ANA CLÁUDIA FERREIRA SOUZA

EFEITOS DO ARSÊNIO EM PARÂMETROS HEPÁTICOS E REPRODUTIVOS DE RATOS WISTAR SAUDÁVEIS E DIABÉTICOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2018

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

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S729e
2018

Souza, Ana Cláudia Ferreira, 1988-
Efeitos do arsênio em parâmetros hepáticos e reprodutivos de ratos Wistar saudáveis e diabéticos / Ana Cláudia Ferreira Souza. – Viçosa, MG, 2018.
ix, 54f. : il. (algumas color.) ; 29 cm.

Orientador: Mariana Machado Neves.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Morfologia (Animais). 2. Toxicologia reprodutiva. 3. Toxicologia hepática. 4. Diabetes. 5. Arsenato. 6. Stress oxidativo. 7. Inflamação. 8. Rato como animal de laborátorio. I. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-graduação em Biologia Celular e Estrutural. II. Título.

CDD 22 ed. 599.35

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APROVADA: 08 de fevereiro de 2018.

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(Orientadora)

Dedico este trabalho aos meus amados pais,

Geraldo e Maria de Lourdes.

AGRADECIMENTOS

A Deus por estar presente em minha vida, iluminar e direcionar meus passos.

Aos meus pais e irmão pelo amor incondicional, pelo exemplo de vida, humildade e caráter. Amo vocês! Obrigada por tudo!

À toda minha família pelo amor que nunca deixaram faltar e pela confiança.

Ao meu noivo Daniel, que me deu todo suporte e apoio durante o doutorado. Obrigada pelo carinho, compreensão e companheirismo durante este período. Obrigada por sempre me incentivar e viver comigo este período tão intenso. Te amo!

À Universidade Federal de Viçosa e ao Programa de Pós-graduação em Biologia Celular e Estrutural pela oportunidade de realização do curso, crescimento profissional e pessoal.

À CAPES pela concessão da bolsa de pesquisa durante o doutorado, incluindo a bolsa de Doutorado Sanduíche no exterior.

À University of Virgínia e ao Cell Biology Department por terem aceitado meu pedido de estágio sanduíche e pelo apoio concedido.

À minha orientadora Mariana Machado Neves, pela orientação, confiança e pelos ensinamentos ao longo destes anos. Muito obrigada!

Ao professor Barry T. Hinton, da University of Virginia, por me receber em seu laboratório durante o período sanduíche e não medir esforços para meu aprendizado e crescimento profissional.

À Angie e Bingfang por me acolherem no laboratório durante o período sanduíche. Obrigada pelos ensinamentos, pela paciência, carinho e por todas as risadas!

Aos professores do curso de Pós-graduação em Biologia Celular e Estrutural, pelos ensinamentos adquiridos durante o curso.

Aos professores Marcos, Izabel, Fabiana e Reggiani pelo pronto comprometimento na formação desta banca avaliadora e pelas contribuições.

À professora Reggiani Vilela Gonçalves pelas contribuições durante a realização deste trabalho.

Ao professor Leandro Licursi de Oliveira por disponibilizar a estrutura do seu laboratório durante a realização das análises enzimáticas.

Ao professor Jorge Dergan por disponibilizar o fotomicroscópio para a aquisição das imagens deste trabalho.

Ao Núcleo de Microscopia e Microanálise da Universidade Federal de Viçosa por disponibilizar a infraestrutura para realização de análises deste trabalho.

À Beth pela atenção e disposição em ajudar.

Aos integrantes do Laboratório de Biologia Estrutural pela amizade, colaboração e momentos de descontração.

Ao Felipe, Marcela, Daniel e Luiz pela valiosa ajuda durante todas as fases deste trabalho, pela amizade e carinho. Obrigada por poder ensinar e aprender todos os dias com vocês! Este trabalho é nosso!

Aos amigos feitos durante os últimos anos em Viçosa. Foi muito bom ter encontrado vocês!

Aos amigos feitos durante o doutorado sanduíche, em especial a Raquel, Juliana, Carol, Maraysa e Natasha por todo carinho, companheirismo, risadas e pela sincera amizade. Sem vocês este período da minha vida não teria sido tão intenso e bem vivido!

As amigas de república pela convivência constante e por serem a minha família em Viçosa!

Aos amigos de Divinópolis que sempre torceram por mim.

Agradeço ainda, a todos com que tive a oportunidade de conviver nestes últimos anos e que muito contribuíram para que eu pudesse chegar até aqui. Muito obrigada por tudo!

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RESUMO

SOUZA, Ana Cláudia Ferreira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Efeitos do arsênio em parâmetros hepáticos e reprodutivos de ratos Wistar saudáveis e diabéticos.** Orientadora: Mariana Machado Neves.

É conhecido que a exposição ao arsênio ou a hiperglicemia pode causar alterações sistêmicas, comprometendo a função e a estrutura dos órgãos. No entanto, não está claro a extensão na qual este metaloide, na presença de uma disfunção metabólica preexistente, poderia afetar a morfologia e função dos órgãos. Portanto, o objetivo deste trabalho foi avaliar parâmetros hepáticos e reprodutivos de ratos normais e diabéticos expostos ao arsênio. Para isso, a diabetes foi induzida em ratos machos utilizando injeção intraperitoneal de estreptozotocina. Os animais saudáveis e hiperglicêmicos receberam solução salina (controle negativo; controle diabetes) ou 10 mg/L de arsênio na forma de arsenato de sódio (controle arsênio; diabetes + arsênio) na água de beber por 40 dias *ad libitum*. A exposição ao arsenato aumentou os níveis de glicose sérica em animais saudáveis comparados aos animais do grupo controle negativo. No entanto, a exposição a este composto de arsênio não intensificou os níveis de glicose já elevados dos animais diabéticos. No fígado, a atividade das enzimas antioxidantes foi reduzida após exposição ao arsenato em animais saudáveis comparados aos animais do grupo controle negativo, enquanto a combinação de diabetes e arsenato agravou a redução da enzima glutationa s-transferase. Consequentemente, os animais expostos ao arsenato apresentaram níveis aumentados de malondialdeido e proteína carbonilada, sendo este aumento intensificado em animais com diabetes pré-existente. Além disso, estes animais apresentaram níveis séricos aumentados de fosfatase alcalina. A exposição ao arsenato causou um processo inflamatório no tecido hepático de animais saudáveis comparados aos animais do grupo controle negativo. Do mesmo modo, a exposição ao arsenato realçou o aumento de infiltrado inflamatório nos animais diabéticos, com alto número de mastócitos e produção de TNF-α. Em adição, alterações vasculares tais como congestão, hemorragia e depósitos de hemossiderina foram intensificados nos animais do grupo diabetes + arsênio, enquanto os estoques de glicogênio foram reduzidos. Em relação ao sistema reprodutor, nós observamos que a exposição ao arsenato reduziu a concentração de testosterona sérica em animais saudáveis e agravou a redução observada em animais diabéticos. O número de espermatozoides no testículo e epidídimo, a produção diária espermática e o tempo de trânsito dos espermatozoides no epidídimo foram reduzidos também em animais saudáveis expostos ao arsenato. Além disso, a exposição a este composto de arsênio

agravou estes parâmetros em ratos diabéticos. A motilidade espermática, a integridade das membranas desta célula e o número de espermatozoides normais foram também reduzidos em animais saudáveis expostos ao arsenato. A combinação de diabetes e arsenato intensificou apenas a redução do número de espermatozoides normais observado nos animais diabéticos. A proporção de arsênio aumentou no testículo e epidídimo de ambos os grupos recebendo arsenato. Além disso, a exposição ao arsenato induziu a um desequilíbrio na atividade das enzimas antioxidantes e no conteúdo de microminerais em animais saudáveis, além de realçar estas mudanças em ratos diabéticos. Mudanças morfológicas ocorreram principalmente nos animais do grupo diabetes + arsênio. Enfim, nossos resultados indicam que a exposição ao arsênio foi capaz de causar intensos danos morfológicos e funcionais no fígado e no sistema reprodutor de ratos Wistar saudáveis. Além disso, estes danos foram intensificados quando animais diabéticos foram expostos ao arsênio. Portanto, mais estudos são necessários para entender melhor o mecanismo de ação do arsênio em animais com doença metabólica pré-existente e os possíveis danos causados por este metaloide na fertilidade e metabolismo dos animais.

ABSTRACT

SOUZA, Ana Cláudia Ferreira, D.Sc., Universidade Federal de Viçosa, February, 2018. **Effects of arsenic in hepatic and reproductive parameters of healthy and diabetic Wistar rats.** Adviser: Mariana Machado Neves

It is known that exposure to either arsenic or hyperglycemia can promote systemic alterations, compromising the structure and function of the organs. However, it is unclear the extent to which this metalloid, in the presence of a preexistent metabolic dysfunction, could affect the morphology and function of the organs. Therefore, the aim of this study was to evaluate hepatic and reproductive parameters of healthy and diabetic rats exposed to arsenic. For this, diabetes was induced in male rats by intraperitoneal injection of streptozotocin. Healthy and hyperglycemic animals received saline solution (negative control; diabetes control) or 10 mg/L arsenic as sodium arsenate (arsenic control; diabetes + arsenic) for 40 days in drinking water *ad libitum*. Arsenate exposure increased serum glucose levels of healthy animals compared to negative controls. However, the exposure to this arsenic compound did not intensify the increased glucose levels of diabetic animals. In the liver, the activity of antioxidant enzymes was suppressed after arsenate exposure in healthy animals compared to negative controls, whereas diabetes and arsenate combination worsened the reduction of glutathione s-transferase enzyme. Consequently, arsenate-exposed animals showed increased malondialdehyde and carbonyl protein levels, being this increase worsened in animals with pre-existent diabetes. Moreover, these same animals had increased alkaline phosphatase serum levels compared to diabetes controls. Arsenate exposure caused an inflammatory process in the liver tissue from healthy rats compared to negative controls. In the same way, exposure to arsenate enhanced the increase of inflammatory infiltrate in diabetic animals, with higher number of mast cells and TNF-α production. In addition, vascular alterations such as congestion, bleeding, and hemosiderin deposition were intensified in diabetes + arsenic animals, whereas glycogen storage was reduced. In reproductive system, we observed that arsenate exposure reduced serum testosterone concentration in healthy animals and worsened the reduction observed in diabetic rats. The sperm numbers in the testis and epididymis, daily sperm production, and sperm transit time were also reduced in healthy animals exposed to arsenate. Moreover, arsenate exposure worsened these parameters in diabetic rats. Sperm motility, integrity of sperm membranes and the number of normal sperm were also reduced in health animals exposed to arsenate. A combination of diabetes and arsenate just intensified the reduction in the number of normal sperm observed in diabetic animals.

The proportion of arsenic increased in the testis and epididymis of both groups receiving arsenate. In addition, exposure to arsenate induced an imbalance in antioxidant enzymes activities and mineral content in healthy animals and enhanced these changes in diabetic rats. Morphological changes occurred mainly in animals co-exposed to diabetes and arsenate. Finally, our results showed that exposure to arsenic was able to cause intense morphological and functional damages to the liver and male reproductive system of healthy rats. Importantly, these damages were intensified by arsenate exposure in livers previously injured by diabetes. Moreover, these damages were intensified when diabetic animals were exposed to arsenic. Therefore, more studies are needed to better understand the mechanism of arsenic action in animals with pre-existent metabolic disease and the possible damages caused by this metalloid in fertility and metabolism of the animals.

INTRODUÇÃO GERAL

Os metais pesados são componentes naturais da crosta terrestre e, como tal, são os agentes tóxicos mais antigos conhecidos pelos humanos, tendo sido usados por milhares de anos. As principais vias de exposição aos metais pesados incluem as fontes naturais, tais como as águas superficiais, processos industriais como a mineração e produtos químicos incluindo os agrotóxicos e aditivos inorgânicos (Ibrahim et al., 2006).

Atualmente, a presença de altas concentrações de metais pesados no ambiente tem constituído um problema global, devido à dimensão dos lançamentos de origem antrópica e o seu contínuo aumento nos diferentes ecossistemas, atribuído em grande parte à industrialização e ao desenvolvimento urbano (Beveridge et al., 1997). Como não possuem caráter de biodegradabilidade, tais metais permanecem nos ciclos biogeoquímicos globais, sendo o das águas naturais o principal meio de condução, podendo haver acumulação na biota aquática em níveis significativamente elevados (Silva, 2002).

Uma vez absorvidos no intestino, os metais pesados podem se ligar a proteínas e serem transportados pelo sangue até os tecidos, onde são biotransformados ou se depositam (Goldhaber, 2003). A toxicidade causada por estes metais deve-se à ocorrência de dois principais mecanismos de ação, a formação de complexos com os grupos funcionais das enzimas, o que prejudica o perfeito funcionamento do organismo, e a combinação com as membranas celulares, o que altera ou impede completamente o transporte de substâncias (Goldhaber, 2003). Além disso, os efeitos tóxicos causados pelos metais podem ser determinados pelo tempo de exposição e pela quantidade de metais ou compostos que se convertem em uma forma biodisponível, causando alterações patológicas, bioquímicas, fisiológicas, comportamentais e reprodutivas (Goyer, 1996; Muniz e Oliveira-Filho, 2006).

O arsênio é um elemento químico amplamente encontrado na natureza. Apesar de ser classificado formalmente como metaloide, por apresentar algumas propriedades dos metais e não metais, este elemento é considerado como metal pesado no contexto da toxicologia (Jomova et al., 2011). O arsênio pode ser encontrado na natureza em diferentes estados de oxidação, tais como +5, +3 e -3, nos quais ele é capaz de formar compostos inorgânicos e orgânicos no ambiente ou dentro do corpo humano. Em combinação com elementos como o oxigênio, enxofre e cloro, este elemento é referido como arsênio inorgânico, e combinado com hidrogênio e carbono ele é chamado de arsênio orgânico (Orloff et al., 2009).

O efeito tóxico das espécies de arsênio depende principalmente de sua fórmula química. Os compostos inorgânicos são considerados 100 vezes mais tóxicos do que as espécies orgânicas (Barra et al., 2000). Dentre os compostos inorgânicos, o arsenito (As^{3+}) e o arsenato (As^{5+}) são formas naturalmente encontradas na natureza. A toxicidade das diversas espécies de arsênio decresce na seguinte ordem: compostos de arsenito inorgânico > compostos de arsenato inorgânico > compostos de arsenito orgânico > compostos de arsenato orgânico (USEPA, 2000). O arsenito é considerado cerca de 60 vezes mais tóxico do que a forma oxidada, arsenato, uma vez que ele reage altamente com grupos tiois endógenos, especificamente ditiois (Neves et al., 2004). No entanto, o arsenato é o composto de arsênio mais encontrado no ambiente, principalmente nas águas superficiais (Jomova et al., 2011).

