Biochimica et Biophysica Acta 1852 (2015) 70–82

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Curcumin ameliorates testicular damage in diabetic rats by suppressing cellular stress-mediated mitochondria and endoplasmic reticulum-dependent apoptotic death

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ARTICLE INFO

Article history:
Received 8 September 2014
Received in revised form 22 October 2014
Accepted 4 November 2014
Available online 11 November 2014

Keywords:
Oxidative and ER stress
Diabetes
Testicular damage
Endoplasmic reticulum and mitochondrial-dependent apoptosis
Antioxidant
Curcumin

ABSTRACT

In the present study, we sought to explore whether curcumin plays any beneficial role against STZ induced testicular abnormalities in diabetic rats, and if so, what possible mechanism it utilizes to provide protection. Exposure to STZ (50 mg/kg body weight, i.p., once) reduced testis-to-body weight ratio, enhanced blood glucose level and intracellular ROS, altered testicular markers, diminished serum testosterone and impaired cellular redox balance. Administration of curcumin at a dose of 100 mg/kg body weight for 8 weeks effectively normalized all the alterations. Curcumin also showed inhibitory effect on the elevation of pro-inflammatory cytokines and translocation of NFκB into the nucleus and promoted the activation of the transcription factor Nrf-2 to provide protection against oxidants. To protect cells from STZ-induced stress-mediated damage, curcumin acted on the key mediators of the apoptotic cell death such as JNK and p38. In addition, this active molecule upregulated Bcl-2 expression, blocked the expression of pro-apoptotic proteins (Bax, Bad and Bid), decreased intracellular Ca^{2+} level, inhibited active caspase cascade and attenuated PARP cleavage. These results suggest that curcumin provides protection against cellular stress-mediated mitochondrial and endoplasmic reticulum-dependent apoptotic death of the testicular cells under diabetic condition and suggests the possibility of using this molecule as a potential therapeutic in the treatment of stress-mediated diabetic testicular dysfunction.

1. Introduction

Diabetes mellitus (DM), a chronic endocrine metabolic disorder, has become a major concern due to its associated complications and rising global incidence [1–3]. It results from either lack of insulin secretion (type-1 diabetes) and/or reduced sensitivity of tissues to insulin (type-2 diabetes) [4]. These events lead to hyperglycemia, and persistent hyperglycemia induces excessive production of reactive oxygen species (ROS) in diabetic subject [5]. Evidence suggests that DM-mediated oxidative stress and altered antioxidant defense of male reproductive system results in infertility/subfertility in the diabetic patient [6–10]. Mammalian sperm cells contain a high amount of polyunsaturated fatty acid that undergoes oxidation to affect sperm motility and fertility [11]. Multiple events, such as irregular hormonal regulation in the process of spermatogenesis, abnormal spermatogenesis, anomalous behavior of sperm, penile erection, ejaculation, germ cell death and varying degree of the testicular lesions are involved in this pathogenesis [12–14]. Along with oxidative stress, recent investigations also depicted the adverse effect of ER stress on male sex organ and fertility in diabetic condition [15]. ER is a eukaryotic cellular organelle present within the cytoplasm and formed by the interconnected network of a series of folded membranes (continue its expansion with the outer membrane of the nucleus) and flattened sacs. SR (sarcoplasmic reticulum) is a type of ER which acts mainly as a store of Ca^{2+} and helps pumping it out on stimulus response. ER plays an important role in protein folding, export and processing. However, sustained hyperglycemia, oxidative stress, excessive Ca^{2+} release and protein load impairs ER homeostasis and results in ER stress, and severe ER stress ultimately causes testicular cell death by activating apoptotic pathways [16]. Since cellular apoptosis is responsible for hyperglycemia-induced oxidative stress-mediated testicular damage in the diabetic pathophysiology, an in vivo model of diabetes has been made to conduct a study employing antihyperglycemic, antioxidative and antiapoptotic remedies to manage this detrimental complication. In the present study, streptozotocin (STZ), a nitrosourea derivative, has been used as a diabetogenic agent that induces

Abbreviations: CAT, catalase; CUR, curcumin; ER, endoplasmic reticulum; GPx, glutathione peroxidase; G6PD, glutathione-6-phosphate dehydrogenase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; MDA, malondialdehyde; MMP, mitochondrial membrane potential; NFκB, nuclear factor kappa B; Nrf-2, nuclear factor erythroid 2-related factor; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositide 3-kinase; ROS, reactive oxygen species; SDH, sorbitol dehydrogenase; SOD, superoxide dismutase; STZ, streptozotocin

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http://dx.doi.org/10.1016/j.bbadis.2014.11.007
0925-4439/

hyperglycemia by the production of excessive free radicals and thus provides a useful model to investigate the efficiency of antioxidant substances [17,18].

For this purpose curcumin, an active yellow colored phenolic pigment of turmeric, isolated from the rhizome of *Curcuma longa*, draws our attention, as it possesses various beneficial biological activities such as antidiabetic, anti-inflammatory, anti-tumor, antioxidant, etc. [19–22]. Besides, this molecule is present in the commonly consumed foodstuff and considered safe compared to the available expensive commercial drugs. Although a number of reports suggest the protective action of curcumin against oxidative stress-mediated cardiomyopathy, neuropathy, nephropathy and hepatic injury; testicular dysfunction [23–29]; etc., no mechanistic approach has yet been taken to describe its detailed beneficial role in oxidative and ER stress-induced testicular damage in diabetes. The present study has been designed to investigate the molecular mechanism of curcumin against hyperglycemia-induced cellular stress-mediated testicular dysfunction. For this purpose, we have determined the effect of curcumin on the sperm count, motility and structural abnormalities in diabetic animals. In addition, the role of this molecule on serum testosterone level and SDH activity; the protein expression of Nrf-2 (stress-induced defense protein), NFκB and upstream signaling components (PI3K, Akt, etc.); ER and oxidative stress-induced apoptotic signaling molecules and stress-induced proteins (JNK and p38) have also been explored. This study might shed some light on the use of curcumin as an effective therapeutic in the treatment of stress-mediated diabetic testicular dysfunction.

