MARCELA NASCIMENTO SERTORIO

PARÂMETROS RENAIS EM RATOS DIABÉTICOS EXPOSTOS AO ARSÊNIO

Dissertação 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 *Magister Scientiae*.

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RESUMO

SERTORIO, Marcela Nascimento, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Parâmetros renais em ratos diabéticos expostos ao arsênio**. Orientadora: Mariana Machado Neves. Coorientadora: Ana Cláudia Ferreira Souza.

Sabe-se que a função renal pode ser influenciada tanto por contaminantes ambientais quanto por doenças metabólicas. No entanto, não está claro como estes fatores, de forma combinada, podem alterar a capacidade de filtração glomerular e de excreção de substâncias tóxicas dos rins. Neste contexto, avaliamos parâmetros morfológicos e funcionais nos rins de ratos diabéticos expostos a arsênio, um potente contaminante ambiental. Ratos Wistar machos saudáveis e ratos diabéticos, modelo experimental induzido por estreptozotocina, foram expostos a 10 mg/L de arsênio na forma de arsenato de sódio via água de beber durante 40 dias. Os constituintes do estroma e parênquima renal foram analisados por meio de morfometria. O dano funcional dos rins foi investigado por meio de marcadores de apoptose e proliferação celular, proporção de elementos químicos, marcadores de estresse oxidativo, e também através da atividade das enzimas do sistema antioxidante endógeno e das proteínas de membrana adenosina trifosfatases. A ingestão de arsenato aumentou as concentrações de glicose sérica em animais saudáveis, no entanto, este aumento não alcançou níveis de hiperglicemia. Nos animais diabéticos, este aumento não foi significativo. O arsenato foi capaz de induzir um aumento notável de nefrose glicogênica nos túbulos distais de animais diabéticos, caracterizada por grande acúmulo de glicogênio nas células tubulares. Além disso, a atividade do sistema antioxidante endógeno foi alterada, e associada a proporção dos elementos ferro, cobre e potássio no tecido renal. Foi observado aumento de peso corporal e nos rins de animais diabéticos expostos a arsenato. Nos animais diabéticos, níveis de malondialdeído, sódio, ureia, creatinina, marcadores de apoptose e proliferação, além do índice renal somático e conteúdo de água nos rins foram alterados pela diabetes isoladamente e não foram agravados pela ingestão de arsenato. Os demais parâmetros, como atividade de superóxido dismutase, níveis de proteínas carboniladas, atividade das proteínas de membrana adenosina trifosfatases e morfologia glomerular não foram modificados significativamente em nenhum dos grupos tratados. Nossos resultados mostraram que a ingestão de água contaminada por arsênio por indivíduos diabéticos possui o potencial para alterar aspectos morfofuncionais renais.

ABSTRACT

SERTORIO, Marcela Nascimento, M.Sc., Universidade Federal de Viçosa, July, 2018. **Renal parameters in diabetic rats exposed to arsenic**. Adviser: Mariana Machado Neves. Co-adviser: Ana Cláudia Ferreira Souza.

It is known that renal function can be disrupted by environmental contaminants or metabolic diseases. However, it is not clear how these combined factors can alter kidney glomerular filtering and excretion of toxic substances. In this framework, we evaluated morphological and functional parameters in the kidneys of diabetic rats exposed to arsenic, a potent environmental contaminant. Healthy male Wistar rats and streptozotocin-induced diabetic rats were exposed to 10 mg/L arsenic as sodium arsenate through drinking water for 40 days. The renal tissue was analyzed using morphometry, mitosis and apoptosis markers, proportion of chemical elements, oxidative stress markers, and activity of antioxidant enzymes and membrane-bound adenosine triphosphatases. Arsenate ingestion increased serum glucose levels in healthy animals. However, this increase did not reach hyperglycemic levels, and in diabetic animals, it was not significant. In diabetic animals, arsenate led to a remarkable increase of glycogen nephrosis in distal tubules. Besides, activity of the antioxidant enzymes catalase and glutathione-s-transferase was disrupted, as well as the proportion of the chemical elements iron, cupper and potassium in renal tissue. In addition, there was an increase in body and kidney weight of diabetic animals exposed to arsenate. However, the levels of malondialdehyde, sodium, urea, creatinine, apoptosis and proliferation markers, besides renal somatic index and water content in the kidneys were altered only by diabetes and were not aggravated by arsenate ingestion. The other parameters, such as superoxide dismutase activity, carbonyl protein levels, activity of membrane-bound adenosine triphosphatases and glomerular morphology were not significantly changed in none of the treated groups. Our results showed that the ingestion of contaminated water with arsenic by diabetic individuals could alter morphofunctional aspects of kidney.

INTRODUÇÃO GERAL

Metais pesados são elementos químicos que existem naturalmente no meio ambiente, fazendo parte da sua composição e manutenção (Gall et al. 2015). No entanto, estes passam a ser considerados poluentes ambientais à medida em que atividades humanas como transformação, transporte e descarte destes metais se dão de forma inapropriada ou em grande quantidade (Gall et al. 2015). Ao serem incorporados aos alimentos e à água, podem ser ingeridos por humanos e animais, passando a causar efeitos tóxicos através de interferência metabólica e mutagênese (Hogan 2010). O grau dessa interferência vai depender da via de administração ou contato, da concentração, do tipo de metal, bem como do tempo de exposição, podendo exacerbar a sintomatologia e a progressão de doenças pré-existentes (Hogan 2010).

O arsênio é um elemento químico potencialmente perigoso para as populações humanas. A principal fonte de contaminação é a água de beber, devido a sua alta solubilidade (Flora 2015). Este elemento químico apresenta propriedades consistentes tanto com metais quanto não metais, sendo classificado como metaloide. Dentro da abordagem toxicológica, é usualmente mencionado como um metal pesado (Jomova et al. 2011). Este elemento pode ser encontrado na natureza nas formas orgânica e inorgânica e em diferentes estados de oxidação (-3, 0, +3, +5), sendo a forma inorgânica a predominante (WHO 2011). O arsênio pode ser mobilizado para o ambiente através do uso industrial na queima de carvão em usinas termoelétricas, escoamento de rejeitos de minas e do uso comercial no processamento de diversos produtos como semicondutores, baterias, preservação de madeira, munições, papel, vidro e pigmentos, além de produtos farmacêuticos e pesticidas (Flora 2015; WHO 2011).

O limite permitido de arsênio presente na água de beber, estabelecido pela Organização Mundial de Saúde, é de 0,01 mg/L (WHO 2011). No entanto, populações de diferentes áreas em todo o mundo estão expostas a níveis mais altos do que o permitido pela legislação. Na América Latina, elevados níveis de contaminação ocorrem na Argentina, Chile e México. Já na Ásia, os principais países com alta exposição ao arsênio são Bangladesh, Camboja, China, Taiwan, Tailândia, Vietnã e Nepal. Não somente países em desenvolvimento são acometidos pelos problemas decorrentes da exposição ao arsênio. Austrália e Estados Unidos são exemplos de países desenvolvidos altamente industrializados e contaminados (Flora 2015). Estudos epidemiológicos em diversas áreas contaminadas mostraram forte associação entre a ingestão de arsênio inorgânico e câncer, lesões na pele, doença cardiovascular, neurotoxicidade e diabetes (WHO 2011).

Fortes associações entre a exposição a altas concentrações de arsênio (≥ 300 μg/l) ingeridas através de água de beber e câncer de bexiga foram observadas, além de associações ao câncer renal (WHO 2011). O rim é o principal órgão excretor e osmorregulador, possui funções metabólicas, hormonais e também de reabsorção e secreção, desempenhando um importante papel na regulação da homeostase. É conhecido como um órgão-alvo da ação tóxica do arsênio, uma vez que a urina é a principal rota de eliminação deste elemento (Fowler 1992). Por receber um alto fluxo sanguíneo, o rim torna-se mais exposto e susceptível à ação deste metal, atuando na sua biotransformação e consequente eliminação (Madden e Fowler 2000). Após absorção intestinal, o arsênio inorgânico é metabolizado através da metilação, onde a redução do arsênio pentavalente para trivalente e adição do grupo metil gera ácido metilarsônico e dimetilarsínico, que são excretados através da urina (Vahter 2002).

