Elisângela Martins dos Santos

EFEITOS DE ATRAZINA COMO DESREGULADOR ENDÓCRINO NA HOMEOSTASE TECIDUAL E NOS PERFIS MORFOLÓGICO E ESTEROIDOGÊNICO DE TESTÍCULOS, DÚCTULOS EFERENTES E PRÓSTATA VENTRAL DE RATOS ADULTOS

Instituto de Ciências Biológicas
Universidade Federal de Minas Gerais
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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de Doutor em Ciências.

Área de concentração: Biologia Celular

Orientadora: Dra. Cleida Aparecida de Oliveira

Instituto de Ciências Biológicas

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Esta tese foi realizada no Laboratório de Biologia da Reprodução do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, sob a orientação da Profa. Dra. Cleida Aparecida de Oliveira, e contou com o auxílio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico e Ministério da Agricultura, Pecuária e Abastecimento (CNPq/MAPA-Edital 64), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) e Pró-Reitoria de Pesquisa da UFMG (PRPq-UFMG).

Universidade Federal de Minas Gerais - Instituto de Ciências Biológicas
Programa de Pós-Graduação em Biologia Celular

ATA DA DEFESA DE TESE DE DOUTORADO DE

ELISÂNGELA MARTINS SANTOS

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Às **quatorze horas** do dia **28 de setembro de 2017**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"EFEITOS DE ATRAZINA COMO DESREGULADOR ENDÓCRINO NA HOMEOSTASE TECIDUAL E NOS PERFIS MORFOLÓGICO E ESTEROIDOGÊNICO DE TESTICULOS, DÚCTULOS EFERENTES E PRÓSTATA VENTRAL DE RATOS ADULTOS "**, requisito final para obtenção do grau de Doutora em Biologia Celular, área de concentração: **Biologia Celular.** Abrindo a sessão, a Presidente da Comissão, **Dra. Cleida Aparecida de Oliveira**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dra. Cleida Aparecida de Oliveira	UFMG	Aprovada
Dr. José Carlos Nogueira	FASEH	Aprovada
Dr. Laércio dos Anjos Benjamin	UFV	Aprovada
Dra. Elizete Rizzo	UFMG	APROVADA
Dra. Tânia Mara Segatelli	UFMG	Aprovada

Pelas indicações, a candidata foi considerada: Aprovada

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 28 de setembro de 2017.**

Drª. Cleida Aparecida de Oliveira (Orientadora) _____

Dr. José Carlos Nogueira _____

Dr. Laércio dos Anjos Benjamin _____

Drª. Elizete Rizzo _____

Drª. Tânia Mara Segatelli _____

Obs: Este documento não terá validade sem a assinatura e carimbo do Coordenador.

Profa. Luciana de Oliveira Andrade
COORDENADORA DO PROGRAMA DE
PÓS GRADUAÇÃO EM BIOLOGIA CELULAR ICB/UFMG

Esta tese foi realizada no Laboratório de Biologia da Reprodução do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, sob a orientação da Profa. Dra. Cleida Aparecida de Oliveira, e contou com o auxílio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico e Ministério da Agricultura, Pecuária e Abastecimento (CNPq/MAPA-Edital 64), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) e Pró-Reitoria de Pesquisa da UFMG (PRPq-UFMG).

"A chave para qualquer problema biológico tem que ser procurada, em última instância, na célula; pois todo o organismo vivo é, ou já foi um dia, uma célula" Wilson, E. B.

"Se fosse fácil achar o caminho das pedras, tantas pedras no caminho não seriam ruins." Gessinger, H.

À minha querida orientadora, que me direcionou e mostrou-me o caminho das pedras!

À minha família, que é meu alicerce, minha base sólida. Queridos, gratidão sempre!

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RESUMO

Atrazina é um potente desregulador endócrino que afeta a esteroidogênese testicular e causa aumento transitório do peso do testículo seguido de atrofia, além de redução de 3β-HSD, redução nos níveis de testosterona e aumento estradiol, sendo postulado que aromatase possa ser alvo desse herbicida. Ainda não se sabe se os efeitos observados nos testículos são reversíveis ou permanentes, nem se esses efeitos são primários ou secundários a alterações em segmentos pós-testiculares, como os dúctulos eferentes, o que objetivamos esclarecer nesse estudo. Para fins de comparação analisamos também a próstata ventral. Foram utilizados ratos Wistar machos, adultos, que receberam dose diária de atrazina 200/mg/Kg durante 7, 15 e 40 dias. Adicionalmente, nós avaliamos se os efeitos de atrazina, poderiam ser reversíveis após interrupção da exposição por 75 dias, período esse maior que o ciclo espermatogênico de ratos (58 dias). A exposição ao atrazina resultou em aumento transitório de peso testicular, dilatação luminal, seguida de atrofia dos túbulos seminíferos, bem como redução de 3β-HSD e aumento de aromatase em células de Leydig. A atrofia testicular e redução de 3β-HSD foram mais pronunciadas após o período de recuperação, em contraste com a expressão de aromatase que retornou ao nível do controle. Além disso, houve aumento de 89%, 76% e 42% nas subpopulações de macrófagos $ED1^{-}/ED2^{+}$ residentes, $ED1^{+}/ED2^{+}$ transitórios e $ED1^{+}/ ED2^{-}$ induzidos, respectivamente, os quais foram positivos para 3β-HSD, indicando a possibilidade do envolvimento destas células na esteroidogênese. A exposição ao atrazina resultou ainda em efeitos discretos na próstata ventral, mas com alterações morfológicas marcantes nos dúctulos eferentes e desequilíbrio da homeostase tecidual, coincidente com aumento da expressão da aromatase. Esses achados enfatizam que os danos testiculares provocados por atrazina podem ser irreversíveis mesmo após um período de recuperação maior que o ciclo espermatogênico, e sugerem que pelo menos parte dos efeitos testiculares provocados por atrazina podem ser secundários a alterações nos dúctulos eferentes.

Palavras-chave: Atrazina, Esteroidogênese, Homeostase tecidual, Testículos, Dúctulos eferentes, Prostata ventral, Rato Wistar

ABSTRACT

Atrazine is a potent endocrine disruptor that affects testicular steroidogenesis, and causes transient increase in testicular weight followed by atrophy, besides reduction of 3β-HSD, decrease in testosterone and increase in estradiol levels, being postulated that aromatase may be a target of atrazine. It is not known whether the effects observed on the testis are reversible or permanent, nor whether these effects are primary or secondary to changes in postesticular segments, as the efferent ductules, what we aim to clarify in this study. For comparison purposes, we also evaluated the ventral prostate. Adult male Wistar rats receiving atrazine at the dosage of 200/mg/Kg for 7, 15, or 40 days were used. Additionally, we evaluated if the effects of atrazine in these target organs could be reversible after discontinuation of the exposure for a period of 75 days, period longer than the spermatogenic cycle of rats (58 days). The exposure to atrazine resulted in transient increased in testicular weight, luminal dilation, followed by seminiferous tubule atrophy, as well as 3β-HSD reduction and increase in aromatase in the Leydig cells. The testicular atrophy and 3β-HSD reduction were more pronounced after the recovery period, in contrast with aromatase that returned to control levels. Moreover, there was increase of 89%, 76%, and 42% in macrophages subpopulations $ED1^{-}/ED2^{+}$ resident, $ED1^{+}/ED2^{+}$ transitory and $ED1^{+}/ED2^{-}$ induced, respectively, which were positive for 3β-HSD, raising the possibility of their involvement on steroidogenesis. The exposure to atrazine resulted in mild effects on the ventral prostate, but remarkable morphological alterations on the efferent ductules and disruption on tissue homeostasis, coincident with increase in aromatase expression. These findings further emphasize that the testicular damages caused by atrazine may be irreversible even after a recovery period longer than the spermatogenic cycle, and suggest that at least part of the testicular effects of atrazine may be secondary to the alterations in the efferent ductules.

Key words: Atrazine, Steroidogenesis, Tissue homeostasis, Testes, Efferent ductules, Ventral prostate, Wistar rat

SUMÁRIO

I. INTRODUÇÃO GERAL E JUSTIFICATIVA

Diversos compostos de ocorrência natural e sintéticos possuem o potencial de desregular a homeostase da fisiologia endócrina. Tais compostos exógenos são denominados desreguladores endócrinos, por interferirem na síntese, estocagem, metabolismo, transporte e eliminação dos hormônios naturais, bem como em seus receptores (Toppari, 2008). Os desreguladores endócrinos podem afetar mais de um hormônio ou componentes diferentes de uma mesma via endócrina, algumas vezes com efeitos antagônicos. São encontrados em produtos diversos tais como anticoncepcionais, pesticidas, plásticos, tintas, detergentes, protetores solares, bem como em produtos alimentícios, incluindo os fitoestrógenos (Norgil Damgaard *et al.*, 2002; Toppari, 2008; Muncke, 2011; Krause *et al.*, 2012). Dentre esses desreguladores endócrinos destaca-se o atrazina.

Atrazina é um componente ativo de herbicidas amplamente utilizado na agricultura, no entanto, é considerado um potente desregulador endócrino por causar distúrbios reprodutivos em vertebrados (Kniewald *et al.*, 2000; Stoker *et al.*, 2000; Trentacoste *et al.*, 2001; Ashby *et al.*, 2002; Hayes *et al.*, 2002; Stoker *et al.*, 2002; Tavera-Mendoza *et al.*, 2002; Betancourt *et al.*, 2006; Swan, 2006; Suzawa e Ingraham, 2008; Rey *et al.*, 2009; Hayes *et al.*, 2010; Hussain *et al.*, 2010; Stanko *et al.*, 2010; Tillitt *et al.*, 2010; Hayes *et al.*, 2011; Papoulias *et al.*, 2014; Richter *et al.*, 2016).

Neste sentido, já foi demonstrado que a exposição à atrazina 200mg/Kg por um curto período de tempo (15 dias) leva a um aumento transitório no peso dos testículos de ratos, seguida de redução significativa após um período mais longo de exposição (40 dias), que resulta na atrofia do órgão (Victor-Costa *et al.*, 2010).

Os efeitos na morfologia testicular ocorreram paralelo à redução dos níveis plasmáticos e testiculares de testosterona e aumento de estradiol (Victor-Costa *et al.*, 2010; Jin *et al.*, 2013).

Paralelo às alterações morfológicas houve redução da enzima 3β-hidroxiesteroide desidrogenase (3β-HSD), o que pode explicar a marcante redução dos níveis de testosterona em animais expostos ao herbicida (Victor-Costa *et al.*, 2010). Especula-se a indução da expressão de aromatase como um mecanismo plausível para explicar o aumento dos níveis de estradiol em animais expostos à atrazina (Crain *et al.*, 1997; Sanderson *et al.*, 2000; Sanderson *et al.*, 2001; Hayes *et al.*, 2002; Sanderson *et al.*, 2002; Heneweer *et al.*, 2004; Hayes *et al.*, 2006; Laville *et al.*, 2006; Sanderson, 2006; Fan *et al.*, 2007a; Fan *et al.*, 2007b; Holloway *et al.*, 2008; Tinfo *et al.*, 2011). Esses dados em conjunto confirmam atrazina como importante fator de risco para o equilíbrio endócrino e sugerem que a inibição de 3β-HSD e indução de aromatase podem representar mecanismos através dos quais esse herbicida afeta a esteroidogênese testicular, levando a infertilidade. No entanto, alguns pontos ainda merecem investigações mais profundas.

As alterações histopatológicas observadas nos testículos, como o aumento transitório de peso correlacionado com a dilatação dos túbulos seminíferos, seguido por grande redução no peso testicular paralelo à completa atrofia do órgão podem estar relacionadas com distúrbios na fisiologia dos dúctulos eferentes, os quais são altamente sensíveis ao desequilíbrio nos níveis de estrógenos e seus receptores (Hess *et al.*, 1997a; Hess *et al.*, 2000; Oliveira *et al.*, 2001). Sabe-se que o distúrbio na reabsorção de fluido pelos dúctulos eferentes

leva a acúmulo de fluido no lúmen e consequentemente, refluxo para o testículo, resultando na dilatação dos túbulos seminíferos seguida de atrofia (Hess *et al.*, 1997a). Desta forma, para melhor entender a ação de atrazina nos testículos torna-se, importante investigar também as possíveis alterações morfológicas e moleculares nas vias espermáticas, como os dúctulos eferentes, para esclarecer se os efeitos testiculares de exposição ao atrazina são primários ou decorrentes de disfunção nas vias espermáticas.

Ainda, após exposição crônica a atrazina, nos testículos foram detectadas células semelhantes a macrófagos no espaço intersticial, marcados fortemente pela enzima 3β-HSD (Martins-Santos, 2013). Sabe-se que, em condições normais, macrófagos desempenham importante papel no sistema genital masculino de mamíferos, estando relacionados com a estimulação da esteroidogênese pelas células de Leydig e com a manutenção do ambiente imunologicamente privilegiado dos testículos (Hutson, 1990; Nes *et al.*, 2000; Lukyanenko *et al.*, 2001; Chen *et al.*, 2002; Lukyanenko *et al.*, 2002; Khan e Rai, 2008). Alterações ultra-estruturais nas células de Leydig e em macrófagos testiculares, bem como na sua interação, foram observadas em ratos adultos expostos ao herbicida atrazina (Victor-Costa *et al.*, 2010). Identificar e esclarecer a presença dessas células seria de grande relevância uma vez que elas podem estar envolvidas com o processo de fagocitose das células de Leydig que sofreram dano, sem, no entanto, descartar a possibilidade de as mesmas estarem engajadas na tentativa de recuperação da esteroidogênese afetada.

As alterações testiculares sugerem desequilíbrio entre proliferação e morte celular, principalmente pela presença de células gigantes e multinucleadas e pela

elevada frequência de figuras de apoptose nos túbulos seminíferos, fato que se soma a resultados anteriores que indicam que a atrazina afeta a homeostase tecidual (Zhang *et al.*, 2011; Song *et al.*, 2015). Aprofundar essas informações, esclarecendo os efeitos do herbicida sobre a proliferação e morte celular em órgãos chave, como os testículos, dúctulos eferentes e próstata, poderão adicionar conhecimentos sobre os possíveis mecanismos que levam aos efeitos adversos da atrazina em tecidos animais.

A próstata é um tecido dependente de andrógenos, porém, já é conhecido que os estrógenos também apresentam papel importante na diferenciação e crescimento da glândula (Ellem e Risbridger, 2010), sendo que o desequilíbrio entre andrógenos/estrógenos pode levar a alterações histopatológicas marcantes, incluindo o desenvolvimento de neoplasias prostáticas (Mcpherson *et al.*, 2006; Mcpherson *et al.*, 2008).

A aromatase é uma enzima determinante para controlar essa homeostase entre andrógenos e estrógenos na próstata. Desta forma, postula-se que atrazina seja indutor da expressão de aromatase em algumas linhagens celulares provenientes de câncer (Sanderson *et al.*, 2000; Sanderson *et al.*, 2001; Sanderson *et al.*, 2002; Heneweer *et al.*, 2004; Fan *et al.*, 2007a; Fan *et al.*, 2007b; Tinfo *et al.*, 2011; Quignot *et al.*, 2012; Thibeault *et al.*, 2014) e correlacionado com alto risco de desenvolvimento de câncer de próstata (Hu *et al.*, 2016). No entanto, não foram encontradas informações sobre os níveis de aromatase em tecido prostático exposto a atrazina.

Torna-se, portanto, importante explorar melhor a possível associação entre atrazina e alterações morfológicas e hormonais na próstata, visando esclarecer se

os efeitos de atrazina se estendem a outras estruturas do sistema genital masculino ou são restritos aos testículos.

II. REVISÃO DE LITERATURA

1. Sistema Genital Masculino

O sistema genital masculino de mamíferos eutérios é composto pelos testículos, vias espermáticas, glândulas anexas e órgão copulador, sendo sua principal função a produção contínua, nutrição e armazenamento temporário de espermatozoides, bem como a condução dos espermatozoides até as vias genitais femininas (Setchell e Breed, 2006).

Testículos são órgãos responsáveis pela produção de espermatozoides, bem como produção dos hormônios sexuais, por meio da esteroidogênese (Russel *et al.*, 1990).

As vias espermáticas incluem túbulos retos, rede testicular, dúctulos eferentes, epidídimos, ductos deferentes e uretra, por onde os espermatozoides produzidos nos testículos são conduzidos até as vias genitais femininas, durante a cópula (Setchell e Breed, 2006).

Próstata, vesículas seminais e glândulas bulbouretrais são as glândulas sexuais anexas de mamíferos eutérios, órgãos responsivos principalmente a andrógenos, cuja principal função é a produção dos componentes do plasma seminal, importante meio pelo qual os espermatozoides serão conduzidos até as vias genitais femininas (Setchell e Breed, 2006), bem como servirá para nutrir e manter os espermatozoides (Mann, 1974).

Os principais órgãos-alvo deste estudo incluem os testículos, dúctulos eferentes e próstata ventral, por esta razão serão abordados de forma mais detalhada a seguir.

1.1 Testículos

Os testículos correspondem à gônada masculina e possuem funções espermatogenica e esteroidegênica, sendo responsáveis tanto pela produção continua de espermatozoides, quanto pela produção de andrógenos, que são essenciais para a manutenção da fertilidade masculina e características sexuais secundárias (Russel *et al.*, 1990; Russell e Franca, 1995). Além disso, produzem os estrógenos a partir da aromatização de andrógenos, e constitui uma das principais fontes desse esteroide nos machos (Carreau *et al.*, 2003; Hess, 2003). Morfologicamente, o testículo é envolto por uma cápsula de tecido conjuntivo denso, denominada túnica albugínea, da qual partem septos fibrosos para o interior do parênquima testicular, dividindo o órgão em lóbulos. O parênquima testicular exibe dois compartimentos principais: (1) o compartimento dos túbulos seminíferos e (2) o compartimento intertubular ou intersticial (Russel *et al.*, 1990; Kerr *et al.*, 2006).

O compartimento dos túbulos seminíferos é formado pelos túbulos seminíferos, nos quais ocorre a espermatogênese, que consiste em um processo cíclico e altamente organizado, em que células germinativas imaturas proliferam, sofrem meiose e se diferenciam para dar origem aos espermatozoides (O'donnell *et al.*, 2001). Cada túbulo seminífero é constituído pela túnica própria e pelo epitélio seminífero, delimitando o lúmen tubular (Russel *et al.*, 1990; Kerr *et al.*, 2006). A túnica própria possui uma ou mais camadas de células mióides contráteis, que varia de acordo com a espécie, e matriz extracelular formada por fibras colágenas, elásticas e substância fundamental amorfa (Bustos-Obregon, 1976; Dym, 1976; Christl, 1990). O epitélio seminífero é constituído por camadas

concêntricas de células germinativas intimamente associadas à célula somática de Sertoli (Hess e Franca, 2008). O processo de desenvolvimento das células germinativas ocorre em estreita interação com as células de Sertoli que exercem funções especializadas relacionadas à manutenção, proliferação e diferenciação das células germinativas, a regulação da espermatogênese (Russell *et al.*, 1993; Skinner e Anway, 2005), bem como conservação de um ambiente imunologicamente privilegiado no testículo (Hess e Franca, 2008).

O compartimento intertubular é formado pelas células de Leydig, associadas a vasos sanguíneos e linfáticos, que são essenciais para o transporte de hormônios e nutrientes no testículo, além de fibras e células de tecido conjuntivo, ocasionalmente mastócitos e, sobretudo, macrófagos (Russell *et al.*, 1998). As células de Leydig possuem abundante retículo endoplasmático liso e mitocôndrias com cristas tubulares, contendo enzimas associadas à síntese de hormônios esteroides (Russel *et al.*, 1990), como a 3β-hidroxiesteroide desidrogenase (3β-HSD), responsável por diversos passos na cascata esteroidogênica, resultando na formação da testosterona (Simard *et al.*, 2005).

1.1.1 Macrófagos Testiculares

No interstício testicular é notável a presença de macrófagos capazes de desempenhar funções intimamente relacionadas ao microambiente testicular, devido à sua alta plasticidade (Mosser e Edwards, 2008). Sabe-se que, em condições normais, macrófagos exercem importante papel na manutenção de um ambiente imunologicamente privilegiado nos testículos, bem como estimulação da esteroidogênese pelas células de Leydig (Hutson, 1990; Nes *et al.*, 2000;

Lukyanenko *et al.*, 2001; Chen *et al.*, 2002; Lukyanenko *et al.*, 2002; Khan e Rai, 2008; Defalco *et al.*, 2015). Macrófagos testiculares expressam 25-hidroxilase (Lukyanenko *et al.*, 2001), enzima que oxida o carbono 25 do colesterol produzindo 25-hidroxicolesterol (Lund *et al.*, 1998; Russell, 2000), um oxisterol que é prontamente convertido em testosterona pelo complexo P450 SCC (*Cholesterol Side-chain clivage complex*) no interior das células de Leydig. Nesse sentido, macrófagos testiculares podem, então, oferecer uma via alternativa para a produção de hormônios esteroides, fornecendo 25-hidroxicolesterol como substrato direto para a cadeia de clivagem lateral (P450scc), produzindo pregnenolona, que será então metabolizada a testosterona (Nes *et al.*, 2000). Além disso, 25-hidroxicolesterol é um fator capaz de induzir a produção de 3β-HSD pelas células de Leydig (Nes *et al.*, 2000; Lukyanenko *et al.*, 2001; Chen *et al.*, 2002; Lukyanenko *et al.*, 2002).

Em ratos, macrófagos testiculares são identificados pela expressão dos receptores CD68 (ED1) e CD163 (ED2), que constituem as populações induzida e residente, respectivamente (Wang *et al.*, 1994). A dinâmica de desenvolvimento de macrófagos no testículo de ratos adultos passa por diversas etapas, como descrito a seguir. Os macrófagos recentemente migrados ou induzidos exibem o fenótipo $ED1^{+}/ED2^{-}$. Estes passam por uma fase transitória na qual expressam ambos os fenótipos $ED1^{+}/ED2^{+}$. Subsequentemente eles adotam um caráter anti-inflamatório e imunossupressor e passam a expressar o fenótipo $ED1^{-}/ED2^{+}$, tornando-se então, residentes no tecido (Hedger, 2002).

1.2 Dúctulos Eferentes

Os dúctulos eferentes, descritos pela primeira vez por De Graaf, em 1668, conectam à rede testicular ao epidídimo (Hermo e Robaire, 1988; Hess, 2002). Os dúctulos partem da rede testicular como delgados ductos individuais, cujo número varia de acordo com a espécie, no caso de ratos, há uma variação de 4 a 8 dúctulos (Ilio e Hess, 1994). São dúctulos retilíneos ou delicadamente ondulados, dispostos paralelamente entre si, mas à medida que se distanciam do seu ponto de origem esses túbulos tornam-se intensamente flexuosos formando assim o cone vasculoso. A partir daí, unem-se para formar um único dúctulo terminal, que é mais retilíneo e estreito e continua-se no ducto epididimário (Jones e Jurd, 1987; Guttroff *et al.*, 1992).