2. Materials and methods

2.1. Chemicals

Curcumin, bovine serum albumin (BSA) and Bradford reagent were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Antibodies were purchased from Abcam (Cambridge, UK), Sigma (Missouri, USA) and Cell Signaling (Cell Signaling Technology Inc.,Danvers, MA). Kits for the measurement of blood glucose were purchased from Span Diagnostic Ltd., Surat, Gujarat, India. Streptozotocin (STZ) and all other chemicals were obtained from Sisco Research Laboratory, Andheri, Mumbai, India.

2.2. Animals and care

Eight-weeks-old adult male Wistar rats, weighing approximate 280-300 g, were purchased from M/S Ghosh Enterprises, Kolkota, India. Animals were acclimatized under laboratory conditions for 2 weeks prior to experiments and were maintained under standard conditions of temperature (23 ± 2 °C) and humidity (50 ± 10%) with an alternating 12 h light/dark cycles. They were fed standard pellet diet (Agro Corporation Private Ltd., Bangalore, India) and water ad libitum. All the experiments with animals were carried out according to the guidelines of the institutional animal ethical committee (IAEC), Bose Institute, Kolkata (the permit number is IAEC/BI/3(I) cert. /2010), and full details of the study were approved by both IAEC and CPCSEA (committee for the purpose of control and supervision on experiments on animals), Ministry of Environment and Forests, New Delhi, India (the permit number is 95/99/CPCSEA).

2.3. Induction of diabetes in the experimental animals

After overnight fasting, diabetes was induced in the experimental rats with a single intraperitoneal injection of streptozotocin (STZ) dissolved in 0.1 M sodium citrate buffer, pH 4.5, at a dose of 50 mg/kg body weight [16]. After 3 days of STZ injection, the fasting blood glucose level was determined using an Advanced Accu-check glucometer (Boehringer Mannheim, Indianapolis, IN, USA). The rats having blood glucose above 300 mg/dL were considered to be diabetic and were used for the experiments as necessary.

2.4. Solvent and route of administration

STZ was administered intraperitoneally in sodium citrate buffer. On the other hand, curcumin was given orally by oral gavages in olive oil.

2.5. Determination of dose- and time-dependent effect of curcumin

Dose- and time-dependent study has been done to determine the effective antidiabetic dose of curcumin. For this purpose, rats were randomly divided into 6 groups. Each group had six rats. First two groups were served as normal control (received only vehicle) and diabetic control (received STZ at a dose of 50 mg/kg body weight). Based on the earlier report [16], the remaining four STZ-exposed diabetic groups were further treated with four different doses of curcumin (40, 70, 100 and 130 mg/kg body weight) daily for 8 weeks or more (data are not shown). The effective dose of curcumin was selected by studying its effect on fasting blood glucose level, serum SDH and testosterone level. Eight weeks treatment with a dose, 100 mg/kg body weight, of curcumin has been taken in consideration as it effectively ameliorate all the above-mentioned alterations. Beyond the effective dose and treatment period, no significant effect was observed compared to the used regimen. Histological study was also done to confirm the effectual protective dose of this active molecule.

2.6. In vivo experimental design

After the determination of effective dose of curcumin, experimental design for the present in vivo study has been summarized as follows (Fig. 1):

Animals were randomly divided into four groups consisting of six animals in each group.

Group 1 (normal control) consists of six animals received only vehicle (olive oil).
Group 2 (curcumin group) consists of six animals received only curcumin at a dose of 100 mg/kg body weight orally, daily for 8 weeks to check whether it has any toxic effect or not.
Group 3 (diabetic control) consists of six animals received STZ at a dose of 50 mg/kg body weight once, intraperitoneally.
Group 4 (STZ + CUR) consists of six animals. After diabetic induction they were treated with curcumin at a dose of 100 mg/kg body weight daily for 8 weeks.

After 8 weeks of curcumin treatment animals were sacrificed.

2.7. Collection of blood, serum and testis

Rats in each group were bled after every 7 days from the lateral vein of the tail and 100 μL of blood was taken for the measurement of blood glucose level. Serum was prepared for the dose- and time-dependent assay of SDH and testosterone. After scarification, blood samples were also drawn from caudal vena cava and incubated at 37 °C for 30 min. To get serum, blood samples were centrifuged at 3000×*g* for 30 min; clear serum was obtained and stored at −80 °C until use. Testes were aseptically taken out from the rats and weighed. These tissues were either stored at −80 °C till further analysis or fixed in 10% buffered formalin for histological assessments.

2.8. Preparation of testicular tissue homogenate

Collected testes were minced, washed and homogenized in a Dounce glass homogenizer in 10 mM HEPES–KOH/1 mM EGTA buffer

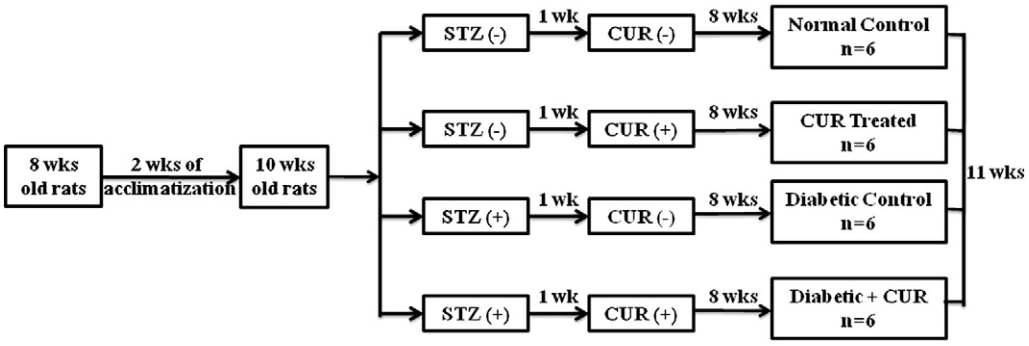

Fig. 1. Schematic diagram of in vivo experimental protocol.

(pH 7.5) containing 250 mM sucrose and supplemented with protease and phosphatase inhibitors. The homogenates were spun down for 10 min at 2,000 g at 4 °C. The supernatant was collected and used for the experiments.

