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Giovana de Moraes Ourique

EFEITO DO RESVERATROL E DA VITAMINA E FRENTE A PREJUÍZOS REPRODUTIVOS CAUSADOS PELO USO DE ÁCIDO VALPRÓICO EM RATOS MACHOS

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

Orientadora: Prof[a]. Dr[a]. Katia Padilha Barreto
Co-orientador: Prof. Dr. Paulo Bayard Dias Gonçalves

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Santa Maria, RS
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Aos meus pais, que sempre serviram de exemplo e nunca

mediram esforços para me apoiar na busca pelos meus objetivos...

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos”
(Isaac Newton)

RESUMO

EFEITO DO RESVERATROL E DA VITAMINA E FRENTE A PREJUÍZOS REPRODUTIVOS CAUSADOS PELO USO DE ÁCIDO VALPRÓICO EM RATOS MACHOS

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O tratamento prolongado com ácido valpróico (VPA) tem sido relacionado com efeitos adversos na função reprodutiva masculina mediada pelo aumento do estresse oxidativo testicular. Neste sentido, este estudo avaliou o possível efeito protetor do resveratrol (RSV, 10 mg/kg i.p.) e da vitamina E (50 mg/kg i.p.) na qualidade dos espermatozoides e em parâmetros de estresse oxidativo no testículo e epidídimo de ratos Wistar tratados com VPA (400 mg/kg, via oral) por 28 dias. O tratamento com VPA causou diminuição da motilidade espermática, acompanhada de aumento no dano oxidativo e diminuição da capacidade antioxidante no testículo e epidídimo. Tanto o RSV quanto a vitamina E foram capazes de prevenir o dano oxidativo e restaurar a capacidade antioxidante dos testículos e do epidídimo, prevenindo a perda de motilidade espermática induzida pelo VPA. Estes dados sugerem que a administração de RSV ou vitamina E pode ser uma estratégia terapêutica para preservar a motilidade dos espermatozoides e a função testicular em pacientes que necessitam de tratamento em longo prazo com VPA. Além disso, este estudo também avaliou o efeito do VPA (1 μM, 10 μM, 100 μM, 1 mM e 3 mM) em espermatócitos em paquíteno e espermátides arredondadas isolados. Nossos resultados demonstraram que, *in vitro*, nas nossas condições experimentais, o tratamento com VPA causou aumento do estresse oxidativo e hiperacetilação da histona H4, sem provocar danos ao DNA em espermatócitos em paquíteno e espermátides arredondadas.

Palavras-chave: Ácido valpróico. Infertilidade masculina. Antioxidantes. Estresse oxidativo. Histonas. Células germinativas.

ABSTRACT

EFFECT OF RESVERATROL AND VITAMIN E AGAINST VALPROIC ACID-INDUCED REPRODUCTIVE DAMAGE IN MALE RATS

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Long-term treatment with valproic acid (VPA) has been associated with adverse effects on the male reproductive function mediated by increased testicular oxidative stress. Thus, this study evaluated the potential protective effect of resveratrol (RSV 10 mg/kg, ip) and vitamin E (50 mg/kg, ip) in sperm quality and oxidative stress parameters in the testis and epididymidis of Wistar rats treated with VPA (400 mg/kg, orally) for 28 days. VPA treatment was associated with decreased sperm motility, increased oxidative damage and decreased antioxidant capacity in testis and epididymidis. RSV and vitamin E were able to prevent oxidative damage and rescue antioxidant capacity of the testis and epididymidis, preventing the impairment of sperm motility induced by VPA. These data suggest the administration of RSV or vitamin E as a therapeutic strategy to preserve sperm motility and testicular function in male patients requiring long-term treatment with VPA. Moreover, this study also evaluated the effect of VPA (1 uM, 10 uM, 100 uM, 1 mM and 3 mM) in isolated pachytene spermatocytes and round spermatids. Our results demonstrated that, *in vitro*, at our experimental conditions, VPA treatment induced oxidative stress and hyperacetylation of histone H4, without causing DNA damage in pachytene spermatocytes and round spermatids.

Keywords: Valproic acid. Male infertility. Antioxidants. Oxidative stress. Histones. Germ cells.

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RESULTADOS COMPLEMENTARES

LISTA DE ABREVIATURAS E SIGLAS

ABAP	2,2'-azobis (2-amidinopropano) *(2,2'-azobis (2-amidinopropane))*
ABAT	GABA transaminase (*GABA transaminase*)
ALDH5A1	Succinato-semialdeído desidrogenase (*succinate-semialdehyde dehydrogenase*)
ALT	Alanina aminotransferase (*alanine aminotransferase*)
AMPc	Adenosina 3',5'-monofosfato cíclico *(adenosine monophosphate)*
AST	Aspartato aminotransferase (*aspartate aminotransferase*),
ATP	Trifosfato de adenosina *(Adenosine triphosphate)*
BRDT	Proteína bromodomínio testicular (*testis-specific bromodomain-containing protein*)
CAT	Catalase (*catalase*)
CDNB	1-cloro-2,4-dinitrobenzeno *(1-chloro-2,4-dinitrobenzene)*
CO_2	Dióxido de carbono *(carbono dioxide)*
CYP	Citocromo P450 (cytochrome P450)
DNA	Ácido desoxirribonucleico (deoxyribonucleic acid)
DNPH	2,4-dinitrofenil-hidrazina *(2,4-dinitrophenylhydrazine)*
DTNB	ácido 5,5'-ditio-bis-(2-nitrobenzóico) (*5,5'-dithiobis-(2-nitrobenzoic acid)*)
EROs	Espécies reativas de oxigênio *(reactive oxygen species, ROS)*
GABA	Ácido γ-aminobutírico (*γ-aminobutyric acid*)
GPx	Glutationa peroxidase (*glutathione peroxidase*)
GR	Glutationa redutase (*glutathione reductase*)
GSH	Glutationa reduzida (*reduced glutathione*)
GSSG	Glutationa oxidada (*oxidised glutathione*)
GST	Glutationa-S-transferase (*glutathione-S-transferase*)
H_2O	Água *(water)*
H_2O_2	Peróxido de hidrogênio (*hydrogen peroxide*)
HATs	Histona acetiltransferases (histone acetyltranferase)
HDACs	Histona desacetilases *(histone deacetylase)*
$HO^{\bullet}$	Radical hidroxila *(hydroxyl radical)*
LOOH	Hidroperóxidos lipídicos (*lipid hydroperoxide*)
LPO	Lipoperoxidação (*lipid peroxidation*)
MDA	Malondialdeído *(malondialdehyde)*
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzido *(nicotinamide adenine dinucleotide phosphate)*
NMDA	N-metil-D-aspartato *(N-methyl-aspartate)*
NPSH	Tióis não proteicos (*non-protein thiols*)
O_2	Oxigênio molecular *(molecular oxygen)*
$^1O_2$	Oxigênio singlete *(singlet oxygen)*

$O_2^{\bullet -}$	Ânion radical superóxido (*superoxide anion*)
$OH^{\bullet}$	Radical hidroxila (*hydroxyl radical)*
OGDH	α-cetoglutarato desidrogenase (*α-ketoglutarate dehydrogenase*)
ROS	Espécies reativas de oxigênio, EROs (*reactive oxygen species*)
RSV	Resveratrol (3,4',5 triidroxiestilbeno) *(3,4',5-triiidroxiestilbene)*
SUCL	Succinato-coenzima A ligase (*succinyl-coenzyme A ligase*)
SOD	Superóxido dismutase (*superoxide dismutase*)
TBA	Ácido tiobarbitúrico *(thiobarbituric acid)*
TBARS	Substâncias que reagem ao ácido tiobarbitúrico (*thiobarbituric acid reactive substances)*
TCA	Ácido tricloroacético *(trichloroacetic acid)*
TRAP	Potencial atioxidante reativo total (*total reactive antioxidant potential*)
UGP	UDP glucuronosiltransferase (*UDP-glucuronosyltransferase*)
VPA	Ácido valpróico *(Valproic acid, 2-propyl-pentanoic acid)*

SUMÁRIO

1. INTRODUÇÃO

1.1 O sistema reprodutor masculino

O sistema reprodutor masculino é responsável pelas seguintes funções: a) produzir, manter e transportar os espermatozoides e seu líquido protetor, o sêmen; b) produzir e secretar hormônios sexuais masculinos responsáveis pela produção de espermatozoides ao longo da vida, bem como a mudanças que ocorrem no corpo masculino ao longo do desenvolvimento, em particular durante a puberdade; c) liberar os espermatozoides no trato reprodutivo feminino durante a relação sexual. Estas funções são realizadas pelos testículos, epidídimo, ductos deferentes, glândulas sexuais acessórias e genitália externa (RUSSELL et al., 1993).

Os testículos são um par de órgãos glandulares ovoides encapsulados por um tecido fibroso, a túnica albugínea. Internamente, eles são divididos em dois compartimentos: os túbulos seminíferos, contendo as células de Sertoli e as células germinativas, e o interstício, o qual contém as células de Leydig, células peritubulares mióides, células endoteliais dos vasos sanguíneos e fibroblastos. As principais funções dos testículos são produzir espermatozoides e sintetizar e secretar andrógenos (hormônios sexuais masculinos), processos chamados de espermatogênese e esteroidogênese, respectivamente (RUSSELL et al., 1993).

1.2 Espermatogênese

O desenvolvimento de células germinativas masculinas é um processo contínuo e complexo, em que células germinativas diplóides arredondadas sofrem sucessivas divisões mitóticas e meióticas, bem como mudanças morfológicas, para a produção de espermatozoides haplóides. Este processo ocorre nos túbulos seminíferos e depende do suporte das células de Sertoli (SHARPE et al., 2003) (Figura 1).

As células de Sertoli são células de forma irregular que ficam na membrana basal dos túbulos seminíferos e têm extensões citoplasmáticas em direção à superfície luminal. Elas estão em contato direto com as células germinativas em desenvolvimento,

e secretam proteínas, tais como fatores de crescimento, proteases, inibidores de proteases, e hormônios necessários para a espermatogênese (SHARPE et al., 2003). As células de Sertoli são lateralmente ligadas entre si através de ”tight junctions”, que formam uma barreira única conhecida como barreira hematotesticular, que divide o epitélio seminífero em compartimentos basal e adluminal (SETCHELL et al., 1969; MITAL et al., 2011). Esta barreira protege as células germinativas em desenvolvimento de agentes citotóxicos potencialmente presentes no sangue. Além disso, a barreira impede que espermatozoides entrem na circulação sanguínea ou no sistema linfático (PEREZ et al., 2014).

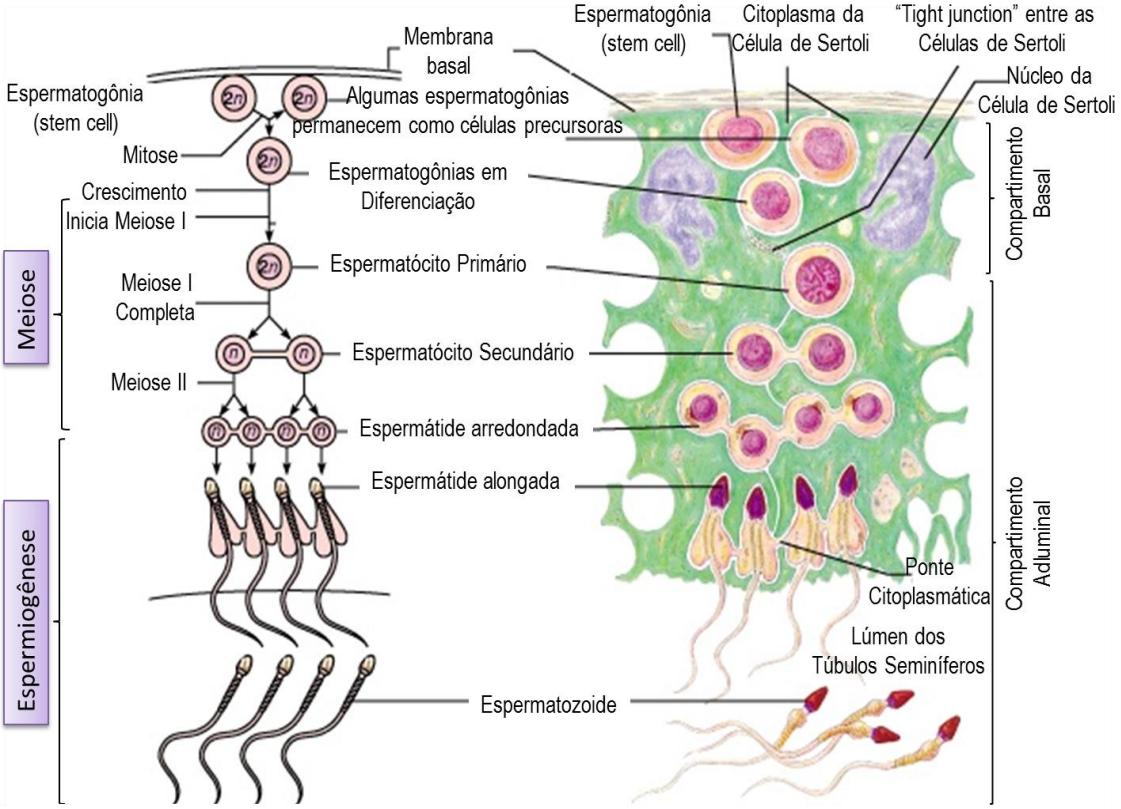

Figura 1. Ilustração esquemática da espermatogênese. Fonte: Adaptado de (http://www.apsubiology.org/anatomy/2020/2020_Exam_Reviews/Exam_5/CH27_Spermatogenesis.htm)

A espermatogênese é um processo complexo e altamente organizado que leva cerca de 35 dias em camundongos, 52 dias em ratos, e 72 dias em seres humanos (CLERMONT, 1963; 1972). Este processo é subdividido em três fases distintas em todas as espécies, a mitose, a meiose, e a espermiogênese, embora a duração de cada

fase seja diferente em cada espécie (CLERMONT, 1972; DE KRETSER et al., 1998). A mitose envolve a divisão mitótica das células germinativas diploides em dois tipos de células-filhas. O primeiro tipo se prolifera para formar duas novas células, mantendo assim a população de células germinativas indiferenciadas. O segundo tipo se prolifera em um processo ordenado em vários tipos de espermatogônias, que dão origem a espermatócitos em pré-leptóteno na última divisão mitótica (CLERMONT, 1972). Subsequentemente, as células de Sertoli facilitam o trânsito dos espermatócitos em pré-leptóteno através da barreira hematotesticular, de tal modo que todas as fases subsequentes da espermatogênese ocorrem no compartimento adluminal (RUSSELL et al., 1993). A prófase meiótica envolve a diferenciação ordenada de espermatócitos em pré-leptóteno para leptóteno, zigóteno, e então espermatócitos em paquíteno. Os espermatócitos em paquíteno então se diferenciam em espermatócitos em diplóteno, que sofrem rapidamente divisão meiótica para produzir espermátides arredondadas (CLERMONT, 1972).

O processo continua com espermiogênese, em que as espermátides arredondadas sofrem alterações morfológicas e bioquímicas, e se transformam em espermátides alongadas, que irão se transformar em espermatozoides. Esta fase final envolve um processo de remodelação da cromatina (em que o DNA nuclear torna-se altamente condensado), a formação do acrossoma e a eliminação do excesso de citoplasma (corpo residual) com a formação simultânea da gota citoplasmática (HESS e DE FRANCA, 2008).

Os espermatozoides recém formados, uma vez liberados das células de Sertoli, migram dos túbulos seminíferos para a rede testicular, em seguida para os ductos eferentes e finalmente alcançam o epidídimo. O epidídimo é responsável pelo transporte, maturação e armazenamento dos espermatozoides. Localiza-se posteriormente ao testículo, no saco escrotal, e desemboca na base do canal deferente, que conduz os espermatozoides até à próstata, onde se mistura com o sêmen originário das vesículas seminais, movendo-se pela próstata até a uretra durante a ejaculação (TURNER et al., 1990). A qualidade do espermatozoide, em termos de motilidade depende das diferenciações pós-testiculares que ocorrem durante o seu trânsito através do epidídimo, que incluem principalmente a migração da gota citoplasmática,

aumento do AMPc e de cálcio intracelular e alterações no estado de fosforilação de proteínas específicas. Outros fatores envolvidos no controle da motilidade dos espermatozoides incluem o ambiente iônico (cálcio, pH, HCO_3^-) e a presença ou a ausência de substratos energéticos específicos para manter a concentração de ATP nos espermatozoides (Gatti et al., 2004). Qualquer alteração na motilidade pode levar a uma penetração ineficiente do espermatozoide através do muco cervical e prejudicar a sua capacidade de alcançar o ovócito (AITKEN et al., 1985).

Este processo é muito sensível a agentes tóxicos e vem sendo alvo de muitos estudos devido ao número crescente de casos de infertilidade idiopática. Muitos fatores têm sido associados ao risco aumentado de infertilidade, incluindo anomalias genéticas, doenças reprodutivas, quimioterapia, radioterapia, e o uso em longo prazo de algumas drogas, entre elas, o ácido valpróico (ROSTE et al., 2005; O'FLYNN O'BRIEN et al., 2010; JUNGWIRTH et al., 2012)

1.3 Ácido valpróico

O ácido valpróico (ácido 2-n-propilpentanóico, VPA) é um ácido carboxílico de estrutura molecular relativamente simples (Figura 2), com baixa solubilidade em água, tradicionalmente utilizado para o tratamento de certos tipos de convulsões (NIH, 2014). Pode ser utilizado sob diversas formas de apresentação como valproato de sódio, divalproato de sódio (composto de partes iguais de ácido valpróico e valproato de sódio) e valproato de magnésio (sal do divalproato), sem apresentar diferença na eficácia (ZARATE et al., 1999).

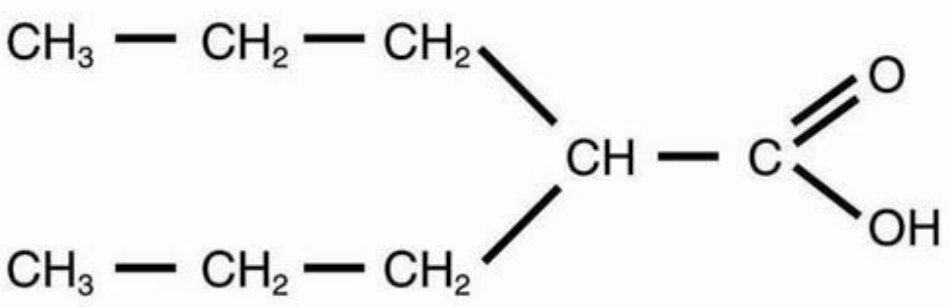

Figura 2. Estrutura química do ácido valpróico
Fonte: NIH, 2014

Desde a descoberta acidental de suas propriedades anticonvulsivantes na França, em 1962, o VPA tornou-se um dos antiepilépticos mais utilizados em diferentes regiões do mundo, podendo ser administrado como monoterapia ou como parte de regimes de politerapia que agrupam vários antiepilépticos. O VPA é geralmente considerado como um agente de primeira escolha em crises generalizadas tônico-clônicas primárias, crises de ausência e crises mioclônicas devido ao seu amplo espectro terapêutico (SEMAH et al., 2004). Atualmente, o VPA também é utilizado na profilaxia da enxaqueca, e para o tratamento de transtornos bipolares, ansiedade, hiperatividade e comportamento impulsivo-agressivo (KRYMCHANTOWSKI et al., 2002; NASRALLAH et al., 2006; JOHANNESSEN LANDMARK, 2008; BOWDEN, 2009; NIH, 2014). Além disto, está sendo utilizado em ensaios pré-clínicos como um agente quimioterápico e mostra efeitos positivos e potencial terapêutico em modelos animais de doença de Alzheimer (QING et al., 2008; NEBBIOSO et al., 2012).

O VPA é administrado principalmente por via oral, embora esteja disponível nas formulações retal e injetável. Em humanos, a absorção por via oral é rápida, total e ocorre primariamente no estômago com pico de absorção de 2 a 3 horas, com exceção do divalproato de sódio, cuja absorção ocorre no duodeno e intestino delgado, prolongando o pico de absorção para até 4 horas (PERUCCA, 2002). O ácido valpróico se liga amplamente (≥ 90%) às proteínas do plasma e, portanto, tem uma depuração muito baixa, com uma meia-vida média que varia de 9 a 16 h (CRAMER e MATTSON, 1979; BRYSON et al., 1983).

Em ratos, a concentração de VPA no soro atinge seu pico entre 5 e 30 minutos após o tratamento (LOSCHER, 1978; 2002). A distribuição do VPA é alta no sangue, moderada no fígado, rim, coração e pulmão, e baixa no cérebro, testículos, gordura e músculo esquelético (DICKINSON et al., 1979). Em ratos, cerca de 63% do VPA encontra-se ligado às proteínas plasmáticas e a sua meia-vida é cerca de 4,6 h (LOSCHER, 1978; DICKINSON et al., 1979; LOSCHER, 2002). O VPA é quase completamente metabolizado antes de ser excretado, o que ocorre principalmente através da urina (GUGLER e VON UNRUH, 1980), apesar de já ter sido demonstrado que o VPA pode ser excretado no leite materno (NAU et al., 1981) e no sêmen (SWANSON et al., 1978).

O VPA tem três principais vias de metabolização no fígado (Figura 3A): é principalmente modificado por glucuronidação (30-50%), com atuação das enzimas UDP-glucuronosiltransferases (UGTs, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B15); β-oxidação mitocondrial é a segunda principal contribuinte para o metabolismo do VPA (cerca de 40%); o restante (cerca de 10%) é metabolizado pelas proteínas do citocromo P450 (CYPs, CYP2A6, CYP2B6, CYP2C9). Diversos metabólitos são formados, sendo o 2-eno-valproato, o 4-eno-valproato e o 2,4-dieno-valproato os principais (TAN et al., 2010; GHODKE-PURANIK et al., 2013).

O VPA é um fármaco com múltiplos e complexos modos de ação, o que explica suas diferentes aplicações farmacológicas (Figura 3B). Os seus efeitos farmacológicos envolvem uma variedade de mecanismos, incluindo principalmente a regulação indireta dos níveis de ácido γ-aminobutírico (GABA), atenuação da excitação neuronal mediada por ativação de receptor de glutamato N-metil-D-aspartato (NMDA), e modulação de canais de sódio, potássio e cálcio voltagem-dependentes, reduzindo assim a neurotransmissão excitatória (PERUCCA, 2002; GHODKE-PURANIK et al., 2013). O GABA é formado a partir do α-cetoglutarato através do ciclo do ácido tricarboxilico, e metabolizado em succinato de semialdeído pela enzima GABA transaminase (ABAT) e, em seguida, a succinato, pela succinato-semialdeído desidrogenase (ALDH5A1). O α-cetoglutarato também pode ser convertido em succinil CoA através da ação de α-cetoglutarato desidrogenase (OGDH), desviando a formação de GABA. O VPA inibe ABAT e ALDH5A1, inibindo a via de degradação do GABA (PERUCCA, 2002; GHODKE-PURANIK et al., 2013).

Além de aumentar os níveis de GABA, o VPA reduz o disparo de neurônios de alta frequência através do bloqueio de canais de sódio, de potássio e de cálcio dependentes de voltagem (incluindo aqueles codificados por CACNA1C, CACNA1D, CACNA1N, e CACNA1F e SCN) (GHODKE-PURANIK et al., 2013). Além disso, o VPA também inibe uma família de enzimas chamadas histona desacetilases (HDAC), mais especificamente HDAC das classes I e II, envolvidas nos processos de condensação da cromatina e controle da expressão gênica (WANG et al., 2013b).

