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# The Antiapoptotic Effects of Sulphurous Mineral Water and Sodium Hydrosulphide on Diabetic Rat Testes

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**Key Words**

Diabetes • Testes • Apoptosis • Bax/Bcl-2 • $H_2S$

**Abstract**

Background/Aims: It is well known that diabetes mellitus is associated with the impairment of testicular function. In the present study, we aimed to study the effects of sulphurous mineral water or sodium hydrosulphide (NaHS) on apoptotic testicular damage in rats with streptozotocin (STZ)-induced diabetes. Methods: Sulphurous mineral water (as drinking water) or NaHS (14 µmol/kg body weight/day, I.P.) was administered for 7 wks to rats with STZ-induced diabetes. Results: Hyperglycaemia, an overproduction of glycated haemoglobin (HbA1C) and a decline in serum insulin, C-peptide and insulin-like growth factor-I (IGF-I) were observed in diabetic rats. A decline in the serum testosterone level and an impairment of spermatogenesis, as indicated by a histopathological examination of diabetic rats, demonstrated significant testicular damage. Sulphurous mineral water and NaHS treatment may have improved the level of testicular GSH by blocking the overexpression of some apoptosis-related regulatory proteins such as Bax/Bcl-2, cytochrome c, caspase-9 and -3, and p53. This anti-apoptotic potential was associated with an increase in serum testosterone level and the amelioration of hyperglycaemia-related biochemical parameters. The histopathological examination was in harmony with the biochemical and molecular findings. Conclusion: Our study provides the first indication that sulphurous mineral water and NaHS may have a novel anti-apoptotic potential that could be a useful treatment in preventing diabetes-induced testicular dysfunction.



## Introduction

Diabetes mellitus is a common health problem and a serious metabolic disorder associated with many functional and physiological complications. Diabetes is frequently associated with sexual dysfunction in men and experimental animals, and it is accepted that infertility is a common complication in approximately 90% of diabetic men [1-3]. The number of young diabetic patients has undergone a significant increase [4, 5]. Therefore, infertility or the reduced ability to impregnate that these young

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diabetic patients experience has become a major concern [6-8]. Low testosterone levels, testicular dysfunction and spermatogenic disruption in the testis have been observed in diabetic men and experimental animals, which can lead to a decrease in libido, erectile dysfunction and a reduction in sperm motility and semen volume. Understanding the mechanisms and signalling pathways underlying diabetes-induced male germ cell death is essential to the development of a strategy to prevent the loss of spermatogenic cells for diabetic patients.

Although the mechanisms involved in the development of such changes have not been thoroughly characterised, an increase in apoptotic cell death in the testicular germ cells of diabetic rats has been reported and has been considered to be the main reason for the infertility of diabetic patients and animals [6-10]. Apoptosis, known as programmed cell death, is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell populations. Control of apoptosis is critical for normal spermatogenesis in the adult testes [11, 12]. The testis is sensitive to cellular damage induced by environmental exposure. Apoptosis of germ cells may occur during nonphysiological stresses such as diabetes, ischemia and hyperthermia [13, 14]. Moreover, current studies have indicated that diabetes-mediated oxidative stress can induce apoptosis [10, 15]. However, few data are available regarding the expression of apoptosis-related proteins in the testes of diabetic animals.

Sulphur (S) is an interesting non-metallic element representing approximately 0.25% of the human total body weight [16, 17]. As a part of the amino acids methionine, cysteine and taurine, S performs a number of functions in enzyme reactions and protein synthesis. It is necessary for the formation of collagen, keratin and taurine. S is also part of other important body chemicals such as insulin, insulin-like growth factor-I (IGF-I), transforming growth factor-β1 (TGF-β1) and glutathione (GSH). For all of these reasons, sulphurous mineral water employed in thermal medicine, containing S in the form of sulphate ($SO_4^{-2}$ > 200 mg/L) and/or hydrogen sulphide ($H_2S$ > 1 mg/L), has a long history of use in the treatment of various clinical conditions, from dermatological and musculoskeletal disorders to aging and age-related degenerative diseases [18-20]. Caraglia et al. [21] demonstrated the anti-oxidant effect of mud therapy in mice with osteoarthritis, showing a significant decrease in the production of endogenous nitric oxide (NO) [22]. In addition to mud and bath therapies, therapies involving drinking water containing S (hydropinic treatments) are employed in thermal medicine, particularly because of their action on gastroenteric and hepatic functions.

Despite its long-standing reputation as a foul-smelling and toxic gas that is associated with the decay of biological matter, $H_2S$ has emerged as an important regulator of a number of cellular signals that regulate metabolism. There appear to be several options for $H_2S$ therapy including $H_2S$ gas, $H_2S$ donors or releasing compounds and $H_2S$ pro-drugs that activate $H_2S$-generating enzymes to increase the circulating and tissue levels of $H_2S$ [23]. Brancaleone et al. [24] demonstrated the progressive decline in vascular reactivity, plasma $H_2S$ levels and vascular $H_2S$ production as the severity of diabetes increased over time in a diabetic mouse model.

Recently, we have shown the first evidence that sulphurous mineral water and sodium hydrosulphide (NaHS), through the action of $H_2S$; have anti-fibrogenic and anti-apoptotic effects on the hearts of rats with streptozotocin (STZ)-induced diabetes [25]. In the present study, we aimed to further investigate the possible beneficial effects of these sulphur-based treatments on testicular apoptosis in diabetic rats through the measurement of the gene expression levels of some of the apoptosis-related regulatory proteins such as Bcl-2 (B-cell lymphoma 2), Bax, cytochrome c, caspase-9 and -3 and p53 (also known as protein 53 or tumour protein 53).

## Materials and Methods

*Drugs and chemicals*

STZ and 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemicals Company, St. Louis, MO, USA. All other chemicals used were of the highest purity and analytical grade.

*Experimental design*

Forty two male Wistar rats weighing 200-220 g were used in this study. Animal care was supervised and approved by the local ethical committee. Animals had free access to rat chow and water throughout the study. Diabetes was induced by a single intraperitoneal injection of STZ, 50 mg/kg body weight, freshly prepared in 0.1 M citrate buffer, pH 4.5 [26]. A normal control group (n = 7) was injected with the appropriate volume of the citrate buffer. During the first 24 h of diabetes induction, STZ-treated rats were allowed to drink 5% glucose solution in order to avoid hypoglycemia resulting from massive destruction of beta cells and release of intracellular insulin associated with STZ treatment [27]. Four days later a blood sample was collected from the tail bleeding and hyperglycemia was confirmed by a blood glucose level ≥ 300 mg/dl. Glucose was determined using a commercial glucometer (Roche Diagnostic Accu-Check test strips, Germany).