2.9. Determination of sperm abnormality, sperm count and motility

For sperm count, sperm abnormality and sperm motility studies, the epididymis was minced with anatomic scissors; a sperm suspension was prepared in phosphate-buffered saline (PBS). Sperm smears were drawn on clean and grease-free slides, dried overnight and then stained with 10% Giemsa (diluted with Sorresons buffer, pH 7.0) for 1 h and subsequently observed. Morphologically abnormal sperm was identified and recorded following the procedure of Wyrobek and Bruce [30]. The same suspension was used for sperm counting and motility using a hemocytometer. 100 μL of the suspension was mixed with an equal volume of 1% Trypan blue in the same medium and number of sperm was counted. Live sperm totally excluded the dye whereas dead sperm had taken up the dye and showed blue head [31].

2.10. Determination of the activities of 3β-HSD and 17β-HSD

Testicular 3β-HSD and 17β-HSD activities were measured spectrophotometrically following the methods of Talalay [32] and Jarabak et al. [33], respectively. For the determination of 3β-HSD activity, the testicular tissue was homogenized in 15% spectroscopic grade 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. One milliliter supernatant was mixed with 1 mL of 100 μM sodium pyrophosphate buffer of pH 8.9 and 30 μg of dehydroepiandrosterone in 40 μL of ethanol and 960 μL of 25% BSA, making a 3 mL of the incubation mixture. The enzyme activity was measured after addition of 0.5 μmol of NAD to the tissue supernatant mixture in a spectrophotometer at 340 nm against a blank (without NAD). One unit of enzyme activity is the amount causing a change in absorbance of 0.001/min at 340 nm. Using the same supernatant prepared for the assay of 3β-HSD, the activity of testicular 17β-HSD was measured. One milliliter supernatant was mixed with 1 mL of 440 μM sodium pyrophosphate buffer of pH 10.2, 40 μL of ethanol containing 0.3 μmol of testosterone and 960 μL of 25% BSA, making a 3 mL volume of the incubation mixture. The enzyme activity was measured after addition of 1.1 μmol NAD to the tissue supernatant mixture in a spectrophotometer at 340 nm against a blank (without NAD). One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

2.11. Measurement of fasting blood glucose, serum insulin, testosterone, TNF-α and IL-1β level

The fasting blood glucose level in the experimental rats was examined periodically during the treatment via tail prick method using an Advanced Accu-check glucometer (Boehringer Mannheim, Indianapolis, IN, USA). Insulin, testosterone, TNF-α and IL-1β concentrations in the serum samples were analyzed using enzyme linked immunosorbent assay (ELISA) performed by the method of Ausubel et al. [34] using standard kits (Span diagnostic Ltd., India).

2.12. Histological evaluation

Testes from the normal and experimental rats were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5 μm thickness were stained with hematoxylin and eosin to evaluate the histological changes under light microscope.

2.13. Biochemical studies

2.13.1. Estimation of lipid and protein damage

Lipid peroxidation in all the experimental sets was assessed by a colorimetric reaction with thiobarbituric acid (TBA) as described by Esterbauer and Cheeseman [35]. The absorbance of thiobarbituric acid reactive substance (TBARS) was measured at 532 nm and its concentration was calculated using the extinction coefficient of MDA which is $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (since 99% of TBARS exists as MDA). Protein carbonyl contents were determined according to the methods of Uchida and Stadtman [36]. The assessment was done based on the formation of protein hydrazone by reaction with 2,4-DNPH. The absorbance was recorded at 365 nm. The results were expressed as nmol of DNPH incorporated/mg protein based on the molar extinction coefficient of $22{,}000\ M^{-1}\ cm^{-1}$ for aliphatic hydrazones.

2.13.2. Measurement of intracellular ROS, cellular metabolites and antioxidant enzymes

Intracellular ROS production was estimated by using 2,7-dichlorofluorescein diacetate (DCF-DA) as a probe using the method of Manna et al. [37]. Briefly, 100 μL of tissue homogenates was incubated with the assay media (20 mM Tris–HCl, 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 30 mM glucose and 5 μM DCF-DA) at 37 °C for 15 min. The formation of DCF was measured at the excitation wavelength of 488 nm and emission wavelength of 510 nm for 10 min by using a fluorescence spectrometer (HITACHI, Model No F7000) equipped with an FITC filter.

GSH and GSSG levels were measured by the method of Hissin and Hilf using o-phthalaldehyde (OPT) as a fluorescent reagent [38]. The method takes advantage of the reaction of GSH with OPT at pH 8 and of GSSG with OPT at pH 12. GSH can be complexed to *N*-ethylmaleimide to prevent interference of GSH with the measurement of GSSG. The fluorescence was determined at excitation wavelength of 360 nm and emission wavelength of 460 nm.

Specific markers related to testicular dysfunction, e.g. sorbitol dehydrogenase (SDH) and glucose-6-phosphate dehydrogenase (G6PD) activities in the tissues, were estimated by the methods of Bergmeyer [39]. SDH is an enzyme, catalyses the oxidation–reduction reaction, involving the interconversion of fructose and sorbitol. The

rate of oxidation of NADH is proportional to the rate of conversion of D-fructose to D-sorbitol. The rate of decrease in absorbance at 340 nm allows the measurement of SDH activity. G6PD is a cytosolic enzyme, which maintains the level of NADPH. G6PD activity was measured by the reduction of 2 mM $NADP^+$ by G6PD, in the presence of glucose 6-phosphate. The enzyme activity was measured by monitoring the increase in absorption at 340 nm. Activities of antioxidant enzymes (SOD, CAT, GST, GR and GPx) in the testicular tissue were determined following the method as described by Rashid et al. [40].

2.14. Measurement of intracellular Ca^{2+}

Testicular cells were isolated by the method of Yeh et al. [41]. Intracellular Ca^{2+} levels were determined using the intracellular Ca^{2+} probe Fluo 3/acetoxymethyl ester (Molecular Probes, Inc), which binds Ca^{2+} with a 1:1 stoichiometry. Samples were incubated in the darkness with 250 nM Fluo-3 for 30 min at 37 °C. Fluorescence was measured at FL1 (530 nm) in a BD Biosciences FACScan flow cytometer with excitation at 488 nm, and Cell Quest software was employed for subsequent data analysis following the method of Das et al. [42].