O arsênio concentra-se naturalmente na crosta terrestre, de onde é lixiviado atingindo as águas superficiais (Vahter, 2008). Além disso, o arsênio tem sido amplamente utilizado com insumo nas indústrias de fundição e manufatura de vidros, assim como na produção de esmaltes, tintas, produtos agrícolas e preservativos de madeira (Gontijo e Bittencourt, 2005). Os humanos são expostos ao arsênio principalmente através das rotas oral e inalatória. A exposição oral ocorre via consumo de água e comida contaminadas. A inalação ocorre devido a exposição ocupacional nas fundições, indústrias de fabricação de vidro e semicondutores (Liu et al., 2001). A exposição a grandes quantidades de arsênio, principalmente a partir de água contaminada, é considerada uma das principais ameaças à saúde em todo o mundo (Li et al., 2012).

A Organização Mundial da Saúde considera o arsênio um elemento químico que apresenta potenciais riscos à saúde humana, sugerindo sua concentração máxima tolerada em águas potáveis de 0,01 mg/L (WHO, 2011). No entanto, é estimado que cerca de 100 milhões de pessoas no mundo estão expostas a níveis de arsênio maiores que 50 µg/L na água de beber (Moon et al., 2012). Certos países, como Índia, Taiwan, China e Bangladesh, tem enfrentado questões graves com a contaminação das suas fontes de água potável pelo arsênio (IARC, 2004). No Brasil, Figueiredo et al. (2007) constataram grande quantidade deste elemento químico liberada para o ambiente devido a mineração de ouro, refino de metais, intemperismo de rochas e associação deste composto ao minério de manganês lavrado nas regiões do Quadrilátero Ferrífero (Minas Gerais), Vale do Ribeira (Paraná/São Paulo) e Santana (Amapá).

A maioria dos mamíferos metaboliza o arsênio inorgânico absorvido via metilação para ácido metilarsônico (MMA) e dimetilarsínico (DMA). A metilação ocorre pela redução do arsênio pentavalente para trivalente e adição do grupo metil (Vahter, 2002;

Naranmandura et al., 2007). Estudos indicam que o fígado é o principal sítio de metilação do arsênio, especialmente após a ingestão, quando o mesmo passa pela circulação hepática. Porém, a atividade de metilação tem sido identificada também em outros órgãos como nos testículos, rins e pulmão (Vahter, 2002). A metilação torna as formas de arsênio inorgânicas menos reativas aos tecidos, facilitando o processo de eliminação por excreção renal (Barra et al., 2000). No entanto, mesmo ocorrendo a metilação, alguns metabólitos do arsênio ainda podem ser depositados nos tecidos (Celik et al., 2008).

É conhecido, que exposições agudas e crônicas ao arsênio causam diversos problemas à saúde humana. Efeitos da exposição aguda incluem náusea, vômito, dor abdominal, encefalopatia e neuropatia (Reddy et al., 2011). Já a exposição crônica pode levar ao surgimento de vários tipos de câncer, doenças cardiovasculares, desordens neurológicas e hepáticas (Ratnaike, 2003; Chen et al., 2009).

O fígado é o maior órgão interno do corpo e desempenha papel crucial na detoxificação do organismo. Ele estoca certas vitaminas e carboidratos digeridos na forma de glicogênio, o que, por sua vez, é quebrado liberando glicose para manter os níveis de açúcar no sangue. Além disso, o fígado é o local de síntese de enzimas, colesterol, bile, vitamina A e outras moléculas necessárias para a homeostase (Ross e Pawlina, 2016). Como o fígado é o principal sítio de metilação do arsênio, exposições repetidas a este metal podem causar acumulação de formas de arsênio no órgão, aumentando a toxicidade hepática (Ratnaike, 2003). Em populações expostas ao arsênio na Índia, elevados níveis de fosfatase alcalina, alanina aminotransferase, aspartato aminotransferase e bilirrubina foram encontrados associado ao aumento do tamanho do fígado nos indivíduos expostos (Mazumder et al., 1998). Estudos experimentais também mostraram que animais expostos a diferentes compostos de arsênio apresentaram alteração das transaminases, desequilíbrio das enzimas antioxidantes e microminerais no fígado, além de diversas patologias no parênquima hepático (Lu et al., 2014; Kharroubi et al., 2014; Souza et al., 2017). Além disso, elevados níveis de espécies reativas de oxigênio (EROs) e produtos de peroxidacão lipídica tem sido observado no fígado de animais expostos ao arsênio (Kharroubi et al., 2014).

Trabalhos com modelos animais também tem demonstrado que a exposição ao arsênio é capaz de afetar o aparelho reprodutor masculino, gerando diminuição da capacidade reprodutiva, inibição da espermatogênese (Pant et al., 2001, Jana et al., 2006) e alterações atróficas do testículo (Ahmad et al., 2008). Ratos expostos ao arsenito e arsenato de sódio na água de beber apresentaram alterações no número de espermatozoides no testículo e epidídimo, na produção diária espermática, nas enzimas

antioxidantes testiculares, além de patologias no testículo (Souza et al., 2016a,b). Camundongos expostos ao arsenato de sódio na água de beber também apresentaram redução na motilidade espermática e em parâmetros de morfologia e integridade destas células (Guvvala et al., 2016). Além disso, foi observado atrofia das células de Leydig (Sanghamitra et al., 2008) e consequente redução na concentração sérica de andrógenos em animais expostos aos compostos de arsênio (Morakinyo et al., 2010), assim como acúmulo de arsênio nos testículos e órgãos sexuais acessórios (Pant et al., 2004).

Estudos sugerem que a toxicidade resultante da exposição crônica ao arsênio está associada com o aumento na formação de EROs e, consequente, estresse oxidativo (Neves et al., 2004; Chang et al., 2007). O arsênio induz mudanças morfológicas na integridade mitocondrial e um rápido declínio do potencial de membrana da mitocôndria. Estas alterações são consideradas o sítio primário de formação de radicais superóxido (Jomova et al., 2011). Em geral, para superar o ataque das EROs, as células animais são equipadas com um sistema de defesa antioxidante intrínseco, sendo a catalase, a superóxido dismutase e a glutationa as principais enzimas envolvidas neste processo. Assim, uma falha no sistema de neutralização das EROs prejudica as células e organelas onde são geradas, podendo acarretar variadas patologias nos tecidos (Reddy et al., 2011).

Recentemente, a exposição ao arsênio também tem sido associada ao desenvolvimento do diabetes (Del Razo et al., 2011; Wang et al., 2014). Sabe-se que o arsenato, a forma pentavalente do arsênio, é capaz de substituir o fosfato em reações bioquímicas no corpo, induzindo alterações no metabolismo da glicose e secreção de insulina e, consequentemente, o desenvolvimento do diabetes (Gonzalez et al., 1995). No entanto, o mecanismo exato de como isto ocorre ainda é pouco entendido.

O diabetes mellitus é uma doença metabólica crônica que afeta cerca de 346 milhões de pessoas no mundo, sendo, portanto, um dos maiores problemas de saúde pública da atualidade (Danaei et al., 2011). Ela é caracterizada por hiperglicemia, que acontece quando as células beta pancreáticas são incapazes de produzir insulina (diabetes tipo 1) ou quando os tecidos apresentam resistência à insulina (diabetes tipo 2) (Zimmet et al., 2001). Os principais sintomas do diabetes são polidipsia, polifagia, poliúria, perda de peso, fadiga e perda de visão. Na maioria dos casos, os sintomas são leves ou podem até estar ausentes. No entanto, a hiperglicemia é suficiente para causar alterações patológicas e funcionais nos tecidos a curto e longo prazo (ADA, 2011).

A diabetes do tipo 1 tem sido amplamente estudada em animais por meio da indução por estreptozotocina (STZ), um antibiótico que promove a destruição das células beta pancreáticas reduzindo a secreção de insulina e, consequentemente, gerando

hiperglicemia (Wu e Huan, 2008). Diversos danos têm sido observados no fígado de animais diabéticos induzidos por STZ. Dentre eles é possível ressaltar o aumento nos níveis de transaminases e peroxidação lipídica, o desbalanço de enzimas antioxidantes e alterações patológicas, tais como congestão e fibrose (Elbe et al., 2015; Oliveira et al., 2016; Hosseini et al., 2017). A redução no peso corporal e do órgão também tem sido relatada com frequência nestes animais, assim como o aumento no peso relativo do órgão, indicando edema ou fibrose (Husin et al., 2017). Estudos epidemiológicos também têm indicado aumento na peroxidacão lipídica e alteração nas enzimas antioxidantes no fígado de indivíduos diabéticos, assim como danos patológicos (Kumawat et al., 2013).

A diabetes também pode afetar a função reprodutiva masculina em vários níveis, podendo prejudicar o controle endócrino da espermatogênese, a própria espermatogênese, a ereção peniana e a ejaculação (Sexton e Jarow, 1997). A doença é reconhecida como uma das causas da disfunção sexual masculina, o que contribui para a subfertilidade. Diversos estudos epidemiológicos têm relatado redução do potencial reprodutivo em homens diabéticos (Echavarria et al., 2007; Delfino et al., 2007). Dados de estudos com animais diabéticos induzidos por STZ também sugerem comprometimento da fertilidade masculina nestes animais, com acentuada redução da fecundidade (Frenkel et al., 1978; Ballester et al., 2004; Scarano et al., 2006), e da qualidade dos espermatozoides (Amaral et al., 2006; Scarano et al., 2006). Estudos com diabetes experimental também apontam uma redução nos níveis de testosterona circulante e no peso dos órgãos reprodutivos (Scarano et al., 2006; Bal et al., 2011), assim como um desequilíbrio na atividade das enzimas antioxidantes e danos patológicos nestes órgãos (Shrilatha e Muralidhara, 2007; Bal et al., 2011, Gobbo et al., 2015).

Portanto, está bem documentado que tanto o diabetes (fator metabólico) quanto a exposição ao arsênio (fator ambiental) podem causar alterações sistêmicas, comprometendo a função e a estrutura dos órgãos. No entanto, não está claro a extensão na qual a exposição ao arsênio na presença de uma doença metabólica pré-existente poderia impactar no organismo. Assim, o objetivo desta tese foi analisar os efeitos do arsênio em parâmetros hepáticos e reprodutivos de ratos Wistar saudáveis e diabéticos. No artigo 1 (*Arsenic exposure: disruption of liver morphology and function in healthy and diabetic rats*) investigamos os efeitos da exposição oral ao arsenato em parâmetros funcionais, enzimáticos e morfológicos do tecido hepático de ratos Wistar saudáveis e diabéticos. Já no artigo 2 (*Arsenate impairs testis functions via nitrosative stress and reduced testosterone levels in healthy and diabetic rats*) avaliamos o efeito deste composto de arsênio em parâmetros reprodutivos de ratos Wistar saudáveis e diabéticos,

focando nos marcadores funcionais do testículo e epidídimo, no balanço das enzimas antioxidantes, no conteúdo mineral e na estrutura morfológica dos órgãos.

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ARTIGO 1

Arsenic exposure: disruption of liver morphology and function in healthy and diabetic rats

*Submetido na revista Oxidative Medicine and Cellular Longevity

Arsenic exposure: disruption of liver morphology and function in healthy and diabetic rats

Abstract Studies have shown that exposure to either environmental toxicants or hyperglycemia may cause hepatic injuries. However, it is unclear the extent to which their combined exposure may negatively influence liver functions. Therefore, we aimed to evaluate morphological and functional hepatic parameters in healthy and diabetic rats exposed to arsenic. Diabetes was induced in male rats by intraperitoneal streptozotocin injection. Healthy and diabetic animals received saline solution (negative control; diabetes control) and 10 mg/L arsenic as sodium arsenate (arsenic control; diabetes + arsenic) for 40 days in drinking water *ad libitum*. The antioxidant enzymes activity was suppressed after arsenate exposure in healthy animals compared to negative controls, whereas diabetes and arsenate combination worsened the reduction of GST enzyme. Consequently, arsenate-exposed animals showed increased malondialdehyde and carbonyl protein levels, being this increase worsened in animals with pre-existent diabetes. Moreover, these same animals had increased ALP serum levels compared to diabetes controls. Arsenate exposure caused an inflammatory process in the liver tissue from healthy rats compared to negative controls. In the same way, exposure to arsenate enhanced the increase of inflammatory infiltrate in diabetic animals, with higher number of mast cells and TNF-α production. In addition, vascular alterations such as congestion, bleeding, and hemosiderin deposition were intensified in diabetes + arsenic animals, whereas glycogen storage was reduced. We concluded that exposure to arsenate was able to cause intense morphological and functional damages to the liver tissue of healthy animals. Importantly, these damages were intensified by arsenate exposure in livers previously injured by diabetes. **Keywords:** arsenate; diabetes; oxidative stress; lipid peroxidation; inflammation; toxicological pathology

1. Introduction

Type 1 diabetes mellitus is generally related to destruction of insulin-producing β-cells by the immune system. It leads to islet dysfunction, with an inadequate production of insulin to lower blood sugar levels, resulting in hyperglycemia and a series of metabolic disorders in the body [1]. In this context, liver is known as an insulin-sensitive tissue primarily susceptible to diabetes effects [2]. Type 1 diabetes can be induced by streptozotocin (STZ), an antibiotic agent that destroys pancreatic β-cells and causes diabetes symptoms [3]. In studies using STZ-induced diabetes several damages were

observed in the liver, such as increase in transaminases levels and lipid peroxidation, antioxidant enzymes imbalance, and pathological alterations [4, 5].

In addition to the large number of studies evaluating the pathogenesis of diabetes, there is a recent interest in understanding how nontraditional risk factors, including environmental toxicants, might contribute to its development [6]. Among the environmental toxicants, arsenic has received great attention [7]. Arsenic is a metalloid widely spread in the environment from natural and anthropogenic sources [8]. Human exposure to inorganic arsenic compounds occurs mainly by drinking water, especially in its pentavalent forms (iAs^V; arsenate) [9]. Arsenic exposure may lead to hepatic disorders that compromises body's detoxification, such as elevation in hepatic enzymes levels, antioxidant status imbalance, and several pathologies on liver parenchyma [10, 11]. Moreover, epidemiological studies in populations exposed to arsenic in drinking water showed that this metalloid induces hepatic damages and increases the risk of developing diabetes [12, 13]. The latter consequence might be related to the effects of arsenic in gluconeogenesis and insulin secretion [14]. Arsenate, for example, can replace phosphate in biochemical reactions in the glucose metabolism due to their similar structure and properties [15].

Although the damages induced by either metabolic disorders or environmental toxicants are extensively studied, it is unclear the extent to which arsenic exposure in the presence of pre-existent diabetes could interfere in liver functions. Since polydipsia is an important symptom of diabetes [1] and drinking water is the main source of arsenic contamination [9], diabetic individuals living in arsenic exposed areas might be suffering extensive health damages, posing a serious problem for public health. In this framework, we aimed to evaluate the effect of arsenic exposure in the liver of healthy and diabetic rats. To that end, diabetes was induced by a single STZ injection, which is a well-characterized model of type 1 diabetes. Indeed, liver is the primary site for detoxification and has crucial role in carbohydrate and lipid metabolism. Thus, we focused on its functional markers, antioxidant enzymes balance, arsenic accumulation, and histopathological parameters.