Diabetic rats were randomly divided into three groups (n = 7 rats in each group). The first group was the untreated-

| Parameter | Unit | Result |
|---|---|---|
| pH | - | 6.69 |
| Conductivity | µS/Cm | 6590 |
| Fixed Residue (at 180°C) | mg/L | 6480 |
| Sulphuric degree | mg/L | 8.4 |
| $CO_2$ | mg/L | 80 |
| $Ca^{2+}$ | mg/L | 403 |
| $Mg^{2+}$ | mg/L | 49 |
| $Na^{+}$ | mg/L | 1300 |
| $K^{+}$ | mg/L | 2 mg |
| $HCO_3^{-}$ | mg/L | 180 |
| $F^{-}$ | mg/L | 1.5 |
| $Cl^{-}$ | mg/L | 1560 |
| $NO_2^{-}$ | mg/L | < 0.01 |
| $SO_4^{2-}$ | mg/L | 844 |
| $NO_3^{-}$ | mg/L | 0.04 |
| Iron | mg/L | 0.006 |

**Table 1.** Physico-chemical characteristics of the sulphurous mineral water of Helwan Kabritage. Reprinted from Arch Biochem Biophys, Vol. 506 (1), El-Seweidy MM, Sadik NA, Shaker OG, Sodium hydrosulfide as potent inhibitors of fibrosis in the heart of diabetic rats, Pages No., 48-57, Copyright (2011), with permission from Elsevier [25].

| Parameter | Primer sequence | PCR product size (bp) |
|---|---|---|
| Cytochrome c | F: TCGAGGTCATGGAGAGAAATGA<br>R: AGTTGCAAGGACGTGCAGTTTA | 227 |
| Caspases 9 | F: ACAAGGCCTTCGACAGTG-3<br>R: 5 -GTACCAGGAACCGCTCTT-3 | 175 |
| Caspases 3 | F:TGAGTTCCTTCCTTTCTTTGTGC<br>R: GAAAGCATCCGGCAATAGGC | 283 |
| Bcl-2 | F: ATGATAACCGGGAGATCGTG<br>R: GACGGTAGCGACGAGAGAAG | 294 |
| Bax | F: CTGAGCTGACCTTGGAGC<br>R: GACTCCAGCCACAAAGATG | 413 |
| p53 | F: CACCTATGATCCTGACAAATACAGG<br>R: TGTAGCATTGAAGAATTGTGCG | 175 |
| β-actin | F:5'TCACCCTGAAGTACCCCATGGAG3'<br>R:5'TTGGCCTTGGGGTTCAGGGGG3 | 150 |

**Table 2.** Sequence of all primers used in the experiment.

diabetic group. The second group was supplied daily with sulphurous mineral water from the Thermal Center of Helwan (Helwan Kabritage, Helwan province; south of Cairo, Egypt), which has a sulphuric degree of 8.4 mg/L, as reported in Table 1 instead of their drinking water [28]. The rats in the third group were intraperitoneally injected with NaHS ($H_2S$ donor), at a dose of 14 µmol/kg body weight/day [29]. Since NaHS was considered a toxic substance, we injected NaHS (14 µmol/kg body weight/day) alone to control rats (n = 7) as the treatment control. Warenycia et al. [30] reported an LD50 value of 15 mg/kg body weight (approximately 192 µmol/kg body weight) for NaHS in rats, which is higher than the dosage we used. Another control group (n = 7) was supplied with sulphurous mineral water to exclude any toxic effect of $H_2S$-rich water consumption.

After 7 wks and immediately before sacrificing, the body weight of each rat was determined. Blood was collected and divided on two specimens; one is processed for serum preparation used for assaying the levels of insulin, IGF-I and C-peptide. The other portion was collected in heparinized tubes with a glycolytic inhibitor and used for the estimation of glycated hemoglobin (HbA1C) % and glucose level. Testes were removed, immediately immersed in ice-cold physiological saline and dried. Sections of testes were used for histopathological examination and the remaining was stored at -30°C until use for of biochemical and molecular assessments.

*Biochemical investigations in blood and serum*

*Assay of glucose, HbA1C, insulin and C-peptide levels.* Plasma level of glucose and the percentage of HbA1C were assayed using commercially available kits provided by Stanbio (San Antonio, TX, USA), according to the methods of Trinder [31] and Abraham et al. [32] respectively. Serum insulin level was measured by immunoradiometric assay using kits provided by Immunotech, France. Serum C-peptide level was measured using solid-phase, two site chemiluminescent immunometric assay using kits supplied by DPC on Immulite analyzer,USA. Serum levels of insulin (µIU/ml) and C-peptide (ng/ml) were calculated by interpolation from a standard curve.

*Assay of IGF-I and testosterone levels.* IGF-I Enzyme - linked Immunoassay (ELISA) Kit (DRG Instruments GmbH, Germany) was used to determine serum IGF-I level. Serum testosterone level was determined using a competitive enzyme immunoassay kit provided by Monobind Inc., Lake Forest, CA 92630 USA. The concentration of the samples was read directly from a standard curve. IGF-I and testosterone levels were expressed as (ng/ml).

*Measurement of testicular GSH level.* GSH level (mg/g wet tissue) in the testes was estimated after de-proteinization and reaction with 5,5-dithionitrobenzoic acid according to the method of Beutler et al. [33].

*Molecular biology assays in the testes*

*RNA extraction and reverse-transcription.* Total RNA was isolated from testes using RNeasy Mini Kit (Qiagen). All RNA preparations were stored at -80°C until use. Total RNA (4 mg) was reverse transcribed in a final volume of 20 uL with 20 mg random hexamer and 200 U MuLV (Promega, Madison, WI), according to the manufacturer's guidelines.