2.15. DNA fragmentation assay for the determination of the mode of cell death

Genomic DNA was isolated from testicular tissues of the experimental rats by phenol–chloroform method and was then electrophoresed on 1.8% agarose gel in presence of ethidium bromide following the method of Huang et al. [43]. The DNA fragmentation (DNA ladder) was visualized by UV light.

2.16. Mitochondria isolation and determination of mitochondrial membrane potential ($\Delta\psi m$)

The prepared testicular tissue homogenates were spun down for 10 min at 2,000 g at 4 °C to discard the pellet. The supernatant was overlaid on 0.75 M sucrose in HEPES buffer and centrifuged for 30 min at 10,000 g. The supernatant was discarded and the mitochondria pellets were resuspended in HEPES buffer and recentrifuged for 10 min at 10,000 g. This supernatant was also discarded and the final mitochondrial pellet was resuspended in PBS. It was stored at −80 °C until use. After isolating fresh mitochondria from the testis, the mitochondrial membrane potential ($\Delta\psi m$) was estimated on the basis of cell retention of the fluorescent cationic probe rhodamine 123 [44,45].

2.17. Assay of cytosolic and mitochondrial cytochrome c

The concentration of cytosolic and mitochondrial cytochrome *c* was measured following the method as described elsewhere with the cytochrome *c* enzyme immunometric assay kit (Minneapolis, USA) [46].

2.18. Western blot analysis

For Western blot analysis, an equal amount of protein (50 μg) from each tissue sample was subjected to SDS–PAGE and transferred to PVDF membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 1% BSA to prevent non-specific binding and then incubated with primary antibodies overnight at 4 °C [47]. A number of primary antibodies such as anti-NFκB (1:250), anti-Lamin B 1 (1: 1,000), anti-IκBα (1:1,000), anti-COX-2 (1:1,000), anti-phospho-PI3K (1: 1,000), anti-phospho-Akt (1: 500), anti-Nrf-2 (1:500), anti-TNF-R1 (1:1,000), anti-phospho-p38 (1:1,000), anti-phospho-JNK (1:1,000), anti-Bad (1:1,000), anti-calpain-1 (1:1000), anti-caspase-12 (1:1000), anti-caspase-3 (1:1,000), anti-Bcl-2 (1:1,000 dilution), anti-caspase-9 (1:1,000 dilution), anti-Bax (1:1,000 dilution), anti-PARP (1:1,000 dilution), anti-caspase-8 (1:1,000 dilution), anti-Bid (1:500) and anti-β-actin (1:1,000 dilution). The membranes were washed in TBST (50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with appropriate HRP-conjugated secondary antibody (1:2,000 dilution) for 2 h at room temperature and developed by the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB) system (Bangalore Genei, India).

2.19. Statistical analysis

Results have been expressed as mean ± SEM, where $n = 6$. Statistical evaluation of data has been done by means of one-way analysis ANOVA, and the group means were compared by Tukey test using Origin8 software (OriginLab, Massachusetts). A *p*-value less than 0.05 have considered as statistically significant.

3. Results

3.1. Evaluation of induced diabetes, alteration of testis-to-body weight ratio and effect of curcumin

Some of the key markers of the diabetic pathophysiology are elevation in the blood glucose level, reduction in serum insulin and decrease in body weight [48,49]. Thus in the present study, diabetes in STZ-exposed rodents was evaluated by the increase in blood glucose level (133%), decrease in serum insulin (80%) and finally reduction in the body weight by 18% (Fig. 2A–D). However, treatment with curcumin reduced blood glucose level in a dose- and time-dependent manner and normalized all the above-mentioned changes optimally at a dose of 100 mg/kg body weight without showing any toxic effect in the control group (Fig. 2A–D). These results indicate that curcumin possesses hypoglycemic activity. Along with this, it has also been observed that curcumin treatment increased the testis weight and testis-to-body weight ratio in the STZ-exposed rats to a considerable extent (Fig. 2E and F, respectively). This discussion suggests that growth reducing activity of STZ in diabetes could be blocked by curcumin.

3.2. Effect of curcumin on testicular function related markers

To check the testicular damage along with the testis weight we measured SDH, G6PD activity and testosterone level in the experimental rats. Former two help in the development of the germinal cells and testosterone is the marker of regulated androgenesis [50]. Reduced activities of SDH, G6PD and level of testosterone were found in the STZ-exposed group compared to normal. In addition, we also performed SDH and testosterone assays to determine the optimum dose and time of curcumin, required for giving protection to testes under hyperglycemic condition. It was found that curcumin effectively increased the activity of SDH and level of testosterone in hyperglycemic rodents at a dose of 100 mg/kg body weight after 8 weeks treatment without affecting normal ones (Fig. 2G–J). Since STZ-induced hyperglycemia is responsible for the reduction of the serum testosterone level, therefore, to investigate the possible mechanism related to this impairment, we determined the activity of 3β-HSD and 17β-HSD. These two testicular enzymes are responsible for the biosynthesis of testosterone. Diminution in the activity of these two enzymes by 63% and 55% was observed in STZ-exposed group. However, curcumin restored these changes as shown in Fig. 2K and L, respectively.

