG, FVC).

Disclosure

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Figure. 1 Weight of rats at day 1, 60 and 110 of life.

Controls=C (n=8); Postnatally androgenized =PostN (n= 7); Prenatally androgenized=PreN (n= 8). (A) ANOVA p<0.0001; a) postnatal versus prenatal adjusted p value p<0.0001; b) control versus prenatal adjusted p value p=0.0001; (B) ANOVA p = 0.01; a) postnatal versus prenatal adjusted p value p = 0.01.

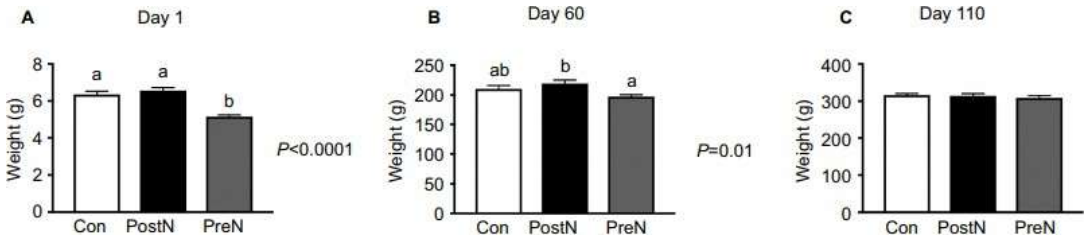


Figure. 2 Oxidative stress markers in controls and pre – and postnatally androgenized rats.

(A) ferric-reducing ability of plasma (FRAP);(B) advanced oxidation protein product (AOPP); (C) total oxidation status (TOS); (D) albumin. Controls=C (n=8); Postnatally androgenized =PostN (n= 7); Prenatally androgenized=PreN (n= 8). (A) ANOVA p=0.03; a) postnatal versus prenatal adjusted p value =0.027; (D) ANOVA p=0.02; a) postnatal versus controls adjusted p value p=0.01.

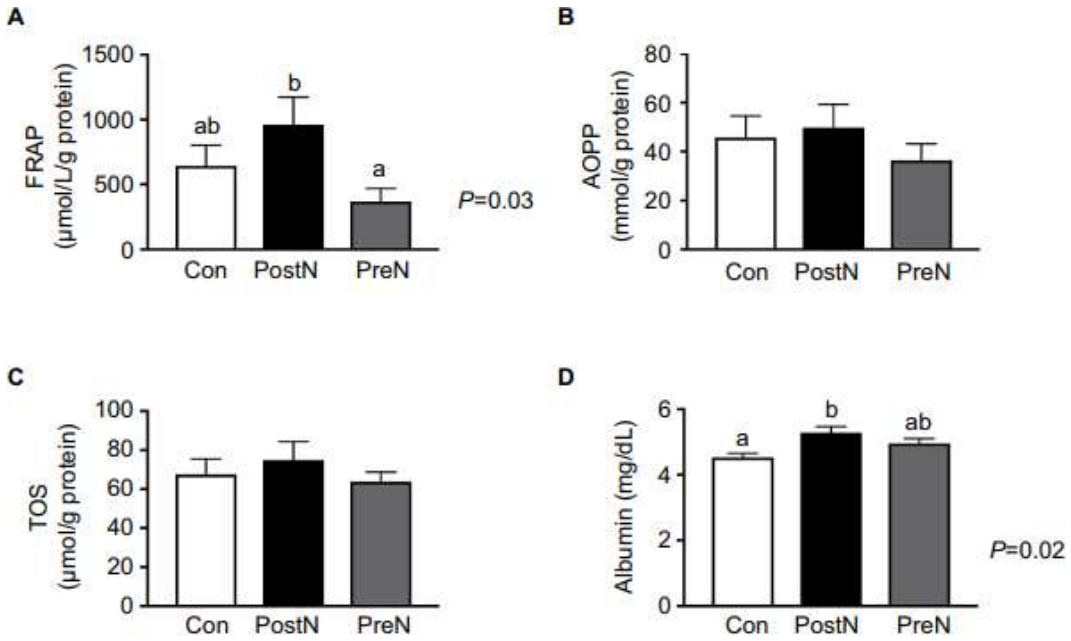


Figure. 3 Serum levels of total Cholesterol.

(A), HDL cholesterol, LDL cholesterol(C), triglycerides (D), Triglygeride-Glucose Index – TyG (E), and glucose (F) in controls, pre – and postnatally androgenized rats. Controls=C (n=8); Postnatally androgenized = PostN (n= 7); Prenatally androgenized = PreN (n= 8). (A) ANOVA p <0.01; a) postnatal versus prenatal adjusted p value = 0.001; (D) ANOVA p = 0.04; a) postnatal versus prenatal adjusted p value = 0.04; (E) ANOVA p=0.02; (F) postnatal versus control adjusted p value = 0.001; postnatal versus control adjusted p value = 0.005, prenatal versus control adjusted p value = 0.003.

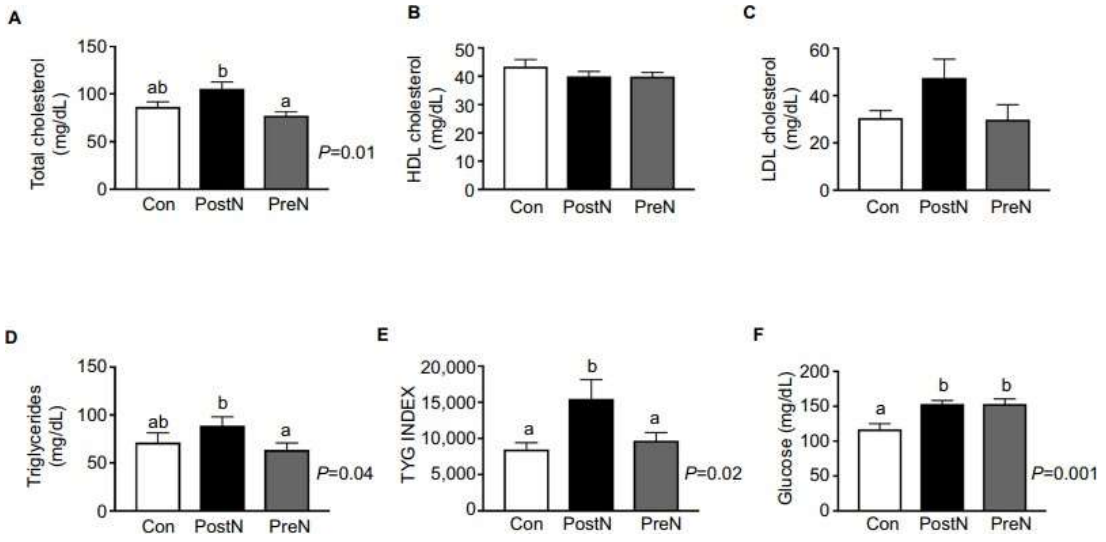


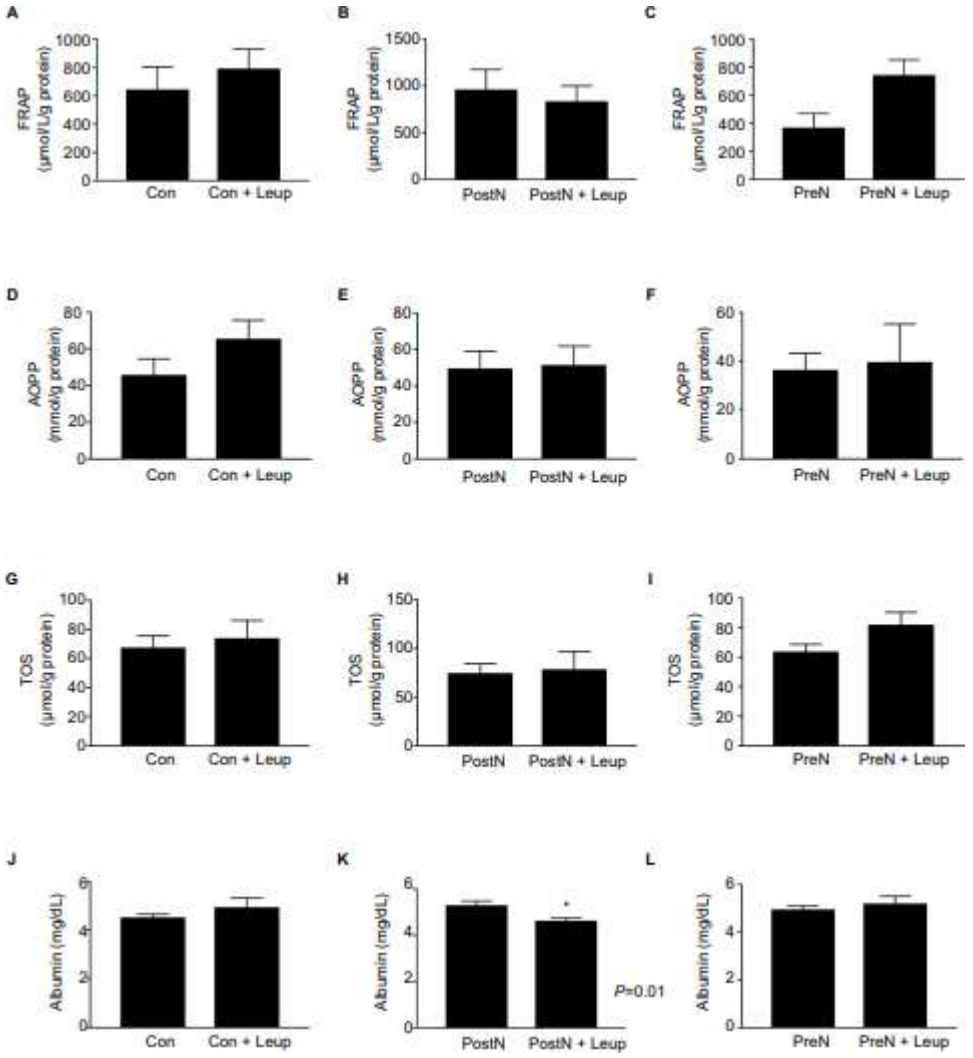
Figure. 4 Metabolic and oxidative stress markers characteristic of postnatally androgenised (PostN) and prenatally androgenised (PreN) rats.

*indicates significant differences with control rats; − indicates similarities with controls.

The full reproductive aspects of these groups (PreN, PostN, Control with and without treatment with GnRH agonists) have been published in a previous study (SERRANO MUJICA et al., 2017). Our results agreed with those of previously studies that employed testosterone propionate (FELS & BOSCH, 1971; HUFFMAN & HENDRICKS, 1981; SLOB et al., 1983; TYNDALL et al., 2012), with few exceptions (SWANSON & WERFF TEN BOSCH, 1965)

		
	PostN group • Anovulatory cycles (anestrous) • Increased number atertic follicles • Decreased corpus luteum	PreN group • Ovulatory cycles (estrous cycles) • Ovary histology (number of atretic and healthy follicles, corpus luteum) similar to control rats
Oxidative stress markers (FRAP, AOPP, TOS, # Albumin)	Increased (FRAP)* Increased (Albumin)*	– –
Glucose	Increased*	Increased*
TyG Index	Increased*	–
Total cholestrol	Increased	–
HDL cholestrol	–	–
Weight	– (d1) – (d90) – (d110)	Decreased (d1)* Decreased (d90) – (d110)

Supplemental Figure. 1 Oxidative stress markers in controls, pre – and postnatally androgenized rats which were treated and not-treated with neonatal leuprolide acetate. (A, B and C) ferric-reducing ability of plasma (FRAP); (D, E and F) advanced oxidation protein product (AOPP); (G, H and I) total oxidation status (TOS); (J, K, and L) albumin. Controls=C (n=8); Controls treated with leuprolide = C+L; Postnatally androgenized = PostN (n=7); Postnatally androgenized treated with leuprolide = PostN+L (n=7); Prenatally androgenized = PreN (n=8); Prenatally androgenized treated with leuprolide =PreN+L (n=4). Results were reported as mean (SEM). Statistical analysis employed Student T test. Significance was assumed at $P < 0.05$.



582 **Supplemental Figure. 2** Biochemical markers.

583 Serum levels of total Cholesterol (A, B and C), HDL cholesterol (E, F and G),
584 triglycerides (I, J and K), Triglygeride-Glucose Index –TyG (M, N and O), and glucose
585 (D, H and L) in controls, pre – and postnatally androgenized rats submitted or not to the
586 neonatal treatment with leuprolide acetate. Controls=C (n=8); Controls treated with
587 leuprolide=C+L (n=6); Postnatally androgenized = PostN (n=7); Postnatally
588 androgenized treated with leuprolide = PostN+L (n=7); Prenatally androgenized = PreN
589 (n=8); Prenatally androgenized treated with leuprolide = PreN+L (n=4).Results were
590 reported as mean (SEM). Statistical analysis employed Student T test. Significance was
591 assumed at $P < 0.05$.
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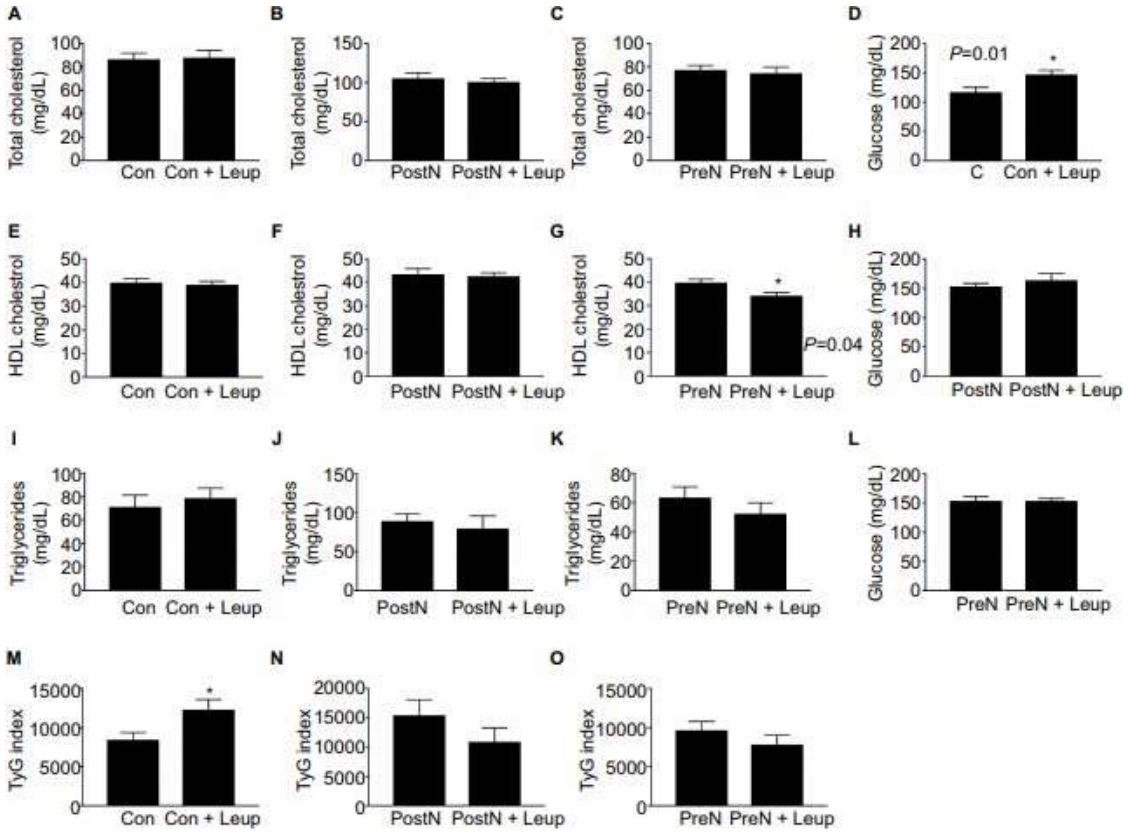

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ARTIGO II

TRABALHO ACEITO PARA PUBLICAÇÃO:

Trabecular Bone is Increased in a Rat Model of Polycystic Ovary Syndrome

Lady Serrano Mujica, Werner Glanzner, Amanda Luiza Prante, Vitor Braga Rissi, Gabrielle Rebeca Everling Correa, Melissa Orlandin Premaor, Alfredo Quites Antoniazzi, Paulo Bayard Dias Gonçalves and Fabio Vasconcellos Comim.