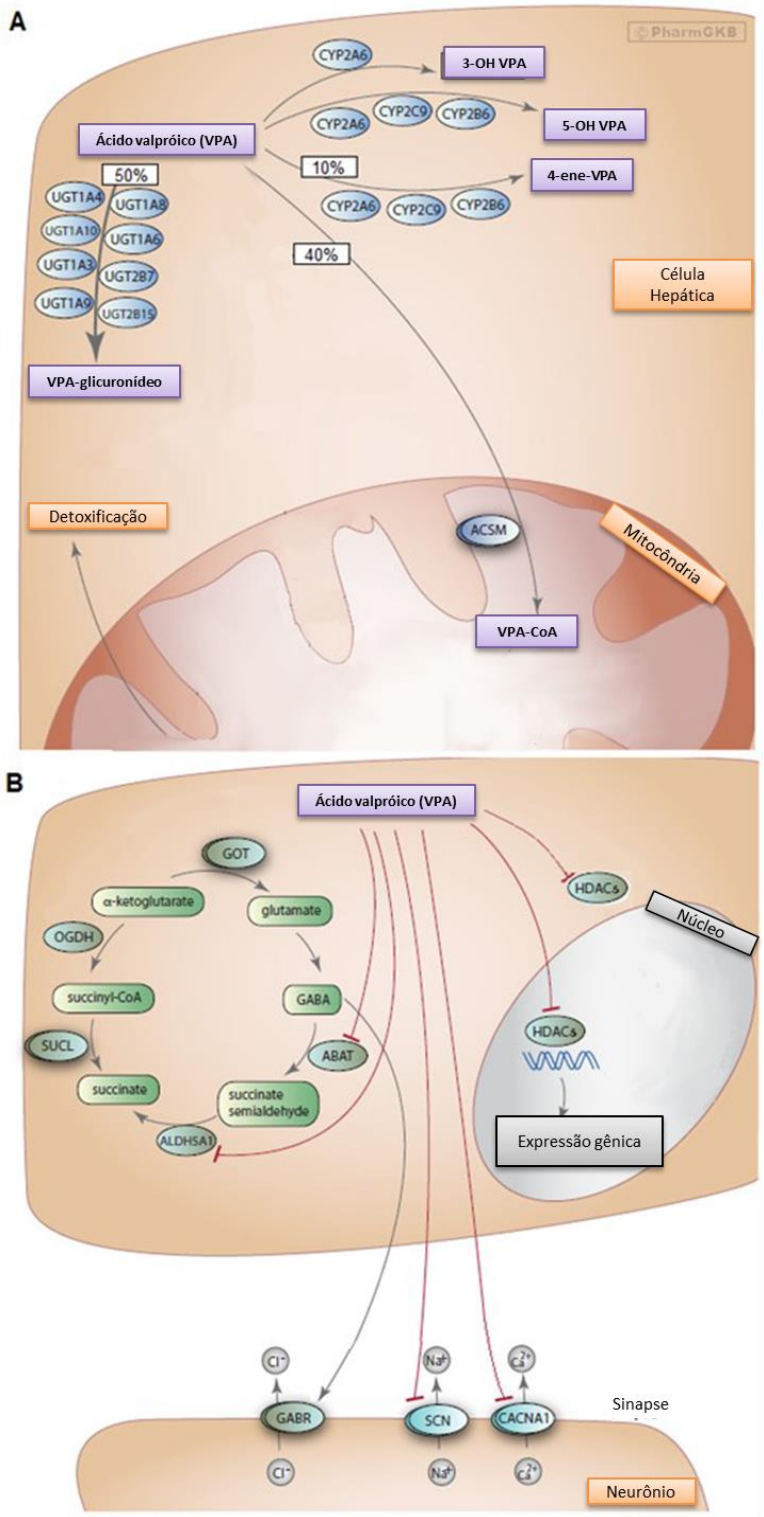

Figura 3. Representação esquemática da farmacocinética e vias farmacodinâmicas do VPA CYP (citocromo P450); UGP (UDP glucuronosiltransferase); 3-OH-VPA (ácido 3-hidroxi-2-propil-pentanóico); 5-OH-VPA (ácido 5-hidroxi-2-propil-pentanóico) 4-OH-VPA (ácido 4-hidroxi-2-propil-pentanóico); HDAC (histona desacetilase); GABA (ácido gama-aminobutírico); ABAT (GABA transaminase); ALDH5A1 (succinato semialdeído desidrogenase); SUCL (succinato-coenzima A ligase); OGDH (α-cetoglutarato desidrogenase). Fonte: Adaptado de Ghodke-Puranik et al., 2013.

1.4 Toxicidade do ácido valpróico no sistema reprodutor masculino

Apesar de ser considerado um fármaco relativamente seguro, o uso do VPA tem sido associado a efeitos adversos sobre os sistemas gastrointestinal, neurológico, hematológico e reprodutivo (PERUCCA, 2002). Em camundongos, o tratamento com VPA durante o período pré-púbere altera a maturação testicular, diminuindo a taxa de espermatogênese e a motilidade espermática, além de causar atrofia testicular (SNYDER e BADURA, 1995). Homens adultos tratados com VPA apresentam uma diminuição na fertilidade (RATTYA et al., 2001; ROSTE et al., 2005). Muitos estudos em animais têm demonstrado que o tratamento contínuo com VPA causa alterações hormonais (ROSTE et al., 2002; KROGENAES et al., 2008), atrofia do testículo, próstata, vesícula seminal e epidídimo (NISHIMURA et al., 2000; ROSTE et al., 2002; KROGENAES et al., 2008), degeneração dos túbulos seminíferos e diminuição da contagem e motilidade dos espermatozoides (NISHIMURA et al., 2000).

O mecanismo exato de toxicidade do VPA sobre o sistema reprodutor masculino ainda não é totalmente conhecido, mas a formação de metabólitos reativos e, principalmente, o desenvolvimento do estresse oxidativo têm sido propostos para explicar a toxicidade do VPA a outros sistemas (CHANG e ABBOTT, 2006). Alguns estudos mostram que o VPA induz a formação excessiva de espécies reativas de oxigênio (EROs), que são responsáveis por efeitos adversos incluindo hepatotoxicidade (TONG et al., 2005; CHANG e ABBOTT, 2006; KIANG et al., 2010), neurotoxicidade (Auinger et al., 2009; Chaudhary e Parvez, 2012), e teratogenicidade (TUNG e WINN, 2011). No testículo, tem sido demonstrado que o tratamento com VPA induz toxicidade às células germinativas, aumenta o dano oxidativo e aumenta o dano ao DNA do espermatozoide, o que sugere que a produção de EROs pode ser um importante mecanismo responsável pela toxicidade do VPA no sistema reprodutor masculino (KHAN et al., 2011).

1.5 Estresse oxidativo

O desenvolvimento e a existência de um organismo em presença de oxigênio estão associados à geração de EROs. Sob condições fisiológicas, a geração das EROs ocorre principalmente durante os processos de oxidação biológica, como a respiração celular acoplada à fosforilação oxidativa, para formação de ATP na mitocôndria (HALLIWELL e GUTTERIDGE, 2007).

A formação dessas espécies ocorre em aproximadamente 5% de todo o processo de redução do oxigênio molecular (O_2) a água (H_2O). A princípio o O_2 é reduzido a H_2O recebendo quatro elétrons de uma só vez pela enzima citocromo oxidase. Entretanto, em razão de sua configuração eletrônica, o O_2 tem uma forte tendência em receber um elétron de cada vez, gerando compostos intermediários altamente reativos. Dentre esses compostos intermediários destacam-se o ânion radical superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxil (OH•) (Figura 4). Quando as EROs apresentam pelo menos um elétron desemparelhado são denominadas radicais livres, que são definidos como qualquer espécie capaz de existir de forma independente e que contenha um ou mais elétrons não-pareados no seu orbital mais externo, característica que lhe confere alta reatividade. Ainda deve-se relatar o oxigênio “singlet”, que representa o estado excitado do oxigênio e também pode causar danos à célula (DEL MAESTRO, 1980; SIES, 1991; YU, 1994; HALLIWELL e GUTTERIDGE, 2007).

O $O_2^{\bullet -}$ é a primeira EROs a ser formada através da redução monovalente do O_2 a H_2O, e a partir do mesmo serão geradas as demais EROs através de reações sequenciais (HALLIWELL, 1996). O H_2O_2 é o segundo intermediário gerado nesse processo. Ele é uma espécie reativa não-radicalar citotóxica, e pode facilmente se difundir entre as células, podendo gerar o OH• através da reação do H_2O_2 com íons de ferro ou cobre (reação de Fenton) ou através da reação do H_2O_2 com o $O_2^{\bullet -}$ (reação de Haber-Weiss) catalisada por íons metálicos (FRIDOVICH, 1974; YU, 1994). O OH• é um dos mais potentes oxidantes em sistemas biológicos, podendo atravessar membranas e reagir com biomoléculas como lipídios, proteínas e DNA (HALLIWELL e GUTTERIDGE, 2007).

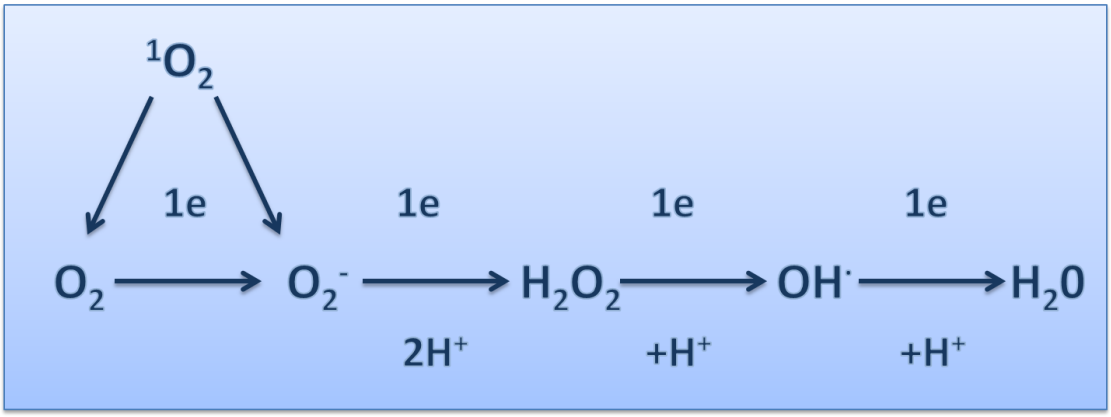

Figura 4. Formação das EROs a partir da redução parcial do oxigênio. $^1O_2$ (oxigênio singlete); O_2 (oxigênio molecular); O_2^- (ânion radical superóxido); H_2O_2 (peróxido de hidrogênio); $OH^·$ (radical hidroxila); H_2O (água); e (eletron); H^+ (próton). Fonte: Adaptado de PAVANATO e LLESUY, 2008.

O potencial reativo das EROs pode ser bem ilustrado pelos processos que desencadeia, como a peroxidação lipídica, as reações oxidativas em proteínas da membrana e o ataque ao DNA, podendo resultar em alterações na estrutura, permeabilidade e função secretora da membrana, mutações, perda funcional e morte celular (SIES, 1991; HALLIWELL, 1996).

A fim de atenuar as consequências da toxicidade causada pelas EROs, os organismos aeróbicos desenvolveram o sistema de defesa antioxidante. Os antioxidantes são a principal linha de defesa e correspondem a quaisquer substâncias que, presentes em baixas concentrações comparadas ao substrato oxidável, retardam ou mesmo impedem a oxidação do substrato (HALLIWELL e GUTTERIDGE, 2007). Os antioxidantes atuam removendo o O_2 presente no meio, impedindo a formação das EROs, quelando metais que catalisam a formação de EROs, induzindo a produção de antioxidantes endógenos e reparando os danos em biomoléculas danificadas (YU, 1994; HALLIWELL e GUTTERIDGE, 2007). O sistema de defesa antioxidante é composto por antioxidantes enzimáticos e não-enzimáticos, que atuam conjuntamente na proteção celular (HALLIWELL e GUTTERIDGE, 2007).

O mecanismo antioxidante enzimático envolve as enzimas que fazem a proteção primária e intrínseca do organismo (Figura 5). Estão incluídas as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e glutationa S-transferase (GST). Através da ação destas enzimas, o organismo

mantém a concentração das EROs dentro dos limites fisiológicos (YU, 1994; HALLIWELL e GUTTERIDGE, 2007). A SOD é responsável pela dismutação do $O_2•^-$ em H_2O_2, o qual é convertido a O_2 e H_2O pela ação da enzima CAT. A GPx, por sua vez, além de remover o H_2O_2, também catalisa a conversão de hidroperóxidos orgânicos a produtos menos reativos, empregando a glutationa reduzida (GSH) como substrato, impedindo, assim, a formação de OH• e, o consequente dano celular. A forma oxidada da glutationa (GSSG) produzida nesse processo é reciclada a moléculas de GSH pela ação da GR, a qual utiliza NADPH como substrato (YU, 1994; HALLIWELL, 1996; HALLIWELL e GUTTERIDGE, 2007). A GST constitui uma família de enzimas de detoxificação de fase II, que catalisa a conjugação da GSH a compostos eletrofílicos, incluindo radicais livres e xenobióticos (HABIG et al., 1974).

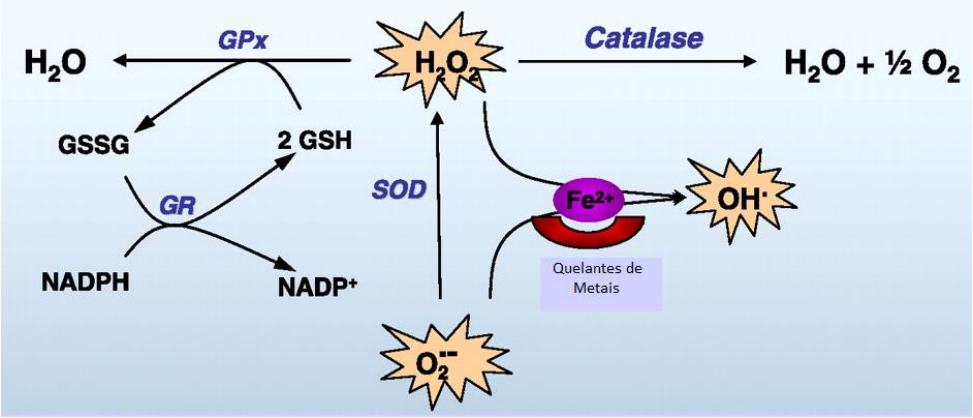

Figura 5. Principais sistemas detoxificadores de EROs O_2 (oxigênio molecular); O_2^- (ânion radical superóxido); H_2O_2 (peróxido de hidrogênio); $OH^·$ (radical hidroxila); H_2O (água); Fe^{2+} (ferro); GPx (glutationa peroxidase); SOD (superóxido dismutase); GR (glutationa redutase); GSSG (glutationa oxidada); GSH (glutationa reduzida); $NADP^+$ (nicotinamida adenina dinucleotídeo fosfato); NADPH (nicotinamida adenina dinucleotídeo fosfato reduzido). Fonte: Adaptado de BASHAN et al., 2009 (Bashan et al., 2009)

O sistema de defesa não-enzimático é formado por antioxidantes lipossolúveis, como, por exemplo o α-tocoferol, assim como por hidrossolúveis, tais como o ácido ascórbico e a GSH (HALLIWELL e GUTTERIDGE, 2007). O α-tocoferol é capaz de inibir as reações de propagação desencadeadas durante a peroxidação lipídica, enquanto o ácido ascórbico além de desempenhar um papel muito importante nos processos de regeneração do α-tocoferol (HUANG e MAY, 2003), é capaz de reagir diretamente com as EROs, como o $O_2•^-$, o H_2O_2 e o OH•, e vários produtos formados

durante a peroxidação lipídica (NIKI, 1991). A GSH, por sua vez, está implicada na reciclagem do ácido ascórbico a partir de sua forma oxidada, o ácido dehidroascórbico (LINSTER e VAN SCHAFTINGEN, 2007).

O desequilíbrio entre os antioxidantes e EROs é denominado estresse oxidativo e ocorre devido ao aumento na velocidade de geração de EROs e/ou diminuição na atividade do sistema de defesa antioxidante, resultando em aumento sustentado das concentrações de EROs (SIES, 1991; PAVANATO e LLESUY, 2008). O estresse oxidativo é resultado de um dos três fatores: (1) aumento na geração das EROs, através da acumulação de intermediários reativos; (2) prejuízo do sistema de defesa antioxidante (inibição de enzimas antioxidantes, depleção de antioxidantes não enzimáticos); (3) incapacidade para reparar o dano oxidativo (HALLIWELL e GUTTERIDGE, 2007).

A consequência do estresse oxidativo mais comumente descrita na literatura científica é a lipoperoxidação. A interação entre radicais livres e lipídeos envolve reações em cadeia em três etapas: iniciação, propagação e terminação. Essas etapas podem ser verificadas através da medida dos hidroperóxidos lipídicos (LOOH) (etapa de propagação) e das substâncias que reagem ao ácido tiobarbitúrico (TBARS) (etapa de terminação) (HALLIWELL e GUTTERIDGE, 2007). Além da lipoperoxidação, podem ocorrer também danos às proteínas, que apresentam muitos sítios reativos que podem ser danificados durante o estresse oxidativo. A formação de grupos carbonil, que se correlacionam diretamente com danos causados às proteínas, também pode ocorrer pelo aumento de EROs que atuam sobre grupos amino das proteínas, alterando sua estrutura e função. Este processo causa alterações conformacionais, diminuição da atividade catalítica de enzimas, e degradação de proteínas por proteases, devido a maior suscetibilidade. As EROs também podem ocasionar danos ao DNA (HALLIWELL e GUTTERIDGE, 2007).

1.6 Estresse oxidativo e fertilidade masculina

Os espermatozoides geram EROs em quantidades fisiológicas, que desempenham um papel importante na função dos espermatozoides durante a

capacitação, reação acrossômica e fusão com o oócito. No entanto, a produção excessiva de EROs no sêmen, acompanhada de uma disfunção nas defesas antioxidantes, resulta em estresse oxidativo, que pode lesar a membrana e o DNA dos espermatozoides, levando a um prejuízo na qualidade seminal (AGARWAL et al., 2006; AGARWAL e SEKHON, 2010).

A espermatogênese é um processo replicativo extremamente ativo, capaz de gerar aproximadamente 1000 espermatozoides por segundo. A alta velocidade de divisão celular inerente a este processo implica em uma alta velocidade de consumo de oxigênio mitocondrial pelo epitélio germinal (AITKEN e ROMAN, 2008). Uma vez que tanto a espermatogênese como a esteroidogênese são vulneráveis ao estresse oxidativo, a baixa tensão de oxigênio que caracteriza este tecido pode ser um importante mecanismo pelo qual os testículos protegem a si mesmos do dano mediado por radicais livres (PELTOLA et al., 1994; CHEN et al., 2005). Adicionalmente, os testículos contêm uma elaborada gama de enzimas antioxidantes e de “scavengers” de radicais livres para impedir que as funções espermatogênica e esteroidogênica deste órgão sejam impactadas pelo estresse oxidativo (AITKEN e BAKER, 2006; AITKEN e ROMAN, 2008).

Estes sistemas de defesa são extremamente importantes, porque o dano oxidativo é reportado como a causa mais comum de bloqueio da função testicular subjacente a consequências patológicas de uma ampla gama de condições que vão desde torsão testicular ao diabetes e exposição à xenobióticos. Tais condições podem causar mudanças no fluxo microvascular testicular, mudança na sinalização endócrina e apoptose das células germinativas (AITKEN e ROMAN, 2008).

As EROs podem causar danos tanto aos espermatozoides quanto aos testículos. Os espermatozoides, por serem ricos em ácidos graxos poli-insaturados, são altamente susceptíveis ao ataque das EROs e à lipoperoxidação, que podem levar a perturbações na membrana e dano ao DNA, resultando em diminuição da motilidade espermática (provavelmente por uma perda rápida de ATP intracelular), dano ao axonema, diminuição na viabilidade espermática, e aumento nos defeitos de peça intermediária, com efeitos deletérios na capacitação espermática e na reação acrossômica (AGARWAL et al., 2006; AGARWAL e SEKHON, 2010). O dano ao DNA também pode

levar a abortos espontâneos (TREMELLEN, 2008). Além disso, a produção de EROs e o estresse oxidativo no testículo podem lesar a membrana mitocondrial e contribuir para a inibição da capacidade esteroidogênica, resultando em diminuição na produção de testosterona pelas células de Leydig (AITKEN e ROMAN, 2008).

Adicionalmente, Agarwal e colaboradores (2006) demonstraram que EROs poderiam servir como marcadores de infertilidade idiopática, uma vez que o estresse oxidativo está diretamente ligado com uma baixa qualidade no sêmen nesta condição. Kao e colaboradores (2008) confirmaram que o estresse oxidativo está diretamente relacionado com a perda de motilidade dos espermatozoides, uma vez que o esperma de homens com infertilidade ou subfertilidade apresentou maior dano oxidativo e menor capacidade antioxidante. Em conjunto, estes dados demonstram fortemente que o estresse oxidativo é uma das principais causas de perda da função espermática (AGARWAL et al., 2006; KAO et al., 2008; AGARWAL e SEKHON, 2010; AITKEN et al., 2014; AITKEN et al., 2015).

Neste sentido, tem aumentado a busca de compostos antioxidantes que possam ser potencialmente utilizados na clínica médica como uma alternativa para diminuir a vulnerabilidade do sistema reprodutor masculino ao dano oxidativo (SOLEAS et al., 2001; KOVACIC e SOMANATHAN, 2010). Entre os compostos de maior interesse, estão os antioxidantes naturais, como o resveratrol (RSV) e a vitamina E.

1.7 Resveratrol

O RSV (3,4',5 triidroxiestilbeno) é um importante composto polifenólico, encontrado principalmente nas uvas e vinhos, que apresenta efeitos benéficos em uma variedade de órgãos e sistemas humanos, devido ao seu alto poder antioxidante (FREMONT, 2000; BHAT et al., 2001).

O RSV é sintetizado naturalmente nas plantas sob duas formas isômeras: trans-resveratrol e cis-resveratrol (Figura 6). O isomêro trans- tem reconhecidas atividades biológicas, e pode facilmente ser convertido para a forma cis- pela luz visível, pois este isômero é mais estável (SOLEAS et al., 2001). O trans-resveratrol foi primeiramente detectado em videiras (*Vitis vinifera*) em 1976 por Landcake e Price, que descobriram

que este composto era sintetizado pelas folhas em resposta a uma infecção fúngica ou a exposição à luz ultravioleta (FREMONT, 2000; BHAT et al., 2001).

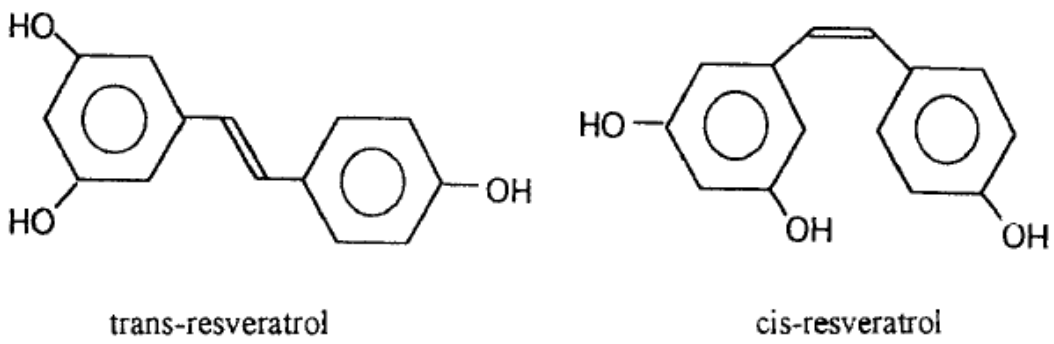

Figura 6. Estrutura química do trans- e cis-resveratrol. Fonte: FRÉMONT, 2000

O interesse neste composto foi estimulado quando estudos epidemiológicos mostraram uma relação inversa entre o consumo de vinho tinto e a incidência de doenças cardiovasculares (FREMONT et al., 1999; FREMONT, 2000). O trans-resveratrol apresenta efeitos benéficos à saúde, incluindo efeitos anticarcinogênicos (JANG et al., 1997; WOO et al., 2007), antiinflamatórios (WU, 2003), proteção cardiovascular (BRADAMANTE et al., 2004) e atividade antioxidante (FREMONT et al., 1999; DOLINSKY et al., 2009; VENTURINI et al., 2010; OURIQUE et al., 2013).

O RSV protege a membrana celular contra a lipoperoxidação e contra danos ao DNA causados por EROs (KASDALLAH-GRISSA et al., 2006; COLLODEL et al., 2011). Os efeitos protetores do RSV contra o dano oxidativo in vivo e in vitro são provavelmente devido à regulação de antioxidantes endógenos dos sistemas celulares, além de atuar como um antioxidante “scavenger” de EROs (LEONARD et al., 2003; SPANIER et al., 2009). O RSV também inibe a formação de radicais livres de oxigênio suprimindo genes pró-oxidantes (DOLINSKY et al., 2009; SPANIER et al., 2009) e induzindo enzimas antioxidantes incluindo SOD, CAT e GPx (UNGVARI et al., 2007; SPANIER et al., 2009). Além disso, o RSV pode quelar o metal de transição cobre, que participa da geração de radicais livres e da lipoperoxidação (BELGUENDOUZ et al., 1997).

In vivo, a suplementação oral com 5 g/kg de RSV se mostrou capaz de proteger os testículos de ratos contra a lipoperoxidação induzida por etanol (KASDALLAH-

GRISSA et al., 2006). Na dose de 10 mg/kg (ip), o RSV foi capaz de impedir a perda de motilidade espermática e prevenir o estresse oxidativo induzido pelo hipertireoidismo (OURIQUE et al., 2013), comprovando a atividade antioxidante deste composto nos testículos. As propriedades de “scavenger” do RSV foram demonstradas *in vitro* em espermatozoides humanos e em células germinativas de ratos, onde 15 μM de RSV impediu a lipoperoxidação induzida por tert-butil hidroperóxido, preservando a cromatina e a membrana plasmática do espermatozoide (COLLODEL et al., 2011).