3.3. Role of curcumin on sperm parameters and testicular morphology of diabetic rodents

Decrease in sperm count, motility and increase in sperm abnormality (the parameters associated with testicular dysfunction) has been observed in the diabetic group compared to the control group (Fig. 3A–C). Combining all, STZ exposure decreased the quantity and quality of semen required to accomplish fertilization. Curcumin (100 mg/kg

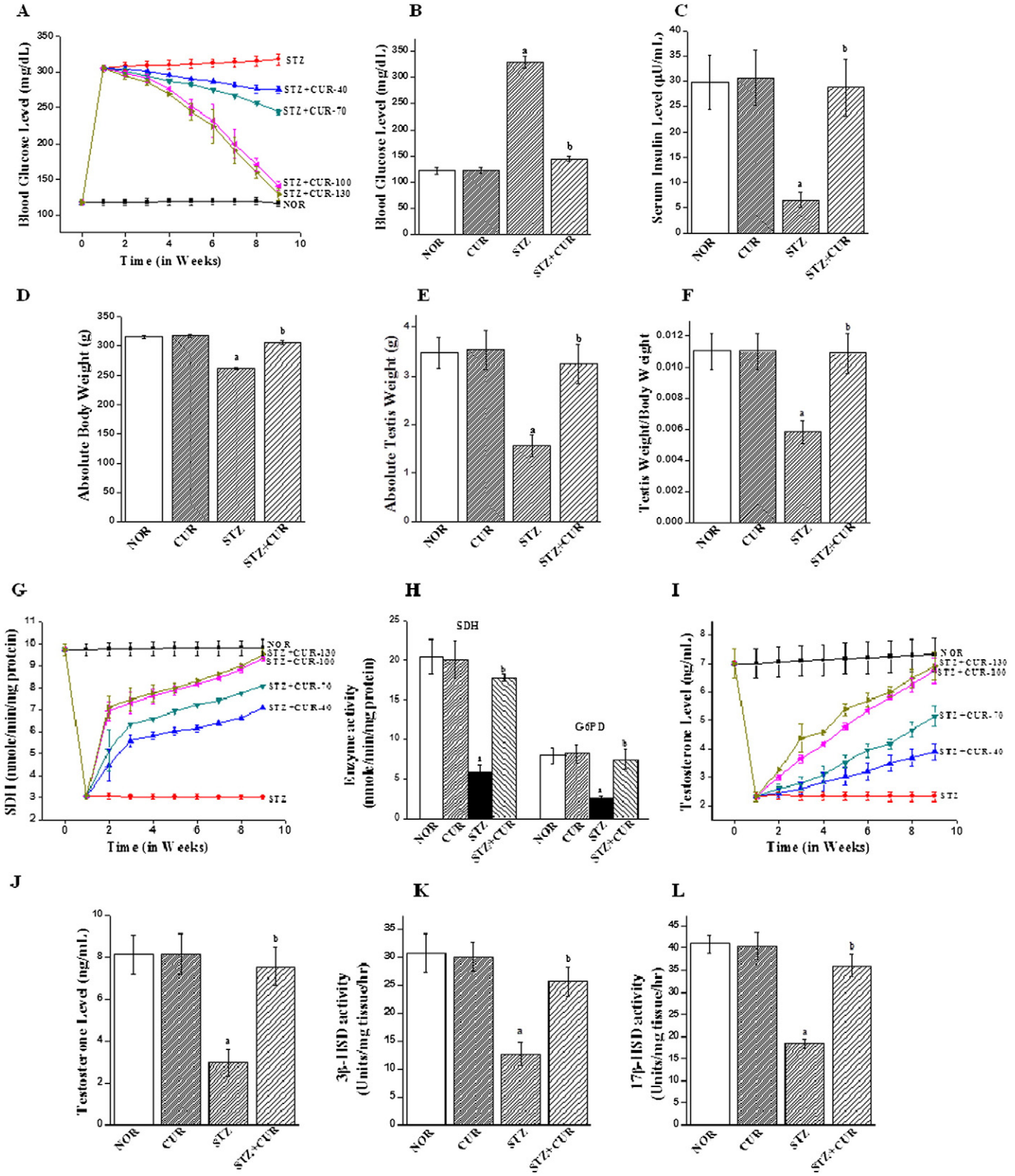

Fig. 2. Effect of STZ and curcumin on blood glucose, testis-to-body weight ratio and serum markers of testis. (A) Dose- and time-dependent effect of CUR on blood glucose level of STZ-induced diabetic rats. NOR: Blood glucose of normal control rats; STZ: Blood glucose of STZ-exposed rats; STZ + CUR-40, STZ + CUR-70, STZ + CUR-100, STZ + CUR-130: blood glucose of CUR-treated diabetic rats for 8 weeks at a dose of 40, 70, 100 and 130 mg/kg body weight. Effect of CUR-100 on (B) blood glucose level, (C) serum insulin level, (D) absolute body weight, (E) absolute testis weight and (F) testis-to-body weight ratio of the experimental rats. NOR: normal rats; CUR: normal rats treated with CUR-100 only; STZ: diabetic control; STZ + CUR: diabetic rats treated with CUR-100. (G) Dose- and time-dependent effect of CUR on serum SDH of STZ-induced diabetic rats. Effect of CUR (CUR-100) on (H) SDH and G6PD enzyme activity, (I) Dose- and time-dependent effect of CUR on serum testosterone level of STZ-induced diabetic rats. Effect of CUR (CUR-100) on (J) testosterone level, (K) 3β-HSD and (L) 17β-HSD activity of the experimental rats. Data are mean ± SEM, for 6 independent experiments for each group and were analyzed by one-way ANOVA, with Tukey test. "a" indicates the significant difference between the normal control and diabetic control groups, and "b" indicates the significant difference between the diabetic control and CUR-treated diabetic groups ($^{a}P < 0.05$, $^{b}P < 0.05$).