O arsenato (As^{v}) é a forma pentavalente do arsênio inorgânico, e particularmente, pode entrar nas células através da competição pelos sítios de ligação do fosfato em proteínas transportadoras (Hoffman 1976). A substituição do fosfato inorgânico pelo arsenato em reações metabólicas gera ligações covalentes instáveis de baixa energia, interferindo e prejudicando o funcionamento dos mecanismos celulares. Ambos se localizam na família 5A da tabela periódica e possuem constantes de dissociação similares, compartilhando, portanto, características físico-químicas semelhantes (Villa-Bellosta e Sorribas 2008).

Estudos experimentais demonstraram que a exposição ao arsenato é capaz de provocar diversas alterações renais. Danos histopatológicos nos glomérulos e túbulos renais, como congestão glomerular, dilatação tubular, mudança na forma dos túbulos, perda da densidade citoplasmática, deformação das estruturas da borda em escova, além de congestão vascular foram reportados (Kharroubi et al. 2014; Rizwan et al. 2014; Yousofvand and Fahim 2015). A exposição ao arsenato também levou à alteração das concentrações de biomarcadores da função renal, como ureia e creatinina (Kharroubi et al. 2014; Shahid et al. 2014), além das concentrações de colesterol, glicose e fosfato inorgânico séricos (Shahid et al. 2014). O arsenato promoveu alterações nas atividades antioxidantes das enzimas superóxido dismutase, catalase e glutationa peroxidase, além de alterações nas concentrações de proteínas carboniladas e peroxidação lipídica, ambos marcadores de estresse oxidativo (Kharroubi et al. 2014, Kharroubi et al. 2015; Kotyzová et al. 2015). A ação do arsênio, portanto, diminui a concentração de enzimas antioxidantes endógenas, aumenta a incidência de espécies reativas de oxigênio e consequentemente, provoca estresse oxidativo nos rins (Robles-Osorio et al. 2015). O arsênio pode ainda

alterar as atividades enzimáticas associadas à membrana, prejudicando o transporte renal de eletrólitos através das proteínas transportadoras de membrana adenosinas trifosfatases (Prabu et al. 2012; Yin et al. 2017) na sua forma mais tóxica, arsenito (As^{III}). No entanto, a literatura de carece de dados acerca dos efeitos específicos do arsenato sobre este parâmetro.

Estudos experimentais com arsenito demonstraram que esta exposição pode causar efeitos pré-diabéticos em indivíduos saudáveis. A exposição ao arsenito induziu danos oxidativos no pâncreas, levando a um quadro de resistência à insulina e consequente hiperglicemia (Izquierdovega et al. 2006), alterou a homeostase da glicose ao danificar células β pancreáticas, aumentando o risco do desenvolvimento de diabetes gestacional em ratas (Bonaventura et al. 2017) e piorou os efeitos diabéticos em indivíduos com diabetes pré-existente ao alterar o metabolismo dos lipídios, a gliconeogênese e a secreção de insulina (Liu et al. 2014). Poucos estudos atestaram as consequências da ingestão de arsenato. Hill et al. demonstrou que a exposição ao arsenato tem efeitos diretos sobre as concentrações de glicose materna e fetais em camundongos, causando intolerância materna à glicose e defeitos no tubo neural fetal (Hill et al. 2009).

Diversos estudos com populações humanas reportaram a correlação entre a exposição ao arsênio e a diabetes (Bräuner et al. 2014; Sung et al. 2015). Indivíduos diabéticos possuíam maiores concentrações de arsênio na urina, e quanto maior a concentração de arsênio, menor o índice de secreção de insulina (Rhee et al. 2013). A diabetes é uma doença metabólica crônica, decorrente da redução na produção ou na ação da insulina no indivíduo. A diabetes do tipo 1 é caracterizada pela destruição autoimune das células β do pâncreas, tornando a produção de insulina insuficiente. Esta condição acomete principalmente crianças e adolescentes, enquanto a diabetes do tipo 2 desenvolve-se em indivíduos obesos com mais de 40 anos de idade e também em jovens sedentários com maus hábitos alimentares. A diabetes do tipo 2 leva o organismo a um quadro de resistência à insulina, e tanto a incapacidade na produção da insulina ou inabilidade do seu uso pelo organismo aumentam os níveis de glicose no sangue. A hiperglicemia a longo prazo é capaz de danificar diferentes órgãos e tecidos, e debilitar as funções orgânicas como um todo (ADA 2009; Alam et al. 2014).

Em consequência de lesão glomerular, a diabetes pode causar danos severos aos rins, ao aumentar a pressão arterial e a albuminúria, causando nefropatia diabética. Diversos estudos demonstraram que a toxicidade renal induzida pela hiperglicemia estabelece, mantém e estimula a progressão da nefropatia diabética, ao promover danos dentro e fora das células (Balakumar et al. 2009; Powell et al. 2013). Fora das células,

ocorre a formação de produtos finais de glicosilação avançada, que prejudicam a degradação de matriz extracelular, estimulando inflamação e ativando a produção intracelular de espécies reativas de oxigênio (Powell et al. 2013). O estresse oxidativo promove aumento da quantidade de células de defesa liberando diversos mediadores inflamatórios, que promovem alterações histológicas como fibrose, hipertrofia e proliferação celular, além de ativar vias de apoptose (Balakumar et al. 2009).

A toxicidade causada pela hiperglicemia também acarreta diminuição da quantidade de glicosaminoglicanas da membrana basal glomerular, diminuição do número total de glomérulos, expansão mesangial e espessamento da membrana basal glomerular (Pourghasem et al. 2015). Alterações histológicas túbulo-intersticiais também foram observadas em rins diabéticos, como hipertrofia tubular, espessamento da membrana basal tubular e infiltração inflamatória, com consequente fibrose intersticial e atrofia tubular, além de vacuolização e deposição de glicogênio nos túbulos renais (Kralik et al. 2009; Tang et al. 2012).

Experimentalmente, o modelo bem estabelecido para induzir os efeitos da diabetes é baseado no uso da droga estreptozotocina, um antibiótico que leva à destruição das células β do pâncreas. Como resultado, os animais tornam-se deficientes na produção de insulina, e portanto, hiperglicêmicos, apresentando polidipsia e poliúria, características da diabetes mellitus tipo 1 humana (Furman 2015). Algumas das alterações características nos rins dos animais deste modelo consiste na alteração nos níveis de ureia e creatinina séricas, acúmulo de glicogênio na alça espessa ascendente e nos túbulos distais, hipertrofia tubular e aumento no peso do órgão (Rasch 1984; Kang et al. 2005).

O estado diabético eleva os níveis de glicose na urina e o desequilíbrio da osmorregulação nos túbulos renais aumenta a saída de água das células, causando diurese osmótica. Desta forma, ocorre um desbalanço eletrolítico no organismo, alterando as concentrações de sódio, potássio, cálcio e magnésio (Liamis et al. 2014). Como a desidratação resultante da diurese leva a um aumento no consumo de água, a água de beber contaminada por arsênio é uma importante questão a ser considerada em áreas contaminadas.

Portanto, tanto fatores ambientais quanto perturbações metabólicas podem contribuir individualmente para progressão da disfunção renal. Sendo assim, é preciso considerar qual a extensão dos danos provocados pela exposição simultânea ao arsênio (poluente ambiental) na presença da diabetes (doença metabólica).