Além da sua propriedade antioxidante, que pode ser uma ferramenta para diminuir o estresse oxidativo e consequentemente, o prejuízo reprodutivo, o RSV também apresenta outros efeitos benéficos sob parâmetros reprodutivos. Revel e colaboradores (2001) demonstraram que a injeção subcutânea semanal de 50 mg/kg de RSV é capaz de proteger o DNA do esperma contra o dano e a apoptose causada por contaminantes ambientais em camundongos. O RSV (30 mg/kg) também é capaz de reduzir a apoptose de células germinativas em testículos de ratos após torção testicular experimental (UGURALP et al., 2005). O tratamento com 50 mg/kg de RSV por 28 dias melhorou a ereção peniana em coelhos e aumentou os níveis de testosterona em ratos, acompanhado de aumento na quantidade e motilidade dos espermatozoides (SHIN et al., 2008).

1.8 Vitamina E

Outro composto com reconhecida atividade antioxidante é a vitamina E, um componente dos óleos vegetais encontrada na natureza em quatro formas diferentes: α, β-, γ- e δ-tocoferol, que se diferenciam pelo número e posição dos grupos metila ligados ao anel fenólico, sendo o α-tocoferol a forma antioxidante amplamente distribuída nos tecidos e no plasma (Figura 7). A vitamina E foi descoberta em 1922 e descrita como fator nutricional considerado especialmente importante na reprodução animal (WANG e QUINN, 1999).

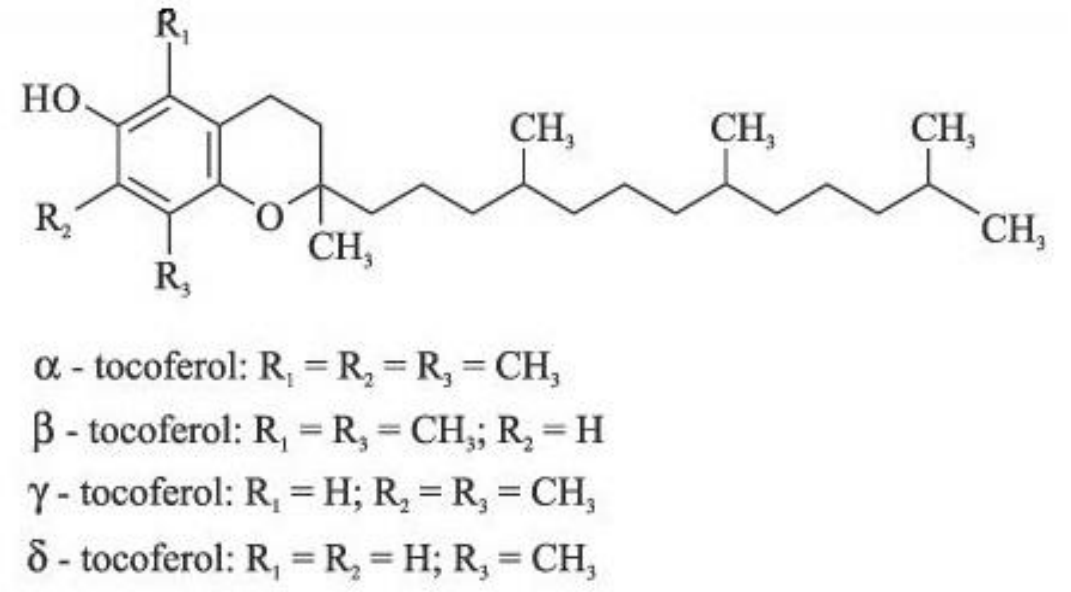

Figura 7. Estrutura química do α, β-, γ- e δ-tocoferol. Fonte: Adaptado de RAMALHO e JORGE, 2006.

A vitamina E é um composto lipossolúvel com papel fundamental na proteção do organismo contra os efeitos prejudiciais das EROs. É um eliminador de radicais peroxila e, provavelmente, o inibidor mais importante das reações em cadeia que vão gerar lipoperoxidação, atuando como “scavenger” de oxigênio singlete, O_2•- e H_2O_2 (HALLIWELL, 1996; HALLIWELL e GUTTERIDGE, 2007). Além disso, a vitamina E tem a capacidade de impedir a propagação das reações em cadeia induzidas pelos radicais livres nas membranas biológicas, impedindo a lipoperoxidação (TRABER e PACKER, 1995).

Os isômeros do tocoferol também contêm uma cadeia lateral fítica, que contribui muito pouco na atividade antioxidante, mas a presença desta cadeia facilita a incorporação e retenção do α-tocoferol nas biomembranas (CHAN E DECKER, 1994). Essa cadeia pode ser saturada, no caso dos tocoferóis, ou insaturada, no caso dos tocotrienóis, perfazendo, então, oito homólogos naturais da vitamina E, que diferem em sua estrutura e atividade biológica. Todas essas moléculas possuem atividade antioxidante, todavia o α-tocoferol tem sido considerado quimicamente e biologicamente mais ativo (SCHNEIDER, 2005).

A vitamina E é um antioxidante lipofílico indispensável para a manutenção da espermatogênese em mamíferos. Está presente em grande quantidade nas células de Sertoli, nos espermatócitos na fase de paquíteno e em menor extensão nas espermátides arredondadas. A deficiência de vitamina E testicular leva a um estado de estresse oxidativo que desregula o processo espermatogênico e a produção de testosterona (AITKEN e ROMAN, 2008). Além disso, a deficiência de vitamina E pode provocar degeneração testicular em várias espécies animais, incluindo os ratos (MARIN-GUZMAN et al., 1997).

Vários pesquisadores têm mostrado que a vitamina E suprime a peroxidação lipídica no testículo, sendo capaz de reverter efeitos adversos decorrentes da exposição testicular a diversos fatores como ozônio, ciclofosfamida, toxinas e contaminantes policlorados bifenilicos (AGUILAR-MAHECHA et al., 2002; MURUGESAN et al., 2005; JEDLINSKA-KRAKOWSKA et al., 2006). De acordo com Aruldhas et al. (2005), a concentração de vitamina E testicular diminui significativamente nos quadros de estresse oxidativo induzido por estímulos pró-oxidantes como o cromo. Além disso, o tratamento com vitamina E é capaz de prevenir danos à fertilidade masculina induzidos por L-tiroxina (SAHOO et al., 2008) e pesticidas (ASTIZ et al., 2013).

Devido às suas propriedades antioxidantes e capacidade de melhorar a função reprodutiva em algumas condições, o RSV e a vitamina E são compostos naturais potencialmente úteis para a prevenção de danos reprodutivos mediados pelo estresse oxidativo induzido pelo VPA.

1.9 Modificação de histonas e espermatogênese

Além do estresse oxidativo, como mencionado anteriormente, o VPA inibe diretamente histona desacetilases (HDACs), e tem sido sugerido que esta interação está relacionada com sua teratogenicidade (PARADIS e HALES, 2013), e poderia estar envolvida com a toxicidade do VPA às células germinativas.

A regulação da expressão gênica celular depende de mecanismos genéticos e epigenéticos; enquanto que o primeiro se baseia unicamente na sequência de DNA, o último é uma rede muito complexa de modificações reversíveis em proteínas e no DNA

que modulam a acessibilidade ao genoma e sua expressão. Mecanismos epigenéticos incluem metilação do DNA e modificação de histonas, especialmente a metilação e acetilação das histonas (STEIN et al., 2010). A acetilação de histonas é uma modificação pós-traducional reversível, que altera a compactação da cromatina e leva a alterações na expressão de genes. A adição de um grupo acetil (acetilação) neutraliza as cargas positivas sobre as histonas, enfraquecendo a interação eletrostática entre histonas e o esqueleto de fosfato do DNA, causando uma maior descompactação da cromatina, que fica mais exposta a fatores de transcrição facilitando a expressão proteica. Por outro lado, a remoção do grupo acetil (desacetilação) leva a um aumento das interações entre histonas e o DNA gerando uma maior compactação da cromatina, o que limita a atividade gênica dificultando a ligação de fatores de transcrição e a expressão proteica (BANNISTER e KOUZARIDES, 2011).

Histona acetiltransferases (HATs) são as enzimas responsáveis pela adição de grupos acetilas nas caudas das histonas, enquanto que HDACs estão envolvidos na sua remoção (DAVIE e SPENCER, 1999). Vários inibidores de HDACs foram identificados ou desenvolvidos sinteticamente (XU et al., 2007). O VPA é um inibidor de HDAC das classes I e II. A inibição de HDACs pelo VPA pode induzir uma hiperacetilação de histonas, levando à atenuação da interação eletrostática entre as histonas e o DNA, o que pode ser associado a uma maior descondensação da cromatina (WANG et al., 2013a) (Figura 8).

Durante a fase haplóide do desenvolvimento das células germinativas, denominado espermiogênese, as espermátides sofrem uma série de transformações morfológicas que levam à formação de espermatozoides altamente especializados. Uma das mudanças mais importantes que ocorrem é a remodelação da cromatina nuclear em uma estrutura altamente condensada. A compactação da cromatina é um fenômeno evolutivo que reduz o tamanho do núcleo do espermatozoide, a fim de proteger o genoma paterno quando os espermatozoides estão no trato reprodutor feminino (MARTINS e KRAWETZ, 2007), sendo essencial para a função espermática e a subsequente embriogênese. Defeitos na cromatina do espermatozoide têm sido associados à infertilidade, falhas no desenvolvimento embrionário, abortos espontâneos

e transmissão de características genéticas indesejáveis para as próximas gerações (CEBESOY et al., 2006; BUNGUM et al., 2007).

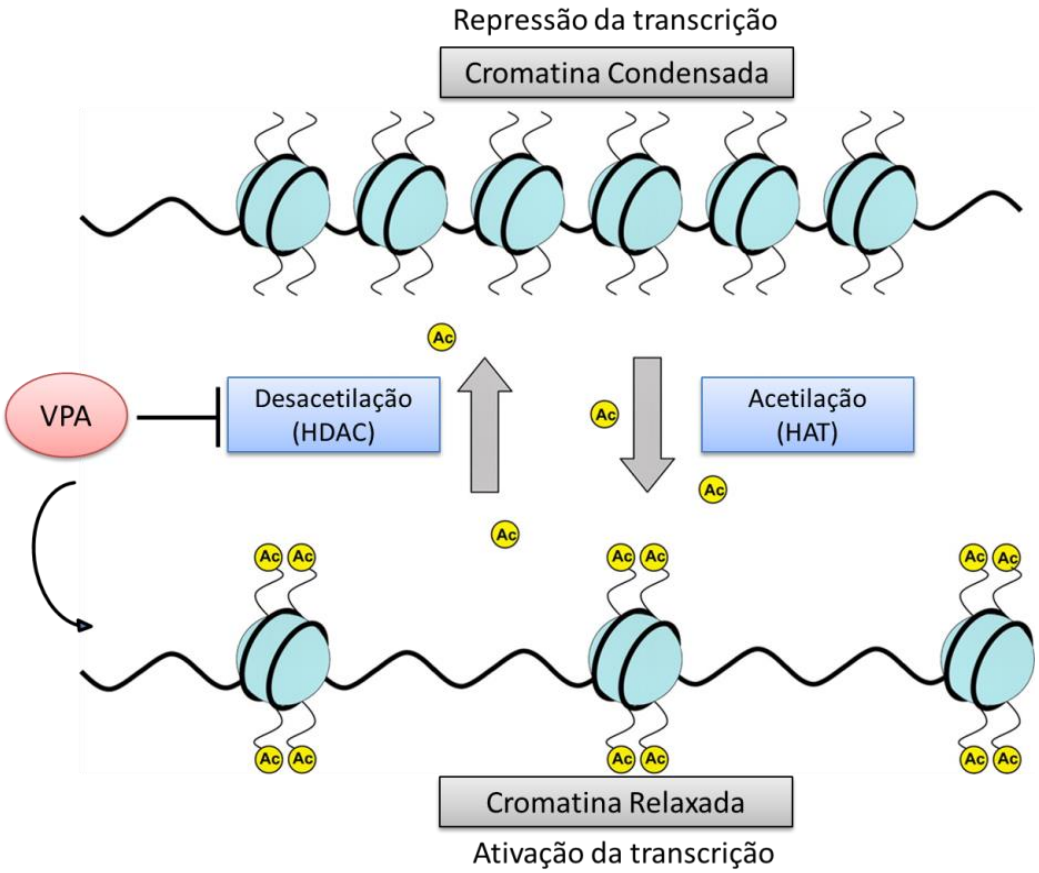

Figura 8. Representação esquemática da acetilação de histonas e possível efeito do VPA. Ac (acetila); HAT (histona acetiltransferases); HDAC (histona desacetilases); VPA (ácido valpróico). Fonte: Adaptado de PONS et al., 2009.

A regulação epigenética é fundamental para o processo de remodelação da cromatina. Em células germinativas pré-meióticas, modificações covalentes das histonas servem para regular a estabilidade dos nucleossomas e controlar a transcrição de genes (KOTA e FEIL, 2010). Em células pós-meióticas, estas modificações servem para a substituição de histonas por protaminas, que são responsáveis por manter a cromatina compactada no espermatozoide (GOVIN et al., 2004). Há evidências do envolvimento do código de histonas na remodelação da cromatina, com modificações estágio-específicas das histonas durante a espermiogênese, que incluem acetilação de histonas H4, metilação e fosforilação de histonas H3, e ubiquitinação da histona H2A (SONG et al., 2011; SHENG et al., 2014; SHIRAKATA et al., 2014). A acetilação das

histonas em resíduos de lisina é de particular interesse, uma vez que orienta a remoção da histona por neutralizar a carga positiva de histonas. Além disso, a acetilação da histona H4 serve como sinalização para o recrutamento da proteína bromodomínio testicular (BRDT), que direciona a remoção das histonas e substituição por protaminas (PIVOT-PAJOT et al., 2003).

O período anterior à compactação da cromatina representa uma janela de vulnerabilidade, em que exposições ambientais podem reprogramar marcadores epigenéticos (Figura 9). Além disso, acredita-se que defeitos nestes estágios de remodelação da cromatina podem afetar a fertilidade em humanos e em modelos animais (BALE, 2015).

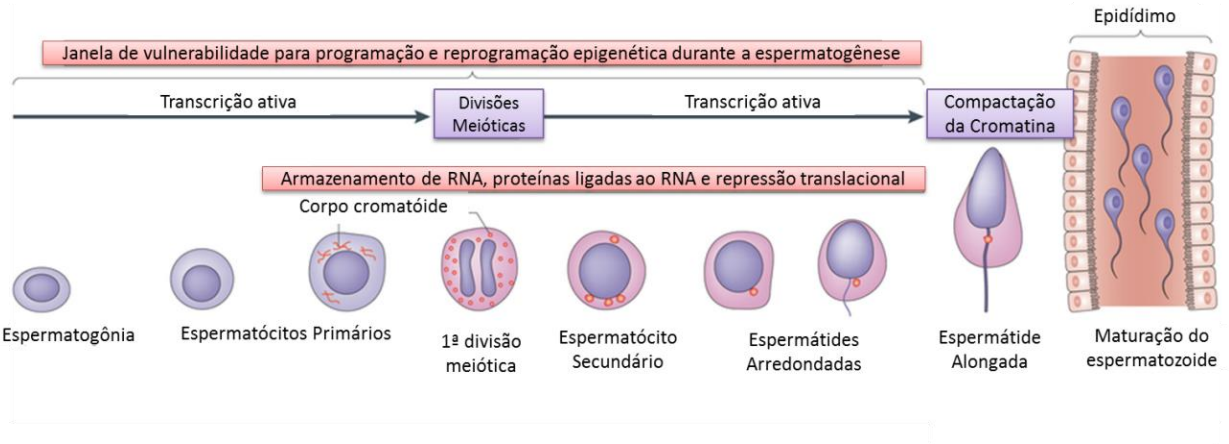

Figura 9. Janela de vulnerabilidade para programação e reprogramação epigenética durante a espermatogênese. Fonte: Adaptada de BALE, 2015

Desta maneira, o VPA, além de causar estresse oxidativo, poderia levar a uma hiperacetilação de histonas nas células espermatogênicas, desestabilizando a compactação da cromatina e afetando a competência funcional do espermatozoide. Adicionalmente, a hiperacetilação de histonas induzida pelo VPA originaria uma cromatina menos condensada, o que poderia aumentar a susceptibilidade ao ataque por EROs, causando dano ao DNA.

Sendo assim, neste trabalho foram investigados os efeitos do RSV e da vitamina E sobre a qualidade dos espermatozoides e sobre parâmetros de estresse oxidativo em testículo e epidídimo de ratos tratados com VPA, bem como o efeito do VPA no estresse oxidativo, na acetilação de histonas e no dano ao DNA em espermatócitos e espermátides isolados.

2. OBJETIVOS

2.1 Objetivo Geral

Investigar o efeito do RSV e da vitamina E frente à toxicidade do VPA na função reprodutiva de ratos machos, bem como o efeito do VPA em células espermatogênicas isoladas.

2.2 Objetivos Específicos

- Verificar o efeito do RSV e da vitamina E no peso do testículo, epidídimo, próstata e vesícula seminal de ratos adultos tratados com VPA;

- Verificar o efeito do RSV e da vitamina E nos níveis plasmáticos de testosterona em ratos adultos tratados com VPA;

- Avaliar o efeito do VPA e da vitamina E na morfologia testicular;

- Avaliar o efeito do VPA e da vitamina E nos níveis plasmáticos de aspartato aminotransferase e alanina aminotransferase;

- Verificar o efeito do RSV e da vitamina E na quantidade e qualidade dos espermatozoides em ratos machos adultos tratados com VPA;

- Verificar o efeito do RSV e da vitamina E nos parâmetros de estresse oxidativo em testículo e epidídimo de ratos adultos tratados com VPA;

- Avaliar a efeito do VPA na produção de EROs, no conteúdo de GSH, na acetilação de histonas e no dano ao DNA em espermatócitos e espermátides isolados.

3. RESULTADOS

3.1 Artigo 1

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Resveratrol prevents oxidative damage and loss of sperm motility induced by long-term treatment with valproic acid in Wistar rats

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ABSTRACT

Valproic acid (VPA) is a drug widely use for the treatment of epilepsy in both children and adults. Evidence suggests that long-term use of VPA may lead to an impairment in the male reproductive function. Oxidative stress is considered to play a major role in VPA associated toxicity. In the present work, we demonstrated that the natural antioxidant compound resveratrol (RSV) can be use to prevent VPA oxidative damage. Wistar rats treated with VPA (400 mg kg^{-1}) by gavage for 28 days showed decrease in sperm motility accompanied by increase in oxidative damage to lipids and proteins. Additionally, VPA administration leaded to depletion of reduced glutathione and decrease in total antioxidant potential in testes and epididymides of Wistar rats. The co-administration of RSV (10 mg kg^{-1}) efficiently prevented VPA pro-oxidant effects. In summary, RSV was shown to protect the reproductive system from the damage induced by VPA. Altogether, our data strongly suggests that RSV administration might be a valuable strategy to minimize reproductive impairment in patients requiring long-term VPA treatment.

1. Introduction

Valproic acid (2-propyl-pentanoic acid, VPA) is an antiepileptic drug used to treat a wide range of epileptic conditions in children and adults (Garcia-Morales et al., 2007; Perucca, 2002). VPA is also utilized for the treatment of other diseases such as migraine (Krymchantowski et al., 2002) and bipolar psychiatric disorders (Nasrallah et al., 2006). Besides these applications, VPA has also been shown to be efficient to treat advanced cancer patients, when combined with other chemotherapeutic regimens (Cinatl et al., 2002; Kortenhorst et al., 2009; Wang et al., 2013). The use of VPA for the treatment of different pathologies revealed that toxic effects are associated with long-term use of this drug as, for example, hepatotoxicity (Chang and Abbott, 2006; Kiang et al., 2010; Tong et al., 2005), neurotoxicity (Chaudhary and Parvez, 2012) and impaired fertility (Bairy et al., 2010; Cohn et al., 1982; Khan et al., 2011; Krogenaes et al., 2008; Nishimura et al., 2000; Rättyä et al., 2001; Røste et al., 2002, 2005; Vijay et al., 2008).

Animal (Bairy et al., 2010; Cohn et al., 1982; Khan et al., 2011; Krogenaes et al., 2008; Nishimura et al., 2000; Røste et al., 2002; Vijay et al., 2008) and clinical (Chen et al., 1992; Rättyä et al., 2001; Røste et al., 2005) studies demonstrated adverse effects of the long term use of VPA on male reproductive function. The exact mechanism of VPA toxicity to the male reproductive system is not well defined, but formation of reactive oxygen species (ROS) and the development of oxidative stress have been proposed to explain some of the adverse effects of VPA, including hepatotoxicity (Chang and Abbott, 2006; Kiang et al., 2010; Tong et al., 2005), neurotoxicity (Chaudhary and Parvez, 2012), and teratogenicity (Tung and Winn, 2011). Furthermore, Khan et al. (2011) demonstrated recently that treatment with VPA is toxic to germ cells. The authors have shown that VPA causes depletion of reduced glutathione (GSH) and increases damage to sperm DNA, which demonstrates thatROS generation might be a key mechanism responsible for the toxicity of VPA in the reproductive system (Khan et al., 2011)

Under physiological conditions, ROS are formed during the metabolism of oxygen, and their concentrations are controlled by

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antioxidant defenses. Several enzymatic systems, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione *S*-transferase (GST), protect the cells against ROS formation. These antioxidant defenses also include non-enzymatic systems as, for example, GSH (Halliwell and Gutteridge, 1999). Oxidative stress occurs when there is an imbalance between production and removal of ROS (Evelson et al., 2001).

Increased oxidative stress is reported as one of the major causes of male infertility. Spermatozoa and testes, being rich in polyunsaturated fatty acids, show a great susceptibility to attack by ROS and oxidative damage (Turner and Lysiak, 2008). Whereas the generation of low levels of ROS is an important component of the signal transduction stimulating capacity of spermatozoa (Ford, 2004), excessive ROS levels induce lipid peroxidation of sperm cell membrane, malfunction of capacitation, impaired acrosome reactions, and loss of motility (Aitken et al., 1985, 2014).

Resveratrol (3,4′,5-triiidroxiestilbene, RSV) is a natural polyphenolic compound found primarily in grapes and wine, presenting a considerable number of beneficial effects in a variety of organs and systems, which are mainly due to its antioxidant activity (Frémont, 2000). Protective effects of RSV against oxidative damage *in vivo* and *in vitro* are likely due to regulation of endogenous antioxidant cellular systems, in addition to acting as an antioxidant scavenger of ROS (Spanier et al., 2009). RSV also inhibits formation of ROS suppressing pro-oxidant genes (Baur and Sinclair, 2006; Dolinsky et al., 2009; Spanier et al., 2009) and inducing antioxidant enzymes including SOD, CAT, and GPx (Spanier et al., 2009; Tanno et al., 2010; Ungvari et al., 2007). Belguendouz et al. (1997) also reported that RSV chelates copper and other transition metals, which are able to generate free radicals and cause lipid peroxidation. Moreover, RSV has been described to prevent damage to male fertility triggered by different oxidative stress inducers like ethanol (Kasdallah-Grissa et al., 2006), *tert*-butil hydroperoxide (Collodel et al., 2011), triiodotironine (Ourique et al., 2013), and iron/ascorbate (Mojica-Villegas et al., 2014). Altogether, these data suggests that RSV may be used as an alternative to minimize reproductive impairment caused by continuous use of VPA.

In the present study, we assess the toxic effect of VPA on male reproductive system, where it caused decrease in sperm motility and vigor. These alterations were associated with increase in lipid peroxidation and oxidative damage to proteins in both testes and epididymides of Wistar rats. The co-administration of RSV was efficient to protect against VPA-mediated toxicity in testes and epididymides. Therefore, we demonstrated that RSV has potential to efficiently improve male fertility in VPA treated individuals.

2. Material and methods

2.1. Chemicals

Valproic acid (Valpakine syrup at a concentration of 200 mg mL^{-1}) was obtained from Sanofi Laboratories (São Paulo, SP, Brazil). RSV was purchased from Pharma Nostra (Chengdu Hawk Bio-Engineering, China), and its purity and structure were previously confirmed using chromatography and nuclear magnetic resonance, respectively. All other reagent-grade chemicals were obtained from Sigma (St Louis, Missouri, USA).

2.2. Animals

All animal procedures were approved by the Animal Ethics Committee of the Federal University of Santa Maria (process 076/2013). Adult male Wistar rats (90 days) were obtained from the Central Animal Breeding Facility of the Federal University of Santa Maria, RS, Brazil. The animals were kept in polypropylene cages with controlled temperature (23 ± 2 °C) and a light-dark cycle of 12 h with access to water ad libitum and to approximately 30 g daily per animal of rodent laboratory chow (Supra, São Leopoldo, RS, Brazil). Animals were acclimated to the experimental conditions for a period of two weeks prior to the commencement of the experiment.