2. Material and Methods

2.1. Animals and ethics statement. Thirty-two male Wistar rats (230-250 g), 70 days old, were provided by the Central Animal Laboratory of the Center of Biosciences and Health of the Federal University of Viçosa. The animals were housed individually in polypropylene cages and maintained under controlled temperature (21 ºC), relative

humidity of 60-70%, and 12-12 h light/dark cycle. They had free access to rat chow and drinking water, which was used for treatment (oral route). The study was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa, Brazil (protocol 61/2016) and was conducted in accordance to the ethical guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

2.2. Experimental design. After 12 h fasting, sixteen rats were randomly selected and diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (Sigma Chemical Co., St Louis, MO, USA) at a concentration of 60 mg/kg body weight (BW) in 0.01 M sodium citrate buffer, pH 4.5 [16]. Blood glucose levels were evaluated 7 days after STZ injection using a glucometer (Acon Laboratories, Inc., San Diego, CA, USA) in samples obtained from the tail vein. Animals having a blood glucose level higher than 250 mg/dL were included in this study. Hyperglycemic rats were randomly divided into two groups (n = 8, each). While animals from diabetes control group received saline solution (0.9 % NaCl), the other group was exposed to 10 mg/L arsenic as sodium arsenate ($Na_2HAsO_4.7H_2O$; Sigma Aldrich Co., St. Louis, MO) in the drinking water *ad libitum* for 40 days (diabetes + arsenic group). In the same way, a set of healthy animals (n = 8) was exposed to 10 mg/L arsenic as sodium arsenate solution *ad libitum* for 40 days (arsenic control group), whereas animals receiving neither sodium arsenate nor STZ (n = 8) were designed as negative control group. Body weight and daily water consumption were assessed in all groups, and clinical signs were observed. During the experimental period, fasting blood glucose was evaluated weekly using test strips and glucometer (Acon Laboratories, Inc., San Diego, CA, USA) in blood samples from the tail vein.

A total of 41.64 mg of arsenate ($Na_2HAsO_4.7H_2O$) was dissolved in 1 L distilled water in order to obtain a total of 10 mg/L arsenic. As previous studies showed that rodents might be less susceptible than human to arsenic toxicity, due to a faster arsenic metabolism and excretion [17], we selected an arsenic concentration higher than that found in most contaminated areas similarly to other studies [10, 11, 18]. Once the biological half-time of inorganic arsenic is about 4 days [19, 20], the exposure time selected herein corresponded to 10 times of arsenic half-time in the body. Saline solution was given to negative and diabetes controls to keep the taste of drinking water similar to arsenate solution.

2.3. Euthanasia, tissue collection and biometric analysis. On the 41th day of experiment, animals were weighed and euthanized by deep anesthesia (ketamine 150 mg/kg i.p. and

xylazine 10 mg/kg i.p.) followed by cardiac puncture. Then, the livers were removed, weighed, and four fragments were quickly removed from them. While two fragments were frozen in liquid nitrogen and stored at -80 ˚C for enzymatic and cytokine assays, the others were immersed in Karnovsky`s fixative solution for 24 h for histopathological analysis and arsenic determination. The remaining liver tissue was used to evaluate the water content per unit weight of tissue (mL/g). For this purpose, liver tissue was dried at 60 ˚C for 96 h, and oedema index was calculated by the difference between wet and dry liver weight [21]. Liver somatic index (LSI, %) was assessed by normalizing the liver weight by the final body weight [22].

2.4. Arsenic determination in the liver. Arsenic proportion in hepatic tissue was assessed by Energy Dispersive X-ray Spectroscopy using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with a x-ray detector system (Tracor TN5502, Middleton, WI, USA) according to Novaes et al. [23]. For that, fragments were dehydrated in ethanol series, submitted to critical point drying (CPD 030, Bal-tec, Witten, North Rhine-Westphalia, Germany), and coated with evaporated carbon (Quorum Q150 T, East Grinstead, West Sussex, England, UK). The analysis was performed at a magnification of 150x using an accelerating voltage of 20 kV and a working distance of 10 mm.

2.5. Functional markers of hepatic damage. Blood samples were taken by cardiac puncture and centrifuged at 2,000 x g for 15 min. Serum was immediately used for the assessment of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) using biochemical kits (Bioclin Laboratories, Belo Horizonte, MG, Brazil) suitable for the BS-200 equipment (Bioclin Laboratories, Belo Horizonte, MG, Brazil) in accordance with the manufacturer`s instructions.

2.6. Oxidative stress markers in the liver. For the analysis of antioxidant enzymes, 100 mg of frozen liver was homogenized in ice-cold phosphate buffer saline (PBS) and centrifuged at 3,500 x g (5 ºC) for 10 min. The supernatant was used for the analysis of superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) activities. SOD activity was estimated by the pyrogallol method based on the ability of this enzyme to catalyze the reaction of superoxide (O^{-2}) and hydrogen peroxide (H_2O_2) [24]. CAT activity was evaluated by measuring the rate of H_2O_2 decomposition according to Aebi [25]. Further, GST activity was estimated spectrophotometrically as described by

Habig et al. [26], and calculated from the rate of NADPH oxidation. Lipid peroxidation was assessed in hepatic tissue analyzing tissue levels of malondialdehyde (MDA). For this, aliquots of the liver (100 mg) were homogenized in PBS and incubated with thiobarbituric acid to evaluate the levels of thiobarbituric acid-reactive substances [27]. Protein oxidation was also analyzed by the quantification of protein carbonyls in liver tissue pellets using the 2.4-dinitrophenylhydrazine (DNPH) method [28]. The total protein level in liver tissue was measured using the Bradford method [29].

2.7. Histopathological and stereological analysis of the liver. After 24 h in Karnovsky`s fixative solution, liver fragments were dehydrated in crescent ethanol series, cleared in xylene and embedded in paraffin. Sections with a thickness of 5 μm were obtained using a rotary microtome (Multicut 2045, Reichert-Jung, Germany) and stained with hematoxylin/eosin (HE) for histopathological and stereological analysis, the best carmine method for glycogen, and toluidine blue for mast cells. To avoid repetitive analysis of the same histological area, sections were evaluated in semi-series using one in every 10 sections. Digital images of hepatic tissue were obtained using a light microscope (Olympus BX-53, Tokyo, Japan) connected to a digital camera (Olympus DP73, Tokyo, Japan). Eighty photomicrographs from HE and carmine method (20x objective) were randomly obtained for each group. For stereological analysis, a test system of 266 points was used in a standard test area. In sections stained with HE points were recorded in liver components (cytoplasm and nucleus of hepatocytes, sinusoidal capillaries, blood vessels and macrophages), inflammatory infiltrate, congestion, bleeding, and hemosiderin deposition. In sections stained with carmine method, points were recorded in glycogen-containing cytoplasmic inclusions. The volume of each component was calculated using the following formula: V = PP/PT x Vo, where PP is the number of points located on the interest structure, PT is the total number of points in the histological area, and Vo is the organ volume [30]. All stereological analyses were performed using ImageJ software (National Institutes of Health). For analysis of mast cells, liver sections stained with toluidine blue were evaluated under a light microscope CX40® (Olympus, Tokyo, Japan) at a magnification of 400x. Ten histological fields were randomly analyzed in each histological section obtaining a total area (AT) of 1.96 mm^2. The number of mast cells per unit histological area was assessed according to the relation QA=Σ mast cells/AT [30].

2.8. Cytokines levels in the hepatic tissue. Fragments of liver tissue previously stored at -80 ˚C were homogenized in ice-cold PBS and centrifuged at 3,500 x g for 10 min at 5 ºC.

The supernatant was collected for cytokine assay. The tumor necrosis factor alfa (TNF-α) and interleukin (IL) 10 were measured using Rat TNF Flex Set and Rat IL-10 Flex Set, respectively (BD Biosciences, San Diego, CA, USA). The data were assessed using the FACS verse flow cytometry (BD Biosciences, San Diego, CA, USA), and analyzed with the FCAP 3.0 software. All cytokines were measured following manufacturer`s instructions.

2.9. Statistical analysis. All the variables analyzed herein were compared between groups using Student's T-test. For validate the liver injuries caused by STZ-induced diabetes, we run a Student's T-test comparing the results from negative control and diabetes control groups. Moreover, we performed two separate Student's T-tests in order to compare the arsenic exposure in the liver of healthy and diabetic animals. The first one comparing data from negative control and arsenic control groups, while the other comparing diabetes control and diabetes + arsenic groups. Differences were considered significant when $P < 0.05$. All tests and graphics were performed using the GraphPad Prism 6.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as mean ± standard error mean (SEM).

3. Results

3.1. Validation of liver injuries caused by STZ-induced diabetes. All the variables of biometric and stereological parameters, hepatic function and oxidative stress markers, as well as cytokine levels significantly changed between negative control animals and STZ-induced diabetic rats. It shows that STZ indeed induced liver injuries in our experimental rats (Tables 1 and 2; Figures 1-5).

3.2. Arsenate exposure altered biometric parameters mainly in diabetic animals. Differently to healthy animals, animals from both diabetic groups presented clinical symptoms including polydipsia, polyuria, loss of weight, and fatigue. Healthy animals receiving arsenate presented higher blood glucose levels than negative control animals ($P < 0.05$; Table 1). In contrast, this variable did not differ between animals from diabetes control and diabetes + arsenic groups ($P > 0.05$; Table 1). In general, diabetic animals consumed more arsenic solution (182.13 ± 4.19 mL) than healthy animals (48.38 ± 4.92 mL) during the experimental period. Based on the average daily solutions intake, animals from arsenic control group ingested 2.42 mg As/kg BW/day, whereas animals from diabetes + arsenic group ingested and 9.10 mg As/kg BW/day. The proportion of arsenic

in the liver was higher in these both groups ($P < 0.05$), being the highest proportion found in diabetic animals exposed to arsenate (Table 1). At the end of the experiment, the final body weight and liver weight in animals from diabetes + arsenic group increased in relation to diabetes control group ($P < 0.05$; Table 1). Those parameters, in turn, did not differ between animals from negative control and arsenic control groups ($P > 0.05$; Table 1). Exposure to arsenate in healthy or diabetic animals did not change the LSI and oedema index ($P > 0.05$; Table 1).

TABLE 1: Blood glucose levels, arsenic proportion, and biometric parameters of healthy and diabetic rats exposed to sodium arsenate in drinking water *ad libitum*.

	Negative control	Arsenic control	Diabetes control	Diabetes + arsenic
Glucose (mg/dL)	56.60 ± 2.49	70.60 ± 2.44*	474.50 ± 32.72†	503.40 ± 40.42
Arsenic proportion (x fold)	1.0	2.6*	1.0	3.3$^{\#}$
Initial body weight (g)	234.30 ± 7.45	242.20 ± 4.52	236.30 ± 2.44	233.10 ± 2.16
Final body weight (g)	350.10 ± 9.16	367.50 ± 7.56	179.90 ± 4.06†	206.00 ± 5.99$^{\#}$
Weight variation (g)	115.80 ± 4.18	125.30 ± 4.53	-56.67 ± 3.39†	-23.73 ± 6.08$^{\#}$
Liver weight (g)	13.78 ± 0.47	14.44 ± 0.47	8.89 ± 0.21†	10.53 ± 0.35$^{\#}$
LSI (%)	3.94 ± 0.09	3.91 ± 0.11	4.86 ± 0.13†	5.05 ± 0.19
Oedema (mL/g)	0.64 ± 0.006	0.65 ± 0.008	0.64 ± 0.004	0.64 ± 0.004

Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. $^{\dagger}$means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. $^{\#}$ means significant difference ($p < 0.05$) between diabetic groups by student`s t-test. LSI: liver somatic index. N = 8 animals/group.

3.3. Impact of arsenate ingestion on liver biochemical markers varied between healthy and diabetic rats. Healthy rats exposed to arsenate showed higher serum levels of AST and ALP when compared to negative controls ($P < 0.05$; Figure 1). On the other hand, arsenate exposure enhanced the increase of ALP serum levels in diabetic animals ($P < 0.05$), whereas AST serum levels remained unchanged in these animals ($P > 0.05$; Figure 1). Further, ALT serum levels were not altered after arsenic exposure ($P > 0.05$; Figure 1).

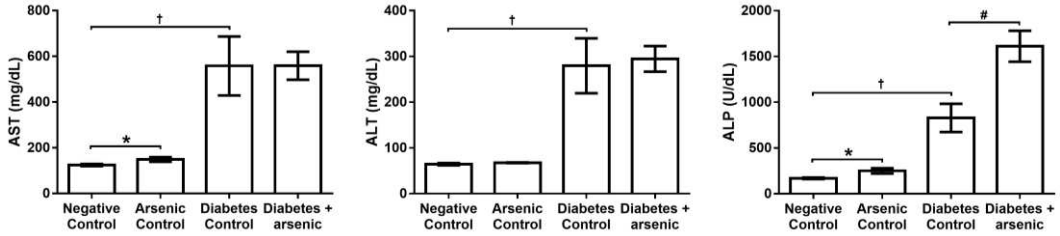

FIGURE 1: Serum levels of hepatic enzymes in healthy and diabetic rats exposed to sodium arsenate in drinking water *ad libitum*. AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase. Negative control: saline solution; Arsenic control: 10 mg/L sodium arsenate; Diabetes control: STZ; Diabetes + arsenic: STZ + 10 mg/L sodium arsenate. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. † means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. #means significant difference ($p < 0.05$) between diabetic groups by student`s t-test. (n = 8 animals/group).

3.4. Arsenate exposure decreases antioxidant defenses enhancing oxidative stress markers. The activities of SOD, CAT and GST decreased in healthy animals exposed to arsenate compared to negative controls ($P < 0.05$; Figure 2). However, when diabetic animals were exposed to arsenate, only GST activity was reduced compared to diabetes control animals ($P < 0.05$; Figure 2). Furthermore, the levels of MDA and carbonyl protein increased in healthy and diabetic rats exposed to arsenate than negative control and diabetes control animals, respectively ($P < 0.05$; Figure 2).