Devido a variações exibidas ao longo de seu comprimento, os dúctulos eferentes são categorizados morfologicamente em três regiões distintas: (1) região proximal ou segmento testicular, (2) região do cone vasculoso ou intermediária e (3) região terminal ou segmento epididimário, também chamada de região distal, por estar mais distante do seu ponto de origem. A região proximal apresenta dúctulos com lúmen amplo, grande quantidade de células não ciliadas e com abundantes lisossomos. A partir da região intermediária para a região distal, o lúmen se estreita, ocorre redução no número de células não ciliadas, bem como de lisossomos citoplasmáticos (Ilio e Hess, 1994).

Histologicamente, os dúctulos eferentes de ratos são formados por uma camada peritubular, constituída por tecido conjuntivo, fibras colágenas e duas a três camadas de células musculares lisas concentricamente arranjadas. São revestidos internamente por um epitélio simples colunar composto por células

ciliadas e não ciliadas (Ilio e Hess, 1994). De modo geral, as células não ciliadas são mais abundantes e caracterizadas principalmente pela presença de numerosas microvilosidades, aparelho endocítico bem desenvolvido, bem como numerosos lisossomos localizados na porção supranuclear do citoplasma, além de núcleo oval, localizado na porção basal. Essas características marcantes estão relacionadas com a principal função atribuída à célula não cilada que é a reabsorção de fluido luminal, além de endocitose (Hermo e Morales, 1984; Veeramachaneni e Amann, 1991; Hermo *et al.*, 1994). A célula ciliada, por sua vez, apresenta citoplasma mais escuro em comparação à célula não ciliada, núcleo localizado na região apical, sendo sua principal característica a presença de numerosos cílios em sua superfície livre, os quais movimentam o fluido luminal em várias direções (Talo, 1981; Chen *et al.*, 1998), o que parece facilitar a absorção de fluidos luminais. Apresentam poucas microvilosidades entre os cílios. O lúmen dos dúctulos eferentes encontra-se aparentemente vazio ou com poucos espermatozoides, que se concentram mais nas porções distais, na medida que ocorre a reabsorção do fluido luminal (Talo, 1981; Ilio e Hess, 1994).

Desta forma, os dúctulos eferentes além de servirem como via de condução dos espermatozoides recém-formados no testículo para o epidídimo, desempenham importante papel na reabsorção de fluido testicular, um processo finamente regulado por estrógenos (Hess *et al.*, 1997a; Hess *et al.*, 2000; Lee *et al.*, 2001; Oliveira *et al.*, 2001; Zhou *et al.*, 2001; Oliveira *et al.*, 2002; Oliveira *et al.*, 2005). Os estrógenos exercem, ainda, papel essencial na manutenção da morfologia e motilidade espermática (Joseph *et al.*, 2010). Ambas as funções dependem da expressão de íons, tais como Na^+/K^+, e proteínas transepiteliais de

transporte passivo de água, denominadas aquaporinas. Juntos, esses fatores estabelecem pH ideal, osmolaridade e concentração espermática adequados no ambiente luminal (Hess *et al.*, 1997a; Zhou *et al.*, 2001; Oliveira *et al.*, 2005). As funções dos estrógenos, presentes em níveis elevados no fluido luminal, são mediadas pelos receptores de estrógenos ERα e ERβ (Hess *et al.*, 1997a), especialmente ERα, presente em níveis elevados no epitélio dos dúctulos eferentes (Hess *et al.*, 1997b). Além disso, a presença da enzima aromatase nos dúctulos eferentes já foi demonstrada (Carpino *et al.*, 2004; Oliveira *et al.*, 2012), destacando a importância da produção local de estrógenos neste segmento. A inativação de receptores de estrógenos e/ou exposição a compostos estrogênicos ou antiestrogênicos estão entre as principais causas de infertilidade em machos, por levar a distúrbios nos dúctulos eferentes (Hess *et al.*, 1997a; Hess *et al.*, 1997b; Lee *et al.*, 2001; Mckinnell *et al.*, 2001; Oliveira *et al.*, 2001; Zhou *et al.*, 2001; Oliveira *et al.*, 2002; Hinton *et al.*, 2011; Nanjappa *et al.*, 2016). Portanto, a ação de estrógenos nesse segmento do trato genital essencial para a manutenção da fertilidade masculina (Hess *et al.*, 1997a; Oliveira *et al.*, 2001; Oliveira *et al.*, 2002).

1.3 Próstata

Em roedores, a próstata é organizada em 4 diferentes lobos bilaterais, os quais são classificados de acordo com a posição anatomotopográfica em relação à uretra de animais quadrúpedes como sendo: próstata ventral, lateral, dorsal e anterior (Risbridger e Taylor et al., 2006). A próstata ventral será o foco deste estudo por ser o lobo que é naturalmente mais acometido por neoplasia

intraepitelial, hiperplasia, atrofia epitelial, bem como dilatação luminal, resultando em alterações morfológicas na estrutura da glândula prostática (Morais-Santos *et al.*, 2015).

Histologicamente, a próstata é constituída por um complexo arranjo de adenômeros túbulo-acinosos ramificados, sustentados por estroma de tecido conjuntivo, contendo nervos, vasos sanguíneos e linfáticos, bem como células do sistema imune (Hayward *et al.*, 1996). Os adenômeros são revestidos por epitélio pseudoestratificado colunar, formado por células secretoras ou luminais, basais e neuroendócrinas, sendo as células luminais as mais abundantes ao longo do epitélio prostático (Risbridger e Taylor, 2006; Signoretti e Loda, 2006).

A próstata é uma glândula dependente de andrógenos, testosterona e, especialmente, diidrotestosterona (DHT), que é produzida localmente via metabolização da testosterona pela enzima 5α-redutase (Thigpen *et al.*, 1993; Torres *et al.*, 2003). Os estrógenos também desempenham importante papel na diferenciação e crescimento da glândula, sendo que o desequilíbrio entre andrógenos/estrógenos pode levar a alterações histopatológicas marcantes, incluindo o desenvolvimento de neoplasias prostáticas (Mcpherson *et al.*, 2006; Mcpherson *et al.*, 2008; Morais-Santos *et al.*, 2015). A importância do equilíbrio adequado no balanço entre andrógenos e estrógenos já tem sido bem demonstrada. Por exemplo, níveis elevados de testosterona associada a ausência de estrógenos levam ao desenvolvimento de hipertrofia e hiperplasia, mas não malignidade. Em contraste, altos níveis de estrógenos e baixos níveis de testosterona podem levar ao desenvolvimento de inflamação e induzir lesões pré-malignas (Ellem e Risbridger, 2010). A manutenção do equilíbrio adequado na

razão entre andrógenos e estrógenos é ainda mais complexa, pois envolve a ação diferencial de ambos os receptores de estrógenos ERα e ERβ na próstata. A ativação de ERα induz proliferação aberrante, inflamação e desenvolvimento de lesões pré-malignas, por outro lado, a ativação de ERβ é relacionada a uma ação anti-proliferativa, anti-inflamatória e, potencialmente, a efeitos anticarcinogênicos que equilibram as ações de ERα bem como de andrógenos. Além disso, a ação de aromatase local é determinante para controlar essa homeostase entre andrógenos e estrógenos na próstata (Ellem *et al.*, 2004; Ellem *et al.*, 2009; Ellem e Risbridger, 2010).

2. Esteroidogênese

A esteroidogênese é o processo pelo qual a molécula de colesterol passa por uma série de transformações para formação de hormônios esteroides, o que ocorre em duas etapas: (1) transporte de colesterol para as mitocôndrias e formação de pregnenolona e (2) metabolismo de pregnenolona por enzimas esteroidogênicas em tecidos específicos, tais como glândulas adrenais, gônadas, tecido adiposo e sistema nervoso central. Na primeira etapa ocorre o transporte de colesterol do citoplasma para o interior da mitocôndria através da proteína StAR (*steroidogenic acute regulatory protein*) juntamente com a proteína translocadora TSPO (*translocator protein*), proteína localizada na membrana mitocondrial externa e que possui elevada afinidade pelo colesterol. Quando transportado para a matriz mitocondrial, o colesterol é clivado em pregnenolona, pela enzima CYP11A (enzima de clivagem da cadeia lateral do colesterol ou *P450scc – side chain cleavage*) (Fig. 1) (Martinez-Arguelles e Papadopoulos, 2010).

A pregnenolona livre difunde para fora da mitocôndria e é usada como precursor imediato para a formação dos hormônios esteroides (glicocorticoides, mineralocorticoides, andrógenos, estrógenos, progestógenos). A partir dessa etapa, o tipo de hormônio produzido vai depender da disponibilidade e ação de enzimas esteroidogênicas tecido-específico, resultando na produção final de diferentes hormônios esteroides com efeitos fisiológicos distintos (Stocco, 2000).

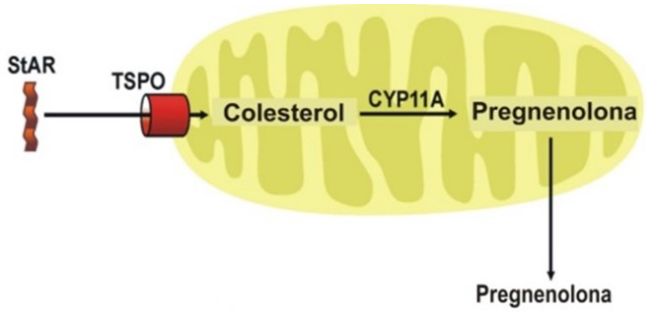

Figura 1: Transporte de colesterol para a mitocôndria e formação de pregnenolona. Adaptado de Martinez-Arguelles e Papadopoulos, 2010 – por Martins-Santos et al., 2013.

Uma das principais enzimas presentes na cascata esteroidogênica é a enzima 3β-hidroxiesteroide desidrogenase (Fig. 2), responsável por vários estágios na via esteroidogênica, incluindo a conversão de pregnenolona em progesterona (Simard *et al.*, 2005). Considerando os testículos, pregnenolona e progesterona são os precursores na biossíntese de testosterona e, subsequentemente, estradiol (Martinez-Arguelles e Papadopoulos, 2010), pela ação da enzima P450-aromatase (Carreau *et al.*, 2003).

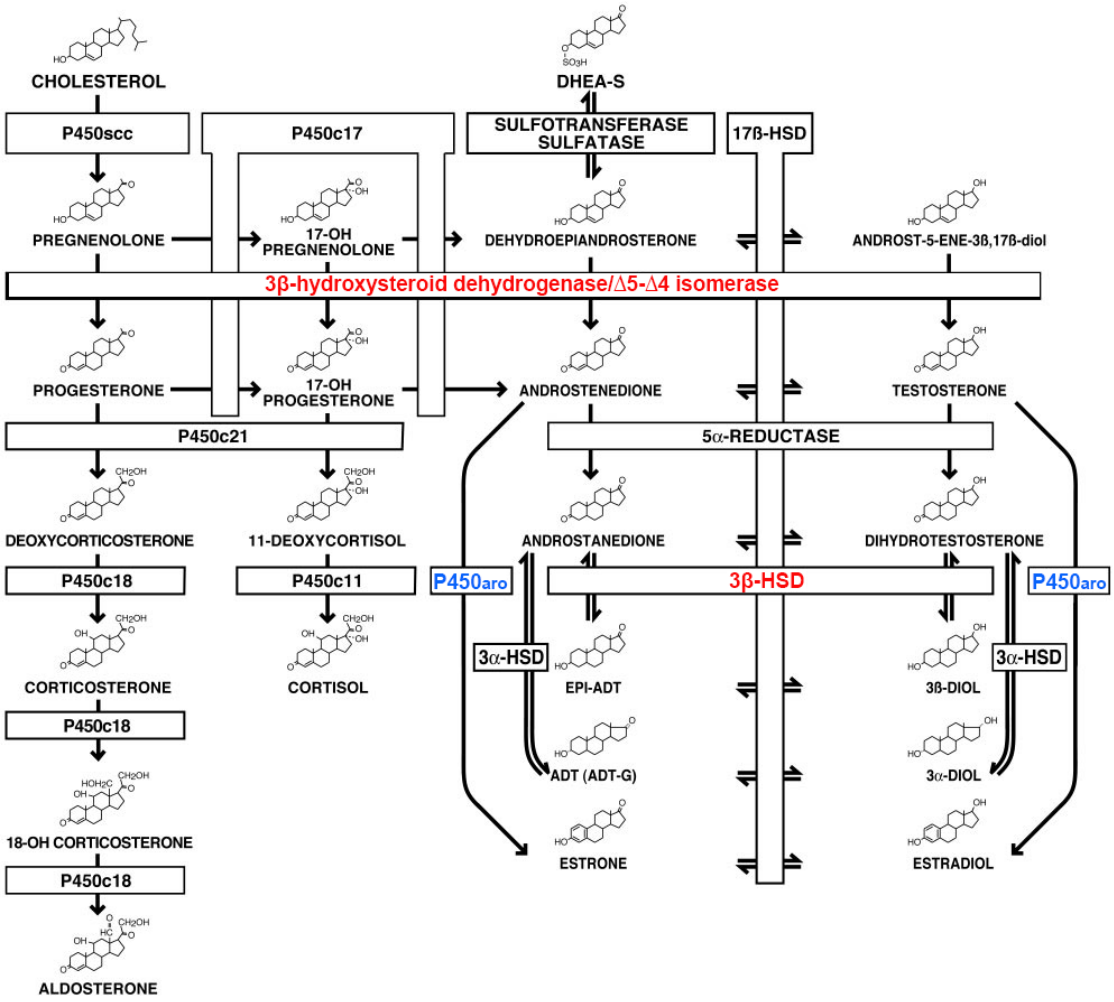

Figura 2: Representação esquemática das principais vias esteroidogênicas em mamíferos. Adaptado de Simard, 2005.

2.1 Enzima 3β-HSD

A enzima 3β-hidroxiesteroide desidrogenase/Δ^5-Δ^4isomerase (3β-HSD) pertence a uma grande família de proteínas denominadas dehidrogenases/redutases. É uma enzima chave na cascata esteroidogênica, reconhecida como um marcador para as células secretoras de esteroides por participar de etapas essenciais na formação de todas as classes de hormônios esteroides (Zhao *et al.*, 1991; Simard *et al.*, 2005).

Em humanos, apresenta duas isoformas, sendo a 3β-HSD tipo I, localizada na placenta e tecidos periféricos como a pele, glândula mamária e próstata, e a isoforma tipo II que é predominantemente expressa na adrenal e gônadas. As isoformas 3β-HSD tipo I e II são codificadas pelos genes *HSD3 B1* e *HSD3 B2*, respectivamente, e compartilham 93,5% de homologia, sendo que a 3β-HSD tipo I tem 372 aminoácidos e a 3β-HSD tipo II, com 371 aminoácidos (Pelletier *et al.*, 1992; Simard *et al.*, 2005). Quatro isoformas para 3β-HSD, com distribuição tecido-específico, são encontradas no rato. As isoformas tipo I e II são expressas no tecido adiposo, gônadas, adrenais, rim, placenta, e útero, enquanto a isoforma tipo III é expressa somente no fígado de machos e a isoforma tipo IV é expressa na placenta e pele (Simard *et al.*, 2005). As enzimas 3β-HSD tipo I e II de ratos possuem 94% de homologia na sequência de aminoácidos, tendo peso molecular de 41,9 e 42,1 kDa, respectivamente. A isoforma tipo I é considerada a mais ativa (Zhao *et al.*, 1991).

Nos testículos, a expressão de 3β-HSD ocorre nas células de Leydig, associada à membrana mitocondrial interna e ao retículo endoplasmático liso (Pelletier *et al.*, 1992). A 3β-HSD marca a diferenciação das células precursoras de Leydig em progenitoras, e à medida que prosseguem em diferenciação para células maduras, a expressão e a atividade de 3β-HSD aumentam concomitante com o aumento do volume celular e com o potencial para secretar testosterona (Mendis-Handagama e Ariyaratne, 2001). Atividade de 3β-HSD nos testículos é essencial para a regulação de esteroidogênese e, subsequentemente, para a manutenção da fertilidade em machos (Rasmussen *et al.*, 2013). Nesse sentido, ressalta-se que a expressão e a atividade enzimática de 3β-HSD, que são

finamente reguladas, podem ser influenciadas por fatores externos e induzir distúrbios reprodutivos (Hurtado De Catalfo *et al.*, 2009; Victor-Costa *et al.*, 2010).

2.2 Enzima Aromatase

A enzima P450 aromatase é classicamente conhecida como responsável pela biossíntese de andrógenos a estrógenos, podendo converter os substratos androstenediona a estrona (E_1), bem como testosterona em estradiol (E_2). Pertence à superfamília do citocromo P450, sendo codificada pelo gene *CYP19*. A aromatase é composta por 503 aminoácidos e possui massa molecular de aproximadamente 55 kDa (Simpson *et al.*, 1994).

A aromatase apresenta-se com um complexo enzimático microssomal composto por duas proteínas, o citocromo P450 aromatase e o NADPH-citocromo P450 redutase. O citocromo P450 aromatase é responsável por ligar e catalisar a modificação do substrato esteroide por meio da ligação ao substrato do C_{19} da cadeia estrutural de hormônios esteroides, catalisando uma série de reações que leva à formação do anel aromático (fenol A), que é típico de estrógenos. O NADPH-citocromo P450 redutase é responsável pela transferência de equivalentes de redutores NADPH para o citocromo P450 (Graham-Lorence *et al.*, 1991; Simpson *et al.*, 1994). O complexo aromatase é amplamente distribuído entre os vertebrados, de maneira especial em mamíferos, localizando-se no retículo endoplasmático liso de vários tipos celulares, sendo encontrado principalmente no tecido adiposo, ossos, fígado fetal, cérebro, placenta e gônadas (Carani *et al.*, 1997).

No sistema genital masculino, a aromatase é expressa nos testículos, nas vias genitais e na próstata (Nitta *et al.*, 1993; Pereyra-Martinez *et al.*, 2001; Carpino *et al.*, 2004; Hejmej *et al.*, 2005; Gist *et al.*, 2007; Oliveira *et al.*, 2012).

Nos testículos de ratos, a atividade aromatase relaciona-se com a idade, sendo localizada principalmente nas células de Sertoli de ratos imaturos e em células de Leydig de ratos adultos (Papadopoulos *et al.*, 1986). Além disso, destaca-se a importância da aromatase no processo de espermatogênese como fonte de estrógenos em machos, devido à expressão desta proteína em células germinativas, bem como em células de Sertoli (Bilinska *et al.*, 2000; Carreau *et al.*, 2003). Desta forma, a presença de aromatase e/ou estrógenos nos testículos é de extrema importância para o processo de diferenciação sexual durante o desenvolvimento, manutenção da reprodução no adulto, bem como para o comportamento sexual (Carani *et al.*, 1997; Honda *et al.*, 1998; Simpson *et al.*, 1999; Carreau *et al.*, 2001). Além disso, estudos com animais deficientes para aromatase ou com administração de inibidores de aromatase e/ou estrógenos mostram a ocorrência de diversos distúrbios reprodutivos, como redução no número de espermátides (Shetty *et al.*, 1998), espermatogênese anormal com interrupção da maturação de células germinativas na fase de espermátides (Fisher *et al.*, 1998) e atrofia testicular seguida de infertilidade (Oliveira *et al.*, 2001). Em adição, ausência de aromatase leva à imobilidade de espermatozoides, destacando a importância de sua produção local, com papel essencial para a motilidade e capacitação de espermatozoides (Lazaros *et al.*, 2011).

Nos dúctulos eferentes, evidências sobre a presença de aromatase são ainda escassas, sendo descritas apenas para o homem e uma espécie de

morcego neotropical *Artibeus literatus,* sendo detectada no epitélio dos dúctulos eferentes em células ciliadas de morcegos (Oliveira et al., 2012), e células ciliadas e não-ciliadas, do epitélio dos dúctulos eferentes do homem (Carpino *et al.*, 2004). Neste sentido, aromatase pode desenvolver importante papel funcional nos dúctulos eferentes, uma vez que esses são altamente sensíveis a alterações nos níveis de estrógenos (Hess *et al.*, 1997a; Hess *et al.*, 2000; Oliveira *et al.*, 2001).

A expressão de aromatase no tecido prostático já foi detectada tanto no epitélio normal e hiperplásico, quanto em presença de malignidade (Matzkin e Soloway, 1992; Hiramatsu *et al.*, 1997; Ellem *et al.*, 2004; Hejmej *et al.*, 2005; Takase *et al.*, 2006; Ho *et al.*, 2008; Castro *et al.*, 2013; Grindstad *et al.*, 2016). No entanto, esses dados são ainda escassos e inconclusivos.

Aromatase já foi detectada restrita ao epitélio prostático normal ou hiperplásico (Hejmej *et al.*, 2005; Takase *et al.*, 2006), ou apenas no estroma de pacientes com hiperplasia benigna ou em presença de malignidade (Hiramatsu *et al.*, 1997; Ellem *et al.*, 2004; Ho *et al.*, 2008). Outros estudos detectaram a proteína no epitélio e no estroma em tecido normal e hiperplásico (Matzkin e Soloway, 1992; Takase *et al.*, 2006), bem como em câncer de próstata (Grindstad *et al.*, 2016). Tais achados são provenientes de estudos realizados em células de linhagem humana e outros provenientes de biópsia em pacientes (Matzkin e Soloway, 1992; Hiramatsu *et al.*, 1997; Ellem *et al.*, 2004; Takase *et al.*, 2006; Ho *et al.*, 2008). Há ainda dados que descrevem a proteína na próstata de equinos (Hejmej *et al.*, 2005).

No rato, aromatase ainda é pouco estudada, sendo descrita no epitélio prostático em condições normais e com aumento da expressão quando expostos

ao desregulador endócrino Bisfenol A (Castro *et al.*, 2013). Embora haja controvérsias quanto à presença de aromatase na próstata, em conjunto esses achados indicam a importância de estrógenos produzidos localmente para regulação da sua histofisiologia.

3. Proliferação Celular e Apoptose

Para manutenção adequada da homeostase tecidual nos organismos vivos é necessário um controle rigoroso nos processos de proliferação, diferenciação e morte das células, que são processos fisiológicos comuns e necessários para sobrevivência (Augenlicht, 1999).

3.1 Proliferação Celular

A proliferação celular é fundamental para o desenvolvimento e sobrevivência de todas as formas de organismos vivos. Em espécies unicelulares, cada divisão celular produz um novo e completo organismo. Por outro lado, em espécies multicelulares, sequências longas e complexas de divisões celulares são necessárias para a formação de um organismo funcional. Em indivíduos adultos a proliferação celular é necessária para o reparo celular e manutenção tecidual (Raff, 1992).