*Primer sequences.* Primers were designed with the Primer3-Blast software (NCBI, USA). All primers were synthesised by the Midland Certified Reagent Company Inc. (Midland, Texas, USA). The sequences of the oligonucleotide primers used for polymerase chain reaction (PCR) and the size of the amplified products are given in Table 2. The sequences of the oligonucleotide primers for each gene obtained from cDNA sequences registered at GeneBank do not share significant sequence homology with other genes, as evaluated by a BLAST search. The gene expression of β-actin was measured as a standard housekeeping gene.

| | Normal | Sulphurous water | NaHS | Diabetic | Diabetic + sulphurous water | Diabetic +NaHS |
|---|---|---|---|---|---|---|
| Final body weight | 310±21 | 250±14 | 252±13.5 | 170±20 $^{a}$ *a1#a2# | 258±19 $^{b\#}$ | 203±11 $^{a\#\#}$ |
| Glycemia | 95.2±3 | 115±7 | 106.8±5 | 412.5±24 $^{a*a1*a2*}$ | 195±18.8 $^{a*a1\#\#a2\#\#b*}$ | 180±15 $^{a\#\#a1\#a2\#b*}$ |
| HbA1C | 5.02±0.37 | 5.4±0.3 | 5.7±0.4 | 11.7±0.66 $^{a*a1*a2*}$ | 8.16±0.5 $^{a\#\#a1\#a2\#\ b\#\#}$ | 8.09±0.8 $^{a\#\#a1\#\ b*}$ |
| Insulin | 25.7±2 | 30.6± 2 | 24.2±1.5 $^{a1\#}$ | 5.7±0.2 $^{a*a1*a2*}$ | 12.2±1.2 $^{a*a1*a2*b\#}$ | 12.5±0.9 $^{a*a1*a2*b\#}$ |
| C-peptide | 2.9±0.17 | 2.9±0.2 | 2.4±0.1 | 1.06±0.07 $^{a*a1*\ a2*}$ | 1.7±0.06 $^{a*a1*a2\#\#\ b\#}$ | 2.02±0.07 $^{a*\ a1*b*}$ |
| IGF-I | 195 ±13 | 255.1±25 | 190.5±10.2 | 93±4.7 $^{a\ \#\#\ a1*\ a2\#\#}$ | 181.3 ±19.6 $^{b\#}$ | 176.6±26 $^{a1\#\ b\#}$ |
| Testosterone | 7.58±1.34 | 7.40±0.85 | 6.82±0.55 | 0.47±0.09 $^{a*a1*a2*}$ | 3.94±0.61 $^{a*a1*a2*b*}$ | 3.54±0.65 $^{a*a1*a2*b*}$ |

**Table 3.** Final body weight (g), Glycemia(mg/dl), HbA1C (%),serum insulin (µIU/ml),C-peptide (ng/ml), IGF-I (ng/ml), testosterone (ng/ml) in studied rat groups**.** Data are represented as means ± SE for seven rats. (a) significant difference from the normal group; (a1) significant difference from the sulphurous water group; (a2) significant difference from the NaHS group (b) significant difference from the diabetic group. The symbols *, ## and # represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively. Reprinted from Arch Biochem Biophys, Vol. 506 (1), El-Seweidy MM, Sadik NA, Shaker OG, Sodium hydrosulfide as potent inhibitors of fibrosis in the heart of diabetic rats, Pages No., 48-57, Copyright (2011), with permission from Elsevier [25].

*PCR experiments*

*Bcl-2.* 5 uL of cDNA was subjected to PCR under the conditions as follows; PCR reaction was carried by adding 50 pmol of each of forward and reverse primer specific to Bcl-2 gene as detailed later. 10 mM dNTPS, 2.5 unit Taq enzyme and PCR 10x buffer. Cycling condition was 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and last extension at 72°C for 10 min.

*Bax.* Thirty-five cycles of PCR, with denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min, were performed.

*Cytochrome c.* Aliquots of cDNA corresponding to 200 ng RNA were amplified in PCR buffer containing, 200 mmol/L dNTPs (Promega), 25 pmol of each primer (MWG Biotech AG, Ebersberg, Germany), and 2 U Taq polymerase (MBI Fermantase) in a final volume of 50 uL. Thirty-five cycles of PCR, with denaturation at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min and last extension at 72°C for 10 min.

*Caspase-9.* Aliquots of the same cDNA were amplified with caspase-9 primers. Each amplification was carried out for 30 cycles, a cycle profile consisting of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min.

*Caspase-3.* 4 µl cDNA were incubated with 30.5 µl water, 4 µl 25 mM $MgCl_2$, 1 µl dNTPS (10 mM), 5 µl 10xPCR buffer, 0.5 µl (2.5 u) Taq polymerase and 2.5 µl of each primer containing 10 pmol. The reaction mixture was subjected to 40 cycles of PCR amplification with denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min.

*p53.* PCR reaction was performed in a total volume of 50 µl in the presence of 2.5 U of Taq DNA polymerase (Promega), 200 µmol/L dNTPs, 25 pmol/L of specific primers. The thermal cycling conditions were 40 cycles with denaturation at 94°C for 1 min, annealing at 56°C for 1min, elongation at 72°C for 2 min with final extension for 10 min at 72°C.

*Electrophoresis and quantification*

The PCR products were electrophoretically resolved on 2% agarose-TBE gel containing 0.5 mg/mL ethidium bromide and run for 1.5 h at 8 V/cm. The gel image was captured using the Eagle Sight Software (Stratagene) system, and the PCR bands were analysed with Molecular Analyst software. The PCR products were then quantified using a quantification kit (Promega Corporation, Madison, WI, USA). This method depends on the purification of the PCR product using a Promega Wizard PCR preps DNA purification kit (Promega Corporation, Madison, WI, USA). The mixture for quantification consisted of DNA quantification buffer, sodium pyrophosphate, NDPK enzyme solution, T4 DNA polymerase and DNA. This mixture was incubated at 37°C for 10 min, at which point 100 µL of Enliten L/L reagent was added. The reaction was immediately read using a luminometer. The same steps were performed on DNAs of known concentration provided by the kit, and a standard curve was performed by plotting the readings of the luminometer against the known concentrations. The readings of the amplified PCR products of the six different genes after using the luminometer were determined based on the standard curve. The results were expressed as µg/mg wet tissue.

*Histopathological studies*

Immediately following sacrifice, testes were removed and stored in 10% formalin in phosphate buffer for 24 h. Testis tissues were processed by routine histological methods and embedded in paraffin blocks. For routine histopathological examination, 5-µm-thick sections were obtained. The sections of testis were stained with hematoxylin-eosin (H&E). For detection of the thickness of seminiferous tubule basement membrane (STBM), 5-µm-thick sections were stained with periodic acid-Schiff. All sections were viewed under a light microscope (Olympus BX51 Tokyo, Japan) equipped with a DP-50 digital camera (Olympus, Tokyo, Japan).

*Measurement of seminiferous tubule diameter (STD).* The 10 most circular seminiferous tubules were randomly identified in each section of the testis, and their diameters were measured with an ocular micrometer using the x10 lens. The mean STD in micrometers was determined for each testis. The images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BX40, Tokyo, Japan), and the images were transferred into the computer using a DP-50 digital video camera (Olympus, Tokyo, Japan). The data were obtained using Leica Qwin 500 software (England).

