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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

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UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

EFEITO DA EXPOSIÇÃO PRÉ-PUBERAL AO ARSÊNIO SOBRE PARÂMETROS MORFOFUNCIONAIS NA PRÓSTATA VENTRAL DE RATOS PUBESCENTES

Ariana Musa de Aquino

Prof. Dr. Wellerson Rodrigo Scarano

Profa. Dra. Juliana Elaine Perobelli

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Prof. Dr. Wellerson Rodrigo Scarano

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1. Próstata. 2. Morfogênese. 3. Puberdade. 4. Arsênito. 5. Toxicologia.

Palavras-chave: Arsenito de sódio ; Morfogênese tardia; Próstata; Puberdade; Toxicologia reprodutiva.

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meu pai, Claudionor Ferreira de Aquino,
principal pessoa responsável por cada
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LISTA DE ABREVIAÇÕES

AR: Receptor de Andrógeno

As: Arsênio

As1: Grupo tratado com 0.01 mg/L

As2: Grupo tratado com 10.0 mg/L

As^{III}: Arsenito

As^{V}: Arsenato

CAT: Catalase

CaP: Câncer de Próstata

Ctrl: Grupo controle

DEs: Desreguladores Endócrinos

DHT: Dihidrotestosterona

DMA^{v}: Íon dimetilarsínico

DPN21: Dia pós-natal 21

DPN23: Dia pós-natal 23

DPN53: Dia pós-natal 53

DPN: Dia pós-natal

EPA: *United States Environmental Protection Agency*

GSH: Glutationa reduzida

IARC: *International Cancer Research*

iAs: Arsênio inorgânico

MDA: Malondialdeído

MMA^{v}: Íon monometilarsônico

MMPs: Metaloproteinases

$NaAsO_2$: Arsenito de sódio

oAs: Arsênio orgânico

OMS: Organização Mundial da Saúde

PCNA: Proliferative Nuclear Cell Antigen

PV: Próstata Ventral

SOD: Superóxido Dismutase

SUG: Seio Urogenital

T: Testosterona

RESUMO

O arsênio é um metaloide associado ao desenvolvimento de algumas patologias, como doenças cardiovasculares, lesões dérmicas e diferentes tipos de câncer. Pouco se sabe sobre a ação do arsênio ou compostos de arsênio na próstata durante o período pré-puberal e puberdade, estágios essenciais para a morfogênese tardia da próstata. Nesse sentido, este estudo teve como objetivo estabelecer se a exposição ao arsenito de sódio ($NaAsO_2$) interfere na morfofisiologia da próstata ventral de ratos púberes. Para isso, 30 ratos machos da linhagem Wistar, no dia pós-natal 23 (DPN23), foram distribuídos, aleatoriamente, em 3 grupos experimentais (n =10/grupo). O grupo controle (Ctrl) recebeu água filtrada (veículo); o grupo As1 recebeu 0.01 mg/L de $NaAsO_2$; e o grupo As2 recebeu 10.0 mg/L de $NaAsO_2$. Todas as soluções foram diluídas na água do bebedouro e estiveram disponíveis aos animais do DPN23 ao DPN53. Os hábitos alimentares e a evolução do peso corpóreo dos animais foram acompanhados durante todo o período experimental. Ao final deste período, os animais foram pesados e, em seguida, eutanasiados (DPN53). Coletou-se o sangue para mensurar os níveis de testosterona. O fígado, os rins e a próstata ventral (PV) foram coletados e pesados. Apenas a PV foi dissecada e destinada às análises histológicas (hemilobo esquerdo) e moleculares (hemilobo direito). Os resultados dos parâmetros analisados durante o período experimental revelaram que o $NaAsO_2$ não foi capaz de causar toxicidade sistêmica em ambos os grupos expostos, bem como alteração nos hábitos alimentares dos animais. No entanto, a análise histológica do estroma prostático demonstrou alteração na quantidade e distribuição do colágeno no grupo As1 e redução na atividade da MMP-2 ativa. Houve redução significativa na expressão do receptor de andrógeno e na expressão da prostateína no grupo As2, assim como aumento da expressão da caspase-3-clivada. No grupo As1, houve redução na expressão de PCNA, provavelmente refletindo uma redução estatisticamente não significativa do AR, mas funcionalmente apta a interferir na proliferação celular. Além disso, houve aumento de malondialdeído (MDA) nos grupos tratados com $NaAsO_2$, sem aumento na atividade das enzimas antioxidantes. Sendo assim, os resultados apontam que a exposição ao $NaAsO_2$, durante a maturação sexual, compromete a homeostasia glandular de ratos púberes em ambas as doses avaliadas nesse trabalho.

Palavras-Chave: Próstata, Morfogênese tardia, Puberdade, Arsenito de sódio, Toxicologia reprodutiva.

ABSTRACT

Arsenic is an endocrine disruptor associated with the development of some pathologies such as cardiovascular diseases, dermal lesions and different types of cancer. Little is known about the action of arsenic or arsenic compounds in the prostate during the prepubertal and puberty period, an essential stage for late morphogenesis of the prostate. Therefore, this study aimed to establish whether exposure to sodium arsenite ($NaAsO_2$) interferes in the morphophysiology of the ventral prostate of pubertal rats. In this study, thirty male Wistar rats, on the postnatal day 23 (PND23), were randomly distributed to 3 experimental groups (n = 10/group). The control group (Ctrl) received only saline solution; the second group (As1) received 0.01 mg/L of NaAsO2; and the third group (As2) received 10.0 mg/L of $NaAsO_2$. All solutions were diluted in drinking water and were available to the animals from DPN23 to DPN53. The eating habits and the evolution of the body weight of the animals were evaluated throughout the experimental period. At the end of this period, the animals were weighed and then euthanized (DPN53). Blood was collected to measure testosterone levels. The liver, kidneys and ventral prostate (VP) were collected and weighed. Only VP was dissected for histological analysis (left hemilobo) and molecular (right hemilobo). The results of the parameters analyzed during the experimental period revealed that $NaAsO_2$ was not able to cause systemic toxicity in both exposed groups nor changes in the eating habits of the animals. However, prostatic stromal analysis showed alterations in the amount and distribution of collagen fibers in the As1 group, as well as a reduction in active MMP-2 activity. There was a significant reduction in the expression of the androgen receptor and in prostatein in the As2 group and in the expression of caspase-3-cleaved. In the As1 group, there was a reduction of PCNA expression, probably reflecting a statistically non-significant reduction of AR, however functionally able to reduce cell proliferation. In addition, there was an increase in malondialdehyde (MDA) in the groups treated with sodium arsenite, with no alteration in the activity antioxidant enzymes. Thus, the results indicate that exposure to $NaAsO_2$, during sexual maturation, compromises the glandular homeostasis of pubertal rats at both doses evaluated in this study.

Keywords: Prostate, Late morphogenesis, Puberty, Sodium arsenite, Reproductive toxicology.

SUMÁRIO

Capítulo I
Revisão da literatura

1. INTRODUÇÃO

1.1. PRÓSTATA

A próstata é uma glândula acessória de suma importância ao sistema genital masculino, uma vez que secreta complexos proteolíticos essenciais na composição do fluido seminal, que ajudam a manter o gradiente iônico e pH ideais para manter a viabilidade dos espermatozoides, ao longo do percurso no sistema genital feminino [1–3].

A próstata humana corresponde a maior glândula acessória do sistema genital masculino, localiza-se inferiormente à bexiga urinária, envolve a parte prostática da uretra, apresenta morfologia compacta sem lobulação aparente e é diferenciada morfologicamente, em três zonas: central, de transição e periférica [4–6] (Figura 1a). Em roedores, a glândula é lobulada, os lobos envolvem a bexiga urinária e também apresenta regiões morfológicas distintas. Dessa forma, a próstata de roedores é diferenciada morfologicamente, em próstata anterior ou glândula coaguladora, próstata ventral, próstata dorsal e próstata lateral, sendo as duas últimas, frequentemente, agrupadas como dorsolateral [7,8] (Figura 1b).

A próstata de ambas as espécies expressam semelhanças no processo de desenvolvimento, sinalização, expressão gênica e na responsividade hormonal, o que torna os ratos importantes modelos experimentais no estudo da morfogênese prostática, bem como nas alterações fisiopatológicas da vida adulta [9]. Apesar de apresentarem características macroscópicas distintas (Figura 1a-b), a próstata de ambas as espécies apresentam características histológicas semelhantes [10], sendo constituída por ductos e ácinos revestidos internamente por epitélio, predominantemente, simples, circundados por estroma fibromuscular [11]. Além disso, as populações celulares são similares e, provavelmente, desempenham funções fisiológicas idênticas ou parecidas [1,12].

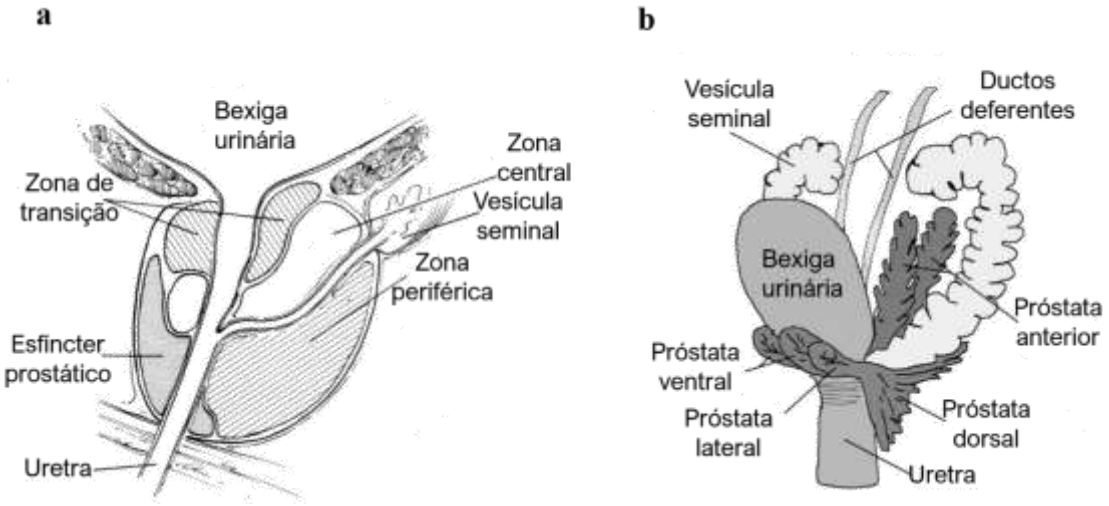


Figura 1: Representação esquemática comparando a próstata humana (a) (Adaptada de Cunha et al., 1978) e da próstata de roedores (b). (Adaptada de McNeal 1969) e reproduzida de Toivanen e Shen, 2017).

Os diferentes lobos e zonas da próstata de roedores e humana, respectivamente, exibem particularidades quanto ao processo de ramificação de ductos e secreção [1,13]. No entanto, ácinos prostáticos pertencentes a um mesmo lobo, apresentam heterogeneidade regional quanto aos tipos celulares, síntese, secreção proteica e resposta a andrógenos [14]. Quanto às diferenças histológicas lobo-específicas, pode-se salientar que a próstata anterior apresenta epitélio densamente pregueado, a próstata dorsal e lateral apresentam ductos revestidos com células colunares baixas dispostas em um epitélio simples, que apresenta pouco pregueamento, e a próstata ventral é composta de epitélio contínuo com pouco pregueado, contendo células que variam de cúbicas a colunares [1,14].

