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Taurine ameliorates oxidative stress induced inflammation and ER stress mediated testicular damage in STZ-induced diabetic Wistar rats

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ABSTRACT

One of the major consequences of diabetes is reproductive dysfunction but the fundamental mechanisms are still poorly known. The objective of the present study was to explore the beneficial role of taurine against streptozotocin induced testicular dysfunctions in diabetic male Wister rats and understanding the underlying intricate molecular mechanisms. Exposure to streptozotocin (50 mg kg^{-1} body weight, i.p., once) elevated blood glucose level, induced testicular histological alterations and reduced testis-to-body weight ratio, serum testosterone, testicular markers and activity of antioxidant enzymes. Generation of ER stress (increased expression of calpain-1, caspase-12 and upregulation of CHOP, GRP78 via eIF2α signaling), translocation of NF κB in the nucleus (leading to the upregulation in the levels of inflammatory cytokines), activation of mitochondria dependent apoptotic pathway and DNA fragmentation were revealed from this study. However, administration of taurine at a dose of 100 mg kg^{-1} body weight for 6 weeks post diabetic induction, successfully ameliorated all these adverse effects. Thus, taurine, as a potential therapeutic agent, may hold promise in preventing oxidative and ER stress mediated diabetic testicular complications in rats.

1. Introduction

Diabetes mellitus (DM) is a leading concern of the modern era for its wide range of physiological complications and prevalence (Dicker, 2010; Ismail, 2008). It refers to a group of chronic metabolic complications, characterized by prolonged hyperglycemia. It can result from either inadequate production of insulin in the pancreas or lack of sensitivity of the body cells to insulin or both (Association, 2014). According to the International Diabetes Federation (IDF), of the 425 million cases worldwide, India has faced 73 million instances of diabetes in 2017 (Snouffer, 2018).

Oxidative stress is triggered under diabetic conditions through the production of free radicals via the polyol pathway, protein glycosylation and glucose auto-oxidation (Moussa, 2008; Nishikimi et al., 1972; Yeon Lee et al., 2010). Reduction in antioxidant levels and/or anomalous increase in reactive oxygen species levels (Dalle-Donne et al., 2003) lead to cellular damage by triggering ER stress, debilitating normal mitochondrial function, disrupting the DNA and enhancing inflammation through activation of NF κB, etc. The damaged organelles elicit the apoptotic signaling pathway as an outcome of oxidative stress (Chowdhury et al., 2016; Liu et al., 2013b; Rashid et al., 2017; Sinha et al., 2013). Several studies have reported the role of oxidative stress, ER stress, NF κB mediated inflammation and apoptosis in the induction of testicular damage (Guo et al., 2017; Rashid and Sil, 2015a).

Sustained hyperglycemia leads to the generation of reactive oxygen species (ROS) (Dalle-Donne et al.) and reactive nitrogen species (RNS) in the body that accelerates cellular oxidative stress and endoplasmic reticulum stress (Khaneshi et al., 2013; La et al., 2009). These

Abbreviations: Bax, Bcl-2-associated X; Bcl-2, B-cell lymphoma 2; CAT, Catalase; CHOP, C/EBP homologous protein; DNPH, Dinitro phenyl hydrazine; ECL, Enhanced chemiluminescence; EDTA, Ethylene diamine tetraacetate; eIF2α, Eukaryotic initiation factor 2α; ELISA, Enzyme linked immunosorbent assay; ER, Endoplasmic reticulum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLUT-2, Glucose transporter 2; GRP78, 78 kDa glucose-regulated protein; HRP, Horseradish peroxidase; HSD, Hydroxysteroid dehydrogenase; ICAM-1, Intercellular adhesion molecule; IL-1β, Interleukin-1β; IL-6, Interleukin-6; MCP-1, Monocyte chemoattractant protein; MDA, Malondialdehyde; NF κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PARP, Poly (ADP-ribose) polymerase; PERK, Protein kinase R like endoplasmic reticulum kinase; PKR, Protein kinase R; ROS, Reactive oxygen species; SDH, Sorbitol dehydrogenase; SOD, superoxide dismutase; STZ, Streptozotocin; TAU, Taurine; TBARS, Thiobarbituric acid reactive substance; TNF-α, Tumor necrosis factor alpha; VCAM-1, Vascular cell adhesion molecule; VDAC, Voltage-dependent anion-selective channel

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complications, accompanied by a feeble internal antioxidant defense machinery causes deleterious alterations in the male reproductive system (John Aitken et al., 1989). Such alterations include testicular complications like abnormal spermatogenesis and oligospermia along with altered histology of ventral prostate, decreased level of reproductive hormones, abnormal penile erection, etc. (Atta et al., 2017; Cameron et al., 1990; Hajizadeh et al., 2014; Kushwaha and Jena, 2012; Liu et al., 2013a; Mu et al., 2018; Scarano et al., 2006; Shrilatha, 2007).

Streptozotocin (STZ), a nitrosourea analogue acts as a diabetogenic agent. It leads to the generation of excessive free radicals thereby leading to the induction of type 1 diabetes in murine models (Lenzen, 2008). Treatment with STZ leads to its selective accumulation and induction of hyperglycemic conditions through mitochondrial dysfunction mediated glucotoxicity in the pancreatic β cells (Szkudelski, 2001; Wu and Yan, 2015).

Naturally occurring antioxidants have attracted immense research interest in recent years for their promising therapeutic role against a broad spectrum of diseases (Banerjee et al., 2018; Bhattacharya et al., 2013; Bhattacharyya et al., 2017; Das and Sil, 2014; Dutta et al., 2018; Ghosh et al., 2015; Pal et al., 2014; Pal et al., 2015; Rashid and Sil, 2015a, b; Sadhukhan et al., 2016; Sil, 2015; Sinha et al., 2007; Sinha et al., 2015). The ameliorative role of taurine and its different derivatives against oxidative stress and various chemical toxin induced pathophysiological conditions including hyperglycemia and related complications is well reported (Abd El-Twab et al., 2016; Das et al., 2012a,b; Liu et al., 2017; Sarkar et al., 2017; Tsounapi et al., 2012; Zhao et al., 2016). Taurine exerts its protective effects mainly through its anti-oxidative and anti-inflammatory properties (Sarkar et al., 2017).

We studied the pathophysiological complications associated with diabetes in STZ induced hyperglycemic condition of male Wistar rats. Our study enlightens the signaling pathways involved in the testicular protective mechanism of taurine, its role against oxidative stress mediated inflammation and apoptotic death of the testicular cells under hyperglycemic condition.

2. Materials and methods

2.1. Chemicals

Taurine (2-aminoethane sulfonic acid) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Chemical Company, (St. Louis, MO) USA. STZ was purchased from Sisco Research Laboratory, Mumbai, India. Bicinchoninic assay (BCA) kit was obtained from Thermo Fisher Scientific, USA. Kit for determining blood glucose was obtained from Roche, Germany. ELISA kits for the measurement of testosterone and serum insulin were purchased from Span Diagnostic Ltd., Surat, Gujarat, India. Antibodies were repurchased from Abcam (Cambridge, UK), Novus Biologicals, U.S.A, Cell Signaling (Cell Signaling Technology Inc., Danvers, MA), U.S.A. and BioBharti Life Sciences Private Limited, India. All other chemicals utilized in this study were of highest experimental grade and purchased from Sisco Research Laboratory.

2.2. Animals

Eight weeks old male Wistar rats, weighing approximately 150–200 g, were used in this study. The animals were acclimatized under standard laboratory conditions (temperature (23 ± 2 °C) and humidity (50 ± 10%) with alternating 12 h light/dark cycles) for two weeks before performing the experiments. The animals were allowed free access to standard pellet diet (Agro Corporation Private Ltd., Bangalore, India) and water ad libitum. All the experiments were conducted following the guidelines approved by the IAEC (Institutional Animal Ethical Committee), Bose Institute, Kolkata [IAEC/BI/3(I)cert./2010] and the study was approved by IAEC, CPCSEA (Committee for the Purpose of Control & Supervision on Experiments on Animals) and Ministry of Environment and Forests, New Delhi, India (1796/PO/Ere/S/14/CPCSEA).

2.2.1. Induction of diabetes in experimental animals

The animals were kept in overnight fasting condition. The experimental rats were then injected with STZ (dissolved in 0.1 M sodium citrate buffer, pH 4.5), once intraperitoneally, at a dose of 50 mg kg^{-1} body weight (Chowdhury et al., 2016). The fasting blood glucose level was assessed in the rats using an Advanced Accu-check glucometer (Roche, Germany) after 7 days from STZ injection. Those animals exhibiting blood glucose level above 300 mg/dL were considered diabetic and used for further experiments.

2.3. Determination of dose and time dependent effects of taurine

Dose and time dependent studies were carried out to determine the effective therapeutic dose of taurine on diabetes related complications. The experimental animals were randomly separated into 6 groups with 6 rats in each group. The first group served as normal control and received only vehicle (water); the second group functioned as the diabetic control receiving a single intraperitoneal injection of STZ at a dose of 50 mg kg^{-1} body weight. The remaining four STZ induced diabetic groups were treated with four different doses of taurine (25, 50, 100 and 150 mg kg^{-1} body weight) daily for another 8 weeks, starting from the onset of diabetes. The doses were selected based on the evidences of previous studies (Das et al., 2009, 2012). Taurine was dissolved in water and administered via oral gavage. After studying the body weight, fasting blood glucose level and serum insulin level of these experimental animals, the effective therapeutic dose of taurine was selected. It was observed that oral administration of taurine at a dose of 100 mg kg^{-1} body weight daily for about 6–7 weeks effectively ameliorated all the above mentioned alterations.

2.4. In vivo experimental design

After determining the effective therapeutic dose of taurine, the *in vivo* study was designed as follows (Table 1):

The animals were divided into 4 groups with 6 animals in each group.

Group 1. CONTROL group: consisted of 6 animals; received only water as vehicle.

Group 2. TAU group: consisted of 6 animals; received only taurine dissolved in water, at a dose of 100 mg kg^{-1} body weight, daily, via oral gavage, for a time span of six weeks.

Group 3. DIA group: consisted of 6 animals; received STZ injection intraperitoneally at a dose of 50 mg kg^{-1} body weight once.

Group 4. DIA + TAU: consisted of 6 animals; after the onset of diabetes, they received taurine at a dose of 100 mg kg^{-1} body weight,

Table 1
In vivo experimental design.

8 weeks	2 weeks	10 weeks	STZ (−)	1	TAU (−)	6	CON	Total
			STZ (−)		TAU (+)		TAU	experiment
old rat	acclimatization	old rat	STZ (+)	week	TAU (−)	weeks	DIA	tenure
			STZ (+)		TAU (+)		DIA + TAU	9 weeks

Table 2
The size of the product and annealing temperature of the primers used for TNF-α, IL-1β, IL-6, MCP-1, ICAM-1, VCAM-1 and β-actin genes.