A proliferação celular consiste em uma sequência organizada de eventos em que a célula duplica seu conteúdo e então se divide em duas, através de uma série de eventos coordenados e altamente regulados, conhecido como ciclo celular (Morgan, 2007).

O ciclo celular compreende o processo completo de replicação do DNA (ácido desoxirribonucleico), mitose e citocinese que leva à produção de duas células-filhas a partir de uma célula-mãe. Este ciclo é tipicamente dividido em quatro fases: **S** (replicação do DNA), **G_2**, **M** (Mitose) e **G_1**. Os eventos de replicação do DNA (fase S) e mitose (fase M) são separados por intervalos de comprimento variável, chamados G_1 e G_2, sendo que o intervalo G_1 ocorre entre a fase M e a fase S, e o intervalo G_2 entre a fase S e a Mitose. O intervalo G_1, a fase S e o intervalo G_2 em conjunto são chamados de interfase (G_1 + S + G_2 = interfase), a qual ocupa a maior parte do ciclo celular. As fases G_1 e G_2 são eventos necessários para o crescimento, controle e progressão do ciclo celular (Alma Howard e Pelc, 1986; Norbury e Nurse, 1992). Se as condições extracelulares forem desfavoráveis, as células podem entrar em estado de repouso, conhecido como G_0 (G zero). Muitas células ficam permanentemente em estado de repouso até que elas ou o organismo morram (Patt e Quastler, 1963; Malumbres e Barbacid, 2001). Se as condições e sinais são favoráveis, a célula se compromete com o crescimento, replicação do DNA, bem como divisão celular, processo no qual ocorre uma cascata de eventos químicos e morfológicos de forma sucessiva e ordenada, fazendo com que a célula avance da fase G_0 para as fases G_1-S e G_2 para a mitose (Malumbres e Barbacid, 2001). Durante a mitose ocorre a divisão celular, onde as células passam pelas fases denominadas prófase, metáfase, anáfase e telófase (Vermeulen *et al.*, 2003).

A fase de replicação do DNA (Fase S) é um intrincado processo que ocupa grande parte do ciclo celular. Para desencadear esse processo, dois fatores são essenciais: a proteína iniciadora, que seleciona o local de início da replicação por

reconhecer uma sequência especifica do DNA, e o replicador, elemento responsável pelo início da replicação (ARS - *autonomous replicating sequences*) (Dutta e Bell, 1997; Bell e Dutta, 2002; Chang *et al.*, 2011). Um fator fundamental para a iniciação da replicação do DNA é um complexo multiproteico denominado complexo de origem de replicação (ORC – *origin recongnition complex*), bem como proteínas iniciadoras CDT1 (*chromatin licensing and DNA replication factor 1*) e CDC6 (*cell division cycle 6*) que se ligam ao ORC nas origens e auxiliam o transporte de um complexo de proteínas denominadas MCM (*Mini-Chromosome Maintenance*), acarretando no início do processo de replicação do DNA (Chang *et al.*, 2011).

O complexo MCM (MCM2-7) é composto por seis proteínas MCM2, MCM3, MCM4 (CD54), MCM5, MCM6 e MCM7 (CD47), sendo que sua disposição forma um heterohexâmero MCM2-7, que possui massa molecular de 560 kDa e está localizado no núcleo ao longo do ciclo celular da maioria dos organismos (Bell e Dutta, 2002). São proteínas essenciais para a replicação do DNA e compõem uma importante ferramenta para estudos sobre o potencial proliferativo celular (Blow e Hodgson, 2002). Nesse sentido, o MCM7, que é indetectável em células quiescentes e tem rápida indução de expressão quando as células são estimuladas a proliferar, tem sido amplamente utilizado como marcador de proliferação celular (Rojiani *et al.*, 2010).

3.2 Apoptose

A apoptose é um tipo de morte celular programada e constitui um mecanismo essencial para o desenvolvimento e regulação do crescimento em

tecidos normais. Considerada um fator vital de vários processos, incluindo o *turnover* normal das células, desenvolvimento e funcionamento apropriados do sistema imunológico, atrofia hormonal-dependente, desenvolvimento embrionário e morte celular induzida por químicos (Gerschenson e Rotello, 1992; Elmore, 2007). A manutenção da homeostase tecidual no sistema genital masculino depende de um adequado balanço entre proliferação e morte celular. Por exemplo, a morte de abundantes células germinativas por apoptose na espermatogênese é um mecanismo fisiológico, enquanto no epitélio das vias genitais e glândulas sexuais, a identificação de células apoptóticas é eventual em condições normais (Oliveira *et al.*, 2009; Shukla *et al.*, 2012).

As células em apoptose possuem características morfológicas distintas, sendo, portanto, bem caracterizadas. As principais características morfológicas são retração celular, perda de aderência com a matriz extracelular e com as células vizinhas, compactação nuclear e condensação citoplasmática, seguida da segmentação do núcleo ou cromossomos em discretos fragmentos (Hacker, 2000). Então, a célula como um todo se rompe em fragmentos envolvidos por membrana, chamados corpos apoptóticos. A superfície dos corpos apoptóticos torna-se quimicamente alterada com exposição da fosfatidilserina para a membrana externa, seguida de fagocitose por células vizinhas, ou por macrófagos, que desempenham importante papel na remoção do tecido (Gerschenson e Rotello, 1992; Hacker, 2000).

Diversas são as alterações bioquímicas que ocorrem nas células em apoptose, bem como são distintas as vias de ativação para desencadeamento do processo apoptótico, o que fornece importantes ferramentas para o estudo de

apoptose tecidual. Por exemplo, a clivagem do DNA cromossomal gera fragmentos que podem ser marcados pela técnica de TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling). O TUNEL é utilizado para a detecção *in situ* de células apoptóticas pela marcação do DNA fragmentado em regiões internucleossômicas específicas. Esse método utiliza a enzima transferase deoxinucleotidil terminal (TdT) que catalisa e transfere um nucleotídeo dUTP para a região livre do grupo 3'hidroxil, presente em fitas fragmentadas de DNA (Huppertz *et al.*, 1999). Outra importante ferramenta para o estudo de células em apoptose é a utilização de marcadores da família de proteases cisteínas, denominadas caspases, que clivam suas proteínas alvo em ácidos aspárticos específicos, conduzindo assim a ativação da via apoptótica mediada por caspases (Degterev *et al.*, 2003).

As caspases são divididas em duas categorias: caspases iniciadoras (caspase-2, -8, -9 e -10) e caspases efetoras (caspase-3, -6, e -7) (Denault e Salvesen, 2002; Boatright e Salvesen, 2003). Para adequada regulação do processo de apoptose, as caspases estão localizadas intracelularmente como precursores inativos, chamados de procaspases. As procaspases são ativadas somente quando estão próximas dos complexos de ativação. Quando ativadas, clivam e ativam caspases executoras, bem como outras proteínas alvo na célula, produzindo uma amplificação irreversível da cascata proteolítica. Além disso, o complexo ativador envolvido depende da origem do estímulo à morte celular, que é classificado como sendo extrínseco, quando induzido por fatores externos, ou intrínseco, em via induzida por fatores internos (Boatright e Salvesen, 2003).

A via extrínseca é iniciada pela ativação de receptores de morte, localizados na membrana celular. Os receptores de morte melhor caracterizados são CD95 (Fas, Apo1), TNFR1 (p55, CD120a), TNFR2, DR3 (DR- *Death Recptors*, Apo3, WSL-1, TRAMP ou LARD), DR4, DR5 (Apo2, TRAIL-R2, TRICK 2 ou KILLER), DR6 e p75 (NGF - *nerve growth factor*), que pertencem à superfamília dos receptores do fator de necrose tumoral (TNF) (Ashkenazi e Dixit, 1998; Sakamaki e Satou, 2009). A ativação dos receptores de morte, que ocorre através de ligantes específicos, leva à formação do DISC (*death-inducing signaling complex*) que é o local de ativação para a caspase-8, e/ou -10, que resultará na ativação de caspases efetoras da apoptose, caspases-3 e -7.

Por outro lado, a estimulação da via intrínseca é iniciada pela permeabilização da membrana mitocondrial e liberação do citocromo *c,* que conduz ao recrutamento e ativação da caspase-9 em um complexo conhecido como apoptossomo. Após a ativação, as caspases iniciadoras então clivam e ativam as caspases efetoras -3 e -7 (Boatright e Salvesen, 2003).

A caspase-3 é a principal caspase efetora, sendo responsável, entre outras funções, pelo reconhecimento e clivagem da subunidade que inibe o fator de fragmentação do DNA (DFF- *DNA fragmentation factor*), liberando sua subunidade ativa, denominada CAD (*Caspase-Activated DNAse*). A subunidade CAD migra para o núcleo, fragmentando o DNA em segmentos de tamanhos típicos da apoptose, com aproximadamente 180 - 200 pares de base (Saraste e Pulkki, 2000). A caspase-3 pode ser ativada tanto pela via intrínseca como pela via extrínseca, sendo, desta forma, considerada como importante marcador em

estudos sobre o processo de morte celular por apoptose, independente da via de ativação (Porter e Janicke, 1999; Snigdha *et al.*, 2012).

4. Atrazina

Atrazina (2-cloro-4-etilamino-6-isopropilamino-S-triazina) é um componente ativo de herbicidas, veiculados comercialmente como: Atrazina Nortox 500 SC, Atrazina Atanor 500 SC, GESAPRIM 500 Ciba-Geigy, dentre outros. Amplamente utilizado para o controle de plantas infestantes nas culturas de abacaxi, cana-de-açúcar, milho, milheto, *Pinus*, seringueira, sisal e sorgo (E.P.A, 2017). Nesse sentido, atrazina desempenha papel significativo, no contexto da alimentação mundial, devido à necessidade de produção alimentar em larga escala. É empregado tanto como agente pré-emergente, bem como agente pós-emergente para controle de plantas infestantes de folhas largas.

O potencial de ação de atrazina deve-se à inibição da fotossíntese pela interrupção do transporte de elétrons no fotossistema II, da reação de Hill (Knauer *et al.*, 2009). Primeiramente, foi postulado que devido ao modo de ação de atrazina ser limitado a fotossíntese e agir exclusivamente em plantas de folhas largas, as espécies animais seriam resguardadas dos efeitos colaterais provocados pelo herbicida. Além disso, o produto é classificado como medianamente tóxico ao ser humano (Anvisa, 2017) e veiculado comercialmente como seguro para as pessoas, bom para o ambiente e para a economia (Syngenta, 2017). No entanto, diversos estudos mostram que atrazina é um potente desregulador endócrino (Hayes *et al.*, 2010; Hussain *et al.*, 2010; Tillitt *et*

al., 2010; Victor-Costa *et al.*, 2010; Hayes *et al.*, 2011; Jin *et al.*, 2013; Abarikwu, 2014; Riffle *et al.*, 2014).

Além do contato ocupacional e contaminação de pessoas e animais que vivem próximas às áreas de disseminação do herbicida, a exposição a esse composto pode ocorrer a longas distâncias através da dispersão atmosférica (Dos Santos *et al.*, 2011), bem como por acúmulo nos corpos d´água (Nwani *et al.*, 2010; Fairbairn *et al.*, 2016; Elias e Bernot, 2017; Qu *et al.*, 2017). Além disso, atrazina tem sido detectado inclusive em produtos alimentícios de consumo diário, como leite, iogurte (Garcia *et al.*, 2012; Li *et al.*, 2013; Yang *et al.*, 2014), arroz, milho, trigo, dentre outros cereais (Zhang *et al.*, 2014; Zhao *et al.*, 2015), resultando na contaminação de pessoas e animais mesmo quando não expostos diretamente.

A questão se faz alarmante pelo fato de que atrazina tem sido evidenciado como causador de desordens reprodutivas nas diferentes classes de vertebrados, incluindo redução da reprodução e desova em peixes (Tillitt *et al.*, 2010; Papoulias *et al.*, 2014; Richter *et al.*, 2016), alteração na razão entre sexos em peixes (Suzawa e Ingraham, 2008; Mac Loughlin *et al.*, 2016), disfunção reprodutiva e alterações moleculares em peixes adultos expostos apenas durante a embriogênese, levando a alterações morfológicas inclusive em sua prole (Wirbisky *et al.*, 2016), indução de feminização em anfíbios (Hayes *et al.*, 2002; Hayes *et al.*, 2010), redução e degeneração de células germinativas primárias do ovário de anfíbios (Tavera-Mendoza *et al.*, 2002; Sai *et al.*, 2016), alterações na arquitetura testicular e nos níveis de testosterona em crocodilianos (Rey *et al.*, 2009), redução dos testículos, túbulos seminíferos e no número de células germinativas em aves

(Hussain *et al.*, 2010). Em mamíferos ocorre, atraso na maturação sexual (Stoker *et al.*, 2000; Trentacoste *et al.*, 2001; Ashby *et al.*, 2002), diminuição do número e motilidade de espermatozoides (Kniewald *et al.*, 2000; Betancourt *et al.*, 2006; Swan, 2006; Song *et al.*, 2014; Komsky-Elbaz e Roth, 2017), redução no peso da próstata e vesícula seminal (Kniewald *et al.*, 2000; Trentacoste *et al.*, 2001; Stoker *et al.*, 2002; Stanko *et al.*, 2010), indução de prostatites (Stoker *et al.*, 2002; Stanko *et al.*, 2010), bem como câncer de próstata (Hu *et al.*, 2016).

Além de animais domésticos e silvestres, atrazina já foi evidenciado como causador de desordens reprodutivas também em humanos, levando assim à redução do sucesso reprodutivo em homens (Swan, 2003; 2006; Hayes *et al.*, 2011), bem como associação com aumento na incidência de câncer de próstata em homens trabalhadores em unidade de produção de atrazina (Maclennan *et al.*, 2002; Sass e Colangelo, 2006).

Na esteroidogênese testicular de roedores já foi demonstrado que atrazina induz a expressão e atividade de aromatase (Jin *et al.*, 2013; Martins-Santos, 2013), bem como diminuição na expressão da enzima 3β-HSD nas células de Leydig (Victor-Costa *et al.*, 2010; Abarikwu, 2014), levando ao desequilíbrio entre os níveis de andrógenos/estrógenos e consequente infertilidade (Victor-Costa *et al.*, 2010; Jin *et al.*, 2013).

Tem sido demonstrado ainda que atrazina afeta a homeostase tecidual, levando a diminuição da proliferação celular, aumento na fragmentação de DNA e aumento na atividade de caspase em cultura celular de fibroblastos humanos (Manske *et al.*, 2004), indução de apoptose em células de peixes (Liu *et al.*, 2006), aumento na incidência de células apoptóticas no mesencéfalo e rim de girinos em

desenvolvimento (Lenkowski *et al.*, 2008), desequilíbrio entre proliferação e morte celular nos testículos de jacarés expostos (Rey *et al.*, 2009), indução de apoptose no baço de camundongos (Zhang *et al.*, 2011), bem como autofagia e apoptose relacionados com efeitos neurodegenerativos em neurônios dopaminérgicos de ratos (Song *et al.*, 2015). Em comum todos estes trabalhos especulam que a intervenção de atrazina sobre a proliferação e morte celular pode funcionar como via adicional da toxicidade desse herbicida em vertebrados.

Desta forma, torna-se importante investigar a morfologia, esteroidogênese, bem como o balanço entre proliferação e morte celular em órgãos chave do sistema genital masculino, como testículos, dúctulos eferentes e próstata ventral, após a exposição ao atrazina. Assim, poderá ser possível esclarecer os mecanismos de ação deste herbicida como desregulador endócrino no sistema genital masculino e suas implicações com o sucesso reprodutivo.

III. OBJETIVOS

1. Objetivo Geral

O presente estudo visa investigar os efeitos de atrazina, como desregulador endócrino, na morfologia e homeostase tecidual e hormonal de órgãos chave do sistema genital masculino de ratos Wistar adultos, tais como testículos, dúctulos eferentes e próstata ventral.

1.1 Objetivos Específicos

Sempre comparando animais expostos ao atrazina e animais controle, pretende-se:

- Determinar o peso corporal e relativo dos órgãos alvo desse estudo;
- Investigar possíveis alterações histopatológicas nos testículos, dúctulos eferentes e próstata ventral;
- Realizar análise morfométrica das possíveis alterações morfológicas encontradas;
- Confirmar a identidade das células semelhantes a macrófagos detectadas nos testículos afetados por atrazina;
- Avaliar possíveis variações na expressão das enzimas esteroidogênicas 3β-HSD e/ou aromatase nos testículos, dúctulos eferentes e próstata ventral;
- Determinar o perfil de proliferação celular e apoptose nos órgãos alvo do estudo.

IV. ARTIGOS

Artigo 1

Persistent testicular structural and functional alterations after exposure of adult rats to atrazine.

Martins-Santos E, Pimenta CG, Campos PRN, Franco MB, Gomes DA, Mahecha GAB, Oliveira CA

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Persistent testicular structural and functional alterations after exposure of adult rats to atrazine

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ABSTRACT

Atrazine is an endocrine disruptor affecting testicular steroidogenesis, and promoting testicular atrophy and 3β-HSD reduction. However, it remains unknown whether these effects are reversible or permanent. To address this issue was the aim of this study. Exposition of rats to 200 mg/kg of atrazine resulted in transient increase in testicular weight, seminiferous tubules dilation and atrophy, and reduction in Leydig cell 3β-HSD. Testicular atrophy and 3β-HSD reduction were more pronounced after the recovery period of 75 days. There was increase in aromatase expression after long-term exposure but it returned to control level after recovery. Moreover, there was increase in $ED1^{-}/ED2^{+}$, $ED1^{+}/ED2^{+}$ and $ED1^{+}/ED2^{-}$ macrophages, in the recovery group. These macrophages were positive for 3β-HSD, thereby raising possibility of their involvement in steroidogenesis. These findings further emphasize the adverse effects of atrazine on male reproduction, highlighting that testicular damages may be irreversible even after a recovery period longer than the spermatogenic cycle.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), an active component found in herbicides commonly used in agriculture worldwide, has been considered a potent endocrine disruptor of testicular steroidogenesis [1–11]. The importance of endocrine disruptors in males reflects the growing body of evidence highlighting the close relationship between these compounds and the increase in male reproductive disturbances of many vertebrates, including humans [12].

Reproductive problems linked to atrazine exposure include demasculinization and feminization in fish, amphibians and reptiles [2,13,14], loss of ovarian germ cells [15,16], testicular degeneration in amphibians [17], structural disruption of testes in fish [18], crocodilians [19], birds [8] and rodents [4,11,20–22], reduction in sperm count and motility [11,21–25], weight reduction of the rat prostate and seminal vesicle [22,26,27], as well as delayed sexual maturation [27–31].

The mechanism of atrazine action is not yet fully understood. A study using short-time (15 days) exposure to 200 mg/kg of atrazine showed a transient increase in the weight of Wistar rat testes, followed by testicular atrophy after prolonged exposure (40 days) [4]. These changes occurred in parallel with a reduction in plasma and testicular levels of testosterone and an increase in estradiol, thus corroborating previous reports that aromatase enzyme may be a potential target for atrazine [9,32–34]. Furthermore, Victor-Costa et al. [4] revealed that the harmful effects of atrazine on the steroidogenic cascade would be more extensive than previously thought. Indeed, atrazine reduced the expression of 3β-HSD on Leydig cells, despite the normal expression of this enzyme on adrenal tissue. This effect on the steroidogenic pathway may represent an additional mechanism by which atrazine affects testicular androgenesis, leading to a reduction in testosterone levels and, consequently, disruption of spermatogenesis.

Considering that atrazine (1) continues to be broadly used in large scale in agriculture across the world, (2) is easily disseminated in the environment, and (3) causes adverse effects on male reproduction by acting as an endocrine disruptive agent, it is paramount to further investigate tissue and hormonal alterations induced by this herbicide. Therefore, the aim of this study was to investigate whether the long-term effects of atrazine on testicular structure and 3β-HSD expression, previously described by Victor-Costa et al. [4], would be reversible after a recovery period longer than the steroidogenesis cycle. In addition, the atrazine effects on testes aromatase expression were also investigated.

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2. Material and methods

2.1. Animals

A total of 55 sexually mature male Wistar rats (100 days old), acquired from the Animal Facility at the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Brazil, was used. The animals were maintained under constant conditions of light (12/12 h light/dark cycles) and temperature (22 °C), receiving pelletized chow (Nuvital Nutrientes S.A., Colombo, PR, Brazil) and water *ad libitum*. The study followed UFMG's animal care and research guidelines of the Institutional Ethical Committee on Animal Use (https://www.ufmg.br/bioetica/ceua/). Experimental procedures were approved by the Institutional Ethical Committee for Animal Experimentation (Protocol number 287/2008).

2.2. Treatment

Daily doses of atrazine (200 mg/kg), diluted in corn oil, were administered by oral gavage, to the rats for 7, 15 and 40 days. The control group received only corn oil, without atrazine. The dose and treatment periods were chosen as a follow-up of previous study showing alterations promoted by atrazine in testicular morphology and steroidogenesis in Wistar rats [4,27,35]. Furthermore, studies concerning *in vivo* effects of atrazine on mammals' testes are routinely executed at this dosage level [1,4,6,9,26,27,31,35]. A 7-day exposure treatment was included for the detection of the early effects of the herbicide. To determine whether the previously reported testicular alterations are reversible, a group of rats who received atrazine for 40 days was maintained in the animal facility for a further 75 days under the same environmental conditions described above but no atrazine exposure (ATZ 40d Rec). The recovery control group (Cont 40d Rec) comprised rats who received corn oil for 40 days and were maintained for a further 75 days in the animal facility without any treatment. This recovery period (75 days) is thought to be sufficient to cover more than one spermatogenesis cycle, which lasts for about 58 days in rats [36].

2.3. Tissue preparation

After each treatment and the recovery period, the rats were weighed, and euthanized under deep anesthesia with intraperitoneal injection of sodium pentobarbital (80 mg/kg) and ketamine chlorydrate (10 mg/kg). Animals from each experimental group were perfused with 10% (v/v) neutral buffered formalin (NBF), the testes were dissected, weighed, immersed and maintained in NBF at 4 °C until needed for histological, immunohistochemical and immunofluorescence analyses. After perfusion with saline solution animals from each group had their testes dissected, weighed, frozen in liquid nitrogen, and stored at −80 °C until needed for Western blotting assays.

In order to investigate possible morphological alterations, NBF-fixed testes' fragments were embedded in glycol methacrylate, sectioned at 3 μm and stained with hematoxylin and eosin (HE), 1% toluidine-blue sodium borate or periodic acid-Schiff (PAS) counterstained with hematoxylin.

2.4. Morphometry

The number of normal, dilated or atrophic seminiferous tubules was counted by using a grid of 100 squares mounted onto the eyepiece of the microscope (Nikon Eclipse E200, Melville, USA). The measurements were taken at 40X magnification in three randomly selected regions of the testes.

Macrophages are known to be PAS-positive in several tissues [37]. Therefore, PAS-stained testes were used to quantify the number of PAS-positive macrophage-like cells in atrazine-treated rats. The quantification was performed at 400X magnification in 10 different testicular areas per animal. Results are expressed as number of PAS-positive macrophage-like cells per mm^2.