De maneira geral, o epitélio prostático é composto por cinco tipos celulares (Figura 2): células epiteliais secretoras, que revestem o lúmen dos ácinos prostáticos, produzem a secreção de proteínas especificas da próstata e expressam altos níveis de receptor de andrógenos (AR); células epiteliais basais, que formam interposição entre células luminais sobre a membrana basal, envolvendo o epitélio e constituem a principal população de células proliferativas do epitélio da próstata; células neuroendócrinas, que secretam hormônios e neurotransmissores que, possivelmente, afetam o desenvolvimento e a manutenção do tecido prostático; células-tronco (*stem cells*) que, mesmo raras, localizam-se na camada de células basais; e, por último, células transitórias amplificadoras (não

apontadas na figura), que expressam características que transitam entre as células basais e secretoras e podem ser células progenitoras ainda não diferenciadas [1,15–17].

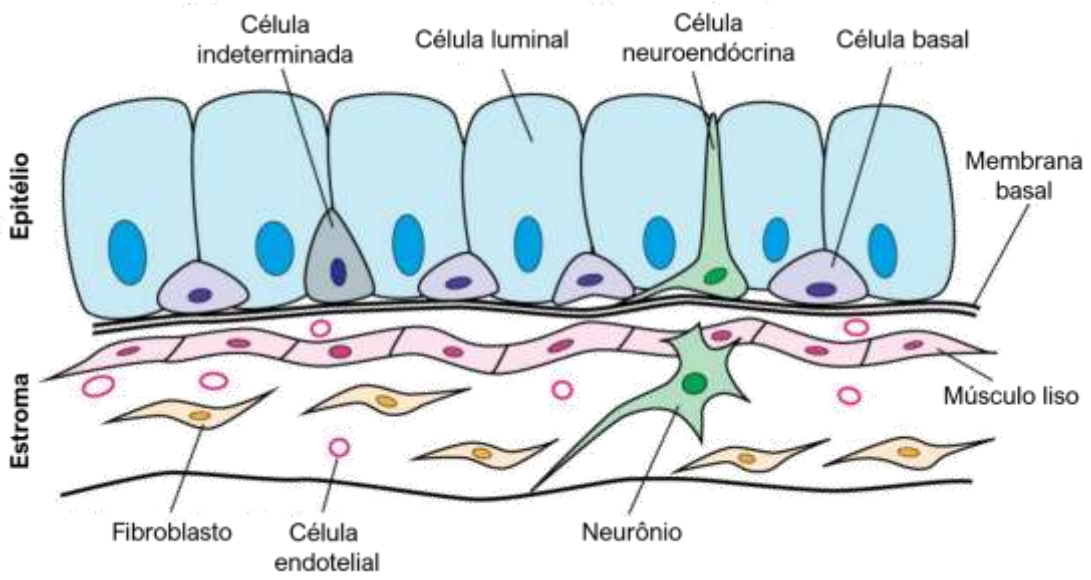


Figura 2: Representação esquemática destacando os tipos celulares que compõem os ductos prostáticos (Adaptado de Toivanen e Shen, 2017).

Envolvendo as estruturas glandulares há estroma conjuntivo vascularizado, com células musculares lisas organizadas concentricamente aos ácinos entremeados às camadas de colágeno, bem como poucas fibras conjuntivas e elásticas [8,18]. Células musculares lisas são estimuladas a se contraírem durante a ejaculação e, em associação aos fibroblastos, sintetizam componentes de matriz extracelular, onde se destacam as fibras colágenas, reticulares e elásticas, que em conjunto, fornecem resistência mecânica e flexibilidade ao tecido, atuando como substrato para ancoragem e migração celular [1,19,20]. A arquitetura do microambiente estromal é extremamente importante, pois direciona os processos de desenvolvimento e diferenciação do epitélio e ajuda a controlar a homeostasia tecidual, fornecendo nutrientes e fatores de crescimento [19,21].

1.2. DESENVOLVIMENTO E MORFOFISIOLOGIA DA PRÓSTATA

Por volta do décimo terceiro e décimo quarto dia de gestação para roedores e nove semanas para humanos, os testículos que já estão estabelecidos, passam a secretar testosterona (T), que é convertida pela 5α-redutase em dihidrotestosterona

(DHT) e passa a estimular os receptores de andrógenos (AR) das células mesenquimais na parte pélvica do seio urogenital (SUG) [1,22], esse evento desencadeia uma cascata de sinalização entre o mesênquima e o epitélio do SUG, estimulando assim, o desenvolvimento inicial da glândula prostática (Figura 3). [10,23,24].

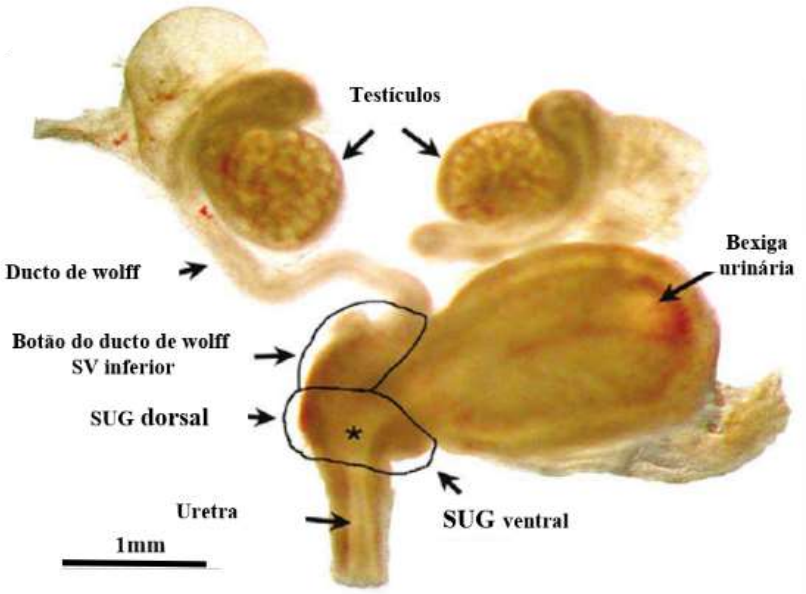


Figura 3 Representação do sistema urogenital masculino de camundongo durante o desenvolvimento embrionário, com destaque na região do seio urogenital em desenvolvimento. Nessa fase, o epitélio do SUG é visto como uma dilatação da uretra (*), circundada por mesênquima nas regiões dorsal e lateral (Adaptada de Marker *et al.*, 2003).

Os primeiros sinais de desenvolvimento emergem da parte pélvica (Figura 3) do SUG, região formada a partir da parte anterior da cloaca [8,10,25]. Células epiteliais brotam do SUG à partir da parte pélvica do SUG, futura região prostática da uretra, para o interior do mesênquima circundante, formam os brotos prostáticos compostos de cordões de células epiteliais (Figura 4) [9,26]. Sob estimulo da DHT, os ARs presentes nas células mesenquimais do SUG, passam a secretar mediadores parácrinos, que atuam sobre os cordões epiteliais, e assim, formam os brotos iniciais da próstata [1,27]. Esses brotos originam os principais ductos prostáticos de todos os lobos específicos: lobo anterior, dorsal, lateral e ventral [28]. Nesse contexto, a formação dos brotos epiteliais e o seu desenvolvimento são eventos dependentes da interação epitélio-mesênquima que, por sua vez, é mediada e regulada por andrógenos [1,15,22] e fatores de crescimento, como I*gf*

(Insulin like Growth Factor), F*gf* (Fibroblast Growth Factor) e E*gf* (Epidermal Growth Factor) [15,29,30]. De modo similar, uma série de sinais parácrinos advindos do epitélio dos brotos induz a diferenciação do mesênquima que circunda os brotos, formando um estroma composto inicialmente por células musculares lisas e fibroblastos [15,29,30].

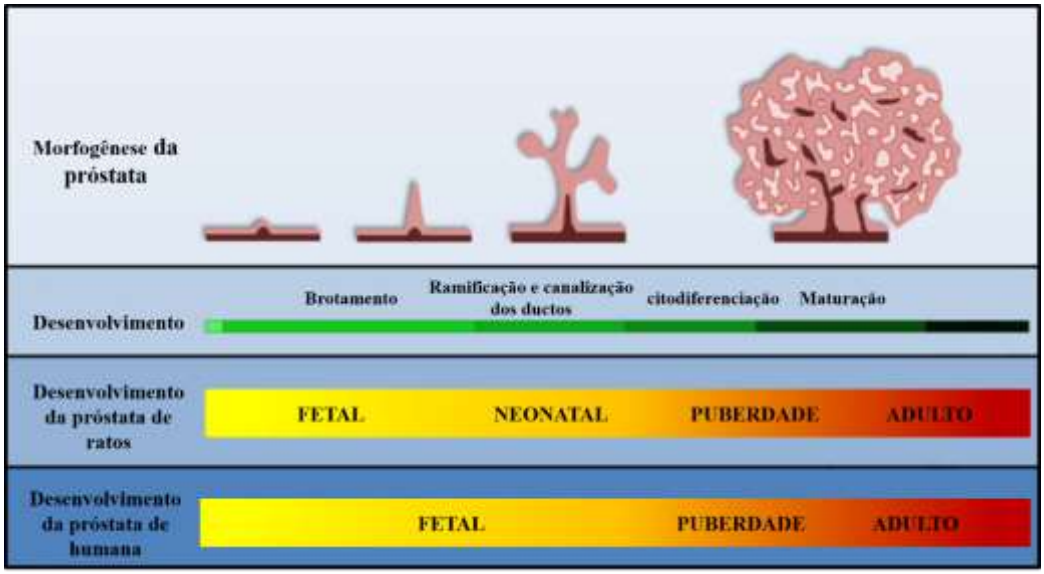


Figura 4: Representação esquemática do desenvolvimento cronológico da próstata de ratos e humanos. (Adaptada de Scarano *et al*, 2018).

Em humanos, grande parte do processo de brotamento, alongamento e ramificação ocorre durante o desenvolvimento intrauterino, mas esses eventos só se completam na vida pós-natal (organogênese tardia) [26]. De maneira oposta, a próstata de roedores é rudimentar ao nascimento, mas logo inicia os processos de bifurcação, ramificação e canalização dos ductos (Figura 4) [13,22]. Ao longo dos quinze primeiros dias pós-natal (DPN15), a próstata do neonato passa por intensas atividades de sinalização morfogênica, onde ocorre aumento significativo na ramificação dos brotos e no peso da glândula [10,13,24,28]. Paralelamente, ocorre o processo de citodiferenciação epitelial e estromal [1].

O período de ramificação e maturação da glândula, sob influência continua dos endógenos circundantes, se estende até a pré-puberdade, período referente a organogênese tardia da próstata [17,28,31–33]. Nessa etapa do desenvolvimento, quaisquer fatores externos que perturbem o equilíbrio hormonal podem desencadear efeitos importantes na vida adulta [23,34]. Na puberdade, estimulado por hormônios, o eixo hipotalâmico-

hipofisário-gonodal passa a liberar sinais moleculares que impulsiona o crescimento e a maturação dos órgãos sexuais e genitálias externas [35,36]. Por corresponder a uma fase em que o processo de desenvolvimento inicial está praticamente estabelecido, a organogênese tardia da próstata tem sido, consideravelmente, negligenciada. No entanto, é importante ressaltar que, neste período, além do aumento expressivo nos níveis hormonais, há importantes mudanças no comportamento sexual, na produção e maturação dos espermatozoides e as glândulas sexuais acessórias passam a produzir e secretar fluido seminal, essenciais para manter a viabilidade das células germinativas [36,37].