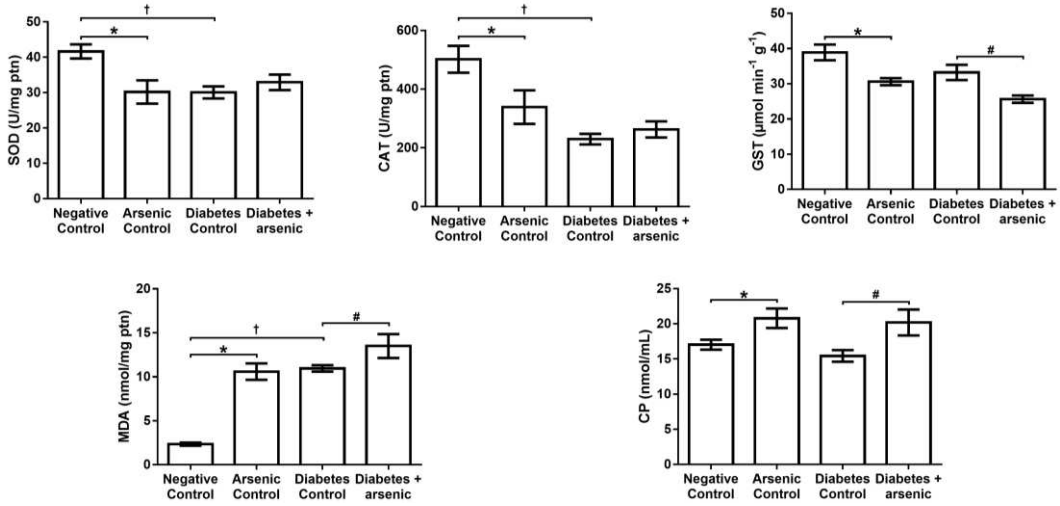

FIGURE 2: Oxidative stress markers in the liver tissue of healthy and diabetic rats exposed to sodium arsenate in drinking water *ad libitum*. SOD: superoxide dismutase; CAT: catalase; GST: glutathione S-transferase; MDA: malondialdehyde; CP: carbonyl protein. Negative control: saline solution; Arsenic control: 10 mg/L sodium arsenate; Diabetes control: STZ; Diabetes + arsenic: STZ + 10 mg/L sodium arsenate. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. † means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. #means significant difference ($p < 0.05$) between diabetic groups by student`s t-test. (n = 8 animals/group).

3.5. Exposure to arsenate intensifies tissue damages in the liver of diabetic rats. Animals from the negative control group showed normal histological appearance of liver parenchyma (Figure 3). In contrast, the liver tissue of diabetes control animals and both groups exposed to arsenate presented several histological changes, such as inflammatory infiltrate, congestion, bleeding, and iron deposition (hemosiderin) (Figure 3). The volume of inflammatory focus, congestion, bleeding, and hemosiderin (Figure 3), as well as the number of mast cells (Figure 4) were increased in the liver tissue of healthy animals exposed to arsenate ($P < 0.05$). Moreover, exposure to arsenate intensified the increase in the volume of these pathologies in diabetic animals ($P < 0.05$). Inversely, there was a reduction in the volume of glycogen-containing cytoplasmic inclusions in the liver of healthy and diabetic animals exposed to arsenate, compared to negative control and diabetes control groups, respectively ($P < 0.05$; Figure 4).

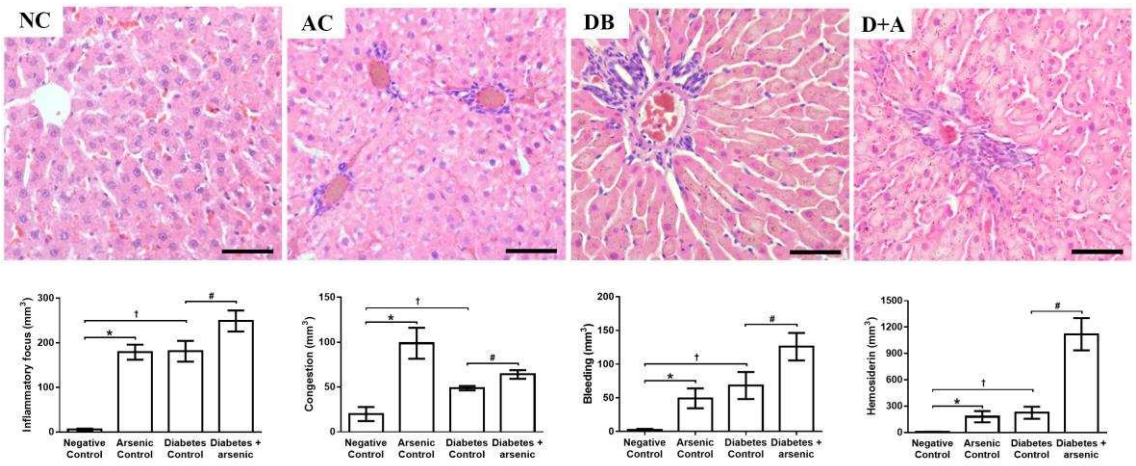

FIGURE 3: Representative photomicrographs and stereological parameters of liver pathologies in healthy and diabetic rats exposed to sodium arsenate in drinking water *ad libitum* (H&E staining, bar = 50 µm). Note in AC, DC and D+A intense damage of liver tissue with inflammatory infiltrate, congestion, bleeding, and hemosiderin deposition. NC: negative control; AC: Arsenic control (10 mg/L sodium arsenate); DC: Diabetes control (STZ-induced diabetic rats); D+A: Diabetes + arsenic (STZ-induced diabetic rats + 10 mg/L sodium arsenate). The graphs show the results of stereological analysis. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. † means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. #means significant difference ($p < 0.05$) between diabetic groups by student`s t-test. ($n = 8$ animals/group).

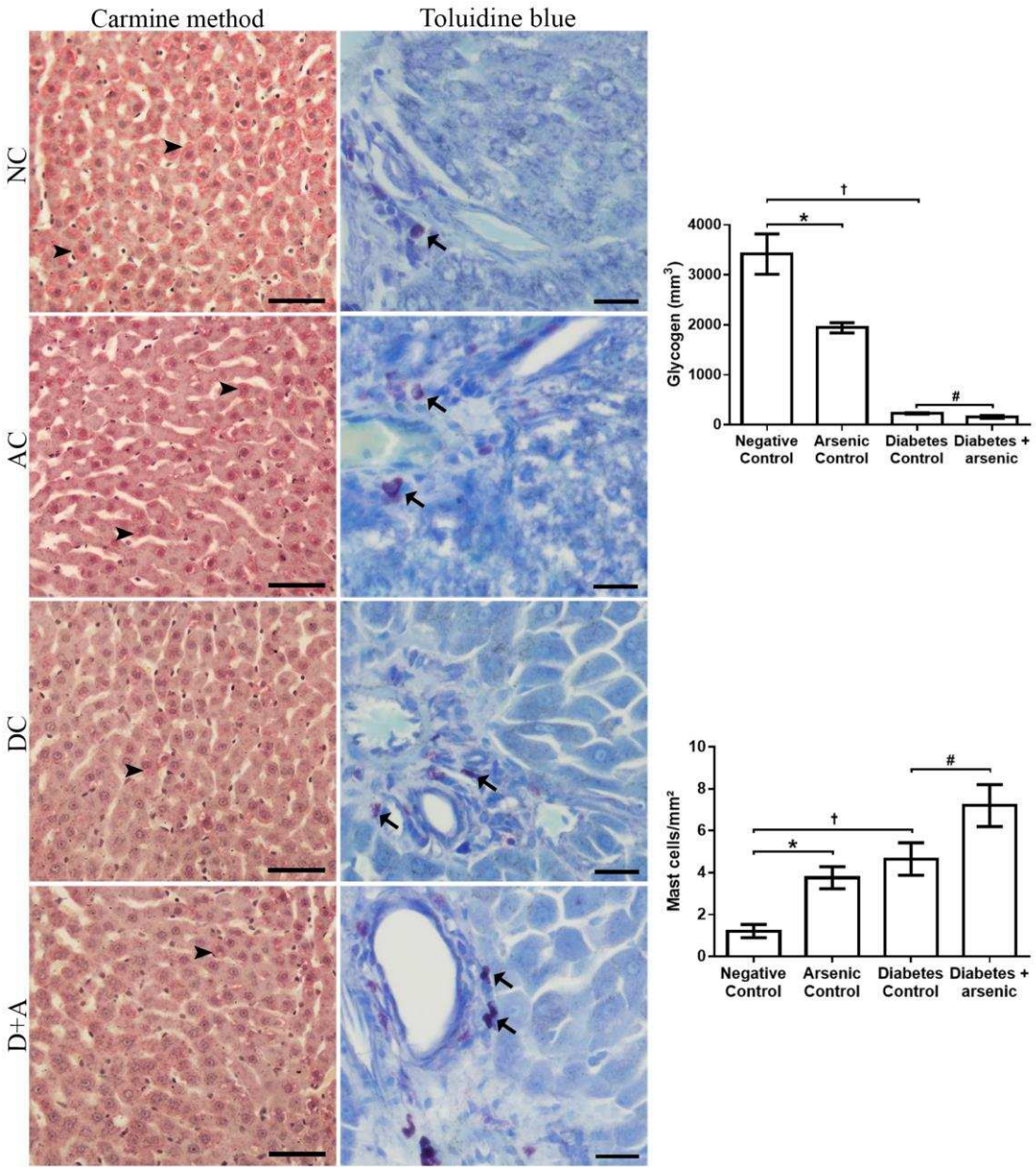

FIGURE 4: Glycogen deposition and mast cell number in the liver of healthy and diabetic rats exposed to sodium arsenate in drinking water *ad libitum*. Cytoplasmic inclusions of glycogen are indicated by arrowheads (Carmine method, bar = 50 µm). Mast cells are indicated by arrows (Toluidine blue staining, bar = 20 µm). NC: negative control; AC: Arsenic control (10 mg/L sodium arsenate); DC: Diabetes control (STZ-induced diabetic rats); D+A: Diabetes + arsenic (STZ-induced diabetic rats + 10 mg/L sodium arsenate). The graphs show the results of stereological analysis. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. † means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. #means significant difference ($p < 0.05$) between diabetic groups by student`s t-test. (n = 8 animals/group).

Stereological analysis also showed differences in liver components between the groups evaluated (Table 2). The volume of hepatocyte nucleus decreased in healthy animals exposed to arsenate compared to negative control ($P < 0.05$). However, arsenate exposure did not worsened the reduction observed in this parameter in diabetic animals ($P > 0.05$; Table 2). On the other hand, the liver of rats from diabetes + arsenic group

presented higher volume of blood vessels and macrophages compared to diabetes control animals ($P < 0.05$). The latter two parameters also increased in healthy rats exposed to arsenate compared to negative controls ($P < 0.05$). Finally, the volume of sinusoidal capillaries was increased in diabetes + arsenic group when compared to diabetes control group ($P < 0.05$).

TABLE 2: Stereological parameters (mm^3) of the liver tissue from healthy and diabetic rats exposed to sodium arsenate in drinking water *ad libitum*.

	Negative control	Arsenic control	Diabetes control	Diabetes + arsenic
V Cytoplasm	7913.41 ± 363.32	8491.14 ± 354.58	5979.28 ± 109.23†	6155.56 ± 268.82
V Nucleus	1983.09 ± 156.51	1493.52 ± 56.01*	1088.32 ± 17.67†	1112.25 ± 38.60
V Hepatocyte	9896.50 ± 511.94	9984.66 ± 397.88	7067.60 ± 112.49†	7267.81 ± 305.17
V Sinusoidal capillaries	3907.75 ± 333.22	3744.66 ± 158.55	2709.96 ± 50.56†	3061.55 ± 130.55$^{\#}$
V Blood vessel	73.98 ± 8.82	208.75 ± 19.70*	146.7 ± 1.70†	192.00 ± 22.05$^{\#}$
V Macrophages	92.79 ± 11.18	156.18 ± 7.00*	123.23 ± 6.56†	150.02 ± 10.97$^{\#}$

Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. $^{\dagger}$means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. $^{\#}$ means significant difference ($p < 0.05$) between diabetic groups by student`s t-test. *V*: volume. N = 8 animals/group.

3.6. Arsenate exposure enhances pro-inflammatory cytokine levels. The levels of TNF-α increased in the hepatic tissue of healthy and diabetic animals exposed to arsenate compared to negative control and diabetes control groups, respectively ($P < 0.05$; Figure 5). However, there was no change in the levels of IL-10 in the groups analyzed ($P > 0.05$; Figure 5).

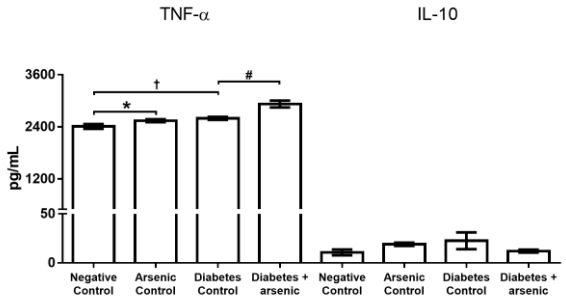

FIGURE 5: Hepatic levels of cytokines in healthy and diabetic rats exposed to sodium arsenate in drinking water *ad libitum*. TNF-α: tumor necrosis factor alfa; IL-10: interleukin 10. Negative control: saline solution; Arsenic control: 10 mg/L sodium arsenate; Diabetes control: STZ; Diabetes + arsenic: STZ + 10 mg/L sodium arsenate. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. $^{\dagger}$ means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. $^{\#}$means significant difference ($p < 0.05$) between diabetic groups by student`s t-test. (n = 8 animals/group).

4. Discussion

In the current study, we evaluate the effect of arsenic, an adverse environmental factor, in the liver of healthy and diabetic animals. Our results provide first evidences for the impact of this metalloid in the rat liver previously injured by a pre-existent metabolic disturbance. In fact, arsenate was able to cause morphological and functional disruption in the liver of healthy rats. Interestingly, the exposure to this arsenic compound in diabetic animals intensified an imbalance in reactive oxygen species (ROS) production due to the reduction in GST activity causing damages to macromolecules and exacerbation of the hepatic injury and inflammation.

Herein, the high level of arsenic observed in the liver of diabetic + arsenic animals may have been a consequence of their high water intake and differential arsenic metabolism. Liu et al. [14] also observed elevated water intake and arsenic deposition in the liver of diabetic mice compared to normal mice. Indeed, the water intake is greater in diabetic animals due to their high blood glucose levels, which increase the risk of dehydration in consequence of metabolic disorders [31]. The water intake in great volumes causes polyuria, which is one of the most classical symptoms in diabetic animals [1]. However, the idea that polyuria could enhances arsenic excretion is not exactly right. It is known that metabolism and excretion of arsenic in diabetic animals is different than in normal animals [14, 32]. Arsenic metabolism in these animals is characterized by higher arsenic methylation [14], which might produce monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}) as well as monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) [33]. However, the concentration of these arsenic metabolites is lower in urine and feces of diabetic animals [14] indicating that these animals retain arsenic metabolites in the body. Thus, the higher water intake as well as the differential arsenic metabolism and excretion in diabetic animals may have contributed to arsenic bioaccumulation in the liver of these animals.