*Statistical analysis*

Statistical analysis was performed using the statistical package for social sciences (SPSS). Data were expressed as means ± standard error of the mean S.E.M. for seven rats. Differences among groups were assessed by one-way analysis of variance (ANOVA). Duncan's test was performed for inter-group comparisons. The Pearson's correlation coefficient was used to determine correlations. The minimum level of statistical signification used was at $P < 0.05$. Significance at P-values <0.001, <0.01 and <0.05 have been given respective symbols in the tables and figures.

## Results

*General features (Table 3)*

The final body weight of diabetic rats was significantly lower than normal rat group ($P < 0.001$), whereas the weight of diabetic + sulphurous mineral water rats was significantly higher than the diabetic group ($P < 0.05$) and similar to controls.

*Alterations in glycaemia, HbA1C and serum levels of insulin, C-peptide, IGF-I and testosterone in the studied groups (Table 3)*

Hyperglycaemia ($P < 0.001$) and an overproduction of HbA1C ($P < 0.001$) were revealed in diabetic rats when compared with normal control rats. The serum levels of insulin (22.2 %, $P < 0.001$), C-peptide (36.5% $P < 0.001$) and IGF-I (47.7%, $P < 0.01$) were significantly lower than the levels in the normal rats. A significant decline (6%, $P < 0.001$) in the serum testosterone level was observed in the diabetic group compared to the normal controls.

Administration of sulphurous mineral water or NaHS to diabetic rats caused a significant reduction in hyperglycaemia ($P < 0.001$ and $P < 0.01$, respectively) and the level of HbA1C ($P < 0.01$ and $P < 0.001$, respectively) vs. the diabetic group. A marked increase in the serum levels of insulin, C-peptide and IGF-1 were detected in the diabetic group treated with either sulphurous mineral water (each $P < 0.05$) or NaHS ($P < 0.05$, $P < 0.001$and $P < 0.05$, respectively) compared with diabetic rats. In addition, sulphurous mineral water restored the serum level of IGF-1 to those of normal rats ($P > 0.5$). A significant difference was detected in the serum insulin level of the NaHS group when compared with the group given sulphurous mineral water ($P < 0.05$). A significant increase in the serum testosterone level was achieved in the diabetic rats treated with either sulphurous mineral water or NaHS when compared with diabetic rats ($P < 0.001$).

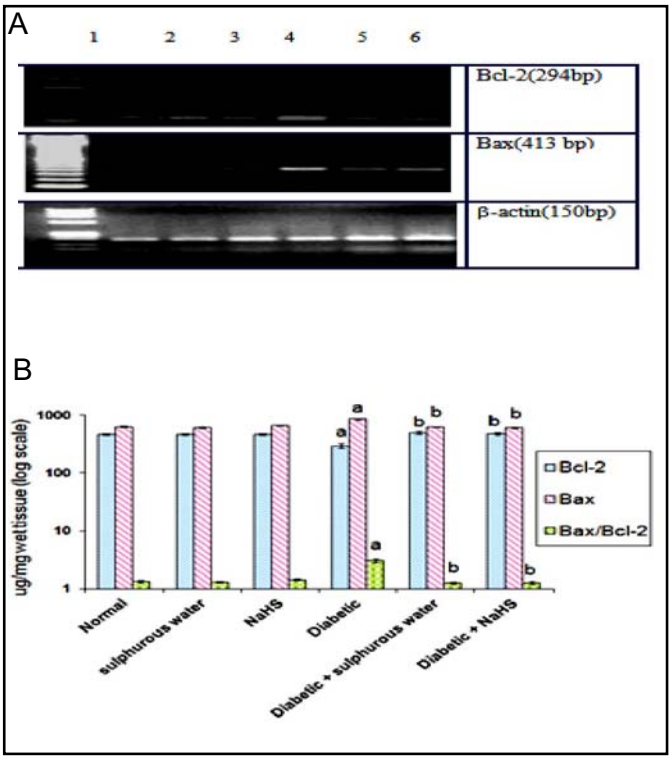


**Fig. 1.** A, Representative agarose gel electrophoresis profiles of mRNA amplification of Bcl-2, Bax and Bax/Bcl-2 and β-actin of the normal (1), sulphurous mineral water (2), NaHS (3), diabetic (4), diabetic + sulphurous mineral water (5) and diabetic + NaHS, (6) groups. B, quantitative representation of testicular gene expression levels. Data are represented as means ± S.E.M for seven rats. (a) significant difference from normal group. (b) significant difference from the diabetic group. Values are statistically significant at $P < 0.001$.

*Alterations in the gene expression levels of apoptotic markers and GSH in the testes of studied groups (Fig. 1-3)*

PCR analysis showed that the expression level of the Bax gene increased, whereas the Bcl-2 level showed a significant reduction; consequently, their ratio (Bax/Bcl-2) significantly increased in the testes of diabetic rats compared with normal controls (Fig. 1, $P < 0.001$). Similarly, the expression levels of cytochrome c, caspase-9 and -3 (Fig. 2) and p53 (Fig. 3A) were significantly increased ($P < 0.001$) in the testes of diabetic rats compared with normal controls. Interestingly, a normalisation of the gene expression of these apoptotic markers except for cytochrome c, which showed a significant decrease, was achieved in the diabetic rats treated with either sulphurous mineral water or NaHS when compared with normal rats ($P < 0.001$). Moreover, a significant decrease in the level of testicular GSH in the diabetic group

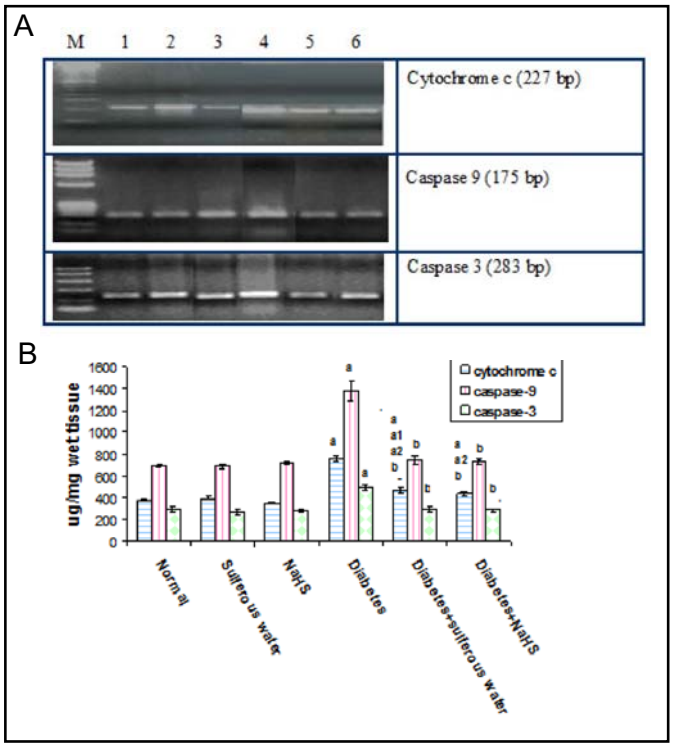


**Fig. 2.** Representative agarose gel electrophoresis profiles of mRNA amplification (A)and the quantitative testicular gene expression levels of cytochrome c, caspase-9 and caspase-3 of the normal (1), sulphurous mineral water (2), NaHS (3), diabetic (4), diabetic + sulphurous mineral water (5) and diabetic + NaHS, (6) groups (B). Data are represented as means ± S.E.M. for seven rats. (a) significant difference from normal group; (a1) significant difference from sulphurous water group;(a2) significant difference from NaHS group (b) significant difference from the diabetic group. Values are statistically significant at $P < 0.001$.