Afecções na glândula têm acometido homens ao longo dos anos, levando à busca pela compreensão e descrição dos fatores ambientais capazes de influenciar sua morfofisiologia ao ponto de acelerar ou retardar os processos de desenvolvimento de desordens prostáticas, como a prostatite, a hiperplasia benigna e o adenocarcinoma prostático e à estudos terapêuticos que possam contribuir para o tratamento e redução da incidência dessas doenças [30,38–40].

Cabe destacar que durante a organogênese tardia, a exposição a um conjunto de moléculas exógenas capazes de perturbar o sistema endócrino, denominadas Desreguladores Endócrinos (DEs), pode interferir na maturação morfofuncional da próstata e, consequentemente, refletir na capacidade reprodutiva do indivíduo, bem como na sensibilidade dos tecidos em desenvolver lesões que podem levar a incidência de patologias crônicas [23,37].

1.3. DESREGULADORES ENDÓCRINOS (DEs)

O intenso uso de substâncias químicas no meio industrial passou a ter destaque devido ao aumento nos índices de contaminantes, potencialmente tóxicos, liberados no meio ambiente diariamente e ao aumento de patologias relacionadas à exposição constante a essas substâncias. Dentre estes compostos, há a classe dos DEs, que são conjuntos de substâncias naturais ou sintéticas com propriedades estruturais semelhantes às moléculas hormonais, que ao entram em contato com organismo, se ligam a receptores específicos e, por consequência, acabam interferindo em atividades importantes como secreção, produção, ligação e transporte de hormônios essenciais para a manutenção da homeostasia

de glandular [38,41,42], o que passa a interferir diretamente na fisiologia de órgãos hormônio-dependentes e desencadeia uma série de distúrbios [23,43].

A semelhança nas propriedades dos hormônios estrogênico ou androgênico permite que os DEs atuem estimulando a proliferação celular, modificando vias de sinalização, inibindo a síntese de hormônios, alterando o metabolismo e modulando receptores específicos [43,44]. Essas alterações, que na maioria das vezes têm efeito anti-androgênico, tendem a interferir com vias importantes e desencadeiam uma série de perturbações no desenvolvimento e na homeostasia de órgãos ou glândulas hormônio-dependentes como a mama, tireoide, pâncreas, fígado e próstata [34,44–47].

Nesse sentido, vários estudos têm como foco a exposição diária a DEs durante o desenvolvimento intrauterino e na infância [37,43], etapas nas quais os sistemas ainda estão se estabelecendo morfofisiologicamente, e perturbações repercutiriam na vida adulta do indivíduo exposto [37]. Contudo, as fases posteriores de desenvolvimento, como a pré-puberdade e a puberdade, também são sensíveis ao ambiente externo, acarretando em importantes alterações endócrinas que podem influenciar a maturação sexual e a vida reprodutiva na vida adulta [23,37].

A ampla maioria dos tóxicos classificados como DEs atuam interferindo diretamente na síntese de testosterona, alterando o crescimento da glândula prostática, bem como a instalação da puberdade e a maturação sexual [23,37,43,48]. Além disso, estudos relacionam o desenvolvimento de afeções prostáticas, o aumento de distúrbios reprodutivos como a infertilidade e o desenvolvimento de patologias, como o câncer, ao estilo de vida e a constantemente exposição aos agentes tóxicos dispersos no ambiente [43,49].

Alguns estudos e dados epidemiológicos apontam que ao longo dos anos, doenças que acometem a glândula prostática+ expressam incidência crescente [50,51] e, de acordo com um levantamento feito por Toivanen e Shen, 2017, quando comparada as demais tecidos sexuais secundários masculino, a glândula prostática apresenta maior susceptibilidade em desenvolver oncogênese. Levando essa questão em consideração e ressaltando que a longevidade é fator determinante na prevalência e incidência da doença, agências como a Sociedade Americana de Câncer e Associação Urológica Americana, passaram a recomendar exames preventivos para diagnóstico precoce das doenças, a partir dos 50 anos de idade, isso porque o número de casos da doenças tem aumentado de maneira significativa em quase todos os países e a taxa de mortalidade tende a aumentar com

diagnósticos tardios [51,52]. Nesse contexto, é importante destacar que o câncer de próstata (CaP) corresponde à segunda maior causa de óbito entre indivíduos do sexo masculino [51–55] e está diretamente associado aos fatores genéticos e ao aumento da expectativa de vida, no entanto, o ambiente ao qual o indivíduo está exposto ao longo da vida, tem influência no surgimento e desenvolvimento do CaP e de outras afecções prostáticas [23,43].

Apesar da literatura não esclarecer de fato a atuação do As e de seus compostos atuando como DEs, há trabalhos que apontam e comprovam interferência, principalmente, dos compostos de iAs no sistema endócrino, por meio da associação aos receptores estrogênicos (ERs) e, por consequência, influenciando na ativação de genes regulados por eles e na regulação gênica de outros receptores [30,56]. Além disso, há trabalhos demonstrando maiores efeitos nas menores doses de exposição e em doses ambientalmente relevantes [30,56–60].

1.4. ARSÊNIO

O arsênio (As) é um elemento químico amplamente disperso no ambiente [61] que, além de agir como desregulador endócrino, e ser associado ao surgimento de determinadas afecções, é classificado como cancerígeno pela *International Cancer Research* (IARC) e *United States Environmental Protection Agency* (EPA) [62]. Normalmente, o As é encontrado em ambientes aquáticos profundos, porém a interferência humana e os processos naturais de intemperismo o dispersaram para os demais ambientes, onde apresenta variáveis níveis de exposição humana [63–65]. As maiores taxas de exposição ocorrem nos meios industriais, além das zonas rurais, onde o hábito de construir poços artesianos, ainda é frequente para irrigar plantações [66–68], ocasionado a intoxicação de milhões de pessoas [64,69]. Estudos recentes investigaram a ação do As e compostos de arsênio em modelos experimentais, principalmente, em órgãos que a literatura aponta como potenciais alvos do arsênio[23,57].

Compostos do As são utilizados na indústria têxtil, indústria farmacêutica, madeireiras, indústrias especializadas na produção de vidros, ração para animais, pesticidas e cigarro [64,68,70,71]. Antes de surgirem estudos apontando seu potencial citotóxico e carcinogênico, o composto chegou a ser usado de forma descontrolada no tratamento de

distúrbios vasculares, neurológicos, no tratamento de lesões dérmicas e de diferentes tipos de câncer [71–73].

Ao entrar em contato com as diversas moléculas dispersas no meio ambiente, como carbono, hidrogênio, ferro, silício, cloro e outros [64,70,71], o As se biotransforma e seus compostos passam a ser classificados em dois subgrupos: Arsênio orgânico (oAs) e Arsênio inorgânico (iAs). O oAs, predominantemente encontrado em organismos advindos dos ambientes aquáticos, apresenta baixos índices de toxicidade, enquanto o iAs é altamente tóxico e está presente nos diferentes ambientes, inclusive em alimentos de consumo diário [33,55,65,74,75]. As classificações de oAs e iAs se diferenciam pelo estado de oxidação e características físico-químicas das substâncias [55].

Os compostos de oAs mais comuns encontros no ambiente atualmente são o ácido arsanílico, ácido monometilarsônico e dimetilarsínico, ácido cacodílico e arsenobetaína. Para os compostos de iAs, os mais comuns são o pentóxido de arsênio, ácido arsênico e arsenato, formas pentavalente do composto. Na forma trivalente, se destacam o trióxido de arsênio, o tricoloreto de arsênio e o arsenito de sódio [55,76]. Sódio arsenato e sódio arsenito são altamente diluídos em água [76], por isso são as formas mais predominante no ambiente.

Por apresentar maior grau tóxico, os compostos de iAs se destacam nas pesquisas que investigam os efeitos da exposição aos compostos de arsênio, suas formas mais estudas são os compostos de íon arsenito (As^{III}), que é a forma trivalente e íon arsenato (As^{V}) forma pentavalente, ambas em estado de oxidação e altamente tóxicas (Figura 5) [77–79]. No interior da célula, o As^{III} se liga aos grupos sulfidrilas e induz modificação estrutural em proteínas, inibi a reação enzimática e interrompe o ciclo celular [68,80,81]. Com relação ao As^{V}, alguns trabalhos apontam que, por apresentar semelhanças estruturais com a molécula de fosfato, ele compete por sítios de ligação e interfere na produção de moléculas importantes, como adenosina 5'-trifosfato (ATP) [80,81]. Quando em contato com o organismos, os compostos de As sofrem biometilação (Figura 6) e passam a se ligar a proteínas, queratinócitos e a alguns grupos de peptídeos, o que facilita a acumulação nos tecidos e órgãos [61,82].

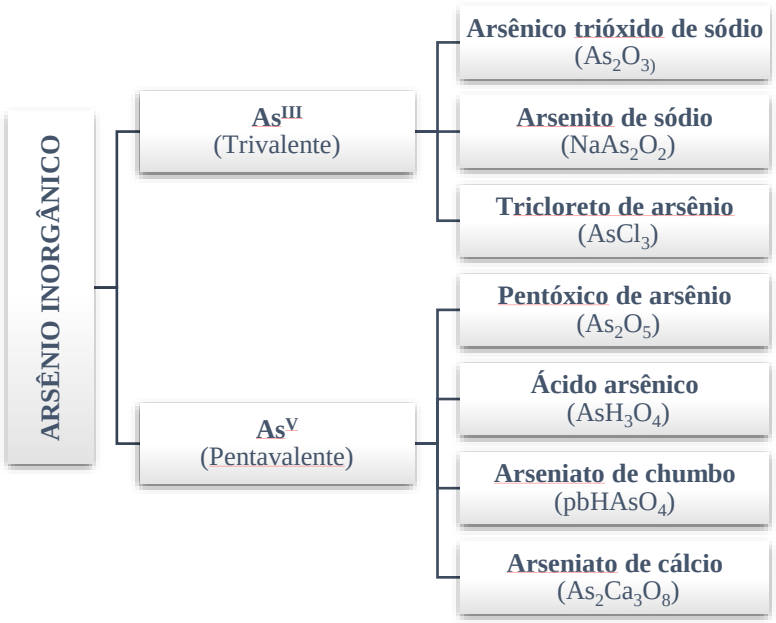


Figure 5: Representação esquemática das formas mais comuns de iAs em estado de oxidação (WHO, 2000; IARC, 2012).

A exposição às diferentes formas do As se dá via respiração, ingestão de água e/ou comida contaminadas ou por contato direto com a pele [63,67,73]. O efeito dessa exposição varia de acordo com alguns fatores como idade, sexo, hábitos alimentares e ambiente ao qual o indivíduo está exposto constantemente [55,76]. A partir desse levantamento, a OMS (2011) revelou que a ingestão e exposição ao As é maior em homens do que em mulheres e crianças, no entanto, mulheres em fase reprodutiva e crianças expressam maior sensibilidade aos efeitos dessa exposição, quando comparados aos homens adultos [43].

A principal fonte de exposição cotidiana ao As e seus compostos são águas naturais, onde o composto ocorre como arsenito (As^{III}) e arsenianto (As^{V}) (figura 5). Nas plantas e nos animais aquáticos/marinhos o composto sofre biometilação facilmente e passa a ser encontrado como MMA, DMA, arsenobetaína (AsB), arsenocolina (AsC) e arseno-açúcares (AsSug)**,** compostos de arsênio orgânico com baixo grau tóxico [33,84]. De acordo com Barra e colaboradores (2000), o grau de toxicidade dos compostos de As seguem a classificação apresentada na figura 7.