Experimental and Clinical Endocrinology & Diabetes, 2020

Abstract

Polycystic ovary syndrome (PCOS) in an intricate disorder characterized by reproductive and metabolic abnormalities that may affect bone quality and strength along the lifespan. The present study analysed the impact of postnatal androgenization (of a single dose of testosterone propionate 1.25 mg subcutaneously at day 5 of life) on bone development and markers of bone metabolism in adult female Wistar rats. Compared with healthy controls, the results of measurements of micro-computed tomography (microCT) of the distal femur of androgenized rats indicated an increased cortical bone volume (VOX BV/TV) and higher trabecular number (Tb.n) with reduced trabecular separation (Tb.sp). A large magnitude effect size was observed in the levels of circulating bone formation (P1NP) at day 60 of life; reabsorption (CTX) markers were similar between the androgenized and control rats at days 60 and 110 of life. Preliminary analysis of gene expression in bone indicated elements for an increased bone mass such as the reduction of the Dickkopf-1 factor (*Dkk1*) a negative regulator of osteoblast differentiation (bone formation) and the reduction of Interleucin 1-b (Il1b), an activator of osteoclast differentiation (bone reabsorption). Results from this study highlight the positive impact of early androgens on bone development with reference to young women with PCOS.

Keywords: polycystic ovary syndrome, bone, microCt, developmental programming, animal models of PCOS

Abbreviations: Dickkopf-1 factor (*Dkk1*); amino-terminal propeptide of type 1 procollagen (P1NP), carboxy-terminal collagen crosslinks (CTX).

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common hyperandrogenic disorders in reproductive-aged women [1-4]. The impact of PCOS on bone development has been a subject of research and conflicting results. Both cross-sectional and longitudinal studies indicated that premenopausal women with PCOS are protected from bone loss [5-7] or at higher risk of bone mass reduction [8-10].

Since a rodent animal model of PCOS replicates many metabolic and reproductive abnormalities observed in women with PCOS, these animals are considered a valuable tool to dissect complex mechanisms in the development of this disorder. In the study of Tamura et al., the treatment with testosterone propionate of young Sprague-Dawley rats

at day 9 of life was effective in increasing the bone mineral density defined by bone densitometry[11]. However, rats in this study were not fully characterized as PCOS rats, as the presence of cycles were not addressed[11].

Therefore, the present work aims to re-visit the role of PCOS in bone metabolism, investigating in an ovary-intact young rodent model of PCOS (exhibiting polycystic ovaries and absence of estrous cycles)[12] the impact of postnatal androgenization on bone markers of formation and reabsorption (P1NP and CTX) and microstructure (micro-CT).

Material and Methods

Ethical Statement

All Institutional and National Guidelines for the care and use of animals were followed. This study was approved by the local Ethics Committee on Animal Use (CEUA-UFSM), under protocol number 100/14, in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Animals

Female Wistar rats (Rattus norvegicus albinus) (n=14) aged 1 day were used in this study. The dams were maintained with their pups until weaning (21 days). The rats were maintained at a temperature of 22ºC, 55% to 65% humidity, and under artificial illumination with a 12-hour light/dark cycle. The rats were fed a standard pellet diet with water given ad libitum, as previously described [12].

Experimental Procedures

Postnatal hormone exposures were performed by the treatment of 5-day-old animals through a subcutaneous (SC) injection of 1.25 mg testosterone propionate (Androgenol™) (androgenized group), and vehicle control 5-day-old animals received 1.25 mg corn oil SC (control group)[12]. Procedures for blood collection and euthanasia were performed as previously described [12].

Acquisition and Analysis of the Distal Femur Images using Micro-CT.

The structural properties of the trabecular and cortical femur were determined with a high-resolution micro-CT system (SkyScan 1272; Bruker micro-CT, Kontich, Belgium). The X-ray tube was set to 50 kV, and the beam was filtered with a 0.5 mm aluminum filter. The sample position and camera settings were adjusted to provide a 3.0-µm isotropic pixel size, and projection images were collected every 0.2°. Reconstructions were done with NRecon (v 1.6.9.8; Bruker micro-CT).

Enzyme-linked Immunosorbent Assay (ELISA)

Procollagen I N-terminal propeptide (PINP) and cross-linked C-telopeptide of type I collagen (CTX) levels (CV <10%) were measured in serum at a single occasion using a specific rat ELISA (Cloud Clone Corp, USA) according to the manufacturers' protocols.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

The femur was collected after euthanasia, cleaned of muscle and soft connective tissues, and stored at -80^{0}C. Before RNA extraction, the specimen was frozen in liquid nitrogen and crushed into powder. Total RNA extraction was performed using Trizol® (Life Technologies, Foster City, CA, United States) as an organic extraction method, following the manufacturer's instructions. The other steps for obtention of RNA samples through cDNA preparation, quantitative real-time RT-PCR and the analysis of gene expression were performed as previously described [12]. The genes of interest and reference are shown in Supplemental Table 1.

Statistical Analysis

The statistical analysis and graphs were performed using the software GraphPad Prism 6.03 (GraphPad Software Inc., San Diego, CA). Comparisons among the two groups were performed using Student's t test. In the absence of a normal distribution, the data were analyzed using a Mann-Whitney test. For the analysis of more than 2 groups having normal data distribution, ANOVA test was performed. Significance was assumed at $p < 0.05$. Additionally, data were also analyzed by Cohen's effect size analysis and effect size with Cohen's d value of 0.8 and above considered as large magnitude differences are

reported [13, 14]. This analysis has been suggested to the study of small samples sizes to reject the null hypothesis[15].

Results

Micro-CT Analysis of Distal Femur

Cortical Bone

The VOX-BV indicating a higher bone volume was significantly higher in androgenized rats (3.28 ± 0.09) versus control rats (2.89 ± 0.07, p=0.01) (Fig 1A). However, other parameters such as VOX-TV (Fig 1D), VOX-BV/TV (Fig 1F), and the measurement of the cortical thickness (Fig1H) were similar between the two groups (P>0.05).

Trabecular Bone

Testosterone-treated rats show marked modifications in trabecular bone of the distal femur. Androgenized rats presented a significantly higher VOX BV/TV (0.41 ± 0.04) versus controls (0.27 ± 0.01, p=0.01) (Fig 1B). These PCOS rats exhibited, respectively, a higher number of trabeculae (7.3 ± 0.74) versus control rats (5.55 ± 0.19, p=0.048) (Fig 1E) and lower trabecular separation (Tb-Sp) (0.13 ± 0.01) versus controls (0.17 ± 0.006, p=0.04) (Fig 1H) (Fig 1G). Trabecular thickness (Tb-Th) was not different between these two groups (Fig 1I). The result of VOX-TV was 8.74 ± 0.33 in androgenized rats versus 0.70 ± 0.47 in controls; VOX-BV (Fig 1C). This contrast in trabecular mass was observed in an example of cross-sectional 2D image (1J, 1K).

Bone Markers

Plasma levels of P1nP and CTX in young rats at day 60 of life were significantly higher than that in adult rats [analysis of variance (ANOVA)] (P1nP p< 0.0005; CTX p<0.0001) (Fig 2A and 2B). At day 60, the mean ± SEM of P1nP was 84.11 ± 11.1 ng/ml in androgenized rats and 62.45 ± 6.84 ng/ml in controls (p=0.11)(Fig 2A);however, a non-significant large magnitude effect size was observed in the levels of circulating bone formation (P1NP) at day 60 of life. CTX value in androgenized rats (d60) was 1694 ± 42.4 pg/ml against 1618 ± 25.5 pg/ml (NS) (Fig 1K). At day 110, the mean ± SEM of P1nP was 16.16 ± 4.05 ng/ml in androgenized rats and 23.85 ± 10.44 ng/ml in controls

(Fig 1J); the CTX value in androgenized rats (d110) was 390.9 ± 0.92 pg/ml against 452.3 ± 129.2 pg/ml (NS) (Fig 2B).

Gene Expression

Supplemental Fig 1 shows the results of gene expression for bone formation and reabsorption factors in rat femurs. We observed at day 110 in androgenized animals, a significant reduction in *Wnt* gene expression (p=0.049) and a non-significant decreased magnitude effect size of the genes of *Dkk1*, *Il1b* in bone. The reduction of *Dkk1* (Negative Regulator of Wnt Signaling) support an increased bone formation, as well the decrease of *Il1b* (an activator of osteoclast differentiation) could imply a reduction in bone reabsorption. However, these figures need to be put in perspective since *Wnt* expression (related to canonical Wnt/beta catetin/TCFL pathway) was also decreased. No differences could be detected for the following genes: *Ctnnb1, Igfr, Sost, Lrp5, Lrp6, Notch, Opg (tnfsf11b), Bmp2I, Bmp2, Tbr2, and Fgfr2.*

Discussion

In the present study, testosterone administration on day 5 of life produced noticeable bone modifications in a rodent model of PCOS. Compared with the control rats, the femur from androgenized female rats exhibited a higher bone volume associated with an increased trabecular number and lower trabecular separation in a micro-CT study.

The results of our study add new complementary information of a previou report that evaluated the impact of androgens in female rodents[11]. In humans as in rats, bone development has a proper timing that is related to the presence of circulating steroids. Although estrogens are the critical factor in bone maintenance, promoting matrix formation and impairing matrix reabsorption, a possible role of androgens on bone metabolism has been reported. Androgens may exert a positive influence on bone metabolism through androgen receptors (AR) present in cells (i.e., osteoblasts, osteoclasts, and osteocytes) or indirectly through its conversion to estradiol[16-19]. The combination of estrogens plus testosterone produced a higher femur bone mass density (BMD), total BMD, increased bone volume fraction, trabecular number, and trabecular thickness in micro-CT compared controls[20]. Studies in ovariectomized mice mimicking cross-sex hormone therapy in humans show that bone acquisition during pubertal

development was not observed with isolated testosterone treatment and absence of estrogens[20].

Results from bone markers indicate a large magnitude effect size in bone formation (P1NP) at day 60 and the absence of differences later, at day 110 of life, suggesting an earlier impact of androgenization in bone mass. These figures may be relevant, since the diagnosis of PCOS cannot be established before 2-3 years after menarche [21]. Indeed, in the study of Bechtold and cols., adolescents between 12.4 to 18 years (mean age of 14.96 ± 1.42 years) had higher trabecular and cortical bone mass compared to healthy reference population[22]. However, most of the studies in late-adolescent and adult women with PCOS show a reduction of P1NP and osteocalcin levels and similar CTX levels [10, 23-26].

In conclusion, the treatment with testosterone propionate during neonatal period lead to an increased cortical bone volume (VOX BV/TV) and higher trabecular number with reduced trabecular separation in the distal femur at adult age. Bone in PCOS rats at day 110 of life show reduction of the Dickkopf-1 factor (*Dkk1*), a negative regulator of osteoblast differentiation (increasing bone formation), and the reduction of Interleucin 1-b, an activator of osteoclast differentiation (decreasing bone reabsorption). These findings highlight the positive impact of androgenization in rat bone development. Further studies are needed in order to evaluate the role of molecular players and its interaction other pathways in bone from androgenized rats before and after puberty.

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Author Contributions

Study Design: FVC, LKSM and PBDG; Study Conduct: FVC, LKSM, ALP, WG, VBR; Data Collection and Analysis of the data: FVC, LKSM, GREC; Data Interpretation and critical reading of the manuscript: FVC, LKSM, MOP, AQA, PBDG; drafting manuscript: FVC and LKSM.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Figure 1 MicroCt analysis

PCOS rat on day 110 exhibited marked increase in trabecular bone characterized by augmentation of VOX BV/TV(T)(1B), trabecular number (1E), and reduction of trabecular separation (1G); these contrasts between control and androgenized rats are represented in a cross-sectional 2D image (1J, 1K). No differences were seen in terms of trabecular thickness (1I) or VOX-TV(T)(1C). Cortical bone parameters, excepting for an increase in VOX-BV(1A), were similar between the groups - VOX-TV(1D), VOX BV/TV(1F), and Cortical Thickness(1H). Androgenized n=6; control n=6.