Desta forma, se torna procedente a hipótese de que animais diabéticos sofrem danos renais mais graves ao serem expostos ao arsênio através da água. Com base no

presente exposto, o objetivo geral deste estudo foi avaliar parâmetros morfológicos, enzimáticos e funcionais no córtex renal de ratos diabéticos expostos ao arsenato de sódio. Para tal, estabeleceu-se como objetivos específicos avaliar a morfologia do tecido renal através de análises morfométricas; quantificar marcadores de apoptose e mitose; determinar a concentração de elementos químicos pertinentes ao funcionamento das células renais, a atividade das enzimas antioxidantes e os níveis dos marcadores de peroxidação lipídica e proteica; analisar a função do transporte renal de eletrólitos; e também, dosar a concentração sérica dos marcadores da função renal.

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ARTIGO

Arsenic exposure aggravates glycogen nephrosis in diabetic rats

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Arsenic exposure aggravates glycogen nephrosis in diabetic rats

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Abstract

It is known that either arsenic exposure or diabetes can alter kidney filtering and excretion. However, it is unclear how these combined factors may influence kidney functions. Therefore, we evaluated morphological and functional kidney parameters in diabetic rats exposed to arsenic. Healthy male Wistar rats and streptozotocin-induced diabetic rats were exposed to 10 mg/L arsenate through drinking water for 40 days. Renal tissue was assessed using morphology, mitosis and apoptosis markers, mineral proportion, oxidative stress markers, as well as the activity of antioxidant enzymes and membrane-bound adenosine triphosphatases. Arsenate ingestion altered glucose levels in healthy animals, but it did not reach hyperglycemic conditions. In diabetic animals, arsenate led to a remarkable increase of glycogen nephrosis in distal tubules. In these animals, additionally, arsenate disturbed the activity of catalase and glutathione s-transferase, besides the proportion of Fe, Cu and K in kidney tissue. Nevertheless, arsenate did not impact negatively other parameters previously altered by diabetes, including levels of malondialdehyde, Na, urea, creatinine, apoptosis and mitosis markers. In conclusion, arsenic exposure and diabetes when combined may impair renal function. Particularly, arsenate may potentialize several mechanisms related to increased glycogen accumulation in tubular renal cells in the diabetic state.

Keywords: arsenate; diabetes; kidney; glycogen nephrosis; nephrotoxicity; toxicologic pathology; environmental pollution

Introduction

Environmental pollution is a matter of great public concern worldwide. Countless toxic substances are disposed every day, everywhere and pollute air, soil and water. Among these toxic substances, arsenic is highlighted as an important contaminant due to its industrial and commercial use in the processing of several products such as ammunition, paper, glass and pigments, as well as pharmaceuticals and pesticides (WHO 2011). Arsenic compounds can be found in different oxidation states (-3, 0, 3 or 5), and arsenate is a pentavalent inorganic form especially occurring in drinking water (IPCS 2001). Hence, kidney could be a target organ for arsenic to exert its toxic effects, as urine is the major route of excretion in the body (Fowler 1992).

Indeed, kidney plays an important role in the biotransformation of inorganic arsenic into organic forms considered less toxic compounds (Madden and Fowler 2000). Accordingly, osmoregulation, secretion, and reabsorption of substances in the kidney can be disrupted (Robles-Osorio et al. 2015). It is well known that arsenic causes oxidative stress, apoptosis, changes in adenosine triphosphatases activity and glucose metabolism, as well as morphological alterations in renal tissue (Kotyzová et al. 2013; Tokumoto et al. 2013; Shahid et al. 2014; Yin et al. 2017). Arsenate can particularly interfere in binding phosphate sites via transporters due to the competition with phosphate groups, impairing in cell mechanisms in multiple pathways (Hoffman 1976).

Bearing in mind the environmental concern itself and all toxicologic pathology regarding arsenic exposure, one question emerges: how organisms already weakened by other diseases would respond to arsenic toxicity? This question becomes more relevant considering that arsenic contamination can occur for a life time in endemic areas (Flora 2015), and its symptoms can be very subtle depending on the arsenic form and concentration. In this context, diabetes is a common metabolic disorder that is reaching epidemic proportions globally. It is characterized by the inability in insulin production or action, resulting in a hyperglycemic state that can impair the activity of the organs, leading to several complications (Alam et al. 2014). Renal toxicity induced by hyperglycemia stablishes, supports and stimulates diabetic nephropathy. High glucose levels in the urine drive to osmoregulatory imbalance in renal tubules and causes osmotic diuresis (Liamis et al. 2014). As the resulting dehydration leads to an increased water consumption, contaminated water by arsenic poses a direct issue to be concerned about in endemic areas, mostly in diabetic individuals poorly treated.

Therefore, it seems reasonable to hypothesize that arsenic exposure aggravates kidney damages previously caused by diabetes. In the present study, we investigated the

possible extent of damages caused by 10 mg/L arsenate exposure in the kidney of diabetic rats. To that end, diabetes was induced by a single streptozotocin injection, which is a well-characterized model of type 1 diabetes (Furman 2015). Thus, we focused on morphology, apoptosis and proliferation markers, mineral concentration, oxidative stress markers, and the activity of antioxidant enzymes and membrane-bound adenosine triphosphatases.

Material and methods

Animals

The use of animals for this study was approved by the Ethics Committee of Animal Use at Federal University of Viçosa, Brazil (protocol 61/2016). Thirty-two male Wistar rats (70 days old) were provided by the Central Animal Laboratory of the Center of Biosciences and Health. The experiment was conducted in accordance to the ethical guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

Experimental design

The animals were individually housed in polypropylene cages under controlled lighting (light/dark cycle) and temperature (21 ºC) with free access to rat chow and drinking water. After 12 h fasting, 16 animals were randomly chosen and diabetes was induced by a single intraperitoneal streptozotocin injection (60 mg/kg in 0.01 M sodium citrate buffer, pH 4.5) (Sigma Chemical Co., St Louis, MO, USA) (Odetti et al. 2003). Seven days later, blood samples from the tail vein were evaluated using a glucometer (Acon Laboratories, Inc., San Diego, CA, USA) in order to determine blood glucose levels. Animals having a blood glucose level higher than 250 mg/dL were included in this study. Thereafter, diabetic animals were divided into two groups (n = 8, each), whereas the other 16 healthy animals were equally divided into other two groups.

Arsenic as sodium arsenate ($Na_2HAsO_4.7H_2O$; Sigma Aldrich Co., St. Louis, MO) at a concentration of 10 mg/L was provided in drinking water *ad libitum* for a group of healthy rats (arsenic control) and diabetic rats (diabetes + arsenic) for 40 days. The other set of diabetic animals (diabetes control) and healthy animals (negative control) received saline solution (0.9 % NaCl) in drinking water *ad libitum* for the same period. During the 40 days of treatment, body weight, daily water intake, and clinical changes were evaluated in all groups. Fasting glucose levels were checked weekly in blood samples from the tail vein, using test strips and glucometer.

Euthanasia and organ collection

After the experimental period, the animals were weighed and euthanized using 150 mg/kg ketamine and 10 mg/kg xylazine by an intraperitoneal injection. The blood was collected by cardiac puncture, and kidneys were removed and weighed. One was frozen in liquid nitrogen and stored at -80 ˚C for biochemical assays and immunofluorescence analysis. The other kidney, in turn, was cut into two fragments. One fragment was used for histopathological and micromineral analysis, whereas the other was used to evaluate the water content in the organ. Renal somatic index (RSI) was calculated using the kidney weight / body weight x 100. For water content calculation, kidney fragments were weighed, and then dried for 96 h at 60 ºC. After drying, the fragments were weighed again.

Markers of renal function

Blood samples collected by cardiac puncture were centrifuged at 2,000 x g for 15 min. The serum was used for quantification of urea and creatinine 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.