2.5. Immunohistochemistry

Immunohistochemical analyses were carried out to investigate possible alterations in aromatase and 3β-HSD expression, as well as to confirm the identity of macrophage-like cells using classical markers of rat macrophages. These include CD68 (ED1; for induced, freshly immigrated, transitory or inflammatory macrophages) and CD163 (ED2; for resident macrophages) [38]. Cell proliferation was also evaluated in atrophic tubules after the recovery period by using minichromosome maintenance protein 7 (MCM7) as marker.

Fragments of NBF-fixed testes and paraffin-embedded, were microwaved in 0.1 M citrate buffer (pH 6.0) for antigen retrieval, followed by incubation with avidin and biotin blocking solution (Avidin/biotin blocking kit; Vector Laboratories, Burlingame, USA), and 10% normal serum. Sections were then incubated, at 4 °C overnight, with the primary antibodies, followed by the biotinylated secondary antibodies set out on Table 1. Negative controls were incubated with phosphate-buffered saline (PBS) instead of the primary antibody. After washing, the sections were incubated with avidin-biotin complex conjugated with peroxidase (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, USA). Immunoprecipitation was performed using diaminobenzidine tetrahydrochloride solution. Sec-

Table 1
Antibodies employed in the immunohistochemistry and Western blotting assays.

Primary Antibodies	Dilution IHC	Dilution WB	Biotinylated Secondary Antibodies	Dilution IHC	Dilution WB
Rabbit anti-aromatase (A7981, Sigma-Aldrich, USA)	1:500	1:1000	Goat anti-rabbit (Dako, Carpinteria, USA)	1:100	1:1000
Goat anti-3β-HSD (P-18, sc-30820, Santa Cruz Biotechnology, CA, USA)	1:500	1:500	Rabbit anti-goat (Dako, Carpinteria, USA)	1:100	1:2000
Mouse anti-CD68 (sc-59103, Santa Cruz Biotechnology, CA, USA)	1:75	–	Goat anti-mouse (Dako, Carpinteria, USA)	1:100	–
Rabbit anti-CD163 (sc-33560, Santa Cruz Biotechnology, CA, USA)	1:75	–	Goat anti-rabbit (Dako, Carpinteria, USA)	1:100	–
Mouse anti-MCM7 (MS-862-P0, Thermo Fisher Scientific, Waltham, USA)	1:1000	–	Goat anti-mouse (Dako, Carpinteria, USA)	1:100	–
Anti-GAPDH (14C10, Cell Signaling, Danvers, USA)	–	1:1000	Goat anti-rabbit (Dako, Carpinteria, USA)	–	1:1000

IHC = immunohistochemistry; WB = Western blotting

tions were slightly counterstained with Mayer's hematoxylin and mounted.

Macrophages positive for ED1/CD68 and ED2/CD163 were quantified in ten different testicular areas per animal, at 400X magnification. The results were expressed as number of positive cells per mm^2. In the recovery group, MCM7 positive spermatogonia as well as Sertoli cells, with visible nucleus, were counted in 50 randomly chosen, nearly circular atrophic seminiferous tubule cross sections per animal [39]. The positive spermatogonia were categorized as isolated, paired or grouped cells (3 or more cells), and the results were expressed as number of positive cells per 100 Sertoli cells.

2.6. Immunofluorescence

Induced ($ED1^+/CD68^+$) and resident ($ED2^+/CD163^+$) macrophages found in the testes of rats from the recovery group and their respective control, were identified by double immunofluorescence labeling. Sections were prepared as for immunohistochemistry. After antigen retrieval the sections were permeabilized in PBS containing 0.5% triton X-100 (v/v), and then incubated with 3% bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, USA), for 90 min. Subsequently, the sections were incubated at 4 °C overnight with a solution containing a mixture of the following primary antibodies: mouse monoclonal anti-CD68 (sc-59103; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution ratio of 1:75, and rabbit monoclonal anti-CD163 (sc-33560, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:100 in PBS supplemented with 1% v/v BSA. After washing, the sections were incubated at room temperature for 60 min with the following secondary antibodies: goat anti-mouse CF555 (Sigma-Aldrich, Saint Louis, USA) and goat anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, Rockford, USA), both diluted 1:100 in PBS supplemented with 1% v/v BSA. Negative controls were performed in the absence of both primary antibodies. Nuclei were identified by 4,6-diamidino-2-phenylindole staining (DAPI-D1306, Life Technology, Carlsbad, USA).

In order to confirm the sub-cellular localization of 3β-HSD in testicular macrophages, 3β-HSD/CD68 (ED1) and 3β-HSD/CD163 (ED2) co-localization experiments were carried out following the same procedures described above, except for the incubation with specific antibodies. The tissues were incubated with a solution containing a mixture of the following primary antibodies: goat anti-3β-HSD (1:100; P-18, SC-30820, Santa Cruz Biotechnology, Santa Cruz, CA, USA), plus either mouse anti-CD68 (1:500; sc-59103, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or mouse anti-CD163 (1:500; sc-58965, Santa Cruz Biotechnology, Santa Cruz, CA, USA), at 4 °C overnight. After washing, the sections were incubated at room temperature for 60 min with the secondary antibody donkey anti-goat Alexa Fluor 488 (Thermo Fisher Scientific, Rockford, USA) plus goat anti-mouse CF555 (Sigma-Aldrich, Saint Louis, USA), diluted 1:300 and 1:100, respectively, in 1% BSA-supplemented PBS (v/v). The sections were examined using an Axio Imager 2 microscope equipped with a HXP illuminator, APOTOME.2 and Axiocam 503 with EC Plan-Neofluar 20X/NA 0.5 and Plan-Apochromat 40X/NA 1.4 objective lenses (Zeiss, Göttingen, Germany). The samples were excited: (1) at 365 nm observed at 420–470 nm to detect the DAPI- stained nuclei, (2) at 450–490 nm observed at 500–550 nm to detect Alexa Fluor 488 and, (3) at 540–560 nm observed at 570–640 nm to detect CF555 staining.

Induced (ED1/CD68) and resident (ED2/CD163) macrophages were quantified in the testes of rats from the recovery groups (ATZ 40d Rec and Cont 40d Rec). To this end, 10 randomly selected testicular areas per group, taken at 400X magnification, were analyzed. The total number of positive cells for each marker was counted individually, as well as those that were co-localized. The results were expressed as number of positive cells per mm^2.

2.7. Western blotting

Expression levels of aromatase and 3β-HSD were confirmed by Western blotting ($n = 3$). Due to the heterogeneous distribution of the testes' weight, in particular from rats chronically exposed to atrazine (40 days), which reflected testicular morphology heterogeneity, the testes from this group were grouped into three sub-categories: small, medium and large testes. Liquid nitrogen-flash frozen testes were macerated and samples of the tissue were homogenized and sonicated in extraction buffer containing 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.5% Nonidet P-40, and a protease-inhibitor cocktail (Sigma-Aldrich, Saint Louis, USA). Proteins were then subjected to continuous electrophoresis, using polyacrylamide gel 10% for aromatase and 12% for 3β-HSD, containing SDS (SDS-PAGE) transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in Tris-buffered saline (TBS) plus 0.5% v/v Tween 20 (TBST) for 60 min and then incubated, overnight, with the primary antibodies (Table 1). This was followed by washing and incubation with the respective biotinylated secondary antibodies (Table 1). The membrane was then incubated with avidin-biotin complex conjugated with peroxidase (Vectastain Standard ABC kit; Vector Laboratories, USA), and finally visualized using 0.01% diaminobenzidine solution containing 0.05% chloronaphtol, 16.6% methanol and 0.04% H_2O_2. The reaction was quenched with deionized water. Band intensities were estimated using Adobe Photoshop CS6 (Adobe Systems Software, Mountain View, USA). The final band intensity was determined by rate between aromatase and GAPDH as well as 3β-HSD and GAPDH.

2.8. Enzyme linked immune sorbent assay-ELISA

ELISA was used for detection of atrazine in plasma and testes. Plasma samples were obtained after centrifugation of total blood (2200*g* for 10 min) in heparin-coated tubes. For testes, liquid nitrogen frozen tissues were macerated in dry ice and the samples ($n = 3$ per group, pooled 50 mg for each, totalizing 150 mg) were homogenized and sonicated (60 s in 250 μL of PBS, pH 7.4). Detection of atrazine on plasma and testes were performed by using commercial Atrazine ELISA kit (Abraxis, Warminster, UK). For the ELISA, it was used 25 μL of sample per well according to the manufacturer's instructions. The sensitivity of the assay was 5.0 ng/mL. The assays were performed in duplicate.

2.9. Statistical analysis

The numerical data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). After performing the Shapiro-Wilk normality test, parametric data were analyzed by Student's *t*-test to compare the means between two groups, or for multiple variance using one-way ANOVA followed by a *post-hoc* Tukey's test to compare more than two experimental groups. Non-parametric data were analyzed using either Mann-Whitney or Kruskal-Wallis plus Dunn's *post-hoc* tests for comparisons between two or more groups, respectively. The data were expressed as mean ± SEM, and differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Body and testes' weights

Rats daily exposed to 200 mg/kg of atrazine for 40 days had a significant reduction in body weight. However, it returned to normal after a 75-day recovery period, without treatment with atrazine (Table 2). There was no change in absolute testicular weights immediately after atrazine exposure. However, a significant decrease was observed following the recovery period. In contrast, the weights of the testes relative to total body weight significantly increased after all treatments, except in the case of the recovery treatment group (ATZ 40d Rec), which presented 58% reduction in the testes' relative weights compared to the testes from the recovery control group (Cont 40d Rec; Table 2).

3.2. Testes morphology

After 7 days of atrazine administration, testicular tissue morphology was similar to that collected from control rats (Fig. 1A and B). Atrazine exposure for 15 and 40 days resulted in heterogeneous testicular morphology, given that normal tubules, dilated tubules or tubules with varying degrees of atrophy were observed in the same sections (Fig. 1C, D, E and F). Atrophic tubules were characterized by loss of germ cells, resulting in seminiferous tubules containing only Sertoli cells. Giant multinucleated bodies and apoptotic cells (Fig. 1D) were frequent in the seminiferous tubules of atrazine-treated rats. In the testes of rats from the Recovery treatment group, all seminiferous tubules had atrophied, mostly displaying Sertoli only cells or just a few spermatogonia (Fig. 1E and F). In some tubules, sporadic spermatogonia in mitosis were observed (Fig. 1E). In order to confirm this finding, the proliferation marker MCM7 was used and the proliferative spermatogonia were counted in the atrophic seminiferous tubule of the recovery group. All spermatogonia remaining in the atrophic tubules were MCM7 positive. These cells were mostly found isolated along the basement membrane (3.6 cells/100 Sertoli cells) (Fig. 2). Fewer paired (0.63 cells/100 Sertoli cells) or grouped (0.07 cells/100 Sertoli cells) MCM7 positive spermatogonia were also seen (Fig. 2). When grouped, at most 4 proliferating spermatogonia were observed.

3.3. P450-aromatase detection

Aromatase positivity was detected in the cytoplasm of spermatogonia, primary spermatocytes and spermatids in the seminiferous tubules of the control rats (Fig. 3A, C and E). More intense staining for the enzyme was detected in spermatocytes, especially at stages 6–8 and stage 1 of the seminiferous epithelium cycle, as determined by the tubular morphology system [40]. Leydig, Sertoli and myoid cells were slightly immunoreactive for the enzyme. There were no detectable changes in the immunoreaction for aromatase after atrazine exposure for 7 and 15 days, whereas after 40 days of exposure stronger immunostaining was detected in the Leydig cells (Fig. 3F). The results were confirmed by Western blotting, as strongest band was detected in the testis after 40 days of atrazine exposure (Fig. 3I and J). In the recovery group, aromatase reactivity became similar to those of controls (Fig. 3G and H).

3.4. 3β-HSD detection

In all control animals, the expression of 3β-HSD was intensely and homogeneously distributed in the cytoplasm of Leydig cells (Fig. 4A, C, E and G). In 7-day atrazine-treated animals, 3β-HSD expression levels in Leydig cells was similar to those of their control counterparts (Fig. 4B). After 15 days of exposure to atrazine, the immunostaining patterns were heterogeneous: some Leydig cells presented staining patterns similar to those found in Leydig cells from the control groups, whereas a reduction in 3β-HSD staining was observed in testes presenting enlarged or atrophic seminiferous tubules (Fig. 4D). Testes of animals treated with atrazine for 40 days showed a significant reduction in the expression of 3β-HSD in Leydig cells (Fig. 4F and I-K). Interestingly, after the 75-day recovery period, 3β-HSD immunoreactivity severely reduced (Fig. 4H). Testes from rats chronically exposed to atrazine followed by a recovery period (ATZ 40d Rec) presented numerous macrophage-like cells strongly positive for 3β-HSD in the testicular interstitium (Fig. 4H).

The Western blots identified two molecular weight bands at 43.0 and 42.7 kDa (Fig. 4I). These correspond to 3β-HSD types II and I, respectively [41]. Corroborating the immunohistochemistry results, there was a detectable decrease in the expression of the 3β-HSD type II isoform after 40 days of exposure to atrazine. Expression levels of the 3β-HSD type I isoform were markedly diminished in the testes of rats exposed to atrazine for 15 days and over (Fig. 4I, J and K).

3.5. Macrophage detection

In the testes exposed to atrazine for 40 days and recovered from the exposure for 75 days (ATZ 40d Rec) there was a significant increase in the number of PAS-positive macrophage-like cells in comparison to the control groups (Fig. 5A–C).

To confirm the identity of the macrophage-like cells, specific markers for induced (ED1/CD68) and resident (ED2/CD163) macrophages were used. Both macrophage populations were detected in the testis interstitium, mainly in the periphery of the seminiferous tubules or nearby Leydig cells (Fig. 5D, E, G and H). A significant increase in $ED1^+$ and $ED2^+$ cell populations was observed in the testes from the recovery treatment group (ATZ 40d Rec; Fig. 5F and I).

Double fluorescent labeling of cells for ED1/CD68 and ED2/CD163 identified three subsets of macrophages in the rats' testes: $ED1^+/ED2^-$, $ED1^-/ED2^+$, and $ED1^+/ED2^+$ cells. These correspond to the induced, resident, and transitory macrophage sub-populations, respectively, found in testes of the recovery treatment group (ATZ 40d

Table 2
Body and testes' weights of Wistar rats treated with atrazine.

Weights (absolute and relative)	Cont 7d	ATZ 7d	Cont 15d	ATZ 15d	Cont 40d	ATZ 40d	Cont 40d Rec	ATZ 40d Rec
BW (grams)	397.5 ± 30.1	328.8 ± 12.9	364.8 ± 30.5	284.0 ± 30.7	456.8 ± 3.6	354.3* ± 14.6	552.3 ± 14.2	539.5 ± 18.5
T (grams)	1.69 ± 0.05	1.69 ± 0.06	1.61 ± 0.05	1.90 ± 0.26	1.81 ± 0.05	1.78 ± 0.11	2.12 ± 0.12	0.84* ± 0.05
T (grams/100 g BW)	0.44 ± 0.02	0.52* ± 0.02	0.45 ± 0.02	0.66* ± 0.05	0.40 ± 0.01	0.50* ± 0.03	0.38 ± 0.02	0.16* ± 0.008

Results are presented as mean ± SEM. BW = body weight; T = testes; Cont = Control group comprising rats receiving corn oil only; ATZ = treated with atrazine 200 mg/kg; Rec = recovery period of 75 days without atrazine; d = days of treatment. $n = 4$; * $P \leq 0.05$ compared to the respective control groups.

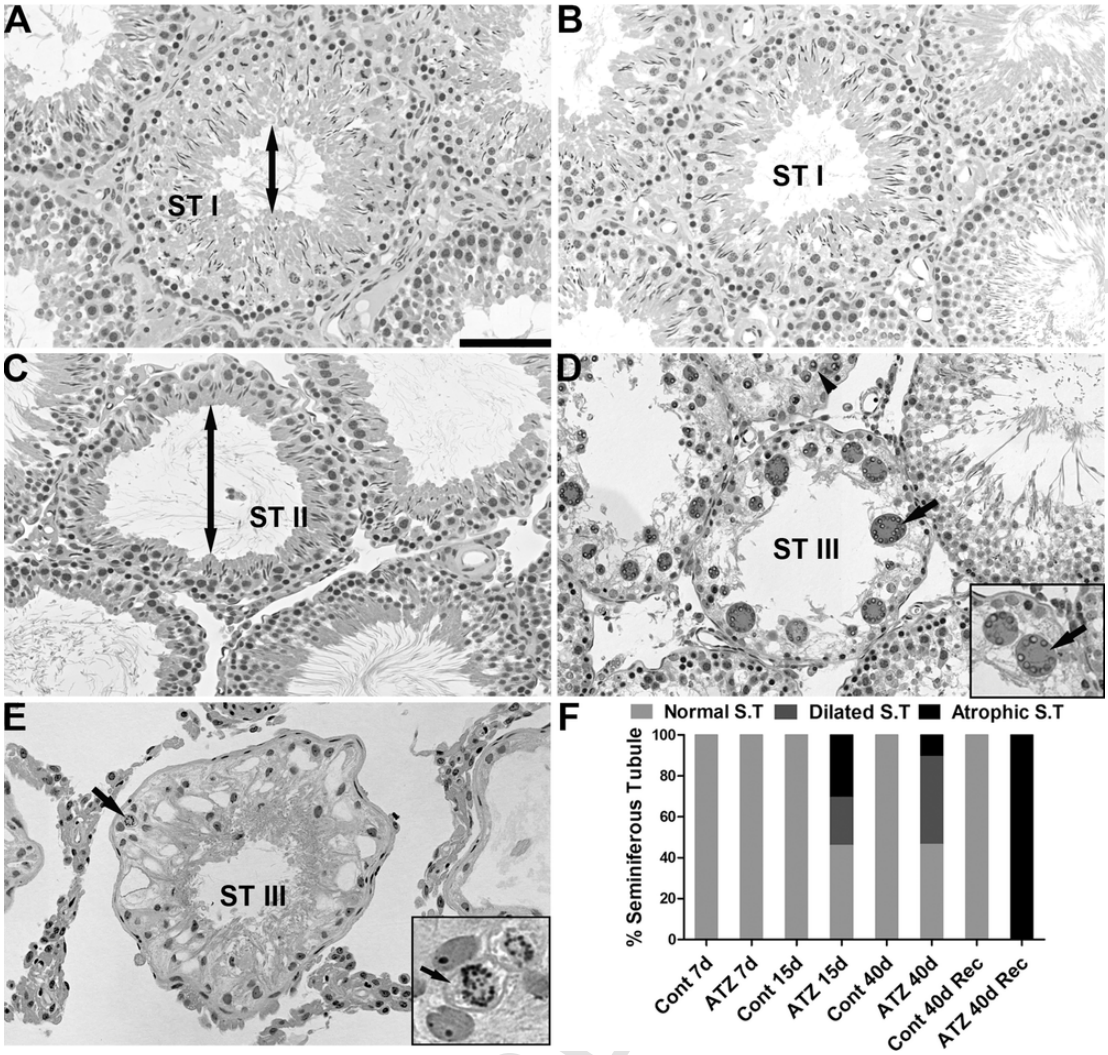

Fig. 1. Effects of atrazine on morphology of Wistar rat testes. **(A)** Representative testes of all control animals showing normal seminiferous tubules (STI). **(B, C and D)** Testes from rats treated with atrazine (200 mg/kg) for 7, 15 and 40 consecutive days, respectively. Arrowhead = apoptotic cells; Arrow and Insert in D = giant multinucleated bodies. STII = dilated seminiferous tubule shown in C; STIII = atrophic seminiferous tubule shown in D. **(E)** Testes of rats treated with atrazine for 40 days followed by a 75-day recovery period (ATZ 40d Rec). Arrow and Insert in E = cell undergoing mitosis. **(F)** Proportion (%) of normal, dilated and atrophied seminiferous tubules (S.T). Cont = Control group comprising rats receiving corn oil only; ATZ = atrazine; Rec = recovery period of 75 days without atrazine; d = days. Double arrows in A and C indicate luminal diameter. Scale bar in A = 100 μm. $n = 4$.

Rec) and control rats (Cont 40d Rec, Fig. 6). A remarkable increase of 89% and 76% in the number of resident and transitory macrophages was observed in the testes of the ATZ 40d Rec group compared to the Cont 40d Rec group. A smaller but still significant increase in the number of induced macrophages was also observed (42%).

Co-localization of 3β-HSD and the macrophage markers showed that 3β-HSD is present in induced and resident macrophages (Fig. 7). Furthermore, 3β-HSD was identified in testicular macrophages of the control and recovery groups.

3.6. Atrazine concentration

High levels of atrazine were detected in the plasma and testis of all experimental groups, compared to controls (Fig. 8).

4. Discussion

The current study investigated the effects of atrazine upon morphology and the physiology of testicular steroidogenesis after a long-term period of exposition followed by a recovery period. Importantly, it reveals, for the first time, the severe effects of prolonged atrazine exposure in the testes of adult rats, even after a recovery period of 75 days post exposure to the herbicide. Morphological changes included transient increase in testicular weight, luminal dilation of seminiferous tubules and testicular atrophy. These morphological changes paralleled a reduction in 3β-HSD, which was detectable after 15 days or more of atrazine exposure. Interestingly, testicular atrophy and 3β-HSD reduction were more pronounced after the recovery period, suggesting that the testicular effects of atrazine are persistent after a period longer than the spermatogenic cycle. Moreover, testes from recovery group animals showed a remarkable increase in testicular 3β-HSD-positive macrophages.