Gene	Primer Sequences (5′-3′)	Annealing Temperature	Amplicon Size (bp)
TNF-α	Fp: CTGAAGTAGTGGCCTGGATTG Rp: GCTGGTAGTTTAGCTCCGTTT	50.5	424
IL-1β	Fp: CTTCCTAAAGATGGCTGCACTA Rp: ATCCCATACACACGGACAAC	50.1	307
IL-6	Fp: CAGAGCAATACTGAAACCCTAGT Rp: TTCTGACCACAGTGAGGAATG	50.5	262
MCP-1	Fp: GTGTCCCAAAGAAGCTGTAGTA Rp: AAGGCATCACATTCCAAATCAC	50.5	297
ICAM-1	Fp: CACCATGCTTCCTCTGACAT Rp: CACTGCTCGTCCACATAGTATT	50.5	283
VCAM-1	Fp: GAGTGCAAGAAGCCAACTAGA Rp: AGCTGCCTACTCAACATTAACA	50.5	258
β-actin	Fp: TCCCTGGAGAAGAGCTATGA Rp: ATAGAGCCACCAATCCACAC	50.7	332

daily, via oral gavage, for a time span of six weeks.

2.5. Collection of blood, serum and testes

After six weeks of treatment, all the animals were sacrificed. The blood was collected and the serum was procured by incubating the collected blood at 37 °C for 30 min followed by centrifugation at 3000*g* for 30 min. The obtained serum was stored at −80 °C for performing various biochemical assays. Testes were removed aseptically, weighed and their sizes compared. The tissues were segregated according to the groups and kept either at −80 °C until further experimentation or were fixed in 10% buffered formalin for further histological studies.

2.6. Determination of fasting blood glucose level, serum insulin level and testes weight to body weight ratio

The blood glucose levels of all the rats were checked regularly using an Advanced Accu-check glucometer (Roche, Germany), at an interval of 7 days by collecting blood from the lateral vein of their tails. The serum insulin levels were determined from the serum samples by enzyme linked immunosorbent assay (ELISA) using standard assay kits (Rashid and Sil, 2015a).

Each experimental rat was weighed before being sacrificed. After dissection, the weight of the dissected testis was determined. Then, the ratio of the weights of the testes to the body weights of the respective animals was obtained.

2.7. Preparation of testis tissue homogenate

Following dissection of the sacrificed experimental rats, the testes were obtained. Then the collected testes were washed properly in PBS and homogenized in a Dounceglass homogenizer using 1/3 (w/v) cold radioimmune precipitation assay (RIPA) lysis buffer [150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), Triton X-100, 50 mM Tris, pH 8.0], supplemented with protease and phosphatase inhibitors (Inhibitor Cocktails, Thermo Fisher Scientific), followed by centrifugation at 12,000 rpm at 4 °C for 10 min. The supernatant obtained was stored for further experimentation.

2.8. Estimation of protein content

Using the BCA (bicinchoninic acid) assay kit, the protein content of the supernatant was measured. The remaining supernatant was aliquoted and stored at −80 °C until further experimentation.

2.9. Assessment of parameters of testicular dysfunction

2.9.1. Determination of testosterone level

The testosterone levels were determined from the serum samples by enzyme linked immunosorbent assay (ELISA) using standard assay kits (Rashid and Sil, 2015a).

2.9.2. Estimation of the activity of 3β-HSD

The activity of testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) was studied by the method of Jarabak et al. (Talalay, 1962). The dissected testicular tissue was homogenized in 15% glycerol containing 5 mmol potassium phosphate and 1 mmol EDTA. The homogenized mixture was centrifuged at 10,000*g* for 30 min at 4 °C and the supernatant was collected. 1 mL supernatant was mixed with 1 mL of 100 μM

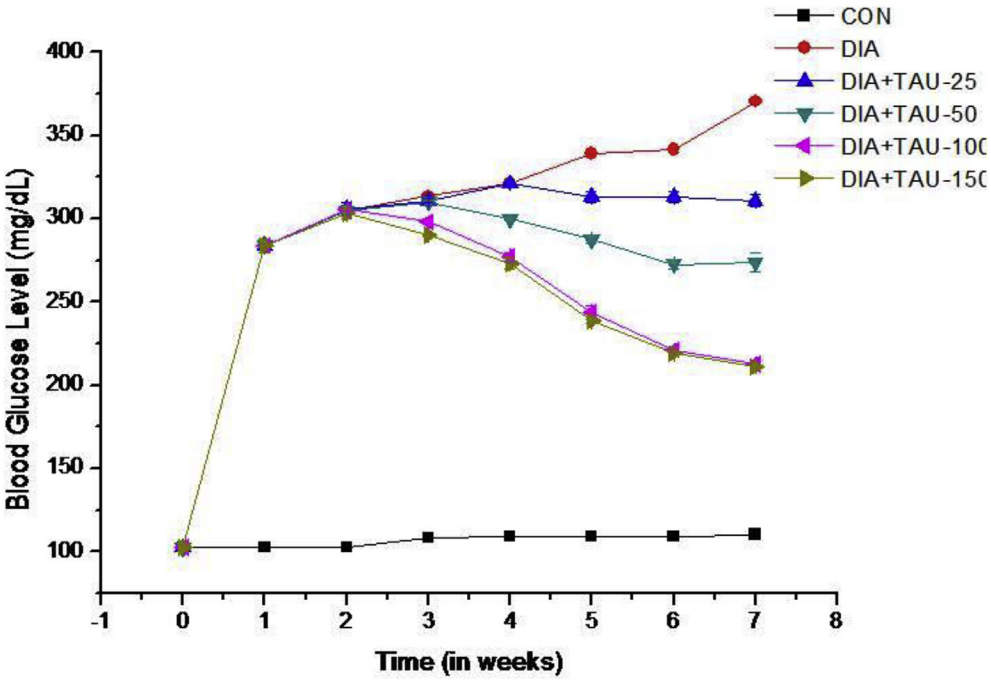


Fig. 1. Dose and time dependent effect of taurine on blood glucose level in STZ treated diabetic rats.
CON: Blood glucose from normal control rats; DIA: Blood glucose from STZ-treated rats; DIA + TAU-25, -50, −100, −150: Blood glucose from diabetic rats treated with TAU for 6 weeks at a dose of 25, 50, 100 and 150 mg kg^{-1} body weight; Values are expressed as mean ± SD, for 6 animals in each group.

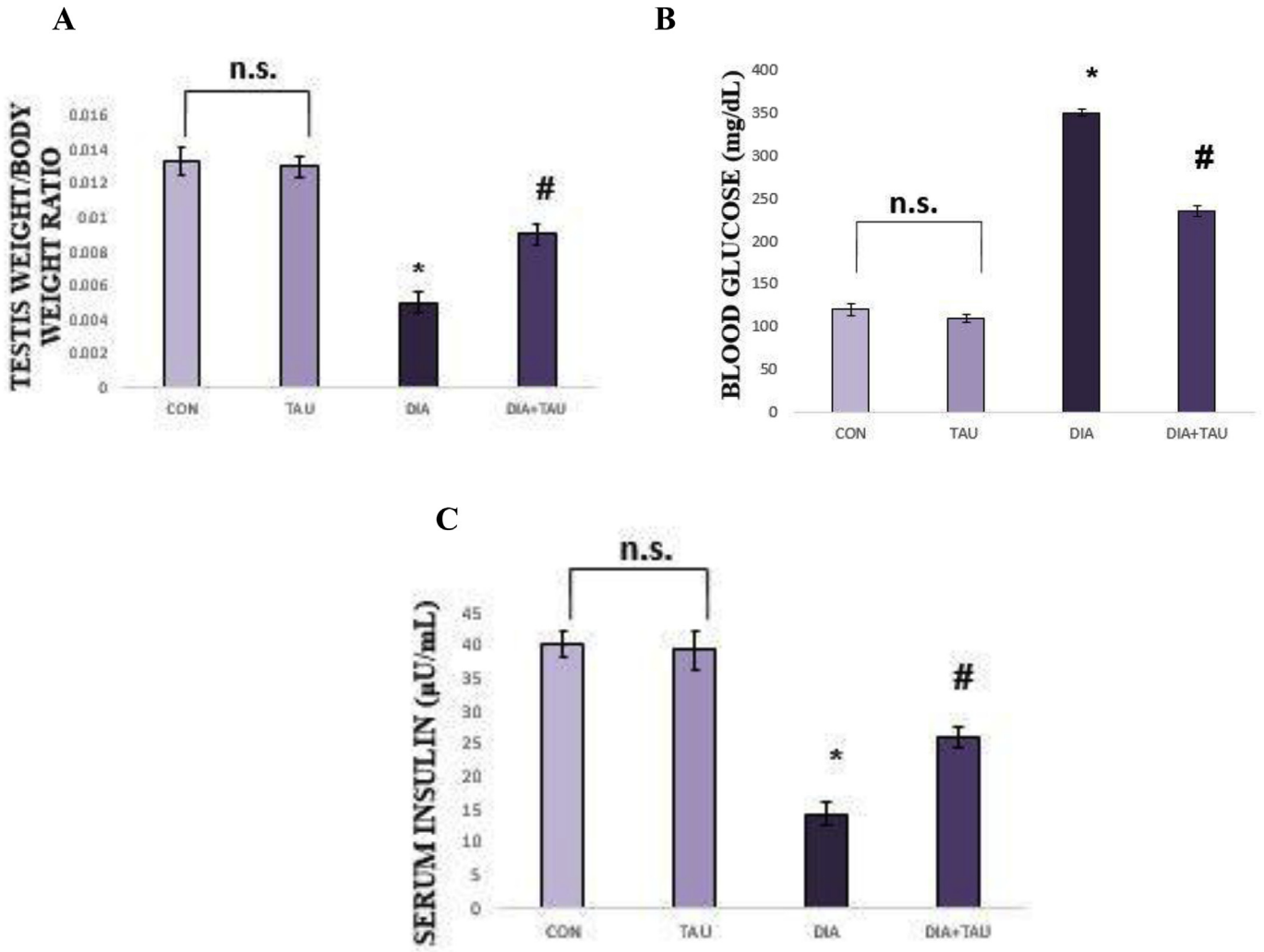


Fig. 2. Effect of taurine on (A) testis weight to body weight ratio, (B) blood glucose and (C) serum insulin level.
CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Values are expressed as mean ± SD, for 6 animals in each group. "*" values differ significantly from CON ($^{*}P < 0.05$); "#" values differ significantly from DIA ($^{\#}P < 0.05$); n.s. represents non-significant difference between CON and TAU.

sodium pyrophosphate buffer, pH 8.9 and 30 µg of dehydroepiandrosterone in 40 µL of ethanol and 960 µL of 25% BSA, making a 3 mL incubation mixture. The enzyme activity was measured spectrophotometrically after addition of 0.5 µmol of NAD to the supernatant mixture at 340 nm against a blank (i.e., without NAD). One unit of enzyme activity is the amount necessary for causing a change in absorbance of 0.001/min at 340 nm. All the experiments were repeated thrice under similar conditions.