Histopathological and stereological analysis

Kidney fragments were immersed in Karnovsky`s fixative solution for 24 h, dehydrated in crescent ethanol series and embedded in glycol methacrylate (Historesin®, Leica, Nussloch, Germany). Histological sections of 3 μm thickness were obtained using rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany). The sections were then stained with hematoxylin/eosin (HE) and periodic acid Schiff (PAS) for histopathological and stereological evaluation under light microscopy. Digital images from the renal cortex PAS-stained at 10x magnification, were capture by brightfield microscope (Olympus BX53, Tokyo, Japan) equipped with a digital camera (Olympus DP73, Tokyo, Japan), and analyzed using the Image-Pro Plus® 4.5 software (Media Cybernetics, Silver Spring, USA). A grid with 266 intersections over a histological field was used to count coincident points over glycogen nephrosis. The number of coincident points over glycogen nephrosis / total number of points in the area x volume of the organ, determined the volume occupied by glycogen nephrosis in the organ. In order to evaluate the number of glomeruli per area, the total number of glomeruli counted in the histological fields was divided by the total area analyzed. Diameter of the glomeruli was measured

for glomerular volume assessment, according to the formula $V = 4/3\pi r^3$, where r corresponds to the mean value of the glomerular diameter divided by 2 (Mandarim-de-Lacerda 2003; Kang et al. 2005). All analyses used 10 digital images of random histological fields for each animal, separated by a 30 μm distance.

Immunofluorescence analysis

Frozen fragments from the renal cortex (n = 5/group) were fixed in 4% paraformaldehyde for 30 min, washed for 30 min, four times, in sodium phosphate buffer 0.1 M at pH 7.2 containing 1% of Triton X-100 (PBST) and incubated at 4°C for 24 h with cleaved-caspase 3 primary antibody (cat #9661; 1:200 dilution; Cell signaling Technology, Inc.) for apoptotic cells detection. Fragments were then washed for 10 min, three times, in PBS and incubated at 4°C for 24 h with FITC-conjugated anti-rabbit IgG secondary antibody (cat #F0382; 1:500 dilution; Sigma-Aldrich). For detection of cell proliferation, fragments were washed for 10 min, three times, with PBS and incubated at 4°C for 24 h with phospho-histone H3 (Ser28) antibody (rat #50-9124-41; ThermoFisher Scientific). Fragments were embedded in glycol methacrylate (Historesin®, Leica, Nussloch, Germany). Sections of 8 μm thickness were obtained using rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany), and then stained with DAPI (4',6-diamidino-2-phenylindole). Slides were evaluated under fluorescence microscopy (Olympus BX53F, Tokyo, Japan) equipped with digital camera (Olympus MX10, Tokyo, Japan). Ten digital images, 40x magnification of random histological fields for each animal, were obtained using the imaging software CellSens. The total number of cells caspase 3-positive and phospho-histone H3-positive counted in histological fields was divided by the total area analyzed.

Analysis of chemical elements

The proportion of the chemical elements arsenic (As), sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), manganese (Mn), zinc (Zn) and selenium (Se) in renal cortex was assessed per area. Fragments were immersed in Karnovsky`s fixative solution for 24 h, dehydrated in ethanol series (80, 90, 95 and 100%), submitted to a 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 by Energy Dispersive X-ray Spectroscopy (EDS) using a scanning electron microscope (Leo 1430VP, Carl Zeiss, Jena, Thuringia, Germany) with an x-ray detector system (Tracor

TN5502, Middleton, WI, USA) at a magnification of 150x, working distance of 10 mm and accelerating voltage of 20 kV.

Antioxidant enzymes activity and oxidative stress markers

The activity of antioxidant enzymes was evaluated in the supernatant of 100 mg of frozen kidney homogenized in ice-cold phosphate buffer saline (PBS) and centrifuged at 3,500 x g at 5 ºC for 10 min. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities, and the oxidative stress markers malondialdehyde (MDA) for lipid peroxidation and protein carbonyl for protein oxidation were assessed. For SOD activity, the pyrogallol method was used based on pyrogallol's ability to catalyze the superoxide (O^{-2}) and hydrogen peroxide (H_2O_2) reaction (Sarban et al. 2005), while CAT activity was estimated by measuring the H_2O_2 decomposition rate (Aebi 1984). The rates of NADPH oxidation were estimated using spectrophotometer for the calculation of GST activity (Habig et al. 1974). Malondialdehyde levels were estimated using 100 mg of kidney fragment homogenized in PBS and incubated with thiobarbituric acid to evaluate the levels of substances able to react with thiobarbituric acid (Buege and Aust 1978). For protein carbonyl quantification, the pellets were used according to the 2.4-dinitrophenylhydrazine (DNPH) method (Levine et al. 1990).

Adenosine triphosphatases (ATPases) activity

Total membrane-bound ATPases activity was measured in the supernatant of 100 mg of frozen kidney previously homogenized in Tris-HCl buffer solution and centrifuged at 3,500 x g at 5 ºC for 10 min. In a microcentrifuge tube, 750 µL of buffer and 250 µL of the mix NaCl 0,1 M, KC1 0,1 M, $MgCl_2$ 0,1 M, $CaCl_2$ 0,1 M, and ATP 0,01 M solution (50 µL of each solution) were added to 50 µL of supernatant. The samples were incubated at 37 ºC for 20 min and the reaction stopped by adding 750 µL of ice-cold 10% TCA (Al-Numair et al. 2015). The samples were centrifuged for 10 min at 1,500 x g and the inorganic phosphorus content in the supernatant was estimated by colorimetric determination (Fiske and Subbarow 1925). The pellets were used for total protein level assessment according to the Bradford method (Bradford 1976), and the total ATPases activity was expressed as µg of phosphorous liberated/min/mg protein.

Statistical analysis

The normality of the results was evaluated by Shapiro-Wilk test, followed by Student's t-test in order to compare the groups separately: negative control x diabetes control, negative control x arsenic control and diabetes control x diabetes-arsenic. Differences were considered significant when $P < 0.05$; The statistical software GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) was used to perform all tests, as well as to design all graphics. Data were expressed as mean ± standard error mean (SEM).

Results

Arsenate changes body and kidney weight in diabetic animals

During the experiment, changes in clinical signs were observed in diabetic animals, particularly. Weight loss, polydipsia and polyuria were the most evident, and diabetic rats had their body and kidney weight increased when submitted to arsenate ingestion ($P > 0.05$), with no changes in RSI ($P < 0.05$; Table 1). Blood glucose levels were altered in healthy animals exposed to arsenate and diabetic animals in comparison to control animals ($P < 0.05$), whereas no significant difference was observed between diabetic animals and those receiving the toxicant ($P > 0.05$; Table 1). Water content in the kidney decreased in diabetic animals in relation to negative control ($P < 0.05$), but it did not differ between animals from diabetes control and diabetes + arsenic groups ($P > 0.05$; Table 1). Finally, urea and creatinine levels increased only in diabetic control group compared to negative controls ($P < 0.05$; Table 1).

Table 1 Blood glucose levels, biometric parameters and markers of renal function of diabetics and non-diabetics rats exposed to 10 mg/L arsenate in drinking water for 40 d

	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
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$^{\#}$
Kidney weight (g)	2.81 ± 0.08	3.00 ± 0.10	2.71 ± 0.05	3.23 ± 0.14$^{\#}$
RSI (%)	0.80 ± 0.01	0.80 ± 0.01	1.48 ± 0.04*	1.54 ± 0.04
Water content (mL/g)	74.80 ± 1.82	72.60 ± 1.93	66.80 ± 1.98*	63.00 ± 2.12
Urea (mg/dL)	51.30 ± 1.52	53.80 ± 2.78	71.90 ± 5.75*	74.70 ± 4.22
Creatinine (mg/dL)	0.49 ± 0.01	0.50 ± 0.01	0.54 ± 0.02*	0.50 ± 0.01

Mean ± SEM. Superscript symbols indicate significant difference ($p < 0.05$) between groups by Student's T-test. †arsenic control x negative control: *diabetes control x negative control. $^{\#}$diabetes+arsenic x diabetes control. RSI: renal somatic index.