2.3. Experimental protocol

Rats (n = 32) were randomly divided in four experimental groups (n = 8 each group) as it follows: (C) control; (RSV) treated with 10 mg kg^{-1} of RSV; (VPA) treated with 400 mg kg^{-1} of VPA; and (VPA + RSV) treated with 400 mg kg^{-1} of VPA and 10 mg kg^{-1} of RSV. VPA was administered daily by gavage at a dose of 400 mg kg^{-1} body weight for 28 days and control groups (C and RSV) received an equal volume of vehicle solution. The dose of 400 mg kg^{-1} (approximately equivalent to the maximum human daily dose on a mg/m^2 basis) of VPA was selected based on the results obtained from different studies carried out for reproductive toxicity in rodents (Khan et al., 2011; Røste et al., 2002; Vijay et al., 2008). Concomitantly with VPA treatment, animals received daily intraperitoneal (i.p.) injections of RSV, freshly prepared in 1% tween 80, at a dose of 10 mg kg^{-1} body weight for 28 days, as used in our previous study (Ourique et al., 2013). Groups C and VPA received i.p. injections of 1% tween 80 in the same conditions.

At the end of the experimental period (four weeks) and 24 h after the last administration, animals were weighed and anesthetised with xylazine and ketamine. Blood was collected through cardiac puncture, and rats were euthanized for removal of their epididymides, testis, prostate, and seminal vesicle which were immediately weighed.

2.4. Assessment of testosterone in serum

Blood was collected in tubes and it was separated using centrifugation (1800*g*, 15 min). Serum was stored at −20 °C for further analysis. Testosterone levels were measured using a competitive electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN). Analyses were performed by the Pasin Laboratory (Santa Maria, RS, Brazil) and expressed as ng dL^{-1}.

2.5. Removal of epididymides and retrieval of spermatozoa

Epididymides were excised and adherent and fat tissues were removed. Cauda epididymidis was collected, transferred to a Petri dishes and immersed in sterile silicone oil, nearby a drop of 200 μL of Fert's medium (114 mM NaCl, 3.22 mM KCl, 0.34 mM NaH_2PO_4, 25 mM $NaHCO_3$, 16 mM $C_3H_5O_3Na$, 0.6% BSA, 0.1% phenol red, 2 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgCl_2{\cdot}6H_2O$, and Milli-Q water). All reagents were adequately preheated. Longitudinal incisions were made in the cauda epididymidis with a fine needle and a scalpel blade to release the spermatozoa. The sperm motility and vigor were evaluated by placing a 4 μL drop of Fert's medium containing the spermatozoa on a slide and then examining the drop using a light microscope (Olympus CX40) at 100×. The percentage of total and progressive motility was estimated from three different fields in each sample, and the mean was used as the final value of motility. Vigor of movement was also estimated, using the following scale: 0, no movement; 1, slight side-to-side movement, no forward progression; 2, rapid side-to-side movement, no forward progression; 3, rapid side-to-side movement, occasional forward progression in spurts; 4, steady, slow forward progression; 5, rapid, steady forward movement (Platz and Seager, 1978).

Epididymal sperm count was determined using Neubauer's hemocytometer. Sperm concentration was expressed as number of sperm per mL of solution containing sperm. To analyse the

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morphology, the sperm were kept in a formaldehyde-citrate solution, and 200 spermatozoa were analyzed from each animal. The analyses were performed using a Leica DMI 4000B inverted microscope with differential interference contrast. Morphological abnormalities of the head (small, amorphous, pinhead or isolated form, i.e., no tail attached), midpiece (without characteristic curvature or broken) and tail (doubled, broken or rolled into a spiral) were examined, and the percentage of normal or abnormal sperm was determined.

2.6. Tissue homogenate preparation

Testes and epididymides were homogenized in 0.1 M phosphate buffer (pH 7.4) using a glass-Teflon grinder. The homogenates were centrifuged at 100*g* for 10 min at 4 °C to discard the nuclei and cell debris, and the resulting supernatant fractions were frozen at −80 °C for further measurements. Protein concentration in the supernatant was determined using the method of Lowry et al. (1951) with bovine albumin as standard.

2.7. Oxidative damage measurements

Lipid peroxidation, as indicated by the amount of lipid hydroperoxides (LOOH), was measured by the xylenol orange method. This technique can detect the primary products of peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. The amount of dye was measured in a spectrophotometer at 560 nm, and results were reported as nmol mg protein^{-1} (Jiang et al., 1991). Lipid peroxidation was also estimated based on the formation of thiobarbituric acid reactive substances (TBARS) in a reaction medium containing 20% TCA and 0.67% TBA. The absorbance was measured at 535 nm and the results were expressed as nmol MDA mg protein^{-1} (Buege and Aust, 1978).

Protein carbonyl content was measured by a method based on the reaction of carbonyls with 2,4-dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound. The standard curve was prepared by using different bovine serum albumin concentrations (0.5–1.5 mg mL^{-1}) and the slope was used to express the levels of carbonyl protein as nmol mg protein^{-1} (Reznick and Packer, 1994).

2.8. Determination of enzymatic and non-enzymatic antioxidants

Total superoxide dismutase (SOD) activity was determined based on the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine/NaOH (pH 10.2). One SOD unit was defined as the amount of enzyme required for 50% inhibition of the adrenochrome formation and the enzyme activity was expressed as USOD mg protein^{-1} (Fridovich, 1974).

Catalase (CAT) activity was evaluated by measuring the decrease in the absorption at 240 nm in a reaction medium consisting of 50 mM phosphate buffer (pH 7.4) and 2 mM hydrogen peroxide (H_2O_2). Results were reported as pmol mg protein^{-1} (Chance et al., 1979).

Glutathione *S*-transferase (GST) activity was assayed based on the conjugation reaction with GSH, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The GST activity was calculated from the changes in absorbance at 340 nm. One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1 μmol of CDNB with GSH per minute at 25 °C, expressed as pmol min^{-1} mg protein^{-1} (Habig et al., 1974).

Non-protein thiols content, an indirect measure of reduced glutathione (GSH), was evaluated after reacting with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The DTNB formed a yellow complex with GSH, and the absorbance was measured at 412 nm. Proteins were eliminated through the addition of 0.5 M perchloric acid and the amount of GSH was expressed as nmol mg protein^{-1} (Ellman, 1959).

2.9. Evaluation of total reactive antioxidant potential (TRAP)

TRAP was measured based on the capacity of the sample to scavenge luminol luminescence induced by thermolysis of 2,2′-azo-bis (2-amidinopropane) dihydrochloride as a source of free radicals. The standard curve was prepared by using different Trolox concentrations (1–4 μmol L^{-1}). The comparison of the induction time after the addition of Trolox and the sample allowed calculation of TRAP as the equivalent of the Trolox concentration necessary to produce the same induction time, expressed as μmol mg protein^{-1} (Evelson et al., 2001).

2.10. Statistical analysis

The results were analyzed using two-way analysis of variance (ANOVA) test followed by Duncan's test. Arcosen was extracted from the data referent to the analysis of spermatozoa before ANOVA test. The level of significance was set at $P < 0.05$. All the analyses were performed using Statistica Software (Stat-Soft, Inc.), version 7.0 and GraphPad Prism (GraphPad Software, Inc.), version 6.01. Results are presented as mean ± standard error of the mean (SEM). Pearson's correlation analysis was performed in order to observe the correlation between sperm motility and different parameters.

3. Results

3.1. Body weight, weight of the reproductive organs, and plasmatic testosterone levels

No statistical significant change was observed in body, testes, epididymides, prostate and seminal vesicle weight among experimental groups. Plasmatic testosterone levels also remained unchanged across the different treatments (Table 1).

3.2. Sperm count, motility and morphology

Sperm concentration remained unchanged across the different treatments (Fig. 1A). VPA reduced significantly sperm motility ($F(1,28) = 13.67$, $P < 0.001$) and sperm vigor ($F(1,28) = 8.34$, $P < 0.05$) compared to control. RSV had no direct effect on sperm motility and vigor, but avoided the effect of VPA on sperm motility ($P < 0.05$), and a significant interaction between VPA and RSV ($F(1,28) = 8.57$, $P < 0.05$) was observed (Fig. 1B and C). The percentage of total morphological abnormalities (Fig. 1D) and defects of head, midpiece and tail did not differ among experimental groups (data not shown).

3.3. Oxidative damage in testes

VPA increased significantly testicular lipid peroxidation, measured by LOOH ($F = (1,28)19.96$, $P < 0.001$) and TBARS ($F(1,28) = 6.32$, $P < 0.05$) compared to control. RSV had no direct effect on TBARS and LOOH levels, but was able to reverse this increase in testicular lipid peroxidation, since VPA + RSV group had lower levels of LOOH ($P < 0.0001$) and TBARS ($P < 0.05$) than VPA group (Fig. 2A and B). A significant interaction was observed between VPA and RSV on LOOH levels ($F(1,28) = 24.92$; $P < 0.0001$). Treatment with VPA caused a significant induction on protein oxidative damage in testes, since levels of carbonyl protein were higher in VPA group compared to control ($P < 0.05$). RSV had no

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Table 1
Effects of VPA and RSV on body weight, on weight of reproductive organs, and on plasmatic testosterone levels.

Parameter	Group			
	Control	RSV	VPA	VPA + RSV
Initial body weight (g)	269.89 ± 4.95	272.75 ± 5.18	269.89 ± 5.62	265.50 ± 4.26
Final body weight (g)	359.33 ± 8.12	349.75 ± 5.10	357.44 ± 8.39	346.50 ± 9.31
Weight of the testes (g)	1.814 ± 0.023	1.840 ± 0.051	1.789 ± 0.030	1.733 ± 0.039
Relative weight of the testes (g 100 g of body wt^{-1})	0.507 ± 0.012	0.527 ± 0.016	0.502 ± 0.012	0.501 ± 0.016
Weight of the epididymides (g)	0.740 ± 0.030	0.759 ± 0.033	0.719 ± 0.022	0.689 ± 0.026
Relative weight of epididymides (g 100 g of body wt^{-1})	0.207 ± 0.009	0.217 ± 0.008	0.202 ± 0.008	0.199 ± 0.007
Weight of the prostate (g)	0.474 ± 0.030	0.459 ± 0.056	0.370 ± 0.037	0.387 ± 0.042
Relative weight of the prostate (g 100 g of body wt^{-1})	0.132 ± 0.009	0.131 ± 0.014	0.115 ± 0.010	0.112 ± 0.012
Weight of the seminal vesicle (g)	0.798 ± 0.052	0.767 ± 0.073	0.932 ± 0.048	0.779 ± 0.055
Relative weight of the seminal vesicle (g 100 g of body wt^{-1})	0.223 ± 0.016	0.219 ± 0.019	0.260 ± 0.012	0.224 ± 0.014
Plasmatic testosterone (ng dL^{-1})	165.67 ± 44.75	141.20 ± 47.30	144.63 ± 27.19	180.23 ± 20.81

Values are expressed as mean ± SEM. There were no significant difference between treatments ($P > 0.05$).

effect itself in this parameter, but prevented the induction of protein damage induced by VPA, since VPA + RSV group presented levels of carbonyl protein lower than VPA group ($P < 0.05$) (Fig. 2C).

3.4. Antioxidant defense in testes

The activity of enzymatic antioxidants SOD, CAT and GST did not differ among the experimental groups (Fig. 2D–F). On the other hand, VPA reduced significantly GSH levels ($F(1,28) = 16.38$, $P < 0.001$) in testes compared to control. RSV had no direct effect on GSH levels and was not able to prevent the effect of VPA (Fig. 2G). TRAP was significantly lower in VPA group compared to the control ($F(1,28) = 14.09$, $P < 0.001$). RSV group did not differ from control, but VPA + RSV had higher TRAP levels than VPA group ($F(1,28) = 10.75$, $P < 0.05$), showing that RSV was able to prevent the decrease in antioxidant potential induced by VPA in testes (Fig. 2H).

3.5. Oxidative damage in epididymides

Treatment with VPA increased significantly LOOH ($F(1,28) = 7.64$, $P < 0.05$), TBARS ($F(1,28) = 8.70$, $P < 0.05$) and carbonyl protein ($F(1,28) = 4.21$, $P < 0.05$) levels in epididymides compared to control. RSV group presented levels of LOOH, TBARS and carbonyl protein similar to control group, but avoided the increase on these parameters induced by VPA ($P < 0.001$, $P < 0.001$ and $P < 0.05$, respectively). ANOVA revealed a significant interaction between treatment with VPA and RSV on TBARS ($F(1,28) = 12.57$; $P < 0.05$) and LOOH ($F(1,28) = 8.61$; $P < 0.05$) levels. (Fig. 3A–C).

3.6. Antioxidant defense in epididymides

The enzymatic antioxidants SOD, CAT and GST presented similar activity in epididymides of different groups (Fig. 3D–F). The same occurred in relation to the level of GSH, which was similar in

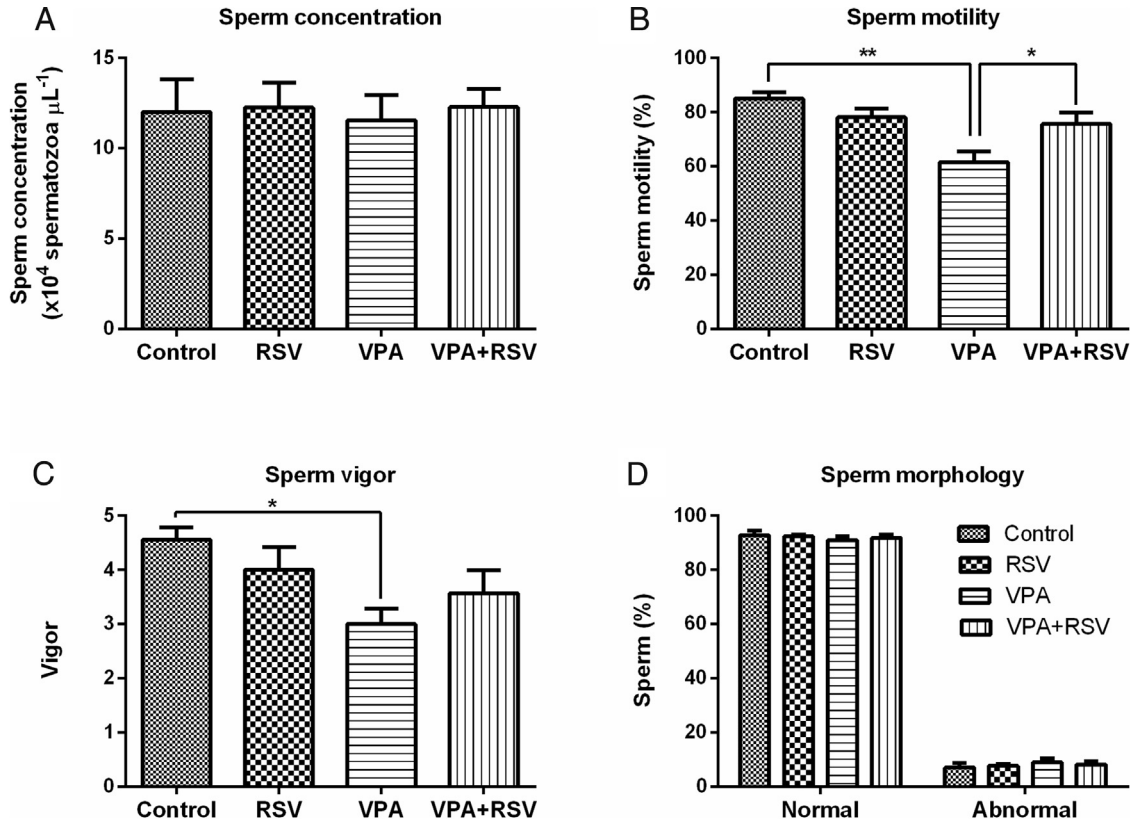

Fig. 1. Effect of VPA and RSV on sperm count, motility, vigor, and morphology. Values are expressed as mean ± SEM (n = 8). Data were analyzed by two-way ANOVA followed by Duncan's multiple comparison test. *Significant difference of indicated groups at $^{*}p < 0.05$; $^{**}p < 0.001$.

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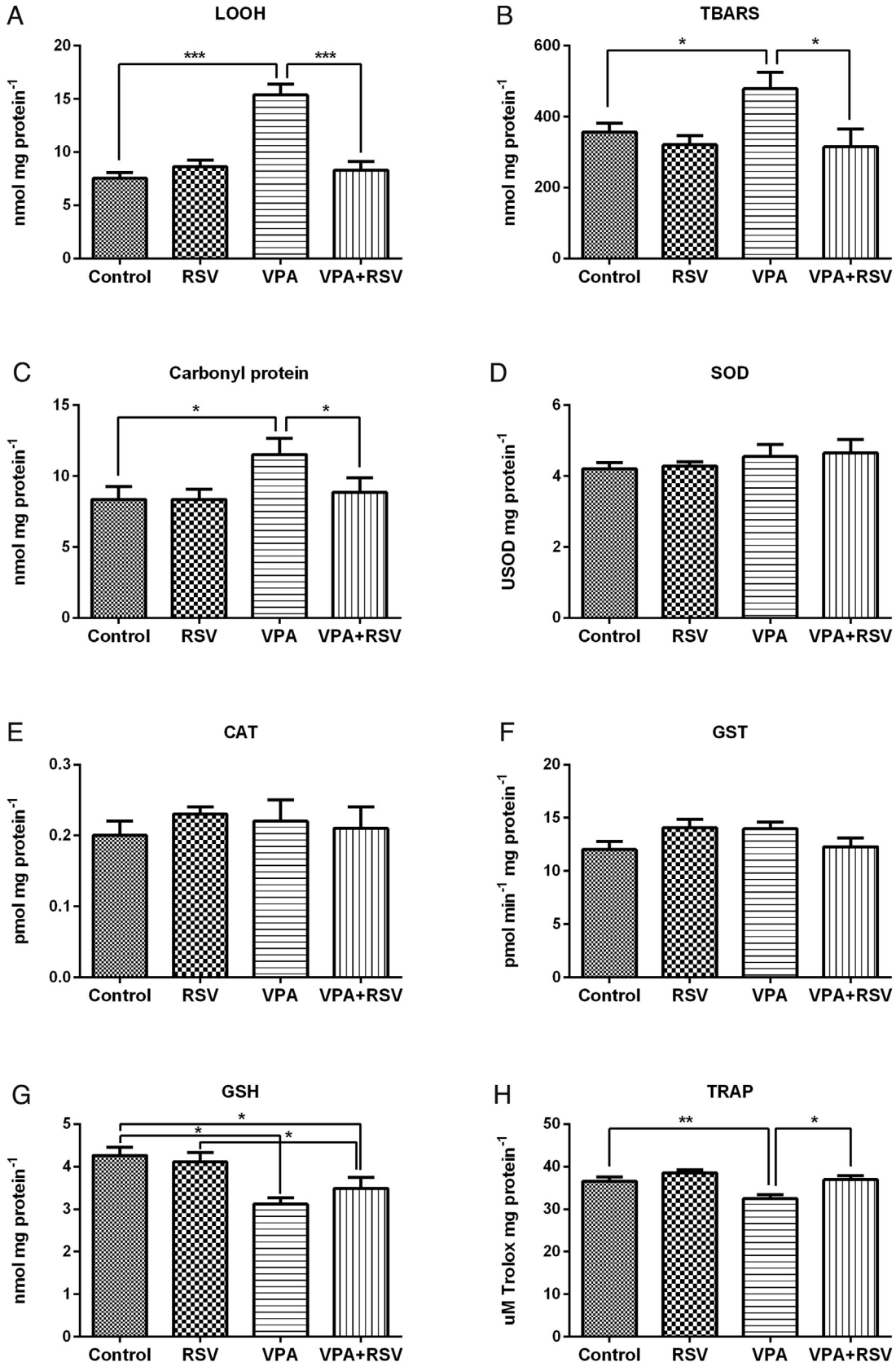

Fig. 2. Effect of VPA and RSV on biomarkers of oxidative stress in **testes** of rats.
TBARS (thiobarbituric acid reactive substances), LOOH (lipid hydroperoxides), SOD (superoxide dismutase), CAT (catalase), GST (glutathione-*S*-transferase), GSH (reduced glutathione), TRAP (total reactive antioxidant potential). Values are expressed as mean ± SEM (n = 8). Data were analyzed by two-way ANOVA followed by Duncan's multiple comparison test. *Significant difference of indicated groups at $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

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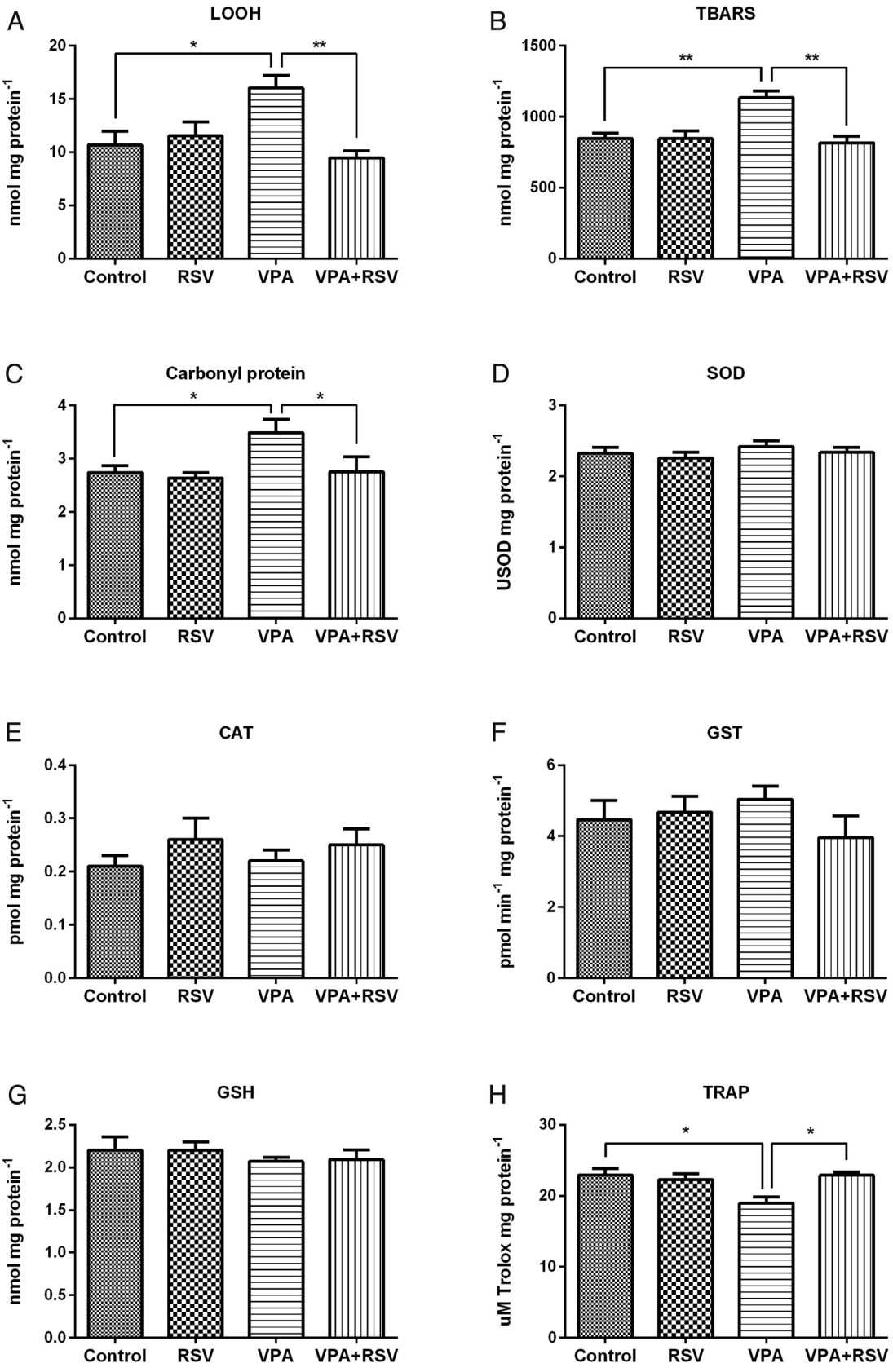

Fig. 3. Effect of VPA and RSV on biomarkers of oxidative stress in **epididymides** of rats.
TBARS (thiobarbituric acid reactive substances), LOOH (lipid hydroperoxides), SOD (superoxide dismutase), CAT (catalase), GST (glutathione-*S*-transferase), GSH (reduced glutathione), TRAP (total reactive antioxidant potential). Values are expressed as mean ± SEM (n = 8). Data were analyzed by two-way ANOVA followed by Duncan's multiple comparison test. *Significant difference of indicated groups at $^{*}p < 0.05$; $^{**}p < 0.001$.

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all experimental groups (Fig. 3G). Anova showed that VPA and RSV had no single effect on antioxidant potential in epididymides. The TRAP was significantly lower in VPA group compared to control ($P < 0.05$). RSV group presented TRAP similar to the control, but VPA + RSV group showed higher TRAP levels than VPA group ($P < 0.05$) (Fig. 3H). There was an interaction between VPA and RSV on TRAP levels ($F(1,28) = 6.78$, $P < 0.05$) in epididymides.