In the present study, exposure to arsenate was able to raise blood glucose levels in arsenic control animals, but it was unable to intensify the increased blood glucose levels of diabetic rats. Several epidemiologic studies have suggested that chronic environmental exposure to inorganic arsenic increase the risk of developing diabetes [12, 13]. The increasing of glucose levels in healthy rats may be explained in two ways. First, the arsenate can replace phosphate in biochemical reactions in the glucose metabolism due to their similar structure and properties, leading to hyperglycemia [15]; second, arsenic may directly affect pancreatic beta cells by oxidative stress induction [34], promoting reduction of insulin transcription and secretion [35]. The latter, however, cannot be

applied to induce an increase in blood glucose levels in STZ-induced diabetic rats since this antibiotic destroys pancreatic β-cells [3]. Moreover, as diabetic animals have differential arsenic metabolism with higher arsenic methylation [14, 32], it is possible that methylated arsenic forms do not interfere in the glucose metabolism and, therefore, do not enhance the increased blood glucose levels of the animals.

Despite diabetic animals presented the lowest body and liver weights, exposure to arsenic increased those weights. The altered glucose metabolism in diabetic animals compromises cell functions, decreasing ATP synthesis and inducing weight loss [36]. The latter, in addition, can be associated with dehydration, catabolism of fats, increased muscle wasting and loss of tissue protein [3]. On the other hand, the arsenic accumulated in the organ may be inducing an initial deposition of collagen, which, in turn, might increase organs weight. Thus, the increase of liver weight in diabetic animals exposed to arsenate might be related to collagen deposition, since there was no oedema in the organ. Moreover, as arsenic can compete with essential elements and bind to metalloproteins [37], it is possible that this element is participating of metabolic reactions in the body, reducing the electrolytic imbalance caused by hyperglycemia, and, consequently, decreasing weight loss in diabetes + arsenic rats.

Herein, either arsenate exposure or diabetes increased the levels of serum hepatic enzymes indicating hepatocytes damages in the animals. Moreover, the combination of arsenate exposure and diabetes intensified the increase of ALP levels. Traditionally, the determination of hepatic transaminases levels has served as a good indicator of cellular injury [21], since the damage in hepatocyte membranes results in the increase of permeability and enzyme releasing into blood vessels [38]. Elevated levels of hepatic enzymes are usually associated with gluconeogenesis, glycogen content imbalance and amino acid metabolism, which are known characteristics of liver injured in diabetic individuals [14, 38]. The combination of diabetes and arsenic may have intensified the enzymes releasing into the circulation due to the oxidation of fatty acids and proteins in hepatocyte membranes, which is common in both diabetes and arsenic exposure [9, 39].

Indeed, the damage in hepatocyte membranes was probably a consequence of lipid and protein peroxidation on liver, since we observed higher levels of MDA and carbonyl protein in diabetic animals exposed to arsenate. Malondialdehyde and carbonyl protein content are frequently used as markers of lipid and protein oxidation, and serve as indicator of tissue oxidant/antioxidant balance [22]. Studies have shown increased levels of MDA in STZ-induced diabetes animals and arsenic-exposed animals [5, 11]. The mechanism by which arsenic and diabetes drive ROS production is still poorly

understood. However, it is known that both factors generate superoxide ions and hydroxyl radicals in the tissues. Those radicals, in turn, interact with cellular lipids and proteins leading to the chain oxidation of cellular components [9, 39]. These molecular alterations might directly affect antioxidant defense systems of cells promoting morphological alterations in the organs.

In light of the foregoing, it is likely that an oxidative overload of hepatic tissue was caused by an imbalance of antioxidant enzymes activities. Excess ROS production may reduce antioxidant proteins expression, a condition known as enzymatic exhaustion [40]. Herein, we observed reduction of SOD, CAT and GST activities in animals exposed to either hyperglycemia or arsenate. Further, a combination of arsenic and diabetes worsened the reduction observed in GST activity. While SOD is a metalloproteinase involved in the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2, representing the first line of enzymatic defense against ROS, CAT catalyzes the decomposition of H_2O_2 to water and oxygen, protecting the cell from oxidative damages generated by H_2O_2. Finally, GST has scavenging potential, once it catalyzes the conjugation of glutathione with several reactive species [41, 42]. As both diabetes and arsenic exposure might induce ROS production in the body, and GST is the last line of enzymatic defenses, it is possible that the combination of these two factors promoted a higher increase of ROS in the liver, leading to GST exhaustion.

As a consequence of oxidative stress process, pathologies emerged in the liver tissue of diabetes control and arsenic control animals and were enhanced in diabetes + arsenic group. In the latter, particularly, the cellular extravasation, characterized by tissue inflammation with high number of mast cells and macrophages volume, may be related to the vascular disorders observed herein. With the increase of inflammation focus, there was a greater release of TNF-α, a pro-inflammatory cytokine that potentiates the process of tissue injury. It is known that inflammatory cells release chemical mediators such as TNF`s and interleukins to attract more cells to the inflammation foci, stimulating collagen synthesis [43]. Thereby, the increase in inflammatory infiltrate along with collagen production might explain the high LSI index in diabetes groups, with no influence of hepatic edema.

Besides, hemorrhage and vascular congestion were other hepatic alterations highly observed in the experimental groups. While bleeding occurred mainly in diabetic animals exposed to arsenate, congestion was intensified in both groups receiving this metalloid. Overall, inflammatory cells produce mediators that increase endothelial cell permeability and oncotic pressure inducing to plasma exudation and, consequently,

bleeding and congestion [44]. Earlier reports described these two alterations in hepatic tissue of either diabetic animals or animals exposed to arsenic [4, 11, 45]. Herein, the combination of diabetes and arsenic enhanced the pathologies in the tissue probably due to intense oxidative stress in the organ, which might induce reduction in its functions. Furthermore, hemosiderin was identified in the liver, especially in the diabetes + arsenic group, reinforcing our findings of vascular overload and consequent change in hepatic morphology. This excessive deposition of iron is an important marker for vascular changes, which is formed due to high hemocataresis of the red blood cells during vascular congestion and hemorrhage [46].

The combination of arsenic and diabetes also affected glycogen metabolism in the animals, enhancing the reduction in glycogen storage in hepatocytes. Taking into account that in our study we used streptozotocin, a drug widely known to promote destruction of pancreatic islets and consequent reduction in insulin production [3], it is easy to understand the reduced glycogen storage in hepatocytes of diabetic animals. Notwithstanding, animals exposed to arsenate also presented low cytoplasmic glycogen storage in their livers. It is known that arsenate, in its protonate state, has similar properties to phosphate and competes for the same binding sites with a preference for arsenate absorption [15, 47]. This competition results in an imbalance in glycogenolysis, glycolysis, and gluconeogenesis reducing glycogen storage on liver [14]. Therefore, a combination of arsenic and diabetes is able to intensify the reduction of glycogen storage observed when animals are exposed to one factor only.

In addition to liver pathologies, changes in the volume of liver components were found in this study. The reduction observed in cytoplasm, nucleus and hepatocytes are possible related to damages in cell membranes and the increase in transaminases levels caused by either diabetes or arsenic exposure. In cases of prolonged stress, free radicals and ROS induce to cell damage that can promote metabolic deficits in macromolecular synthesis and cell death, reducing cell density in the organ [48]. The combination of arsenic and diabetes did not intensify the reduction in these parameters. However, it was able to increase the volume of sinusoidal capillaries and blood vessels. The increase in the volume of sinusoidal capillaries might be associated with deposition of extracellular matrix components in response to intense oxidative stress generated by arsenic exposure and diabetes [49]. On the other, the increased volume of blood vessels found herein might be associated to the prominence of the central vein, which is a hallmark of hepatic venous hypertension and might be a consequence of sinusoidal congestion [50] intensified in healthy and diabetic animals exposed to arsenate.

In conclusion, our results show that the exposure to arsenate induces oxidative stress and macromolecules damages in the liver of healthy rats. Moreover, its negative effects are intensified in livers previously injured by diabetes. These alterations induce an intense liver inflammation, and impairs liver vital processes, such as detoxification and lipid and carbohydrate metabolism, that are important for body homeostasis. Therefore, additional studies should be undertaken to investigate the possible effects of diabetes and arsenate exposure in molecular pathways and genes expression involved on liver function.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank Bioclin Laboratories for kindly provide biochemical kits used in this work and Núcleo de Microscopia e Microanálise of the Federal University of Viçosa by assistance in flow cytometer and SEM. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Fundação de Amparo à Pesquisa do Estado de Minas Gerais (APQ-00685-14), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (448455/2014-5).

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ARTIGO 2

Arsenate impairs testis functions via nitrosative stress and reduced testosterone levels in healthy and diabetic rats

*Submetido na revista Scientific Reports

Arsenate impairs testis functions via nitrosative stress and reduced testosterone levels in healthy and diabetic rats

Abstract It is know that exposure to either arsenic or diabetes can induces male reproductive damages. However, their combined effects on male reproductive organs are still unclear. Therefore, the present study investigated morphological and functional parameters of the testis and epididymis in healthy and diabetic rats exposed to arsenate. Arsenate exposure reduced serum testosterone concentration in healthy animals and worsened the reduction observed in diabetic rats. The sperm numbers in the testis and epididymis, daily sperm production, and sperm transit time were also reduced in healthy animals exposed to arsenate. Moreover, arsenate exposure worsened these parameters in diabetic rats. Sperm motility, integrity of sperm membranes and the number of normal sperm were also reduced in health animals exposed to arsenate. A combination of diabetes and arsenate just intensified the reduction in the number of normal sperm observed in diabetic animals. The proportion of arsenic increased in the testis and epididymis of both groups receiving arsenate. In addition, exposure to arsenate induced an imbalance in antioxidant enzymes activities and mineral content in healthy animals and enhanced these changes in diabetic rats. Testicular pathologies occurred mainly in animals co-exposed to diabetes and arsenate. Our results indicated that arsenate exposure promoted several damages to male reproductive functions in healthy rats, mainly by impairs testosterone levels and induces nitrosative stress in the organs. Moreover, these damages were intensified when diabetic animals were exposed to this arsenic compound. **Keywords** Arsenic; Diabetes; Epididymis; Testis; Sperm transit time; Reproductive toxicity

Introduction

Arsenic is a sulfhydryl-reactive metalloid widely spread in the environment from natural and anthropogenic sources. The water is considered the main source of arsenic contamination, since natural processes, such as weathering and soil erosion, as well as agricultural and occupational routes are responsible for releasing this element into the ground water[1]. Although arsenic can exist in organic and inorganic forms with different oxidation states, the inorganic arsenic predominates in surface water sources, especially in its pentavalent state (arsenate)[2].

It is estimated that about 100 million people worldwide are exposed to arsenic via drinking water[3]. Epidemiological studies have shown that arsenic exposure induces low sperm quality and erectile dysfunction in men, culminating in infertility[4,5]. Experimental

studies, in turn, have reported a reduction in the testosterone concentration[6,7], sperm parameters[8-10], and alterations in antioxidant enzymes and testis tissue architecture[11]. Recently, arsenic exposure has also been associated to developing diabetes[12,13]. It is known that arsenate, a pentavalent arsenic form, can replace phosphate in biochemical reactions, inducing alterations in glucose metabolism and insulin secretion[14]. However, the etiopathogenesis of this mechanism remains poorly studied.

Diabetes is a chronic metabolic disease that happens when pancreatic beta cells are unable to produce insulin (type 1 diabetes) or when the tissues present insulin resistance (type 2 diabetes)[15]. Diabetes induced by streptozotocin (STZ) is a common model for study of type 1 diabetes. STZ is an antibiotic agent that destroys pancreatic beta cells reducing insulin production, and consequently promoting hyperglycemia[16]. Several damages were observed in male reproductive organs of STZ-induced diabetic animals, such as antioxidant enzymes imbalance, pathological damages, decreased testosterone concentration and sperm quality[17-19]. In the same way, epidemiological studies have reported reproductive impairment in diabetic men[20,21].

Despite the male reproductive impairing is well documented in either diabetic individuals or animals exposed to arsenic, it is unclear the extent to which exposure to arsenic in the presence of pre-existent diabetes could impact male reproductive functions. Considering that polydipsia is an important symptom of diabetes[22] and drinking water is the main source of arsenic contamination[1], diabetic individuals living in arsenic exposed areas might be suffering extensive health damages, posing a serious problem for public health. Therefore, the aim of this study was to evaluate the influences of arsenic in morphological and functional parameters of the testis and epididymis in healthy and STZ-induced diabetic rats using drinking water. Indeed, STZ-induced diabetes is a well-characterized model of type 1 diabetes, and arsenate is the major arsenic compound found in drinking water with structural properties that could contribute for developing diabetes. Thus, we focused on functional markers, antioxidant enzymes balance, micromineral content, and histopathological parameters of the male reproductive organs as an attempt to better understand the diabetic effects of arsenic.

Methods

Animals. Adult male Wistar rats (n=40, 70 days old) were supplied by the Central Animal Laboratory of the Center of Biosciences and Health of the Federal University of Viçosa. During the experiment, the animals were allocated individually in polypropylene cages, and maintained under controlled temperature (21 ºC) and lighting conditions (12-12h

light/dark). Food and drinking water were provided *ad libitum*, being the later used for oral treatment. The study was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa (protocol 61/2016), and was conducted in accordance to National Council for the Control of Animal Experimentation.

Experimental design. The animals were randomly divided into 4 groups (n=10, each):
Negative control: Healthy rats received saline solution in drinking water;
Arsenic control: Healthy rats received 10 mg/L arsenic as sodium arsenate in drinking water;
Diabetes control: STZ-induced diabetic rats received saline solution in drinking water;
Diabetes + arsenic: STZ-induced diabetic rats received 10 mg/L arsenic as sodium arsenate in drinking water.

Diabetes in overnight fasted rats of diabetes control and diabetes + arsenic groups was induced by a single intraperitoneal injection of STZ (Sigma Chemical Co., St Louis, MO, USA) at a concentration of 60 mg/kg body weight in 0.01M citrate buffer, pH 4.5[18]. Glucose dosage was performed seven days after STZ administration using a glucometer (Acon Laboratories, Inc., San Diego, CA, USA) in blood samples obtained from the tail vein. Only animals having glucose levels higher than 250 mg/dL were used in the experiment. Saline solution or sodium arsenate ($Na_2HAsO_4.7H_2O$; Sigma Aldrich Co., St. Louis, MO) were administered *ad libitum* daily for 40 days in drinking water. As previous studies showed that rodents might be less susceptible than human to arsenic toxicity, due to a faster arsenic metabolism and excretion[23], we selected an arsenic concentration higher than that found in arsenic exposed areas similarly to other studies[9-11]. The exposure time selected in the present study was 10-fold higher than the biological half-time of inorganic arsenic in the body, which is considered 4 days[24,25]. Saline solution was provided to negative and diabetes controls to keep the taste of the drinking water similar to arsenic solution. Daily water consumption was evaluated in all groups. Moreover, blood glucose levels and body weight were recorded regularly throughout the study and prior to euthanasia.

Euthanasia and tissue collection. After 40-days experimentation, animals were euthanized by deep anesthesia (ketamine 150 mg/kg and xylazine 10 mg/kg, intraperitoneal). Next, a median laparotomy was made and the chest cavity was opened to collect blood by cardiac puncture. The testes, epididymides, ventral prostate, and seminal vesicle (without coagulating gland) were removed, dissected, and weights

(absolute and relative to the body weights) recorded. The right epididymides and testes were used for sperm transit time determination, biochemical analysis, and testosterone assay, while the left epididymides and testes were used for histology, sperm evaluation, and micromineral determination. The ventral prostate was frozen in liquid nitrogen and stored at -20 ˚C for fructose content analysis according to the Mann[26] methodology.