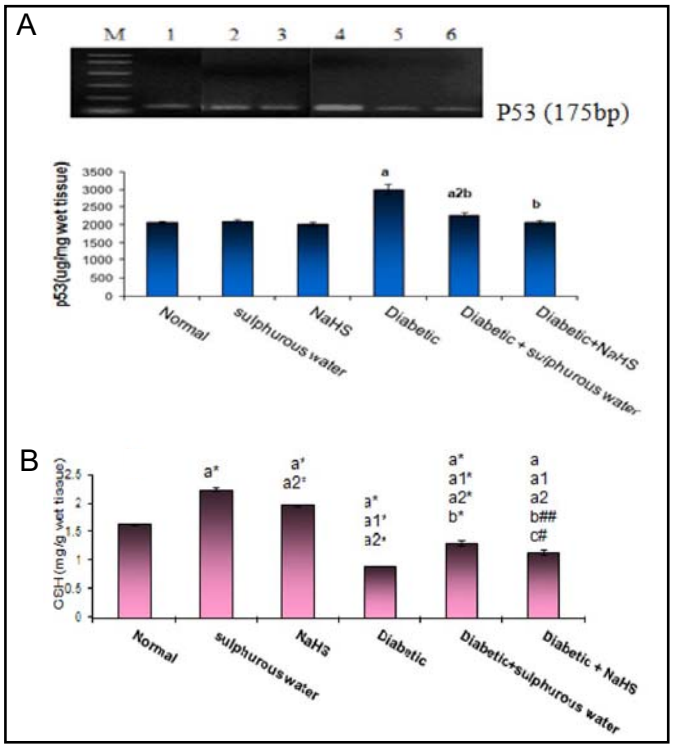


**Fig. 3.** (A) Representative agarose gel electrophoresis profiles of mRNA amplification and the quantitative testicular gene expression of p53 of normal (1), sulphurous mineral water (2), NaHS (3), diabetic (4), diabetic + sulphurous mineral water (5) and diabetic + NaHS, (6) groups; (B) GSH level in different studied groups. Data are represented as means ± S.E.M. for seven rats. (a) significant difference from normal group; (a1) significant difference from sulphurous water group;(a2) significant difference from NaHS group (b) significant difference from the diabetic group. (c) significant difference from the diabetic + sulphurous water group. The symbols *, ## and # represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.

was observed compared to normal controls (Fig. 3B, $P < 0.001$). Both treatments caused significant increase in the testicular GSH level when compared with diabetic rats ($P < 0.001$and $P < 0.01$, respectively). Overall, no statistically significant differences were observed between the sulphurous mineral water and NaHS-treated non-diabetic groups and the normal control group, except in the level of testicular GSH ($P < 0.001$).

*Correlations*

Some important strong correlations were observed that are likely due to the diabetic condition and suggest that apoptosis represents an active mechanism that contributes to testicular dysfunction in diabetes. In the diabetic group (Table 4), the level of Bcl-2 gene expression was found to be negatively correlated with Bax/Bcl-2 (r = -0.87), caspase-9 (r = -0.85), caspase-3 (r = -0.80) and p53 (r = -0.96). Moreover, the level of p53 gene expression was negatively correlated with caspase-9 (r = -0.80) and caspase-3 (r= -0.87).

| Parameters | Bcl-2, r | p53, r |
|---|---|---|
| Bax / Bcl-2 | -0.87$^{\#}$ | - |
| caspase- 9 | -0.85$^{\#}$ | -0.80$^{\#}$ |
| caspase-3 | -0.80$^{\#}$ | -0.87$^{\#}$ |
| p53 | -0.96$^{\#\#}$ | - |

**Table 4.** Correlation of testicular gene expression levels Bcl-2 and p53 with the other apoptotic parameters in the diabetic group. $^{\#\#}$significant at $P < 0.01$; $^{\#}$Significant at $P < 0.05$.

On the other hand, in the diabetic group treated with sulphurous mineral water, negative correlations were revealed between the serum testosterone level and caspase-3 (Fig. 4A; r = - 0.83) and the gene expression level of Bcl-2 and that of Bax/Bcl-2 (Fig. 4B; r = -0.87). Moreo-

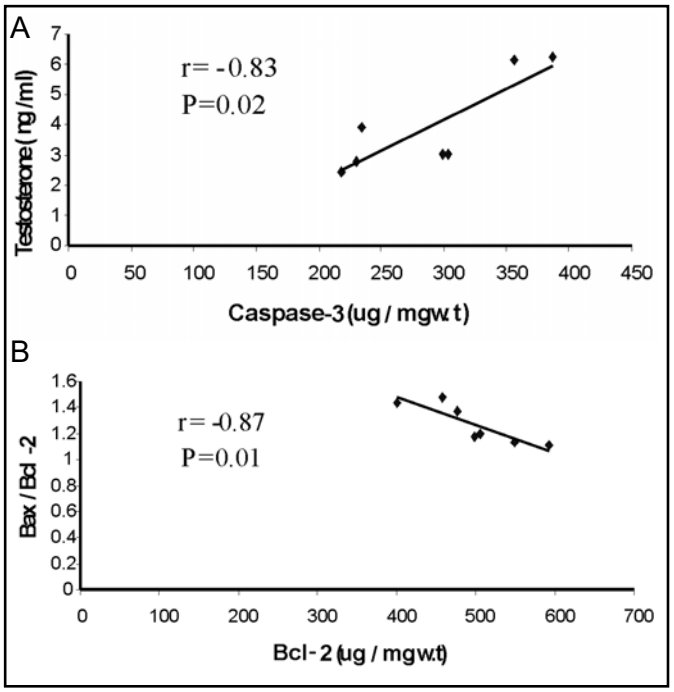


**Fig. 4.** Correlation of testicular caspase-3 gene expression level with serum testosterone level (A) and gene expression level of testicular Bcl-2 with Bax/Bcl-2(B) in the diabetic group treated with sulphurous mineral water.

ver, in the diabetic group treated with NaHS, the level of cytochrome c gene expression was positively correlated with Bax (r = 0.78), Bax/Bcl-2 (r = 0.90) and p53 (r = 0.77) and negatively correlated with Bcl-2 (r = -0.88); Bcl-2 was negatively correlated with Bax/Bcl-2 (r = -0.95) and p53 (r = -0.85) as shown in Table 5.