3.7. Correlation between sperm motility and oxidative damage

Pearson's correlation analysis showed a negative correlation between sperm motility and TBARS ($R^2 = 0.1460$, $P = 0.0309$) and LOOH ($R^2 = 0.2296$, $P = 0.0055$) in testes. A negative correlation was also observed between sperm motility and TBARS ($R^2 = 0.2600$, $P = 0.0029$) and LOOH in epididymides ($R^2 = 0.2856$, $P = 0.0016$) (Fig. 4).

4. Discussion

RSV was able to prevent the impairment of sperm motility, decreased the oxidative damage, and increased antioxidant potential in testes and epididymides of rats subject to long-term treatment with VPA.

Although been considered safe, several clinical (Chen et al., 1992; Rättyä et al., 2001; Røste et al., 2005) and experimental animal studies (Bairy et al., 2010; Cohn et al., 1982; Khan et al., 2011; Krogenaes et al., 2008; Nishimura et al., 2000; Røste et al., 2002; Vijay et al., 2008) have demonstrated that long-term VPA treatment can affect male fertility. More recently, it was described that the adverse effect of VPA on male reproductive system is associated with oxidative stress (Khan et al., 2011), which is clearly one of the major causes of defective sperm function and male infertility (Aitken et al., 1985, 2014; Aitken and Roman, 2008; Kao et al., 2008). In this regard, we used the antioxidant RSV as an alternative to minimize reproductive impairment caused by VPA.

Some studies report that high doses of VPA may cause atrophy of sexual organs and endocrine alterations in rats (Nishimura et al., 2000; Røste et al., 2002) and goats (Krogenaes et al., 2008). In our study, there was no significant difference on the weight of the reproductive organs as well as testosterone levels among the experimental groups. These observations support the idea that VPA effects on male fertility and the protection promoted by RSV are not testosterone-related. In fact, we observed a trend on reducing testes, epididymides and prostate weight, and testosterone levels following VPA treatment, however, such differences were not statistically significant. Røste et al. (2002) reported that rats treated for three months with high doses of VPA (800 mg kg^{-1}) had a decrease in testicular weight and LH, FSH and testosterone levels. In the same study, no changes were observed in rats treated with low VPA doses (400 mg kg^{-1}) for a similar period. Similarly, in a study by Nishimura et al. (2000), no effects were observed in a group of rats receiving VPA at doses of 250 mg kg^{-1} for 10 weeks, but, at doses of 500 mg kg^{-1}, the weight of testes and accessory glands were decreased. Thus, difference on VPA doses and duration of the treatment in our experimental design (400 mg kg^{-1} for 28 days) can explain the fact that the reproductive organ weights and testosterone levels remain the same in our VPA treated group.

Sperm motility is one of the most important parameters used in evaluation of sperm quality. Spermatozoa acquire movement

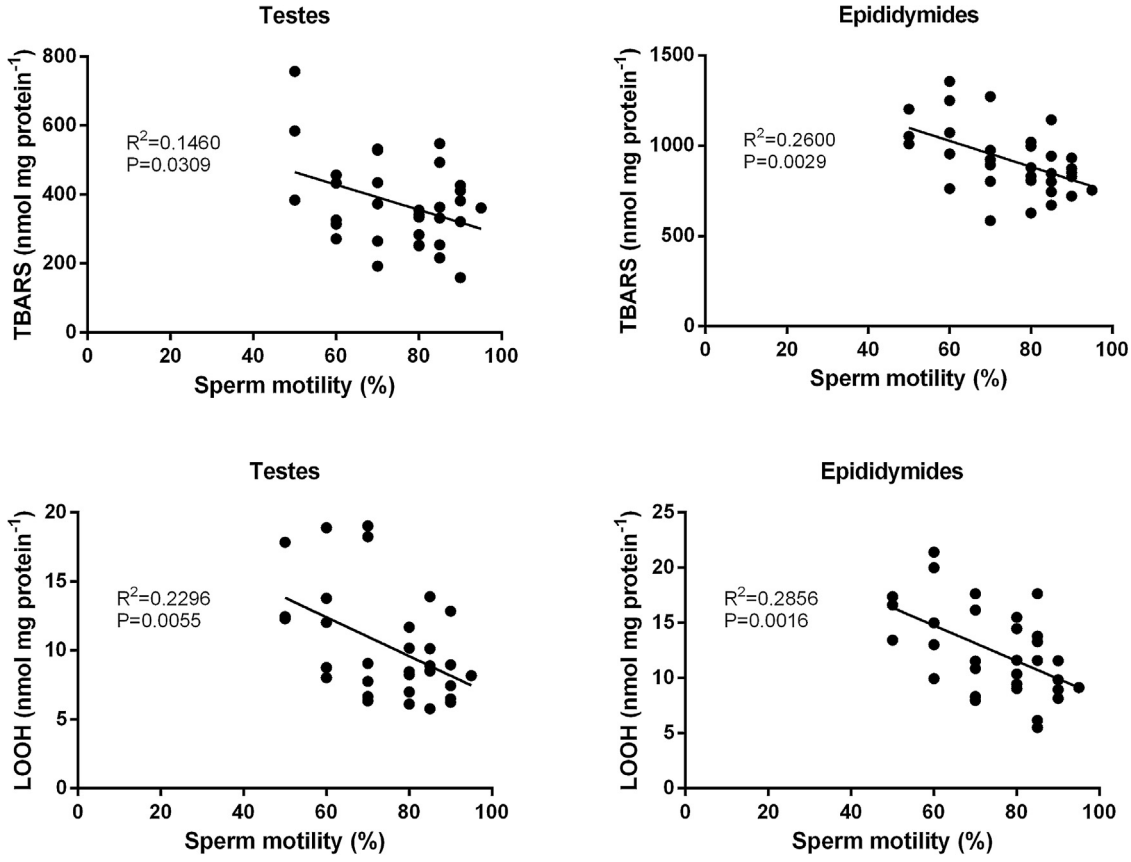

Fig. 4. Pearson's correlation analysis showing the correlation between sperm motility and oxidative damage in testes and epididymides of rats (n = 32). TBARS (thiobarbituric acid reactive substances), LOOH (lipid hydroperoxides).

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during its transit through epididymal duct (Gatti et al., 2004). Alterations in motility capacity might lead to an inefficient sperm penetration of cervix mucus and impair its ability to reach the oocyte (Aitken et al., 1985). Our results demonstrate that VPA treatment decreased sperm motility and vigor, while no significant difference was observed in concentration and morphology of the sperm. These data agree with clinical studies indicating that long-term VPA treatment is associated with poor quality of semen in humans, particularly impaired motility (Chen et al., 1992). Moreover, we observed that treatment with RSV prevented the decrease of sperm motility induced by VPA, without altering sperm motility in animals non-treated with VPA. It was already demonstrated that RSV per se did not cause any change in motility of spermatozoa, but prevented the loss of sperm motility induced by oxidative stress in other conditions such as hyperthyroidism (Ourique et al., 2013) and iron/ascorbate treatment (Mojica-Villegas et al., 2014). These results are in agreement with our results, which demonstrate that RSV does not alter sperm function under physiological conditions, but protects the sperm when subjected to a harmful agent such as VPA.

One of the major causes of defective sperm function is oxidative stress (Aitken et al., 1985, 2014; Aitken and Roman, 2008; Kao et al., 2008). At the level of testes, ROS can damage germ cells and reduce the capacity of the germinal epithelium to differentiate spermatozoa with normal function. On the epididymides, ROS can affect spermatozoa and induce damage to lipid and proteins, decreasing sperm motility (Aitken and Roman, 2008; Kao et al., 2008). In fact, our results showed that sperm motility was negatively correlated with TBARS and LOOH levels in testis and epididymides, demonstrating that loss of sperm motility is associated with lipid peroxidation.

Lipid peroxidation is one of the most important free radicals reactions and the mostly utilized oxidative stress biomarker. If unopposed with an efficient antioxidant defense system, peroxidation of the phospholipids of the plasma membrane can potentially lead to severe cell damage (Finker and Holbrook, 2000). Our results showed that long-term treatment with VPA increased lipid peroxidation in testes and in epididymides, demonstrated by higher levels of LOOH and TBARS. Khan et al. (2011) had already demonstrated that treatment with VPA at the same conditions induced the same effect in testes. Moreover, we found that VPA treatment caused oxidative damage to the proteins, as detected by a marked increase of carbonyl protein formation in testis and epididymides. Therefore, our data suggests that ROS formation, mediated by VPA administration, induces lipid and protein oxidative damage, which is reflected in sperm reduced motility and vigor.

The co-administration of RSV protected testes and epididymides from VPA-induced oxidative damage to lipids and proteins, as indicated by the decrease in LOOH, TBARS and protein carbonyl levels. It is known that RSV inhibits effectively lipid peroxidation of cellular membranes, as well as protein oxidation due to its ability to directly scavenge various ROS, including superoxide and hydroxyl radicals (Leonard et al., 2003). Thus, we believe that this protective effect of RSV on sperm motility may be attributed to its scavenging properties. The treatment with RSV did not modify the biomarkers of oxidative damage in animals non-treated with VPA, since RSV group did not differ from control, which reveals that RSV protects rat testes and epididymides from oxidative changes induced by a harmful substance as VPA but did not alter these variables in healthy animals.

Changes in antioxidant defense may also be involved on the process of oxidative stress that cause the reproductive impairment induced by VPA. Long-term treatment with VPA caused diminution on GSH content in testes and decrease on TRAP in testes and epididymides. Decrease in GSH levels in testes after VPA treatment, also demonstrated by Khan et al. (2011), may represent a depletion of this antioxidant due to its increased utilization in removing H_2O_2 and other peroxides produced in excess due to oxidative stress. Moreover, it has been reported that long-term treatment with VPA increases clearance of selenium, cooper and zinc (Tabatabaei et al., 1999). These metals are cofactors for glutathione and other endogenous free radicals scavengers (Tabatabaei et al., 1999), which could also explain depletion of GSH and decrease of TRAP in animals treated with VPA. This deficiency in GSH levels and TRAP can expose testes and epididymides to oxidative damage and contribute to the impairment of sperm motility and vigor, which ultimately affects the overall reproductive function.

RSV was not able to prevent depletion of GSH in testes, but restored TRAP in testes and epididymides of rats treated with VPA. TRAP measurement includes a variety of compounds bearing different reactive centers (phenols, thiols) with widely different hydrophobicity, which allows trapping of both hydrophobic and hydrophilic radicals (Evelson et al., 2001). Increase on TRAP by RSV in testes and epididymides of rats treated with VPA supports the role of this compound as a radical scavenger, which may contribute to the protective effect of RSV against oxidative damage induced by VPA.

The molecular mechanisms by which VPA can trigger oxidative stress in testis and epididymides are not clear. Evidence suggests that VPA inhibits the activity of histone deacetylase (HDAC) enzymes (Wang et al., 2013). This inhibition would lead to a detrimental alteration in the cellular gene expression profile and/or induce a conformational change in chromatin exposing DNA to damage (Rajender et al., 2011). RSV, on the other hand, is shown to positively regulate the levels and activity of the sirtuins, which are members of class-III HDAC (Lakshminarasimhan et al., 2013; Tanno et al., 2010). Our work demonstrates that RSV mitigates the toxic effects of VPA in testis and epididymides. Further studies are necessary to investigate if epigenetic alterations are in fact related to the protective effects promoted by RSV in our experimental model. Besides epigenetics changes, VPA is also shown to promote inflammation (Oktay et al., 2015). RSV is a well known anti-inflammatory molecule (Dudka et al., 2012). Therefore, the measurement of different inflammatory markers would be also of extremely importance to further elucidate the molecular mechanism behind RSV protection against VPA mediated reproductive impairment.

Altogether, our data represents the first evidence in the literature showing the RSV capacity to protect testes and epididymides against VPA toxicity and consequent impairement of sperm motility in Wistar rats. More importantly, our results strongly suggest RSV as an alternative to minimize reproductive impairment in male patients requiring long-term treatment with VPA.

Conflict of interest

The authors declare that there are no conflicts of interest.

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3.2 Artigo 2

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Protective effect of vitamin E on sperm motility and oxidative stress in valproic acid treated rats

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ABSTRACT

Long-term administration of valproic acid (VPA) is known to promote reproductive impairment mediated by increase in testicular oxidative stress. Vitamin E (VitE) is a lipophilic antioxidant known to be essential for mammalian spermatogenesis. However, the capacity of this vitamin to abrogate the VPA-mediated oxidative stress has not yet been assessed. In the current study, we evaluated the protective effect of VitE on functional abnormalities related to VPA-induced oxidative stress in the male reproductive system. VPA (400 mg kg^{-1}) was administered by gavage and VitE (50 mg kg^{-1}) intraperitoneally to male Wistar rats for 28 days. Analysis of spermatozoa from the cauda epididymides was performed. The testes and epididymides were collected for measurement of oxidative stress biomarkers. Treatment with VPA induced a decrease in sperm motility accompanied by an increase in oxidative damage to lipids and proteins, depletion of reduced glutathione and a decrease in total reactive antioxidant potential on testes and epididymides. Co-administration of VitE restored the antioxidant potential and prevented oxidative damage on testes and epididymides, restoring sperm motility. Thus, VitE protects the reproductive system from the VPA-induced damage, suggesting that it may be a useful compound to minimize the reproductive impairment in patients requiring long-term treatment with VPA.

1. Introduction

Infertility and impaired fertility have been a concern through ages and represent significant clinical problems (WHO, 2000). It is estimated that infertility affects 8–12% of couples worldwide, with a male factor contributing to approximately 40–50% of the cases (Brugh and Lipshultz, 2004; WHO, 2000). In 30–45% of these cases, the reason for abnormal semen parameters is not identified (idiopathic male infertility) (Pierik et al., 2000). However, numerous factors have been associated with increased risk of infertility including, but not limited to, genetic anomalies, reproductive diseases, cancer diagnoses requiring chemotherapy, radiation or surgery, and long-term use of some drugs (Jungwirth et al., 2012; O'Flynn O'Brien et al., 2010).

Valproic acid (2-propyl-pentanoic acid, VPA) is a FDA approved drug used as anticonvulsant, in the treatment of bipolar disease and also for migraine prophylaxis (Haddad et al., 2009; Loscher, 2002). More recently, VPA has been proposed in clinical trials for treatment of human disorders such as retinitis pigmentosa (Kumar et al., 2014), myelodysplastic syndrome, acute myelogenous leukaemia (Issa et al., 2015), and prostate cancer (Sharma et al., 2008). Despite its beneficial effects in the treatment of many diseases, VPA-mediated toxicity to the male reproductive system is a real concern for patients. Cases of infertility have been reported in male subjects taking VPA for epilepsy (Roste et al., 2005; Xiaotian et al., 2013; Yerby and McCoy, 1999). Both animal (Girish et al., 2014; Hamza and Amin, 2007; Nishimura et al., 2000) and human (Chen et al., 1992; Roste et al., 2005; Xiaotian et al., 2013; Yerby and McCoy, 1999) studies demonstrated that VPA has many adverse effects to male fertility, including inhibition of the sperm motility.

The mechanism by which VPA toxicity leads to impaired sperm

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motility is not fully known. A growing body of evidence suggests that oxidative stress is responsible for VPA toxicity (Hamza and Amin, 2007; Khan et al., 2011). Treatment of rats with VPA induced a decrease in sperm motility accompanied by increased oxidative stress in testes (Hamza and Amin, 2007). VPA induced germ cell toxicity is associated with sperm DNA damage and oxidative stress in testes (Khan et al., 2011). Some organs withstand stress better than others, with the testes known to be particularly sensitive to oxidative stress (Tomascik-Cheeseman et al., 2004). Therefore, it is not surprising that VPA-induced oxidative stress promotes significant damage in this organ.

Both the testes and the sperm cells are rich in polyunsaturated fatty acids, which increase their susceptibility to attack by reactive oxygen species (ROS) and consequently oxidative damage. At the level of the testes, oxidative stress can disrupt the steroidogenic capacity of Leydig cells, and the ability of the germinal epithelium to differentiate into spermatozoa with normal function. At the level of the spermatozoa, oxidative stress affects the motility of these cells and their capability to support normal embryonic development (Agarwal et al., 2006; Aitken and Baker, 2006; Aitken and Roman, 2008).

Many investigators have used a variety of antioxidants, including vitamin E (α-tocopherol, VitE), to prevent the increase of oxidative stress in testes (Hsu et al., 1998; Patra et al., 2011). It has been recognized that VitE is a powerful lipophilic antioxidant that is vital for the maintenance of mammalian spermatogenesis (Johnson, 1979), being present in particularly high amounts in Sertoli cells and pachytene spermatocytes and, to a lesser extent, in round spermatids (Yoganathan et al., 1989). Deficiency of VitE leads to oxidative stress levels that are incompatible with normal spermatogenesis and testosterone production (Johnson, 1979). VitE also counteracts testicular oxidative stress induced by exposure to prooxidants such as cadmium (El-Demerdash et al., 2004; Sen Gupta et al., 2004), alcohol (Maneesh et al., 2005), L-thyroxine (Sahoo et al., 2008), and pesticides (Astiz et al., 2013). Moreover, VitE has been shown to exhibit promising protective effects against VPA-induced teratogenicity in animal models and to alleviate the hepatotoxicity of VPA mainly through its anti-inflammatory and antioxidant actions (Al Deeb et al., 2000; Baran et al., 2006; Chen et al., 2014; Hsieh et al., 2014; Jurima-Romet et al., 1996). These observations strongly suggest that VitE may be an important protective agent for the maintenance of male fertility in conditions where it has been adversely affected by oxidative stress. However, VitE capacity to prevent VPA-induced toxicity in the reproductive system has not been determined so far.

Evaluation of reproductive toxicity in rats exposed to a high dose of VPA and the search for alternatives that counteract this toxicity may provide valuable information with clinical implications. The purpose of the present study was to evaluate the protective effect of VitE against VPA-induced reproductive toxicity in male Wistar rats.

2. Materials and methods

2.1. Chemicals

VPA (Valpakine syrup at a concentration of 200 mg mL^{-1}) was obtained from Sanofi Laboratories (São Paulo, SP, Brazil). VitE and all other reagent-grade chemicals were obtained from Sigma (St Louis, Missouri, USA).

2.2. Animals

Male Wistar rats (90 days) were maintained under standard laboratory conditions: 23 ± 2 °C and light-dark cycle of 12 h; free access to tap water and about 30 g daily per animal of rodent chow (Supra, São Leopoldo, Rio Grande do Sul, Brazil). Animals were acclimatized to the experimental conditions for a period of two weeks prior to the commencement of experiments. The study was approved by the Animal Ethics Committee (CEUA) of the Federal University of Santa Maria, Rio Grande do Sul, Brazil (process #076/2013).

2.3. Experimental design

2.3.1. Groups

Rats (n = 32) were randomly divided into four experimental groups, composed of eight animals each, and treated as follows:

- Control: received both VPA and VitE vehicles;
- VPA (VPA group): received VPA treatment and VitE vehicle;
- VitE (control group treated with VitE): received VPA vehicle and VitE treatment;
- VPA + VitE (VPA group treated with VitE): received both VPA and VitE treatments.

2.3.2. VPA and VitE doses

VPA was administrated by gavage as a syrup at a dose of 400 mg kg^{-1}. A vehicle solution was prepared with the same components as the syrup, except for VPA. The dose of 400 mg kg^{-1} (approximately equivalent to the maximum human daily dose on a mg/m^2 basis) of VPA was selected based on the results obtained from different studies carried out for reproductive toxicity in rodents (Khan et al., 2011; Roste et al., 2002; Vijay et al., 2008).

VitE was dissolved in 1% tween 80 solution and administered by intraperitoneal injection at a dose of 50 mg kg^{-1}. This dose was based on previous studies showing protective effects of VitE against oxidative stress in testes and sperm, where no side effects were reported (Astiz et al., 2013; Krishnamoorthy et al., 2007). The dose is approximately equivalent to 480 mg per day for an adult of 60 kg. In a previous study, VitE did not show any adverse effects in doses up to 2146 mg per day. The same study concluded that up to 1073 mg per day of VitE was safe for most adults (Hathcock et al., 2005). To avoid stress associated with multiple daily administrations by gavage, we decided to administer VitE intraperitoneally. Although intraperitoneal injection is considered a parenteral route of administration, the pharmacokinetics of substances administered intraperitoneally are similar to those seen after oral administration, because the primary route of absorption is into the mesenteric vessels, which drain into the portal vein and pass through the liver (Turner et al., 2011).

All treatments (VPA, VitE or their vehicles) were freshly prepared, being administrated on a daily basis through four weeks.

2.3.3. Blood collection and euthanasia

At the end of the experimental period (four weeks) and 24 h after the last treatment, animals were weighed and anesthetised with xylazine and ketamine. Blood was collected through cardiac puncture, and the rats were euthanized by decapitation for the epididymides, testis, prostate, and seminal vesicle removals, which were immediately weighed.

2.4. Retrieval and evaluation of spermatozoa

Epididymides were collected and freed from the adherent and fat tissues. Cauda epididymidis was separated from the rest of the epididymidis and transferred to petri dish containing sterile silicone oil and a 200 μl drop of Fert's medium. All reagents were preheated. Longitudinal incisions were made in the cauda epididymidis with a fine needle and a scalpel blade to release the

spermatozoa. The sperm motility and vigor were evaluated by placing a 4 μl drop of Fert's medium containing the spermatozoa on a slide and then examining the drop using a light microscope Olympus CX40 (Olympus, Shinjuku-ku, Tokyo, Japan) at 100X. The percentage of total and progressive motility was estimated from three different fields in each sample, and the mean was used as the final value of motility. Vigor of movement was also estimated, using the following scale: 0, no movement; 1, slight side-to-side movement, no forward progression; 2, rapid side-to-side movement, no forward progression; 3, rapid side-to-side movement, occasional forward progression in spurts; 4, steady, slow forward progression; 5, rapid, steady forward movement (Platz and Seager, 1978).

Epididymal sperm count was determined using Neubauer's haemocytometer. Sperm concentration was expressed as the number of sperm per mL of solution containing sperm. To analyse the morphology, sperm were kept in a formaldehyde-citrate solution, and 200 spermatozoa were analysed from each animal. The analyses were performed using a Leica DMI 4000B inverted microscope with differential interference contrast. Morphological abnormalities of the head (small, amorphous, pinhead or isolated form, i.e., no tail attached), midpiece (without characteristic curvature or broken) and tail (doubled, broken or rolled into a spiral) were examined, and the percentage of normal or abnormal sperm was determined.

2.5. Sample preparation

Blood was collected in tubes and it was separated using centrifugation (1800 g, 15 min). The serum was stored at −20 °C for further analysis.

The right testes and epididymides were homogenized in a glass-Teflon grinder, using 0.1 M phosphate buffer (pH 7.4) containing 1 mmol l^{-1} phenylmethylsulfonyl fluoride. The homogenates of each tissue were centrifuged at 100 g for 10 min at 4 °C to eliminate nuclei and cell debris and supernatants were frozen at −70 °C for further measurements (Ourique et al., 2013).

2.6. Testicular histology

The left testis were removed and fixed in 4% buffered formaldehyde solution for 24 h for histological examination. The samples were embedded in paraplast, cut in 5 μm thick sections and stained with hematoxylin and eosin (HE) for light microscopic evaluation. Six aleatory fields of each section of the testes were qualitatively analysed.

2.7. Assessment of testosterone, alanine transaminase, and aspartate transaminase in serum

Testosterone levels were measured in serum using a competitive electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN). Results were reported as ng dl^{-1}. Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were measured using colorimetric assays (Labtest, Lagoa Santa, MG, Brazil).

2.8. Estimation of lipid peroxidation in testes and epididymides

Lipid peroxidation, as indicated by the amount of lipid hydroperoxides (LOOH), was measured by the xylenol orange method (Jiang et al., 1991). This technique can detect the primary products of peroxidation using the oxidation of Fe^{2+} by LOOH in an acidic medium with xylenol orange dye, which forms a complex with Fe^{3+}. For the assay the following reagents were added sequentially at a final concentration: 20 mol l^{-1} methanol, 100 μmol l^{-1} xylenol orange, 25 mmol l^{-1} sulfuric acid, 4 mmol l^{-1} butylated hydroxytoluene, 250 μmol l^{-1} ferrous sulfate, to a total of 0.45 ml. Sample aliquots (0.05 mL) were then added and incubated at room temperature for 30 min, and the absorbance at 560 nm was then read. The results were reported as nmol mg $protein^{-1}$.

Lipid peroxidation was also estimated based on the formation of thiobarbituric acid reactive substances (TBARS) (Buege and Aust, 1978). The amount of malondialdehyde (MDA) produced was used as an index of lipid peroxidation. Briefly, the sample was mixed with 20% TCA and 0.67% TBA, and heated for 1 h at 100 °C. After cooling, the precipitate was removed by centrifugation. The absorbance of the organic phase was measured using a spectrophotometer Biospectro SP 220 (Biospectro, São Paulo, SP, Brazil) at 535 nm, and the results were expressed as nmol MDA mg $protein^{-1}$.