Testicular and serum testosterone assays. The blood collected by cardiac puncture was centrifuged at 2,000 x g for 15 min, and the serum was used for the quantification of testosterone. The assay was performed at the Tecsa® Veterinary Laboratory in Belo Horizonte/Brazil using the chemiluminescence methodology. For the analysis of testicular testosterone, 100 mg of testes parenchyma were homogenized in ice-cold phosphate buffer saline (PBS) and centrifuged at 3,500 x g (5 ºC) for 10 min. Testosterone concentration was assessed in the supernatant using Testosterone ELISA kit (ADI-900-065, ENZO Life Sciences, Plymouth, PA, USA) in accordance with the manufacturer`s instructions.

Daily sperm production per testis, sperm number and transit time in the epididymis. The right testes (n = 5/group) were decapsulated and homogenized for 3 min in ice-cold saline-triton (0.9% NaCl and 0.05% Triton X-100). The homogenate was diluted and used to count the number of homogenization-resistent spermatids in each sample using Neubauer chambers (4 fields per animal). Daily sperm production (DSP) was assessed by dividing the number of spermatids per testis by 6.1, which is the number of days that spermatids at stage 19 are present in the seminiferous epithelium[27]. In the same way, caput/corpus and cauda portions of the right epididymis (n = 5/group) were homogenized, and sperms were counted as described for testis. The transit time of sperm through the epididymis was calculated by dividing the number of sperms in each epididymal portion by the DSP[27].

Sperm parameters. Immediately after euthanasia, portions of cauda region from left epididymides (n = 5/group) were cut into small pieces and added 500 µl Tris-citric-fructose (Tris 3.025 g, citric acid 1.7 g, fructose 1.25 g, and distilled water 100 ml) to enable release of spermatozoa. Aliquots of this fluid were collected for sperm analysis. For the assessment of sperm motility, 10 µl of sperm suspension was placed between the slide and coverslip, previously heated to 37 °C, and 100 spermatozoa were examined under phase contrast microscope (Bioval L-1000B, Brazil) at 400x magnification. The

sperm cells were classified as motile or immotile[28]. The sperm morphology was assessed using 50 µl of sperm suspension fixed in 100 µl 4% buffered formaldehyde. The samples were smeared in glass slides and analyzed under phase contrast microscope (Bioval L-1000B, Brazil) at 1000x magnification, and 200 sperms were evaluated. Morphological abnormalities were classified as defects in the head, midpiece, and tail[29]. Finally, the structural integrity of plasma and acrosomal membranes was assessed in the sperms using carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) in accordance to the Harrison and Vickers[30] methodology. Two hundred sperms were evaluated under EVOS FL fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA) at 400x magnification, and classified as intact or injured sperm membranes.

Assessment of oxidative and nitrosative stress markers. For the analyses of oxidative and nitrosative stress markers, 100 mg of right testes and epididymides (n = 5/group) were homogenized in PBS and centrifuged at 3,500 x g (5 ºC) for 10 min. The supernatant was then used for measure the activity of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and nitric oxide (NO). SOD activity was determined in accordance to the method of Dieterich et al.[31]. The activity of CAT was assayed according to the Aebi[32] methodology using hydrogen peroxide as the substrate. The activity of GST was evaluated spectrophotometrically as described by Habig et al.[33] and calculated from the rate of NADPH oxidation. The nitric oxide levels were indirectly determined by the quantification of nitrite/nitrate levels in the tissues according to the Griess methodology[34]. The status of lipid peroxidation was determined by analyze the tissue levels of malondialdehyde (MDA). Briefly, 100 mg of the testes and epididymides (n = 5/group) were homogenized in PBS and incubated with thiobarbituric acid to evaluate the levels of thiobarbituric acid-reactive substances[35]. Protein oxidation was also evaluated in these same organs by the quantification of protein carbonyls in tissue pellets using the 2.4-dinitrophenylhydrazine (DNPH) method[36]. Finally, protein concentration was measured in accordance to the Bradford method[37].

Mineral microanalysis. The mineral content in testis and epididymis tissue (n = 5/group) was assessed by Energy Dispersive X-ray Spectroscopy using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with a x-ray detector system (Tracor TN5502, Middleton, WI, USA) as described by Novaes et al.[38]. The analysis was performed at 150x magnification, using an accelerating voltage of 20 kV and a working distance of 10 mm. The proportion of arsenic (As), iron (Fe), manganese

(Mn), zinc (Zn), copper (Cu), and selenium (Se) was evaluated and expressed as a mean value.

Histopathological evaluation. The left testes and epididymides (n = 5/group) were removed and fragments selected from the testis and caput and cauda epididymis were immersed in Karnovsky fixative solution for 24 hr. Fixed tissue samples were dehydrated in crescent ethanol series, and embedded in glycol methacrylate (Historesin®, Leica, Nussloch, Germany). Histological sections (3 μm) were stained with toluidine blue-sodium borate (1%), and histopathological analysis was performed using light microscope (Olympus CX40, Tokyo, Japan).

Testes morphometry and stereology. For morphometric and stereological analysis, digital images of the testes parenchyma (10x and 40x objectives) were obtained using a light microscope (Olympus BX-53, Tokyo, Japan) connected to a digital camera (Olympus DP73, Tokyo, Japan), and analyzed using the Image-Pro Plus® 4.5 (Media Cybernetics, Silver Spring, USA) software. Using a grid with 266 intersections over 10 histological images from testes parenchyma, the proportion of testis components (tubule and intertubule) was determined based on the total number of intersections[11]. The volume of each testicular component, expressed in milliliters, was also calculated by multiplying the volumetric proportion of each component by the testicular volume, divided by 100. Considering the testis density as one, the testicular weight was considered the same of its volume[39]. The tubular diameter and epithelium height of the seminiferous tubules were measured in 30 tubules per animal at ×100 magnification.

To analyze the volumetric proportion of the intertubular components (lymphatic space, blood vessels, nucleus and cytoplasm of Leydig cells, macrophages and connective tissue), 1,000 intersections were counted on intertubular images randomly obtained at 400x magnification, using a grid with 266 intersections. The proportion of each component was calculated based on the total of intersections[11]. The diameter of the Leydig cell nucleus was measured in 30 cells per animal and the nuclear volume was calculated using the following formula: $4/3\pi R^3$ (R = nuclear diameter/2). The cytoplasm volume was estimated by multiplying the volumetric proportion of cytoplasm by the nuclear volume, divided by the nuclear proportion. Adding these values, the individual Leydig cell volume was determined. The number of Leydig cells per testis was estimated dividing the total Leydig cell volume per individual Leydig cell volume[40].

Statistical analysis. All the variables analyzed herein were compared between groups using Student's T-test. For validate the injuries caused by STZ-induced diabetes, we run a Student's T-test comparing the results from negative control and diabetes control groups. Moreover, we performed two separate Student's T-tests in order to compare the arsenic exposure in reproductive parameters of healthy and diabetic animals. The first one comparing data from negative control and arsenic control groups, while the other comparing diabetes control and diabetes + arsenic groups. Differences were considered significant when $P < 0.05$. All tests and graphics were performed using the GraphPad Prism 6.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as mean ± standard error mean (SEM).

Results

Biometric parameters and blood glucose levels. Exposure to arsenate increased the blood glucose levels of healthy rats, but did not enhance the high blood glucose level of STZ-induced diabetic rats (Table 1). During the experimental period, STZ-induced diabetic animals ingested more arsenic solution (182.13 ± 4.19 mL) than healthy animals (48.38 ± 4.92 mL). Based on the average daily solutions intake, animals of arsenic control and diabetes + arsenic groups ingested 2.42 mg As/kg BW/day and 9.10 mg As/kg BW/day, respectively. At the end of the experiment, diabetes control animals had lower body weight and absolute weights of the testis, epididymis, seminal vesicle and prostate than negative control animals (Table 1). Exposure to arsenate reduced prostate weight in healthy animals and intensified the decrease of prostate weight in STZ-induced diabetic rats. Moreover, arsenate exposure induced a slightly increase in body weight of diabetes + arsenic animals compared to diabetes controls. The relative weight of testis and epididymis were higher in rats of diabetes control group compared to negative controls, while the relative weight of seminal vesicle and prostate were reduced. Exposure to arsenate induced a decrease of testis, epididymis and prostate relative weights in diabetes + arsenic group compared to diabetes control (Table 1).

Table 1. Serum glucose levels, biometric data, and prostate fructose content of healthy rats and STZ-induced diabetic rats exposed to arsenate in drinking water *ad libitum*

Parameters	Negative control	Arsenic control	Diabetes Control	Diabetes + arsenic
Serum glucose levels (mg/dL)	56.60 ±2.49	70.60 ± 2.44*	474.50 ± 32.72	503.40 ± 40.42#
Initial body weight (g)	223.51 ± 8.41	243.65 ± 6.29	233.83 ± 3.30	233.59 ± 3.40
Final body weight (g)	352.45 ± 9.98	357.55 ± 9.48	182.55 ± 7.95†	206.88 ± 9.51#
Testis (g)	1.71 ± 0.08	1.68 ± 0.08	1.32 ± 0.06†	1.35 ± 0.04
Testis (g/100g)	0.49 ± 0.02	0.46 ± 0.02	0.72 ± 0.01†	0.65 ± 0.01#
Epididymis (mg)	694.50 ± 18.17	666.00 ± 21.05	404.00 ± 20.83†	424.00 ± 20.73
Epididymis (mg/100g)	195.30± 4.77	184.90 ± 3.59	210.80± 7.34†	193.70 ± 2.75#
Seminal vesicle (mg)	935.20 ± 65.09	838.20 ± 52.03	136.20 ± 14.96†	170.40 ± 28.41
Seminal vesicle (mg/100g)	256.86 ± 19.91	224.57 ± 17.82	74.20 ± 11.45†	81.46 ± 13.08
Ventral prostate (mg)	418.20 ± 20.17	335.50 ± 35.68*	175.20 ± 16.90†	120.00 ± 19.47#
Ventral prostate (mg/100g)	121.70 ± 4.53	94.39 ± 6.96*	96.01 ± 8.23†	57.32 ± 8.54#
Fructose (mg/g prostate)	0.43 ± 0.03	0.26 ± 0.01	1.19 ± 0.22†	0.81 ± 0.06

Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. †means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. # means significant difference ($p < 0.05$) between diabetic groups by student`s t-test.

Prostate fructose content. The ventral prostate fructose levels were increased in animals of diabetes control group compared to negative control. However, exposure to arsenate did not increase prostate fructose levels in both healthy and STZ-induced diabetic rats (Table 1).

Testicular and serum testosterone. Arsenate exposure reduced serum testosterone concentration in healthy animals compared to negative controls and intensified the reduction found in STZ-induced diabetic rats (Fig. 1). On the other hand, exposure to arsenate did not affect the testicular testosterone concentration in health rats, but it was able to intensify the reduction of this hormone in the testis of diabetic rats (Fig. 1).

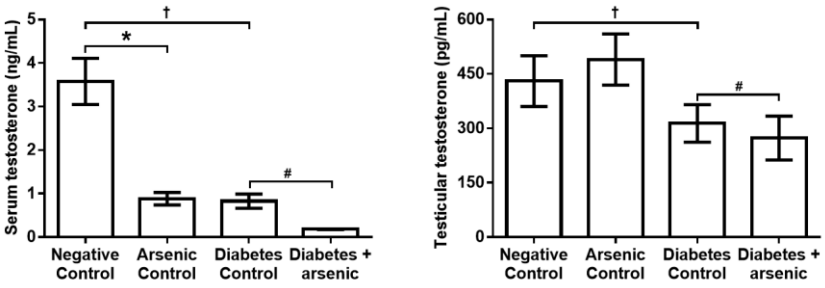

Figure 1. Testicular and serum testosterone concentration in healthy and STZ-induced diabetic rats exposed to arsenate in drinking water *ad libitum*. Negative control: saline; Arsenic control: 10 mg/L sodium arsenate; Diabetes control: STZ; Diabetes + arsenic: STZ + 10 mg/L sodium arsenate. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. †means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. # means significant difference ($p < 0.05$) between diabetic groups by student`s t-test.

Daily sperm production per testis, sperm number and transit time in the epididymis. The number of mature spermatids in the testis and per g testis, and daily sperm production decreased in animals of arsenic control and diabetes control group compared to negative control. Exposure to arsenate further decrease these parameters in diabetes + arsenic animals in comparison to diabetes control (Table 2). In the caput/corpus and cauda epididymis, the number of sperm cells was also reduced in arsenic control and diabetes control animals in relation to negative controls, even when the sperm counting was analyzed per g epididymis (Table 2). In the same way, arsenate exposure intensified this reduction in STZ-induced diabetic rats. The sperm transit time in caput/corpus and cauda epididymis was reduced in diabetes control rats compared to negative controls. However, exposure to arsenate was unable to change this parameter in healthy and STZ-induced diabetic rats (Table 2).

Table 2. Sperm count in testis and epididymis and sperm parameters of healthy and STZ-induced diabetic rats exposed to arsenate in drinking water *ad libitum*

Parameters	Negative control	Arsenic control	Diabetes control	Diabetes + arsenic
Sperm count				
Spermatid number ($x10^6$/testis)	220.92 ± 8.21	176.13 ± 6.41*	147.50 ± 10.86†	96.32 ± 6.85#
Spermatid number ($x10^6$/g testis)	167.83 ± 6.91	127.50 ± 4.60*	145.90 ± 6.456†	86.51 ± 8.36#
Daily sperm production ($x10^6$/testis/day)	36.22 ± 1.35	28.87 ± 1.05*	24.18 ± 1.78†	15.79 ± 1.12#
Caput/corpus epididymis sperm number ($x10^6$/organ)	116.41 ± 6.33	91.60 ± 4.87*	60.28 ± 7.59†	35.70 ± 2.92#
Caput/corpus epididymis sperm number ($x10^6$/g organ)	384.68 ± 17.17	271.48 ± 8.55*	248.00 ± 17.50†	147.89 ± 3.87#
Sperm transit time in the caput/corpus epididymis (days)	3.22 ± 0.17	3.18 ± 0.17	2.46 ± 0.15†	2.27 ± 0.13
Cauda epididymis sperm number ($x10^6$/organ)	215.69 ± 12.48	167.99 ± 12.79*	50.80 ± 8.24†	38.77 ± 6.00#
Cauda epididymis sperm number ($x10^6$/g organ)	831.21 ± 30.74	604.57 ± 28.98*	424.74 ± 37.98†	256.50 ± 17.15#
Sperm transit time in the cauda epididymis (days)	5.97 ± 0.33	5.85 ± 0.54	2.49 ± 0.39†	2.49 ± 0.36
Sperm parameters				
Sperm motility (%)	71.25 ± 4.27	57.00 ± 4.89*	46.25 ± 1.85†	48.00 ± 4.36
Normal shaped sperm (%)	98.25 ± 0.37	91 ± 2.07*	96.25 ± 0.58†	93.5 ± 1.02#
Sperm with intact membranes (%)	73.40 ± 1.33	13.20 ± 2.50*	5.40 ± 1.25†	11.00 ± 3.58

Mean ± SEM. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. †means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. # means significant difference ($p < 0.05$) between diabetic groups by student`s t-test.