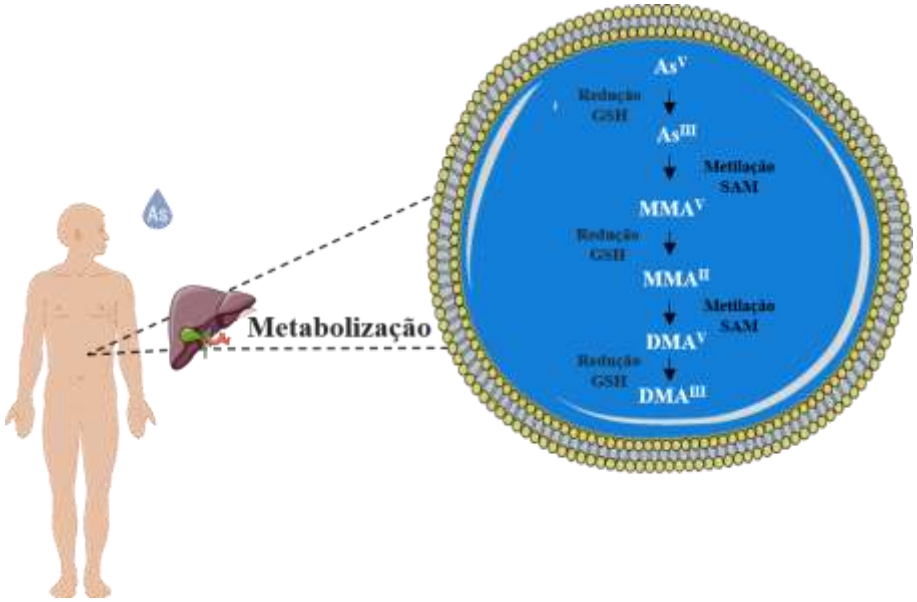


Figura 6: Esquema da metabolização de arsênio inorgânico no organismo. Ao entrar em contato com os hepatócitos, a glutationa redutase (GSH) reduz arseniato ($As^{V)}$ para arsenito (As^{III}). O As^{III} é metilado por meio da S- adenosilmetionina (SAM) e forma o íon monometilarsônico (MMA^V). MMA^V é então reduzido para a forma de MMA^{III} pela GSH, que sobre ação da SAM também sofre metilação e forma o íon dimetilarsínico (DMA^v). DMA^v é reduzido e eliminado através da urina (Barra *et al.*,2000; IARC, 2004).

A Organização Mundial da Saúde (OMS) recomenda o índice máximo de iAs detectado em água potável seja ≤ 10.0 mg/L, porém estudos apontam que em algumas regiões esse valor chega a 100 mg/L [62,64,85]. Este aumento nos níveis detectados em água potável chama atenção porque, recentemente, o composto passou a ser encontrado nos cereais, legumes, carnes entre outros alimentos [64,67,68,70,73,86].

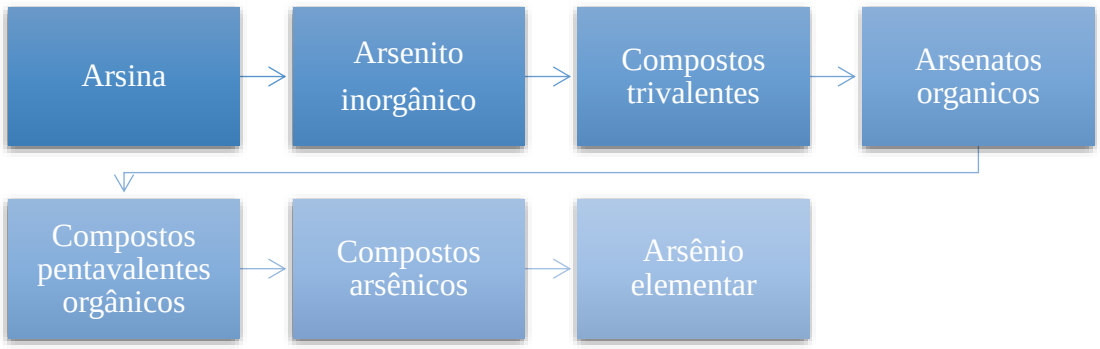


Figura 7: Ordem decrescente do grau de toxicidade dos compostos de arsênio Barra, 2000; Cornelis *et al.*, 2005).

Mesmo sob baixos níveis, o iAs é capaz de se acumular nos rins, pulmões e pele [67,78,87] e apresentar efeitos biológicos adversos como lesões na pele, diabetes, problemas no sistema nervoso, redução na pigmentação da pele, distúrbios reprodutivos, problemas cardiovasculares, entre outros [67,83]. A literatura comprova associação direta da exposição a compostos de iAs com o desenvolvimento de câncer de pele [66,73], pulmão, rim, bexiga urinária e fígado [67,78,88].

A exposição ao iAs está relacionada ao aumento na incidência de distúrbios reprodutivos em indivíduos do sexo masculino [58], como menor contração do ducto epididimário, redução nos níveis de testosterona, diminuição na produção e na qualidade espermática; e também à oncogênese prostática [58,89–91].

Embora estudos *in vitro* demonstrem que, mesmo sob baixos níveis, o iAs seja capaz de induzir transformação maligna em células epiteliais prostáticas [75,91], são escassos os dados na literatura avaliando o efeito dessa exposição no microambiente prostático em modelos animais [62,90,92]. Além disso, a maioria dos estudos não avalia os mecanismos moleculares que podem ser alterados no microambiente prostático de ratos púberes em resposta à interação com $NaAsO_2$.

JUSTIFICATIVA E RELEVÂNCIA DO TEMA

A próstata é uma glândula de importância ímpar para o sistema genital masculino e, quando exposta a agentes tóxicos, sobretudo desreguladores endócrinos, apresenta alta sensibilidade, podendo sofrer importantes modificações histofisiológicas capazes de desequilibrar o microambiente prostático, resultando em distúrbios adaptativos ou patologias crônicas.

Diante da ampla dispersão do arsênio no ambiente e do seu potencial carcinogênico, é provável que o composto tenha influência sobre a morfofisiologia do sistema genital masculino, por vias hormônio-responsivas e de citotoxicidade direta do composto.

Levando em consideração que a próstata adulta é resultado de eventos morfogênicos advindos dos períodos pré e pós-natal, destacando a importância do processo de crescimento e maturação durante a fase pré-puberal, esse estudo buscou esclarecer se a exposição ao arsenito de sódio é capaz de perturbar a morfogênese tardia e comprometer a morfofisiologia prostática ainda na maturação sexual da glândula.

REFERÊNCIAS

NOTA: As referências da introdução geral encontram-se juntamente com as referências do capítulo II.

Capítulo II
Artigo científico

ARSENIC EXPOSURE DURING PREPUBERTY ALTERS PROSTATE MATURATION IN PUBESCENT RATS

Ariana M. Aquino[1], Cristiane F. Pinho[1], André T. A.G. de Freitas[1], Giovanna C. Salata[1], Paloma C Medeiros1, Larissa Lucena Périco Leonardo[1], O. Mendes[2], Luís A. Justulin[1], Glaura S. A. Fernades[4], Juliana E. Perobelli[3], Wellerson R. Scarano[1].

[1]*Department of Morphology, Sao Paulo State University - UNESP, Botucatu, SP, Brazil*, 18618-689.[2]*Graduate Program in Animal Science, Univ Oeste Paulista, Presidente Prudente, SP, Brazil*, 19050-920.[3]*Department of Marine Science, Instituto de Biociências, Univ.Federal de São Paulo, Santos, Brazil*, 11015-020. [4]*Department of General Biology, State University of Londrina, Londrina, Brazil*, 86057-970.

***Corresponding author:**
Wellerson Rodrigo Scarano, PhD
Departamento de Morfologia (Department of Morphology)
Instituto de Biociências – UNESP – Institute of Biosciences
Botucatu, SP, Brazil
CEP 18618-970
Tel: +55 14 3880-0475
E-mail: wellerson.scarano@unesp.br

AR: Androgen Receptor; As: Arsenic; CAT: Catalase; Ctrl: Control group; DEs: Endocrine disrupters; DHT: Dihydrotestosterone; DNMT1: DNA (cytosine-5)-methyltransferase 1 DPN21: Postnatal day 21; DPN23: Postnatal day 23; DPN53: Postnatal day 53; DPN: Postnatal day; EPA: United States Environmental Protection Agency; GSH: Reduced glutathione; IARC: International Cancer Research; iAs: Inorganic arsenic; IL-6: interleukin 6; IL-10: interleukin 10 MDA: malondialdehyde MMP-2: Metalloproteinase 2; $NaAsO_2$: Sodium arsenite; oAs: Organic arsenic; WHO: World Health Organization; PCNA: Proliferative Nuclear Cell Antigen; VP: Ventral Prostate; SIRT1: Sitiun1 SOD: Superoxide dismutase; T: Testosterone.

Abstract

Arsenic is a toxic compound associated with the development of some diseases and different types of cancer. Little is known about the action of arsenic compounds in the prostate during the prepubertal and puberty period, late morphogenesis of the prostate. This study evaluated the exposure to sodium arsenite in the morphophysiology of the ventral prostate of rats, during pubertal period. Male Wistar rats at PND23, were randomly distributed into three experimental groups (n=10/group). The Ctrl group (saline solution); As1 group (0.01 mg/L of $NaAsO_2$); As2 group (10.0 mg/L of $NaAsO_2$) that received the diluted solution in drinking water from PND23 to PND53. Histological and molecular analyzes showed a developmental delay in As1 group and important ophysiological alterations in As2 group. The results indicate that exposure to $NaAsO_2$, during sexual maturation, compromises the glandular homeostasis of pubertal rats at both doses evaluated in this study.

Keywords: Prostate, late morphogenesis, puberty, sodium arsenite, reproductive toxicology.

Highlights

- Arsenite toxicity was tested on prostate of prepubertal rats
- Low dose exposure to $NaAsO_2$ compromises the sexual maturation of the gland
- High dose exposure to $NaAsO_2$ compromises functional activity of the gland
- Both exposures may compromise the prostate at puberty

1. INTRODUCTION

Arsenic (As) is a widely dispersed chemical element that has been studied in biological systems due to its activity as an endocrine disrupter (ED) and mainly to act as a carcinogenic agent (Benbrahim-Tallaa and Waalkes, 2008). Arsenic is usually found in deep aquatic environments, but human interference and natural processes have dispersed it to other environments, where it exhibits varying levels of exposure [63–65].

By contacting the other several molecules in the environment such as carbon, hydrogen, iron, silicon, chlorine and others [64,70,71], arsenic is biotransformated into two subgroups: Organic Arsenic (oAs) and Inorganic Arsenic (iAs). The oAs is predominantly found in organisms from aquatic environments and it has low toxicity rates; while iAs is highly toxic and it can be found in different environments and products, including food of daily consumption [33,55,65,93]; additionally, exposure to iAs by ingestion of contaminated food and water is more aggressive compared to skin or air contact [67].

The iAs, even at low levels, it is able to accumulate in the kidneys, lungs and skin [67,78,87] and present adverse biological effects such as skin lesions, diabetes, nervous system problems, reduction in skin pigmentation, reproductive disorders, cardiovascular problems among others [67,83]. Several reports have showed that iAs can promote reproductive disturbances in males [58], such as: decreased contraction of the epididymal duct, reduction in testosterone levels, decreased production and sperm quality; and also, to induce prostatic oncogenesis [58,89–91].

The prostate is an accessory gland of the male genital system with essential importance for reproductive success, since it secretes essential proteolytic complexes in the composition of seminal fluid [1–3]. Under the stimulation of androgens secreted by the

testis of the fetus, the first signs of the development of the human prostate and rodents appear [22], extending to puberty when the males complete their sexual maturation. The period of ramification, cytodifferentiation and maturation in the prostate of rodents extends until pre-puberty, period referring to late organogenesis of the prostate [28]. At this stage of development, any external factors that disturb the hormonal balance could trigger important effects in adult life [23,34].