2.9. Determination protein carbonyl in testes and epididymides

Protein carbonyl content was measured by a method based on the reaction of carbonyls with 2,4-dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound. The standard curve was prepared by using different bovine serum albumin concentrations (0.5–1.5 mg mL^{-1}) and the slope was used to express the levels of carbonyl protein as nmol mg $protein^{-1}$ (Reznick and Packer, 1994).

2.10. Assay of total superoxide dismutase

Total superoxide dismutase (SOD) activity was determined based on the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine/NaOH (pH 10.2). One SOD unit was defined as the amount of enzyme required for 50% inhibition of the adrenochrome formation and the enzyme activity was expressed as USOD mg $protein^{-1}$ (Fridovich, 1974).

2.11. Assay of catalase

Catalase (CAT) activity was evaluated by measuring the decrease in the absorption at 240 nm in a reaction medium consisting of 50 mM phosphate buffer (pH 7.4) and 2 mM hydrogen peroxide (H_2O_2). Results were reported as pmol mg $protein^{-1}$ (Chance et al., 1979).

2.12. Assay of glutathione S-transferase

Glutathione-S-transferase (GST) activity was assayed based on the conjugation reaction with GSH, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig et al., 1974). Sample aliquots (0.05 mL) were added to 0.6 mL of the assay mixture containing 100 mmol l^{-1} phosphate buffer (pH 6.5), GSH and CDNB at a final concentration of 1 mmol l^{-1} each. The activity was calculated from the changes in absorbance at 340 nm. It was expressed as pmol min^{-1} mg $protein^{-1}$.

2.13. Assay of non-protein thiols

Non-protein thiols (NPSH) represent an indirect measure of reduced glutathione (GSH). Proteins were eliminated by adding 0.25 mmol l^{-1} perchloric acid to the homogenates and centrifuging the mixture at 700 g for 5 min. To the supernatants (0.05 mL), 0.45 mL of 0.2 mol l^{-1} phosphate buffer (pH 8.0) and 0.18 mmol l^{-1} 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at a final concentration were added and vortexed. The DTNB formed a yellow complex with GSH, and the absorbance was measured at 412 nm. NPSH content was expressed as nmol mg $protein^{-1}$ (Ellman, 1959).

2.14. Evaluation of total reactive antioxidant potential

Total reactive antioxidant potential (TRAP) was measured based on the capacity of the sample to scavenge luminol luminescence induced by thermolysis of 2,2′-azo-bis (2 amidinopropane) dihydrochloride as a source of free radicals. The standard curve was prepared by using different Trolox concentrations (1–4 μmol l^{-1}). The comparison of the induction time after the addition of Trolox and the sample allowed calculation of TRAP as the equivalent of the Trolox concentration necessary to produce the same induction time, expressed as μmol mg $protein^{-1}$ (Evelson et al., 2001).

2.15. Estimation of protein

Protein was determined by the method of Lowry et al. (1951) by using bovine serum albumin as standard (Lowry et al., 1951).

2.16. Statistical analysis

The results are expressed as the mean ± standard error. Levene's test was used to determine homogeneity of variance. Two-way analysis of variance followed by Duncan post-hoc test was done to evaluate the differences between the groups. The data referent to the analysis of spermatozoa were transformed (arcsine transformation) before ANOVA test. The statistical analyses were performed using the software Statistica 7.0 (StatSoft, Inc., Tulsa, Oklahoma, USA) and GraphPad Prism 6.01 (GraphPad Software, Inc.). Differences were considered significant at $p < 0.05$.

3. Results

3.1. VPA and VitE have no effect on body and sexual organs weight, on hepatic enzymes levels, and on plasmatic testosterone levels

Final body weight, absolute weight and relative weight of testes, epididymides, prostate and seminal vesicle were not different among treatments (Table 1). The hepatic enzymes ALT and AST did not differ among the experimental groups (Table 1).

VPA and VitE treatments did not change plasmatic testosterone levels (Fig. 1A).

3.2. VitE prevents loss of sperm motility and vigor induced by VPA

Sperm concentration was not affected by treatment with VPA (Fig. 1B). However, motility and vigor of spermatozoa significantly decreased in rats treated with VPA compared to control groups. The sperm motility and vigor did not differ among VitE and control group and this vitamin avoided the alteration in these parameters provoked by VPA ($P < 0.05$) (Fig. 1C and D). The percentage of abnormal sperm (Fig. 1E) as well as abnormalities of the head, midpiece and tail of spermatozoa did not differ among the experimental groups (Fig. 1F).

3.3. VPA and VitE have no effect on testicular morphology

Light microscopical analysis of HE stained testicular sections assessed qualitative morphologic characteristics of germ, Sertoli and interstitial cells. In our experimental conditions, there were no alterations on spermatogenesis and on morphology of the seminiferous tubules among the experimental groups (Supplementary Fig. 1).

3.4. VitE prevents VPA-induced oxidative damage in testes and epididymides

Lipid peroxidation was determined based on LOOH and TBARS levels. TBARS and LOOH in testes and epididymides were significantly higher in the VPA group than in the control group ($P < 0.05$). The VPA + VitE group presented testicular and epididymal levels of LOOH and TBARS significantly lower than VPA ($P < 0.05$) (Fig. 2A and B).

Oxidative damage to proteins was measured based on the levels of carbonyl proteins formed, which was significantly higher in testes and epididymides of the VPA group than in the control group ($P < 0.05$). Rats from VPA + VitE group presented carbonyl protein levels on testes and epididymides significantly lower than VPA ($P < 0.05$) (Fig. 2C).

3.5. VPA and VitE induce no change on the activity of enzymatic antioxidants in testes and epididymides

There was no significant difference on the activity of the enzymatic antioxidants SOD, CAT and GST on testes and epididymides among the experimental groups (Fig. 3A, B and C).

3.6. VitE prevents VPA-induced decrease on GSH levels in testes

The levels of non-enzymatic antioxidant GSH were significantly lower in the testes of the VPA group than in the control group ($P < 0.05$). VitE treatment recovered GSH levels of VPA-treated animals (Fig. 4A).

GSH levels did not differ among the experimental groups in epididymides (Fig. 4A).

Table 1
Effects of valproic acid (VPA) and vitamin E (VitE) on body weight and weight of reproductive organs of rats.

Parameter	Group			
	Control	VitE	VPA	VPA + VitE
Initial body weight (g)	271.00 ± 4.79	275.28 ± 5.27	272.89 ± 5.08	273.75 ± 4.66
Final body weight (g)	355.67 ± 6.97	368.43 ± 10.59	359.89 ± 8.07	345.00 ± 5.91
Testicular weight (g)	1.818 ± 0.023	1.748 ± 0.057	1.788 ± 0.028	1.776 ± 0.072
Relative testicular weight (g 100 g of body $weight^{-1}$)	0.503 ± 0.012	0.478 ± 0.008	0.498 ± 0.011	0.514 ± 0.016
Epididymal weight (g)	0.765 ± 0.035	0.812 ± 0.020	0.751 ± 0.035	0.718 ± 0.031
Relative epididymal weight (g 100 g of body $weight^{-1}$)	0.212 ± 0.010	0.216 ± 0.004	0.209 ± 0.011	0.209 ± 0.010
Prostate weight (g)	0.474 ± 0.030	0.489 ± 0.057	0.413 ± 0.038	0.388 ± 0.042
Relative prostate weight (g 100 g of body $weight^{-1}$)	0.132 ± 0.009	0.131 ± 0.014	0.115 ± 0.010	0.112 ± 0.011
Seminal vesicle weight (g)	0.825 ± 0.069	0.881 ± 0.081	0.921 ± 0.044	0.788 ± 0.054
Relative seminal vesicle weight(g 100 g of body $weight^{-1}$)	0.228 ± 0.019	0.237 ± 0.024	0.257 ± 0.011	0.228 ± 0.013
Serum alanine transaminase level (U l^{-1})	28.34 ± 1.77	26.56 ± 4.51	35.79 ± 4.37	31.17 ± 4.15
Serum aspartate transaminase level (U l^{-1})	113.7 ± 10.05	101.6 ± 11.11	92.5 ± 7.69	111.6 ± 9.64

All values are expressed as mean ± SEM (n = 8). There were no significant difference between treatments ($P > 0.05$).

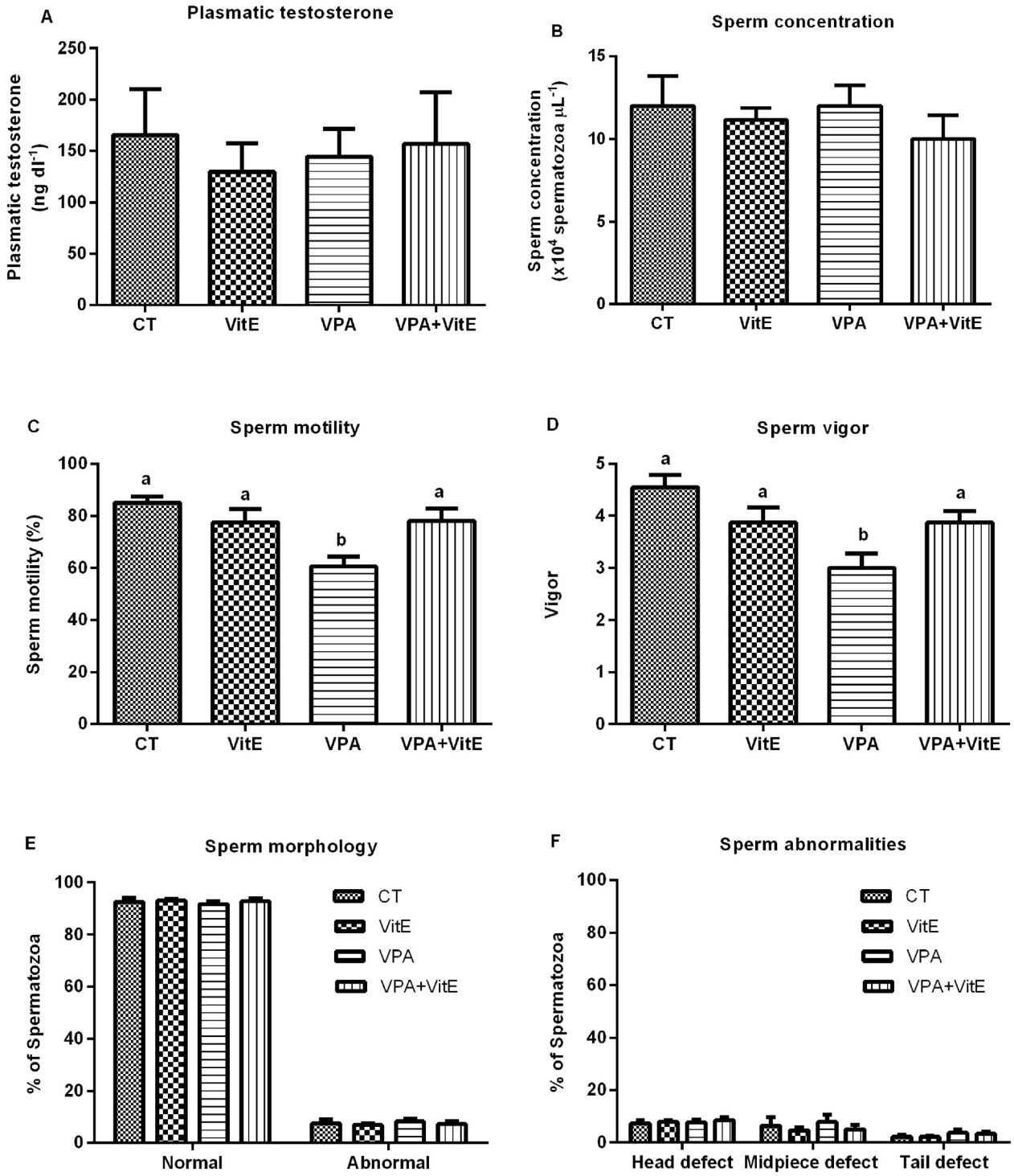
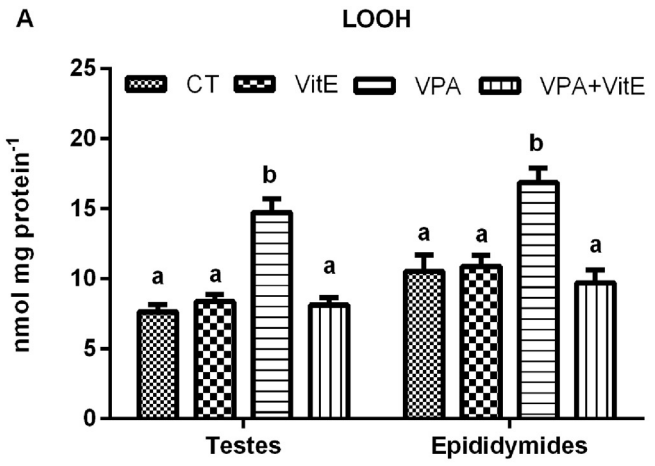


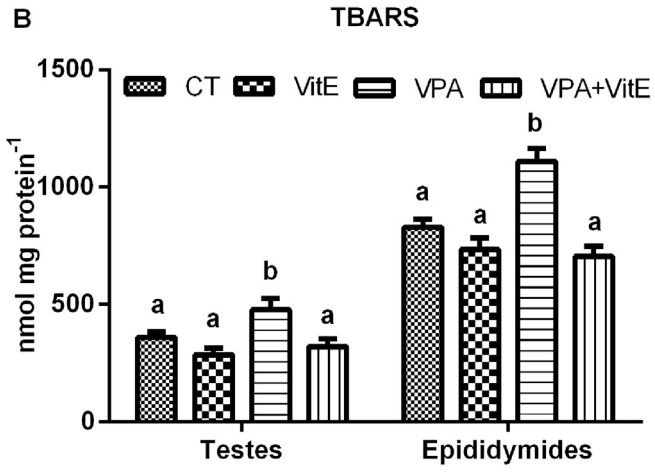
Fig. 1. Effect of valproic acid (VPA) and vitamin E (VitE) on plasmatic testosterone levels and on sperm count, motility, vigor and morphology. All values are expressed as mean ± SEM (n = 8), values having different superscripts are significantly different (P < 0.05).

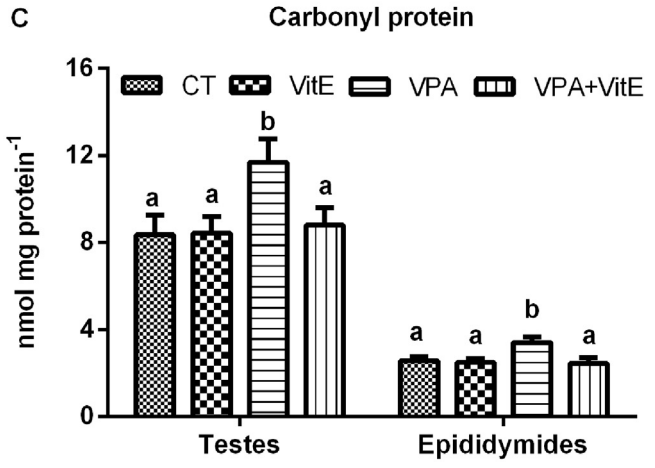
3.7. VitE improves antioxidant potential in testes and epididymides of VPA-treated animals

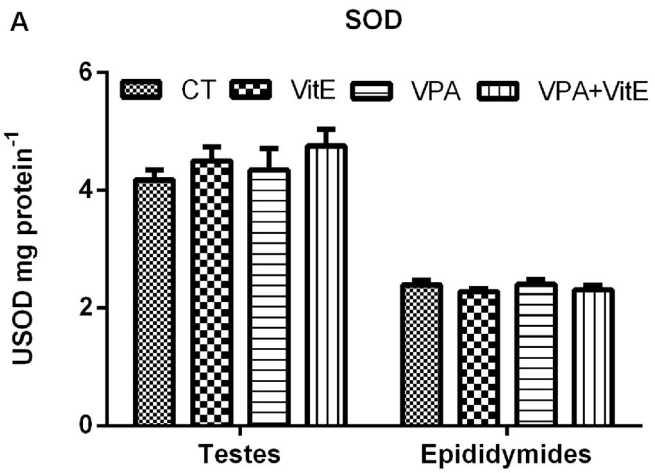
Antioxidant potential was analysed in testes and epididymides by TRAP. In testes, TRAP did not differ between VitE and control groups. Rats treated with VPA presented significantly lower TRAP than the control ones (P < 0.05) and this decrease was avoided by VitE (P < 0.05) (Fig. 4B).

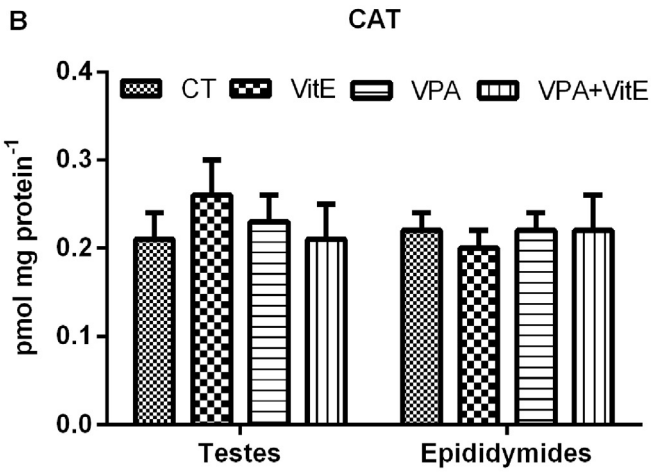
Epididymal TRAP was significantly lower in VPA than in control group (P < 0.05). TRAP was higher in the epididymides of VitE-treated animal than the in the control ones (P < 0.05). Rats from VPA + VitE group presented epididymal TRAP significantly higher than VPA group (P < 0.05) (Fig. 4B).











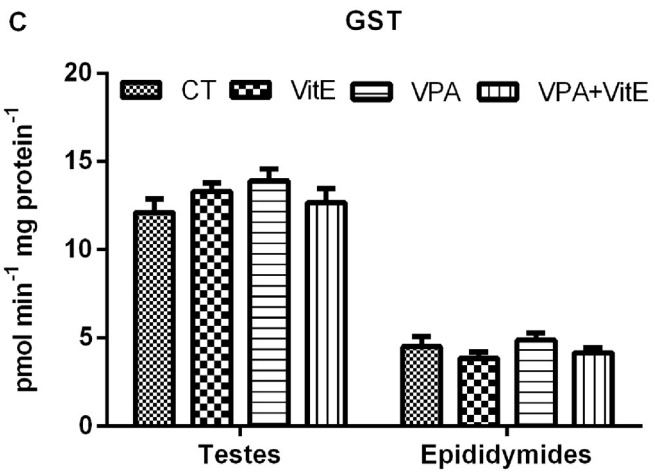


Fig. 2. Effect of valproic acid (VPA) and vitamin E (VitE) on biomarkers of oxidative damage in testes and epididymides. TBARS (thiobarbituric acid reactive substances), LOOH (lipid hydroperoxides). All values are expressed as mean ± SEM (n = 8), values having different superscripts are significantly different ($P < 0.05$).

Fig. 3. Effect of valproic acid (VPA) and vitamin E (VitE) on enzymatic antioxidants in testes and epididymides of rats. SOD (superoxide dismutase), SOD (superoxide dismutase), CAT (catalase), GST (glutathione-S-transferase). All values are expressed as mean ± SEM (n = 8), values having different superscripts are significantly different ($P < 0.05$).

4. Discussion

Our data demonstrate that treatment with VitE significantly reduces the severity of reproductive toxicity induced by VPA in male Wistar rats. To our knowledge, this is the first study that

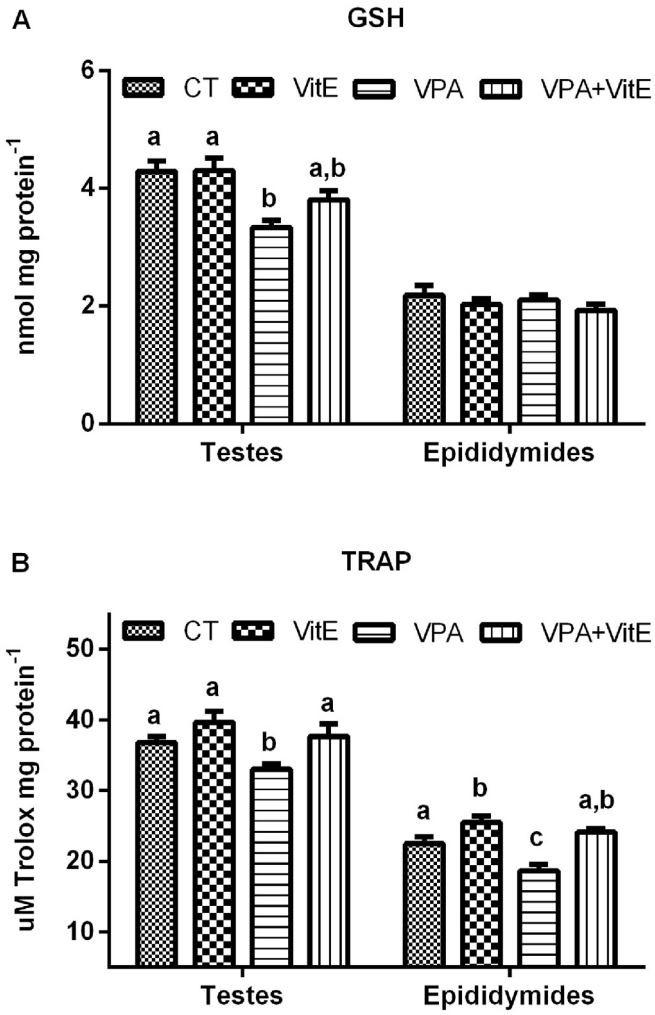


Fig. 4. Effect of valproic acid (VPA) and vitamin E (VitE) on enzymatic antioxidants in testes and epididymides of rats. GSH (reduced glutathione), GSH (reduced glutathione), TRAP (total reactive antioxidant potential). All values are expressed as mean ± SEM (n = 8), values having different superscripts are significantly different ($P < 0.05$).

evaluates the protective effects of an antioxidant vitamin against VPA-induced testicular and epididymal damage in experimental animals.

Sperm motility is one of the most important parameters used in evaluation of sperm quality. Fertile spermatozoa results from a series of successive and complex morphological and surface modifications, wherein the main morphological changes occur during spermatogenesis. Through this process, the surface of the germ cells is organized in specific domains that are defined by distinct subsets of non-exchangeable lipids and proteins (Gatti et al., 2004). After being liberated into the testicular fluid, spermatids start a long journey through the epididymidis. Sperm quality, in terms of motility and oocyte recognition, will be strongly dependent on the subtle post-testicular differentiations that occur during its transit through epididymal duct (Gatti et al., 2004). Alterations in motility capacity might lead to inefficient sperm penetration of the cervix mucus and impair its ability to reach the oocyte (Aitken et al., 1985).

Oxidative stress is one of main factors that contribute to loss of sperm motility and male infertility. One of the major cellular sources of ROS in semen is sperm cells. From the earliest stages of the development, male germ cells are able to produce small amounts of ROS, such as the superoxide anion, hydrogen peroxide, and nitric oxide which are involved in the sperm chromatin condensation, adjusting the number of germ cells by induction of apoptosis or proliferation of spermatogonia (Agarwal et al., 2006; Agarwal and Sekhon, 2010; de Lamirande et al., 1997; Fisher and Aitken, 1997). In mature sperm, ROS play an important role in the capacitation, acrosome reaction, mitochondrial stability and sperm motility. ROS can also mediate cell signalling functioning as second messengers (Agarwal and Sekhon, 2010); however, high concentrations of these free radicals can directly damage sperm cells (de Lamirande et al., 1997). The testicular membrane and sperm cells are very susceptible to attack by ROS since they are rich in polyunsaturated fatty acids, which may result in decreased sperm motility (Sheweita et al., 2005).

In the present study, we observed that co-treatment with VitE is able to counteract the VPA-induced impairment of sperm motility and vigor, which are sensitive biomarkers of testicular damage. The reported VPA-induced decrease of sperm motility was similar to what was shown in previous studies in humans (Chen et al., 1992; Isojarvi et al., 2004) and animals (Cohn et al., 1982; Isojarvi et al., 2004; Nishimura et al., 2000; Snyder and Badura, 1995). This reduction in sperm motility was not associated with testosterone, since VPA treatment did not cause alteration in testosterone levels and weight of the reproductive organs (testes, epididymides, seminal vesicle and prostate). These findings are in agreement with a previous study that demonstrated no alteration on testicular weight and testosterone concentrations using the same dose of VPA (400 mg kg^{-1} day^{-1}) (Roste et al., 2002).