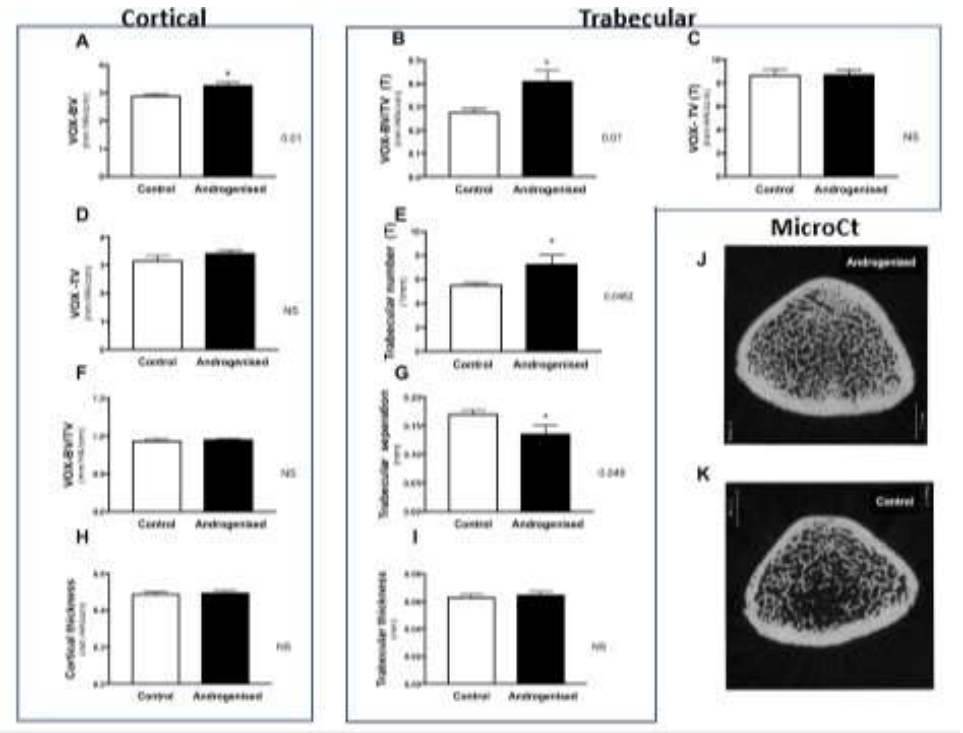
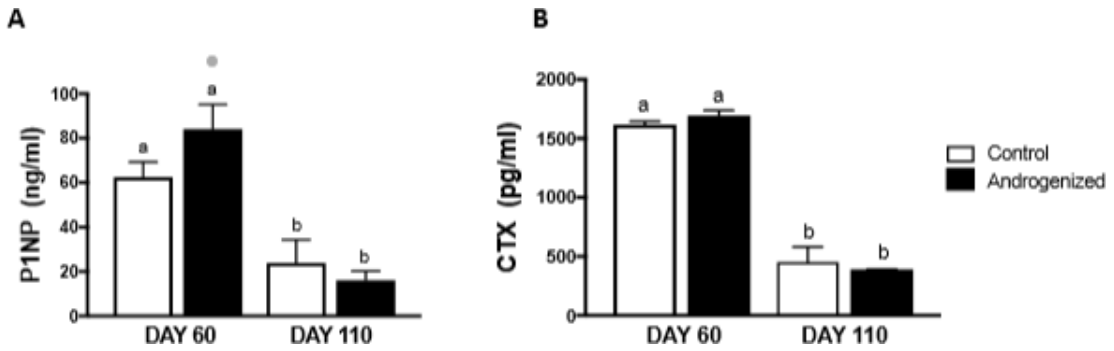


Figure 2 Analysis of bone markers of female control and androgenized rats at days 60 and 110 of life

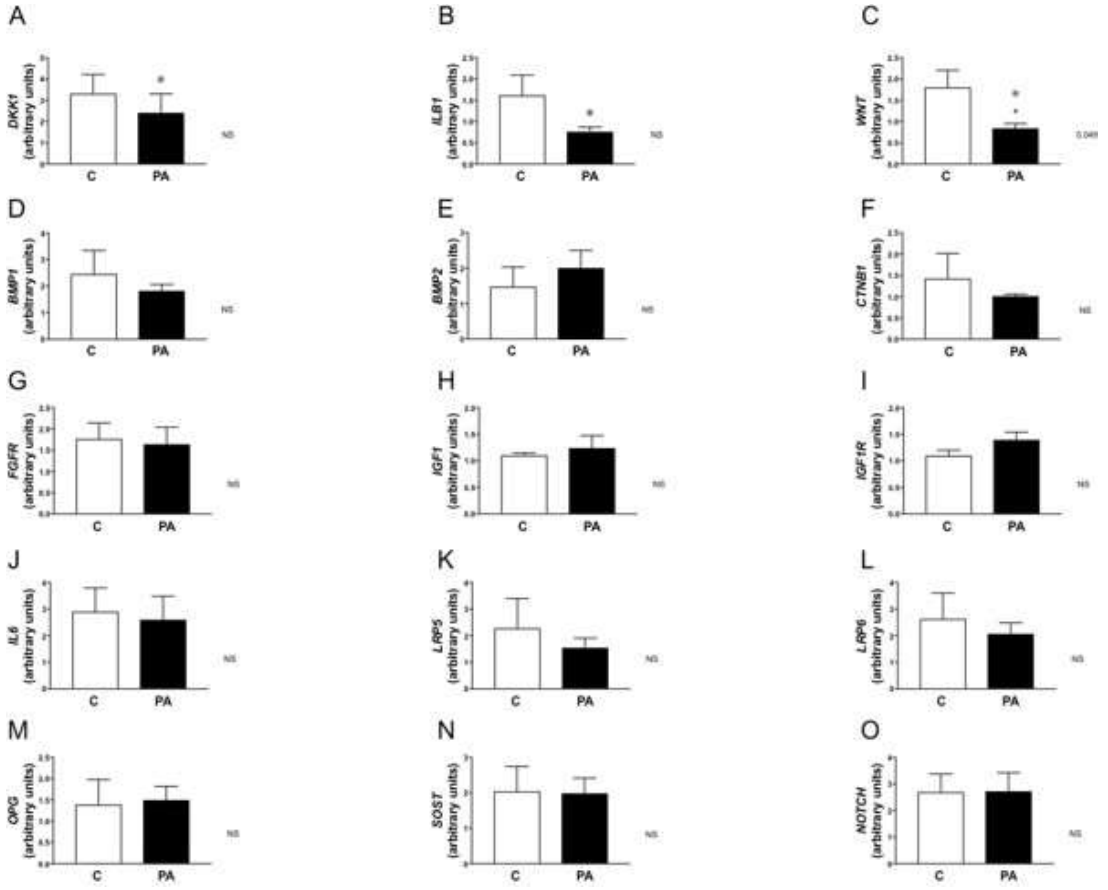
Plasma levels of bone formation (P1nP) and bone reabsorption (CTX) were significantly higher in younger (day 60) than older animals (110 of life). An increased Cohen effect size (•) for P1nP was observed in androgenized animals versus control at day 60.



Supplemental Table. 1 Genes of interest and reference.

Gene	Reference Sequence	Forward primer	Reverse primer
Ar	NM_012502.1	CTGGATGGGACTGATGGTATTT	CAGGTCAGGTGCAAAGTAGAG
Dkk1	NM_001106350.1	ATGCCCTCTGACCACAGCCATT	CACCGTGGTCATTGCCAAGGT
Igf1	NM_052807.2	CAATATCACAGACCCGGAAGAG	CGATACGGTACAGAGTGAAAGG
Ctnb1	NM_053357.2	CTCAGATGGTGTCTGCCATAG	TGGTGGGAAAGGTTGTGTAG
Igfr	NM_052807.2	CAATATCACAGACCCGGAAGAG	CGATACGGTACAGAGTGAAAGG
Sost	NM_030584.1	GGCAAGCCTTCAAGAATGATG	GGTCTGGTTGTTCTCTAGTTCC
Lrp5	NM_001106321.2	TTGTCATCTCTGGCCTTGTATC	CCTGCCAGAAGAGAACCTTAC
Lrp6	NM_001107892.1	CTTGCTGGGCGACTATGTTTA	GTCTGGCAGCTGGTCTATTATG
Notch	NM_001105721.1	ATACGCCTGTGGCAGAATAAG	CCATGGTCTGACATTCCTCATC
Opg	NM_012870.2	GCACCCTGAGAAAGAGGATATT	GGGATGACACAGAAGATAGTAGAAG
IL6	NM_012589.2	GGTTTGCCGAGTAGACCTCA	GTGGCTAAGGACCAAGACCA
IL1B	NM_031512.2	AAAGAAGGTGCTTGGGTCCT	CAGGAAGGCAGTGTCACTCA
Bmp2	NM_017178.1	ATCCACTCCACAAACGAGAAA	CCACATCACTGAAGTCCACATA
Tbr2	XM_017596193.1	GGACATTAACACTGAGGAGTACAG	GGTCCATCTGGAAAGACGTTAG
Fgfr2	NM_001109892.1	CCTCATCCCAAGATGCCTTAAT	GAATGTGACGGGTGTGTAACT
Ciclophilin A	NM_017101.1	GAAAGAAGGCATGAGCATTGTG	GCCCGCAAGTCAAAGAAATTAG
Gpdh	NM_017008.4	AGACAGCCGCATCTTCTTGT	CCGTTCACACCGACCTTCA

Supplemental Figure 1 Gene expression for bone formation and reabsorption factors



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ARTIGO III

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

Ovariectomy improves metabolic and oxidative stress marker disruption in androgenized rats: relevance to postmenopausal PCOS

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Metabolic Syndrome and Related Disorder, 2020

Abstract

Purpose: This study aims to evaluate metabolic and oxidative stress markers in a postmenopausal rat model of PCOS.

Methods: Wistar Rats were divided in 4 groups: Control OVX (n=9); Control SHAM (n=9); Androgenized OVX (n=10) and Androgenized SHAM (n=10). Female rats were androgenized during the neonatal period and compared to controls. Surgery (ovariectomy or SHAM procedure) was performed at day 100 and euthanasia at day 180 of life. Bodyweight, lipids, glucose, TyG index, and oxidative stress markers (TOS, TAC, NOX, FRAP AOPP) were addressed.

Results: Androgenized SHAM rats exhibited a higher total, LDL cholesterol, triglycerides, TyG index (an insulin resistance marker), and increased total oxidant status (TOS), FRAP, and albumin in comparison to control SHAM rats. These abnormalities disappeared after ovariectomy despite the fact that ovariectomized androgenized rats became heavier than the other three groups.

Conclusion: Ovariectomy improved metabolic and oxidative stress markers in a rat model of PCOS.

Key words: PCOS, animal models, oxidative stress, metabolism, postmenopausal, developmental programming

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Conflicts of interest: the authors have no conflicts of interest for this publication

Ethics approval: This study was approved by the local Ethics Committee on Animal Use (CEUA-UFSM), under protocol number 1619180917

Availability of data: all data from this study is available at: https://figshare.com/articles/Databank_Metabolic_and_Oxidative_stress_Markers_in_a_Postmenopausal_rat_model_of_PCOS/12198498

Author's contribution:

Study Design: FVC, LKSM, RNM and AQA; Study Conduct: FVC, LKSM, CSS; Data Collection and Analysis of the data: FVC, LKSM, RNM, CS; Data Interpretation and critical reading of the manuscript: FVC, MOP; drafting manuscript: FVC and LKSM

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Introduction

Polycystic ovary syndrome (PCOS) affects women during their entire lifespan[1,2]. Evidence from the literature has shown conflicting results about the existence of an increased risk for cardiovascular and metabolic disease in postmenopausal women with PCOS. While several studies in PCOS during the menacme have revealed a disruption in the oxidative stress, a key element for the development of the cardiometabolic disease, there is a lack of information of this condition in PCOS women after the menopause when a significant reduction in steroid secretion occurs[3,4].

Animal models replicate many abnormalities (reproductive and metabolic) seen in women with PCOS[5]. However, no study has addressed the impact of ovariectomy on lipids, glucose, and oxidative stress markers altogether, which is the aim of the present study. As shown below, ovariectomy performed in androgenized adult female rats leads to an improvement of the metabolic profile (LDL, triglycerides, TyG index) and total oxidant status (TOS) after 80 days.

Material and Methods

Animals

Female Wistar rats (Rattus norvegicus albinus) (n=38) aged one day were used in this study. The protocol of androgenization and animal care (including blood collection and euthanasia) was executed as previously described [6,7]. Briefly, the dams were maintained with their pups until weaning (21 days). The rats were maintained at a

temperature of 22ºC, 55% to 65% humidity, and under artificial illumination with a 12-hour light/dark cycle; they receive a standard pellet diet with water given ad libitum[7,6]. Postnatal treatments were performed by the administration of a subcutaneous (s.c) injection of 1.25 mg testosterone propionate (Androgenol™) (androgenized group), or vehicle control -1.25 mg corn oil s.c (control group) in pups at day 5 of life. Surgery (ovariectomy or SHAM procedure) were performed at day 100 of life under general anesthesia (ketamine and isoflurane) associated with pre and post-surgical analgesia with tramadol chloride (Tramadol™, Pfizer, Sao Paolo, Brazil) and ketoprofen (Ketofen, Merial, Paulinia, Brazil). SHAM rats were submitted to full characterization of estrous cycles by vaginal smear cytology and were euthanized approximately at day 180 during metestrus/diestrous [7,6]. Ovariectomized rats were euthanized at day 180. In both cases, the animals were transferred and then anesthetized with isoflurane plus administration of tramadol chloride (Tramadol™, Pfizer, Sao Paolo, Brazil) intramuscularly (20–40 mg/kg). Blood samples were collected before the animals were finally sacrificed using cardiac puncture under deep anesthesia in the absence of pedal and corneal reflexes. Blood samples were centrifuged at four ^{0}C and 5000 rpm/4696 g (Sorvall-Thermo Scientific, Asheville, NC, USA) for 15 min to separate the solid blood components from the serum and plasma (EDTA), and stored at -80 ^{0}C. Overall, rats were divided in 4 groups: 1) “Control OVX”, (n=9); 2) “Control SHAM” (n=9); 3) “Androgenized OVX”(n=10); and 4) “Androgenized SHAM” (n=10).

Laboratory measurements

Total blood cholesterol, high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) triglyceride, albumin, and glucose were measured enzymatically in serum using a commercial assay kit (Labtest Diagnostics®, Lagoa Santa, Brazil). Markers of oxidative stress, ferric-reducing ability of plasma (FRAP), advanced oxidation protein product (AOPP), Total oxidation status (TOS), Total Antioxidant Capacity (TAC), nitric oxide (NOX) were performed exactly as previously published [6,8]

Statistical analysis

Statistical analysis of the data was executed with the support of the software GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Comparisons among the

groups were performed by ANOVA, followed by post hoc comparisons by the Tukey test. Differences between independent variables of two groups were accessed by the Student T-test or Mann-Whitney test according to the presence or absence of a normal distribution. Significance was assumed at $P < 0.05$.