The prepubertal and pubertal periods have been little approached in studies of exposure to EDs, because at this stage the genital system is practically established. However, it is worth noting that in addition to the significant increase in hormonal levels in this period, important changes in sexual behavior, germ cell production occur and the accessory sex glands secrete seminal fluid [36,37]. In addition, exposure to EDs during late morphogenesis could interfere with morphofunctional maturation of the prostate, and therefore reflect on the individual's reproductive capacity, as well as to increase the gland sensitivity in developing lesions at long term. Thus, the purpose of this study was to test our hypothesis that arsenic exposure during prepuberty could alter the prostate maturation process and to modify morphological and functional important parameters that are essential to reproductive success after the puberty.

2. MATERIAL AND METHODS

2.1. Animals

Thirty male Wistar rats aged 21 days old were provided from CEDEME- Center for Development of Experimental Models for Medicine and Biology, Federal university of São Paulo - UNIFESP. The animals were housed under controlled environmental conditions (temperature: 22 ± 2°C; relative humidity: 55 ± 20%; 12/12-h light–dark cycle; and continuous air exhaust) and were provided free access to water and standard chow diet

(NUVILAB-CR1-Nuvital-PR) were randomly distributed into three experimental groups (n = 10/group): Control (Ctrl), only the vehicle was offered (filtered drinking water); As1 (low dose; sodium arsenite (AsNaO2), 0.01 mg/L of drinking water); As2 (high dose; AsNaO2), 10 mg/L of drinking water). $AsNaO_2$ was acquired from Sigma Aldrich (St. Louis, Mo, USA, ref. V000351-250G). All groups were exposed to their respective treatments for 30 continuous days (from PND23 to PND53) through drinking water. Selected doses in this protocol were based on experimental design adopted by da Cunha de Medeiros et al. (2018), and they reflect the range of environmental contamination, mainly via water and food, according World Health Organization.

Throughout the experimental period the animals were evaluated for water consumption, feed intake and physical status. From the PND30, the animals were examined daily to verify the onset of puberty (preputial separation). At PND53, the animals were anesthetized by the association of xilasin and ketamine and decapitated; blood was collected by rupture of the cervical vessels. Kidney and liver were weighed to indirectly assess systemic toxicity. Ventral prostate (VP) was weighed, and both left and right ventral prostate hemilobes were assigned to histological and molecular analyzes, respectively.

2.2. Testosterone Levels

Blood was collected in heparinized tubes. The blood was centrifuged at 3,000 rpm, 4°C, for 20 minutes and plasma was separated. Testosterone levels was performed by electrochemiluminescence. All the samples were dosed in the same assay, to avoid inter-assay errors. The lowest detection limit was 0.064 ng/mL, with a 4% intra-assay variation.

2.3. Light Microscopy

VP fragments collected on PND53 (n=10/group) were fixed by immersion in Methacarn [94] for 3 hours and included in Paraplast Plus® (Sigma-Aldrich, St. Louis, Mo, USA). Histological sections (5μm) were obtained and collected on silanized slides and stored until the moment of use. Histological sections were stained with hematoxylin-eosin (H&E) for morphological analysis, Gömöri reticulin for reticular fibers analysis and picrosirius to evidence collagen fibers. The slides were analyzed and microscopic fields

scanned using an image analyzer system (Image-Pro-Plus software version 4.5 for Windows) connected to a Leica DM 2500 microscope.

2.4. Stereological and Morphometric Analysis

The H&E stained sections were studied to measure the relative proportion of the tissue components: epithelium, lume and stroma luminal compartments, based to the Weibel score system which consists of a test system of lines and points in a with 168 points (WEIBEL, 1963). 10 digitalized images (20x) were taken from random fields of two different heights of the histological slides of all the animals (n = 200 images/group).

Epithelial cells height was measured using the LeicaQuwin V3 software coupled to the LeicaTM DMLB 80 microscope (Leica Microsystems, Nussloch, Germany). 10 acini per slide (x200) were analyzed and for each acinum, 5 measurements were performed in different regions (n=50 fields/slide). Thus, the mean height of the epithelium was obtained per group and the data were compared among the experimental groups.

2.5. Relative volume of collagen

Histological sections stained by picrosirius were analyzed to measure the relative volume of collagen in the prostate. Five randomized histological fields (x200) from two different depth of VP fragment (n = 50 histologic fields/group) were examined. The slides were analyzed at LeicaQuwin V3 software coupled to the LeicaTM DMLB 80 microscope (Leica Microsystems, Nussloch, Germany), where the system was programmed to recognize only red color and estimates the percentage of collagen (red stained) per histological field.

2.6. Immunohistochemistry

PV histological sections were submitted to the antigen retrieval in the humid environment at 100°C in 0.1M citrate buffer for 30-45 minutes. After washing, the slides were submitted to the endogenous peroxidase blockade in hydrogen peroxide/methanol and to block non-specific proteins (3% BSA + 1% goat serum) for 1 hour at room temperature.

After, sections were incubated with polyclonal anti-androgen receptor antibody (sc-816-Santa cruz® Biotechnology, Inc., USA) at a 1:100 dilution overnight at 4°C. After washing with PBS, the slides were incubated for 1 hour at room temperature with goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, CA), diluted 1:200 in 1% BSA in PBS. Chromogen color development was carried out with 3'3-diaminobenzidine tetrahydrochloride, slides were counterstained with Harri's hematoxylin.

2.6. Extraction and Quantification of Protein

PV frozen samples were mechanically homogenized with RIPA extraction buffer, plus proteases inhibitor (Sigma-Aldrich®, USA), in a Tureaux type homogenizer (Ultra Stirrer-Ultra80) in 3 cycles of 10 seconds around 4°C. The homogenate was centrifuged at 14,000 rpm for 20 minutes at 4°C, and the supernatant was collected. Protein concentration was determined by the Bradford method [95] on 96-well polystyrene plates and reading of absorbance was performed on Biochrom microplate reader (Holliston, Massachusetts, USA).

2.6.1. Gelatinolytic activity (Zimography)

The gelatinolytic activity of the metalloproteinases was analyzed by zymography with polyacrylamide containing gelatin according to the procedure performed by Scarano *et al*., (2009). The relative molecular weight of the bands was determined according to the molecular weight standard. Semiquantitative analyzes by densitometry of the bands were performed using ImageJ® for Windows software and the values of integrated optical density (IOD) obtained were normalized by means of fold and compared among the experimental groups.

2.6.2. Western Blotting

Five different protein samples (50 μg) for each group were separated on SDS-PAGE. Following the electrophoresis, the proteins were transferred to nitrocellulose membranes or Polyvinylidene Difluoride (PVDF) (only Caspase-3-cleaved). The

nonspecific binding of proteins was blocked by incubating the membrane in 5% non-fat milk in TBST buffer for 90 min at room temperature. The membranes were incubated with the respective primary antibody in 1% non-fat milk or 3% BSA (only Caspase-3-cleaved) in TBST (1:350-1,000) overnight at 4°C: DNMT1 (ab13537- Abcam® Inc.,USA); AR (N20) (sc-816- Santa cruz® Biotechnology, Inc., USA); SIRT1 (sc-15404-Santa Cruz® Biotechnology, Inc., USA); PCNA (sc-56-Santa Cruz® Biotechnology, Inc., USA); IL-6 (ab6672- Abcam® Inc.,USA); IL-10 (ab6672- Abcam® Inc.,USA); Caspase-3-cleaved (D175- Cell Signalig Technology, Inc. USA); Prostatein (7821-1009 – BioRAD); ß-Actin (sc-47778-Santa Cruz® Biotechnology, Inc., USA).

The membranes were then incubated with a specific secondary antibody conjugated with peroxidase, which was diluted (1:10,000 – 20,000) in TBST for 1 h (IgG goat-antirabbit, ab97051 and IgG goat-anti-mouse, ab97023, Abcam® Inc., USA). The immunoreactive components were revealed by GE Amersham ECL chemiluminescent substrate (GE Healthcare). Analyses were done in five different biological samples per group. To calculate the mean and SEM, the optic density of band was used as the unit of measure with software Image J (version 1.33u—National Institutes of Health, USA), and normalized by β-actin values and the results were normalized from fold change and expressed as means ± SD.

2.6.3. Measurement of reduced glutathione (GSH)

Reduced glutathione (GSH) was measured according to the method described by Tietze (1969). Briefly, GSH levels were determined using 5,50-dithiobis 20-nitro benzoic acid in supernatant from prostate homogenates (50 mg mL-1; n=5/group) and evidenced by formation of a yellow color. GSH levels were measured at 412 nm and results are expressed as mMmg-1 tissue [97].

2.6.4. Determination of superoxide dismutase (SOD) activity

The activity of superoxide dismutase was determined according to a method established by Winterbourn e colaboradores (1975). Aliquots containing 5µg of proteins

were homogenized with RIPA buffer and protease inhibitor cocktail. After homogenization, the samples were centrifuged at 13680 g for 15 min at 4 ºC. The centrifuged samples were diluted in phosphate buffer (0.1 M; pH 7.4) in a 1:20 ratio. In 100 µl of the homogenate were added 150 µl of cocktail containing: hypoxanthine, xanthine oxidase and nitrobluetetrazolium (NBT) in a ratio 1:1:1. The absorbance was read every minute for 10 min, 37ºC, at 560 nm. The integral was multiplied by 800 and results were expressed as U/g of tissue.

2.6.5. Determination of catalase (CAT) activity

Catalase activity was measured according to the method described by Aebi, 1984. Aliquots containing 15µg of protein were diluted in sample buffer (KH_2PO_4, 25 mM, pH 7,5; EDTA 1mM; BSA 1%) in a 1:20 ratio. The plate contained 2wells with catalase, used as a positive control. A 20 µL of the homogenate was placed in a 96-well plate with 100 µL of assay buffer (KH2PO4, 250 mM, pH 7.0); 30 µL of methanol and 20 µL of hydrogen peroxide (35.3 mM). The plate was incubated for 20 min at room temperature. After this period, 30 µL of potassium hydroxide (10 mM) and 45 µL of Purpald® was added and the plate was incubated for more 10 min at room temperature. After that, 15 µL of potassium periodate (65.2 mM) was added and the plate was incubated for more 5 min at room temperature. At the end, the absorbance determination was done in a spectrophotometer at 540 nm. The concentration was multiplied by 4000 and results were expressed as U/g of tissue.

2.7. Malondialdehyde (MDA) measurement by high performance liquid hromatography (HPLC)

The determination of the levels of lipid peroxidation was carried out by the technique of determination of cytoplasmic levels of malondialdehyde (MDA) by high performance liquid chromatography (HPLC) [96]. Fragments of the prostate of 5 animals were stored in cryovials at a temperature of -80 ° C. MDA levels were determined from a standard curve. The standard MDA solution (3 ml of 0.1M HCl in 3µL of 1,1,3,3 tetraetoxipropano (TEP) was kept for 10 minutes in boiling water, it was subsequently left

for 5 minutes in an ice bath. To determine the calibration points and held serial dilutions of the standard solution of MDA in order to obtain the linear regression equation of the straight line and the MDA sample concentration calculations. The method used was adapted from Oliveira e Cecchini (2000), which uses 160μL of the sample plus 100 ul of 0.5 M perchloric acid and incubated on ice for 10 minutes to precipitate the proteins. Next, were incubated with 100 ul of TBA for 30 minutes in boiling water and then cooled in an ice bath to occur interruption of the reaction. To stabilize the pH of the sample was used 100 ul of 1 M NaH2PO4 buffer samples were centrifuged at 5000rpm at 4 ° C for 10 minutes. The HPLC mobile phase consisting of 65% 50 mM KH2PO4 buffer and 35% HPLC grade methanol. The supernatants were filtered and injected into the HPLC. Readings were taken at 535 mm during a 11-minute run with flow 0.8 mL / minute, and the results were expressed as nM MDA.