2.9.3. Estimation of the activity of 17β-HSD

The activity of testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) was determined using the protocol of Jarabak et al. (1962). The dissected testicular tissue was homogenized in 15% glycerol containing 5 mmol potassium phosphate and 1 mmol EDTA. The homogenizing mixture was centrifuged at 10,000*g* for 30 min at 4 °C and the supernatant was subsequently collected. 1 mL supernatant was mixed with 1 mL of 440 µM sodium pyrophosphate buffer, pH 10.2, 40 µL of ethanol containing 0.3 µmol of testosterone and 960 µL of 25% BSA, making a 3 mL incubation mixture. After addition of 1.1 µmol NAD to the supernatant mixture, the enzyme activity was measured spectrophotometrically at 340 nm against a blank (without NAD). One unit of enzyme activity is equivalent to an alteration in absorbance of 0.001/min at 340 nm. All the experiments were repeated thrice under similar conditions.

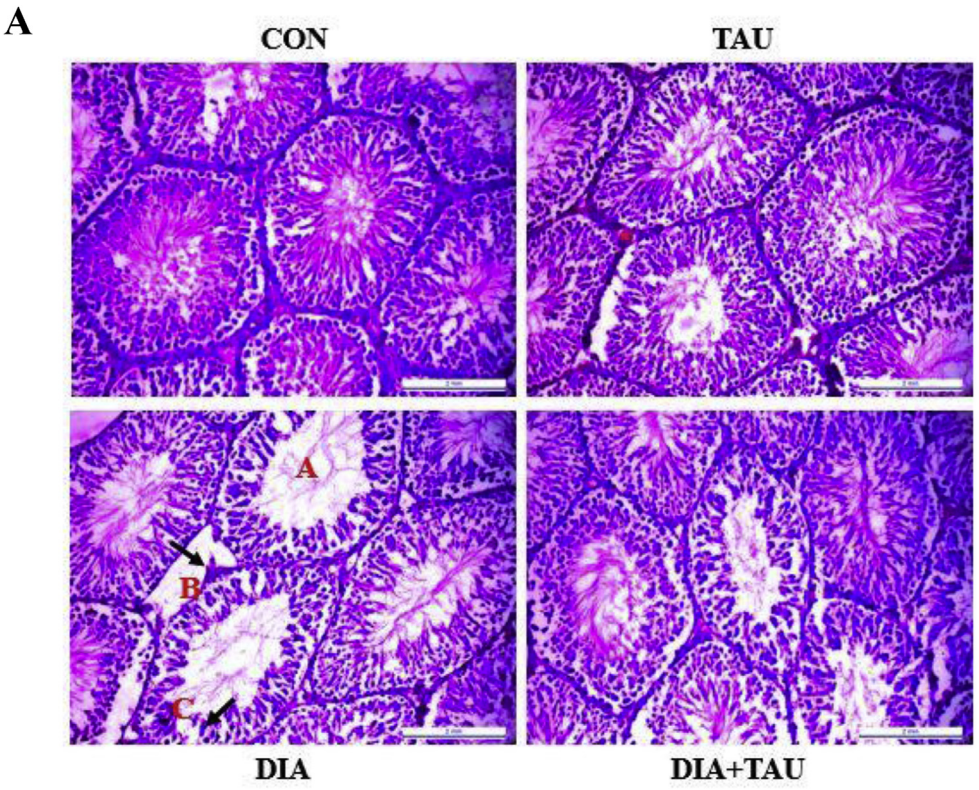
2.9.4. Estimation of sorbitol dehydrogenase (SDH) activity

Sorbitol dehydrogenase activity was estimated following the protocol described by Bergmeyer (Pant and Srivastava, 2003). SDH is an enzyme which acts as a marker of testicular dysfunction since it catalyzes the interconversion of D-fructose and D-sorbitol through redox reaction. The rate of conversion of D-fructose to D-sorbitol is directly proportional to the rate of oxidation of NADH. The SDH activity was measured spectrophotometrically in terms of the rate of decrease in absorbance at 340 nm. All the experiments were repeated thrice under similar conditions.

2.10. Determination of biochemical markers for the detection of oxidative stress

2.10.1. Assessment of lipid peroxidation

Lipid peroxidation assay, in terms of malondialdehyde (MDA), was estimated according to the spectrophotometric method described by Esterbauer and Cheeseman (1990). The experiment was carried out by a colorimetric reaction with thiobarbituric acid (TBA) and the



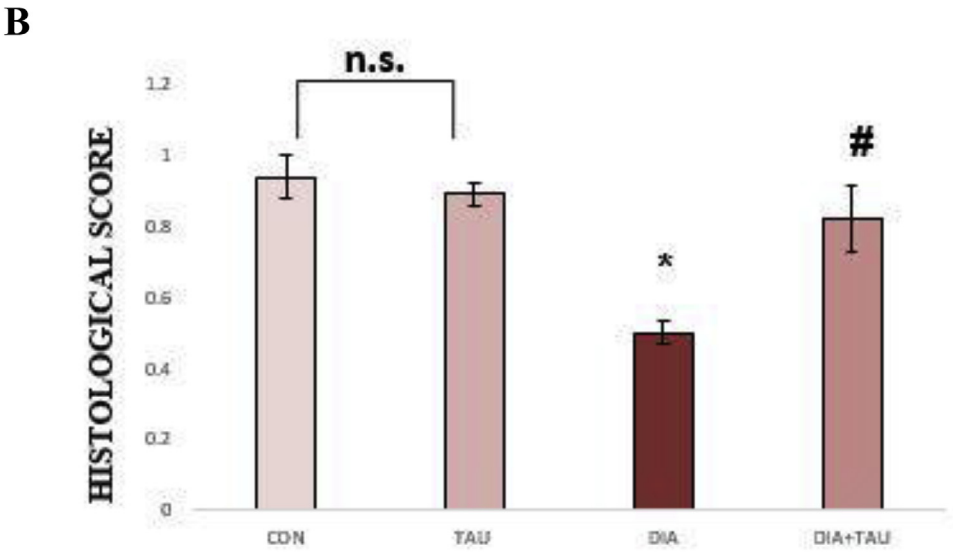


Fig. 3. Effect of taurine on diabetes induced testicular damage.
Sections of testes tissues of rats were stained with hematoxylin-eosin (magnification: 40×). In DIA group, A: loss of centrally located spermatozoa, B: loss of Leydig cells, C: disruption of the germinal epithelium and loss of Sertoli cells. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Arrows indicated site of damage of testicular tissue. Values are expressed as mean ± SD, for 6 sections in each group. "*" values differ significantly from CON ($^{*}P < 0.05$); "#" values differ significantly from DIA ($^{\#}P < 0.05$); n.s. represents non-significant difference between CON and TAU.

experimental samples. After thiobarbituric acid reactive substance (TBARS) formation, the absorbance was measured spectrophotometrically at 532 nm and the concentration was determined using the extinction coefficient of MDA which is $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (since 99% of TBARS exists as MDA). All the experiments were repeated thrice under similar conditions.

2.10.2. Determination of protein carbonyl content

To determine protein carbonyl content, the protocol of Uchida and Stadtman (1993) was adopted. The samples were treated with an equal volume of 0.1% (w/v) 2, 4-dinitrophenylhydrazine (DNPH) in 2 N HCl and incubated for 1 h at room temperature and then treated with 20% trichloroacetic acid (TCA). After centrifugation, the obtained precipitates were washed three times with ethyl acetate. Then they were dissolved in 8 M guanidine hydrochloride in 133 mM of Tris buffer, containing 13 mM EDTA. The absorbance was measured in a spectrophotometer at 365 nm. The results were expressed in terms of nmol of DNPH incorporated/mg protein, according to the molar extinction coefficient of aliphatic hydrazones i.e., 22,000 $M^{-1}\ cm^{-1}$. All the experiments were repeated thrice under similar conditions.

2.10.3. Evaluation of superoxide dismutase (SOD) activity

To estimate SOD activity, the method proposed by Nishikimi et al. (1972) and later modified by Kakkar et al. (1984) was adopted. Testis tissue homogenate samples containing 5 µg protein was mixed with sodium pyrophosphate buffer, PMT and NBT. The reaction was being