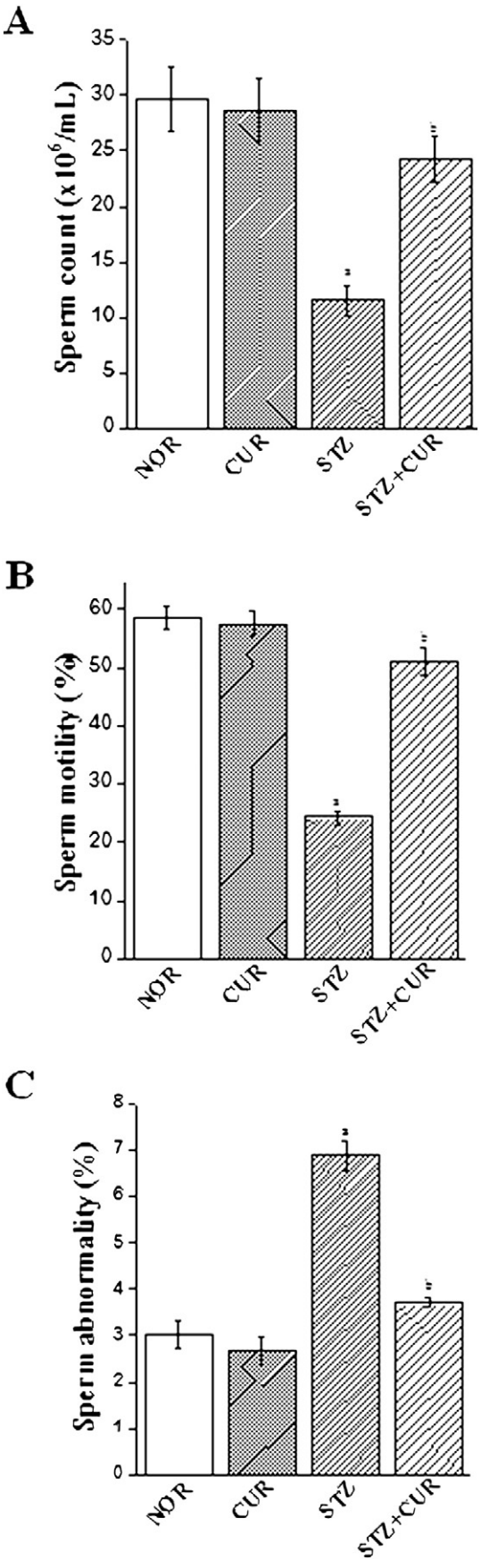

Fig. 3. Effect of STZ and curcumin on sperm parameters. (A) Sperm count, (B) sperm motility and (C) sperm abnormality of the experimental rats. NOR: normal rats; CUR: normal rats treated with CUR-100 only; STZ: diabetic control; STZ + CUR: diabetic rats treated with CUR-100. Data are mean ± SEM, for 6 independent experiments for each group and were analyzed by one-way ANOVA, with Tukey test. "a" indicates the significant difference between the normal control and the diabetic control groups, and "b" indicates the significant difference between the diabetic control and CUR-treated diabetic groups ($^{a}P < 0.05$, $^{b}P < 0.05$).

body weight), however, significantly minimized the adverse effect of STZ and increased the efficacy of semen for its function.

Histological assessment of the testicular tissue showed atrophy of seminiferous tubule, loss of testicular cells (such as Leydig and Sertoli cells), sloughing of centrally located spermatozoa and disappearance of spermatids in the seminiferous tubular lumen in the diabetic rats as shown in Fig. 4. However, dose-dependent treatment of these animals with curcumin for 8 weeks attenuated these changes and provided optimal protection at a dose of 100 mg/kg body weight, suggesting the protective action of curcumin against oxidative stress-mediated testicular damage in diabetic rats.

3.4. Effect of curcumin on ROS generation; oxidative stress-associated markers and cellular antioxidant capacity

Intracellular ROS level, MDA (end product of lipid peroxidation), protein carbonyl content, activities of antioxidant enzymes and cellular metabolites (GSH/GSSG ratio) express the state of oxidative stress within the cell. STZ exposure caused increase in intracellular ROS by 155% (Fig. 5A), and enhanced level of oxidative stress related markers MDA (140%) and protein carbonyl (210%) has also been found in this group (Fig. 5B and C, respectively). In addition, diabetic animals also showed decreased activities of antioxidant enzymes (Fig. 5D) and impaired cellular redox balance in tissues in favor of oxidative stress by decreasing GSH, increasing GSSG and diminishing GSH/GSSG ratio within the testes (Fig. 5E and F, respectively). These results collectively reflect the antioxidant capability of curcumin against oxidative stress in diabetic complications.

3.5. Effect of curcumin on STZ-induced upregulation of pro-inflammatory cytokines TNF-α and IL-1β

As pro-inflammatory cytokines play an important role in the initiation and progression of diabetic testicular dysfunction [15], we investigated the level of TNF-α and IL-1β in the experimental animals. Results showed that both the cytokines TNF-α and IL-1β get elevated (by 135% and 150%, respectively) in STZ-exposed groups; curcumin administration, however, restored these alterations to a significant extent suggesting the anti-inflammatory potential of this active molecule (Fig. 6A).

3.6. Effect of curcumin on STZ-induced apoptotic death of the testicular cells

Earlier evidences showed that alterations in the testes related parameters in favor of oxidative stress lead to testicular cell death [51] . In order to investigate the mode of STZ-induced testicular cell death under diabetic condition, we performed DNA fragmentation assay with the genomic DNA of the experimental animals. It was found that STZ exposure caused fragmentation of DNA in testicular cells (Fig. 6B). However, treatment with curcumin effectively attenuated DNA fragmentation in STZ administered group.

3.7. Effect of curcumin on nuclear translocation of NFκB and activation of Nrf-2 by PI3K/Akt-mediated pathway

NFκB is a transcription factor that plays a vital role in the regulation of inflammation, cell division and apoptosis. TNF-α is responsible for the increased degradation of IκBα and translocation of p65 subunit