After oral administration, VPA is rapidly absorbed from the gastrointestinal tract and rapidly distributed to various tissues, with maximum serum levels in rats occurring between 5 and 30 min after treatment (Loscher, 1978). Studies in rats showed that VPA is highly distributed in the blood, moderately in the liver, kidney, heart and lung, and low in the brain, testis, fat, and skeletal muscle (Dickinson et al., 1979). In rats, about 63% of VPA is found bound to plasmatic proteins and its half-life is about 4.6 h (Dickinson et al., 1979; Loscher, 1978). VPA is almost completely metabolised before excretion, which is mainly via urine (Gugler and von Unruh, 1980). Moreover, it has been demonstrated that VPA can be excreted into breast milk (Nau et al., 1981), as well as into semen (Swanson et al., 1978). Loscher and Nau (1983) demonstrated that some VPA metabolites can be accumulated in some regions of the brain after prolonged treatment in rats (Loscher and Nau, 1983) but, to our knowledge, there is no data available in the literature about the accumulation of VPA in testes. Some studies have demonstrated that treatment with VPA induce changes in testicular morphology, such as degeneration of seminiferous tubules and depletion of germ cells (Hamza and Amin, 2007; Khan et al., 2011; Roste et al., 2001), however, we did not observe histological alterations on testes following VPA and/or VitE treatment. Spermatogenesis appeared to be normal in our experimental conditions, which corroborate our findings of normal sperm count and morphology, suggesting that VPA-induced loss of sperm motility is an event posterior to sperm production in the testes. Moreover, we also evaluated serum levels of the hepatic enzymes ALT and AST, because liver function and vitamin homeostasis are also of importance in sperm function. The hepatic enzymes analysed, ALT and AST were not altered with VPA and VitE treatments, which demonstrate that reduction in sperm motility was not associated with liver dysfunction, in our experimental model.

Oxidation products of lipids and proteins have been shown as reliable biomarkers of oxidative stress (Dalle-Donne et al., 2003). VPA was previously reported to increase lipid peroxidation in testes (Hamza and Amin, 2007; Khan et al., 2011), but such an effect has not been evaluated on epididymidis until our present study. The

epididymidis plays an essential role in sperm motility. In the epididymidis, the site of sperm capacitation and maturation, oxidative stress results in detrimental effects that could explain how motility of spermatozoa is affected by VPA. In our study, VPA-induced reproductive toxicity was associated with elevated oxidative stress on testes and epididymides, as evidenced by the increased levels of testicular and epididymal TBARS, LOOH and carbonyl protein content. Increased lipid peroxidation and altered membrane function can affect sperm motility and cause sperm dysfunction, which has been suggested to be a consequence of a rapid loss of intracellular ATP leading to decreased sperm viability (Cummins et al., 1994; Sheweita et al., 2005). Treatment with VitE protected against VPA oxidative damage in the testes and epididymides, decreasing levels of TBARS, LOOH and carbonyl protein. The lipophilic character of VitE enables it to locate itself in the interior of the cell membrane bilayer, where it rapidly reacts with fatty acid peroxyl radicals, the primary products of lipid peroxidation, and intercepts the chain reaction (Schneider, 2005).

When the cellular antioxidant defences are saturated, ROS levels increase potentially damaging all types of biological molecules (Dalle-Donne et al., 2003). Although in the present study VPA treatment did not change the activity of the measured antioxidant enzymes, it caused depletion on GSH content in testes and decreased the antioxidant capacity, evaluated by TRAP, in testes and epididymides. GSH is required for many critical cell processes, and can be considered one of the most important agents of the cellular antioxidant defence system, protecting the cell against damage from exposure to oxidizing agents (Ballatori et al., 2009; Halliwell and Gutteridge, 2007). ROS-mediated decrease in the levels of GSH during sperm production has been shown to cause disruption in the membrane integrity of spermatozoa, leading to instability of sperm midpiece, and consequent defect in sperm motility (Ochsendorf et al., 1998; Ursini et al., 1999). It has been suggested that decrease in the GSH levels, following VPA treatment, might be due to the exhaustion of GSH stores and the increase in oxidative stress (Khan et al., 2011).

In our study, VitE increased the GSH levels in testes and the total antioxidant capacity in testes and epididymides. This enhanced GSH content is essential to maintain the appropriate cellular redox state and promote the protective effect of VitE. Supplementation of VitE and increase in its total cellular levels stimulates free radical scavenging, which may also explain the increase in total antioxidant capacity of testes and epididymides in rats under VitE treatment.

Previous studies had already reported that the administration of VitE prevented ROS-induced impairment in sperm motility (El-Demerdash et al., 2004; Sonmez et al., 2007). In addition, some studies have shown a protective effect of VitE against VPA-induced hepatoxicity (Jurima-Romet et al., 1996) and teratogenicity (Al Deeb et al., 2000; Baran et al., 2006; Chen et al., 2014; Hsieh et al., 2014). Jurima-Romet et al. (1996) demonstrated that hepatocyte susceptibility to VPA metabolite-mediated cytotoxicity depends on cellular GSH homeostasis and is preventable by VitE. Al Deeb et al. (2000) observed that VitE supplementation decreases VPA-induced neural tube defects. Baran et al. (2006) showed a protective effect of VitE against skeletal anomalies induced by VPA. Chen et al. (2014) also proved that proteomic and genomic teratogenicity elicited by VPA is preventable by VitE (Chen et al., 2014). In addition, Hsieh et al. (2014) found that VitE can rescue VPA-mediated teratogenicity by supressing ROS formation and GSH regeneration (Hsieh et al., 2014). Taken together, these results suggests that VitE is potentially useful for the prevention of impairment of sperm motility induced by VPA, mainly due to its potential to upregulate GSH content and cellular antioxidant capacity, preventing oxidative stress.

5. Conclusion

The protective effect of VitE against VPA-induced male reproductive toxicity in experimental animals has not been evaluated before. Our data strongly suggest that VitE rescues sperm from VPA-induced impairment motility through the upregulation of GSH content, increase in the cellular non-enzymatic antioxidant response, and suppression of oxidative damage in testes and epididymides. Therefore, VitE is a strong candidate molecule to be administered to patients requiring long-term treatment with VPA if proved effective in clinical trials.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2016.07.011.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.07.011.

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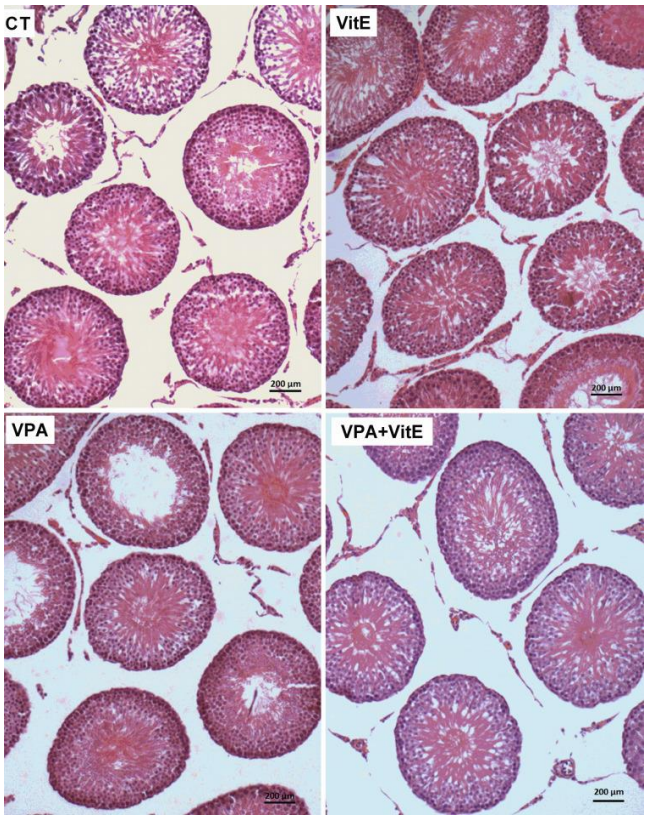
Appendix A. Supplementary data



Supplementary Fig. 1. Histological evaluation of the seminiferous epithelium of Wistar rats from control group (CT), treated with vitamin E (VitE), treated with valproic acid (VPA) and co-treated with valproic acid and vitamin E (VPA + VitE) showing normal morphology of germ, Sertoli and interstitial cells. Tissues were stained with hematoxylin-eosin and analysed at 100× magnification.

3.3 Resultados complementares

Os resultados complementares estão apresentados em formato de resumo expandido.

Effect of Valproic Acid on Production of ROS, Thiol Content and Histone Acetylation in Pachytene Spermatocytes and Round Spermatids

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Effect of Valproic Acid on Production of ROS, Thiol Content and Histone Acetylation in Pachytene Spermatocytes and Round Spermatids

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INTRODUCTION

Valproic acid (2-propyl-pentanoic acid, VPA) is a well-known drug widely used for the treatment of epileptic seizures, mania episodes in bipolar disorder, and in the prophylaxis of migraines (Krymchantowski et al., 2002; Nasrallah et al., 2006). More recently, preclinical trials using VPA have shown its potential as a chemotherapeutic agent in the treatment of different types of cancer. Additionally, VPA has shown promising results when employed in animal models for the treatment of Alzheimer's disease (Duenas-Gonzalez et al., 2008; Nebbioso et al., 2012; Qing et al., 2008). However, VPA widespread use raised concern about adverse effects in male reproductive system related to long-term use in patients. Such secondary potential toxic effect of VPA was demonstrated in both animal and clinical studies (Girish et al., 2014; Ourique et al., 2016a; Ourique et al., 2016b; Roste et al., 2005). Therefore, further investigation is of the outmost importance to fully exanimate all detrimental effects that might be associated to VPA use at systemic level and, more specifically, to male reproductive system.

The exact mechanism by which VPA promotes toxicity to the male reproductive system is not yet well understood, but previous studies showed that impaired motility of spermatozoa is associated with VPA-mediated oxidative damage to lipids and proteins, depletion of reduced glutathione (GSH) and decrease of total reactive antioxidant potential in testes and epididymidis of male rats (Khan et al., 2011; Ourique et al., 2016a; Ourique et al., 2016b). These results suggest that reactive oxygen species (ROS) generation may be the key mechanism responsible for VPA toxicity to male reproductive system.

Furthermore, VPA is known to be an inhibitor of class I and II histone deacetylases (HDACs). Suppression of HDACs function leads to increase in histone tails acetylation and consequent chromatin descondensation, caused by attenuation of the electrostatic charge interactions between histones and DNA (Chateauvieux et al., 2010). During spermatogenesis and sperm maturation, there are several epigenetic modifications, such as methylation, acetylation and replacement of histones by protamine, which are essential for the functional competence of the sperm (Shirakata et al., 2014). The inhibition of HDACs by VPA can affect germ cells, leading to alterations of gene expression. The descondensation of chromatin would also increase DNA susceptibility to ROS attack and damage, which may be associated with additional alteration in gene expression with potential adverse effects to germ cells and untoward progeny outcomes.

The aim of this study was to determine ROS production, histone acetylation and DNA damage in pachytene spermatocytes and round spermatids after VPA treatment, in order to elucidate a potential mechanism by which VPA treatment alters normal sperm function.

METHODS

Animals. Male Brown Norway rats of 4 months of age were purchased from Harlan (Indianopolis, IN) and housed under controlled light conditions (12L:12D) in the Animal Resources Centre of McGill University. Animals were provided with food and water ad libitum and all animal care and handling were done in accordance with the guidelines outlined by the Canadian Council on Animal Care (McGill Animal Resources Centre protocol #2144).

Germ Cell Isolation and Separation. Rats were euthanized by CO_2 asphyxiation and decapitation, and their testes were removed for isolation of spermatogenic cells. Spermatogenic cells were obtained through cell separation using the STA-PUT velocity sedimentation method as described by Bellve et al. (Bellve et al., 1977) and modified by Aguilar-Mahecha et al. (Aguilar-Mahecha et al., 2001). Briefly, both testes were removed, decapsulated, and digested by incubation with collagenase (Sigma). After a brief washing, seminiferous tubules were further digested by incubation with trypsin (type 1; Sigma) and DNase I (type 1; Sigma). After dissociation with flamed glass Pasteur pipettes in the presence of DNase, cells were filtered through a 70 μm nylon mesh and washed with RPMI (RPMI medium 1640; Life Technologies) containing 0.5% bovine serum albumin (BSA). Following centrifugation and filtration through a 56 μm nylon mesh, 5.6 x 10^8 cells suspended in 25 ml of RPMI medium containing 0.5% BSA were loaded into a velocity sedimentation cell separator apparatus and separated by unit gravity sedimentation with a 2%–4% BSA

gradient in RPMI. Fractions of pachytene spermatocytes and round spermatids were identified by phase-contrast microscopy. Fractions with more than 80% purity were pooled.

Isolated Germ Cells Culture and VPA Treatment. After germ cell separation, pachytene spermatocytes and round spermatids were seeded (2.5 x 10^5 cells/well) into 96-well culture plates (Costar 3595; Corning Life Sciences) in phenol red-free Dulbecco's modified Eagle medium (DMEM/F12 medium; Life Technologies), supplemented with HEPES, lactic acid, fetal bovine serum, streptomycin, and penicillin G, as adapted from the method of LaSalle et al. (La Salle et al., 2009). Cells were cultured overnight for approximately 13 h at 32ºC in 5% CO_2. Isolated germ cells were treated with different concentrations (1 µM; 10 µM; 100 µM; 1 mM; 3mM) of VPA (Valproic Acid Sodium Salt P4543; Sigma) or media-only (controls) for 1 hour (T13-T14). Following each treatment, an aliquot of cells was incubated with each different fluorogenic probe. An aliquot of cells was prepared for Comet assay (see below). Another aliquot was collected, pelleted, and stored at -80ºC for further protein extraction.

High Content Screening: Cell Viability Analysis, Cellular ROS, and Cellular Thiol Content. Following culture and treatment, aliquots of isolated cells were removed from culture plates and transferred to 1.5 ml microcentrifuge tubes. An aliquot of cells was incubated in the nuclear stain Hoechst (2,50-bi-1H-benzimidazole, 20-[4-ethoxyphenyl]-5-[4- methyl-1-piperazinyl]; Invitrogen), Calcein-AM (C3100MP; Invitrogen) and the fluorogenic probe CellROX DeepRed Reagent (Invitrogen) for 30 min at 32ºC; another aliquot of cells was incubated with NuclearMask Deep Red Stain (Invitrogen) and the fluorogenic probe ThiolTracker Violet (Invitrogen) for 30 min at 32ºC. Following incubation, cells were washed in Live Cell Imaging solution (Invitrogen) and transferred to a 96-well Cell Carrier plate (PerkinElmer) with an optically clear bottom. The plate was centrifuged at 1200 rpm at 4ºC for 5 min and immediately scanned by the Operetta high content imaging system (PerkinElmer) at 20x magnification with 15 fields of view per well. The image analysis software Columbus 2.2 (PerkinElmer) was used to quantify the mean fluorescent signals from individual cells in each well.

Protein Extraction and Western Blotting. Total protein was extracted from control and VPA-treated pachytene spermatocytes and round spermatids by using a radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with proteinase inhibitor cocktail (Sigma) and phosphatase inhibitor (Active Motif) at a concentration of 10 µl/ml of RIPA. Samples were homogenized in RIPA (100 µl/1x10^6 cells) by sonication for 3–5 sec on ice. Homogenates were centrifuged at 5000 rpm at 4ºC for 10 min. The supernatant was transferred into a new tube and protein concentrations were determined by Bradford assay using a protein assay reagent (Bio-Rad). Samples were resolved in SDS polyacrylamide (w/v) gradient (4%–12%) Bis- Tris gels (Invitrogen) at 200 V for 35 min using MES (2-[N-morpholino] ethanesulfonic acid) SDS running buffer (Invitrogen). Gels were transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Bio-Sciences Inc.) using NuPAGE transfer buffer (Invitrogen) at 100 V for 1 h. Membranes were washed briefly with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and blocked with 5% non-fat milk in TBS-T. Proteins were detected using antibodies specific for H4K12ac (04-112; Millipore; 1:5000 dilution) and H4 (ab177840; Abcam; 1:1000 dilution) in 5% nonfat milk in TBS-T and incubated overnight at 48ºC. Primary antibodies were followed by horseradish peroxidase-linked

secondary antibody donkey anti-rabbit immunoglobulin G (NA93V; GE LifeSciences; 1:50000 dilution), incubated for 2 h at room temperature. Protein bands were detected by electrochemiluminescence prime Western blotting detection reagent (GE LifeSciences).

Comet assay. The extent of DNA damage in isolated pachytene spermatocytes and round spermatids elicited by exposure to VPA was assessed using an alkaline comet assay according to methods published by Albert et al. (Albert et al., 2016) with modifications detailed below. After cultured, isolated pachytene spermatocytes and round spermatids were collected and centrifuged at 500 ×g for 5 min at 4ºC, and the culture media was removed and replaced by an equivalent volume of filtered 1x PBS. The number of cells in each sample was counted using a hemocytometer, and the sample was diluted to a concentration of 1.5 x 10^5 cells/ml in PBS, aliquoted and stored at -80ºC until further use. For a positive control, samples were centrifuged at 500 ×g for 5 min at 4ºC, all of the media was removed and replaced by an equivalent volume of DNAse solution (100 KU/ml DNAse; Sigma). Tubes were placed at 37ºC for 20 min, centrifuged at 500 ×g for 5 min and washed with 1x PBS three times. The sample was diluted to a concentration of 1.5 x 10^5 cells/ml in PBS, aliquoted and stored at -80ºC. Low melting point agarose 0.5% (Sigma) was prepared in PBS using a 85ºC water bath for 1 h and cooled down to 37ºC in an incubator overnight. Fifty microliters of each sample were diluted in 500 µl of 37ºC warm low melting point agarose, pipetted up and down to ensure proper mixing, and 20 µl of this mix were immediately spread in each well of a 96-well CometSlide TM plate (Trevigen). Samples were left on ice for 20 min and subsequently immersed in the following solutions: (i) a lysis solution containing 10% dimethylsulfoxide (DMSO; Fisher Scientific), 1 mM DTT and 1% Triton X-100 (Sigma) for 60 min at 4ºC; (ii) a 4 mM lithium 3,5-diiodosalicylate (LIS; Sigma) solution for 90 min at room temperature; (iii) an alkaline solution (1 mM EDTA; pH 12.1) for 45 min at room temperature. Electrophoresis was then carried out using the Comet Assay horizontal electrophoresis system (Trevigen) at 4ºC at 0.7 V/cm for 5 min in 1x TBE (pH 8). Slides were fixed in 70% ethanol for 5 min at 4ºC, air-dried at room temperature, and protected from light for 48-72 h prior to scoring. Comets were stained using 20 µl of 1:10000 SYBR Gold nucleic acid gel stain (Life Technologies) in TBE, and imaged using a 10x objective in the automated Operetta high content imaging system (PerkinElmer). About 15 pictures/well were taken. Images were analyzed with the ColumbusTM software (PerkinElmer). Samples were run in duplicate, and about 50 pachytene spermatocytes and 100 round spermatids were analyzed per well and scored for Comet tail parameters.

Statistical analyses. Statistical analyses were conducted using Prism version 6 software (GraphPad Software, Inc., LaJolla, CA). All morphology and protein expression data sets were analysed statistically using the Mann-Whitney U-test with Bonferroni's multiple-comparison correction. The comet data set was analyzed one-way ANOVA and post hoc Dunnet's multiple comparison test. The minimum level of significance was $p < 0.05$.

RESULTS

Using live cell staining with Hoechst (nuclear), Deep Red Nuclear Mask (nuclear) and Calcein-AM (cytoplasmic), global changes in nuclear and cytoplasmic morphology were determined to address the effect of VPA in pachytene spermatocytes and round spermatids of rats. As presented in Table 1, no effect on cell count, cell survival, and cell morphology was observed after the cells were treated with concentrations of VPA ranging from 1μM to 3mM. Similarly, VPA treatment had no effect on Calcein-AM intensity, a marker of cell viability (Fig. 1).

Table 1. Cell count, nucleus area, nucleus roundness, cell survival, Hoechst and Nuclear Mask intensities after 1 hour treatment of pachytene spermatocytes and round spermatids with different concentrations of VPA. Values express the mean ± SEM of 5 independent experiments.

	Control	VPA 1μM	VPA 10μM	VPA 100μM	VPA 1mM	VPA 3mM
Pachytene Spermatocytes						
Number of Cells	712.2±120.5	833.6±164.3	772.2±188.4	750.2±177.2	653.4±116.0	742.8±140.3
Cell Survival (% live cells)	92.2±2.4	93.0±1.8	86.1±6.7	94.9±1.2	92.2±3.3	94.7±1.1
Nucleus Area [μm²]	129.2±5.1	129.8±4.5	129.3±4.7	129.7±5.1	129.1±4.5	129.6±4.6
Nucleus Roundness	0.94±0.01	0.95±0.006	0.94±0.01	0.94±0.01	0.94±0.01	0.95±0.01
Hoechst intensity	1669.1±236.0	1603.9±174.9	1510.0±175.4	1602.9±210.3	1572.0±202.3	1685.4±198.7
Nuclear Mask intensity	93.4±18.6	92.4±19.7	96.3±19.6	96.1±19.4	95.4±18.6	102.4±24.2
Round Spermatids						
Number of Cells	1550.8±252.1	1333.2±209.7	1429.2±298.2	1399.4±183.4	1487.8±116.4	1413.2±184.2
Cell Survival (% live cells)	86.1±3.2	89.9±1.6	85.4±3.4	87.2±3.9	88.9±2.4	89.8±1.9
Nucleus Area [μm²]	55.5±2.4	56.0±2.7	55.6±2.4	56.2±2.2	56.3±2.6	56.1±2.6
Nucleus Roundness	0.96±0.02	0.96±0.01	0.96±0.02	0.96±0.02	0.96±0.02	0.97±0.01
Hoechst intensity	1533.1±181.0	1508.5±202.9	1511.6±217.0	1312.1±177.9	1460.7±164.5	1589.2±203.8
Nuclear Mask intensity	101.3±16.1	102.8±17.9	101.6±17.9	102.1±17.0	105.9±19.5	112.4±20.9

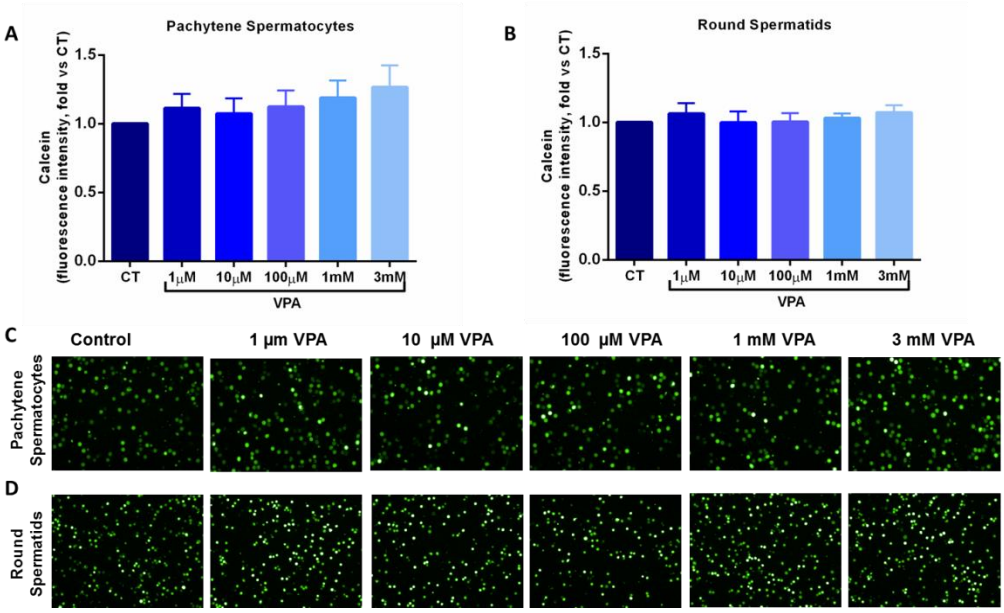

Figure 1. The mean of cytoplasmic Calcein intensity measured in spermatocytes (A) and spermatids (B), with representative images of spermatocytes (C) and spermatids (D) treated with different concentration of VPA for 1 hour. Values express the mean ± SEM change over Control of 5 independent experiments; Mann-Whitney U-test; n=5; Magnification of 20X.

Pachytene spermatocytes treated with 1 mM and 3 mM of VPA display increased ROS production when compared to those from control group (Fig. 2A and 2C). Round spermatids presented a significant increase in ROS production after VPA treatment at all the concentrations tested (1 μM, 10 μM, 100 μM, 1 mM and 3 mM) (Fig. 2B and 2D).

In pachytene spermatocytes, a significant increase on thiols content, which is an indirect measurement of GSH levels, was observed after treatment with 1 μM, 100 μM, 1 mM and 3 mM of VPA (Fig.3A and 3C). Round spermatids treated with VPA (1 μM, 10 μM, 100 μM, 1 mM and 3 mM) show significant increase on thiol content when compared to those from control group (Fig. 3B and 3D).