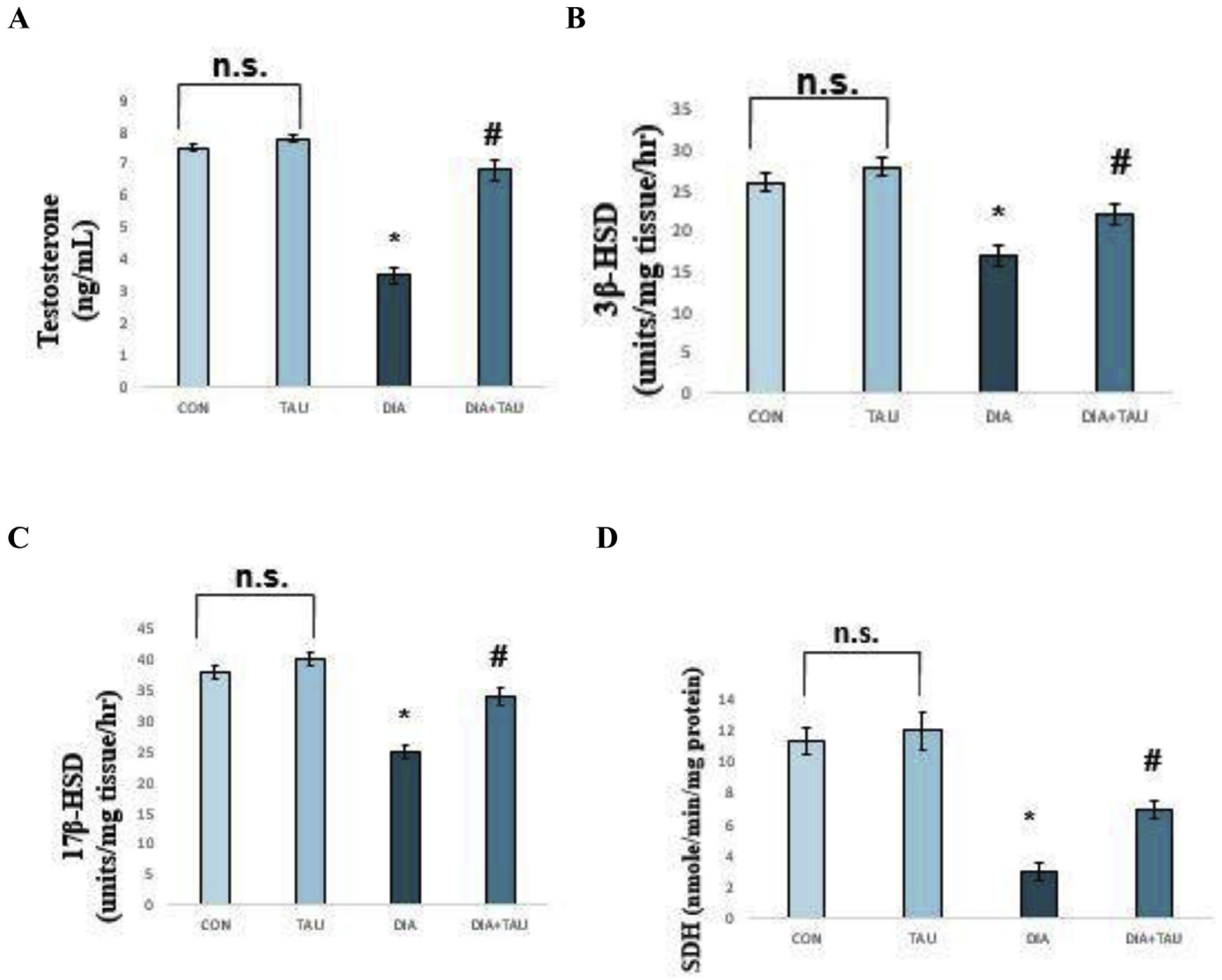


Fig. 4. Effect of taurine on testicular markers.
Effect of taurine on the status of (A) testosterone, (B) 3β-HSD, (C) 17β-HSD and (D) SDH in diabetic rats. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Values are expressed as mean ± SD, for 6 animals in each group. "*" values differ significantly from CON (*$P < 0.05$); "#" values differ significantly from DIA ($^{\#}P < 0.05$); n.s. represents non-significant difference between CON and TAU.

initiated by the addition of NADH. At 30 °C, the reaction mixture was incubated for 90s followed by the addition of 1 mL of glacial acetic acid for stopping the reaction. The absorbance of the generated chromogen was determined at 560 nm. One unit of SOD activity can be defined as the enzyme concentration which is required to inhibit chromogen production by 50% in 1 min under assay conditions. All the experiments were repeated thrice under similar conditions.

2.10.4. Evaluation of catalase (CAT) activity

Following the protocol of Bonaventura et al. (1972), tissue homogenate containing 5 µg protein was mixed with 2.1 mL of 7.5 mM H_2O_2 and the decrease in absorbance was then measured at 240 nm spectrophotometrically at 25 °C for about 10 min. One unit of catalase activity can be defined as the amount of enzyme which can reduce 1 µmol of H_2O_2 per minute. All the experiments were repeated thrice under similar conditions.

2.10.5. Estimation of GSH/GSSG ratio

The level of GSH and GSSG were estimated following the protocol of Hissin and Hilf (1976). In this method, GSH and GSSG were allowed to react with o-phthalaldehyde (OPT), a fluorescent reagent at pH 8 and 12 respectively. GSH can be complexed to N-ethylmaleimide to prevent interference of GSH in the measurement of GSSG. The fluorescence was determined spectrophotometrically at excitation wavelength of 360 nm and emission wavelength of 460 nm. All the experiments were repeated thrice under similar conditions.

2.11. DNA fragmentation analysis to determine the mode of cell death

To assess the extent of DNA fragmentation, phenol-chloroform method described by Sellins and Cohen (1987) was adopted. Genomic DNA isolated from testicular tissues of the control and experimental rats were subjected to 1.5% agarose gel electrophoresis.

2.12. Extraction of RNA and reverse transcriptase PCR (RT-PCR)

RNA was extracted from the testis tissue of all groups of rats using the TRIzol reagent (Invitrogen, Carlsbad, USA), following the

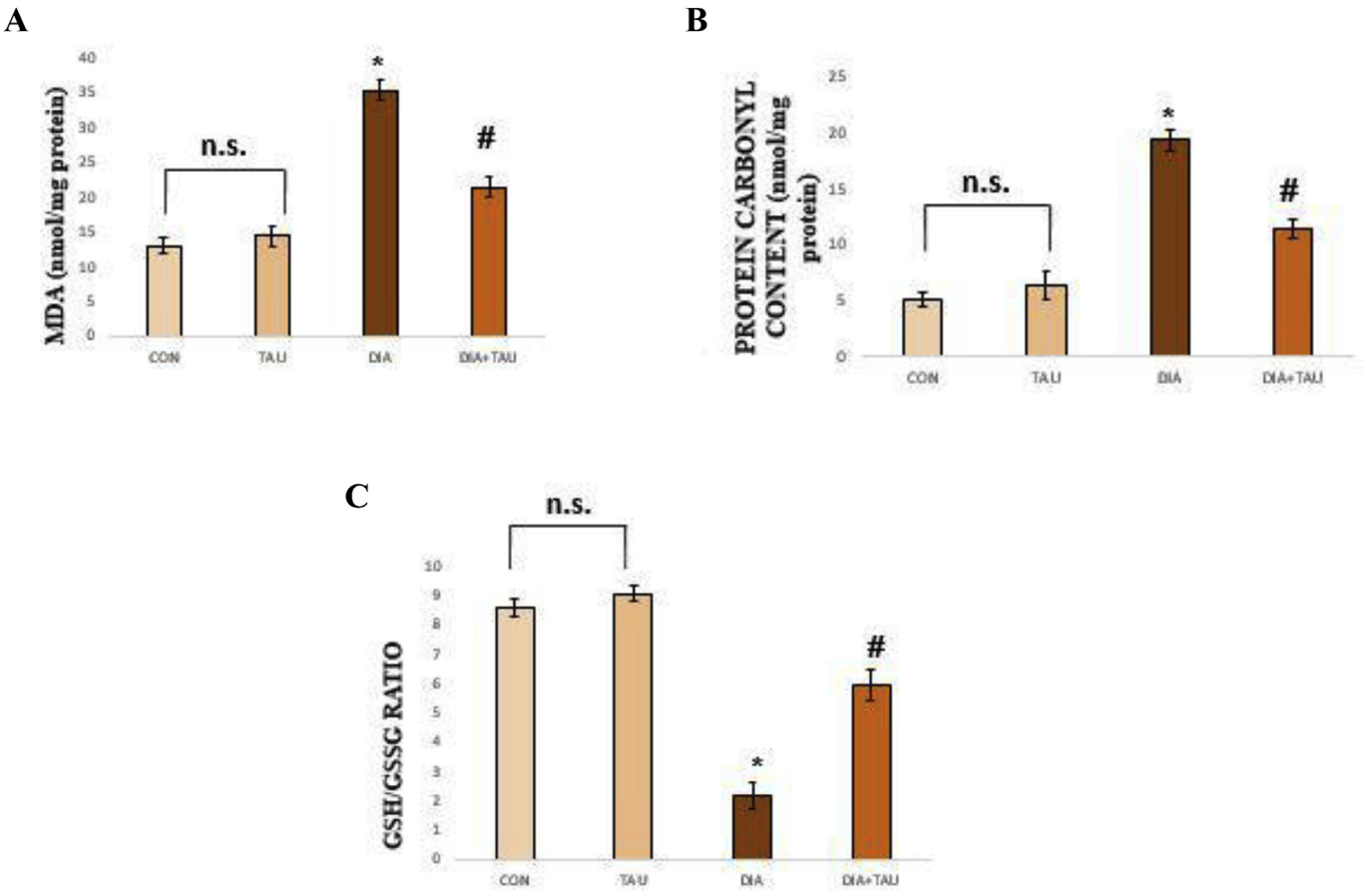


Fig. 5. Effect of taurine on oxidative stress related parameters.
Effect of taurine on the status of (A) lipid peroxidation, (B) protein carbonylation and (C) GSH:GSSG ratio in diabetic rats. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Values are expressed as mean ± SD, for 6 animals in each group. "*" values differ significantly from CON (*P < 0.05); "#" values differ significantly from DIA ($^{\#}$P < 0.05); n.s. represents non-significant difference between CON and TAU.

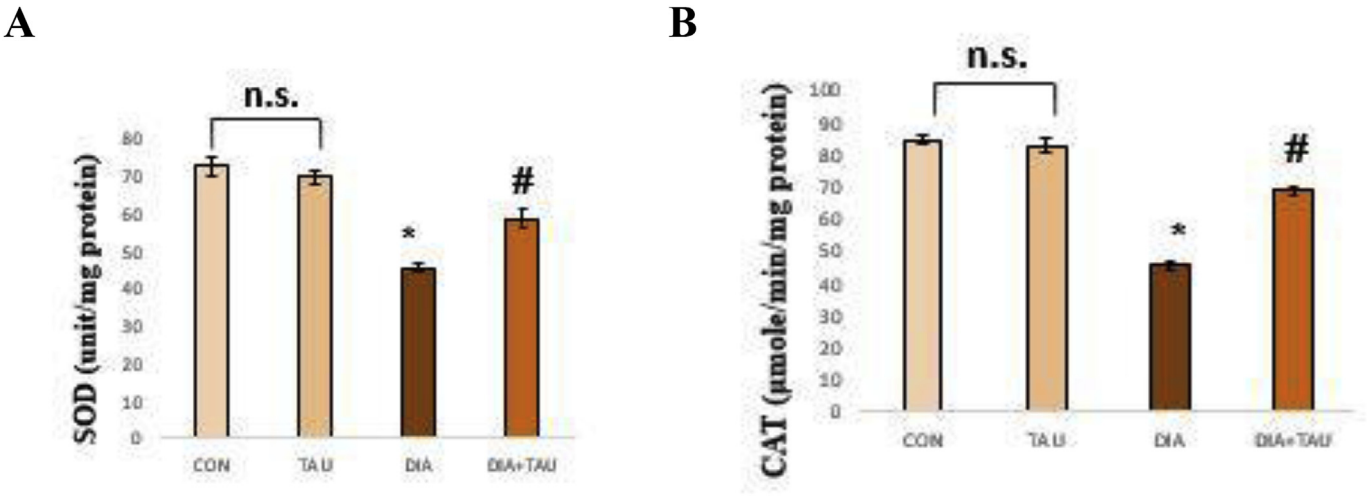
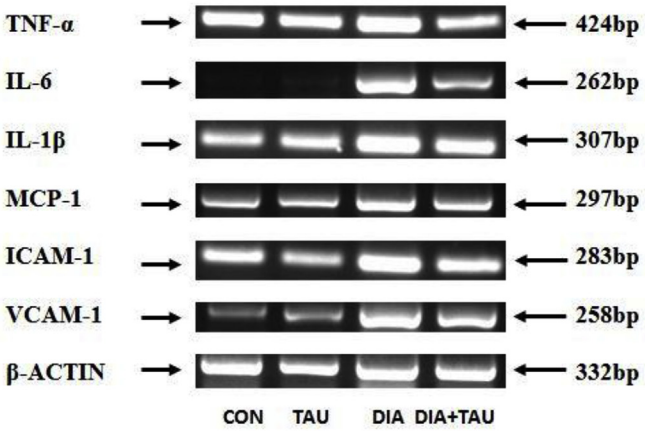


Fig. 6. Effect of taurine on the antioxidant enzymes.
Effect of taurine on the status of (A) SOD and (B) CAT activity. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Values are expressed as mean ± SD, for 6 animals in each group. "*" values differ significantly from CON (*P < 0.05); "#" values differ significantly from DIA ($^{\#}$P < 0.05); n.s. represents non-significant difference between CON and TAU.