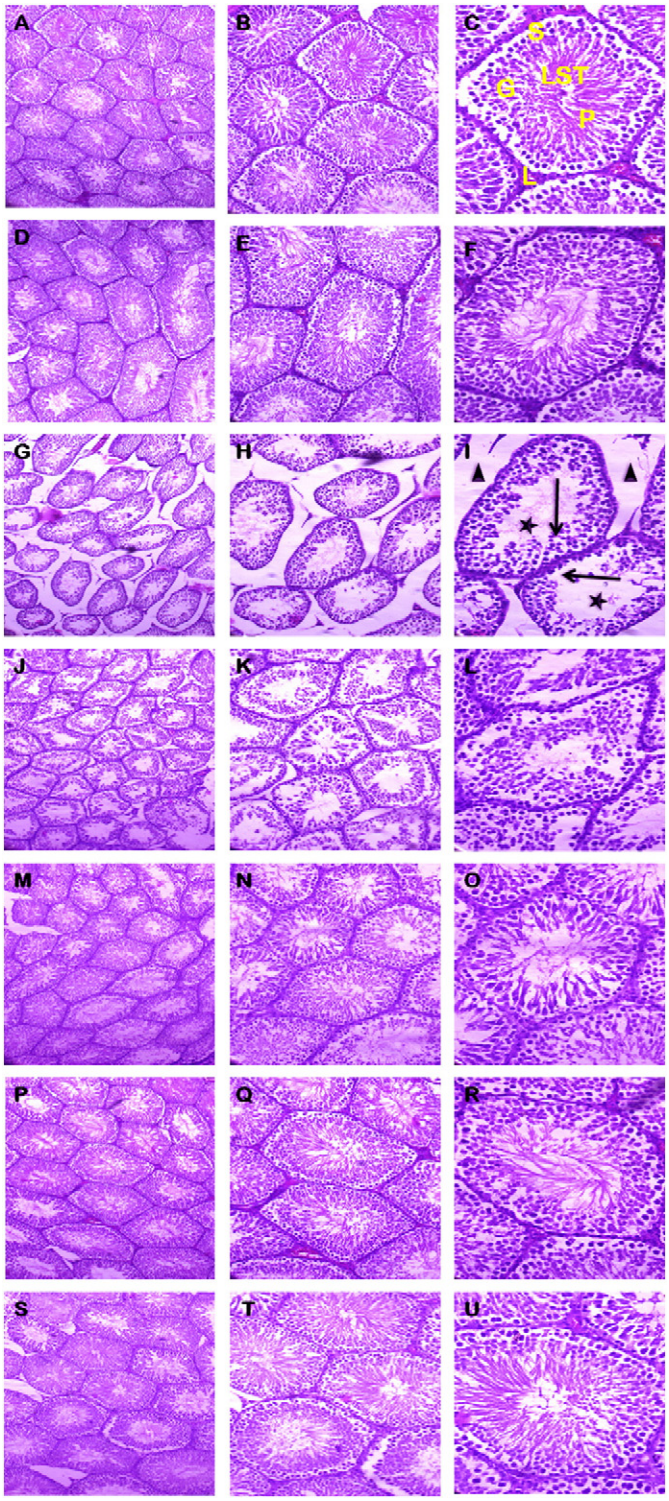

Fig. 4. Dose-dependent effect of CUR on histopathological changes in testicular tissue (stained with hematoxylin and eosin dye). (A–C) Testicular section from normal rat testis, A (×100), B (×200), C (×400); (D–F) testicular section from animals treated with CUR-100 only, D (×100), E (×200), F (×400); (G–I) testicular section from the diabetic group, G (×100), H (×200), I (×400); (J–U) testicular section from the animals treated with CUR at a dose of 40 (J–L), J (×100), K (×200), L (×400); 70 (M–O), M (×100), N (×200), O (×400); 100 (P–R), P (×100), Q (×200), R (×400) and 130 (S–U) mg/kg body weight after diabetic induction, S (×100), T (×200), U (×400). Normal control shows spermatogonia (G), cytoplasm of Sertoli cells (S), Leydig cells (L), lumena of seminiferous tubule (LST) and spermatocytes (P). In the STZ-intoxicated group, triangles indicate loss of leydig cells, arrows indicate loss of Sertoli cells and asterisks indicate loss of centrally located spermatozoa, whereas CUR treatment shows normal morphology.

of NFκB into the nucleus. In the present study, elevated level of NFκB in nucleus (by 2.8-fold), decreased level of it in cytosol (by 1.4-fold) and reduced level of IκBα (by 1.6-fold) in the testis of diabetic rats were observed. We have also found increased expression of COX-2 (by 5.8-fold) at the downstream of NFκB (Fig. 6C). In the diabetic group, low levels of another transcription factor Nrf-2 (2.5-fold) (a protein responsible for cellular defense) and decreased expression of phospho-PI3K (by 2.5-fold) along with phospho-Akt (by 3-fold) without any change in the expression of the total protein (data are not shown) have also been detected compared to the normal at the upstream of Nrf-2. However, the administration of curcumin significantly restored all the changes and brought them back to normal (Fig. 6C).

3.8. Effect of curcumin on TNF-α and its receptor-mediated cell death pathway

TNF-α induces both cell death and proliferation. In order to find out the role of TNF-α in testicular pathophysiology, we have measured the expression of its receptor TNF-R1. Our data showed that STZ exposure caused increase in the expression of TNF-R1 (by 3-fold), cleaved caspase-8 (by 2.8-fold) and proapoptotic protein Bid (by 4.3-fold), whereas curcumin attenuated diabetes-induced elevation of these three proteins (Fig. 6C), suggesting its inhibitory effect on TNF-α-dependent extrinsic pathway of apoptosis.

3.9. Effect of curcumin on the phosphorylation of stress proteins and stress proteins induced apoptosis

In STZ-induced diabetic testicular dysfunction JNK and p38 play an important role and helps in the induction of apoptosis [52]. In order to investigate the protective role of curcumin, we did immunoblotting of both phosphorylated JNK as well as phosphorylated p38 in experimental animals. Increased expression of p-JNK (by 2.4-fold) and p-p38 (by 4.9-fold) was found in the diabetic group as shown in the densitometric analysis of these two proteins (Fig. 6C). Curcumin administration markedly decreased the expression of both the proteins and protects cells from JNK and p38-mediated apoptotic damages.

3.10. Effect of curcumin on STZ-induced endoplasmic reticulum/sarcoplasmic reticulum-dependent apoptotic pathway

Oxidative stress increases the stress of ER/SR and stressed ER results in the release of Ca^{2+} to cytosol. Increased intracellular Ca^{2+} might bring cell death. In order to determine the role of curcumin against ER/SR-dependent testicular cell death, we have measured intracellular Ca^{2+} along with the expression of calpain-1 and cleaved caspase-12 as these are the crucial players of ER/SR-mediated apoptosis. In the diabetic group elevated level of intracellular Ca^{2+} (Fig. 7A), enhanced expression of calpain-1 (by 2.6-fold) and cleaved caspase-12 (by 3.5-fold) were observed (Fig. 7A and B, respectively); administration of curcumin, however, normalized all the changes and protects cells from ER-mediated apoptosis in hyperglycemic condition.