Arsenate aggravates glycogen nephrosis in diabetic animals

Histological sections of renal cortex from negative control and arsenic control groups did not show any apparent pathology nor morphology alteration in proximal tubules and glomerulus (Fig. 1). However, it was possible to observe glycogen nephrosis in the distal tubules of animals from both diabetic groups. The volume occupied by glycogen in the tubules increased even more in the diabetes + arsenic rats when compared to diabetes control animals ($P < 0.05$; Fig. 1). Glomerular parameters such as number of glomeruli per area and glomerular volume did not change between groups ($P > 0.05$; Fig. 2).

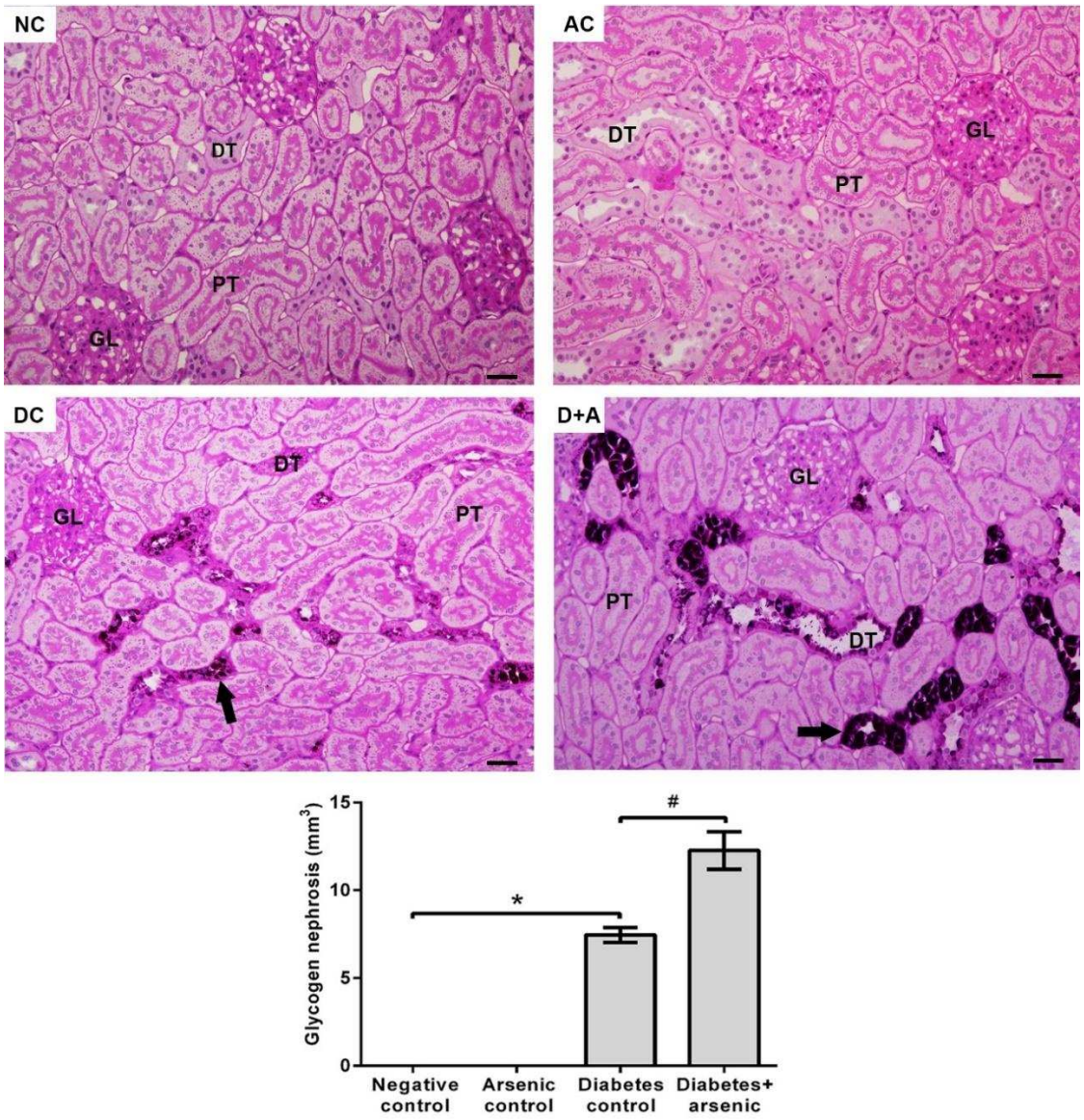

Fig. 1 Glycogen nephrosis in renal cortex of diabetics and non-diabetics rats exposed to arsenate in drinking water for 40 d. NC: negative control (saline solution); AC: arsenic control (10 mg/L sodium arsenate); DC: diabetes control (STZ-induced diabetic rats); D+A: diabetes + arsenic (STZ + 10 mg/L sodium arsenate). Arrow: tubular glycogen deposition. DT: distal tubules; PT: proximal tubules; GL: glomeruli. Superscript symbols indicate significant difference ($P < 0.05$) by Student's t-test: *diabetes control x negative control. #diabetes + arsenic x diabetes control. PAS staining. Scale bar: 20 µm. n = 8 animals/group

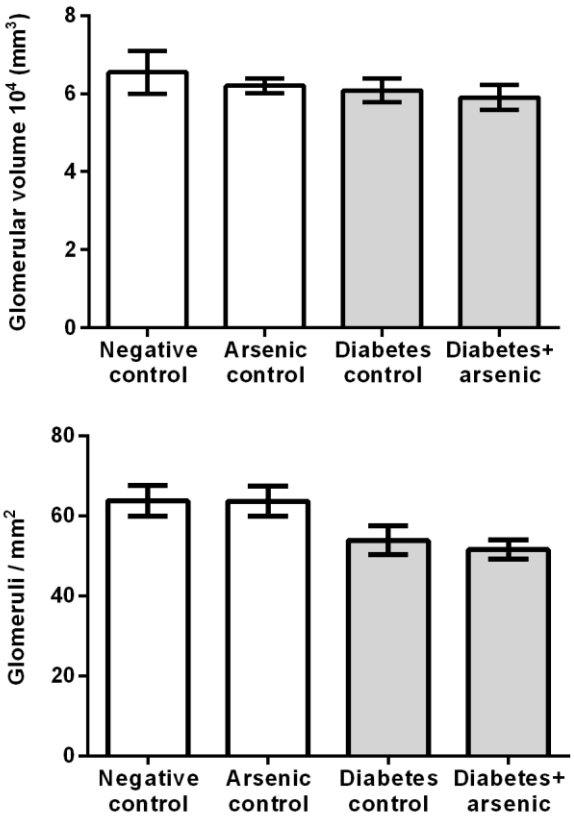

Fig. 2 Glomerular volume and glomeruli per area in renal cortex of diabetics and non-diabetics rats exposed to 10 mg/L arsenate in drinking water. $p > 0.05$. (n = 8 animals/group)

Diabetes increases apoptosis and cell proliferation

Apoptosis and cell proliferation labeling were detected in animals from all groups evaluated (Fig. 3). However, there was a statistically significant increase in these cell markers in the kidney of diabetes control animals compared to the negative controls ($P < 0.05$). Arsenate exposure did not increase the number of labeled cells in diabetic animals compared to their controls (Fig. 3).