A



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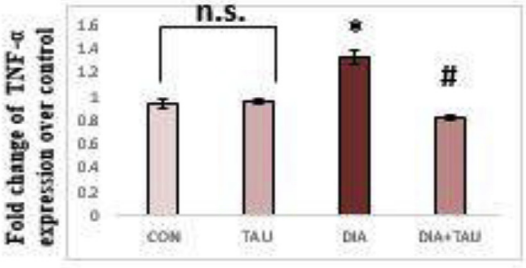

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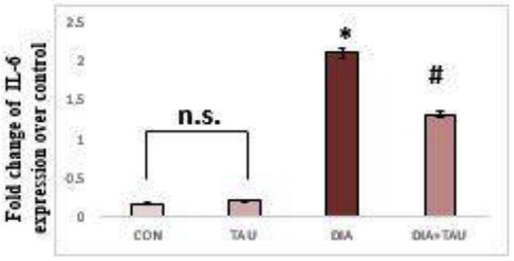

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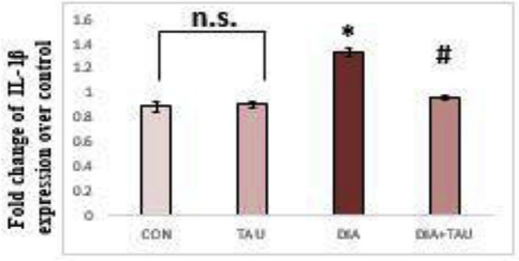

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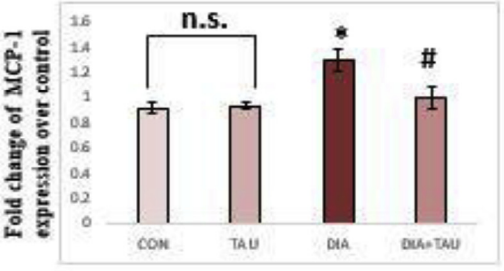

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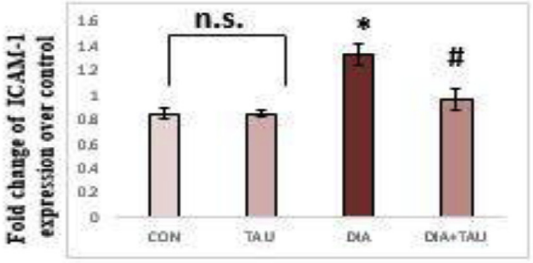

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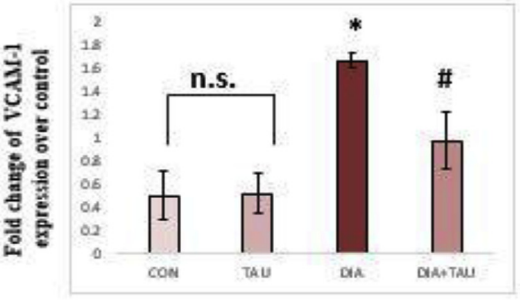

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Fig. 7. Effect of taurine on the level of testicular inflammatory cytokines, adhesion molecules and chemokines.
RT-PCR analyses (TNF-α, IL-1β, IL-6, MCP-1, ICAM-1, VCAM-1) in the tissue of the experimental rats (B i-vi) Densitometric analyses of the concerned molecules. Densitometric analysis data are represented as the mean ± SEM of three different experimental sets, *P < 0.05 vs. CON; #P < 0.05 vs. DIA. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes).

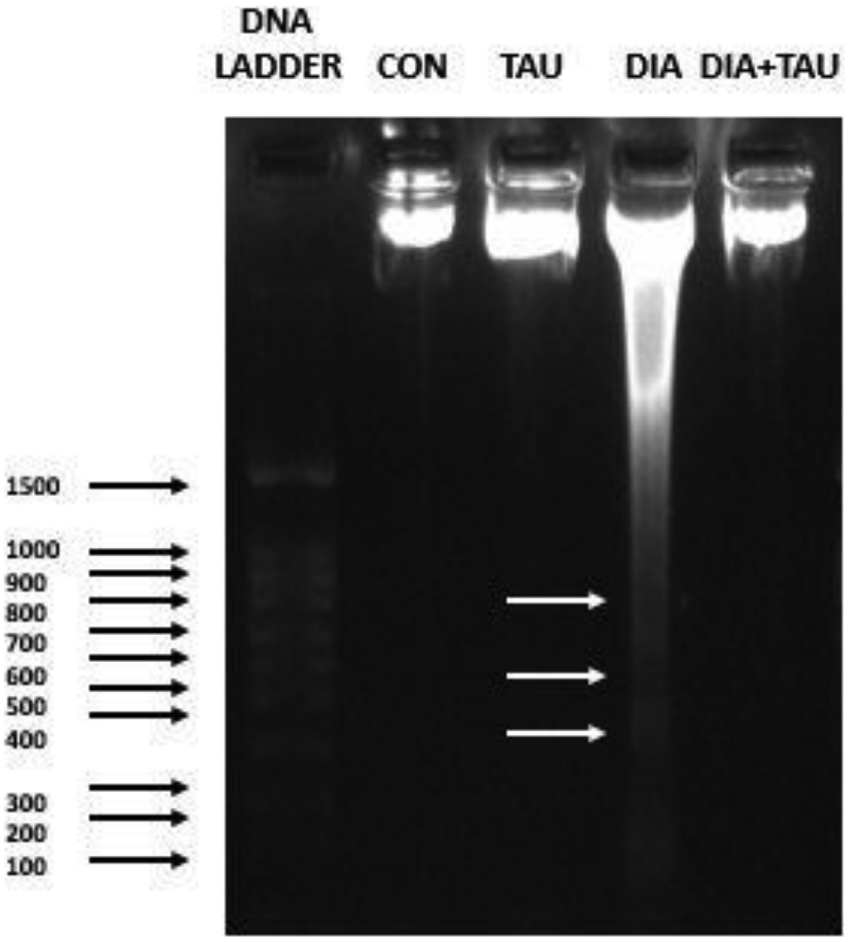
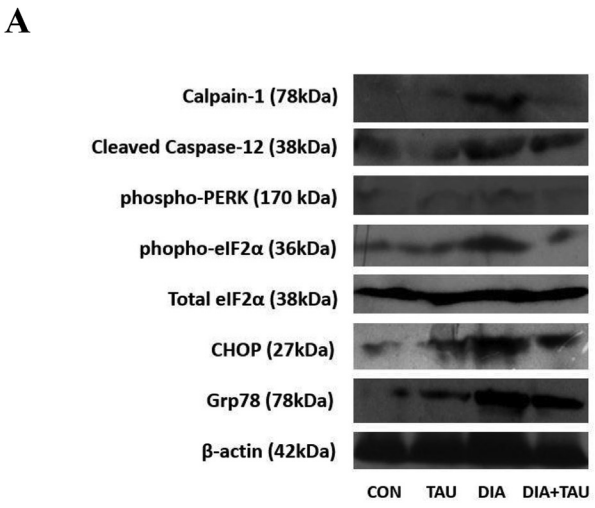


Fig. 8. Effect of taurine on apoptotic cell death.
DNA fragmentation assay on agarose/ethidium bromide gel. Arrows indicate the DNA fragments. Results represent one of the three independent experiments. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes).

manufacturer's protocol. RNA concentration was measured spectrophotometrically using nanodrop, Hellma Tray-Cell Type 105.810 (Hellma Analytical). 2 μg of RNA from each sample was converted into cDNA using Thermo Scientific Versoc DNA synthesis kit (Thermo Scientific, USA). Thermal cycling was performed as follows: initial denaturation at 95 °C for 5 min, followed by a set of 35 cycles of 95 °C for 30s, melting temperature (Tm°C) for 30s, and 72 °C for 45s. After completion of 35 cycles, the DNA extension time was specified at 72 °C for 5 min. The final products were subjected to electrophoresis in 1.5% agarose gel. The size of PCR amplified product and annealing temperatures of the primers used for TNF-α, IL-1β, IL-6, MCP-1, ICAM-1, VCAM-1, MCP-1 and β-actin genes are given in Table 2. To ensure that the amplified products were not obtained from contaminated genomic DNA, a control without reverse transcriptase (containing all the RT-PCR reagents except the reverse transcriptase) was also performed; no product was observed in the minus-reverse transcriptase control sample.

2.13. Subcellular fractionation of testis tissue to separate nuclear, mitochondrial and cytoplasmic fractions

The method of Cox and Emili with slight modification was followed to obtain subcellular fractionation of testis tissue (Manna et al., 2010; Priyadarsini et al., 2003). The organ was washed in cold PBS and then homogenized in 5 vol of cold 250-STMDPS Buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 1 mM PMSF, 25 μg spermidine) containing protease and phosphatase inhibitors. It was then centrifuged at 800*g* for 15 min at 4 °C. The supernatant (Sup I) was collected and to isolate the nuclear proteins, 250-STMDPS buffer was added to the pellet, homogenized again, followed by another round of centrifugation at 800*g* for 15 min at 4 °C. The pellet (nuclei) thus obtained was suspended in five volumes of protease and phosphatase inhibitor supplemented buffer, NET (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA, 1% Triton-X-100, 1 mM PMSF, 20% glycerol, 1 mM DTT). This was then incubated for another 30 min with vortexing occasionally at 4 °C and finally lysed through sonication. After that, it was subjected to centrifugation at 14,000*g* at 4 °C for 25 min. The supernatant was collected and was used for