Results

Weight of animals

Both Androgenized groups (OVX and SHAM) were heavier than control groups at day 100 of life (ANOVA $p = 0.01$). After surgery, only Androgenized OVX rats continue to have a significant gain in weight in comparison with the other three groups, including Androgenized SHAM (ANOVA $p= 0.0001$). At day 180 of life, the weight in Androgenized OVX (mean $\pm$ SD) was 377.8 $\pm$ 37.2 g versus was 289.5 $\pm$ 9.75 g in Control OVX, 306.6 $\pm$ 10.48 g in Androgenized SHAM and 289.9 $\pm$ 11,64 g in Control SHAM.

Metabolic profile

Differences in the metabolic parameters between Androgenized SHAM and Control SHAM rats are shown in Fig 1. Androgenized SHAM rats exhibited a significant higher total cholesterol (mean $\pm$ SD) of 141.5 $\pm$ 15.1 mg/dl versus 94.89 $\pm$ 2.36 mg/dl (Student t test $p=0.01$)(Fig 1A), elevated LDL cholesterol (mean $\pm$ SD) of 47.6 $\pm$ 4.89 mg/dl versus 35.3 $\pm$ 1.50 mg/dl (Student t test $p=0.03$)(Fig 1B), and triglycerides (mean $\pm$ SD) of 199.3 $\pm$ 36.2 mg/dl versus 77.16 $\pm$ 11.1 mg/dl (Student t test $p=0.005$)(Fig 1D). Androgenized SHAM rats also show a significant elevation of TyG index, considered a marker of insulin resistance: in Androgenized SHAM rats TyG was 141.5 $\pm$ 15.1 and in Control SHAM rats was 94.89 $\pm$ 2.36 (Student t test $p=0.01$) (Fig 1F). No differences were observed in relation to basal glucose in the four groups (Fig 1E). Remarkably, after ovariectomy, those differences reported above disappeared (Fig 1 A, B, D, and F). Exceptionally, Androgenized OVX rats presented a lower HDL levels (mean $\pm$ SD) of 77.2 $\pm$ 5.75 mg/dl against 103.0 $\pm$ 14.2 mg/dl in Control OVX (Student t test $p=0.01$) (Fig 1C).

Oxidative stress markers

Examination of total oxidant status (TOS) showed a significant elevation in Androgenized SHAM rats (mean $\pm$ SD) of 38 $\pm$ 3.29 µmol/g protein versus 24.4 $\pm$ 3.69

µmol/g in Control OVX (Student t test p=0.01) (Fig 2A). Relative to direct oxidation markers, Androgenized SHAM rats had a significant increase in ferric-reducing ability of plasma (FRAP) (mean ± SD) of 623.8 ± 96.1 µmol/L.g protein^{-1} against 375.9 ± 28.5 µmol/L.g protein^{-1} in Control OVX (Student t test p=0.03) (Fig 2E). This last result contrasts with a reduced advanced oxidation protein product (AOPP) in comparison to Control SHAM rats (Fig 2F). After ovariectomy, FRAP levels became significantly reduced in Androgenized OVX rats (mean ± SD) of 221.3 ± 13.3 µmol/L.g protein^{-1} against 385.7 ± 72.7 µmol/L.g protein^{-1} in Control OVX (Student t test p=0.03) (Fig 2E); no differences in TOS or AOPP were observed (Fig 2A and 2F).

Antioxidant status was addressed through the total antioxidant capacity (TAC), albumin (an antioxidant molecule which removes hydroxyl radicals with its reduced (–SH) cysteine residue), and the levels of nitric oxide (NOX). Postnatal treatment with testosterone induced a higher increase in albumin levels (Fig 2C) despite no elevations in TAC or NOX (fig 2B and 2D).

Discussion

The present report shows, for the first time, that ovariectomy improved metabolic and oxidative stress markers in a postmenopausal rat model of PCOS. As a whole, these findings highlight the importance of steroids in the development of lipid and redox disorders[4].

We explored a new possible phenotype of postmenopausal rat PCOS, combining the protocol of neonatal androgenization with the realization of ovariectomy, usually employed to simulate the menopausal condition. This unique approach supports an investigation of the consequences of developmental programming in the absence of supraphysiological concentrations of androgens. Previous studies tried to answer specific questions in postmenopausal rats. For example, in the elegant study of Torres Fernandez (2019) focused on evaluation the impact of liraglutide in PCOS, rats were treated continuously with a pellet of dihydrotestosterone up to 17 months of age[9]. These animals exhibited higher HOMA-IR, TC, LDL, HDL and triglycerides that decreased after the treatment of liraglutide [9]. Another study, also evaluated the consequences of chronic hyperandrogenemia through DHT treatment in female rats with intact ovaries reporting adverse results on blood pressure, renal function, and glucose levels [10].

Our study has some strengths: Firstly, it employed a validated animal model of PCOS, according to previous publications[7,6]. Secondly, SHAM animals were euthanized only during metestrous/ diestrous phases in order to reduce bias in oxidative stress markers due to the influence of circulating estrogen. Thirdly, it followed OVX rats for more than 60 days in conditions of estrogenic depletion (estrogen levels were undetectable in all OVX animals – data not shown). Limitations from this study came from the lack of measurement of other inflammatory markers and key adipokines (such as adiponectin, leptin, and irisin), androgens, and the absence of a dynamic test of glucose/insulin metabolism.

In conclusion, our study shows that ovariectomy in androgenized rats improves the metabolic profile (lipids and oxidative stress) despite a significant gain of weight. More studies will be necessary to address the importance of androgens/estrogens on redox state balance and the consequences on adipokines, fat depots and inflammation.

Figure . 1 Changes in lipids, glucose, TyG index in androgenized and control rats subdivided in OVX and SHAM groups.

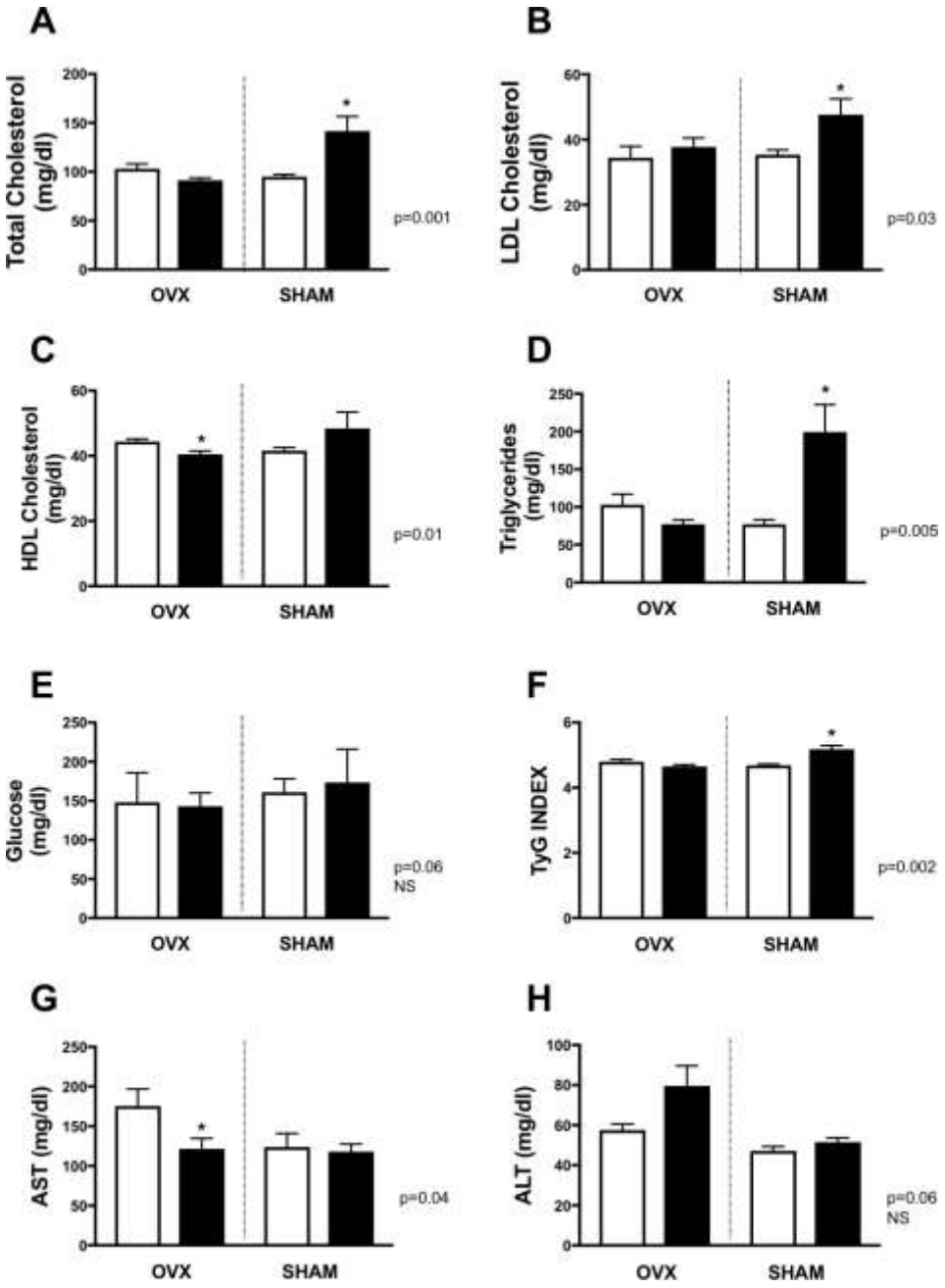
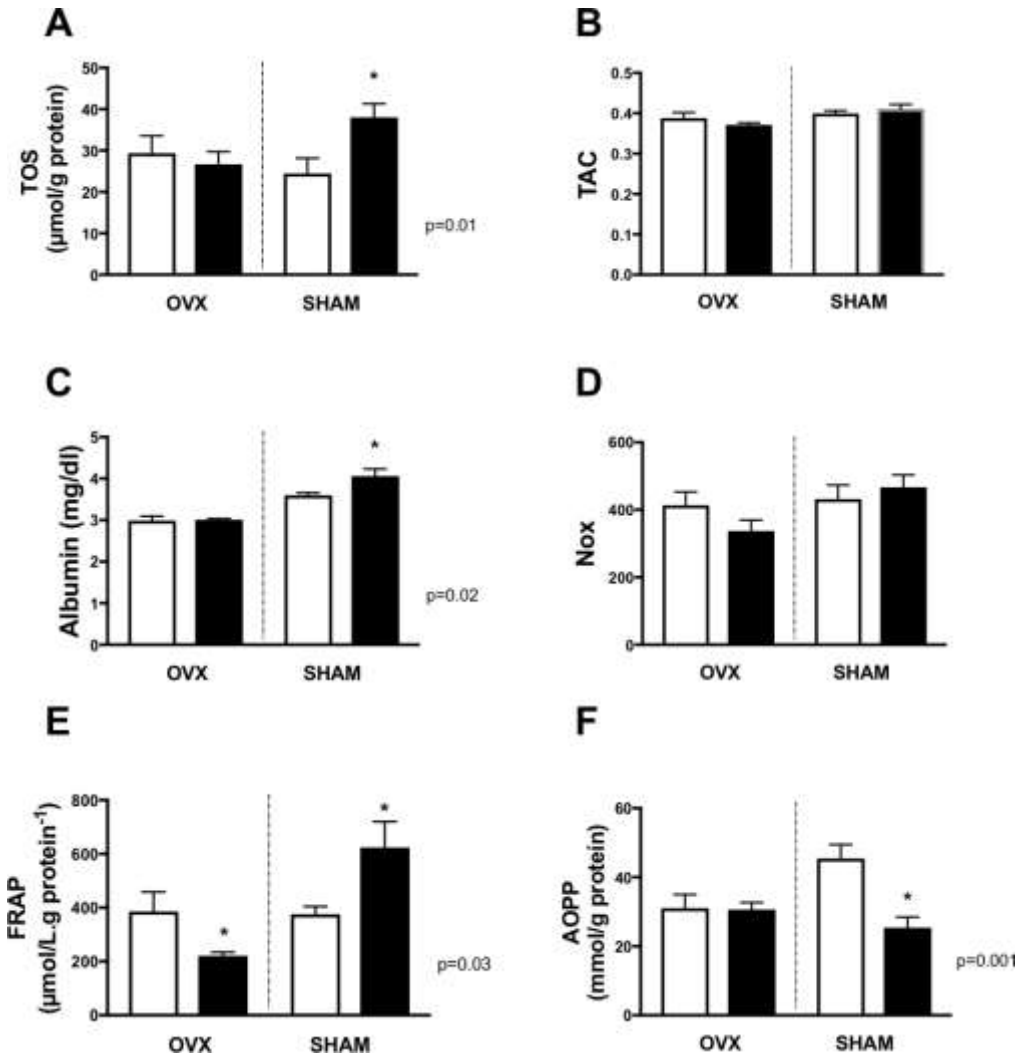


Figure . 2 Changes in oxidative stress markers in androgenized and control rats subdivided in OVX and SHAM groups.

Black columns = androgenized rats
White columns= control rats
Statistical significance ($p < 0.05$) is indicated by (*) symbol.



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251

3. Conclusão

Com a realização deste trabalho, conclui-se que o modelo pós-natal de androgenização com propionato de testosterona foi superior ao modelo pre-natal na capacidade de produzir alterações importantes no perfil metabólico e em alguns marcadores de estresse oxidativo.

As alterações metabólicas observadas como (aumento de glicemia, índice TyG e colesterol LDL) bem como os desarranjos do estresse oxidativo (aumento de FRAP) são consistentes com anormalidades descritas em mulheres com SOP.

A realização de ooforectomia mostrou-se capaz de melhorar muitas das alterações reportadas tanto metabólicas quanto de estresse oxidativo, ainda que animais androgenizados e submetidos a ooforectomia tenham exibido maior peso.

Outros achados relevantes em ratas androgenizadas no quinto dia de vida foram os relacionados a modificações no metabolismo ósseo. O tratamento com propionato de testosterona durante o período neonatal levou a um aumento no volume do osso cortical (VOX BV / TV) e um maior número trabecular com redução da separação trabecular no fêmur distal na idade adulta. Entretanto, o processo de aumento do volume trabecular isoladamente, não garante que a resistência óssea esteja aumentada em ossos longos que dependem da qualidade e quantidade de osso cortical e caracterização funcional através do teste mecânico. Mais estudos precisam ser realizados para avaliar o papel dos atores moleculares e suas interações com outras vias nos ossos de ratos androgenizados antes e depois da puberdade.

Anexos

Anexo. 1 Oxidative stress and metebolic markers in pre and posnatal polycystic ovary syndrome rat protocols.