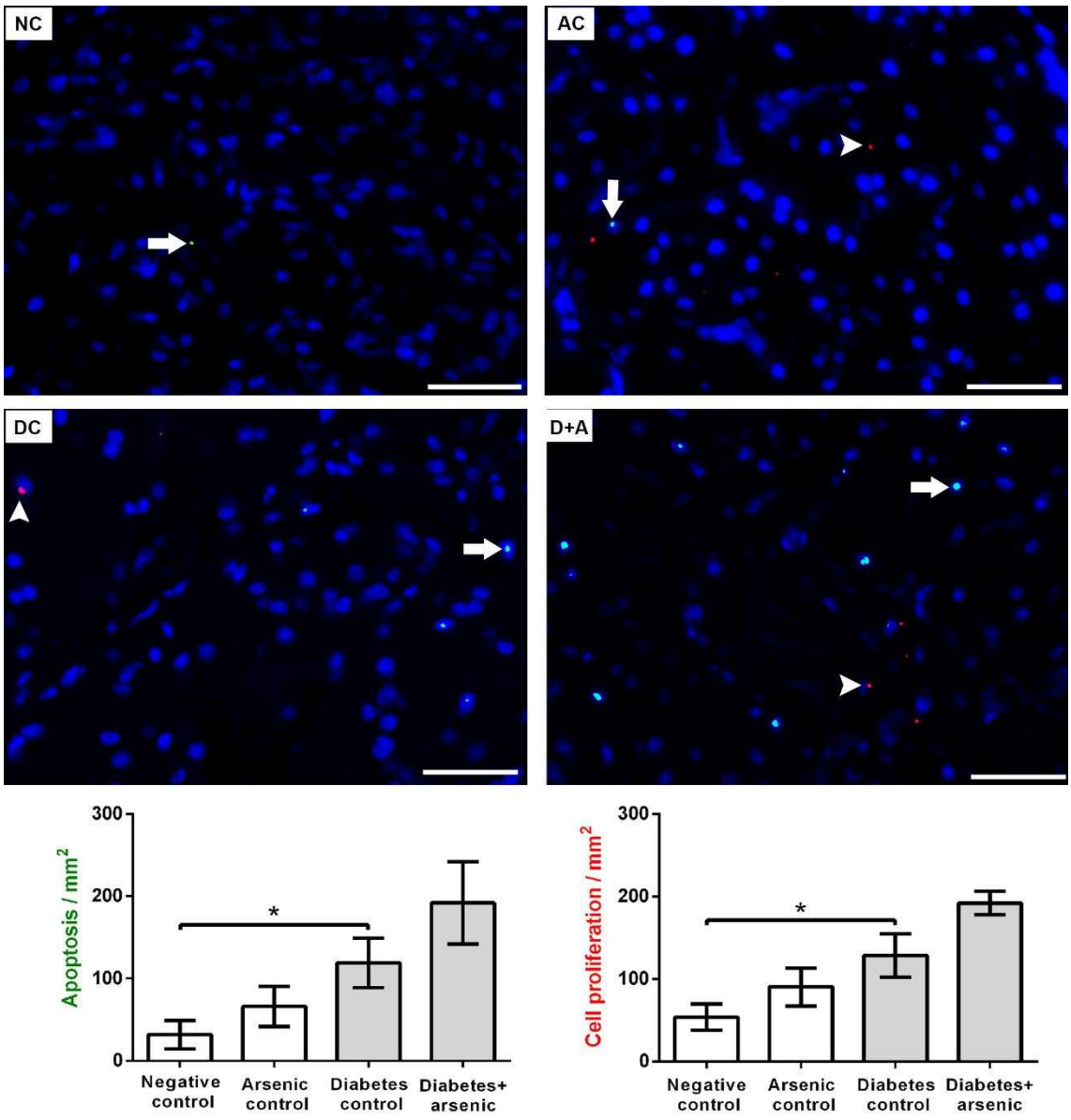

Fig. 3 Apoptosis and cell proliferation in renal cortex of diabetics and non-diabetics rats exposed to arsenate in drinking water for 40 d. NC: negative control; AC: arsenic control (10 mg/L sodium arsenate); DC: diabetes control (STZ-induced diabetic rats); D+A: diabetes + arsenic (STZ + 10 mg/L sodium arsenate). Arrow: apoptotic cells (green); arrow head: proliferative cells (red). Nuclei are stained with DAPI. Superscript symbols indicate significant difference ($P < 0.05$) by Student's t-test: *diabetes control x negative control. Scale bar: 20 µm. n = 5 animals/group.

Arsenic changed the proportion of K, Fe and Cu in diabetic animals but it did not accumulate in kidney

The proportion of As in the kidney remained practically unchanged in all groups after the experimental period ($P > 0.05$; Fig. 4). However, Na proportion increased in diabetes control animals when compared to negative controls ($P < 0.05$; Fig. 4). The proportion of K, in turn, decreased between these same groups. Further, the proportion of this element increased in diabetic animals exposed to arsenate when compared to diabetic control ($P < 0.05$; Fig. 4). Fe had its proportion increased while Cu decreased in diabetes

+ arsenic group when compared to diabetes controls ($P < 0.05$; Fig. 4), in contrast to the results observed in diabetic control animals and animals from negative control group ($P > 0.05$; Fig. 4). The proportion of Ca and Mg did not change between groups, as well as Mn, Zn and Se ($P > 0.05$; Fig. 4).

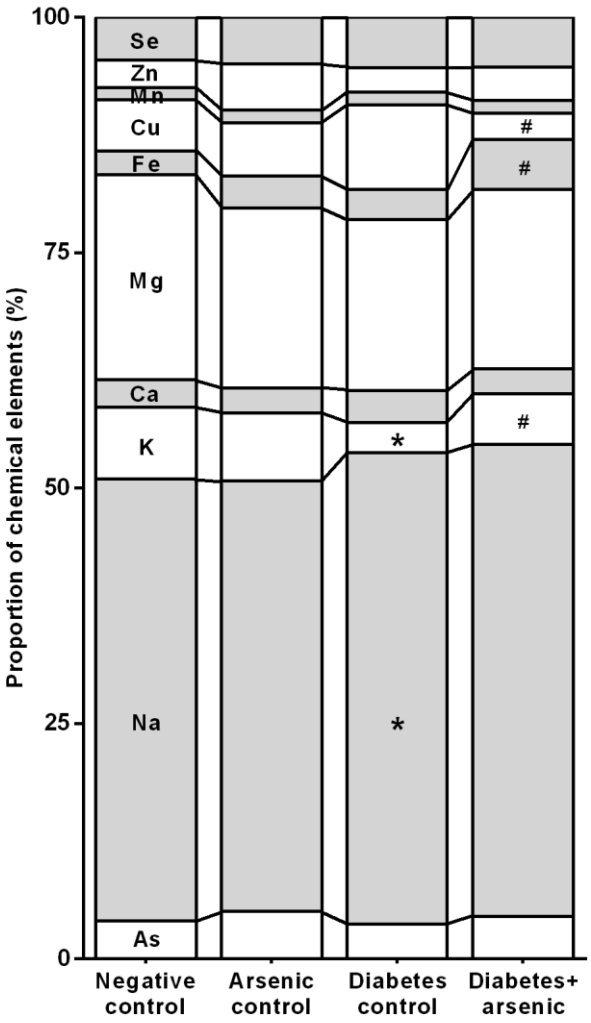

Fig. 4 Proportion of chemical elements in the kidney of diabetics and non-diabetics rats exposed to 10 mg/L arsenate in drinking water. As: arsenic; Na: sodium; K: potassium; Ca: calcium; Mg: magnesium; Fe: iron; Cu: copper; Mn: manganese; Zn: zinc; Se: selenium. Symbols indicate significant difference ($P < 0.05$) by Student's t-test: *diabetes control x negative control. #diabetes + arsenic x diabetes. n = 5 animals/group.

Arsenic altered the antioxidant enzymes activity in diabetic rats, and it did not change total ATPase activity and oxidative stress markers

The activity of SOD in the kidney did not change between groups ($P > 0.05$; Fig. 5). However, CAT activity decreased in diabetes control and diabetes + arsenic groups compared to negative control and diabetes control groups, respectively ($P < 0.05$; Fig. 5). Moreover, GST activity was increased in diabetes + arsenic animals when compared to diabetes controls ($P < 0.05$; Fig. 5).