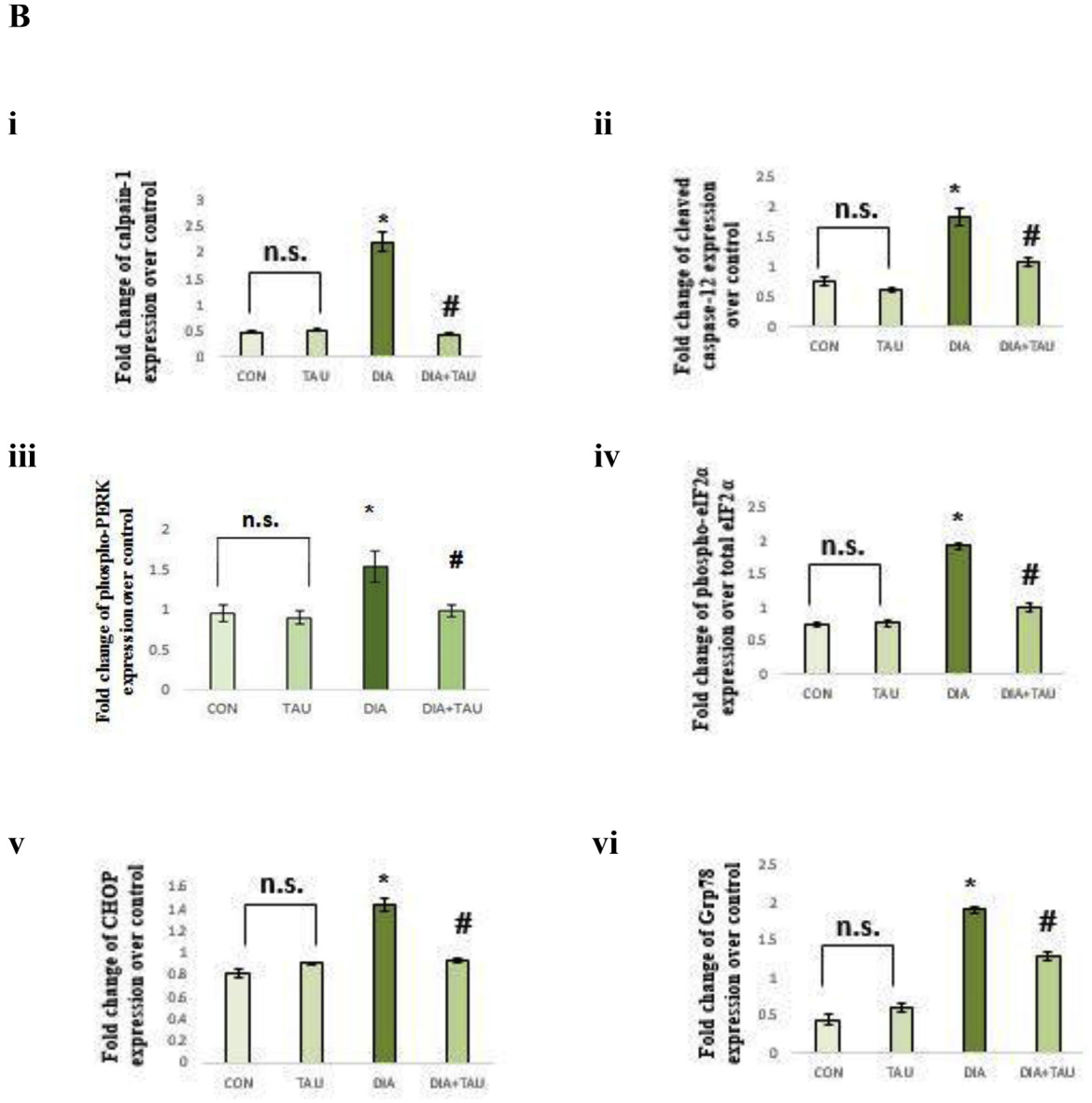


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Fig. 9. Effect of taurine on the activation of ER stress related pathway.
Immunoblot blot analyses of calpain-1, cleaved Caspase-12, phospho-PERK, phospho and total eIF2α, CHOP and Grp78 in the testicular tissue of experimental rats (B i-vi) Densitometric analyses of the concerned molecules. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Densitometric analysis data are represented as the mean ± SEM of three different experimental sets, *P < 0.05 vs. CON; #P < 0.05 vs. DIA.

immunoblotting with respect to NF κB and Lamin B1. The supernatant fraction (Sup I) was then centrifuged at 4 °C at 6,000*g* for 15 min for isolation of mitochondrial proteins. The supernatant thus obtained (Sup II) was stored and the pellet (containing mitochondrial proteins) was suspended in 5 vol of protease and phosphatase inhibitor supplemented buffer, ME (20 mM Tris-HCl pH 7.8, 0.4 M NaCl, 15% glycerol, 1.5% Triton-X-100, 1 mM PMSF, 1 mM DTT) and lysed through sonication. Then, it was again centrifuged at 4 °C at 14,000*g* for 30 min. The supernatant obtained was used for immunoblotting with respect to cytochrome *c* and VDAC. The supernatant (Sup II) was centrifuged at 14,000*g* at 4 °C for 30 min and collected to obtain the cytoplasmic fractions which were in turn used for immunoblotting with respect to NF κB, cytochrome *c* and β-actin. Lamin B1 and VDAC were used as marker proteins for the nuclear and mitochondrial fractions of the testis tissue respectively.

2.14. Immunoblot analysis

For immunoblot analyses, equal concentration of proteins from the whole tissue lysate of each sample of all the four groups were taken and resolved in 10–12% SDS-PAGE following the standard protocol (Rashid et al., 2013). Then they were transferred to PVDF membrane followed by blocking for 45 min at 37 °C in 1% BSA to avoid non-specific binding. Then the membranes were incubated with the following anti-cleaved caspase 12 (1:1000), anti-calpain-1 (1:1000), anti-phospho PERK (1:1000), antiphosho-eIF2α (1:1000), anti-total eIF2α (1:1000), anti-CHOP (1:1000), anti-GRP78 (1:1000), anti-NF κB (1:1000), anti-I κB α, anti-Lamin B1 (1:1000), anti-VDAC (1:1000), anti-cytochrome C (1:1000), anti-Bax (1:1000), anti-Bcl-2 (1:1000), anti-cleaved caspase 9 (1:1000), anti-cleaved caspase 3 (1:1000), anti-PARP (1:1000) and anti-β-actin (1:1000) primary antibodies overnight at 4 °C. Primary antibodies were detected against HRP conjugated secondary antibody (1:20,000) using the HRP substrate ECL solution.

2.15. Histological assessment

The testes obtained from all the four groups were fixed in 10% buffered formalin and processed for paraffin sectioning. Sections of approximately 5 mm thickness were then stained with hematoxylin and eosin to assess the histological alterations under light microscope.

Histological scoring of each section of all the 4 groups were performed based on the following criteria of ranks defined as follows: germinal epithelium well organized-10, disruption of germinal epithelium-5, presence of centrally located spermatozoa-8, loss of centrally located spermatozoa −4, presence of Leydig cells-6, loss of Leydig cells-3, presence of Sertoli cells-4 and loss of Sertoli cells-2. Based on the above mentioned criteria, each section of all the 4 groups were ranked with respect to the highest possible rank of 28, such that the final histological scoring ranged between 0 and 1.

2.16. Statistical analysis

All results have been expressed in terms of mean ± SEM (n = 6). One-way analysis of variance (ANOVA) was used to evaluate all the statistical data. To compare the group means, Tukey test was adopted by utilizing the OriginPro 8 software (OriginLab, MA). A p-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of taurine on fasting blood sugar level of STZ-induced diabetic Wister rats: dose and time dependent study

To find out the effective therapeutic dose of taurine on blood sugar level under hyperglycemic condition, a dose and time dependent study was performed. 100 mg kg^{-1} body weight was determined to be the most effective dose in reducing the elevated blood sugar level, increasing the serum insulin level and restoring normal testis weight to body weight ratio as compared to the normal control and diabetic control group, when administered for 6 weeks post diabetic induction, by oral gavage (Fig. 1). However, a higher dose could not exhibit any added advantage.

3.2. Effect of taurine on body weight to testis weight ratio, blood glucose and serum insulin level

STZ induced diabetic rats exhibited significant elevation of blood sugar level, reduction in the level of serum insulin, testis weight to body weight ratio compared to the normal control group as characteristic features of hyperglycemia. Oral treatment of taurine for 6 weeks at a dose of 100 mg kg^{-1} body weight could significantly reduce the elevated blood sugar level to the normal level and effectively restore the serum insulin level and the testis weight to body weight ratio compared to the diabetic control group (Fig. 2).

3.3. Taurine restores the diabetes induced morphological alterations of testis

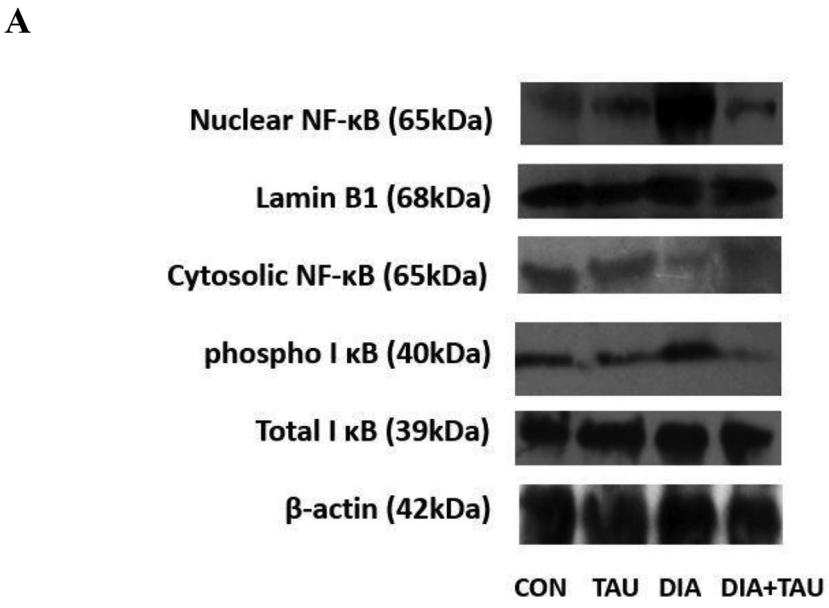
Evaluation of histological sections of testes of STZ induced diabetic rats showed that the hyperglycemic condition can lead to loss of spermatids, disappearance of testicular cells like Leydig and Sertoli cells, sloughing of centrally located spermatozoa and the disruption of germinal epithelium. However, post diabetic treatment with taurine efficiently improved such alterations (Fig. 3A). At the same time, CON and TAU groups showed no significant difference in morphology pointing towards lack of toxic side effect of taurine itself. The reduced histological score of the DIA group with respect to DIA + TAU group supported the above stated observation (Fig. 3B).

3.4. Effect of taurine on the markers of testicular dysfunction

In our study, we found that in STZ induced diabetic rats, both the activity of SDH and the level of testosterone got reduced compared to the normal control group. Our study also included the estimation of the activity of 3β-HSD and 17β-HSD, enzymes that help in biosynthesis of testosterone. In STZ administered rats, the activity of these two enzymes were significantly reduced compared to the normal control group. Treatment with taurine for 6 weeks at a dose of 100 mg kg^{-1} body weight post diabetic induction could significantly increase the activity of SDH, level of testosterone and the activity of 3β-HSD and 17β-HSD compared to the diabetic control group (Fig. 4).