*Histopathological analysis*

*Evaluation of haematoxylin-eosin staining.* The testes of normal controls showed normal features of spermatogenesis with a complete seminiferous tubule cell series (Fig. 5A). The histological structure of the testes in the sulphurous mineral water and NaHS-treated non-diabetic groups resembled that of the normal rats (Fig. 5B and C). The seminiferous tubule structure in the diabetic rats was found to be disrupted, and there was a considerable decrease in the spermatogenic cell series (Fig. 5D, E, F, and G). The number of spermatogenic cells in the diabetic rats treated with either sulphurous mineral water or NaHS was increased compared to the group with diabetes, and there was an improvement in the seminiferous tubule structure (Fig. 5H and I).

*Evaluation of periodic acid-Schiff staining.* While STBM in the normal controls (Fig. 6A) and sulphurous mineral water and NaHS-treated non-diabetic groups (Fig. 6B and C) were observed to be normal, an increase in

| Parameters | Cytochrome c, r | Bcl-2,r |
|---|---|---|
| Bcl-2 | $-0.88^{\#}$ | - |
| Bax | $0.78^{\#}$ | - |
| Bax / Bcl-2 | $0.90^{\#\#}$ | $-0.95^{\#\#}$ |
| p53 | $0.77^{\#}$ | $-0.85^{\#}$ |

**Table 5.** Correlation of testicular gene expression levels of cytochrome c and Bcl-2 with the other apoptotic parameters in the diabetic group treated with NaHS. $^{\#\#}$significant at $P < 0.01$; $^{\#}$Significant at $P < 0.05$

thickness was found in the diabetic group (Fig. 6D and E). On the other hand, the thickening in the diabetic rats treated with either sulphurous mineral water (Fig. 6F) or NaHS (Fig. 6G) was found to be less than that observed in the diabetic group.

*STD.* The STD values of each group are shown in Fig. 6H. It was observed that STD was significantly decreased in diabetic rats compared with the normal control group ($P < 0.01$). However, the STD value was significantly increased in the diabetic rats treated with either sulphurous mineral water ($P < 0.001$) or NaHS ($P < 0.05$) compared with the diabetic rats. The restoration of STD values was also achieved in both treatment groups compared with the normal group ($P < 0.05$).

## Discussion

In the present study, we found that diabetes caused testicular dysfunction that was mainly mediated by increased apoptosis. The significant decreases in body weight, STD and testicular injury shown by histopathological examination in our diabetic rats are consistent with previous studies [9, 10, 12]. Furthermore, the diabetic animals in our study exhibited decreased levels of serum testosterone. A decline in the serum testosterone level is known to induce germ cell apoptosis and abnormal spermatogenesis as previously reported [34-37].

To better understand the pathway leading to testicular apoptosis in diabetes, we investigated the expression of some apoptosis-related genes in the testes of rats with diabetes. We observed that diabetes increases the gene expression levels of Bax, cytochrome c, caspase-9 and -3 and p53 and decreases that of Bcl-2. Apoptosis is controlled in part by the Bcl-2 family of regulatory proteins, such as Bcl-2, Bax and others. Bcl-2 can prevent or delay many forms of apoptosis [38]. Bax binds to and antagonises the protective effect of Bcl-2 [39]. In this sense, the increased ratio of Bax to Bcl-2 expression ob-

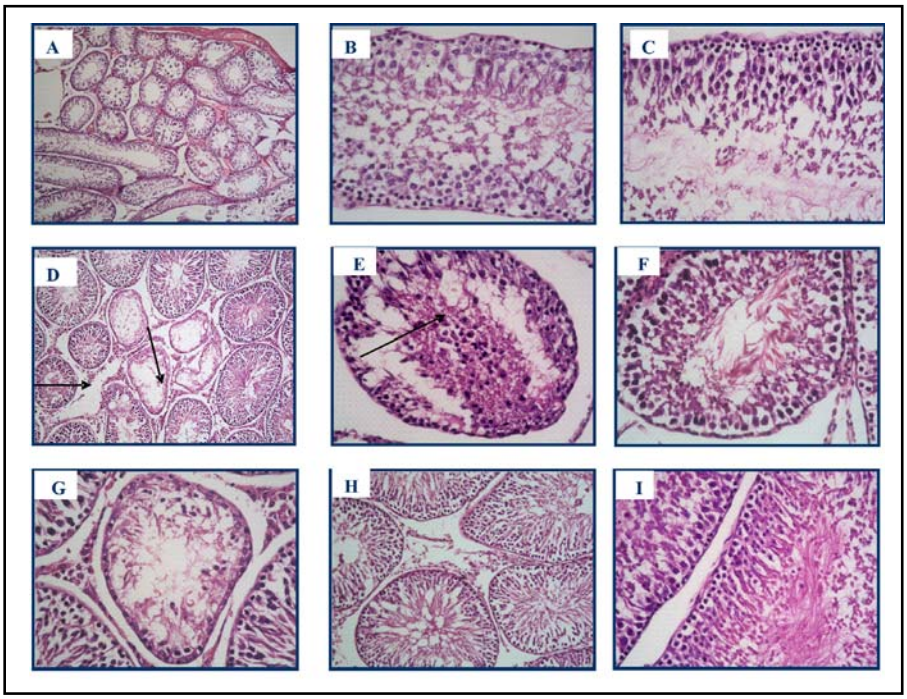


**Fig. 5.** Photomicrograph of (H&E) stained sections of testis from rats of the normal control (A), sulphurous mineral water (B), NaHS(C), diabetic (D, E, F and G), diabetic + sulphurous mineral water (H) and +NaHS(I) groups. A (×400), B and C (×400) groups presented normal architecture with active spermatogenesis. The diabetic group showed disintegrated seminiferous tubules (D, x100), impaired spermatogenesis, hypoplasia and dispersion of Leydig cells, interstitial edema, and increased interstitial space, failure of spermiation (E and F×400), detached spermatotogenic cells (G,×400). Diabetic rats treated with either sulphurous mineral water (H, x200) or NaHS(I,400x) show nearly normal architecture.