Levels of MDA in kidney tissue were increased in diabetes control group when compared to negative controls ($P < 0.05$), while no changes were observed in arsenic control and diabetes + arsenic groups when compared to negative control and diabetes control, respectively ($P > 0.05$; Fig. 5). CP levels did not differ between groups as well as the activity of membrane-bound ATPases ($P > 0.05$; Fig. 5).

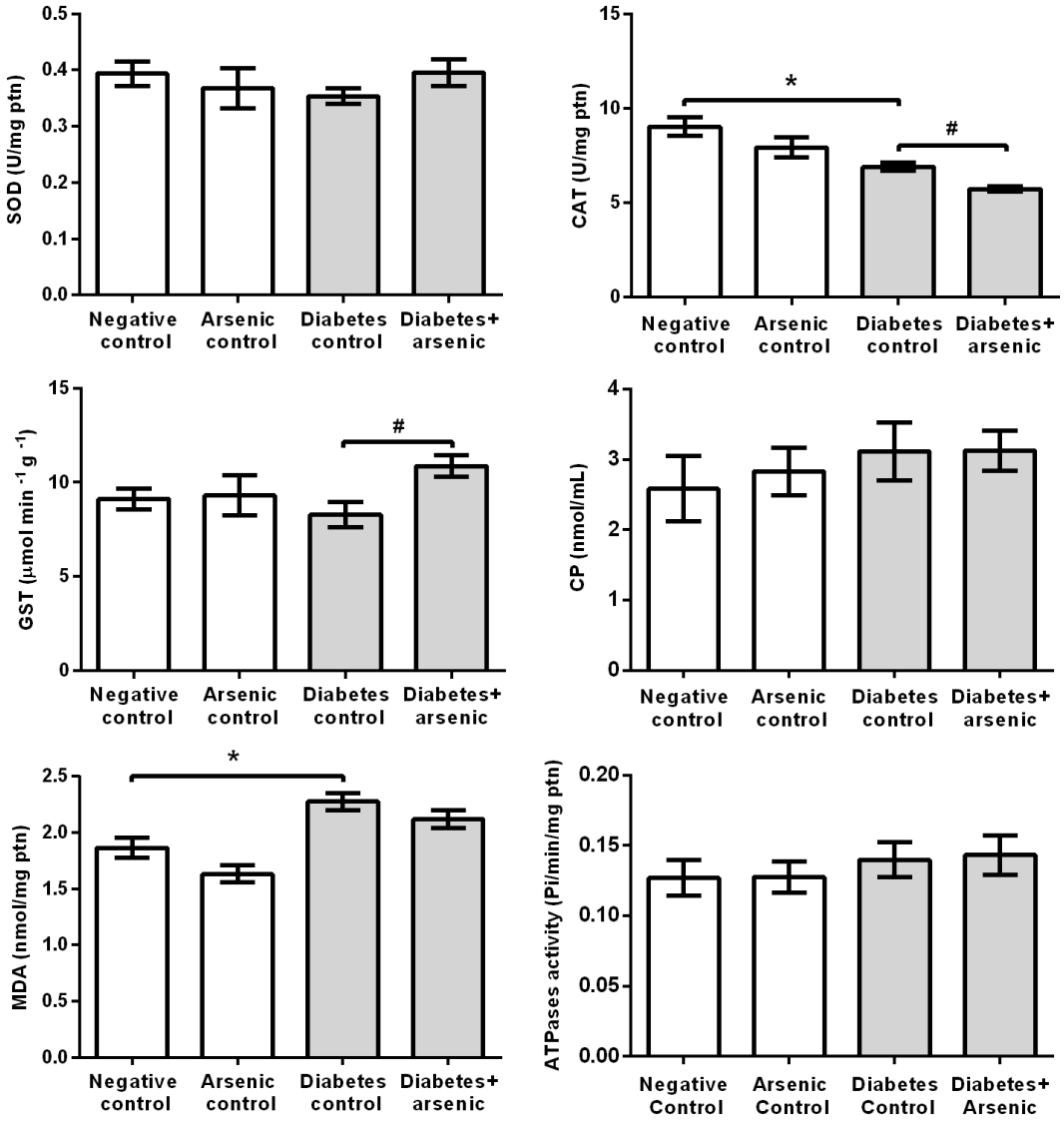

Fig. 5 Antioxidant enzymes activity, oxidative stress markers and total ATPase activity in the kidney of diabetics and non-diabetics rats exposed to 10 mg/L arsenate in drinking water. SOD: superoxide dismutase; CAT: catalase; GST: glutathione S-transferase; MDA: malondialdehyde; CP: carbonyl protein. Symbols indicate significant difference ($P < 0.05$) by Student's t-test: *diabetes control x negative control. #diabetes + arsenic x diabetes. n = 8 animals/group.

Discussion

This study investigated the effects of arsenate exposure over morphological and functional parameters in the kidney of streptozotocin-induced diabetic rats. Our results showed that the combination of a preexistent diabetic state and arsenate ingestion disturbed the activity of catalase and glutathione s-transferase, besides the proportion of Fe, Cu and K in renal tissue and the aggravation of diabetic glycogen nephrosis, the remarkable feature observed in this study.

Glycogen nephrosis is a morphological alteration characterized by glycogen accumulation in renal tubular cells (also called Armanni-Ebstain lesion). It is commonly observed in the thick ascending limbs and distal tubules of streptozocin-induced diabetic rats (Rasch 1984; Holck and Rasch 1993). Herein, a great increase of this lesion was mainly observed in diabetic rats exposed to 10 mg/L sodium arsenate. Several mechanisms have been proposed to explain the glycogen accumulation in tubular renal cells. Glucose filtered by the glomeruli is reabsorbed by the proximal tubules and so, the higher glucose levels of diabetic animals can overwhelm the capacity of these tubules. This way, glucose become available for the distal segment, which is not able to handle glucose efficiently. The distal segment contains higher levels of glucose metabolism enzymes that can lead to glycogen accumulation (Ritchie and Waugh 1957; Rasch and Osterby 1989). In addition, glucose-6-phosphate (G6P) is an allosteric activator of glycogen synthase and was found to be in higher levels in these glycogen-filled cells (Cammisotto et al. 2008). The activity of the protein phosphatase 2A (PP2A) was also found to be higher. The activation of PP2A leads to AMP-activated protein kinase (AMPK) dephosphorylation that, in turn, activates glycogen synthase (Vallon and Komers 2011). Interestingly, serum glucose levels were higher in healthy animals receiving arsenate, and although not significant, serum glucose levels were increased in diabetic animals receiving arsenate. This slight increase may have been enough to influence major glycogen accumulation in the animals. Moreover, arsenate is capable of competing with phosphate groups, disrupting glucose-6-phosphatase activity, and consequently G6P breakdown (Rizwan et al. 2014; Shahid et al. 2014). Arsenic can also activate PP2A and impair AMPK activity *in vitro* (Zhu et al. 2014).

In the current study, additionally, diabetic animals receiving arsenate did not lose weight as diabetic control animals. This fact may be related to its interaction to lipid metabolism. Fatty acids are increased in diabetes due to insulin deficiency that stimulates lipolysis in adipose tissue (Guder et al. 1992). Hyperglycemia could compensate glucose uptake with glycogen accumulation due to competition with free fatty acids in circulation

in the diabetic state (Randle et al. 1994). Thus, diabetes is able to disrupt lipid metabolism and induce tubular changes (Guder et al. 1992). Cammisotto et al. (2008) showed that adiponectin signaling is impaired in diabetes. Globular adiponectin binds the adiponectin receptor 1 in distal tubular cells and activates AMPK, suppressing glycogen synthase in normal state. In diabetic rats, this signaling is altered and AMPK activity is reduced, compromising glycogen synthase regulation. Furthermore, arsenic was found to impair adiponectin (Song et al. 2017) and free fatty acids levels in plasma (Muthumani and Prabu 2014; Afolabi et al. 2015), which might have contributed to the increased glycogen accumulation.