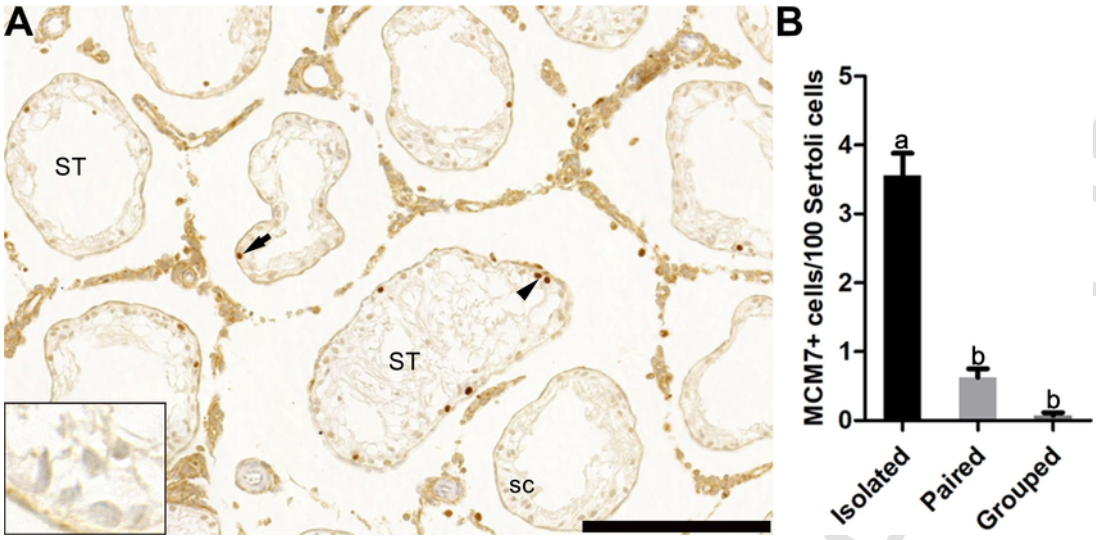

Fig. 2. Effects of atrazine on cell proliferation in Wistar rats' testicular atrophy after the recovery period. **(A)** MCM7 positivity was found in the spermatogonia remaining in the atrophic seminiferous tubules, whereas Sertoli cells were negative. Arrow = isolated spermatogonia; arrowhead = paired spermatogonia; SC = Sertoli Cells; ST = seminiferous tubule; Scale bar = 200 μm; Insert = negative control. **(B)** The graph shows the proportion of isolated, paired and grouped MCM7 positive spermatogonia per 100 Sertoli cells; $n = 3$; Different letters represent significant differences ($P < 0.05$).

We observed a significant reduction in body weight following chronic exposure to atrazine. This finding corroborates previous studies reporting that atrazine exposure at doses higher than 50 mg/kg leads to a reduction in rat body weight [4,9,22,27,35]. Furthermore, we also found that the rats' body weights returned to normal after the recovery period, indicating that the time interval used was sufficient to recover this effect of the herbicide. A similar result was recently described for pre-pubertal rats exposed to the same dosage of atrazine [35]. Loss of body weight has been associated with reduction in food intake after exposure to atrazine [27,31]. We cannot rule out this possibility for the present results; however, other studies using alternative approaches have confirmed that, despite the loss of weight, atrazine has direct effect on testis and androgen biosynthesis, a factor that also interfere in the body mass [31,42].

Atrazine exposure periods of seven days and over resulted in increased relative testicular weight in parallel with an increase in the proportion of seminiferous tubules with dilated lumens, as observed in rats exposed for 15 and, even more prominently, 40 days of atrazine. Interestingly, seminiferous tubules from atrazine-treated rats presented both atrophied and dilated regions. This simultaneous increase in luminal diameter and atrophy is in agreement with previous reports describing the effects of atrazine exposure [4]. Increases in testicular weight and seminiferous tubule luminal diameter associated with fluid accumulation in the testis has been a common finding in studies of compounds affecting the balance between estradiol and testosterone, pointing out possible effects in the excurrent ducts, especially the efferent ductules [4,43–45]. The main function of the efferent ductules is reabsorption of fluid coming from the testis, accomplished under control of estrogens [44–50]. Disruption in fluid reabsorption leads to fluid accumulation in the ductule lumen and subsequent reflux to the testis [44–46]. Altogether, these results warrant further study concerning effects of atrazine on efferent ductules.

The recovery group presented a severe reduction in testes' weight, which correlated with a reduction in the total number of atrophic and Sertoli-only seminiferous tubules. Occasionally, a few atrophic tubules with spermatogonia undergoing mitosis were observed. However, spermatocytes were not detected, thus indicating that, although there was an attempt to recover spermatogenesis, the effects caused by atrazine were not reversible within the time period investigated (75 days). Accordingly, testicular weight did not recover after cessation of pre-pubertal exposure to atrazine [35]. The recovery of spermatogenesis depends on proliferation and differentiation of spermatogonia. Previous works on mice have shown long-term survival of testicular stem cell and recovery of the spermatogenesis following cytotoxic insult [51–53]. Interestingly, exposure of rat to similar agents induce testicular atrophy, despite the presence of undifferentiated spermatogonia, which fail to differentiate and recover the spermatogenesis, resembling results for human [39,54–63]. In some rat strains, the block of spermatogonial differentiation is complete even at moderate doses of cytotoxic agents [61], thus pointing out that interstrains and interspecies differences do exist. We presently found that indeed most proliferative spermatogonia remaining in the tubules were seen as isolated cells, and rarely as paired or grouped spermatogonia, thus indicating that they possibly belong to the undifferentiated population [59]. Therefore, the presence of these residual spermatogonia in tubules without postspermatogonial cells suggests a similar long-lasting blockage of spermatogenesis promoted by atrazine. However, considering that the spermatogonial differentiation block may remain irreversible for long time [63], recovery at later times should not be ruled out.

Changes in aromatase expression in testes of rats occurred after long-term exposure to atrazine (40 days). Aromatase has long been investigated as a possible target for atrazine, even though the results are still confusing, as some studies have found increase in mRNA and enzyme activity, whereas others could not detect alteration [12–14,23,32–35,64–78]. One obstacle for conciliatory interpretation is that most data were obtained from *in vitro* studies and confined to mRNA and/or enzyme activity, whereas fewer were focused in the protein level. Herein we show for the first time that the protein level is increased in the Leydig cells of adult testes, just after longer treatment, but returned to control level after recovery. Similar recovery has been found for aromatase activity in prepuberal rat testes [34]. Collectively, these findings raise the possibility that this enzyme may not be the primary target for atrazine in rat testis. In fact, others have also shown that exposure to atrazine does not change rat testicular

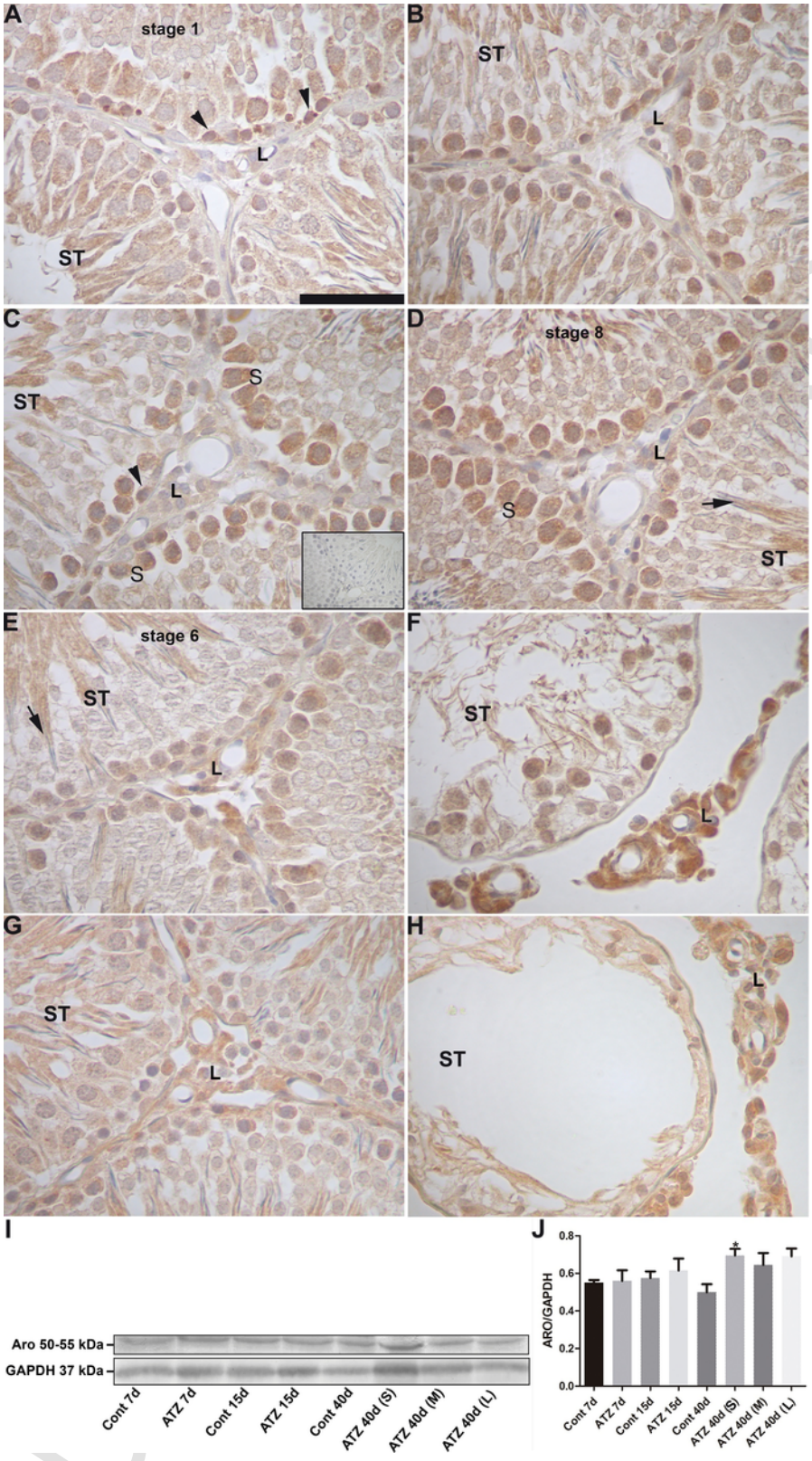

Fig. 3. Aromatase expression in testes of Wistar rats treated with atrazine. **(A, C, E and G)** Control rats receiving corn oil for 7, 15, 40 days and 40 days followed by a recovery period of 75 days without atrazine, respectively. Insert in C = negative control. **(B, D and F)** Rats treated with atrazine 200 mg/kg for 7, 15, 40 days, respectively; **(H)** Rats treated with atrazine for 40 days followed by a 75-day recovery period (ATZ 40d Rec); **(I)** Western blotting of aromatase; GAPDH was used as the internal control; **(J)** Densitometry of the

50–55 kDa band corresponding to aromatase. Arrowheads = spermatogonia; S = primary spermatocytes; arrows = spermatids; L = Leydig cell; Cont = Control; ATZ = treatment with 200 mg/kg of atrazine; d = days; (S) small; (M) mddle; (L) large testis; ST = seminiferous tubule; Scale bar in A = 50 μm. *n* = 3; * P ≤ 0.05 compared to the respective control.

aromatase activity or CYP19 mRNA, suggesting change in steroid metabolism or elimination but not biosynthesis [34,79].

The data regarding 3β-HSD in Leydig cells of rats following atrazine exposure corroborate previous studies presenting a reduction in the levels of this key enzyme in androgen biosynthesis [4,10,80]. We also found that 3β-HSD expression levels reduced further after a recovery period of 75 days without atrazine. These data further support the hypothesis that falling levels of 3β-HSD may represent an alternative mechanism by which atrazine affects testicular androgenesis, and lead to changes in spermatogenesis [4]. Evidence that atrazine affects not only the final steps of androgen metabolism has been well documented. In this regard, atrazine exposure causes a decrease in the mRNA levels of StAR (steroidogenic acute regulatory protein), SF1 (steroidogenic factor 1), TSPO (translocator protein), phosphodiesterase 4B, P450scc (cytochrome P450 cholesterol side chain cleavage enzyme), CYP17A1, 3β-HSD (3β-hydroxysteroid dehydrogenase) and 17β-HSD [1,6,9,74,81]. Information concerning the hormonal environment of adult testes undergoing recovery from atrazine exposure is still lacking, thus making the data presented herein important to further our understanding about the mechanisms related to the endocrine disruption promoted by this herbicide. Interestingly, high levels of atrazine were detected in rat testes as well as blood plasma from 7 days of treatment onward, paralleling the effects on 3β-HSD and testicular architecture.

Subsequently to chronic exposure to atrazine, there was a marked increase in the abundance of macrophage-like cells strongly positive for 3β-HSD in the testicular interstitium. The identity of these cells was confirmed by testing for the presence of ED1/CD68 and/or ED2/CD163, characterizing induced ($ED1^+/ED2^-$) and resident ($ED1^-/ED2^+$), as well as a transitory ($ED1^+/ED2^+$) macrophage sub-population. A similar increase in testicular macrophage population has been previously described in rats treated with EDS (ethane dimethane sulphonate), which is a Leydig cell-specific cytotoxin [38,82]. In both cases, the increase in macrophage count paralleled a drastic reduction in testosterone [4,82]. Ultrastructural changes in Leydig cells and macrophages, as well as their interaction, have been characterized following atrazine [4].

It is known that under normal conditions, macrophages play multiple roles in the testes, including stimulation of Leydig cell steroidogenesis [83–90]. In this regard, testicular macrophages produce 25-hydroxycholesterol, which can be converted to testosterone in Leydig cells [85,89–91]. Furthermore, 25-hydroxycholesterol induces 3β-HSD activity in Leydig cells [85]. Therefore, the observed increase in macrophage count following chronic exposure to atrazine might be the body's attempt to re-stimulate Leydig cell-mediated steroidogenesis that was impaired during the atrazine exposure period by overriding the initial stages of testosterone biosynthesis. Further studies are needed to confirm this hypothesis.

The presence of 3β-HSD in the cytoplasm of testicular macrophages is noteworthy. Indeed, the expression of 3β-HSD in testicular macrophages, to the best of our knowledge, has not been described to date. In fact, 3β-HSD has been considered a marker for Leydig cells [7]. We cannot exclude the possibility that these macrophages are removing dead Leydig cells from the interstitium. However, previous work showed that testicular macrophages in atrazine-treated rats presented fewer lysosomes than controls [4], suggesting that they may not be performing the classical role of phagocytosis. Furthermore, 3β-HSD presence was observed in testicular macrophages both after atrazine exposure and under normal conditions, thereby raising the possibility of their involvement in steroidogenesis. Data supporting the hypothesis that macrophages from various other tissues under experimental conditions have the ability to synthetize steroid hormones have been previously reported [92–95].

5. Conclusion

The results reported herein further highlight the adverse effects of atrazine upon testicular morphology and physiology, and suggest that testicular damage caused by chronic exposure of adult rats to atrazine persist even after a recovery period longer than the spermatogenic cycle.

Acknowledgements

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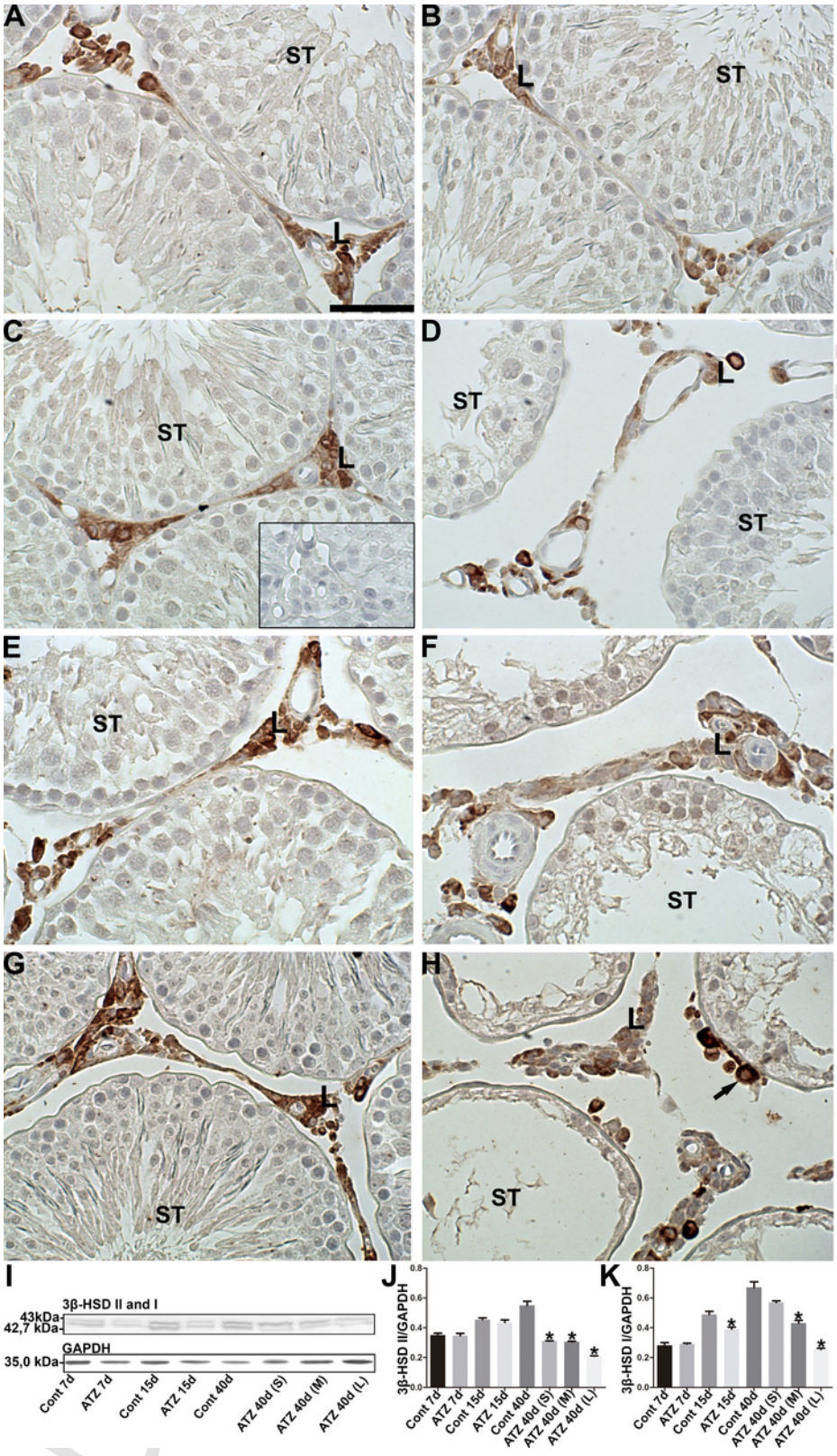

Fig. 4. Analysis of 3β-HSD expression in testes of Wistar rats treated with atrazine. **(A, C, E and G)** Control rats receiving corn oil for 7, 15, 40 days and 40 days followed by a recovery period of 75 days without atrazine, respectively. Insert in C = negative control. **(B, D and F)** Rats treated daily with atrazine 200 mg/kg for 7, 15, 40 days, respectively; **(H)** Rats treated with atrazine for 40 days followed by a 75-day recovery period (ATZ 40d Rec). Arrows indicates macrophage-like cells. **(I)** Western blotting of 3β-HSD; GAPDH was used as the internal control. **(J and K)** Densitometry of the 43.0 and 42.7 kDa bands corresponding to 3β-HSD II and 3β-HSD I, respectively. Cont = Control; ATZ = treatment with

200 mg/kg of atrazine; d = days; (S) small; (M) middle; (L) large testis; L = Leydig cell; ST = seminiferous tubule. Scale bar in C = 100 μm. $n = 3$; * $P \leq 0.05$ compared to the respective control.

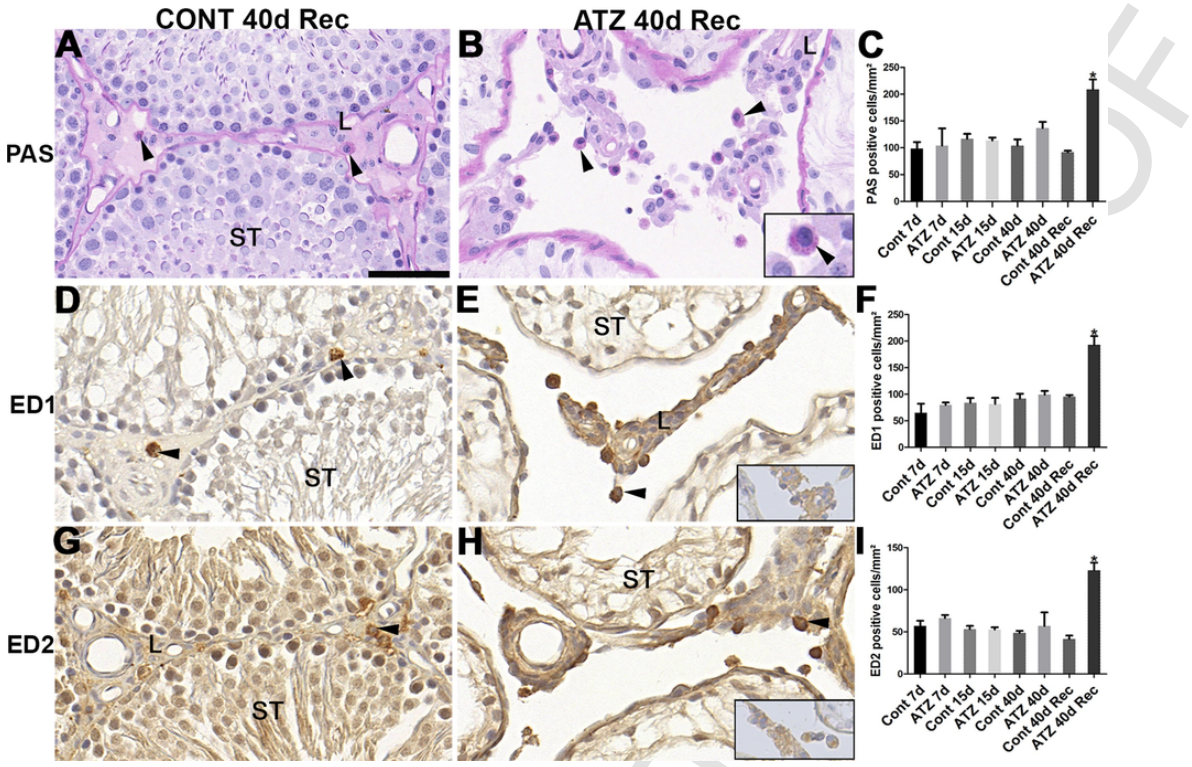

Fig. 5. Effects of atrazine on testicular macrophage-like cells of Wistar rats. **(A)** PAS-positive macrophage-like cells (arrowheads) in the testes of Cont 40d Rec (rats receiving corn oil only) and **(B)** ATZ 40d Rec rats (treated with 200 mg/kg atrazine for 40 days followed by a 75-day recovery period); Insert in B = detail of macrophage-like cell. **(C)** Number of PAS-positive macrophage-like cells per mm^2. **(D and E)** Immunohistochemistry assay showing the localization of ED1/CD68 macrophages in the testes of control and recovery group rats. **(F)** Number of $ED1^+$ macrophages per mm^2. **(G and H)** Immunohistochemistry showing the localization of ED2/CD163 macrophages in the testes of Cont 40d Rec and ATZ 40d Rec groups. **(I)** Number of $ED2^+$ macrophages. Inserts in E and H = negative controls; Cont = rats receiving corn oil only; ATZ = treatment with atrazine 200 mg/kg; Rec = recovery period of 75 days without atrazine; d = days; arrowhead = positive cells; L = Leydig Cells; ST = seminiferous tubule; Scale bar in A = 50 μm; $n = 4$; *$P \leq 0.05$ compared to the respective control.