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ORIGINAL RESEARCH

Oxidative stress and metabolic markers in pre- and postnatal polycystic ovary syndrome rat protocols

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Background: Several studies have described an enhanced inflammatory status and oxidative stress balance disruption in women with polycystic ovary syndrome (PCOS). However, there is scarce information about redox markers in the blood of androgenized animal models. Here, we evaluated the serum/plasma oxidative stress marker and metabolic parameter characteristics of prenatal (PreN) and postnatal (PostN) androgenized rat models of PCOS.

Materials and methods: For PreN androgenization (n=8), 2.5 mg of testosterone propionate was subcutaneously administered to dams at embryonic days 16, 17, and 18, whereas PostN androgenization (n=7) was accomplished by subcutaneously injecting 1.25 mg of testosterone propionate to animals at PostN day 5. A unique control group (n=8) was constituted for comparison.

Results: Our results indicate that PostN group rats exhibited particular modifications in the oxidative stress marker, an increased plasma ferric-reducing ability of plasma, and an increased antioxidant capacity reflected by higher albumin serum levels. PostN animals also presented increased total cholesterol and triglyceride–glucose levels, suggesting severe metabolic disarrangement.

Conclusion: Study findings indicate that changes in oxidative stress could be promoted by testosterone propionate exposure after birth, which is likely associated with anovulation and/or lipid disarrangement.

Keywords: animal models of PCOS, oxidative stress, prenatal, postnatal

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder impacting 5%–10% of women at reproductive age.[1–3] Although PCOS has been identified mainly by reproductive features (oligo-amenorrhea, hyperandrogenism, polycystic ovary appearance), other metabolic and inflammatory conditions, including disruption in cholesterol and glucose levels and the oxidative stress balance, have also been reported.[4–10] A recent publication has found that total oxidative stress and antioxidant capacity were increased in PCOS against controls.[11] In this study, which also evaluated the four phenotypes of PCOS, a higher oxidative stress was related to increased androgens, plasma glucose, and triglycerides, and decreased apoA_1 concentrations.[11]

Studies in rodent models replicate many of the abnormalities observed in PCOS women and, for this reason, have been used to explore the pathophysiological basis of the disorder.[12–17] Currently, there is scarce information about the redox state in the blood of PCOS rats, once the majority of the studies have focused most of their attention on the ovaries and liver, fat, and muscle tissues.[17,29,33]

Therefore, the aim of this study was to compare the oxidative stress profile in two different phenotypes obtained by prenatal (PreN) and postnatal (PostN) androgenization

protocols (both with testosterone propionate). This research worked with an anestrous rat model of PCOS (PostN androgenized rat or PostN group), an estrous rat model (PreN androgenized rat or PreN group), and an androgenized PostN estrous rat model (PostN L group).[18,19]

Our results indicate that modifications in the oxidative stress markers in the blood occurred in the presence of severe reproductive and metabolic disarrangements observed in the female rats submitted to PostN androgenization with testosterone propionate.

Methods

Animals

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Santa Maria (UFSM), Brazil, under protocol number 100/14. The procedures with animals were in agreement with the guidelines of the Brazilian National Council of Control of Animal Experimentation that follows the "Principles of Laboratory Animal Care" established by the National Institutes of Health, Bethesda, MD, USA.

Overall, 46 female Wistar rats (*Rattus norvegicus var. albinus*) were used in this study and housed at the Laboratory of Biotechnology and Animal Reproduction, BioRep, UFSM. The animals were maintained at a temperature of 22°C, 55%–65% humidity under artificial illumination on a light–dark cycle of 12:12 h, with daylight from 7 am to 7 pm. Food and water were given ad libitum.

A total of 30 female rats were submitted to the protocol for synchronization of estrus. They received an intraperitoneal injection of 10 IU of equine chorionic gonadotropin (eCG; Folligon™; Intervet, São Paulo, Brazil), followed 48 h later by 10 IU of human chorionic gonadotropin (hCG; Pregnyl™; Organon, Cascavel, Brazil), and were placed with a male for 24 h. Matches were controlled; vaginal plug was checked every 12 h. Observation of the vaginal plug was considered as the first day of pregnancy. Female rat pups were divided into four groups for androgenization by treatment with testosterone propionate or two control groups. Dams were maintained with their pups until weaning (21 days). PreN hormone exposure was accomplished by the treatment of pregnant dams during embryonic days 16, 17, and 18 through a subcutaneous injection of 2.5 mg testosterone propionate (Androgenol™; Hertape Calier, Juatuba, Brazil) (PreN group), whereas vehicle control exposures were accomplished by similar treatment of pregnant dams with 2.5 mg of corn oil (control PreN). PostN hormone exposures were performed by the treatment of 5-day-old animals through a subcutaneous injection of 1.25 mg testosterone propionate (PostN group), whereas vehicle control PostN 5-day-old animals received a subcutaneous injection of 1.25 mg of corn oil (control PostN).[19] The final groups were as follows: PreN (n=8), PostN (n=7), and control group (control PreN with control PostN) (n=8). Another androgenized group, PostN leuprolide (PostN L n=7), included the treatment with an intramuscular (im) injection of 0.40 mg of leuprolide acetate depot (Lectrum™; Sandoz International GmbH, Holzkirchen, Germany) in 2-day-old rats before PostN androgenization with testosterone propionate. Information of other groups of leuprolide treatment including the number of animals per group (eg, PreN androgenized with leuprolide) is available in the Supplementary materials.

Euthanasia and sample collection

At the age of 110 days, the animals were transferred and then anesthetized with isoflurane by administering tramadol chloride (Tramadol™; Pfizer, São Paulo, Brazil) intramuscularly (20–40 mg/kg). Between 9 am and 10 am, blood samples were collected before the animals were finally sacrificed using cardiac puncture under deep anesthesia in the absence of pedal and corneal reflexes. Blood samples were centrifuged at 4°C and 5000 rpm/4696× *g* (Sorvall-Thermo Scientific, Asheville, NC, USA) for 15 min to separate the blood solid components from the serum and plasma (ethylenediaminetetraacetic acid) and stored at −80°C.

Laboratory measurements

Total blood cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) triglyceride, albumin, and glucose were measured enzymatically in serum using a commercial assay kit (LabTest Diagnostics, Lagoa Santa, Brazil).

Ferric-reducing ability of plasma (FRAP)

FRAP was assessed as previously described.[20] In brief, the FRAP reagent was freshly prepared and warmed at 37°C by mixing the following solutions: 1) 0.3 M sodium acetate buffer solution (pH 3.6), 2) 10 mM 2,4,6-tripyridyl-1-5-triazine in 40 mM HCl solution, and 3) 20 mM $FeCl_3$ solution at the ratio of 10:1:1 (v/v/v). Plasma (10 µL) was incubated with 90 µL of FRAP reagent in a microplate for 30 min at room temperature in the dark. Subsequently, the level of absorbance of the mixture was measured at the wavelength of 595 nm using a spectrophotometer. The FRAP values were calculated by using a calibration standard curve of $FeSO_4$ (0–2000 µM). All measurements were performed at the same time. The intra-assay coefficient of variation was between 1% and 2%.

Measurements of advanced oxidation protein product (AOPP) levels in serum

Samples were prepared as follows: in a tube, 20 µL of serum from each rat was diluted into 100 µL in phosphate-buffered saline, followed by the addition of 10 µL of 1.16 M KI and 20 µL of absolute acetic acid. The absorbance of the reaction mixture was immediately read using a SpectraMax 1601 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 340 nm against a blank containing 100 µL of phosphate-buffered saline, 20 µL of acetic acid, and 10 µL of KI solution.[21] As the linear range of chloramine-T absorbance at 340 nm is between 0 and 100 µM, AOPP concentrations were expressed in µM chloramine-T equivalents. All measurements were performed at the same time. The intra-assay coefficient of variation was 4%.

Total oxidation status (TOS)

TOS of serum was measured using a colorimetric measurement method.[22] Briefly, 225 µL of Reagent 1 (xylenol orange 150 µM, NaCl 140 mM, and glycerol 1.35 M in 25 mM H_2SO_4 solution, pH 1.75) was mixed with 35 µL of serum sample, and the absorbance of each sample was read spectrophotometrically at 560 nm as a sample blank. Subsequently, 11 µL of Reagent 2 (ferrous ion [5 mM] and *o*-dianisidine [10 mM] in 25 mM H_2SO_4 solution) was added to the mixture for ~3–4 min. After mixing, the last absorbance was read at 560 nm. The analytical sensitivity of the method was found to be 0.0076 absorbance/amount (AX/µM). The assay was calibrated with H_2O_2, and the results are expressed in terms of micromolar H_2O_2 equivalent per liter (µmol H_2O_2 equiv/L). The detection limit of the method was determined by evaluating the zero calibrator 10 times. All measurements were performed at the same time. The intra-assay coefficient of variation was 6.5%.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons among the groups were performed by analysis of variance (ANOVA) followed by post hoc (Tukey) comparison test. In the absence of a normal distribution, verified by Shapiro–Wilk test, the data were analyzed by a Kruskal–Wallis test, followed by Dunn's post hoc test. Proportion among groups was compared by the Fisher's test. Differences between independent variables of two groups were accessed by the Student's *t*-test or Mann–Whitney *U*-test according to the presence or absence of a normal distribution. Significance was assumed at $P<0.05$.

Results

Weight of the animals

PreN androgenized rats showed a lower weight at first PostN day (mean ± SD; 5.16±0.21 g) in comparison with PostN androgenized (6.56±0.4 g) and control (6.35±0.44 g) groups ($P < 0.001$) (Figure 1A). At day 60, PreN rats continued to be lighter than PostN and control rats: the mean ± SD of weight was 197.3±8.0 g for PreN, 219.5±1.5 g for PostN, and 210.5±13.3 g for control rats ($P=0.01$) (Figure 1B). Finally, at 110 days, all groups displayed similar weights: the mean ± SD of 308.8±15.2, 314±15.0, and 316.5±10.67 g, respectively (Figure 1C). Groups subjected to leuprolide acetate treatment did not exhibit any modifications in the total weight (data not shown).

Oxidative stress markers and antioxidant capacity (albumin) in serum and plasma

PostN group rats displayed a significant increase in FRAP, a marker of direct oxidation, compared to PreN group rats. The mean ± SD of FRAP in PreN was 369.2±103 µmol/L/g protein, whereas that in the PostN group was 962±210.8 µmol/L/g pro-

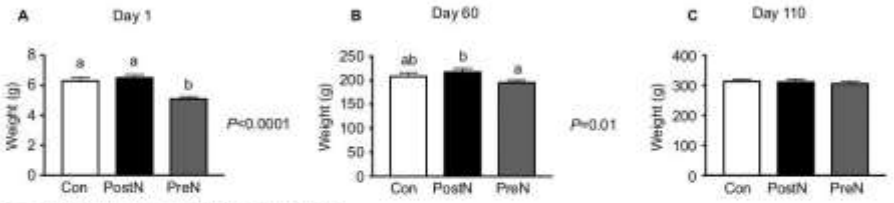

Figure 1 Weight of rats at days 1 (**A**), 60 (**B**), and 110 (**C**) of life.
Notes: Con (n=8); PostI androgenized (n=7); PreN androgenized (n=8). (**A**) ANOVA P<0.0001; (**B**) ANOVA =0.01. Distinct letters indicate a statistical significant difference (adjusted P level <0.05) obtained with multi-comparison Tukey's test.
Abbreviations: ANOVA, analysis of variance; Con, controls; PostN, postnatal; PreN, prenatal.

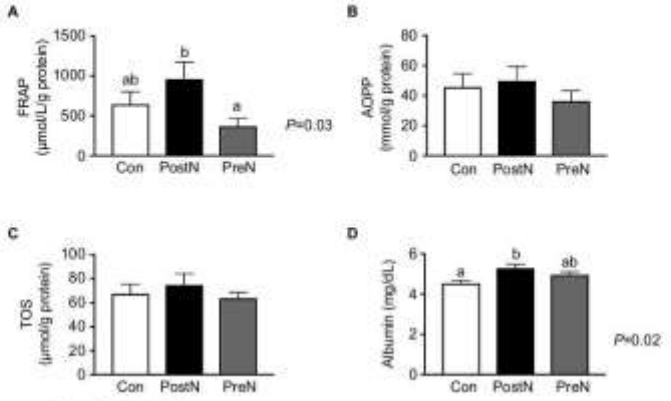

Figure 2 Oxidative stress markers in C, PreN, and PostN rats.
Notes: Values of oxidants FRAP (**A**), AOPP (**B**), TOS (**C**), and anti-oxidant, albumin (**D**) in the blood of female rats. Con (n=6); PostN androgenized (n=7); PreN androgenized (n=8). (**A**) ANOVA P=0.03; (**D**) ANOVA P=0.02. Distinct letters indicate a statistical significant difference (adjusted *P* level <0.05) obtained with multi-comparison Tukey's test.
Abbreviations: ANOVA, analysis of variance; AOPP, advanced oxidation protein product; Con, controls; FRAP, ferric-reducing ability of plasma; PostN, postnatal; PreN, prenatal; TOS, total oxidation status.

tein (*P*=0.03) (Figure 2A). The mean ± SD of FRAP in controls was 644.3±258.6 µmol/L/g protein. The difference between PostN and controls did not reach statistical significance.

AOPP, another marker of direct oxidative stress, was comparable among the three groups (Figure 2B). The PreN, PostN, and control values were similar, with the mean ± SD of 36.46±20.1, 49.75±23.32, and 4585±213 mmol/g protein, respectively.

Values for TOS, which estimates the final oxidant status, were superimposed among the three groups. As shown in Figure 2C, similar features were observed in the PreN (mean ± SD 63.78±14.68 µmol/L/g protein), PostN (mean ± SD 74.77±24.9 µmol/L/g protein), or control (mean ± SD 67.5±20.9 µmol/L/g protein) groups. Notably, neonatal treatment with leuprolide acetate showed no effect on FRAP, AOPP, or TOS in all groups (Figure S1A–I).

Levels of serum albumin, a surrogate marker of anti-oxidant capacity, were elevated in the PostN group (mean ± SD 5.28±0.18 mg/dL) versus the control group (mean ± SD 4.53±0.12 mg/dL) (*P*=0.01). Intermediate values (mean ± SD) of 4.95±0.4 were exhibited in the PreN group (Figure 2D). The treatment with leuprolide in the PostN L group was associated with a significant reduction in albumin levels in comparison with PostN rats (Figure S1K). These changes did not occur with controls (Figure S1J) or with PreN rats (Figure S1L).