Nevertheless, RSI did not change in diabetic animals exposed to arsenate, probably due to the increase in kidney weight. Changes in kidney weight, tubular length and diameter of tubular cells are characteristics of streptozotocin-induced diabetes (Rasch 1984). In the latter, these alterations are attributed to the damage caused to the tubular cells, leading to a compensatory hypertrophy and increase in the number of cells (Kang et al. 2005). Herein, the significant increase in the expression of the mitosis marker phospho-histone H3 occurred in diabetic animals, as well as those exposed to arsenate though the difference between them was not statistically significant.

Our findings showed significant increase of caspase 3 activation in diabetic control group. Indeed, cell death in glycogen-accumulating cells can be mediated by Fas/Fas-L pathway with activation of caspase-3 (Bamri-Ezzine et al. 2003). As oxidative stress can trigger cell death, MDA was used herein as a marker for lipid peroxidation. In this sense, MDA levels increased in diabetic animals, reflecting decreased levels of the antioxidative enzyme CAT. This fact probably influenced the high levels of urea and creatinine found in this group, which are important markers for renal damage.

In contrast, no differences were observed between diabetic animals and those exposed to arsenate in relation to the expression of apoptosis and mitosis markers. Although the increased number of cells expressing those markers in the kidney of diabetic animals exposed to arsenate, the high variance between samples was probably the main reason for this result. Arsenic is known by its deleterious effects in renal tubular cells through several routes leading to apoptosis, including activation of caspase-3 (Robles-Osorio et al. 2015). Moreover, arsenate was able to impair CAT activity in our diabetic rats. The activity of antioxidant enzymes lies on the availability and mobilization of essential minerals (Soetan et al. 2010). CAT requires Fe or Cu to play its role in tissue detoxification through Fenton reaction (Valko et al. 2016), and its levels were found disturbed in this study. Mn and Zn are required for SOD activity in cytosol and

mitochondria respectively (Brasileiro Filho 2008), and neither their levels, nor SOD activity were disrupted by diabetes or arsenate exposure. Moreover, the concentration of Se, an essential mineral for GST activity (Zwolak and Zaporowska 2012), was not impaired in the groups evaluated. However, the activity of the antioxidative enzyme GST was increased in diabetes rats exposed to arsenate, which may have slowed apoptosis down through oxidative stress in these animals, which could be also indicated by the unchanged MDA levels found.

Although Na e K concentrations were disturbed in the renal tissue, no change was observed between groups regarding total ATPases activity, neither Ca e Mg concentrations. Higher concentration of Na in diabetic state can occur due to the high concentration of glucose in the glomerular ultrafiltrate, once they are co-transported in proximal tubular cells (Vallon and Komers 2011). Increase in Na and glucose concentration inside the cell leads to an osmotic change and consequently, to polyuria and polydipsia (Liamis et al. 2014). Polyuria caused by the osmotic diuresis leads to dehydration and decreased water content could be observed in the kidney of diabetic groups. Na overload as well as the increase in tubular flow are able to promote greater K secretion in distal tubules (Welling 2013; Udensi and Tchounwou 2017). These facts were observed in our diabetes animals and consequently, decreased the K concentration in the tissue. In this study, arsenate was not able to increase Na levels in diabetic neither healthy rats. However, arsenate increased K levels in diabetic animals. Interestingly, K is required to glycogen synthesis and storage, and glycogenesis was found to be impaired in the kidney of K-depleted rats (Schaefer et al. 1985). It might be possible that K was not secreted as an adaptive response to the impaired glycogenesis in distal tubules of diabetic rats exposed to arsenate, contributing to glycogen accumulation in the cells.

Nevertheless, glomerular changes were not observed in this study. In streptozotocin-induced diabetic rats, tubular changes can be seen within days whereas markedly glomerular changes can be seen in long-term experiments (Nannipieri et al. 2001; Singh et al. 2018). The duration of our study was not enough to cause these marked changes and it could also have influenced the lack of arsenic accumulation in kidneys. None of the groups exposed to arsenate presented any significant difference when compared to controls. In spite of that, the differences observed in other several parameters indicate that arsenate was able to exert its toxic effects, especially regarding glycogen nephrosis in diabetic animals. A long-term experiment could lead to glomerular changes both because of diabetes complications and arsenate exposure. This way, urinary excretion of arsenic would decrease and arsenic would probably accumulate in the tissue.

Furthermore, long-term experiments show the progression from glycogen tubular cells to clear cell renal cell carcinoma (ccRCC), indicating glycogen nephrosis as a preneoplastic lesion in streptozotocin-induced diabetic rats and spontaneous diabetic rats (Dombrowski et al. 2007; Ribback et al. 2015). In hematoxylin-eosin stained sections, the glycogen-filled tubules appear empty and resemble ccRCC. In addition, ccRCC presented high levels of glycogen synthase activity and lack of levels of glucose-6-phosphatase activity. Glycogen nephrosis and ccRCC presented the same expression patterns of growth factors, receptors and signal transduction proteins (Dombrowski et al. 2007). PI3K/AKT/mTOR pathway is linked to carcinogenesis and related metabolic alterations on it were found to be activated in both human ccRCC and in rat glycogen nephrosis (Ribback et al. 2015). Of note, several studies have reported that arsenic activates PI3K/AKT/mTOR pathway, as well as its carcinogenic effects *in vitro*, in rats and humans (Chen and Costa 2018). In humans, ccRCC can be related to disturbed adiponectin levels (Wang et al. 2016) and arsenic-related renal cell carcinoma susceptibility can be increased by adiponectin gene polymorphisms (Hsueh et al. 2018).

Conclusion

Altogether, arsenate may potentialize several mechanisms related to increased glycogen accumulation in tubular renal cells in the diabetic state. In this study, glycogen metabolism disturbance due to arsenate exposure could be indicated by changes in glucose levels and K concentration. Obviously, other changes in carbohydrate metabolism, as well as changes in lipid metabolism cannot be ruled out. For this reason, further experiments and analyses should be performed in order to clarify the exact mechanism leading to this increased pattern of glycogen nephrosis. Finally, arsenate exposure and diabetes when combined could impair renal function.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

MNS, ACFS and MM-N designed the research study. MNS, ACFS, DSSB, FCS, LOGE and KMF performed the experiments. MNS analyzed the data, prepared the figures and wrote the manuscript. ACFS, LLO and MM-N. contributed to discussion and reviewed the manuscript critically. All authors read and approved the final manuscript.

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CONCLUSÕES GERAIS

Os resultados deste trabalho indicam que a exposição a 10 mg/L de arsenato na água de beber altera alguns parâmetros renais já comprometidos pela diabetes induzida através da estreptozotocina em ratos. A alteração marcante observada neste estudo consiste no grande aumento do acúmulo de glicogênio nos túbulos distais destes animais. Além disso, o arsenato foi capaz de modificar a atividade das enzimas antioxidantes catalase e glutationa s-transferase, a concentração dos elementos químicos ferro, cobre e potássio, e ainda, aumentar o peso corporal e dos rins de animais diabéticos.

O aumento de peso dos rins pode ser associado ao aumento na intensidade de nefrose glicogênica. Esta patologia pode estar correlacionada à alteração na concentração de potássio renal, uma vez que este elemento é requerido no metabolismo do glicogênio. A alteração na porcentagem dos elementos ferro e cobre, necessários para ação antioxidante da enzima catalase, demonstra que o arsenato tem o potencial para comprometer a defesa antioxidante do organismo. No entanto, as alterações observadas em animais diabéticos que foram expostos ao arsenato não interferiram nos demais parâmetros analisados.

Contudo, outros experimentos e análises devem ser realizados para a elucidação mais detalhada dos mecanismos de ação do arsenato sobre animais diabéticos, especialmente os mecanismos que modulam o aumento da concentração de glicogênio nos túbulos renais e suas consequências para o estado diabético.