2.8. Statistical Analysis

All statistical analyses were carried out using GraphPad Prism® software (version 5.00; Graph Pad, Inc., San Diego, CA). The data was analyzed by normality test "Shapiro-Wilk". Parametric data were analyzed by ANOVA followed by the ‘Tukey's Multiple” test. The nonparametric analysis was performed, by “Mann-Whitney’s” test or “Kruskal-Wallis” test followed by “Dunns test”. Differences were considered statistically significant when p-value was < 0.05.

3. RESULTS

There was no change in water and feed intake among groups during the experimental period (Table 1). Data for those parameters did not express variations among the experimental groups (Table 1). At the end of the experimental period (PND53), there were no difference in the final weight gain, in the relative weight of the organs and in testosterone levels among the groups (Table 1).

At PND53, few variations were observed in VP morphology. VP acini (intermediate/distal region) consisted of simple secretory high epithelium, with cells ranging from cuboid to cylindrical, supported on the basal lamina and surrounded by a

thin fibromuscular stroma, containing smooth muscle cells closely associated with connective tissue. The acini of the distal region of VP (highlighted in Figures 1a-i) did not demonstrated many variations, in generally the acinis showed are well dilated acini, with secretion product in the lumen and high epithelium with secretory ephithelial cell.

Table 1: Hormonal, consumption and weights parameter of experimental groups

	Experimental groups		
Parameters	**Control (n=10)**	**As1 (n=10)**	**As2 (n=10)**
Water consumption (mL)	53.76±14.0	50.03±16.13	53.39±13.81
Preputial separation (days)	45.22±0.95	46.8±0.73	41.1±0.26
Body weight (g)	193.3±23.27	194.6±24.86	195.7±20.39
Prostate weight (g)	0.051±0.010	0.048±0.013	0.047±0.008
Kidney weight (g)	0.91±0.03	0.90±0.04	0.89±0.4
Liver weight (g)	11.76±1.26	11.9±1.0	11.9±1.4
Testosterone (ng/dL)	295.4±311.2	295.5±327.9	390.1±214.9

Data were expressed as mean ± SEM; $p<0.05$.

There was no alteration in the relative proportion of tissue constituents: stromal, luminal and epithelial compartments, among experimental groups at PND53 (Table 2). However, there was a decrease in epithelial height in the As1 group in relation to the Ctrl group (Table 2).

Table 2: Stereological and morphometric analyzes of the prostate of the animals at PND53

	Experimental groups		
Parameters ***Relative proportion of the constituents of VP (%)***	**Control (n=10)**	**As1 (n=10)**	**As2 (n=10)**
Lumen	39.2±9.1	41.5±7.9	38.0±7.5
Epithelium	43.1±9.2	39.4±8.6	43.5±7.5
Stroma	17.6±4.8	19.4±8.2	18.4±4.6
Epithelium height (um)	17.9±2.7	16.4±1.9*****	16.9±2.5

Data were expressed as mean ± SEM. Asterisk represents statistically significant ($p<0.05$).

No significant variations were observed in relation to the distribution and arrangement of the collagen fibers (Figure 2d-f) and reticular fibers (Figure 2g-i) in the VP at PDN53. However, when the groups were compared in relation to the amount of collagen fibers, measured by red-stained (picrosirius), there was a decrease in the collagen amount in As1 compared to the Ctrl (Figure 1j).

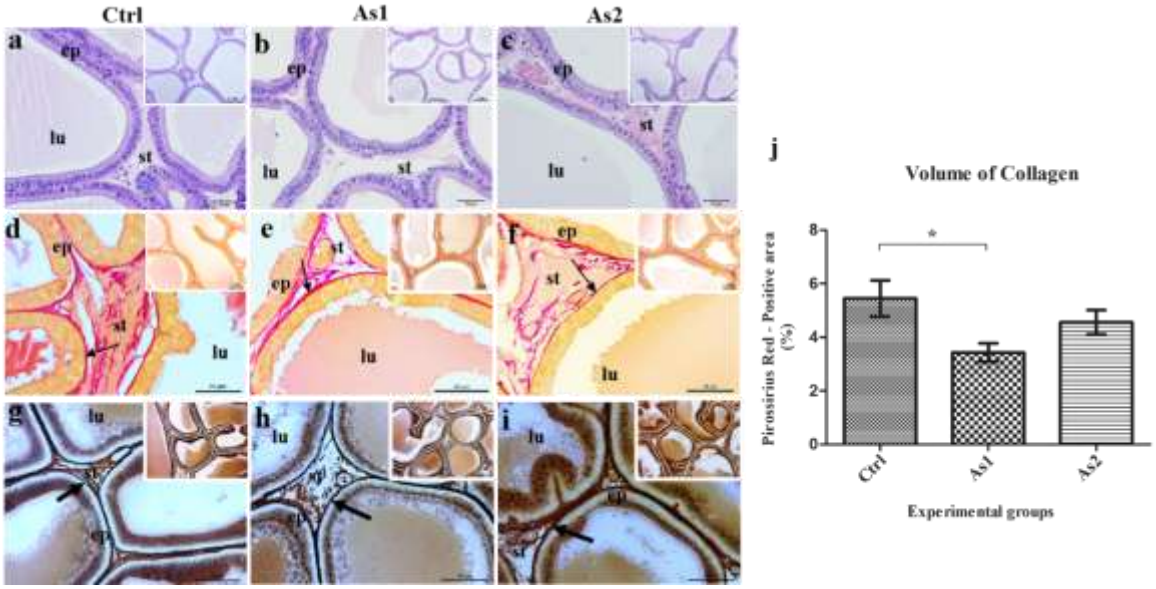


Figure 1: Histological sections of VP at PND53 and details stained by H&E (a-c), picrosirius (d-f) and Gömöri reticulin (f-i). Abbreviations: lu: lumen; ep: epithelium; st: stroma. Fine arrows point to collagen fibers and thick arrows point the reticular fibers. Volume of collagen fibers present in the fibrillar stroma of VP of the different groups (n =10/group) (J). Data were expressed as median ± interquartile range. Bar with asterisk represent statistically significant difference (* $p<0.05$).

The metalloproteinase 2 (MMP-2) gelatinolytic activity in VP at PND53 showed an increase in the MMP-2 intermediate form in As1 group compared to the Ctrl group and a decrease in the active form in As1 group when compared to the other groups (Figure 2b).

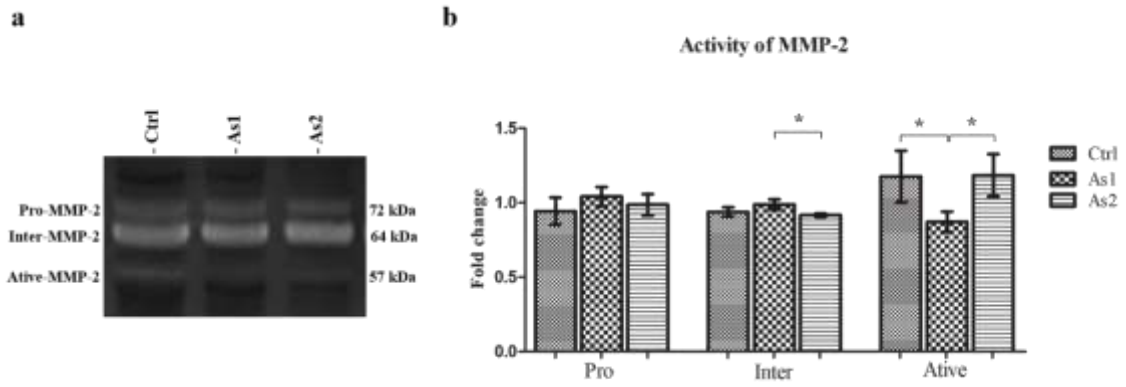


Figure 2: a. Representative image of the expression of enzymatic activity at PND53. **b.** Enzyme activity for MMP-2 (n = 5/group). Data were expressed as mean ± SEM. Bars with asterisks represent a statistically significant difference (* $p<0.05$).

According to the results obtained from the western blotting technique, it was observed that both androgen receptor and prostatein expression decreased according with $AsNO_2$ exposure dose, however only in As2 group there was a significant reduction in both proteins (Figures 3a-b). Cell proliferation, as measured by PCNA expression, decreased in As1 group when compared to the Ctrl group and As2 group (Figure 3c). On the other hand, the apoptotic activity analyzed by Caspase-3-cleaved expression revealed a higher apoptosis index in the As2 group, compared to the other two experimental groups (Figure 3e). SIRT1 expression, a protein associated with antioxidant response, was significantly decreased in the As1 group when compared to the Ctrl group (Figure 3d). DNA methylation was indirectly measured by DNMT1 expression and our results showed a higher DNMT1 expression in As2 compared to other groups (Figure 3f). To assess whether continuous intake of $NaAsO_2$ was able to increase inflammatory process in the VP of the exposed animals, we analyzed the expression of anti-and pro-inflammatory cytokines IL-10 and IL-6. The results showed a decrease in IL-10 in the As2 group when compared to the Ctrl group (Figure 5g) and an expressive increase in IL-6 in As2 compared to As1 (Figure 3h).