VPA increased reactive oxygen species thereby causing oxidative stress in pachytene spermatocytes and round spermatids, which is according with previous studies reporting that oxidative stress plays an important role in VPA-induced decrease on sperm motility (Ourique et al., 2016a; Ourique et al., 2016b). Furthermore, as mentioned above, VPA directly inhibits HDACs and it has been suggested that this interaction is related to its teratogenicity (Paradis and Hales, 2013), and could be involved in the toxicity of VPA on germ cells.

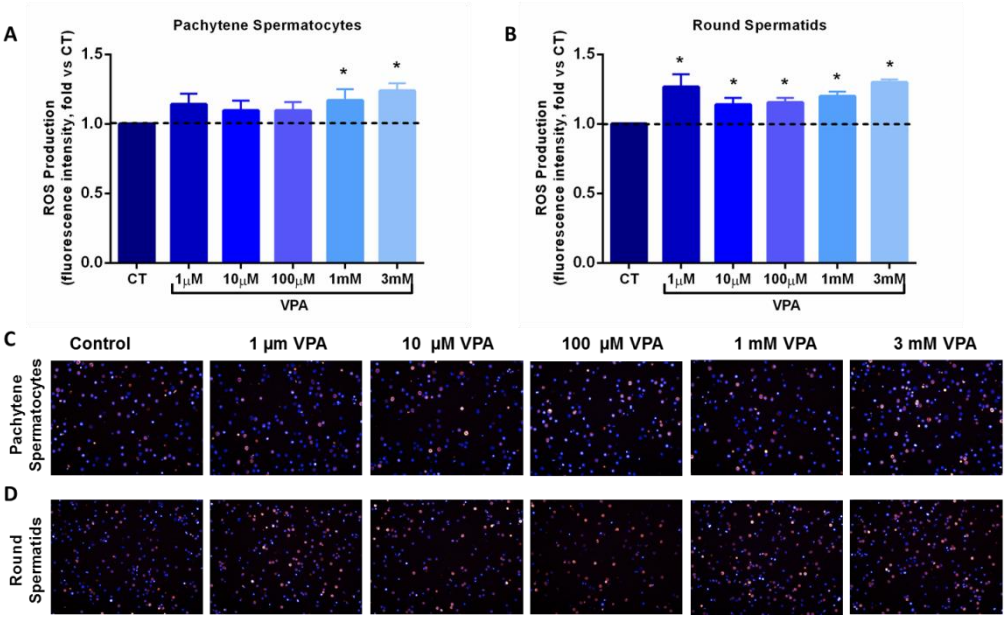

Figure 2. The mean ROS intensity measured in spermatocytes (A) and spermatids (B), with representative images of spermatocytes (C) and spermatids (D) treated with different concentration of VPA for 1 hour. The images show ROS detected with CellROX DeepRed Reagent as a red cytoplasmic fluorescence and the nuclei are visualized using Hoechst (blue). Values express the mean ± SEM change over Control of 5 independent experiments; Mann-Whitney U-test; n=5; *P<0.05. Magnification of 20X.

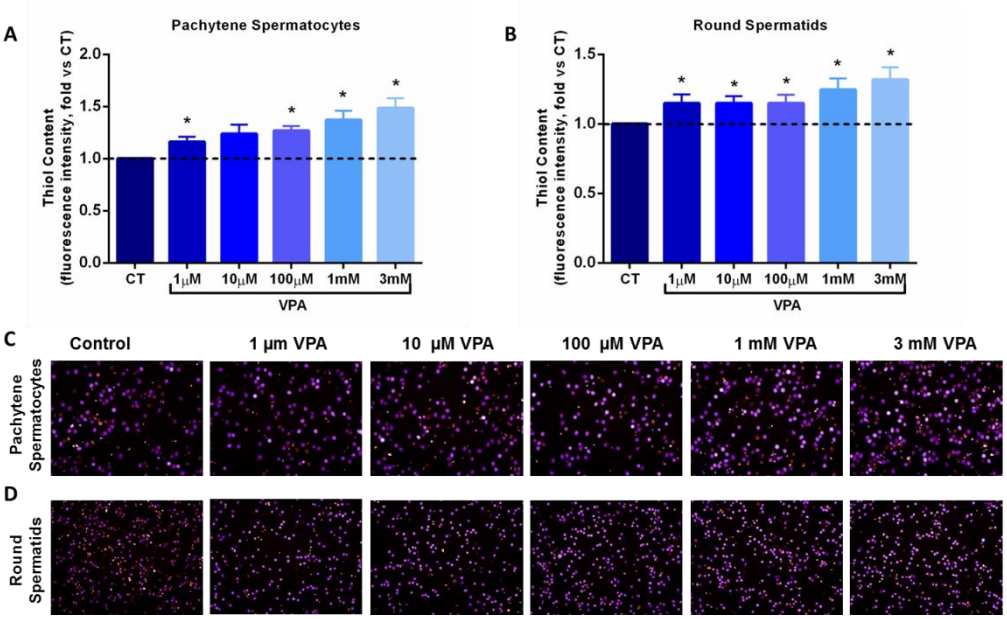

Figure 3. The mean Thiol Content intensity measured in spermatocytes (A) and spermatids (B), with representative images of spermatocytes (C) and spermatids (D) treated with different concentration of VPA for 1 hour. The images show Thiol Content detected with ThiolTracker Violet Reagent as a violet cytoplasmic fluorescence and the nuclei are visualized using Nuclear Mask DeepRed (red). Values express the mean ± SEM change over Control of 5 independent experiments; Mann-Whitney U-test; n=5; *P<0.05 .Magnification of 20X.

Using Western blot analysis, we determined whether VPA treatment affected histone H4 protein expression and its acetylation in pachytene spermatocytes and round spermatids. In pachytene spermatocytes, a significant increase in the expression of histone H4 acetylated on lysine 12 (H4K12ac) was observed after VPA treatment at the concentrations of 1μM and 3 mM, whereas unacetylated H4 protein expression level was not affected (Fig. 4A). In round spermatids, there was a clear trend of increase in H4K12ac levels at all VPA concentrations tested (Fig. 4B), with this increase being statistically significant at concentrations of 10 μM and 3 mM. VPA treatment did not affect the corresponding unacetylated H4 protein in round spermatids (Fig. 4B).

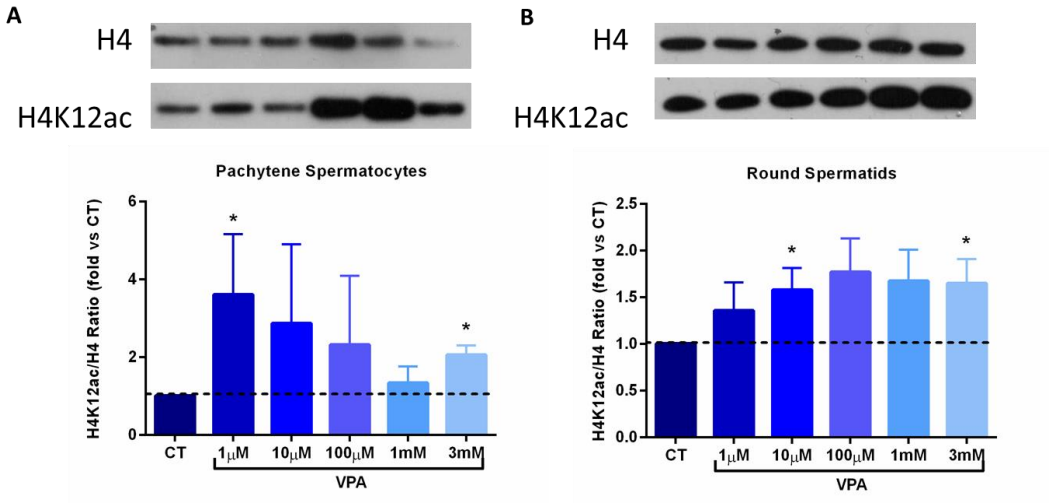

Figure 4. VPA induction of histone 4 lysine 12 acetylation. H4K12 acetylation was used as a marker of HDAC inhibition and normalized to H4. Western blot of whole-cell protein extracts from pachytene spermatocytes (A) and round spermatids (B) treated with different concentrations of VPA for 1 hour. Values express the mean ± SEM change over Control; Mann-Whitney U-test; n=3 (pachytene spermatocytes) and n=6 (round spermatids); *P<0.05.

Using Comet assay, we determined whether VPA treatment affected the level of DNA damage after 1 hour of treatment. There was no alteration in Comet tail extent moment, percentage of DNA in the tail, and tail length in both pachytene spermatocytes (Fig. 5A and 5C) and round spermatids (Fig. 5B and 5D), which suggests that VPA, at the concentrations tested, does not cause DNA damage in these cells.

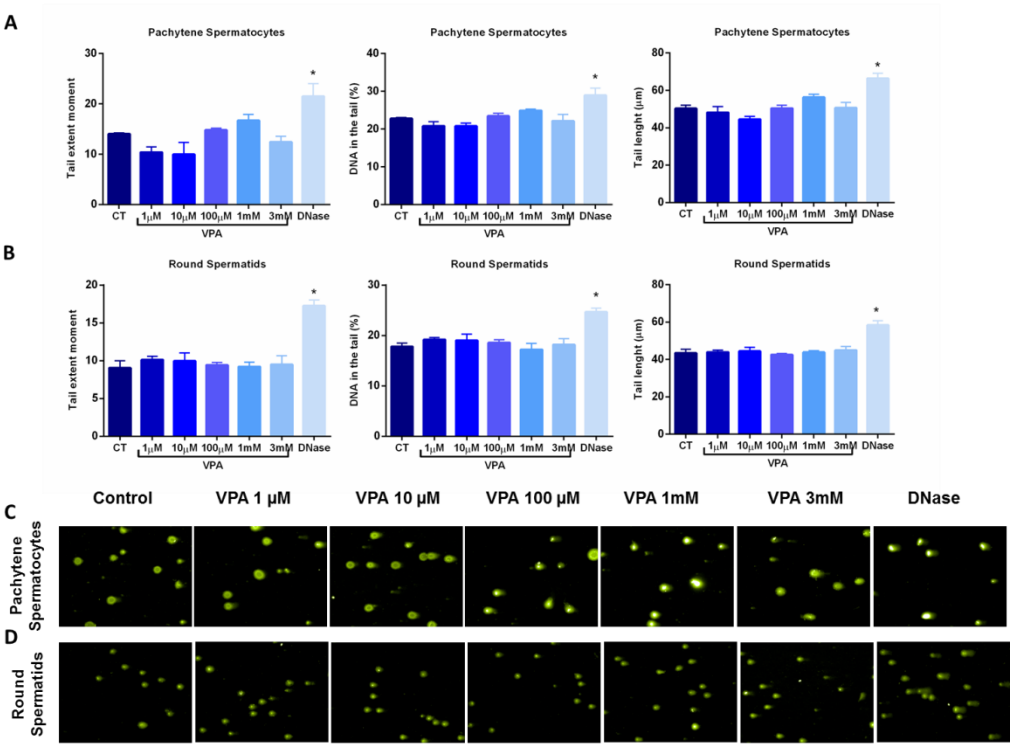

Figure 5. Tail length, % of DNA in the tail and tail extent moment in pachytene spermatocytes (A) and round spermatids (B) with representative images of pachytene spermatocytes (C) and round spermatids (D) treated with different concentrations of VPA and subjected to Comet assay DNase treatment was used as positive control. Values express the mean ± SEM of 3 independent experiments. *Denotes that data are significantly different from CT at $P<0.05$. Dunnett's multiple comparisons test. Magnification of 20X.

CONCLUSION

Our results demonstrated that VPA treatment (1 hour) is associated with increased oxidative stress and histone acetylation in pachytene spermatocytes and round spermatids, without causing DNA damage in those germ cells.

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4. DISCUSSÃO GERAL

O VPA é um fármaco amplamente utilizado no tratamento da epilepsia, sendo também eficaz como profilaxia na enxaqueca e em distúrbios psiquiátricos bipolares, de humor e de ansiedade (EVERS, 2008; BOWDEN, 2009). Atualmente, o VPA tem sido utilizado em ensaios pré-clínicos como um agente quimioterápico e tem mostrado efeitos positivos e potencial terapêutico em modelos animais da doença de Alzheimer (QING et al., 2008; NEBBIOSO et al., 2012). Diante disto, é amplamente pertinente avaliar sua toxicidade, bem como sua eficácia clínica.

Dentre os efeitos adversos associados ao uso contínuo de VPA, há um aumento da preocupação com a toxicidade do VPA sobre a função reprodutiva masculina, o que já foi demonstrado tanto em estudos com animais (COHN et al., 1982; SNYDER e BADURA, 1995; NISHIMURA et al., 2000; ISOJARVI et al., 2004) quanto em estudos clínicos (CHEN et al., 1992; ISOJARVI et al., 2004). O mecanismo exato da toxicidade do VPA à função reprodutiva masculina ainda não está completamente elucidado, mas um dos principais mecanismos propostos é o estresse oxidativo (KHAN et al., 2011), que é claramente uma das principais causas da infertilidade masculina (AITKEN e ROMAN, 2008; KAO et al., 2008; AITKEN et al., 2014; AITKEN et al., 2015). Neste sentido, nós utilizamos os antioxidantes RSV e vitamina E como alternativas para minimizar os efeitos adversos do VPA na função reprodutiva masculina.

A diminuição da motilidade espermática é relatada como um dos principais efeitos tóxicos do VPA em animais (COHN et al., 1982; SNYDER e BADURA, 1995; NISHIMURA et al., 2000) e em humanos (CHEN et al., 1992; ISOJARVI et al., 2004). No presente estudo, o tratamento com VPA induziu uma diminuição significativa na motilidade e no vigor dos espermatozoides, sem causar alteração na contagem e na morfologia espermática. O co-tratamento com RSV ou Vitamina E foi capaz de impedir a diminuição da motilidade e do vigor espermático induzido por VPA, possivelmente pela ação antioxidante destes compostos.

Alguns estudos demonstram que o tratamento com altas doses de VPA pode causar atrofia dos órgãos sexuais e alterações endócrinas em ratos (NISHIMURA et al., 2000; ROSTE et al., 2002). Røste e colaboradores. (2002) relataram que ratos tratados

com elevadas doses de VPA (800 mg/kg) por três meses apresentaram diminuição do peso dos testículos e dos níveis de LH, FSH e testosterona, mas estas alterações não foram observadas em ratos tratados com uma dose mais baixa de VPA (400 mg/kg) pelo mesmo período. Da mesma forma, Nishimura e colaboradores (2000) demonstraram que o tratamento com 500 mg/kg de VPA por dez semanas causou diminuição do peso dos testículos e das glândulas acessórias em ratos, mas o tratamento com doses de 250 mg/kg pelo mesmo período não causou nenhuma alteração nestas variáveis. No nosso estudo, o tratamento com 400 mg/kg de VPA por quatro semanas não causou alteração significativa no peso dos órgãos reprodutores, bem como nos níveis de testosterona. Assim, a dose utilizada e duração do tratamento parecem definir os efeitos do VPA no peso dos órgãos reprodutivos e nos níveis de testosterona. Alguns estudos têm demonstrado que o tratamento com VPA induz alterações na morfologia testicular, como degeneração dos túbulos seminíferos e diminuição das células germinativas (ROSTE et al., 2001; HAMZA e AMIN, 2007; KHAN et al., 2011). No entanto, no nosso modelo experimental, o tratamento com VPA não causou nenhuma alteração significativa na morfologia dos testículos, bem como na quantidade e na morfologia dos espermatozoides.

Uma das principais causas da diminuição da motilidade espermática é o estresse oxidativo (AITKEN e ROMAN, 2008; KAO et al., 2008; AITKEN et al., 2014). Embora uma certa quantidade de EROs seja essencial para as funções celulares normais, tais como sinalização celular, e manutenção da homeostase, e processos como a capacitação espermática, que permite que o espermatozoide fertilize o óvulo (FORD, 2004), um grande problema surge quando EROs são geradas em excesso. A acumulação de EROs pode causar danos a macromoléculas, incluindo lipídios, proteínas e ácidos nucleicos, tanto em nível de testículo quanto em nível de epidídimo (AITKEN et al., 2014). No testículo, as EROs podem lesar as células germinativas e reduzir a capacidade do epitélio germinal em diferenciar espermatozoides com função normal. No epidídimo, as EROs em excesso podem danificar lipídios e proteínas da membrana dos espermatozoides, alterando a permeabilidade celular e diminuindo a motilidade espermática (AITKEN e ROMAN, 2008; KAO et al., 2008; AITKEN et al., 2014). De fato, nossos resultados demonstram que o tratamento com VPA induz um

aumento do dano oxidativo a lipídios e proteínas no testículo e no epidídimo, o que se reflete na diminuição da motilidade dos espermatozoides.

Tanto o RSV como a vitamina E foram capazes de impedir o dano oxidativo induzido pelo VPA no testículo e no epidídimo. Acreditamos que o RSV inibe a peroxidação lipídica das membranas celulares, bem como a oxidação de proteínas devido à sua capacidade de eliminar diretamente algumas EROs, incluindo $O_2\bullet^-$ e OH• (LEONARD et al., 2003). Já a vitamina E, devido ao seu caráter altamente lipofílico, é capaz de posicionar-se no interior da membrana celular, onde reage rapidamente com radicais peroxila e impede a propagação da peroxidação lipídica (SCHNEIDER, 2005).

Além do dano oxidativo, o tratamento com VPA causou uma diminuição do conteúdo de GSH no testículo, bem como da capacidade antioxidante total no testículo e no epidídimo. A GSH é essencial para muitos processos celulares, e pode ser considerado como um dos agentes mais importantes do sistema de defesa antioxidante, protegendo a célula contra o dano oxidativo (HALLIWELL e GUTTERIDGE, 2007; BALLATORI et al., 2009). A diminuição dos níveis de GSH mediada por EROs durante a espermatogênese induz a uma perturbação na integridade da membrana dos espermatozoides, podendo resultar em perda de motilidade (OCHSENDORF et al., 1998; URSINI et al., 1999). A diminuição da GSH pode representar uma depleção deste antioxidante devido à sua maior utilização na remoção de H_2O_2 e outros peróxidos produzidos em excesso devido ao estresse oxidativo induzido pelo VPA. Além disso, tem sido relatado que o tratamento crônico com VPA aumenta a depuração de selênio, cobre e zinco (TABATABAEI et al., 1999). Estes metais atuam como cofatores para a glutationa e outros “scavengers” de radicais livres endógenos, o que poderia contribuir para a depleção de GSH e diminuição da capacidade antioxidante total em animais tratados com VPA. Esta deficiência na capacidade antioxidante pode expor o testículo e o epidídimo ao dano oxidativo e contribuir para a diminuição da motilidade dos espermatozoides, o que acaba afetando a função reprodutiva em geral.

O RSV e a vitamina E foram capazes de melhorar os níveis de GSH e aumentar significativamente a capacidade antioxidante do testículo e do epidídimo nos ratos tratados com VPA. O aumento da capacidade antioxidante total pelo VPA e pela

vitamina E reforça o papel destes antioxidantes como “scavengers” de radicais livres, o que contribui para o efeito protetor contra o dano oxidativo induzido pelo VPA.

Em conjunto, estes dados demonstram que o RSV e a vitamina E recuperam a motilidade espermática em animais tratados com VPA, através do aumento da capacidade antioxidante e supressão do dano oxidativo no testículo e no epidídimo. Estudos anteriores já haviam relatado que o RSV (KASDALLAH-GRISSA et al., 2006; COLLODEL et al., 2011; OURIQUE et al., 2013; MOJICA-VILLEGAS et al., 2014) e a vitamina E (EL-DEMERDASH et al., 2004; SONMEZ et al., 2007) são capazes de prevenir a perda de motilidade espermática mediada por EROs em outras situações. Além disso, Jurima-Romet e colaboradores (1996) demonstraram que a vitamina E protege os hepatócitos da citotoxicidade mediada por metabólitos do VPA por manter a homeostase celular de GSH. Este mecanismo é evidenciado por Chen e colaboradores (2014) e Hsieh e colaboradores (2014), que demonstraram que o RSV e a vitamina E são capazes de prevenir efeitos teratogênicos do VPA por suprimir a formação de EROs e aumentar os níveis de GSH.

Além de aumentar os níveis de EROs e causar estresse oxidativo, o VPA é um potente inibidor de HDACs das classes I e II (PHIEL et al., 2001). HDACs são enzimas que removem os grupos acetila a partir de resíduos de lisina de histonas (bem como outras proteínas não-histonas), levando a efeitos sobre a expressão gênica e sinalização celular. Durante a espermatogênese e a maturação do espermatozoide, ocorrem modificações epigenéticas, como metilação, acetilação e substituição de histonas por protaminas, que são essenciais para a competência funcional do espermatozoide. Por este motivo, as células espermatogênicas representam um período de vulnerabilidade, quando marcadores epigenéticos podem ser reprogramados, alterando a expressão gênica (BALE, 2015). Uma vez que não foram encontrados dados na literatura, se torna importante avaliar os efeitos do VPA nas células espermatogênicas.

Com o objetivo de elucidar o efeito do VPA sobre as células espermatogênicas, nós avaliamos os efeitos do tratamento com baixas concentrações de VPA (1 μM, 10 μM, 100 μM, 1 mM e 3 mM) *in vitro* sobre a viabilidade celular, produção de EROs,

conteúdo de tióis, acetilação de histonas e dano ao DNA em espermatócitos em paquíteno e espermátides arrendondadas isolados.

Nossos resultados demonstram que após uma hora de tratamento, baixas concentrações de VPA não causam alteração morfológica nas células avaliadas, nem alteram a viabilidade celular. No entanto, o VPA já é capaz de induzir um aumento na produção de EROs e no conteúdo de tióis em espermatócitos e espermátides arrendondadas, comprovando que o VPA altera o estado redox, causando estresse oxidativo nas células germinativas. Adicionalmente, o tratamento com VPA induziu um aumento na acetilação da histona H4 em espermatócitos e espermátides arrendondadas. A inibição de HDACs pelo VPA pode induzir uma hiperacetilação de histonas, levando à atenuação da interação eletrostática entre as histonas e o DNA, o que pode ser associado à descondensação da cromatina. A descondensação da cromatina, por sua vez, pode aumentar a susceptibilidade ao ataque de EROs e danos ao DNA, o qual pode estar associado com alterações na expressão de genes, efeitos adversos sobre as células germinativas, abortos espontâneos e resultados indesejáveis na prole. Nas concentrações utilizadas o VPA não causou dano ao DNA em espermatócitos e espermátides arrendondadas. Khan e colaboradores (2012) observaram dano ao DNA em espermatozoides de ratos tratados com VPA. No entanto, não foram encontrados na literatura dados referentes ao efeito do VPA em células germinativas isoladas.

Mais estudos são necessários para avaliar os efeitos do VPA sobre as células espermatogênicas, uma vez que alterações epigenéticas neste período podem não só afetar a fertilidade e o desenvolvimento embrionário, mas também a transmissão de características genéticas indesejadas para as próximas gerações.

5. CONCLUSÕES

5.1 Conclusão Geral

Nossos dados demonstram que tanto o RSV quanto a vitamina E são capazes de prevenir a perda de motilidade espermática induzida pelo uso de VPA através da manutenção do conteúdo de GSH, aumento da capacidade antioxidante, e supressão do dano oxidativo nos testículos e epidídimo. Desta maneira, o co-tratamento com RSV ou vitamina E pode ser uma alternativa para pacientes que necessitam de tratamento prolongado com VPA, com o objetivo de preservar a motilidade dos espermatozoides e a função testicular.

Além disso, nossos resultados demonstram que o tratamento com VPA *in vitro* está associado com estresse oxidativo e hiperacetilação de histonas em espermatócitos em paquíteno e espermatídes arredondadas, sem provocar danos ao DNA.

Verificar o efeito do RSV e da vitamina E no peso do testículo, epidídimo, próstata e vesícula seminal de ratos adultos tratados com VPA;

5.2 Conclusões Específicas

- O VPA, o RSV e a vitamina E não afetam os níveis plasmáticos de testosterona em ratos adultos;
- O VPA e a vitamina E não possuem efeito na morfologia testicular;
- O VPA e a vitamina E não afetam os níveis plasmáticos de aspartato aminotransferase e alanina aminotransferase;
- O RSV e a vitamina E previnem a diminuição na motilidade espermática induzida pelo VPA, sem afetar a quantidade e a morfologia dos espermatozoides;
- O RSV e a vitamina E previnem o dano oxidativo induzido pelo VPA em testículos e epidídimo através do aumento dos níveis de GSH e melhora na capacidade antioxidante total destes órgãos;
- O VPA induz aumento na produção de EROs, no conteúdo de GSH e na acetilação de histonas em espermatócitos e espermátides isolados, sem causar dano ao DNA.

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COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Efeito do resveratrol e da vitamina E frente a prejuízos reprodutivos causados pelo uso de valproato de sódio em ratos machos adultos."

Número do Parecer: 076/2013

Pesquisador Responsável: Profa. Kátia Padilha Barreto

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 12/12/2013.

Santa Maria, 12 de dezembro de 2013.

Prof. Dr. Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais- UFSM

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