Sperm evaluation. Sperm motility and the percentage of sperm with intact membranes decreased in healthy animals exposed to arsenate and in diabetes control rats compared to negative controls. However, exposure to arsenate did not intensify this reduction in diabetes + arsenic animals (Table 2). On the other hand, arsenate exposure reduced the number of sperm with normal morphology in healthy animals and enhanced the decrease observed in STZ-induced diabetic rats (Table 2).

Oxidative and nitrosative stress markers. In the testis, the activity of CAT was reduced in arsenic control and diabetes control rats compared to negative controls, but no changes were observed between diabetes + arsenic and diabetes control groups (Fig. 2). On the other hand, GST activity was reduced in both groups receiving arsenate, while their NO_3/NO_2 levels were increased when compared to negative control and diabetes control groups, respectively (Fig. 2). Moreover, SOD activity was reduced only in diabetes control animals, whereas protein carbonyl content was higher in these animals in relation to negative control group. No changes were observed in these parameters when healthy and STZ-induced diabetic rats were exposed to arsenate. Further, the levels of MDA in the testis did not change in the animals analyzed (Fig. 2).

In the epididymis, in turn, SOD activity was slightly increased in diabetes control animals compared to negative controls, while exposure to arsenate induced a higher increase of this enzyme in healthy and STZ-induced diabetic rats (Fig. 2). The activity of CAT also increased in arsenic control and diabetes control animals compared to negative control rats. However, exposure to arsenate reduced CAT activity in diabetes + arsenic animals in relation to diabetes control (Fig. 2). On the other hand, exposure to arsenate induced an increase in GST activity and NO_3/NO_2 levels in healthy and STZ-induced diabetic rats, when compared to negative control and diabetes control groups, respectively. Protein carbonyl content and MDA levels did not change in the groups analyzed (Fig. 2).

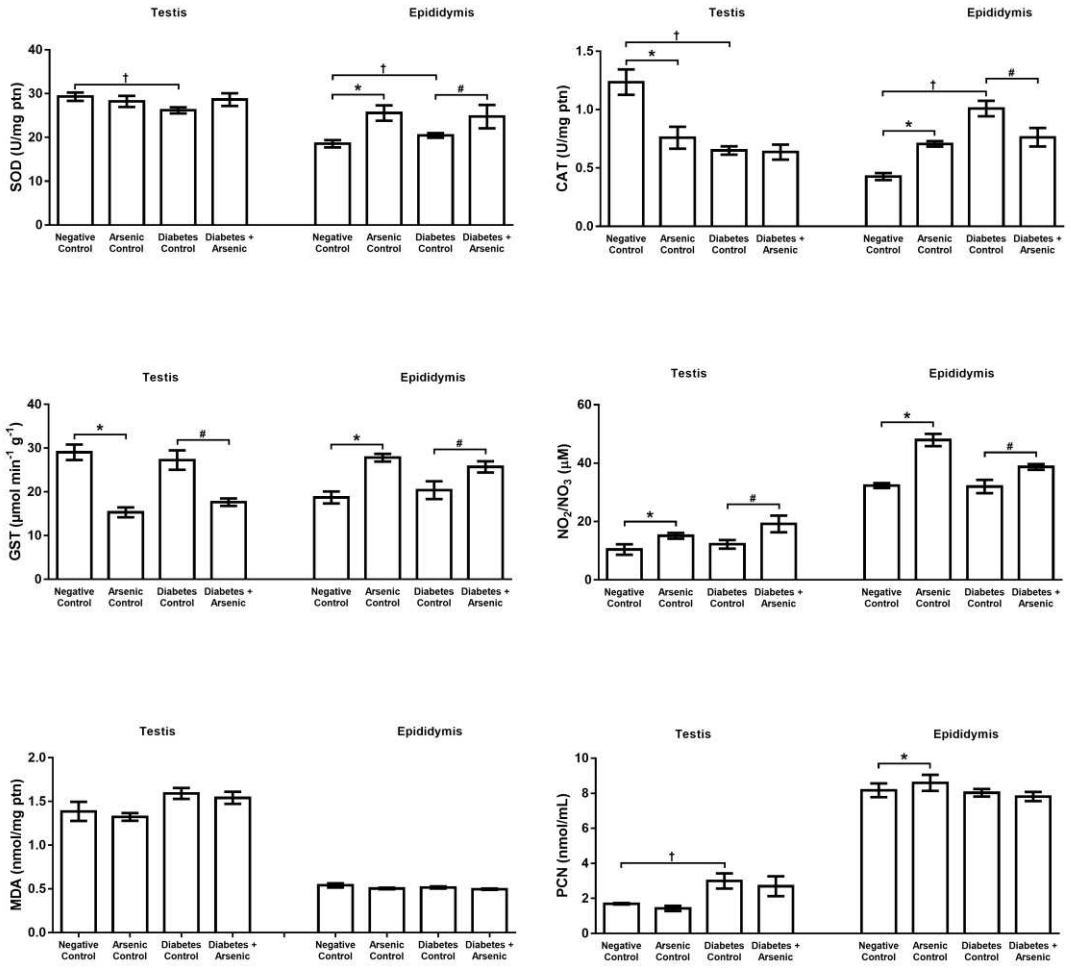

Figure 2. Oxidative and nitrosative stress markers in the testis and epididymis of healthy and STZ-induced diabetic rats exposed to arsenate in drinking water *ad libitum*. SOD: superoxide dismutase; CAT: catalase; GST: glutathione S-transferase; NO_2/NO_3: nitrite/nitrate levels; MDA: malondialdehyde; PCN: carbonyl protein. Negative control: saline; Arsenic control: 10 mg/L sodium arsenate; Diabetes control: STZ; Diabetes + arsenic: STZ + 10 mg/L sodium arsenate. Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. †means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. # means significant difference ($p < 0.05$) between diabetic groups by student`s t-test.

Micromineral content. The analysis of mineral content in testis and epididymis tissue presented marked changes on the elements evaluated (Fig. 3). In the testis, the proportion of As and Zn increased in rats of arsenic control and diabetes + arsenic groups, while Fe proportion was reduced in these groups, when they were compared to negative control and diabetes control groups, respectively (Fig. 3). The proportion of Se and Mn decreased in arsenic control and diabetes control animals compared to negative controls. Moreover, exposure to arsenate worsened the decrease of these elements in STZ-induced diabetic rats (Fig. 3). Diabetes control animals had lower Cu proportion than negative control

animals. However, exposure to arsenate increased the proportion of this element in healthy and STZ-induced diabetic rats, when compared to negative control and diabetes control group, respectively (Fig. 3).

In the epididymis, the proportion of As also increased in both groups receiving arsenate (Fig. 3). The concentration of Mn and Fe was lower in diabetes control animals compared to negative controls. However, exposure to arsenate did not change the proportion of these elements in healthy and STZ-induced diabetic rats (Fig. 3). On the other hand, arsenate exposure reduced Cu proportion in arsenic control rats in relation to negative controls, while STZ-induced diabetic rats had increase in this element proportion mainly when exposed to arsenate (Fig. 3). Moreover, the proportion of Se was reduced in arsenic control and diabetes control groups in comparison to negative control animals. Arsenate exposure was able to enhance this reduction in STZ-induced diabetic rats, as well as induce a decrease in Zn proportion in these animals compared to diabetes control (Fig. 3).

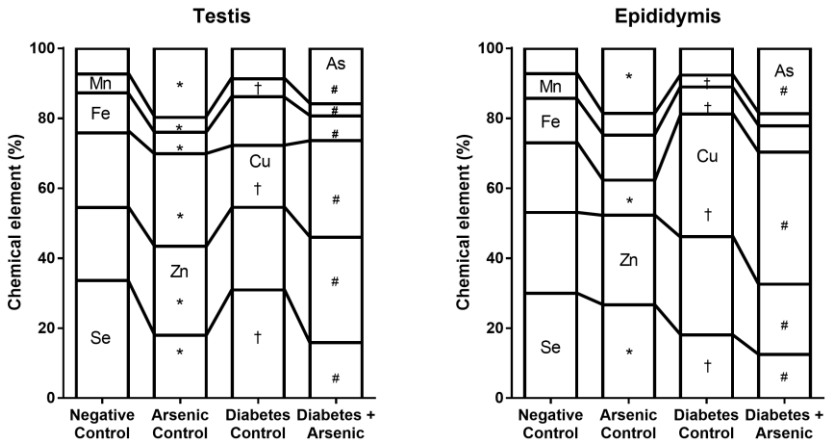

Figure 3. Mineral proportion in the testis and epididymis of healthy and STZ-induced diabetic rats exposed to arsenate in drinking water *ad libitum*. As: arsenic; Mn: manganese; Fe: iron; Cu: copper; Zn: zinc; Se: selenium. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. †means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. # means significant difference ($p < 0.05$) between diabetic groups by student`s t-test.

Histopathological analysis. The testis parenchyma showed normal tissue architecture in negative control rats (Fig. 4). On the other hand, animals of diabetes control and both groups exposed to arsenate presented vacuolization in the seminiferous epithelium, especially in animals from diabetes + arsenic group (Fig. 4). Caput and cauda epididymis tissues from negative and arsenic controls did not reveal any apparent alteration that could be attributed to treatments. However, diabetes control and diabetes + arsenic animals showed germ cells in the lumen of both epididymal regions (Fig. 4)

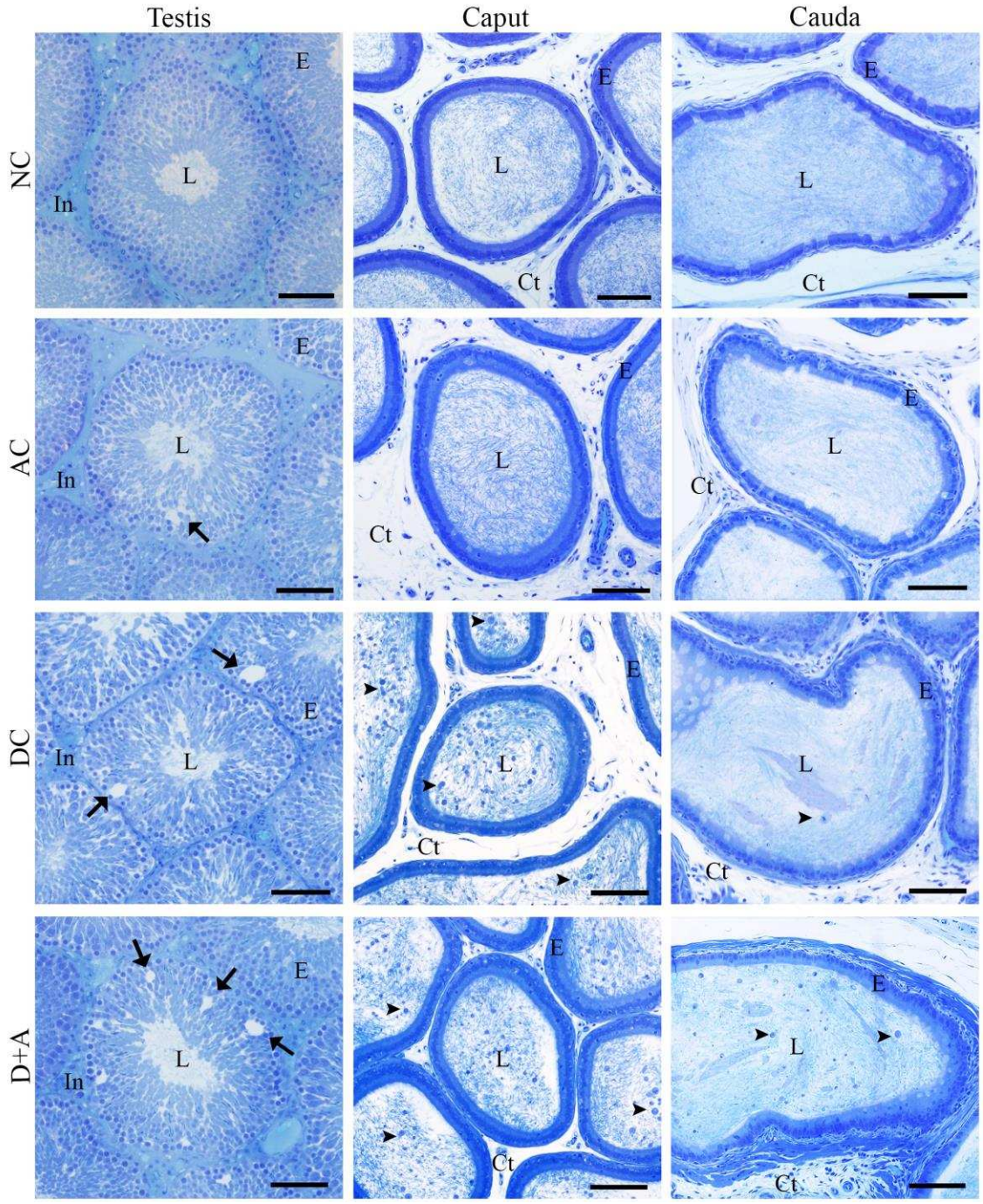

Figure 4. Histological sections from testes and epididymis of healthy and STZ-induced diabetic rats exposed to arsenate in drinking water *ad libitum*. NC: negative control (saline); AC: arsenic control (10 mg/L sodium arsenate); DC: diabetes control (STZ); DA: diabetes + arsenic (STZ + 10 mg/L sodium arsenate). E = epithelium, L = lumen, In = intertubule, Ct = connective tissue, Arrows = vacuoles, Arrowhead = germ cells. Toluidine blue. Scale bars = 100 µm.

Testis morphometry and stereology. The percentage of tubules increased in diabetes control animals, but was reduced in arsenic controls compared to negative control animals. Inversely, the percentage of intertubule was lower in diabetes control rats and higher in arsenic control rats in relation to negative control. Arsenate exposure did not

worsen these parameters in diabetes + arsenic group in relation to diabetes controls (Table 3). The animals of diabetes control group also presented reduction in tubule and intertubule volumes when compared to negative control. Moreover, arsenic control animals had higher intertubule volume than negative control animals (Table 3). Exposure to arsenate also did not intensify the changes observed in STZ-induced diabetic rats. No differences were observed in the experimental groups with respect to tubular diameter and epithelium height of the seminiferous tubules (data not shown).