3.5. Effect of taurine on oxidative stress related parameters

In testicular tissues of STZ induced diabetic rats, the malondialdehyde level, protein carbonyl content increased and GSH/GGSG ratio decreased significantly than in normal control group. After



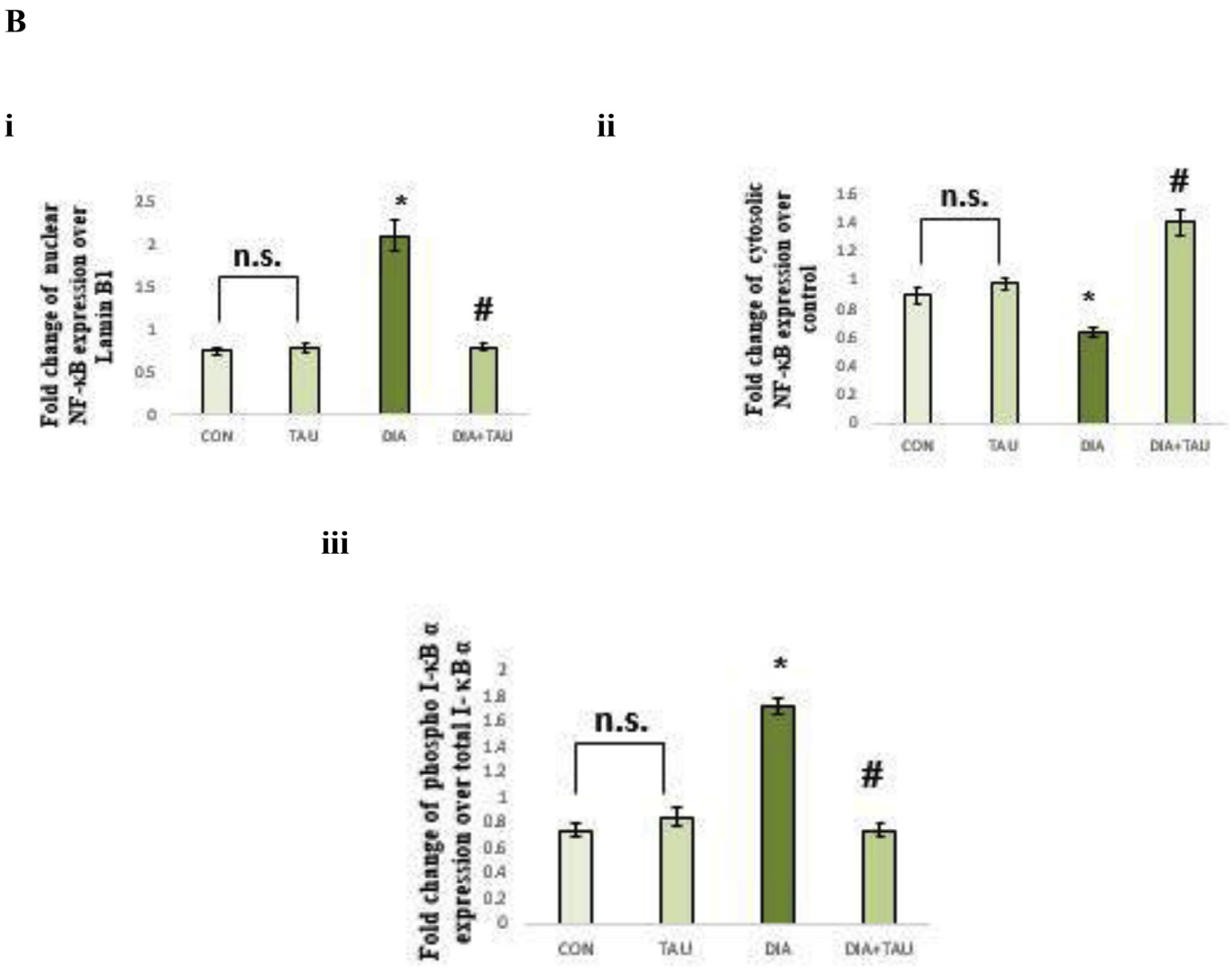
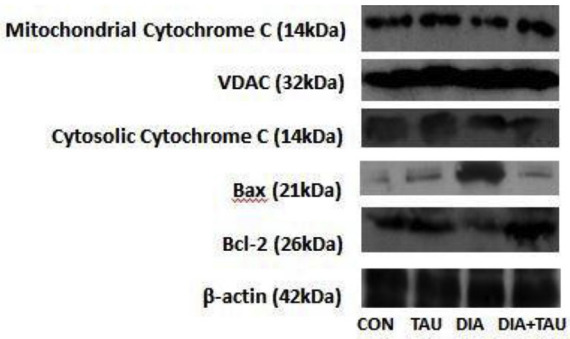
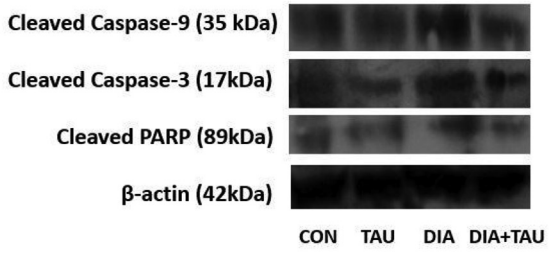


Fig. 10. Effect of taurine on the activation of NF κB mediated pathway.
Immunoblot blot analyses of nuclear and cytosolic NF κB, phospho and total I κBα in the testicular tissue of experimental rats (B i-iii) Densitometric analyses of the concerned molecules. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Densitometric analysis data are represented as the mean ± SEM of three different experimental sets, *P < 0.05 vs. CON; #P < 0.05 vs. DIA.

A





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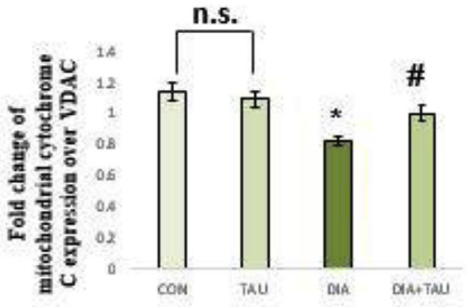

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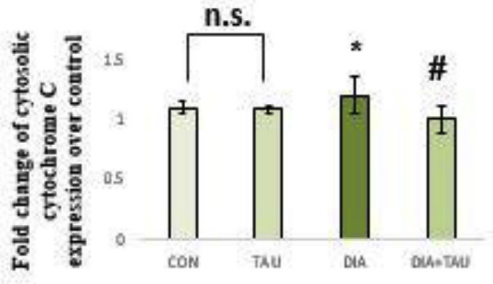

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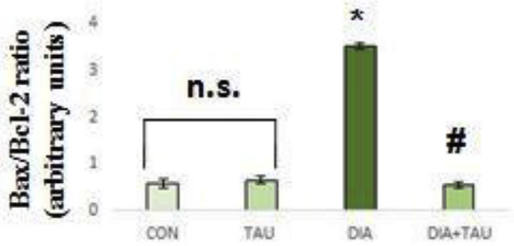

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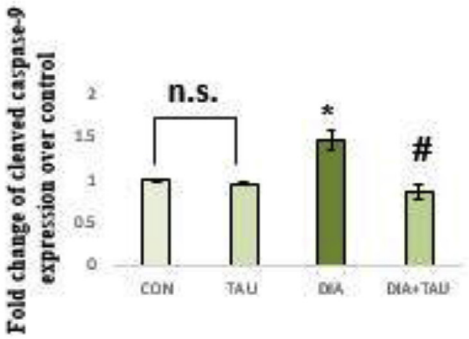

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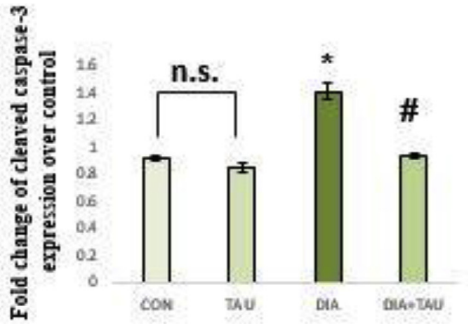

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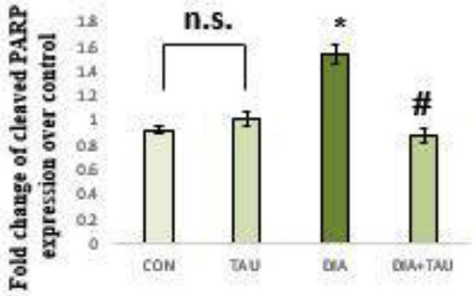

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Fig. 11. Effect of taurine on mitochondria dependent apoptotic pathways.
Immunoblot blot analyses of Bax, Bcl-2, cytosolic cytochrome-C, mitochondrial cytochrome-C, cleaved Caspase-9, cleaved Caspase-3 and cleaved PARP in the testicular tissue of experimental rats (B i-vi) Densitometric analyses of the concerned molecules. CON: received only water; Taurine (TAU): received only taurine at a dose of 100 mg kg^{-1} body weight, daily, orally for 6 weeks; Diabetic control: received STZ (50 mg kg^{-1} body wt, intraperitoneally); (DIA + TAU): treated with taurine (100 mg kg^{-1} body wt, daily for 6 weeks post induction of diabetes). Densitometric analysis data are represented as the mean ± SEM of three different experimental sets, *P < 0.05 vs. CON; #P < 0.05 vs. DIA.