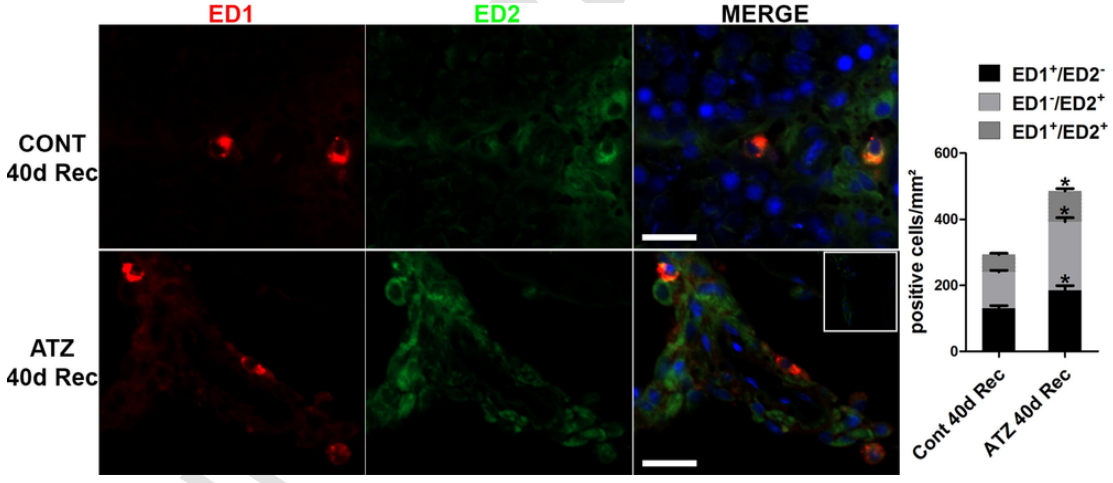

Fig. 6. Immunofluorescence showing macrophage sub-populations in the testes of adult Wistar rats. Co-localization of induced ED1/CD68 (Red) and resident ED2/CD163 (Green) macrophages in the testes of ATZ 40d Rec (rats daily exposed to 200 mg/kg of atrazine for 40 days followed by 75 days without the herbicide) and Cont 40d Rec (rats receiving corn oil only); Insert corresponds to negative control; Scale bars = 20 μm. The graph shows the number of induced ($ED1^+/ED2^-$), transitory ($ED1^+/ED2^+$) and resident ($ED1^-/ED2^+$) macrophages in the testes of control and atrazine-treated rats. Cell nuclei are stained with DAPI (Blue). $n = 3$; *$P \leq 0.05$ compared to the respective control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

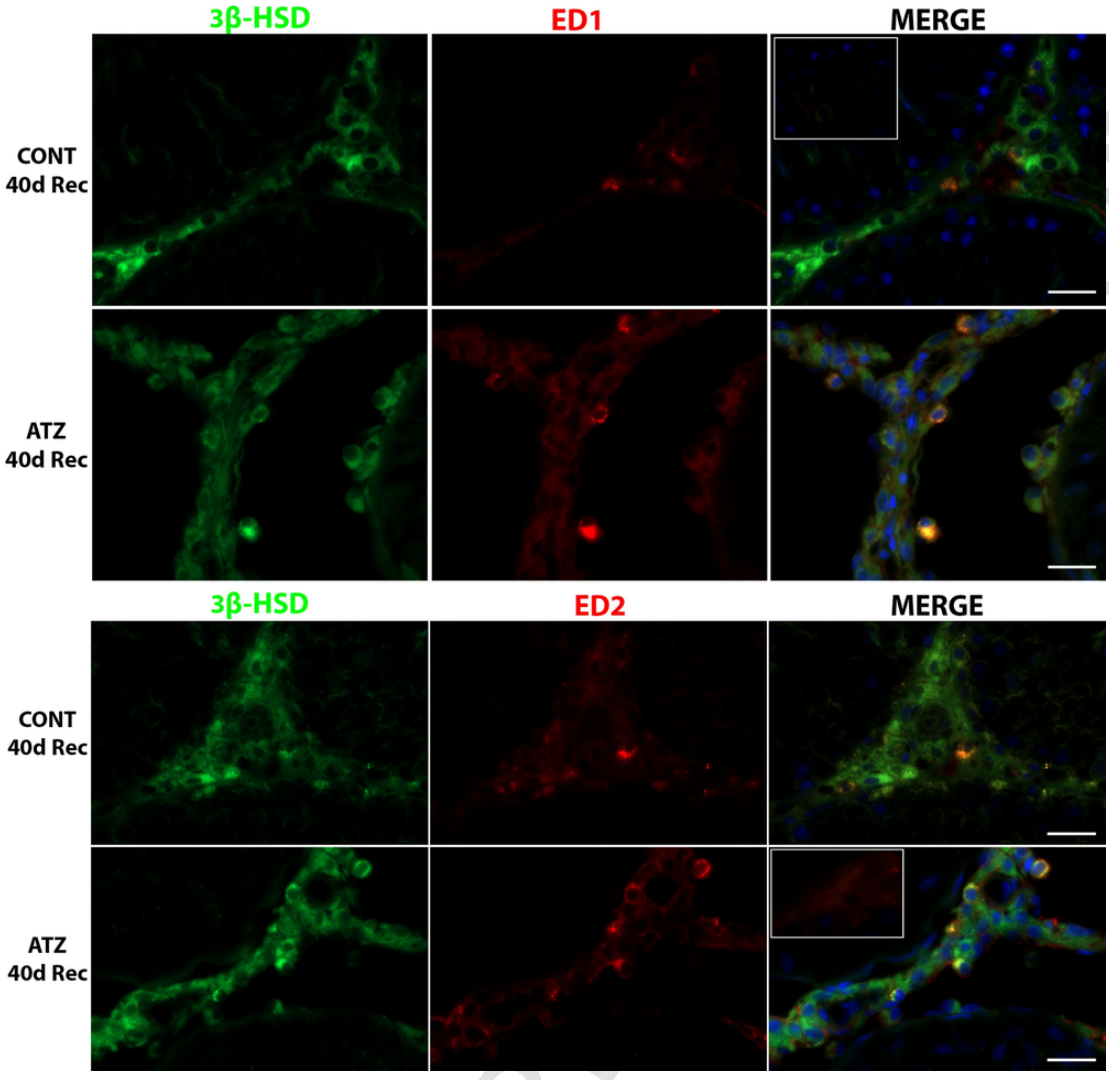

Fig. 7. Immunofluorescence showing the sub-cellular localization of 3β-HSD enzyme in Wistar rats' testicular macrophages. Co-localization of 3β-HSD (Green) and induced ED1/CD68 (top panel) or resident ED2/CD163 (bottom panel) macrophages (Red) in the testes of ATZ 40d Rec (rats exposed daily to 200 mg/kg of atrazine for 40 days followed by a 75-day recovery period without atrazine) and Cont 40d Rec (rats receiving corn oil only). Cell nuclei are stained with DAPI (Blue). Inserts correspond to negative controls; Scale bars = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

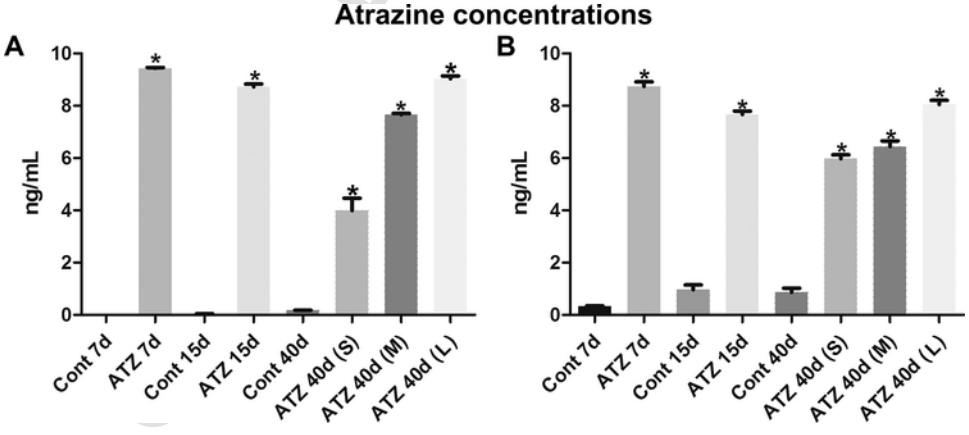

Fig. 8. Concentration of atrazine in **(A)** plasma and **(B)** testes of adult Wistar rats. Cont = Control; ATZ = treatment with 200 mg/kg of atrazine; d = days; (S) small; (M) middle; (L) large testis; L = Leydig cell; ST = seminiferous tubule. $n = 3$; * $P \leq 0.05$ compared to the respective control.

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Artigo 2

Atrazine affects the morphophysiology, tissue homeostasis and aromatase expression in the efferent ductules of adult rats with mild alterations in the ventral prostate.

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Abstract: The widely used herbicide atrazine is a potent endocrine disruptor known to cause increased aromatase expression and transient increase in testicular weight followed by remarkable testis atrophy. However, whether the effects of atrazine on the testes are primary or secondary to dysfunctions in other components of male reproductive tract remains unknown. Given the high sensitivity of the efferent ductules to estrogen imbalance and the similarity to alterations previously described for other disruptors of these ductules function, and the testicular alterations observed after atrazine exposure, we hypothesized that the efferent ductules could be a target for atrazine. Herein we characterized the efferent ductules and the ventral prostate of adult Wistar rats treated with 200mg/kg/day of atrazine for 7, 15, and 40 days. Additionally, we evaluated if the effects of atrazine in these organs could be reduced after discontinuation of the treatment. Atrazine exposure resulted in mild effects on the ventral prostate, but remarkable alterations on the efferent ductules, including luminal dilation, reduced epithelial height, and disruption of the epithelial homeostasis, which coincides with increased aromatase expression. Together with our previous data, these results suggest that at least part of the testicular effects of atrazine may be secondary to the alterations in the efferent ductules.

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September 05, 2017

Jacob de Boer
Co-Editors-in-Chief
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Dear Dr. Jacob de Boer

Please find enclosed the manuscript by Martins-Santos et al. entitled **"Atrazine affects the morphophysiology, tissue homeostasis and aromatase expression in the efferent ductules of adult rats with mild alterations in the ventral prostate"** to be considered for publication in Chemosphere.

This is a pioneering study showing effects of atrazine on the morphology of the efferent ductules and in the prostate. Atrazine exposure resulted in mild effects on the ventral prostate, but remarkable alterations on the efferent ductules, including luminal dilation, reduced epithelial height, and disruption of the epithelial homeostasis, which coincides with increased aromatase expression. Together with our previous data (Victor-Costa et al., 2010; Martins-Santos et al., 2017), these results suggest that at least part of the testicular effects of atrazine may be secondary to the alterations in the efferent ductules.

This is an original study that has not been submitted, accepted or published either in part or as a whole. The manuscript was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability. We appreciate your time considering our manuscript and look forward to receiving your editorial decision.

Yours sincerely,

Prof. Dr. Cleida Aparecida Oliveira

Highlights

Atrazine alters efferent ductules morphology with mild effects on rat ventral prostate.

Atrazine induces aromatase expression in the efferent ductules but not in the ventral prostate.

Atrazine effects in testis may be secondary to alterations in the efferent ductules.

Atrazine affects the morphophysiology, tissue homeostasis and aromatase expression in the efferent ductules of adult rats with mild alterations in the ventral prostate

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Abstract

The widely used herbicide atrazine is a potent endocrine disruptor known to cause increased aromatase expression and transient increase in testicular weight followed by remarkable testis atrophy. However, whether the effects of atrazine on the testes are primary or secondary to dysfunctions in other components of male reproductive tract remains unknown. Given the high sensitivity of the efferent ductules to estrogen imbalance and the similarity to alterations previously described for other disruptors of these ductules function, and the testicular alterations observed after atrazine exposure, we hypothesized that the efferent ductules could be a target for atrazine. Herein we characterized the efferent ductules and the ventral prostate of adult Wistar rats treated with 200mg/kg/day of atrazine for 7, 15, and 40 days. Additionally, we evaluated if the effects of atrazine in these organs could be reduced after discontinuation of the treatment. Atrazine exposure resulted in mild effects on the ventral prostate, but remarkable alterations on the efferent ductules, including luminal dilation, reduced epithelial height, and disruption of the epithelial homeostasis, which coincides with increased aromatase expression. Together with our previous data, these results suggest that at least part of the testicular effects of atrazine may be secondary to the alterations in the efferent ductules.

Key words: Atrazine, Efferent ductules, Aromatase, Endocrine disruptor, Prostate, Tissue homeostasis

1 Introduction

Exposure to estrogenic or antiestrogenic compounds and impairment of estrogen receptors by genetic or chemical inactivation are among the main causes of male infertility and have been shown to cause major dysfunction of the efferent ductules (Hess *et al.*, 1997a; Hess *et al.*, 2000; Lee *et al.*, 2001; Mckinnell *et al.*, 2001; Oliveira *et al.*, 2001; Zhou *et al.*, 2001; Oliveira *et al.*, 2002; Lee *et al.*, 2009; Hess *et al.*, 2011; Nanjappa *et al.*, 2016). The efferent ductules are the segment of the male reproductive tract presenting the highest levels of estrogen in the luminal fluid and the highest expression of estrogen receptor in the epithelium (Hess *et al.*, 1997b). Their main function is to reabsorb fluid coming from the testis, which occurs under the control of estrogens (Hess *et al.*, 1997a; Hess *et al.*, 2000; Lee *et al.*, 2001; Oliveira *et al.*, 2001; Zhou *et al.*, 2001; Oliveira *et al.*, 2002; Oliveira *et al.*, 2005). Disturbance in fluid reabsorption leads to the accumulation of fluid in the ductule lumen and consequent reflux to the testis, thus resulting in luminal dilation of the seminiferous tubules followed by testicular atrophy and, consequently, infertility (Hess *et al.*, 1997a; Oliveira *et al.*, 2001; Oliveira *et al.*, 2002).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a widely used herbicide shown to be a potent endocrine disruptor and cause adverse effects on the male genital system (Kniewald *et al.*, 2000; Betancourt *et al.*, 2006; Hayes *et al.*, 2006; Swan, 2006; Suzawa e Ingraham, 2008; Rey *et al.*, 2009; Hayes *et al.*, 2010; Belloni *et al.*, 2011; Hayes *et al.*, 2011). Potential risks for animal health include the increase in estrogen and reduction in the testosterone levels (Stoker *et al.*, 2000; Friedmann, 2002; Victor-Costa *et al.*, 2010). Among the

48 effects of atrazine on male rats are testicular alterations, such as a transient
49 increase in weight followed by a remarkable testicular atrophy (Victor-Costa *et al.*,
50 2010), with a concurrent increase in aromatase immunoexpression in Leydig cells
51 (Martins-Santos *et al.*, 2017). Although evidence suggests that this key enzyme for
52 estrogen production could be a target for the herbicide (Crain *et al.*, 1997;
53 Sanderson *et al.*, 2000; Sanderson *et al.*, 2001; Sanderson *et al.*, 2002; Heneweer
54 *et al.*, 2004; Laville *et al.*, 2006; Sanderson, 2006; Fan *et al.*, 2007a; Fan *et al.*,
55 2007b; Holloway *et al.*, 2008; Tinfo *et al.*, 2011), atrazine's mechanism of action
56 remains to be elucidated. Moreover, most of the atrazine's effects on aromatase
57 were demonstrated *in vitro* (Sanderson *et al.*, 2000; Sanderson *et al.*, 2001;
58 Sanderson *et al.*, 2002; Heneweer *et al.*, 2004; Betancourt *et al.*, 2006; Laville *et*
59 *al.*, 2006; Fan *et al.*, 2007a; Fan *et al.*, 2007b; Holloway *et al.*, 2008; Suzawa e
60 Ingraham, 2008; Higley *et al.*, 2010; Tinfo *et al.*, 2011; Quignot *et al.*, 2012b; Fa *et*
61 *al.*, 2013; Caron-Beaudoin *et al.*, 2016).
62 One important question that remains unanswered is whether the atrazine
63 effects in the testis are primary or secondary to changes in other testicular
64 segments of the male reproductive tract. Given the high sensitivity of the efferent
65 ductules to estrogen imbalance, the similarity to the alterations previously
66 described for other disruptors of these ductules function (Nakai *et al.*, 1992; Nakai
67 *et al.*, 1993; Hess, 1998; Gotoh *et al.*, 1999), and the testicular alterations
68 observed after atrazine exposure, we hypothesized that the efferent ductules could
69 be a target for atrazine. We investigated this hypothesis by evaluating the effects of
70 atrazine on the morphology, aromatase expression, cell proliferation, and
71 apoptosis of the efferent ductules. For comparison purposes, we also evaluated

the effects of atrazine on the ventral prostate, which is an important target for androgens and estrogens (Ellem e Risbridger, 2010).

2 Materials and Methods

2.1 Animals

Sexually mature male Wistar rats (100 days old), acquired from the Animal Facility at the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Brazil, were used in this study. The rats were maintained under constant conditions of light (12/12h light/dark cycles) and temperature (22 ºC), receiving pelletized chow (Nuvital Nutrientes S.A., Colombo, PR, Brazil) and water *ad libitum.* All experimental procedures followed the guidelines of UFMG's animal care and research of the Institutional Ethical Committee on Animal Use (https://www.ufmg.br/bioetica/ceua/). The study was approved by the Institutional Ethical Committee for Animal Experimentation (Protocol number 287/2008).

2.2 Treatment

For 7, 15, and 40 days, sexually mature male Wistar rats received a daily dose of 200 mg/kg of atrazine (Gesaprim 500 Ciba Geigy, Syngenta, São Paulo, Brazil) diluted in corn oil. The control group received only corn oil. The dosages and treatment periods were based in previous studies showing alterations in testicular morphology and steroidogenesis (Victor-Costa *et al.*, 2010; Martins-Santos *et al.*, 2017).

In order to determine if the possible alterations caused by atrazine are permanent or transitory, after the 40-days treatment, a group of rats was maintained in the animal facility under the same environmental conditions for further 75 days (ATZ 40d Rec), without any treatment. Control of the recovery group (Cont 40d Rec) consisted of rats receiving only corn oil for 40 days and maintained for further 75 days (Martins-Santos *et al.*, 2017).

2.3 Tissue preparation

Subsequently to each treatment and recovery periods, the rats were weighed, anesthetized with intraperitoneal injection of sodium pentobarbital (80 mg/kg) and ketamine chlorydrate (10 mg/kg) and then perfused intracardially with Ringer solution, followed by 10% (v/v) of neutral buffered formalin (NBF). After fixation, the efferent ductules and ventral prostate were dissected, immersed in NBF, and stored at 4°C. Fragments of both segments were routinely processed for histological and immunohistochemical analyses.

2.4 Histology and morphometry

The tissue fragments were dehydrated with ascending concentrations of ethanol solutions, embedded in paraffin (Histosec pastilhas, Merck KGaA, Darmstadt, Germany), cut into 5-µm-thick sections and placed on glass slides. The sections were stained with hematoxylin and eosin (HE), and 1% toluidine blue-sodium borate solution for histological analysis.

The luminal diameters of the proximal and distal efferent ductules were measured at the widest region of five randomly chosen transversal sections per animal, by

using a scale of 1000 µm coupled to the eyepiece of the microscope (Nikon Eclipse E200, Melville, USA). Measurements were made at 400X magnification.

The height of the proximal efferent ductules epithelium was measured from the basement membrane to the microvillus tip, in areas of straight sections from 25 cells with evident nuclei (in five tubule sections). Pictures were taken by using Pannoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary). The proximal area was selected for this measurement based on previous studies showing that this is the region more sensitive to disruption on the estrogen responsive system (Oliveira *et al.*, 2002).

2.5 Immunohistochemistry

Immunohistochemistry was performed to detect aromatase in the efferent ductules and the ventral prostate and to determine possible alterations in cell proliferation and apoptosis by using MCM7 and caspase-3 as markers, respectively.

To this end, fragments of NBF-fixed tissues were embedded in paraffin, cut into 5-µm-thick sections, mounted on glass slides, dewaxed, re-hydrated in graded ethanol, and incubated in methanol containing 0.6% H_2O_2 to inactivate the endogenous peroxidase. After microwaving in 0.1 M citrate buffer, pH 6.0, for antigen retrieval, the endogenous biotin activity was blocked by avidin and biotin-blocking solution (avidin/biotin blocking kit; Vector Laboratories, Burlingame, USA). To block non-specific antibody binding, the sections were incubated with 10% normal goat serum, followed by an overnight incubation at 4°C with the primary antibodies: polyclonal rabbit anti-human aromatase (Sigma-Aldrich, Saint Louis,

142 USA), diluted 1:500; monoclonal mouse anti-CDC47/MCM7 (Ab-2, Thermo Fisher
143 Scientific, Rockford, USA), diluted 1:500; or polyclonal rabbit anti-cleaved caspase-
144 3 (Millipore Corporation, California, USA), diluted 1:200. Negative controls were
145 incubated with phosphate buffer saline (PBS) instead of the primary antibodies.
146 Tissues were then incubated for 1 hour with a biotinylated secondary antibody:
147 goat anti-rabbit (for aromatase and caspase-3) or goat anti-mouse (for MCM7)
148 (Dako, Carpinteria, USA), both diluted 1:100. This step was followed by incubation
149 with avidin-biotin complex conjugated with peroxidase (Vectastain Elite ABC kit –
150 Vector Laboratories, Burlingame, USA), for 30 min. The immunoreaction was
151 visualized using 3,3 diaminobenzidine containing 0.01% hydrogen peroxide in 0.05
152 M Tris-HCl buffer, pH 7.6. Sections were counterstained with Mayer's hematoxylin
153 and mounted.
154
155 **2.6 TUNEL**
156 Apoptotic cells were also investigated in the efferent ductules by TUNEL
157 (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling), using the
158 ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore Corporation,
159 California, USA). The assays were performed according to the manufacturer's
160 instructions, with some modifications described in (Gonzaga *et al.*, 2017).
161
162 **2.7 Quantitative analyses**
163 The intensity of the cytoplasmic immunoreaction for aromatase in the
164 epithelium was measured in five randomly chosen areas of the proximal and distal
165 efferent ductules and the ventral prostate, for each animal, using the Adobe

Photoshop CS6 (Adobe Systems Software, Mountain View, USA). The images were taken at 40X magnification, using the Pannoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary), converted to grayscale mode and inverted. The average number of pixels was measured in the apical cytoplasm of six non-ciliated cells of the efferent ductules and luminal cells of the prostate per area, thus totalizing 30 cells per animal (Oliveira *et al.*, 2007; Oliveira *et al.*, 2013).

The number of MCM7, caspase-3, and TUNEL positive cells were counted among 100 epithelial cells from five randomly chosen areas of the proximal and distal efferent ductules and the ventral prostate as in (Gonzaga *et al.*, 2017). Positive and negative cells were counted on images taken at 400X magnification, using the Pannoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary). The results were expressed as percentage of positive epithelial cells.