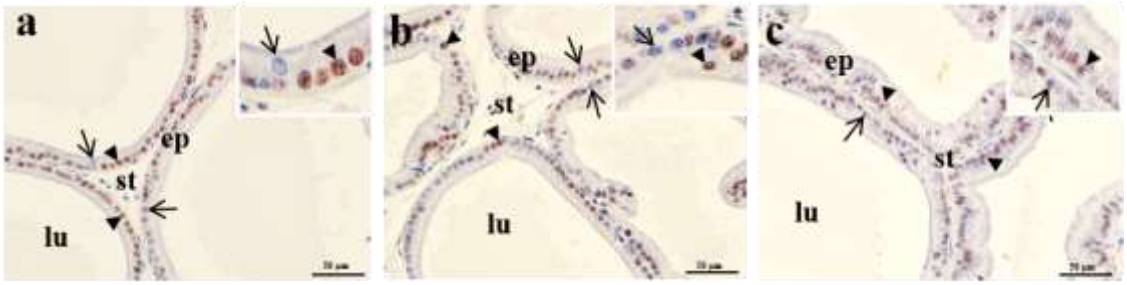
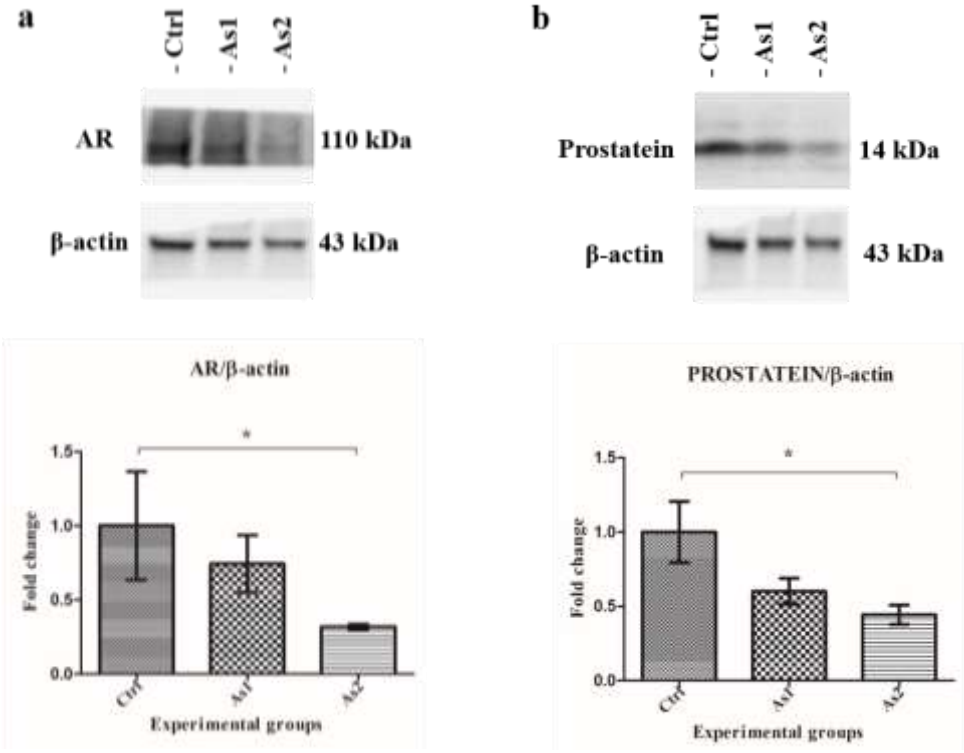
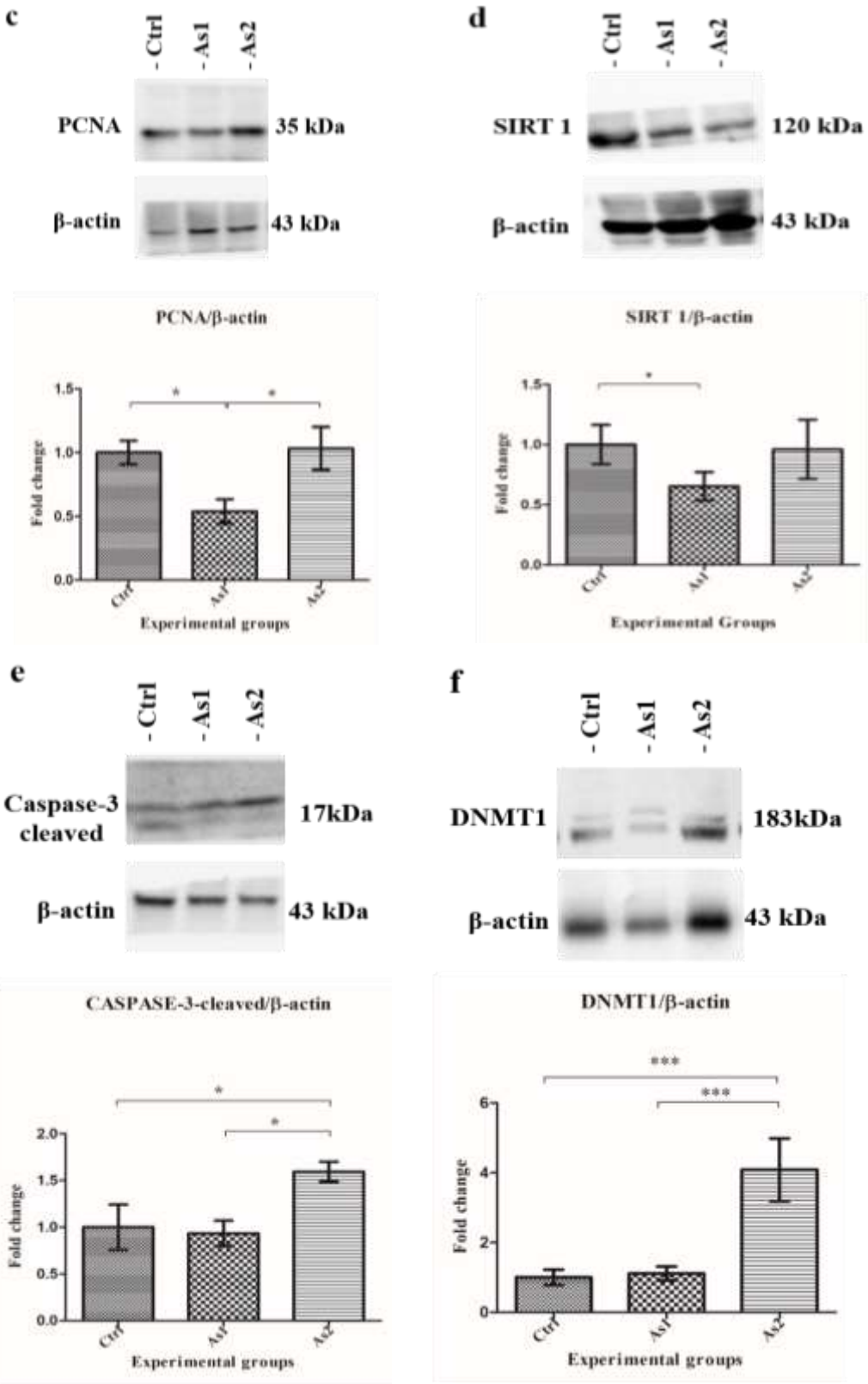


Figure 2: Representative histological sections of VP at PND53 and details immunostained for AR (a-c). Abbreviations: lu: lumen; ep: epithelium; st: stroma. Arrowhead point to nuclei positive for AR and fine arrows point to nuclei negative.




c
- Ctrl
- As1
- As2
PCNA
35 kDa
β-actin
43 kDa
PCNA/β-actin
Fold change
Experimental groups
d
- Ctrl
- As1
- As2
SIRT 1
120 kDa
β-actin
43 kDa
SIRT 1/β-actin
Fold change
Experimental Groups
e
- Ctrl
- As1
- As2
Caspase-3 cleaved
17kDa
β-actin
43 kDa
CASPASE-3-cleaved/β-actin
Fold change
Experimental groups
f
- Ctrl
- As1
- As2
DNMT1
183kDa
β-actin
43 kDa
DNMT1/β-actin
Fold change
Experimental groups

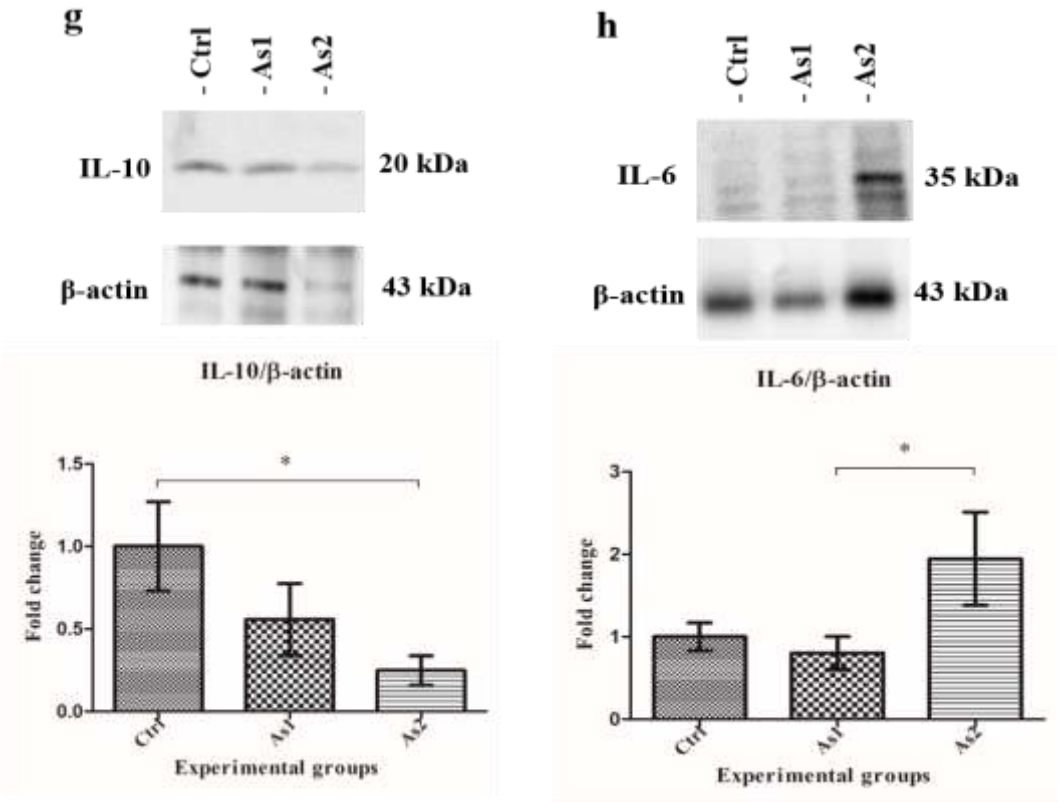


Figure 3: Image and plot for expression of the proteins AR (a), prostatein (b), PCNA (c), SIRT1 (d), Caspase-3-cleaved (e), DNMT1 (f), IL-10 (g) and IL-6 (h), measured from protein extracts of VP at PND53 (n = 5 / group); followed by fold change for the proteins. Data were expressed as median ± interquartile range. The target bands were quantified and normalized by β-actin expression. Bars with asterisks represent a statistically significant difference (* $p<0.05$; *** $p<0.001$).

The redox state on the VP microenvironment at PND53 was evaluated by the activity of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in the tissue (Figure 4). The result showed no significant changes among the experimental groups.

Lipid peroxidation in the tissue was measured by the Malondialdehyde (MDA) levels. The result showed a significant increase of MDA in both groups exposed to $NaAsO_2$, however, this increase was more pronounced in As1 group (figure 5).

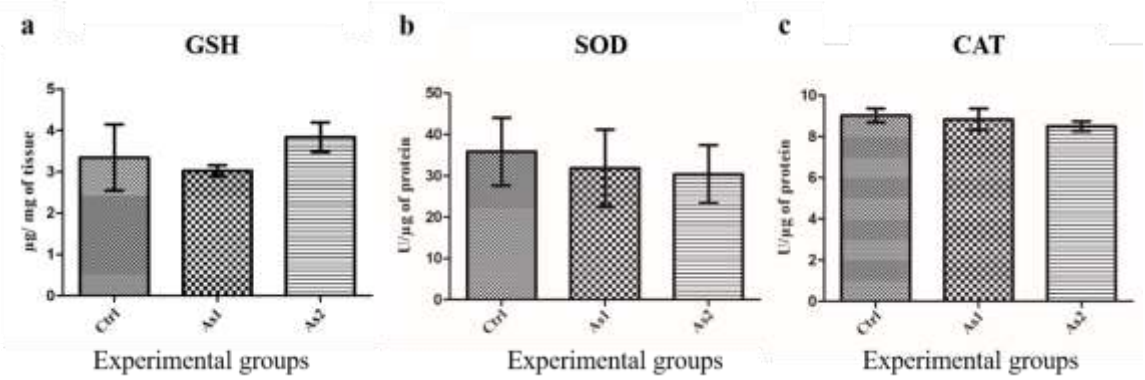


Figure 4: GSH, SOD and CAT expression of the VP at PND53 of the different groups (n = 5/group). Data were expressed as mean ± SEM (* $p<0.05$).