Table 3. Testicular morphometry and stereology of healthy and STZ-induced diabetic rats exposed to arsenate in drinking water *ad libitum*

	Negative control	Arsenic control	Diabetes control	Diabetes + arsenic
Tubule				
Tubule (%)	88.88 ± 0.85	86.36 ± 0.34*	91.84 ± 0.76†	90.25 ± 0.64
Tubular volume (mL)	2.84 ± 0.15	2.70 ± 0.14	2.28 ± 0.09†	2.23 ± 0.08
Intertubule (%)	11.12 ± 0.85	13.64 ± 0.34*	8.16 ± 0.76†	9.75 ± 0.63
Intertubule volume (mL)	0.35 ± 0.03	0.43 ± 0.02*	0.20 ± 0.02†	0.24 ± 0.02
Intertubule				
Connective tissue (%)	3.76 ± 0.29	3.80 ± 0.34	3.38 ± 0.54	2.34 ± 0.26
Lymphatic space (%)	57.30 ± 0.76	56.62 ± 0.69	56.36 ± 1.09	54.90 ± 1.43
Blood vessel (%)	11.06 ± 0.53	13.86 ± 0.66*	14.92 ± 0.48†	15.62 ± 0.92
Macrophages (%)	1.26 ± 0.25	2.70 ± 0.24*	3.40 ± 0.26†	3.50 ± 0.20
Leydig cell (%)	26.62 ± 0.94	23.02 ± 0.70*	21.94 ± 1.13†	23.64 ± 0.72
Leydig Cell				
Nuclear volume (μm^3)	175.03 ± 10.44	185.86 ± 5.40	190.83 ± 6.31	191.44 ± 7.85
Cytoplasmatic volume (μm^3)	337.18 ± 21.22	351.59 ± 15.35	207.50 ± 8.43†	248.13 ± 23.36
Cell volume (μm^3)	512.21 ± 31.49	537.44 ± 12.27	398.33 ± 11.77†	439.57 ± 26.76
Number of cells/testis ($x10^6$)	92.26 ± 7.05	90.98 ± 3.15	55.10 ± 4.66†	65.92 ± 7.15

Mean ± SEM. *means significant difference ($p < 0.05$) between arsenic control and negative control groups by student`s t-test. †means significant difference ($p < 0.05$) between diabetes control and negative control groups by student`s t-test. # means significant difference ($p < 0.05$) between diabetic groups by student`s t-test.

In the intertubule, the percentage of macrophages and blood vessels increased in arsenic control and diabetes control animals in relation to negative controls, while Leydig cell percentage was reduced in these same groups. Exposure to arsenate did not worsen these changes in diabetes + arsenic animals compared to diabetes control (Table 3). The cellular and cytoplasm volume of Leydig cells as well as their number were decreased in

animals from diabetes control relative to negative control rats (Table 3). Arsenate exposure was unable to change these parameters in healthy and STZ-induced diabetic rats. The other parameters analyzed remained unchanged in the experimental groups (Table 3).

Discussion

The present study evaluated the effects of arsenate exposure on morphological and functional parameters of male reproductive organs in healthy and STZ-induced diabetic rats. Our results showed that arsenate caused an intense reduction in serum testosterone levels in healthy animals and intensified the reduction in serum and testicular testosterone in diabetic animals. Consequently, the low levels of testosterone were not enough to support the sperm production and the number of these cells in the testis and epididymis of both groups receiving arsenate. Arsenate exposure also caused an antioxidant enzyme imbalance and consequent nitrosative stress, which was intensified in diabetic animals and might have been responsible by testicular pathologies. On the other hand, the sperm quality was impaired by arsenate exposure without strong alteration when environmental and metabolic disturbance act at the same time.

The marked reduction in serum testosterone levels observed in diabetes control and arsenic control animals corroborates previous studies using experimental diabetes or arsenic exposure[6,7,41]. It is known that either diabetes or arsenic can promote atrophy of Leydig cells inducing a reduction in testosterone levels[6,42]. Furthermore, reduction in this hormone has been associated with absence or diminution of serum insulin levels in male diabetic rats[43], and inhibition of androgen production in male rats exposed to arsenic probably by affecting pituitary gonadotrophins[7]. Thus, a combination of arsenic and diabetes effects might explain the intense reduction of serum and testicular testosterone observed in diabetes + arsenic animals. However, exposure to arsenate was unable to reduce testicular testosterone in healthy animals. Probably, these animals are being able to retain testosterone inside the testis, despite their lower serum testosterone levels, as an attempt to maintain paracrine functions necessary in the organ.

The absolute and relative weights of organs such as testis, epididymis, seminal vesicle and prostate are good indicators to evaluate the risks induced by toxicants on male reproductive system and are related to androgens levels. The significant reduction observed in the absolute weight of the reproductive organs in diabetes control animals is in accordance with previous studies[17-19], and suggests compromised growth of these androgen-dependent organs probably due to impaired serum testosterone levels[44].

Exposure to arsenate was able to reduce absolute and relative prostate weights of healthy animals and worsen the reduction in these parameters in STZ-induced diabetic rats. Probably this also occurred in response to low levels of testosterone, once prostate is an organ highly dependent on this hormone. The increased prostate fructose content observed in STZ-induced diabetic rats along with the reduction in relative weights of prostate and seminal vesicle corroborates the compromised growth of the reproductive organs due to lack of androgen. Fructose content has been used to evaluate androgen-dependent accessory sex gland function, and an increase in this parameter indicates reduction in androgen levels[26]. Elevated fructose levels were previously observed in seminal vesicle of diabetic rats[43] indicating low levels of testosterone in these animals. Exposure to arsenate did not intensify the elevated fructose levels in diabetes + arsenic rats, probably because arsenic metabolites did not affect fructose metabolism in the organ.

In keeping with the reduced testosterone levels, we observed a decrease in testicular and epididymal sperm counts as well as DSP in the testis of arsenic control and diabetes control animals. All these decreases were intensified in diabetic animals exposed to arsenate. Reduction in these parameters was previously observed in studies using either experimental diabetes or arsenate exposure separately[10,17]. These decreases might be explained by alterations in the spermatogenesis process[6,17]. It is known that testosterone is a crucial hormone to maintain the spermatogenesis process and releasing of the sperm[45]. Thus, the decrease in serum testosterone concentration induced by both diabetes and arsenate exposure may have led to alterations in the spermatogenesis and intensified the low sperm counts in diabetes + arsenic animals. In addition, as a consequence of the decrease in sperm reserves, the sperm transit time was accelerated in caput/corpus and cauda epididymis of STZ-induced diabetic rats. The sperm transit time is a process highly regulated by androgens, which controls the viscosity of the epididymal luminal fluid and the contractility of the duct. Therefore, the greater reduction in testicular and serum testosterone concentration in diabetes control animals may explain the accelerated sperm transit time in these animals. However, despite the decreased sperm counts observed in arsenic control animals, exposure to arsenate was unable to intensify the reduction in sperm transit time in STZ-induced diabetic rats. The accelerated transit of sperm through the epididymis promotes lower exposure of these cells to epididymal microenvironment, which is very important to sperm maturation[46]. Thus, the greater decrease in sperm motility and integrity of sperm membranes observed in this study might be a consequence of reduced sperm transit time in the epididymis. Furthermore, the reduction of sperm motility in healthy animals receiving arsenate might be due to binding of arsenic to

sulfhydryl or thiol groups on sperm proteins or the inhibition of enzymes involved in sperm motility[47]. However, despite arsenate exposure has not intensified the reduction of sperm transit time observed in diabetic animals, the exposure to this arsenic compound induced a slight reduction in the number of normal sperm in these animals compared to diabetes controls.

Sperm damages, such as decreased fertilization capacity and altered membrane functions have been also related to imbalance in antioxidant defenses[48]. In this work, we observed marked alterations in antioxidant enzymes activities in the testis and epididymis. Exposure to arsenate was able to decrease CAT and GST activity and increase NO_2/NO_3 levels in healthy rats as well as worsened these parameters in STZ-induced diabetic rats. Reduction in testicular antioxidant enzymes was also observed in studies using STZ-induced diabetic mice[18] and in rats exposed to arsenite and arsenate in drinking water[11]. The decrements observed in antioxidant enzymes might be due to failure of adaptive mechanism to act against reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by diabetes and arsenic exposure in the organ. The overproduction of these species in the testis along with the elevated NO_2/NO_3 levels indicates that the organ is suffering nitrosative stress, which might explain the pathologies observed in the tissue and the damages in sperm membrane found herein. On the other hand, in the epididymis, exposure to arsenate was able to induce an increase in the antioxidant enzymes in healthy animals and enhanced these parameters in diabetic rats. This indicates that arsenic might has different modes of action depending on the organ. Herein, the increase in antioxidant enzymes indicates an adaptation on part of the antioxidant system to counteract oxidative stress. However, the upregulation of these epididymal antioxidant defenses did not prevent the increase of NO_2/NO_3 levels in the organ, also indicating nitrosative stress.

It has been reported that there is a relationship between antioxidant enzymes and trace elements balance in the organs. Superoxide dismutase activity requires Mn in the mitochondria, and Zn and Cu in the cytosol[49]. On the other hand, Fe is necessary for CAT activity and Se in essential in activating glutathione peroxidase[50,51]. In our study, arsenic control and diabetes control animals had an imbalance on testicular and epididymal micromineral content. Moreover, exposure to arsenate worsened the micromineral changes of diabetic rats. Probably this imbalance was due to increased arsenic proportion observed in the testis and epididymis of these animals. It is known that arsenic can compete with trace elements and bind to metalloproteins altering body homeostasis[52]. Furthermore, both arsenic exposure and diabetes induce increased ROS production, which in turn, has been related to metabolic homeostasis of the minerals[53]. Thus, it seems

that arsenic can cause and worsen the micromineral imbalance by a combination of the process cited above. Moreover, in this work the changes on micromineral content were unable to support antioxidant enzymes functions in both organs.

Herein, despite diabetic animals had ingested more arsenic solutions than healthy rats, both animals had similar arsenic accumulation in the testis and epididymis. It is know that polydipsia is an important symptom of diabetes[22] and drinking water is the main source of arsenic contamination[1]. Thus, it is easy to understand the higher water intake in STZ-induced diabetic animals. Moreover, it has been reported that diabetic individuals have a different metabolism and excretion of arsenic[54], which can explain the similar arsenic accumulation in the organs, despite the higher arsenic solution ingestion by diabetic rats. However, the arsenic accumulated in the organs along with ROS and RNS generated during its metabolism may have been responsible by structural alterations found in testis. The vacuolization observed in the seminiferous epithelium is in accordance with previous studies evaluating arsenate exposure in drinking water[11] and experimental diabetes[18] in the testis of rats. According to Creasy[55], the vacuolization might be an answer of Sertoli cells to injuries induced by ROS and RNS in the organ, and is usually followed by degeneration and detachment of the germ cells. Herein, the increased NO_2/NO_3 levels observed in diabetes + arsenic animals might have been responsible by the intense vacuolization in these animals. As a consequence of this pathology, we also observed detached germ cells in the epididymis, indicating that the detached cells from the testis have already followed to the epididymis along with the testicular fluid. Further, the pathologies observed in this study can be related to imbalance in micromineral content in the organs. It is known that trace elements such as selenium and zinc are required to maintain testicular structure and spermatogenesis in rats[56]. In this study, both microminerals were altered in the testis.

Besides the morphological damages observed in the testes, we also observed alterations in its stereological parameters. However, a combination of arsenate and diabetes was unable to intensify these changes. We can suggest that the increased proportion of blood vessels found in arsenic control and diabetes control groups might be related to a beginning of an angiogenic process in the organ. It is possible that the ROS and RNS generated by either arsenic exposure or diabetes induced endothelial cell damages, leading to formation of new vessels in the organ. Furthermore, the increased ROS and RNS production in the testis might be associated to the elevated proportion of macrophages found in the organ as well as with the reduction in Leydig cell proportion and number observed herein. Reduction in Leydig cell proportion was previously reported

in animals exposed to either arsenic or diabetes[6,42], and might indicate cell atrophy, which can be related to the decrease in testosterone concentration found in the animals. Probably, a combination of arsenate exposure with pre-existent diabetes had no synergistic action and, therefore, did not intensify the changes observed in stereological parameters when they acted separately.

Herein, as showed in previous studies[17-19], we observed a significant loss in body weight and a greater increase in glucose levels in STZ-induced diabetic rats. Moreover, the blood glucose levels were increased in healthy animals receiving arsenate. This increase might be explained due to similarity between arsenate and phosphate, which allows that arsenate replace phosphate in biochemical reactions in the glucose metabolism leading to hyperglycemia[14]. However, arsenate exposure did not worsen the higher glucose levels already present in diabetic animals. Considering, that diabetic animals have differential arsenic metabolism with higher arsenic methylation[54], it is possible that methylated arsenic forms were unable to affect glucose metabolism and intensify the increased blood glucose levels in these animals. Moreover, once diabetic animals have lower arsenic excretion, the arsenic accumulated in the organs might be inducing an initial deposition of collagen, which, in turn, might be responsible by changes in relative organs weight and final body weights observed in diabetes + arsenic animals.

Conclusions

Our results showed that arsenate exposure impaired testosterone levels and induced nitrosative stress in the testis and epididymis of healthy rats. Moreover, these negative effects of arsenate were intensified in animals with pre-existent diabetes. The reduction in testosterone concentration might have been responsible by the decrements in daily sperm production and sperm counting in the testis and epididymis, while nitrosative stress induced histopathological damages in the testis. Consequently, these alterations on male reproductive parameters can suggest that adverse effects on fertility might be occurring. Therefore, additional studies are needed to better understand the mechanisms of action of arsenic in diabetic animals and the influences of this combination in fertility parameters of the animals.

Acknowledgments

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors are grateful to “Núcleo de Microscopia e Micoanálise”

of the Federal University of Viçosa by assistance in EDS analysis, and Tecsa Laboratory for hormonal analysis.

Competing financial interests. The authors declare no competing financial interests.

Data availability. All data generated or analyzed during this study are included in this published article.

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CONCLUSÃO GERAL

Os resultados desta tese indicam que a exposição oral ao arsenato de sódio causou diversos danos nos parâmetros hepáticos e reprodutivos de ratos Wistar saudáveis. Além disso, a maior parte destes danos foram intensificados quando animais diabéticos foram expostos a este composto de arsênio. Em geral, a exposição ao arsênio concomitante a presença do diabetes induziu um intenso desequilíbrio na atividade das enzimas antioxidantes, deixando os tecidos vulneráveis ao ataque das espécies reativas de oxigênio e espécies reativas de nitrogênio, o que, por sua vez, promoveu o aparecimento de inúmeras patologias. Além disso, no sistema reprodutor, a presença destes dois fatores induziu uma acentuada redução na testosterona, a qual pode ter sido responsável pelos severos danos funcionais encontrados no testículo. Dessa forma, mais estudos são necessários para se compreender melhor o mecanismo de ação do arsênio em animais com doença metabólica pré-existente, além das influências deste metaloide na fertilidade e funções hepáticas destes animais.