Biochemical variables

Total cholesterol levels were significantly reduced in PreN rats (mean ± SD 77.25±11.4 mg/dL) in comparison with those in PostN rats (mean ± SD 100.7±11.71 mg/dL) (*P*=0.01) (Figure 3A). Differences between the total cholesterol levels in the control group (mean ± SD 86.43±5.25) versus PostN almost reached significance (*P*=0.052). HDL-C and LDL-C levels were similar between the three groups (Figure 3B and C). Notably, the HDL levels decreased in the PreN group after neonatal leuprolide treatment (Figure S2G); other variables in PreN rats, such as glucose (Figure S2L), total cholesterol (Figure S2C), triglycerides (Figure S2K), and triglyceride–glucose (TyG) index (Figure S2O), did not modify after leuprolide treatment. PostN rats, in turn, did not show any metabolic modification after administration of leuprolide acetate (Figure S2B, F, H, J, and N).

Triglyceride levels were increased in the PostN group (mean ± SD 88.8±9.3) compared to those in the PreN group (mean ± SD 63.5±7.2) (Student's *t*-test, *P*=0.04). Overall, no changes in the triglyceride levels were identified in the three different groups (Figure 3D), although a significant higher glucose was observed in both androgenized rodent models (*P*=0.001) (Figure 3F). The product of TyG was increased in PostN rats in comparison with that in the two other groups (*P*=0.02) (Figure 3E).

As shown in Figure S2M, control rats treated with leuprolide acetate (control L) exhibited an increase in the TyG index, suggesting a worsening of metabolic control (Figure S2D). No other changes regarding total cholesterol, HDL-C, or triglycerides were reported in control rats (Figure S2A, E, and I).

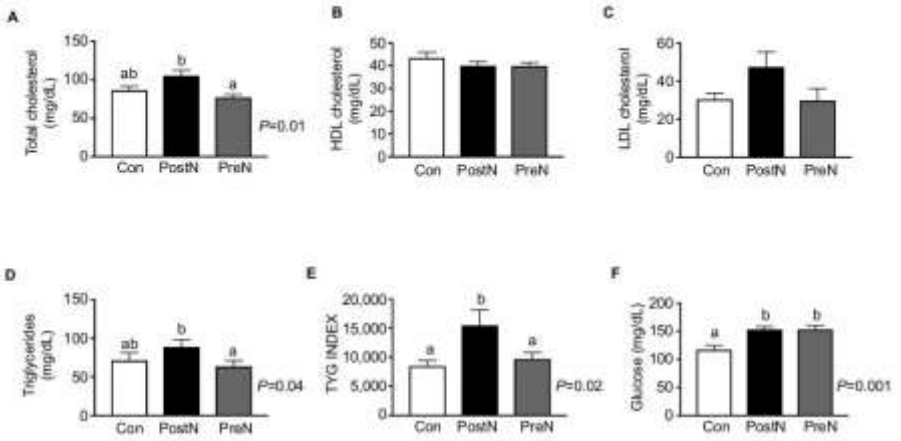

Figure 3 Serum levels of total cholesterol (**A**), HDL cholesterol (**B**), LDL cholesterol (**C**), triglycerides (**D**), TyG index (**E**), and glucose (**F**) in Con, PreN, and PostN androgenized rats.

Notes: Con (n=8); PostN androgenized (n=7); PreN androgenized (n=8). (**A**) ANOVA $P=0.01$; (**D**) ANOVA $P=0.04$; (**E**) ANOVA $P=0.02$; (**F**) ANOVA $P=0.001$. Distinct letters indicate a statistical significant difference (adjusted P level <0.05) obtained with multi-comparison Tukey's test.

Abbreviations: ANOVA, analysis of variance; Con, controls; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PostN, postnatal; PreN, prenatal; TyG, triglyceride-glucose.

Figure 4 summarizes the main dissimilarities between PreN and PostN androgenized rat protocols in our study.

Discussion

Women with PCOS most frequently exhibit dyslipidemia, glucose intolerance/diabetes mellitus, and increased oxidative stress marker levels. Our study evaluated whether different protocols of androgenization leading to normal and abnormal reproductive and metabolic rat phenotypes could be associated with a particular lipid status and redox balance in the blood. We show that a combination of changes in oxidative stress (increased direct oxidation and increased antioxidative profile) was observed in PostN rats exhibiting anovulation/anestrous and increased TyG, whereas this phenomenon was not observed in ovulatory/estrous PreN rats.

All groups of rats achieved a similar final weight at the end of the study. As a result, it was possible to avoid possible bias related to higher adipose accumulation, which is a predominant characteristic of rodents following the use of implants with dihydrotestosterone (DHT),[13,23] letrozole,[23–26] or other miscellaneous protocols.[27–29] We also observed an earlier weight reduction in PreN group rats at birth and day 60. This finding was consistent with previous reports of rats androgenized with testosterone propionate prenatally.

An increase in direct oxidation, FRAP, was identified in the plasma of androgenized animals in our study. This result contrasts with the work of Daneasa et al (2016),[30] where the serum levels of malondialdehyde (MDA), a lipid peroxidation marker, were similar between letrozole-treated rats and its controls. No modifications in MDA were also reported in the ovary tissue of Sprague–Dawley rats submitted to free testosterone (2 or 5 mg) administration,[13] although an increase in lipid peroxidation products of the ovary was found in letrozole rats by another study.[24]

In the present study, antioxidant capacity was estimated in the serum of androgenized animals and controls through the levels of albumin. Albumin is capable of scavenging hydroxyl radicals with its reduced (−SH) cysteine residue (Cys34) and, therefore, considered as one of the major antioxidant elements in the blood of humans and rats.[31–36] We identified increased serum albumin levels in PostN rats compared with controls and the PreN group. In a previous study, no differences in glutathione peroxidase (GPx), another antioxidant marker, were described between letrozole rats and controls.[30] However, an increase in the antioxidant capacity measured by catalase activity and superoxide dismutase or GPx has been identified in the ovary of PCOS rats.[17,24,30]

		
	PostN group • Anovulatory cycles (anestrous) • Increased number atretic follicles • Decreased corpus luteum	PreN group • Ovulatory cycles (estrous cycles) • Ovary histology (number of atretic and healthy follicles, corpus luteum) similar to control rats
Oxidative stress markers (FRAP, AOPP, TOS, # Albumin)	Increased (FRAP)* Increased (Albumin)*	– –
Glucose	Increased*	Increased*
TyG Index	Increased*	–
Total cholestrol	Increased	–
HDL cholestrol	–	–
Weight	– (d1) – (d90) – (d110)	Decreased (d1)* Decreased (d90) – (d110)

Figure 4 Metabolic and oxidative stress markers' characteristics of PostN and PreN rats.
Notes: *Significant differences with control rats. – indicates similarities with controls. The full reproductive aspects of these groups (PreN, PostN, and control with and without treatment with GnRH agonists) have been published in a previous study.[9] Our results agreed with those of previous studies that employed testosterone propionate,[12,13,43,44] with few exceptions.[45]
Abbreviations: AOPP, advanced oxidation protein product; FRAP, ferric-reducing ability of plasma; GnRH, gonadotropin-releasing hormone; HDL, high-density lipoprotein; PostN, postnatal; PreN, prenatal; TOS, total oxidation status; TyG, triglyceride-glucose.

One reason for the divergences among experimental studies may be based on the existence of several approaches for the development of animal models of PCOS. Because of the diversity and limitation of rodent models, it has been claimed that there is no "gold standard" reproducing all aboormalities seen in PCOS.[37] For this reason, caution is necessary to avoid an indiscriminate generalization of the meaning of PreN and PostN models regarding the presented data.

Our results suggested a dual augmentation in oxidative and antioxidative statuses that agreed with some findings in the blood of women with PCOS. In a previous meta-analysis, the mean of MDA, a direct oxidant marker, was ~40% higher in PCOS than in controls.[38] Other direct oxidants such as dimethylarginine and homocysteine and nitric acid were also increased. However, in the same study, antioxidant markers were reduced (glutathione), increased (superoxide dismutase activity), or equal (total antioxidant capacity) to controls.[38]

A recent study (544 PCOS and 468 control women) showed that all four typical phenotypes of PCOS based on the Rotterdam criteria were associated with higher TOS and oxidative stress index (OSI) in comparison with control women.[11] Increased oxidative stress in PCOS was related to higher plasma glucose and triglycerides.[11] Remarkably, all oligo-anovulatory PCOS women, except PCOS women with presumed regular cycles, show an increased total antioxidant capacity in the serum.[11] In our study, anovulatory rats (PostN) presented an increased TyG index. TyG, the product of triglycerides with glucose, has been considered in humans and in rodents as a surrogate marker of insulin resistance and metabolic syndrome.[39–42] We showed that only PostN rats exhibited statistically significant higher TyG indices than controls, which may represent an additional link toward the disruption of the oxidative stress markers.[45]

Although the comparison of two models (PreN and PostN) with an extra ovulatory control (PostN L rats) consisted in one strength of the present study, weakness should be considered as well. The realization of euthanasia in rats at different estrous cycles may also had an impact of estrogens on oxidative stress markers. Other limitations of our research from our point of view were related to sample size, the absence of subgroups (lean, obese/young, aged), and the lack of inclusion of more antioxidant stress markers to the study.

Conclusion

The results presented suggest that an increased direct oxidation and an increased antioxidative capacity could be associated with PostN treatment with testosterone propionate (PostN), which is usually linked with anovulatory cycles and insulin resistance estimated by higher TyG. Androgenized rats treated with leuprolide acetate (PostN L) and presenting

estrous cycles did not exhibit modifications in biochemical status or increased direct oxidation (FRAP) in plasma but showed a reduced antioxidant capacity estimated by albumin serum levels. PreN androgenization, in turn, was related to a lower weight at birth but a less harmful phenotype. Altogether, these findings continue to support the central role of androgen excess, anovulation, and insulin resistance as the key factors to trigger redox abnormalities in PCOS. Additional studies of the impact of weight gain, high-glucose/high-fat diet,[41] or simply the long-term follow-up will improve the comprehension of the intricacy mechanisms of oxidative stress in PCOS.

Acknowledgment

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Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

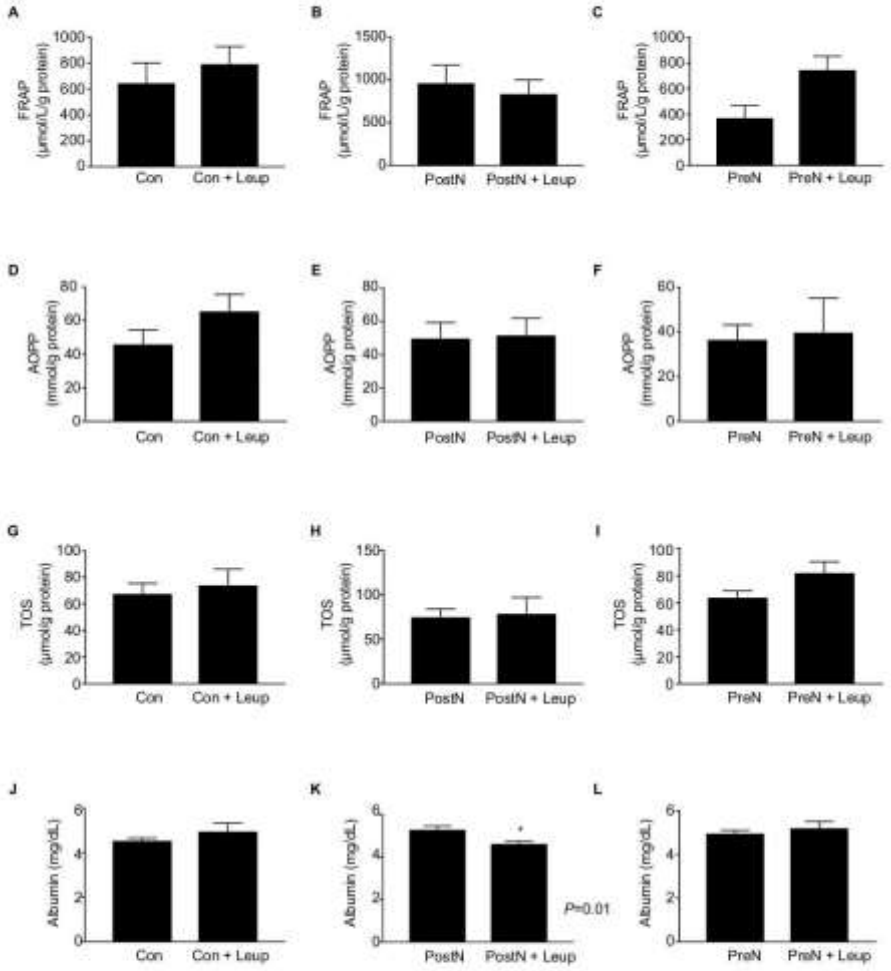


Figure S1 Oxidative stress markers in C, PreN, and PostN androgenized rats, which were treated and not treated with neonatal leuprolide acetate.
Notes: (**A–C**) FRAP; (**D–F**) AOPP; (**G–I**) TOS; (**J–L**) Albumin. Con (n=8); Con + Leup, Con treated with leuprolide; PostN androgenized (n=7); PostN + Leup, PostN androgenized treated with leuprolide (n=7); PreN androgenized (n=8); PreN + Leup, PreN androgenized treated with leuprolide (n=4). Results were reported as mean (SEM). Statistical analysis used Student's *t*-test. *Significance was assumed at $P<0.05$.
Abbreviations: AOPP, advanced oxidation protein product; Con, controls; FRAP, ferric-reducing ability of plasma; Leup, leuprolide; PostN, postnatal; PreN, prenatal; SEM, standard error of the mean; TOS, total oxidation status.