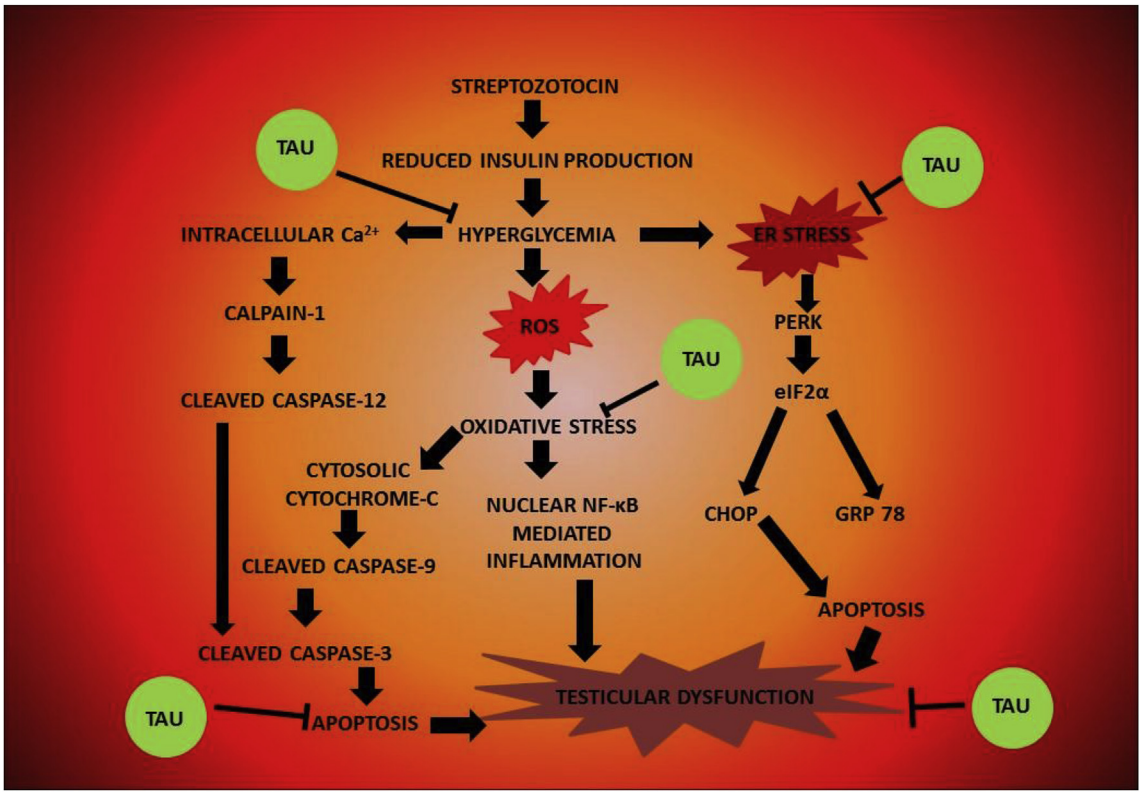

Fig. 12. Schematic diagram of the therapeutic effects of taurine against testicular damage in diabetes.

treatment with taurine, the level of lipid peroxidation, protein carbonylation and GSH/GSSG ratio got altered compared to the diabetic control group (Fig. 5).

3.6. Effect of taurine on the antioxidant enzymes

The activity of superoxide dismutase (SOD) and catalase (CAT) were found to be significantly decreased in the diabetic control group compared to normal control group. On the other hand, taurine treatment for 6 weeks post diabetic induction was able to significantly increase the activities of the said antioxidant enzymes compared to the diabetic control group. However, the normal control and only taurine treated groups showed no significant difference (Fig. 6).

3.7. Effect of taurine on the level of adhesion molecules, chemokines and inflammatory cytokines

The expression of TNF-α, IL-1β, IL-6, MCP-1, ICAM-1 and VCAM-1 were significantly increased in the STZ-induced diabetic rats compared to the normal control group. Treatment with taurine for 6 weeks at a dose of 100 mg kg^{-1} body weight post diabetic induction could significantly ameliorate these pathophysiological alterations compared to the diabetic control group (Fig. 7).

3.8. Effect of taurine on apoptotic cell death

To determine the mode of cell death in the testis of STZ-induced diabetic rats, agarose gel electrophoresis was carried out with the genomic DNA obtained from the experimental rats. In diabetic control group, characteristic DNA ladder was observed as the conformational signal of apoptosis, whereas taurine successively attenuated this pathophysiological condition in the DIA + TAU group (Fig. 8).

3.9. Effect of taurine on ER-stress mediated apoptotic pathway

From the immunoblot data, upregulation of calpain-1, cleaved caspase 12, p-PERK, p-eIF2α/total eIF2α ratio, GRP78 and CHOP were scrutinized in the STZ-induced diabetic animals compared to the normal control group. Post treatment with taurine effectively amended these alterations, thereby protecting the cells from ER-stress induced cellular apoptosis (Fig. 9).

3.10. Effect of taurine on the activation of NF κB mediated pathway

Reduced expression of cytosolic NF κB and increased expression of I κB and nuclear NF κB was observed in the diabetic control group (Fig. 10). Administration of taurine (DIA + TAU), however, significantly altered such effects.

3.11. Effect of taurine on mitochondria dependent apoptotic pathways

Immunoblot analyses revealed downregulation in the level of mitochondrial cytochrome C and upregulation in the levels of Bax/Bcl-2 ratio, cytosolic cytochrome-C, cleaved caspase-9, cleaved caspase-3 and

cleaved PARP in the diabetic control groups. DIA + TAU groups showed significant alterations in the expression of these proteins (Fig. 11).

4. Discussion

Prolonged maintenance of hyperglycemia over a long period of time induces oxidative stress in diabetic rats through the activation of the polyol pathway, glucose auto-oxidation and protein glycosylation (Chikezie et al., 2015; Chowdhury et al., 2016; Ghosh et al., 2018). In the present study, the ameliorative role of taurine against diabetes associated oxidative and ER stress induced testicular damage was investigated owing to its already reported potent therapeutic properties (Abd El-Twab et al., 2016; Liu et al., 2017; Sarkar et al., 2017; Tsounapi et al., 2012; Zhao et al., 2016).

Hyperglycemia induced oxidative stress and ER stress elicits pathophysiological complications by augmenting inflammatory and apoptotic pathways (Zhao et al., 2011; Zhang et al., 2013). Diabetes induced testicular damage follows similar mechanisms (Rashid and Sil, 2015a). Administration of STZ was found to increase blood glucose level, decrease serum insulin level and reduce testis weight to body weight ratio and such observations were significantly reversed following treatment with taurine, signifying its anti-diabetic properties.

High level of testosterone is essential for the maintenance of normal physiological status of seminiferous tubules and regulation of spermatogenesis (Smith and Walker, 2014). SDH catalyzes conversion of sorbitol to fructose, thereby providing the energy necessary for the maintenance of normal metabolic functions of the sperm cells (Prasad et al., 1995). 3β-HSD and 17β-HSD are enzymes which catalyze androgenesis (Jana et al., 2006). Reduction in the level of testosterone along with the activity of SDH, 3β-HSD and 17β-HSD are the key signs of diabetes induced testicular damage (Rashid and Sil, 2015a). Our observations in conformity with such a report in STZ treated rats indicated towards testicular damage. Such alterations were significantly reversed following administration of taurine.

Presence of Sertoli cells, Leydig cells, centrally localized spermatozoa and well organized germinal epithelium are key histological features of the testis (Rashid and Sil, 2015a). Histological studies revealed that the testes suffered from loss of Sertoli cells, diminution of Leydig cells, lack of centrally localized spermatozoa and disruption of germinal epithelium in the diabetic rats. Administration of taurine significantly restored the normal histology of testes pointing towards its potency in ameliorating diabetes mediated testicular damage.

Oxidative stress plays a major role in the induction of diabetic complications associated with treatment of STZ (Ghosh et al., 2018). Reduction in the activity of antioxidant enzymes such as SOD and CAT along with GSH/GSSG ratio and elevation of protein carbonyl content and MDA in STZ treated rats were significantly reversed following administration of taurine owing to its potent antioxidant properties. This indicates that taurine protects against diabetic complications by reducing oxidative stress.

Diabetes mediated testicular damage induces elevated translocation of the transcription factor NF κB into the nucleus thereby eliciting inflammatory response (Rashid et al., 2017). In our study, taurine was observed to be able to significantly ameliorate these alterations and inhibit NF κB mediated inflammatory response. ROS enhances NF κB mediated inflammation along with the increase in the levels of proinflammatory cytokines, chemokines and adhesion molecules (Rashid et al., 2017). In our study, the significantly elevated expression of TNF-α, IL-1β, IL-6, MCP-1, ICAM-1 and VCAM-1 in STZ treated rats were significantly reduced following administration with taurine by virtue of its potent anti-inflammatory properties.

ER stress mediated apoptotic death of testicular cells is reported in hyperglycemic conditions (Morishima et al., 2002; Rashid and Sil, 2015a). ER stress induces phosphorylation mediated activation of PERK which in turn activates eIF2α. It then activates CHOP, a downstream molecule. PERK also releases GRP78, which is associated with unfolded protein response (Morishima et al., 2002). Increase in intracellular Ca^{2+} levels in diabetic rats also leads to the activation of calpain-1 which in turn activates caspase-12 (Rashid and Sil, 2015a). In the present study, it was observed that STZ treated rats exhibited increased level of expression of p-PERK, p-eIF2α, GRP78, calpain-1, cleaved caspase-12 and CHOP. Taurine effectively altered such changes thereby inhibiting ER stress mediated apoptotic death of testicular cells.

Programmed cell death or apoptosis is a genetically organized pathway for suicidal attempt of cells under different pathophysiological conditions. Apoptotic cellular death is associated with caspase mediated degradation of DNA by DNases, giving it a ladder like pattern (Baron et al., 1994). Apoptosis is a key feature of diabetes associated oxidative stress induced testicular damage (Manna et al., 2010; Rashid and Sil, 2015a). In our study, DNA fragmentation analyses revealed the occurrence of apoptotic testicular cell death and administration of taurine efficiently restored DNA from oxidative damage by virtue of its anti-apoptotic activity.

Apoptotic cell death is a characteristic of diabetic complications (Singh et al., 2012). Oxidative stress mediated hyperglycemia induced apoptosis may follow the intrinsic mitochondrial pathway with the disruption in the balance between proapoptotic Bax and antiapoptotic Bcl-2 proteins which reduces the loss of mitochondrial membrane potential following the release of cytochrome C in the cytosol. It then subsequently activates caspase-9 and caspase-3. Caspase-3 in turn initiates apoptosis by activating DNases and cleaving PARP (Ghosh et al., 2018; Rashid et al., 2017). In our study, STZ treated rats also exhibited increased expression of the proapoptotic Bax and decreased level of antiapoptotic Bcl-2, augmentation of the release of cytochrome C from mitochondria to cytosol along with increased level of cleaved caspase-9, 3 and PARP. Administration of taurine, however, altered these changes prompting towards its role in suppressing the mitochondrial dependent pathway of apoptosis.

Thus, our findings suggest that taurine ameliorates diabetic testicular damage by inhibiting oxidative and ER stress mediated inflammatory and apoptotic pathways.

5. Conclusion

In conclusion, it can be stated that taurine has the potential to provide protection to testes from diabetes induced oxidative (alterations in the level of antioxidant enzymes) and ER stress (eIF2α signaling cascade) and inflammation (NF κB signaling cascade) and apoptosis mediated damage by circumventing hyperglycemia, improving the status of the markers of testicular damage, regulating intracellular redox balance, inhibiting inflammatory response and ER stress induced mitochondrial dependent apoptotic pathway (Fig. 12). Thus, the study provides a detailed insight into the molecular mechanisms, by which, taurine, through its hypoglycemic, antioxidant, anti-inflammatory and anti-apoptotic properties can ameliorate testicular complications associated with diabetes.

Conflicts of interest

The authors declare no conflict of interest.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.055.

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