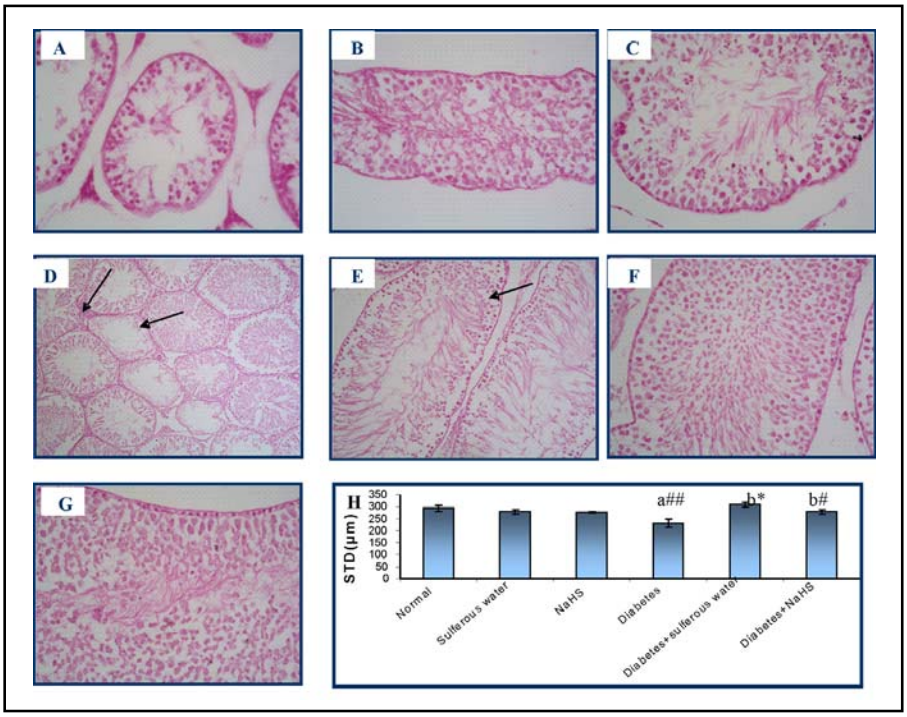


**Fig. 6.** Representative photomicrographs of periodic-acid-Schiff-stained sections in the testes of the normal control group (A, ×400), sulphurous mineral water (B, ×400) and NaHS-treated (C, ×400) nondiabetic groups, diabetic (D, ×100 and E, ×400), diabetic + sulphurous mineral water (F,×400) and diabetic +NaHS (G, ×400) groups. The thickness of the STBM in the diabetic group (arrows) increased when compared with the normal control group, but sulphurous mineral water and NaHS reduced this increase in thickness (arrows). H; Quantitative analysis of STD represented as means ± S.E.M (n=7). (a) significant difference from normal; (b) significant difference from the diabetic group.The symbols *, ## and # represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively. STBM; seminiferous tubule basement membrane, STD; seminiferous tubule diameter.

served in our diabetic rats appears to determine cell susceptibility to apoptosis when the microenvironment is not conducive to survival. Shifting the balance of Bcl-2 family members toward pro-apoptotic effectors will enhance cytochrome c release from mitochondria. When cytochrome c is released from the mitochondria into the cytosol, it is responsible for activating caspase-9, which further activates caspase-3 and executes the apoptotic program [40]. Caspase-3 can cleave vital cellular proteins or activate additional caspases by proteolytic cleavage [41]. Activation of caspase-activated DNase is integrally involved in DNA degradation [42].

The increased expression of Bax and caspase-3 in the testes of diabetic rats in our study is in agreement with previous findings demonstrated by Koh [12]. The author attributed these increases to the c-Jun NH2-terminal kinase (JNK), which is critical for increased Bax expression in response to stress [43]. JNK is a classic stress-activated protein kinase involved in apoptotic signal transduction.

The permeability of the mitochondrial outer membrane is essential for the initiation of apoptosis through this pathway. Proteins belonging to the Bcl-2 family appear to regulate the membrane permeability to ions and possibly to cytochrome c as well [44]. Mitochondria play an important role in the apoptotic process. The mitochondrial dysfunction induced by oxidative stress can lead to the release of cytochrome c and then caspase activation, which results in apoptotic cell death [45]. It has been previously demonstrated that the induction of testicular apoptotic cell death in diabetic rats [9] is mainly caused by diabetes-increased oxidative stress in the testis [15, 37, 46].

Mammalian sperm cells present a specific lipidic composition, with a high content of polyunsaturated fatty acids, plasmalogens and sphingomyelins. The lipids in spermatozoa are the main substrates for peroxidation, and Aitken et al. [47] have shown that excess amounts of reactive oxygen species (ROS) and free radicals have adverse effects on sperm motility and fertility. Furthermore, oxidative damage to the lipids and DNA of spermatozoa is associated with declining motility and diminished fertility of human sperm [48, 49].

Our study also strongly suggests the possibility of the involvement of oxidative stress in diabetes-induced testicular apoptosis due to the decline in the testicular GSH level observed in the diabetic group. GSH is the main and most prevalent cellular anti-oxidant and can act directly or through GSH-related anti-oxidant enzymes. The stability and capacity of anti-oxidant status during chronic diabetes seriously influences the outcome of the long-term complications caused by oxidative stress [50].

In this context, an increased gene expression level of p53 was found in the testes of diabetic rats compared to normal controls. It has been reported that p53 exerts its apoptotic effect through increasing the expression of the p53-upregulated modulator of apoptosis (PUMA), also known as Bcl-2-binding component 3 (BBC3) [51, 52]. PUMA is involved in p53-dependent and -independent apoptosis induced by a variety of signals. After activation, PUMA interacts with anti-apoptotic Bcl-2 family members, thus freeing Bax, which is then become able to send the apoptotic signal to the mitochondria ultimately leading to cell death [53]. p53 becomes activated in response to a myriad of stress types, which include but are not limited to DNA damage and oxidative stress [54]. The critical event leading to the activation of p53 is its phosphorylation by a variety of protein kinases. These protein kinases are known to respond to several types of stress, such as membrane damage and oxidative stress.

Testicular dysfunction in the diabetic group was also revealed in histological examination by the atrophy of the seminiferous tubules, decreased tubule diameters and reduction of the spermatogenic cell series. Seminiferous tubule atrophy and the decrease in spermatogenic cells are indicators of spermatogenesis failure [9, 55]. Our results are consistent with those of previous studies [15]. The thickness of the STBM plays an important role in spermatogenesis. Diabetes increases thickening of the STBM [56], and this thickness is accompanied by a decreased rate of sperm production and an overall reduction in the size of seminiferous tubules.