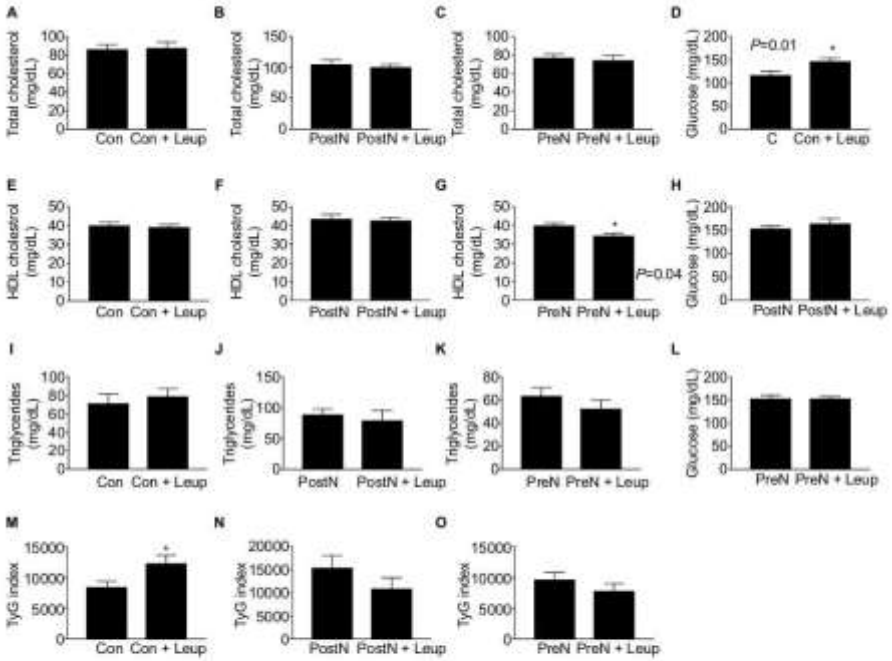
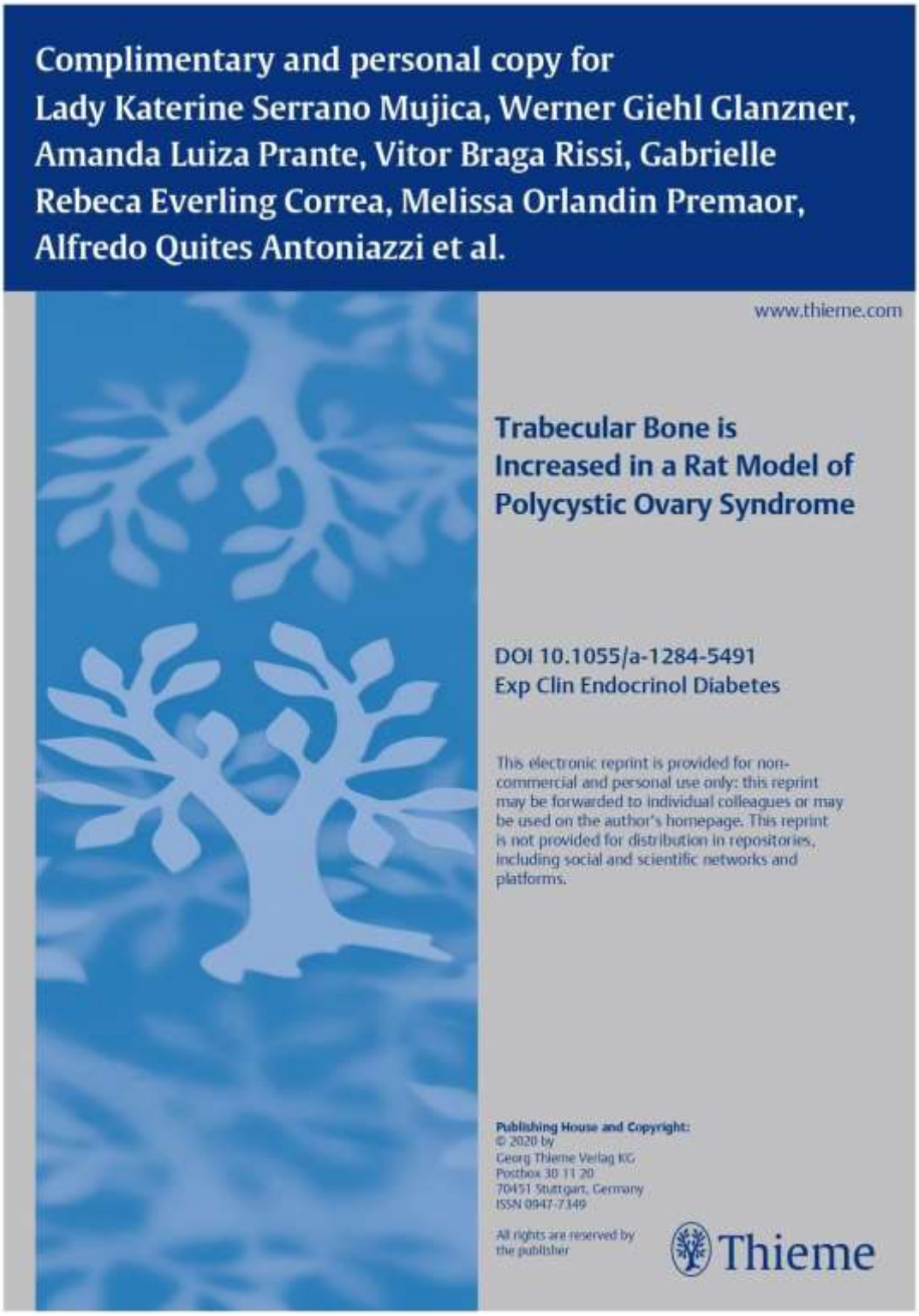


Figure S2 Biochemical markers.

Notes: Serum levels of total cholesterol (**A**–**C**), HDL cholesterol (**E**–**G**), triglycerides (**I**–**K**), TyG index (**M**–**O**), and glucose (**D**, **H**, and **L**) in Con, PreN, and PostN rats submitted or not submitted to the neonatal treatment with leuprolide acetate. Con (n=8); C + Leup, Con treated with leuprolide (n=6); PostN androgenized (n=7); PostN + Leup, PostN androgenized treated with leuprolide (n=7); PreN androgenized (n=8); PreN + Leup, PreN androgenized treated with leuprolide (n=6). Results were reported as mean (SEM). Statistical analysis used Student's *t*-test. *Significance was assumed at $P<0.05$.

Abbreviations: Con, controls; HDL, high-density lipoprotein; Leup, Leuprolide; PostN, postnatal; PreN, prenatal; SEM, standard error of the mean; TyG, triglyceride–glucose.

Anexo. 2 Trabecular Bone is Increased in a Rat Model of Polycystic Ovary Syndrome.



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Trabecular Bone is Increased in a Rat Model of Polycystic Ovary Syndrome

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Key words

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ABSTRACT

Polycystic ovary syndrome (PCOS) is an intricate disorder characterized by reproductive and metabolic abnormalities that may affect bone quality and strength along with the lifespan. The present study analysed the impact of postnatal androgenization (of a single dose of testosterone propionate 1.25 mg subcutaneously at day 5 of life) on bone development and markers of bone metabolism in adult female Wistar rats. Compared with healthy controls, the results of measurements of micro-computed tomography (microCT) of the distal femur of androgenized rats indicated an increased cortical bone volume voxel bone volume to total volume (VOX BV/TV) and higher trabecular number (Tb.n) with reduced trabecular separation (Tb.sp). A large magnitude effect size was observed in the levels of circulating bone formation Procollagen I N-terminal propeptide (P1NP) at day 60 of life; reabsorption cross-linked C-telopeptide of type I collagen (CTX) markers were similar between the androgenized and control rats at days 60 and 110 of life. The analysis of gene expression in bone indicated elements for an increased bone mass such as the reduction of the Dickkopf-1 factor (*Dkk1*) a negative regulator of osteoblast differentiation (bone formation) and the reduction of Interleukin 1-b (il1b), an activator of osteoclast differentiation (bone reabsorption). Results from this study highlight the possible role of the developmental programming on bone microarchitecture with reference to young women with PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common hyperandrogenic disorders among women of reproductive age [1, 2]. The impact of PCOS on bone development has been a subject of research that has reported conflicting results. Both cross-sectional and longitudinal studies have indicated that premenopausal women with PCOS are protected from bone loss [3–5] or are at a higher risk of bone mass reduction [6–8]. The difficulties in addressing the effects of PCOS on bone are based on different phenotypes of the syndrome and could be related to the variables that influ-

ence mineral metabolism, such as diet, anovulation, alimentary disorders, insulin resistance, and steroid milieu.

Rodents have been employed in translational studies of osteoporosis in humans and also for investigating developmental programming in PCOS. A rodent model of PCOS replicates many metabolic and reproductive abnormalities that are observed in women with PCOS; therefore, these animals are considered a valuable tool to dissect complex mechanisms in the development of this disorder. In a study by Tamura et al., treatment with testosterone propionate in young Sprague-Dawley rats, at day 9 of life, was effective in increasing their bone mineral density (BMD), which was defined by bone densitometry [9]. However, rats in this study were not fully characterized as PCOS rats as the presence of cycles was not addressed [9]. Rats can be considered a valuable model for studying PCOS because rats that are subjected to testosterone treatment during the neonatal period manifest acyclicity, anovulation, polycystic ovaries, an increased proportion of atretic follicles, insulin resistance, and dyslipidemia [10, 11]. Therefore, our study aimed to reexamine the role of PCOS in bone metabolism by investigating an ovary-intact young rodent model of PCOS (that exhibited polycystic ovaries and fewer estrous cycles) [10]. Additionally, we examined the impact of postnatal androgenization on bone markers of formation and reabsorption (P1NP and CTX) and microstructure (micro-CT).

Material and Methods

Ethical statement

All Institutional and National Guidelines for the care and use of animals were followed. This study was approved by the local Ethics Committee on Animal Use (CEUA-UFSM), under protocol number 100/14, in accordance with ARRIVE guidelines.

Animals

Female Wistar rats (Rattus norvegicus albinus) (n = 14) aged 1 day were used in this study. The dams were with their pups until weaning (21 days). The rats were maintained at a temperature of 22 °C, 55–65 % humidity, and under artificial illumination with a 12-h light/dark cycle. The rats were fed a standard pellet diet with water given ad libitum, as previously described [10].

Experimental procedures

Postnatal hormone exposures were performed by the treatment of 5-day-old animals through a subcutaneous (SC) injection of 1.25 mg testosterone propionate (Androgenol™) (androgenized group), and vehicle control 5-day-old animals received 1.25 mg corn oil SC (control group) [10]. Retro-orbital blood collection was performed at day 60 using a micro-hematocrit capillary tube, under deep anesthesia (isoflurane); at day 110 of life, blood was collected by cardiac puncture after euthanasia as previously described [10].

Acquisition and analysis of the distal femur images using micro-CT

The structural properties of the trabecular and cortical femur were determined with a high-resolution micro-CT system (SkyScan 127, Kontich, Belgium). The X-ray tube was set to 50 kV, and the beam was filtered with a 0.5 mm aluminum filter. The sample position and camera settings were adjusted to provide a 3.0-µm isotropic pixel size, and projection images were collected every 0.2°. Reconstructions were done with NRecon (v 1.6.9.8; Bruker micro-CT).

Enzyme-linked immunosorbent assay (ELISA)

Procollagen I N-terminal propeptide (PINP) and cross-linked C-telopeptide of type I collagen (CTX) levels (CV < 10 %) were measured in serum at a single occasion using a specific rat ELISA (Cloud Clone Corp, USA) according to the manufacturers' protocols.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

The femur was collected after euthanasia, cleaned of muscle and soft connective tissues, cut in two halves, and stored at − 80 °C. Before RNA extraction, the bone medulla was manually removed and washed several times with PBS, and the specimen was frozen in liquid nitrogen and crushed into powder. The total RNA was obtained using Trizol® (Life Technologies, Foster City, CA, United States), following the manufacturer's instructions. After that, the samples were treated with DNase I™ (Life Technologies, United States) and RNA samples were quantified in a spectrophotometer NanoDrop (ND1000, Thermo Scientific) with a wavelength of 260 nm. Total RNA (1 µg) was treated with DNase™ (DNAse Amplification Grade I - Invitrogen) at 37 °C within 5 min to digest any contaminating DNA. The reverse transcription reaction to cDNA was performed according to the manufacturer's instructions using the iScript cDNA Synthesis Kit™ (Bio-Rad). After obtained, cDNAs were kept in a − 20 °C freezer until the gene expression was checked. Two genes of reference were employed: *Cyclophilin A* (NM_017101.1) and *Gapdh* (NM_017008.4). Details of primers and genes of interest used in this study are disposed of in the (▶ **Table 15**). The qRT-PCR reactions were performed in duplicate in a total volume of 20 µL (including 10 ng of cDNA, 10 µL of TaqMan Universal PCR Master Mix, and 1 µ of the assays) and were carried out on CFX384 real-time PCR systems (Bio-Rap) (Hercules, CA). The cycle conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 10 s, and 60 °C for 1 min. The data were analyzed using Bio-rap CFX Manager Software (version 3.0), and Ct values were transformed to quantities using the comparative Ct method (ΔΔCt, Life Technologies), as previously described [12].

Statistical analysis

The statistical analysis and graphs were performed using the software GraphPad Prism 6.03 (GraphPad Software Inc., San Diego, CA). Comparisons among the two groups were performed using Student's t test. In the absence of a normal distribution, the data were analyzed using a Mann-Whitney test. For the analysis of more than 2 groups having normal data distribution, the ANOVA test was performed. Significance was assumed at $p<0.05$. Additionally, data were also analyzed by Cohen's effect size analysis and effect size with Cohen's d value of 0.8 and above considered as large magnitude differences are reported [12, 13] This analysis has been suggested to the study of small sample sizes to reject the null hypothesis [14].