2.8 Statistical analysis

Data obtained from quantitative studies were analyzed using GraphPad Prism (GraphPad Software, San Diego, California, USA). The data were submitted to Shapiro-Wilk normality test and investigated for multiple variances using one-way ANOVA and a post-hoc Tukey's test to compare more than two populations or Student's t-test was used to compare the means between two groups. Nonparametric data were analyzed using the Mann-Whitney and Kruskal-Wallis followed by Dunn's post-hoc tests for comparisons between two or more groups, respectively. The data were expressed as the mean ± SEM, and differences were considered significant at $p \leq 0.05$.

3 Results

3.1 Atrazine treatment alters efferent ductules histology with mild effects on the ventral prostate of male rats

After 7 days of treatment, the morphology of the efferent ductules from rats exposed to atrazine was similar to the controls (Fig. 1), except for a slight decrease in epithelial height (Fig. 1S). After 15 and 40 days of atrazine exposure, the rats exhibited luminal dilation (Fig. 1) and further reduction in epithelial height in most efferent ductules (Fig. 1S). The luminal dilation increased 58% and 34% in the proximal ductules and 119% and 57% in the distal ductules after 15 and 40 days of treatment, respectively (Fig. 1T-U). The epithelial height reduced 50% and 54% in the proximal ductules after 15 and 40 days of treatment, respectively (Fig. 1Q-S). After 40 days, we also observed luminal sloughed epithelial cells (insert in Fig. 1O).

On the other hand, the luminal diameter of proximal and distal ductules significantly decreased (61% and 18%, respectively) after the recovery period (Fig. 1T-U). We also observed a 15% reduction in the epithelial height of the proximal efferent ductules of rats of the recovery group in comparison with the control (Cont 40d Rec) (Fig. 1S).

The morphology of the ventral prostate varied considerably among the animals after atrazine exposure and for each period of treatment, since we observed glands with normal appearance and glands with the presence of cystic or hyperplasic acini. Foci of inflammatory cells were frequent in the stroma, especially after long-term treatment (40 days), and were still present after the recovery period (Fig. 2).

3.2 Aromatase expression is altered in the efferent ductules but not in the ventral prostate following atrazine treatment of male rats

Aromatase was immunodetected in the cytoplasm of non-ciliated epithelial cells of the efferent ductules in all the animals, whereas staining of ciliated cells was intermittent (Fig.3). The groups treated with atrazine, including the recovery group (ATZ 40d Rec), presented increased cytoplasmic aromatase staining at both proximal and distal regions of the efferent ductules in comparison with the controls (Fig. 3).

Immunoreaction was positive for aromatase in the cytoplasm of the luminal cells of the ventral prostate, intermittent in the basal cells, and not detected in the peritubular smooth muscle cells (Fig. 4A-E). No detectable changes were observed in the pattern and intensity of the aromatase immunoreactivity in the luminal cells after atrazine treatment (Fig. 4F).

3.3 Cell proliferation increases in efferent ductules and decreases in the ventral prostate of atrazine-treated male rats

Cell proliferation in the efferent ductules and ventral prostate was assessed using the MCM7 marker. In control animals, the protein was detected in the nuclei of some cells along the efferent ductules epithelium, mainly in non-ciliated cells (Fig. 5). Cells were found more frequently dividing perpendicular to the basement membrane (Fig. 5C) or in small groups (Fig. 5K), whereas some cells were found dividing parallel to the basement membrane (Fig. 5J). After exposure to atrazine, cell proliferation increased (Fig. 5Q-R). The pattern of cell division was similar to that found in control groups, which also showed cellular divisions parallel and

perpendicular to the basement membrane. Many cells were dividing in parallel, thus resulting in rows of MCM7 positive cells. In the recovery groups, we observed few cells positive for MCM7 in atrazine-treated and control animals, in both the proximal (Fig. 5 D and H) and distal regions (Fig 5 L and P) of the efferent ductules.

Considering the scarcity of information regarding cell proliferation in the normal efferent ductules, we compared the proliferation rate from control rats at different ages (107, 115, 140 and 210 days old). We detected a gradual decrease in cell proliferation with aging, which was statistically significant at the proximal and distal efferent ductules (Fig. 5S). When the proliferation rates of proximal versus distal region were compared, we observed a higher proliferation rate at the distal efferent ductules at all the time points analyzed (Fig. 5S).

In atrazine-exposed rats, cell proliferation was also reduced in the ventral prostate epithelium. The index of MCM7 positive cells was smaller in the gland of treated animals when compared with the controls, with rates of 85.6%, 90.8%, and 86.5% at 7, 15, and 40 days of exposure, respectively (Table 1). After the recovery period, cell proliferation in the ventral prostate of atrazine-treated animals (ATZ 40d Rec) returned to normal levels.

3.3 Apoptosis increases in the distal efferent ductules of atrazine-treated male rats and in the ventral prostate of animals subjected to a recovery period

Apoptosis in the efferent ductules was assessed using the marker caspase-3 and TUNEL. Caspase-3-positive cells were undetectable in the epithelium of the

proximal ductules and scarce (0.02%) in the distal ductules of control animals (Table 1). After exposure to atrazine, the amount of caspase-3 positive epithelial cells was variable, as in some animals these cells were undetectable whereas in others they appeared increased, especially in the distal ductules. As a result of this heterogeneity, statistical significance was reached just for distal efferent ductules of animals treated for 40 days, even though the number of cells was 4 to 4.5 times greater in the treated group. After recovery, the number of caspase-3 positive cells was similar when ductules from treated and control rats, at both proximal and distal regions, were compared.

The distribution pattern of TUNEL-positive cells was similar to that observed for caspase-3. After all treatment periods, a trend towards increased positivity was observed in both the proximal and the distal efferent ductules and reached statistical significance after 40 days of exposure (Table 1). The percentage of TUNEL-positive cells was similar in the recovery groups of atrazine-treated and control animals

Caspase-3-positive cells were rare or undetectable in the epithelium of the ventral prostate of control and treated animals, except in the recovery group (ATZ 40d Rec), in which a significant increase of caspase-3-positive cells was observed when compared to the respective control (Table 1).

4 Discussion

In the present study, we investigated whether the atrazine effects previously observed in the testis (Victor-Costa *et al.*, 2010; Martins-Santos *et al.*, 2017) were primary or secondary to changes in other male reproductive tract segments. We

show that exposure to atrazine results in increased luminal diameter, reduced epithelial height, increased aromatase expression, and disrupted rates of cell proliferation and apoptosis in the rat efferent ductules, whereas the effects on the ventral prostate were mild. Given that similar alterations in the efferent ductules lead to dilation and testicular atrophy (Hess *et al.*, 1997a; Oliveira *et al.*, 2001; Oliveira *et al.*, 2002), our results favor the hypothesis that the testicular changes in rats exposed to atrazine may be, at least in part, secondary to the alterations in the efferent ductules.

The significant increase observed in the luminal diameter and the reduction in the epithelial height closely resemble those associated with impairment in luminal fluid reabsorption in rodent efferent ductules induced by alteration in the estrogen responsive system (Hess *et al.*, 1997a; Oliveira *et al.*, 2001; Zhou *et al.*, 2001; Oliveira *et al.*, 2002; Cho *et al.*, 2003; Oliveira *et al.*, 2003; Nanjappa *et al.*, 2016). Non-reabsorbed fluid accumulates in the lumen and returns to the testis, ultimately causing testicular damages, such as seminiferous tubules dilation and atrophy, followed by a reduction in sperm and infertility (Hess *et al.*, 1997a; Oliveira *et al.*, 2001; Oliveira *et al.*, 2002). Our present and previous results (Victor-Costa *et al.*, 2010; Martins-Santos *et al.*, 2017) suggest that similar mechanism may explain the effects observed in the efferent ductules and testis following exposure to atrazine. Interestingly, after the recovery period, the luminal diameter of the efferent ductules was greatly reduced when compared to controls. Although there was a clear trend for recovery of the epithelial height, it remained lower than controls after recovery. Reduction in the diameter and epithelial height of the

309 efferent ductules coincides with testis atrophy (Martins-Santos *et al.*, 2017), thus
310 emphasizing the intricate relationship between testis and efferent ductules.
311 The morphological changes promoted by the herbicide in the efferent
312 ductules were similar to those found in other experimental models of androgen and
313 estrogen imbalance (Mckinnell *et al.*, 2001; Oliveira *et al.*, 2001; Oliveira *et al.*,
314 2002; Cho *et al.*, 2003). Stimulation of aromatase has long been speculated as a
315 possible mechanism of atrazine action on the male reproductive system (Crain *et*
316 *al.*, 1997; Sanderson *et al.*, 2000; Sanderson *et al.*, 2001; Hayes *et al.*, 2002;
317 Sanderson *et al.*, 2002; Heneweer *et al.*, 2004; Hayes *et al.*, 2006; Laville *et al.*,
318 2006; Sanderson, 2006; Fan *et al.*, 2007a; Fan *et al.*, 2007b; Holloway *et al.*, 2008;
319 Tinfo *et al.*, 2011; Quignot *et al.*, 2012b; Jin *et al.*, 2013; Thibeault *et al.*, 2014;
320 Caron-Beaudoin *et al.*, 2016; Martins-Santos *et al.*, 2017). Indeed, we found a
321 remarkable increase in aromatase immunoreactivity in the efferent ductules after
322 atrazine exposure. This increase was detected as early as 7 days after the
323 treatment and remained high even after the recovery period, in contrast to the
324 testis, which showed aromatase expression increase only after 40 days of
325 exposure (Martins-Santos *et al.*, 2017). These data suggest that aromatase may
326 be a direct target of atrazine on the efferent ductules.
327 Since the cell proliferation profile has not been previously established in the
328 epithelium of rodent efferent ductules, we first characterized MCM7 expression
329 under normal conditions. Few proliferative cells were observed in the efferent
330 ductules of adult rats and they were mainly non-ciliated cells, thus confirming that
331 this is a stable tissue during adulthood. Cell division occurred both parallel and
332 perpendicular to the basement membrane. In the epididymal duct, these patterns

333 of division are known to result in increased duct length and diameter, respectively
334 (Hinton *et al.*, 2011). We observed regional variations in cell proliferation, as higher
335 rates were found in the distal efferent ductules when compared to the proximal.
336 These results are in agreement with data showing that expression of the androgen
337 receptor (AR) and the estrogen receptor ERα, two potent proliferation factors, is
338 higher in the distal efferent ductules (Oliveira *et al.*, 2003). Additionally, we showed
339 that the proportion of cell proliferation significantly decreased in the efferent
340 ductules of older compared to younger animals. Although age-related changes in
341 cell proliferation were still uncharacterized in the efferent ductules, similar
342 morphological alterations and decreased proliferative activity have been previously
343 reported in other segments of the male reproductive tract, such as the epididymis
344 (Clermont e Flannery, 1970; Calvo *et al.*, 1999).

345 After exposure to atrazine, we observed increased cell proliferation in the
346 proximal and distal efferent ductules in comparison with the control at the same
347 time points. In contrast, apoptosis remained unaffected in the experimental groups,
348 except after 40 days of treatment, when apoptosis was higher in the treated
349 animals, especially in the distal ductules. This disruption in the rates of cell
350 proliferation and apoptosis after atrazine exposure corroborates other findings that
351 this herbicide may affect tissue homeostasis in vertebrate species (Liu *et al.*, 2006;
352 Lenkowski *et al.*, 2008; Rey *et al.*, 2009; Zhang *et al.*, 2011; Song *et al.*, 2015) and
353 suggests that this may be another possible mechanism of action for atrazine on
354 animal tissues. Furthermore, cell proliferation and apoptosis in the recovery group
355 (ATZ 40d Rec) returned to levels similar to the controls, thus indicating that the

356 discontinuation of atrazine treatment was sufficient to re-establish tissue
357 homeostasis.
358 Atrazine effects in male extragonadal organs, such as the prostate, are not
359 well established. Herein, we showed variable changes in the morphology of the
360 ventral prostate, especially regarding inflammation in the stroma after long-term
361 exposure. These results are in agreement with previous data showing that atrazine
362 acts as a prostatitis inductor in male rats (Stoker *et al.*, 1999; Stanko *et al.*, 2010).
363 Atrazine exposure also reduced the prostate proliferation index in all the
364 experimental groups, further emphasizing the above-mentioned capability of this
365 herbicide to disrupt tissue homeostasis and its potential ability to promote tumor
366 malignancy in the prostate (Hu *et al.*, 2016). Following the cessation of exposure,
367 cell proliferation returned to levels similar to the controls, suggesting that the
368 effects may be at least partially reversible.
369 It is known that atrazine may be an inductor of aromatase in several human
370 cancer cell lines (Sanderson *et al.*, 2000; Sanderson *et al.*, 2001; Sanderson *et al.*,
371 2002; Heneweer *et al.*, 2004; Fan *et al.*, 2007a; Fan *et al.*, 2007b; Tinfo *et al.*,
372 2011; Quignot *et al.*, 2012b; Thibeault *et al.*, 2014; Caron-Beaudoin *et al.*, 2016),
373 and correlated with an elevated risk of prostate cancer development (Hu *et al.*,
374 2016). Surprisingly, we found that expression of this enzyme was not altered in the
375 ventral prostates of rats exposed to atrazine, indicating that aromatase induction
376 may be restricted to the efferent ductules and testis (Martins-Santos *et al.*, 2017).
377 Aromatase is knowingly tissue-specifically regulated (Mahendroo *et al.*, 1993;
378 Simpson e Davis, 2001). Likewise, atrazine induction of aromatase is also tissue-
379 specific and appears to involve direct binding to the steroidogenic factor 1 (SF-1)

(Fan *et al.*, 2007a; Fan *et al.*, 2007b; Suzawa e Ingraham, 2008). In this sense, tissues expressing SF-1 may be more susceptible to atrazine, which could explain the tissue-specific effects observed on aromatase induction, as SF1 is expressed in the testis and epididymis (Pezzi *et al.*, 2004; Rivest *et al.*, 2010), but is lacking in the normal prostate tissue (Lewis *et al.*, 2014). Although this may offer a plausible explanation for the variable responses to the herbicide, further studies would be required to confirm this hypothesis.

5 Conclusion

In summary, this study revealed that atrazine effects on the ventral prostate are mild, but exposure to this herbicide leads to remarkable alterations on the efferent ductules, including luminal dilation and disruption of the epithelial homeostasis, which coincides with increased aromatase expression. Together with our previous data (Victor-Costa *et al.*, 2010; Martins-Santos *et al.*, 2017), these results suggest that at least part of the testicular effects of atrazine may be secondary to the alterations in the efferent ductules.

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Figure legends

Figure 1: Effects of atrazine on the morphology of the efferent ductules of Wistar rats. **(A - D, I - L)** Representative efferent ductules of control animals. (**E - G, M - O)** Efferent ductules of rats treated with atrazine (200 mg/kg/day) for 7, 15, and 40 consecutive days, respectively. **(H and P)** ED of rats treated with atrazine for 40 days followed by a 75-day recovery period (ATZ 40d Rec). **(Q, R and S)** Effects of atrazine on the epithelial height of efferent ductules: **(Q)** normal epithelium; **(R)** reduced epithelial height; **(S)** Graphical representation of the epithelial height of the proximal efferent ductules. **(T and U)** Graphical representation of effects of atrazine on luminal diameter of the **(T)** proximal and **(U)** distal efferent ductules. Scale bar in A (= B to P) = 200 µm; Scale bar in Q (= R) = 50 µm; Double arrows indicate luminal diameter; * = stroma; *SPZ* = sperm; Insert in O indicate sloughed epithelial cells; Cont = Control; ATZ = atrazine; Rec = recovery group; d = days of treatment; n = 4; * $P \leq 0.05$ compared to the respective control.

Figure 2: Effects of Atrazine on morphology of the ventral prostate of Wistar rat. **(A)** Representative image of the ventral prostate of control animals. **(B, C, D and E)** Ventral prostate of rats treated with atrazine 200mg/Kg/day for 7, 15, and 40 days, respectively. **(F)** Ventral prostate of rats treated with atrazine for 40 days followed by a 75-day recovery period (ATZ 40d Rec). Scale bar in A = 200 µm; * = stroma; L = lumen; arrow = inflammatory foci.

Figure 3: Effect of atrazine on expression of aromatase in the efferent ductules of Wistar rats. **(A - D, I - L)** Representative efferent ductules of control animals. **(E - G, M - O)** Efferent ductules of rats treated with atrazine (200 mg/kg/day) for 7, 15, and 40 consecutive days, respectively. **(H and P)** Efferent ductules of rats treated with atrazine for 40 days followed by a 75-day recovery period (ATZ 40d Rec). **(Q and R)** Graphical representation of the quantification of aromatase expression in proximal **(Q)** and distal **(R)** efferent ductules. Scale bar in A = 50 µm; Insert in P = negative control; Arrow and insert in C = Ciliated positive cells; hollow arrow and insert in G = Ciliated negative cells; ED = Efferent ductules; Cont = Control; ATZ = atrazine; Rec = recovery group; d = days of treatment; n = 4; * P ≤ 0.05 compared to the respective control.

Figure 4: Effects of atrazine on aromatase expression in the ventral prostate of Wistar rat. **(A)** Representative image of the ventral prostate of the control animals. **(B - D)** Ventral prostate of rats treated with atrazine 200mg/Kg/day for 7, 15, and 40 days, respectively. **(E)** Ventral prostate of rats treated with atrazine for 40 days followed by a 75-day recovery period (ATZ 40d Rec). **(F)** Graphical representation of quantification of aromatase expression in the ventral prostate. Scale bar in A = 50 µm; Insert in B and arrow = positive basal cell; hollow arrow = negative basal cell; Insert in E = negative control; L = lumen; Ep= epithelium positive for aromatase. n = 4; * P ≤ 0.05 compared to the respective control.

Figure 5: Effects of atrazine on proliferative MCM7 positive cells in the efferent ductules of Wistar rat. **(A - D, I - L)** Representative efferent ductules (ED) of control

906 animals. **(E - G, M - O)** ED of rats treated with atrazine (200 mg/kg) for 7, 15 and
907 40 consecutive days, respectively. **(H and P)** ED of rats treated with atrazine for 40
908 days followed by a 75-day recovery period (ATZ 40d Rec). **(Q and R)** Graphical
909 representation of quantification of MCM7 positive cells in the **(Q)** proximal and **(R)**
910 distal ED. **(S)** Graphical representation of quantification of MCM-7 positive cells in
911 the control proximal (P) versus distal (D) ED at different ages. Scale bar in A = 50
912 µm; Insert in B = detail of a positive nuclei; Insert in C = cells dividing perpendicular
913 to the basement membrane; Insert in J = cells dividing parallel to the basement
914 membrane; Insert in P = negative control; Arrowheads = positive cells; Cont =
915 Control; ATZ = atrazine; Rec = recovery; d = days; n = 4; Different letters indicate P
916 ≤ 0.05 among ages in the same region. * P ≤ 0.05 compared to respective age
917 (proximal versus distal).

Table 1
Click here to download Table: TABLE 1.pdf

Table 1: Effect of atrazine on cell proliferation and apoptosis in the ventral prostate and efferent ductules of adult male Wistar rats.

	Cont 7d	ATZ 7d	Cont 15d	ATZ 15d	Cont 40d	ATZ 40d	Cont 40d Rec	ATZ 40d Rec
MCM7 VP	31.67 ±20.42	4.54* ±2.55	28.90 ±19.35	2.64* ±2.01	13.87 ±5.29	1.87* ±1.29	3.40 ±2.60	5.93 ±4.49
Caspase-3 ED proximal	0.00 ± 0.00	0.02 ± 0.04	0.00 ± 0.00	0.04 ± 0.08	0.00 ± 0.00	0.02 ± 0.02	0.00 ± 0.00	0.01 ± 0.02
ED distal	0.02 ± 0.04	0.08 ± 0.07	0.02 ± 0.04	0.09 ± 0.13	0.00 ± 0.00	0.09* ± 0.08	0.02 ± 0.04	0.02 ± 0.02
VP	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	0.00 ± 0.00	0.12* ± 0.17
TUNEL ED proximal	0.00 ± 0.00	0.03 ± 0.05	0.01 ± 0.02	0.11 ± 0.12	0.01 ± 0.02	0.12* ± 0.04	0.00 ± 0.00	0.01 ± 0.02
ED distal	0.04 ± 0.04	0.07 ± 0.12	0.00 ± 0.00	0.08 ± 0.14	0.00 ± 0.00	0.05* ± 0.02	0.00 ± 0.00	0.01 ± 0.02

* $P \leq 0.05$ compared to the respective control. Cont = Control; ATZ = atrazine; Rec = recovery; d = days; n = 3 - 4; ED= efferent ductules; VP = ventral prostate.