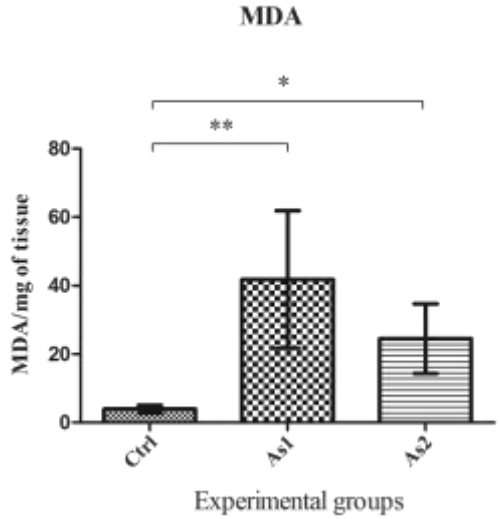


Figure 5: MDA expression of VP at PDN53 of the different groups (n = 5/group). Data were expressed as mean ± SEM. Bars with asterisks represent a statistically significant difference (* $p<0.05$; ** $p<0.01$).

4. DISCUSSION

Prostate diseases are responsible for the major causes of morbidity and mortality among old men in the world [54,100]. Some studies have related the increasing incidence of these pathologies with the increase of daily exposure to the different EDs dispersed in the environment [22,31]. In addition, epidemiological data have associated the exposure to iAs to disorders in the prostatic microenvironment and to the development of prostate lesions [101], in individuals whose exposure occurs in adulthood or in individuals exposed

during gestation. In this study, we analyzed the effect of exposure to iAs, by the main exposure route, at the minimum and maximum levels of contamination that have been tolerated by the control agencies [55,60,83], during sexual maturation of juvenile rats.

In this study, no changes were observed in the eating habits of the animals submitted to constant exposure to $NaAsO_2$ when compared to the control group, which justifies the similar pattern in the evolution of body weight throughout the experimental period. In addition, the analysis of the weight of liver and kidneys, did not show any variation among the groups. These results agree with the data obtained in similar experimental protocols [60] and other protocols involving exposure to arsenic [57–59]. When associated with similarity in final body weight and analyzes of dietary habits among experimental groups, these data demonstrate that the treatment, as it was proposed (dose and time of exposure), was not able to induce clinical or systemic toxicity in juvenile animals.

At puberty, with a marked increase in the androgen levels, the hypothalamic-pituitary-gonodal axis starts to release molecular signals that stimulate the growth and maturation of the testicles, prostate and external genitalia [35,36]. The prostate is highly dependent of the androgen receptors (AR) stimulated by testosterone [1] and EDs generally alter the dynamics of the endocrine glands through interaction with hormone receptors [30,34]. Our results showed that there was no change in circulating testosterone levels. Souza e colaboradores 2016 e Medeiros e colaboradores 2018 did not identify variations in testosterone levels in adult and pubertal animals exposed at the same concentrations adopted for this study. However, the non-alteration of circulating levels of testosterone during the experimental protocol and immaturity of the steroidogenesis system at PND53 [59,60] may explain the similarity in prostate weight among the experimental groups.

Although the stereological results did not reveal alterations in the compartments: epithelium, stroma and lumen; more specific parameters were assessed to evaluate the integrity of the epithelial and stromal microenvironment. Both prostatic stromal and epithelial cells, when exposed to an atypical environment, can have their function altered and secrete a variety of paracrine substances such as cytokines and growth factors, which interfere with the balance and architecture of the gland. In addition, it was observed reduction in the stromal collagen quantity, as well as, in the metalloproteinase 2 activity, and in secretory epithelium height of in As1 group compared to control.

The stromal microenvironment participate of the prostate homeostasis, providing nutrients and growth factors [19,21]. Among the different matrix components, metalloproteinases (MMPs), enzymes responsible for modeling and degrading protein components [46,102,103], help to maintain tissue architecture integrity [104,105]. Santos et al. (2017) pointed out that the reduction in MMP-2 activity in the VP of pubertal animals may result in the accumulation of collagen fibers in the extracellular matrix, which would make the stromal environment more reactive. However, our results showed that As1 group presented lower collagen quantity and reduction in active-MMP-2 activity compared to other groups. Probably the reduction of MMP-2 activity in this group is related to a decrease in the collagen amount; in other words, MMP-2 would have less substrate to degrade in these animals, which would reduce its activity in relation to the other groups. In addition to those data, there was a reduction in the cell proliferation index, as well as in the secretory epithelium height in As1 related to the other groups. Taken together, these data reinforce the hypothesis that there was a delay in the late development of the prostate.

Inorganic arsenic compounds (iAs) may act in different ways, for example, by inhibiting cell proliferation, inducing apoptosis or even stimulating differentiation [79]. The analysis of the proliferative activity of the experimental groups, measured by the PCNA expression, showed a decrease in the proliferative activity of the As1 group compared to the other groups, which may indicate that the exposure is inhibiting proliferation and, consequently, delaying the development and maturation of the prostate. Similar procedure was observed in the mammary gland of rats exposed to iAs [107]. So, a reduction of approximately 25% in AR and 40% in prostatein expression indicates a significant functional reduction, and probably, the impaired cellular turnover in the prostate from As1 group. In addition, previous data have shown that some chemical agents are capable of producing severe effects in low doses when compared to the high doses, leading to effects where dose-response relation is not linear and progressive [32,60,108].

Trivalent arsenic metabolites are reactive and act as inhibitors of GSH, an important enzyme in the strategy of protecting the cellular environment against toxic effects, since it acts as an antioxidant and anti-inflammatory enzyme [79,81]. Inhibition of this enzyme leads to an imbalance in the oxidation-reduction pathway of cells [75,81] and directly interferes with the mechanism of glandular homeostasis. In this context, Shen et al (2013) proposed that glutathione is a key enzyme in modulating the antiproliferative and

proapoptotic mechanisms of exposure to arsenic trioxides (As). Arsenic acts by inhibiting DNA repair enzymes and decreased glutathione levels, which may directly affect the cellular protection mechanisms against reactive oxygen species [109]. Our data for the redox status showed that, although SIRT 1 levels were reduced in the As1 group, the activity of the main antioxidant enzymes like GSH, SOD and CAT in the prostate of animals submitted to $AsNaO_2$ exposure was not altered. However, MDA levels were expressly increased in both arsenic-treated groups, which clearly indicates that the increase in lipid peroxidation is directly associated with exposure to the toxicant. In addition, Choudhury (2016a) also identified increased MDA in the thymus of mice exposed to different doses of $NaAsO_2$.

MDA is a product of lipid peroxidation produced from high free radicals [111]. Previous report showed that rats exposed to $NaAsO_2$ had higher rates of lipid peroxidation in response to accumulation of the substance in the testes [112], which probably may be occurring in the prostate in our model. Different metals can induce oxygen free radicals (ROS) generation, which influence in pathways that cause oxidative damage in macromolecules and inactivate enzymes that act as antioxidants, increasing tissue damage [111]. In this context, it is probable that $NaAsO_2$ induces an oxidative stress status by increasing lipid peroxidation; and therefore, it would possible to associate this stress condition with reduction in SIRT1expression and cell proliferation in As1 and increase in apoptosis index in As2. This imbalance in the lipid peroxidation status make the cells more susceptible to methylation when exposed to $AsNaO_2$ [75,109].

iAS compounds, especially in trivalent forms, stimulate apoptosis through activation of caspases 3 and 9 by oxidative stress [78,79]. Besides that, one of the mechanisms of the iAs biomethylation process utilizes S-adenosylmethionine (SAM) as a methyl donor and this reduction of SAM can induce DNA hypoxylation, leading to significant damage [62,75]. Hinhumpatch (2013) suggest that children exposed to iAs continuously may have disorders such as oxidative DNA damage and inability to repair DNA [113,114]. According Svedruzic (2011) , DNMT1, the main mammalian DNA methyltransferase, is an enzyme with multiple regulatory functions that can control cell cycle regulation, DNA methylation and apoptotic cell activity. Our results showed DNMT1 expression and apoptosis index increased and reduction in androgen receptor expression. It is known that DNMT1, important methyltransferase, is mainly related to DNA

methylation and cell cycle control, therefore it can be hypothesized that the reduction of androgen receptor levels may be associated with inhibition of transcription sites for AR, since circulating levels of testosterone among the groups were similar. In addition, oxidative damage caused by increased lipid peroxidation would have interfered with DNA repair mechanisms, which would accentuate the mechanism of cell death by apoptosis in As2 animals.

The prostatic epithelium is composed of secretory epithelial cells that produce prostatic fluid and express high levels of androgen receptors, which determine the magnitude of the secretory process in the prostate [1,15,16]. The prostatein is an androgen dependent protein, being the main protein produced and secreted by the ventral prostate, and its major function is to inhibit the phagocytic activity of macrophages, and therefore, it has an important antioxidant function of the seminal fluid [2]. Our results showed a reduction in the amount of prostateín, revealing a dose-dependent response, where only the highest exposure (As2) was able to significantly alter its expression. This result resembles to the result found for the AR expression, where the As2 group showed reduction of that protein, and demonstrates that there was a significant reduction of the functional activity of the prostate in this group. Furthermore, it is possible to affirm that sodium arsenite, by compromising the expression of androgen receptors by mechanisms independent of the availability of the ligand, as previously described, ends up interfering in androgen dependent cellular pathways, a factor indicative of compromised functional activity.

According to Choudhury (2016b), high doses of arsenic activate NF-κB in response to increased oxidative stress. NF-kB may activate or inhibit the expression and activity of anti-inflammatory cytokines [116], on the other hand, anti-inflammatory interleukins inhibition, such as IL-10, leads to the increase of pro-inflammatory interleukins [117,118], which generates inflammation and can be harmful to tissue . Our results showed increase in IL-6 expression and IL-10 reduction in As2 group. During the process of acute inflammation, cells of the immune system present in the prostatic stroma stimulate the production of cytokines mediating initial processes of inflammation (IL-1, IL-6 and IL-8)[117,119]. The exacerbated increase of IL-6 is commonly found under stress conditions [119] and rapidly induces acute immune system response [120]. It is possible that the increase of IL-6 is related to the reactive species of oxygen increase caused by lipid peroxidation as it was observed in our results. In contrast, IL-10 is an anti-inflammatory

cytokine with a key role in the modulation of immune responses, as it inhibits the action of leukocytes and limits the damage caused by inflammation [116]. According Iyer e Cheng [121] IL-10 deficiency, in long term, tends to be harmful to tissue, which in combination with increased molecular perturbations caused by the higher $NaAsO_2$ exposure dose may be rendering the tissue vulnerable to early development of lesions.

By evaluating the data obtained, it is possible to hypothesize that the sub chronic exposure to $AsNaO_2$ at an environmentally low dose during late prostate morphogenesis, although not presenting important systemic toxic effects, compromised the prostate maturation, where molecular markers signaling to an important structural and functional deficit. These data are similar to studies evaluating low-dose exposure of other metals and chemicals such as cadmium, bisphenol, phthalates, methylmercury [23,32,43] and other studies that revealed imbalances and tissue damage in the lower doses of exposure to $NaAsO_2$ [60,110]. Although the animals exposed to the highest concentration of $AsNaO_2$ showed little structural impairment, changes observed in As2 animals were important, since they demonstrate imbalance in important markers for the physiology of the gland. In addition, as observed by Choudhury [110], that evaluating doses ranging from 0.01 to 0.1 mg/L in the thymus of Swiss mice exposed to $AsNaO_2$, the highest dose of exposure presented a more intense cytotoxic potential.

In view of the results obtained and emphasizing that, together with other components, the product of prostatic secretion maintains germ cell viability, we can infer that continuous exposure to sodium arsenite could compromise male fertility at puberty and in long term, make the prostatic tissue more susceptible to oncogenesis, however, this latter hypothesis needs to be better explored.

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