Results

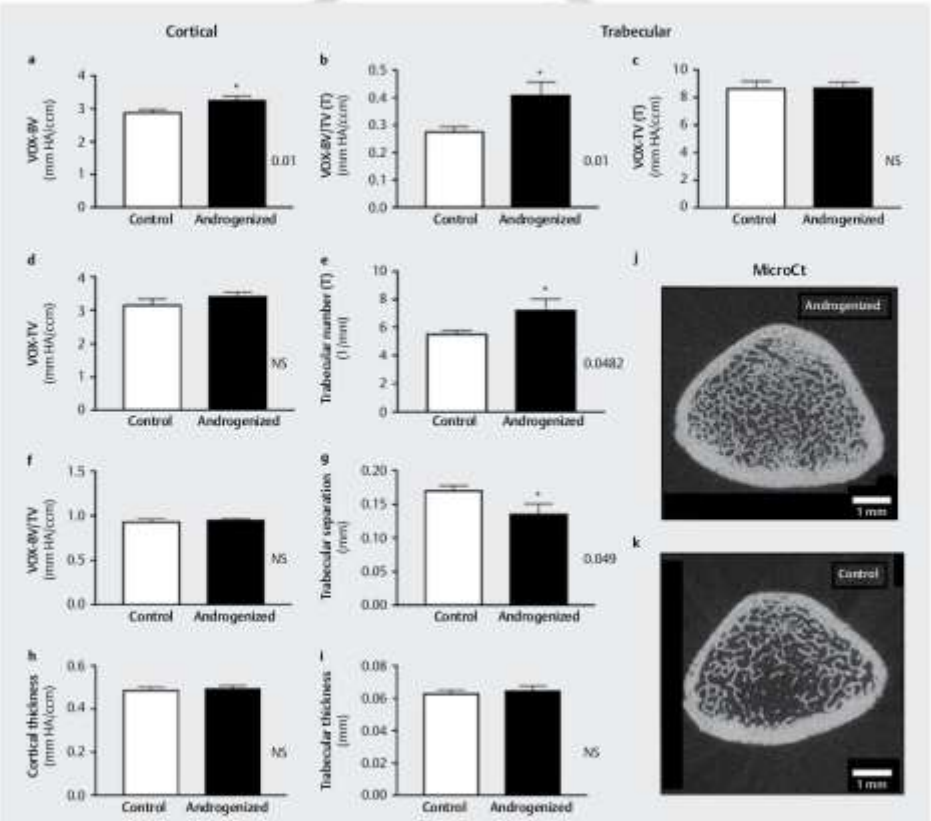
Micro-CT analysis of distal femur

Cortical Bone

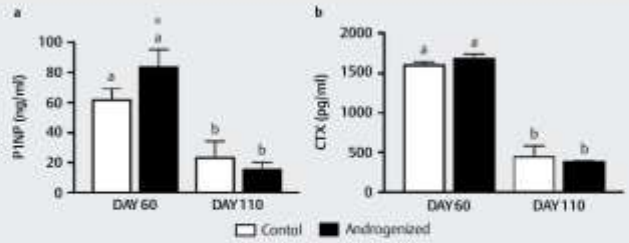
The VOX-BV indicating a higher bone volume was significantly higher in androgenized rats (3.28 ± 0.09) versus control rats (2.89 ± 0.07, p = 0.01) (▸ **Fig. 1a**). However, other parameters such as VOX-TV (▸ **Fig. 1d**), VOX-BV/TV (▸ **Fig. 1f**), and the cortical thickness (▸ **Fig. 1h**) were similar between the two groups (P > 0.05).

Trabecular bone

Androgenized rats show marked modifications in trabecular bone of the distal femur. This group presented a significantly higher VOX BV/TV (0.41 ± 0.04) versus controls (0.27 ± 0.01, p = 0.01) (▸ **Fig. 1b**). Androgenized rats exhibited, respectively, a higher number of trabeculae (7.3 ± 0.74) versus control rats (5.55 ± 0.19, p = 0.048) (▸ **Fig. 1e**) and lower trabecular separation (Tb-Sp) (0.13 ± 0.01) versus controls (0.17 ± 0.006, p = 0.04) (▸ **Fig. 1h**) (▸ **Fig. 1g**). Trabecular thickness (Tb-Th) was not different between these two



▸ **Fig. 1** MicroCt analysis of Androgenized rat on day 110. Androgenized rat on day 110 exhibited a marked increase in trabecular bone characterized by augmentation of the voxel bone volume to total volume, VOX BV/TV(T) **b**, trabecular number **e**, and reduction of trabecular separation **g**; these contrasts between control and androgenized rats are represented in a cross-sectional 2D image **j**, **k** where more trabeculae are observed in androgenized rats. No differences were seen in terms of trabecular thickness (i) or the voxel trabecular volume VOX-TV (T) **c**. Cortical bone parameters, excepting for an increase in the voxel bone volume VOX-BV **a**, were similar between the groups - the voxel trabecular volume VOX-TV **d**, voxel bone volume to total volume VOX BV/TV **f**, and Cortical Thickness **h**. Androgenized n = 6; control n = 6.



▶ **Fig. 2** Analysis of bone markers of female control and androgenized rats at days 60 and 110. Plasma levels of bone formation (P1nP) **a** and bone reabsorption (CTX) **b** were significantly higher in younger (day 60) than older animals (110 of life). An increased Cohen effect size (•) for P1nP was observed at day 60 suggesting a higher bone formation in androgenized rats versus control. Results are expressed in mean + SEM. Day 60: both groups n = 4; day 110: Androgenized n = 4; control n = 8.

groups (▶ **Fig. 1l**). The result of VOX-TV was 8.74 ± 0.33 in androgenized rats versus 0.70 ± 0.47 in controls; VOX-BV (▶ **Fig. 1c**). This contrast in trabecular mass was observed in a cross-sectional 2D image (1j, 1K).

Bone markers

Plasma levels of P1nP and CTX in young rats at day 60 of life were significantly higher than that in adult rats [analysis of variance (ANOVA)] (P1nP $p < 0.0005$; CTX $p < 0.0001$) (▶ **Fig. 2a,b**). At day 60, the mean ± SEM of P1nP was 84.11 ± 11.1 ng/ml in androgenized rats and 62.45 ± 6.84 ng/ml in controls ($p = 0.11$) (▶ **Fig. 2a**); however, a non-significant large magnitude effect size was observed in the levels of circulating bone formation (P1NP) at day 60 of life. CTX value in androgenized rats (d60) was 1694 ± 42.4 pg/ml against 1618 ± 25.5 pg/ml (NS) (▶ **Fig. 1k**). At day 110, the mean ± SEM of P1nP was 16.16 ± 4.05 ng/ml in androgenized rats and 23.85 ± 10.44 ng/ml in controls (▶ **Fig. 1j**); the CTX value in androgenized rats (d110) was 390.9 ± 0.92 pg/ml against 452.3 ± 129.2 pg/ml (NS) (▶ **Fig. 2b**).

Gene expression

▶ **Fig. 1S** shows the results of gene expression for bone formation and reabsorption factors in rat femurs. We observed at day 110 in androgenized animals, a significant reduction in *Wnt* gene expression ($p = 0.049$) and a non-significant decreased magnitude effect size of the genes of *Dkk1*, *Il1b* in bone. No differences could be detected for the following genes: *Ctnnb1*, *Igfi*, *Sost*, *Lrp5*, *Lrp6*, *Notch*, *Opg (tnfsf11b)*, *Bmp2i*, *Bmp2*, *Tbr2*, *and Fgfr2*.

Discussion

In the present study, testosterone administration on day 5 of life produced noticeable bone modifications in a rodent model of PCOS. When compared with control rats, the femur from androgenized female rats exhibited a higher bone volume associated with an increased trabecular number and a lower trabecular separation on micro-CT.

The results of our study, however, do not explain why these changes occurred. Besides the fact that the administration of testosterone during the neonatal period increases androgen levels, this occurs shortly, and no hyperandrogenism is observed at adult age, in spite of other reproductive manifestations of PCOS [10]. Therefore, further studies regarding developmental programming on bone formation need to be conducted. Analyzing genes that control bone, at day 110 of life, revealed a reduction of *Dkk1* (negative regulator of Wnt signaling) that supports an increase in bone formation and a decrease in *Il1b* (an activator of osteoclast differentiation) indicating a reduction in bone reabsorption. However, Wnt expression, related to canonical Wnt/β-catenin in/TCFL pathway, was also decreased; this indicated a reduction in osteoblast differentiation, proliferation, and mineralization.

The timings during which changes develop in bone metabolism are also relevant. According to our results, an effect size of large magnitude in bone formation (P1NP) occurred at day 60 of life, but not at day 110, suggesting an earlier impact of androgenization on bone mass. In humans, as in rats, bone development occurs at a proper time owing to the presence of circulating steroids. Androgens may exert a positive influence on bone metabolism through androgen receptors (AR) that are present in cells such as osteoblasts, osteoclasts, and osteocytes or indirectly through its conversion to estradiol [15–18]. It is known that the combination of estrogens and testosterone produces a higher femur BMD, total BMD, increased bone volume fraction, trabecular number, and trabecular thickness as assessed by micro-CT, when compared to controls [19]. Nevertheless, a positive effect of progesterone on trabecular bone, which is suggested in the literature [20], cannot be definitively excluded, because androgenized rats are reported to remain at metestrus for a longer period of time when progesterone levels are increased. At present, the relevance of these findings needs to be cautiously extrapolated for adolescents with PCOS. For example, in a study conducted by Bechtold and colleagues, adolescents with PCOS aged between 12.4 and 18 years (mean age, 14.96 ± 1.42 years) showed higher trabecular and cortical bone mass compared to the healthy reference population [21]. However, studies in late-

adolescent and adult women with PCOS reveal a reduction in bone mass, P1NP, and osteocalcin [8, 22–25].

In conclusion, treatment with testosterone propionate during the neonatal period led to an increase in the cortical bone volume (VOX BV/TV) and a higher trabecular number with reduced trabecular separation in the distal femur during adulthood. Despite the limitations of our study related to a lack of analysis of the axial skeleton and the absence of a mechanical test to address the strength of the bones, our findings highlight the positive impact of androgenization on rat bone development. Further studies need to be conducted to evaluate the role of molecular players and their interactions with other pathways in the bones of androgenized rats before and after puberty.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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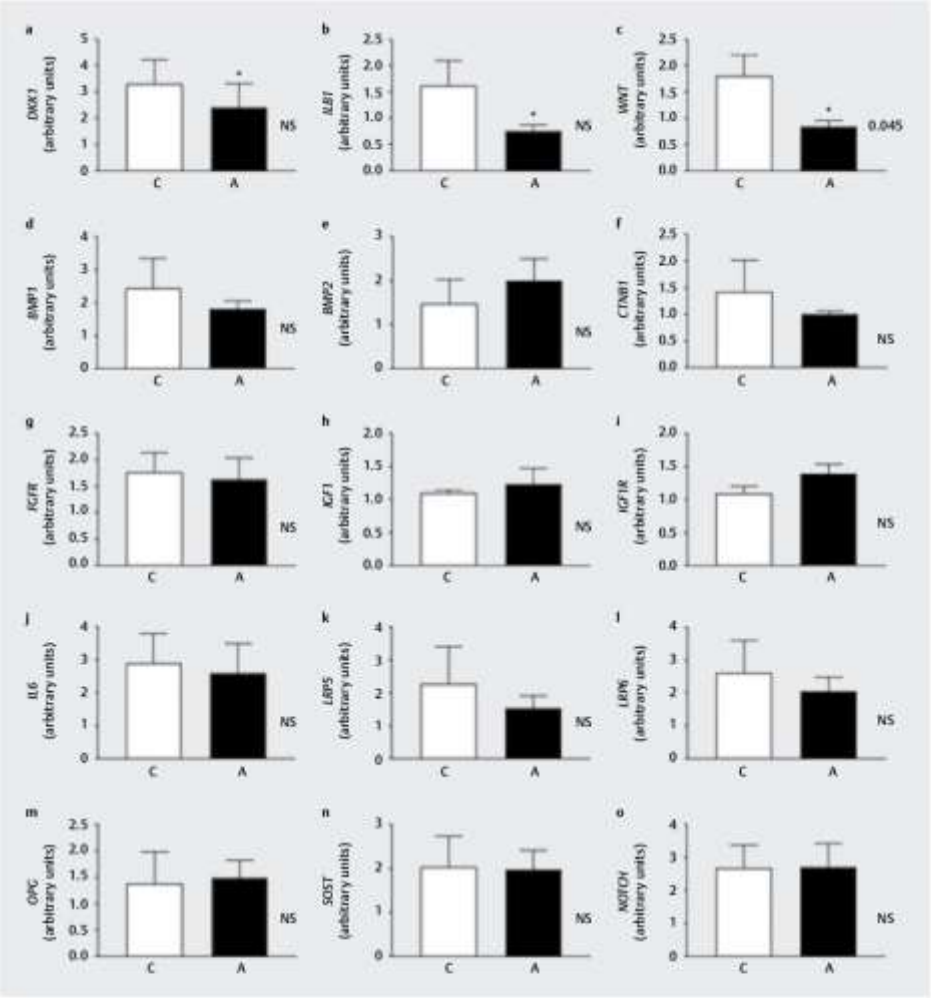
Supplementary Material

▶ **Table 1S** List of the primers used for detection and quantification of gene transcripts.

Gene	Reference Sequence	Forward primer	Reverse primer
Ar	NM_012502.1	CTGGATGGGACTCATGGTATTT	CAGGTCAGGTGCAAAGTAGAG
Dkk1	NM_001106350.1	ATGCCCTCTGACCACAGCCATT	CACCGTGGTCATTGCCAAGGT
Igf1	NM_052807.2	CAATATCACAGACCCGGAAGAG	CGATACGGTACAGAGTGAAAGG
Ctnb1	NM_053357.2	CTCAGATGGTGTCTGCCATAG	TGGTGGGAAAGGTTGTGTAG
Igfr	NM_052807.2	CAATATCACAGACCCGGAAGAG	CGATACGGTACAGAGTGAAAGG
Sost	NM_030584.1	GGCAAGCCTTCAAGAATGATG	GGTCTGGTTGTTCTCTAGTTCC
Lrp5	NM_001106321.2	TTGTCATCTCTGGCCTTGTATC	CCTGCCAGAAGAGAACCTTAC
Lrp6	NM_001107892.1	CTTGCTGGGCGACTATGTTTA	GTCTGGCAGCTGGTCTATTATG
Notch	NM_001105721.1	ATACGCCTGTGGCAGAATAAG	CCATGGTCTGACATTCCTCATC
Opg	NM_012870.2	GCACCCTGAGAAAGAGGATATT	GGGATGACACAGAAGATAGTAGAAG
Il6	NM_012589.2	GGTTTGCCGAGTAGACCTCA	GTGGCTAAGGACCAAGACCA
Il1B	NM_031512.2	AAAGAAGGTGCTTGGGTCCT	CAGGAAGGCAGTGTCACTCA
Bmp2	NM_017178.1	ATCCACTCCACAAACGAGAAA	CCACATCACTGAAGTCCACATA
Tbr2	XM_017596193.1	GGACATTAACACTGAGGAGTACAG	GGTCCATCTGGAAAGACGTTAG
Fgfr2	NM_001109892.1	CCTCATCCCAAGATGCCTTAAT	GAATGTGACGGGTGTGTAACT
Cyclophilin A	NM_017101.1	GAAAGAAGGCATGAGCATTGTG	GCCCGCAAGTCAAAGAAATTAG
Gapdh	NM_017008.4	AGACAGCCGCATCTTCTTGT	CCGTTCACACCGACCTTCA



▶ **Fig. 15** Gene expression for bone formation and reabsorption factors in androgenized and control rats at day 110 of life. **a** DKK1, **b** ILB1, **c** WNT, **d** BMP1, **e** BMP2, **f** CTNB1, **g** FGFR, **h** IGF1, **i** IGF1R, **j** IL6, **k** LRP5, **l** LRP6, **m** OPG, **n** SOST, **o** NOTCH. Results are expressed in mean ± SEM. Androgenized n = 6; control n = 6.