Figure 1

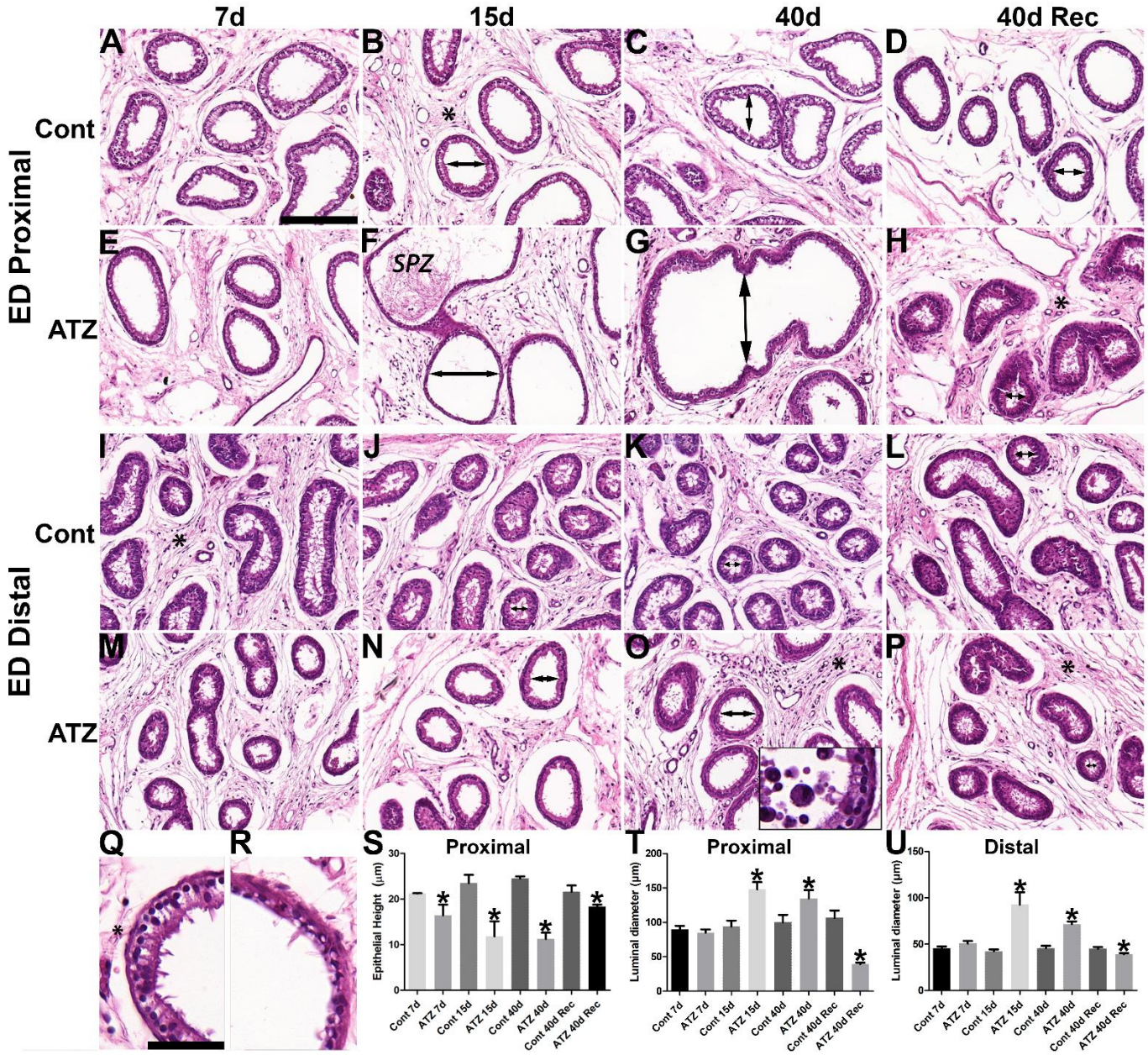

7d
15d
40d
40d Rec
ED Proximal
Cont
ATZ
ED Distal
Cont
ATZ
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
SPZ
Proximal
Epithelial Height (µm)
Proximal
Luminal diameter (µm)
Distal
Luminal diameter (µm)
Cont 7d
ATZ 7d
Cont 15d
ATZ 15d
Cont 40d
ATZ 40d
Cont 40d Rec
ATZ 40d Rec

Figure 2

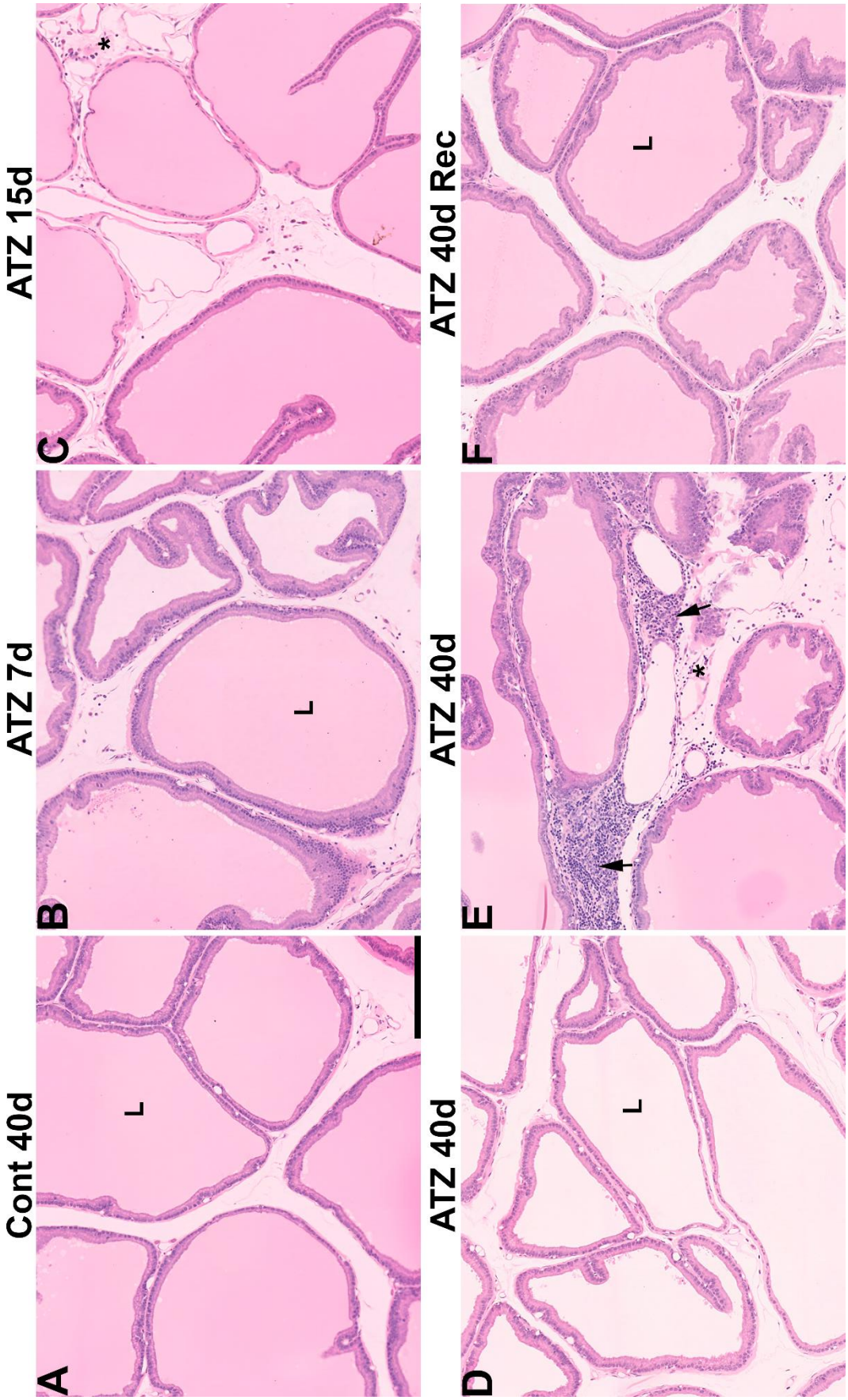


Figure 3

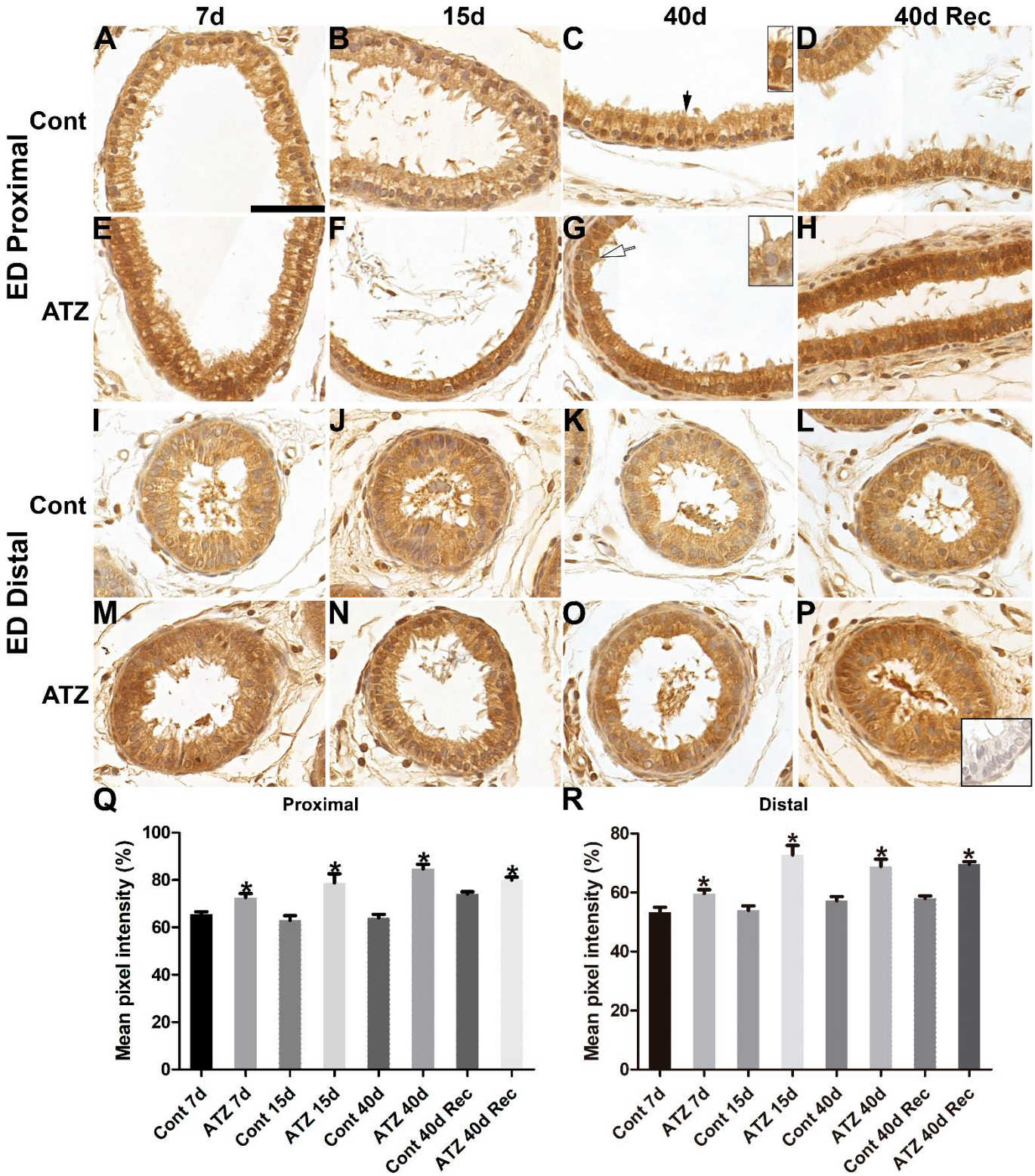

7d
15d
40d
40d Rec
A
B
C
D
Cont
ED Proximal
E
F
G
H
ATZ
I
J
K
L
Cont
ED Distal
M
N
O
P
ATZ
Q
Proximal
R
Distal
Mean pixel intensity (%)
100
80
60
40
20
0
Cont 7d
ATZ 7d
Cont 15d
ATZ 15d
Cont 40d
ATZ 40d
Cont 40d Rec
ATZ 40d Rec
Mean pixel intensity (%)
80
60
40
20
0
Cont 7d
ATZ 7d
Cont 15d
ATZ 15d
Cont 40d
ATZ 40d
Cont 40d Rec
ATZ 40d Rec

Figure 4

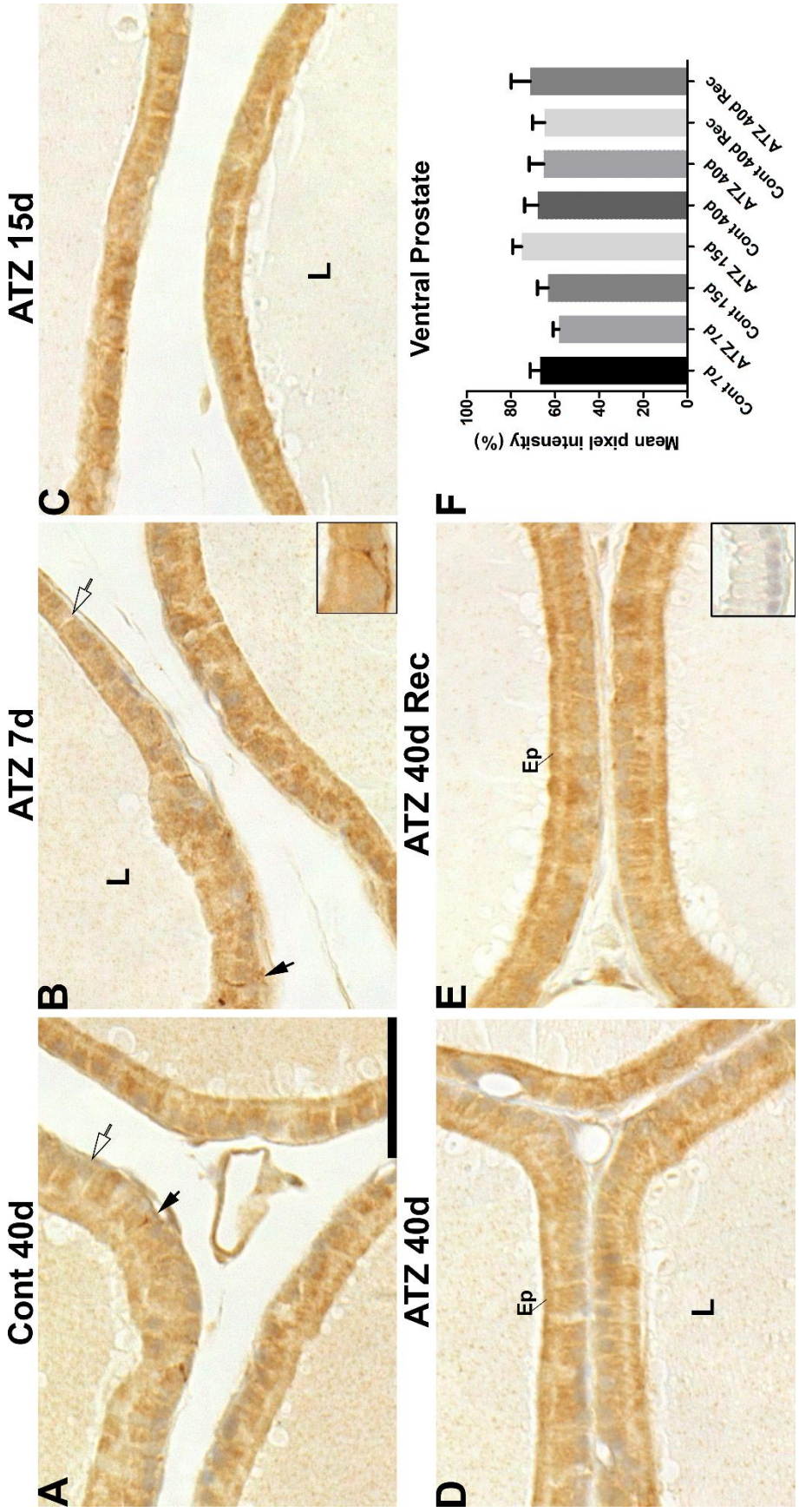
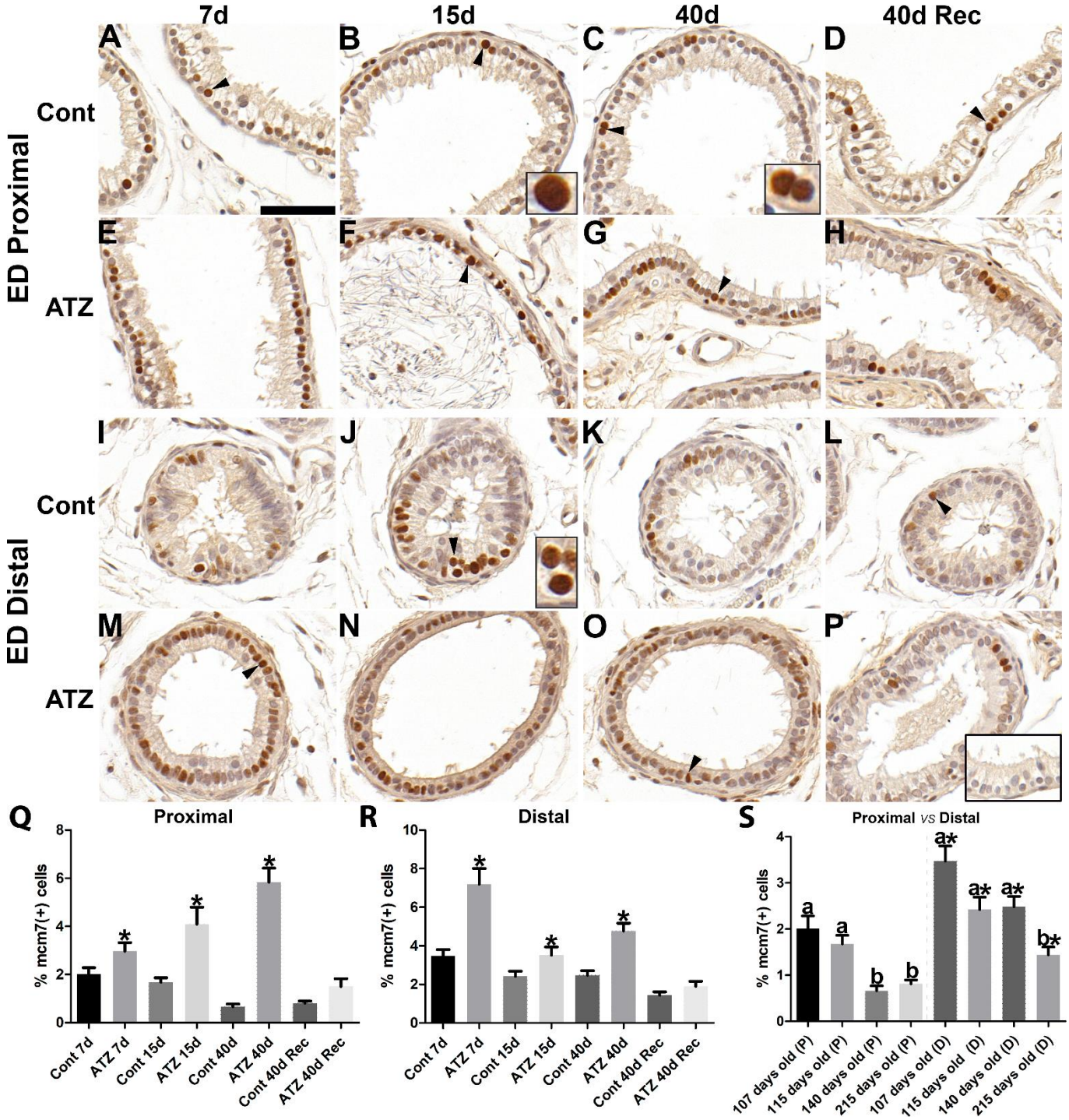

A
Cont 40d
B
ATZ 7d
L
C
ATZ 15d
L
D
ATZ 40d
Ep
L
E
ATZ 40d Rec
Ep
F
Ventral Prostate
Mean pixel intensity (%)
0
20
40
60
80
100
Cont 7d
ATZ 7d
Cont 15d
ATZ 15d
Cont 40d
ATZ 40d
Cont 40d Rec
ATZ 40d Rec

Figure 5


7d
15d
40d
40d Rec
A
B
C
D
Cont
ED Proximal
E
F
G
H
ATZ
I
J
K
L
Cont
ED Distal
M
N
O
P
ATZ
Q
Proximal
% mcm7(+) cells
Cont 7d
ATZ 7d
Cont 15d
ATZ 15d
Cont 40d
ATZ 40d
Cont 40d Rec
ATZ 40d Rec
R
Distal
% mcm7(+) cells
Cont 7d
ATZ 7d
Cont 15d
ATZ 15d
Cont 40d
ATZ 40d
Cont 40d Rec
ATZ 40d Rec
S
Proximal vs Distal
% mcm7(+) cells
107 days old (P)
115 days old (P)
140 days old (P)
215 days old (P)
107 days old (D)
115 days old (D)
140 days old (D)
215 days old (D)

V. DISCUSSÃO GERAL

O presente estudo acrescenta informações relevantes sobre os efeitos do desregulador endócrino atrazina no perfil morfológico, esteroidogênico e de homeostase tecidual, de órgãos genitais masculinos de ratos Wistar adultos, como testículos, próstata e dúctulos eferentes, além de reforçar alguns dados prévios obtidos por nosso grupo de pesquisa nos testículos (Victor-Costa *et al.*, 2010; Martins-Santos, 2013).

Os resultados mostraram redução precoce de 3β-HSD e posterior indução da expressão de aromatase nos testículos de ratos. Atrofia testicular e redução de 3β-HSD foram pronunciadas após o período de recuperação, sugerindo efeitos prolongados e possivelmente irreversíveis. Após o período de recuperação, foi notável a presença de células semelhantes a macrófagos fortemente positivas para 3β-HSD nos testículos atróficos. A identidade dessas células foi confirmada pelo uso de marcadores específicos para os macrófagos induzidos (ED1/CD68) e residentes (ED2/CD163). Houve aumento de 89%, 76% e 42% nas subpopulações de macrófagos $ED1^{-}/ED2^{+}$ residentes, $ED1^{+}/ED2^{+}$ transitórios e $ED1^{+}/ ED2^{-}$ induzidos, respectivamente. Esses macrófagos foram positivos para 3β-HSD, indicando possibilidade de envolvimento dessas células na esteroidogênese local.

Além disso, a exposição ao atrazina causou alterações nos dúctulos eferentes, que incluem aumento do diâmetro luminal, redução da altura epitelial, bem como aumento da expressão de aromatase, proliferação celular e apoptose. Estes resultados revelam distúrbios na morfofisiologia dos dúctulos eferentes após a exposição ao atrazina, semelhantes a alterações previamente demonstradas, as quais mostram que distúrbios na reabsorção de fluido pelos dúctulos eferentes, função finamente regulada por estrógenos, leva a acúmulo de fluido no lúmen e

consequente refluxo para o testículo, resultando assim em dilatação luminal dos túbulos seminíferos e consequente infertilidade (Hess *et al.*, 1997a; Hess *et al.*, 2000; Oliveira *et al.*, 2001), sugerindo que pelo menos parte dos efeitos testiculares causados por atrazina podem ser de fato secundárias a disfunções nos dúctulos eferentes.

Por outro lado, as alterações morfológicas na próstata ventral foram mais discretas. Além disso, encontramos que a expressão de aromatase não é alterada na próstata ventral de ratos expostos ao atrazina, ressaltando que esse efeito pode ser restrito aos testículos e dúctulos eferentes. Corroborando esta hipótese, Rivest et al (2010), já haviam demonstraram que atrazina induz a expressão de aromatase apenas no epidídimo. Sabe-se que a aromatase é regulada de modo altamente tecido-específico (Mahendroo *et al.*, 1993; Simpson e Davis, 2001), sendo que os tecidos que expressam SF1 (*steroidogenic factor 1*) parecem ser mais suscetíveis aos efeitos de atrazina. Considerando que SF1 não é expresso em tecido prostático normal (Lewis *et al.*, 2014), mas é expresso nos testículos e epidídimos (Pezzi *et al.*, 2004; Rivest *et al.*, 2010), embora seja especulativa, essa diferença pode oferecer uma explicação plausível para a divergência na resposta dos tecidos ao herbicida.

VI. CONCLUSÃO GERAL

Os resultados obtidos enfatizam os efeitos adversos de atrazina sobre a morfofisiologia dos testículos, dúctulos eferentes e, de maneira mais branda, na próstata ventral, sugerindo que as alterações testiculares causadas pelo herbicida podem ser secundárias a disfunções nos dúctulos eferentes. Em conjunto, esses resultados revelam os testículos e dúctulos eferentes como importantes alvos da ação deste desregulador endócrino em machos, e consequente infertilidade.

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