Hyperglycaemia leads to the increased production of free radical intermediates via at least four different routes, defined as follows: increased glycolysis, intercellular activation of the sorbitol (polyol) pathway, auto-oxidation of glucose and non-enzymatic protein glycation [57, 58]. Therefore, the increased HbA1C level observed in our study represents a key point in diabetes-associated complications [59, 60]. In this context, the reduced levels of IGF-I and C-peptide in the serum of diabetic rats in our study may contribute to apoptosis. Recently, Li et al. [61] reported that enhanced IGF-I signalling inhibits glucose-induced endothelial cell apoptosis by reducing mitochondrial dysfunction. Recent data suggest that the IGF-axis may in turn influence the risk of type 2 diabetes through the anti-inflammatory effects of IGF-I [62]. Interestingly, the serum C-peptide has previously been demonstrated to enhance the gene expression of IGF-I, its receptor and that of the insulin receptor itself [63]. Despite being ignored for many years, C-peptide has recently been demonstrated as a peptide hormone in its own right, independent of insulin, possibly acting through a G protein-coupled membrane receptor. The study of Al-Rasheed et al. [64] provides evidence for the ability of the C-peptide, acting via Gαi, to protect against apoptosis in kidney proximal tubular cells.

The effects of drinking therapies involving $H_2S$-rich waters on the body redox status have been poorly investigated. A recent study [28] has reported an improvement in the bodily redox status in healthy volunteers undergoing a cycle of sulphurous mineral water drinking therapy and suggested major benefits from sulphurous water consumption in reducing biomolecule oxidation, possibly furnishing valid protection against oxidative damage. The protective effects of sulphurous mineral water administration against the free-radical damage of lipids and proteins may potentially be related to the significant increase in total -SH levels [28]. Kimura et al. [65] showed that endogenously produced $H_2S$ can protect neurons

from oxidative injury by increasing the intracellular concentrations of GSH. This effect depends on the ability of $H_2S$ to increase the activity of γ-glutamylcysteine synthetase (GCS) and to upregulate cystine transport, resulting in an increase in GSH levels. In addition, $H_2S$ itself might be involved in the reduction of thiols, thus being directly implicated in redox reactions as an anti-oxidant.

The endogenous production and physiological function of $H_2S$ in the pancreas have been previously studied, resulting in the identification of both cystathionine-β-synthetase (CBS) and cystathionine γ-lyase (CSE) in rat pancreatic tissues [66]. The study of Brancaleone et al. [24] reported the enzymatic impairment of $H_2S$ biosynthesis in the setting of diabetes mellitus associated with impaired vascular reactivity. Endogenous $H_2S$ production is significantly impaired in aortic tissue of non-obese diabetic mice [24]. Indeed, $H_2S$ is permeable to plasma membranes, as its solubility in lipophilic solvents is approximately five-fold greater than that in water; for this reason; $H_2S$ is now considered a gasotransmitter able to induce specific cellular responses [67, 68]. Rinaldi et al. [68] reported that $H_2S$ prevents apoptosis of human polymorphonuclear leukocytes (PMN) via the inhibition of p38 and caspase 3. In this regard, $H_2S$ has been shown to modulate metabolic state [69], vascular reactivity, mitochondrial function, cellular redox status and apoptosis [70]. Many studies have indicated that exogenous administration of the $H_2S$ donor NaHS to rats with myocardial injury reduces the mortality rate and the accumulation of plasmatic lipid peroxidation markers such as malondialdehyde (MDA) and conjugated dienes. At the same time, $H_2S$ may inhibit the formation of protein carbonyls induced *in vitro* by hypochlorous acid [30, 71].

In the present study, glycaemia, HbA1C, insulin, C-peptide, IGF-I, testosterone and testicular GSH levels were reversed in diabetic rats treated with either sulphurous mineral water or NaHS, when compared with diabetic rats, indicating a return towards normal conditions. It appears that by preventing the redox imbalance, sulphurous mineral water and NaHS counteract overexpression levels of Bax as well as the subsequent increase in Bax/Bcl-2, cytochrome c, caspase-9, caspase-3 and p53 and elevate Bcl-2. These results can collectively be attributed to the anti-oxidant and antiapoptotic effects of $H_2S$ suggested previously [68]. These results are consistent with previous studies that demonstrated that anti-oxidants prevent testicular dysfunction mediated-apoptosis [15]. Interestingly, our current results are also in harmony with our earlier results that showed the anti-apoptotic and antifibrinogenic effects of sulphurous mineral water and NaHS in the hearts of diabetic rats [25].

It is worth mentioning that sulphur is necessary for the formation of insulin and IGF-I and that this element is provided by sulphurous mineral water and NaHS [72]. In addition to its observed effect as an anti-oxidant due to its $H_2S$ content, sulphurous mineral water may combat the state of hypomagnesaemia often encountered in diabetes [73] because this water is enriched with Mg. By counteracting the overexpression of apoptotic factors in the testicular tissue of diabetic rats, sulphurous mineral water or NaHS treatment reversed STZ-induced testicular dysfunction. In addition, in the present study, microscopic examination revealed that treatment with either sulphurous mineral water or NaHS reduced the increase in the STBM thickness, increased the STD and improved spermatogenesis and testicular damage.

It appears that further investigation of $H_2S$ will provide exciting research opportunities for many years to come. $H_2S$ can contribute to a balanced oxidant-anti-oxidant status and thus may provide a useful therapeutic option to reduce the testes injury associated with patients with diabetes mellitus. In conclusion, our results indicate that STZ-induced diabetes leads to apoptotic cell death in the testes via activation of the Bax pathway, cytochrome c, caspase-9 and -3 and p53. The observed comparable anti-apoptotic effects of sulphurous mineral water and NaHS in the testes of diabetic rats suggests an additional therapeutic approach to diabetic testicular dysfunction. To our knowledge, this study is the first that reports the useful effects of either sulphurous mineral water or NaHS in testicular dysfunction in diabetic rats. Taken together, the results of our study suggest that $H_2S$ donors may be considered to be a novel and applicable approach in preventing the testicular dysfunction often associated with diabetes.

## Abbreviations

JNK (c-Jun NH2-terminal kinase); CBS (cystathionine-β-synthetase); CSE (cystathionine γ-lyase); ECM (extracellular matrix); GCS (γ-glutamylcysteine synthetase); HbA1C (glycated hemoglobin); GSH (reduced glutathione); $H_2S$ (hydrogen sulphide); IGF-I (insulin like growth factor-I); MDA (malondialdehyde); NO (nitric oxide); PMN (polymorphonuclear leukocytes); ROS (reactive oxygen species); TGF-β1t (ransforming growth factor-β1); NaHS (sodium hydrosulphide); STZ (streptozotocin).

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