3.11. Effect of curcumin on STZ-induced mitochondria-dependent apoptotic pathway

Mitochondria play a crucial role in electron transport and oxidative phosphorylation. Fall in mitochondrial membrane potential (MMP), release of cytochrome *c* from mitochondria to cytosol, alteration in the ratio of Bax-Bcl-2 and activation of subsequent caspase cascade are the key signaling mechanism that takes part in mitochondrial-dependent apoptotic pathway. In the present study, reduction in MMP, elevation in cytosolic cytochrome *c* (by 2.8-fold), diminution in mitochondrial cytochrome *c* (by 1.8-fold), increment in pro-apoptotic protein (Bad by 2.6-fold), enhancement in the ratio of pro and anti-apoptotic proteins (Bax-Bcl-2 by 3.1-fold), activation of cleaved

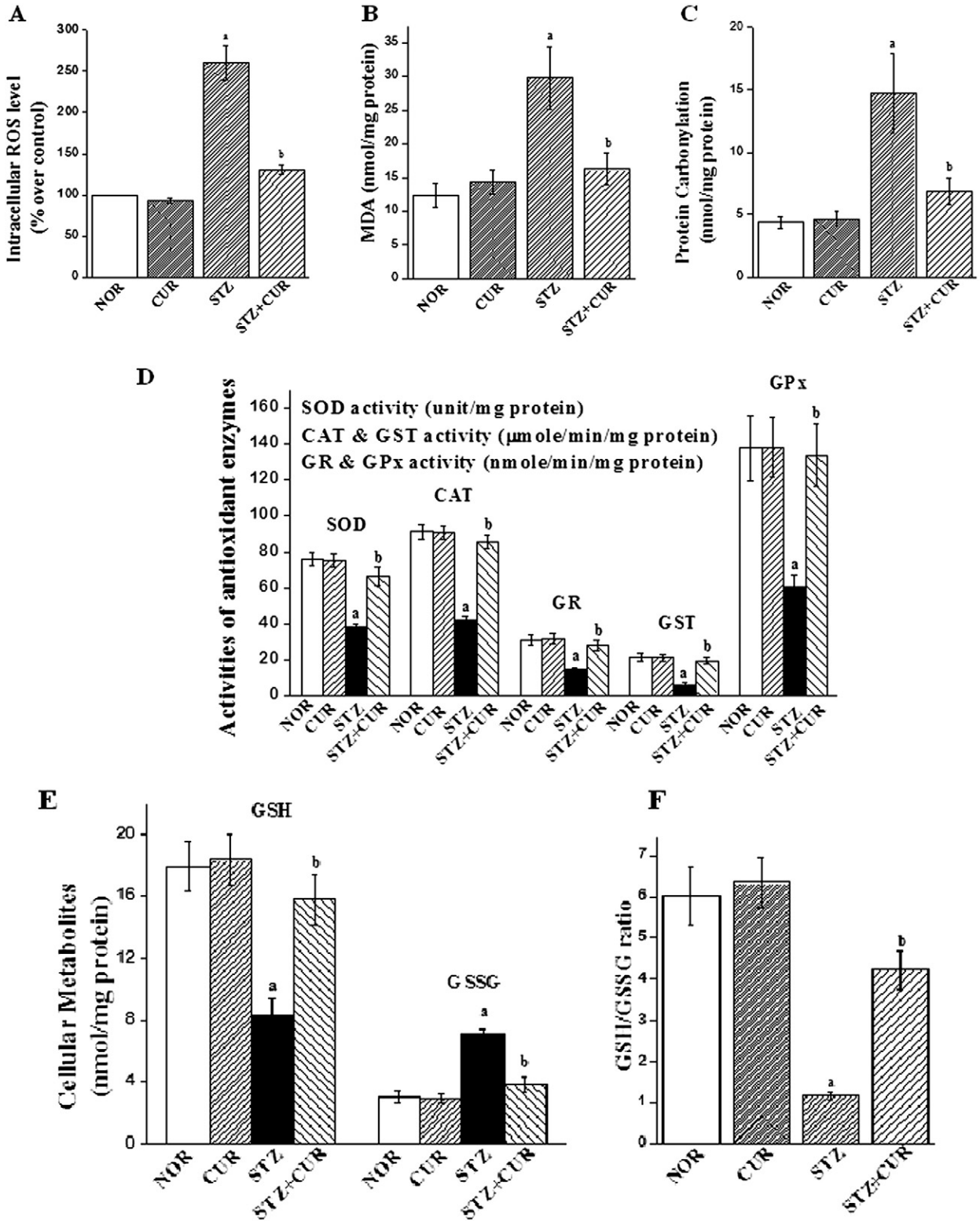

Fig. 5. Effect of CUR (CUR-100) on oxidative stress related parameters. (A) ROS level, (B) MDA level, (C) protein carbonylation, (D) activity of antioxidant enzymes, (E) cellular metabolites (GSH and GSSG) level and (F) Redox (GSH/GSSG) ratio in the testicular tissue of the experimental rats. NOR: normal rats; CUR: normal rats treated with CUR (CUR-100) only; STZ: diabetic control; STZ + CUR: diabetic rats treated with CUR. Each column represents mean ± SEM, $n = 6$. "a" indicates the significant difference between the normal control and diabetic control groups, and "b" indicates the significant difference between the diabetic control and CUR-treated diabetic groups ($^{a}P < 0.05$, $^{b}P < 0.05$).

caspases (9 and 3, by 4.6- and 3-fold, respectively) and augmentation of cleaved PARP (by 2.3-fold) were detected in STZ-induced diabetic rats (Fig. 8A–C, respectively). Curcumin effectively attenuated all the alterations and by suppressing mitochondrial-dependent apoptotic pathway, helped cells to bring back their normal physiological state.

4. Discussion

Prolonged hyperglycemia is a causative factor for the manifestation of oxidative and ER stress in diabetic patients [48,53,54]. In this patho-physiology, glucose autooxidation and protein glycosylation result in

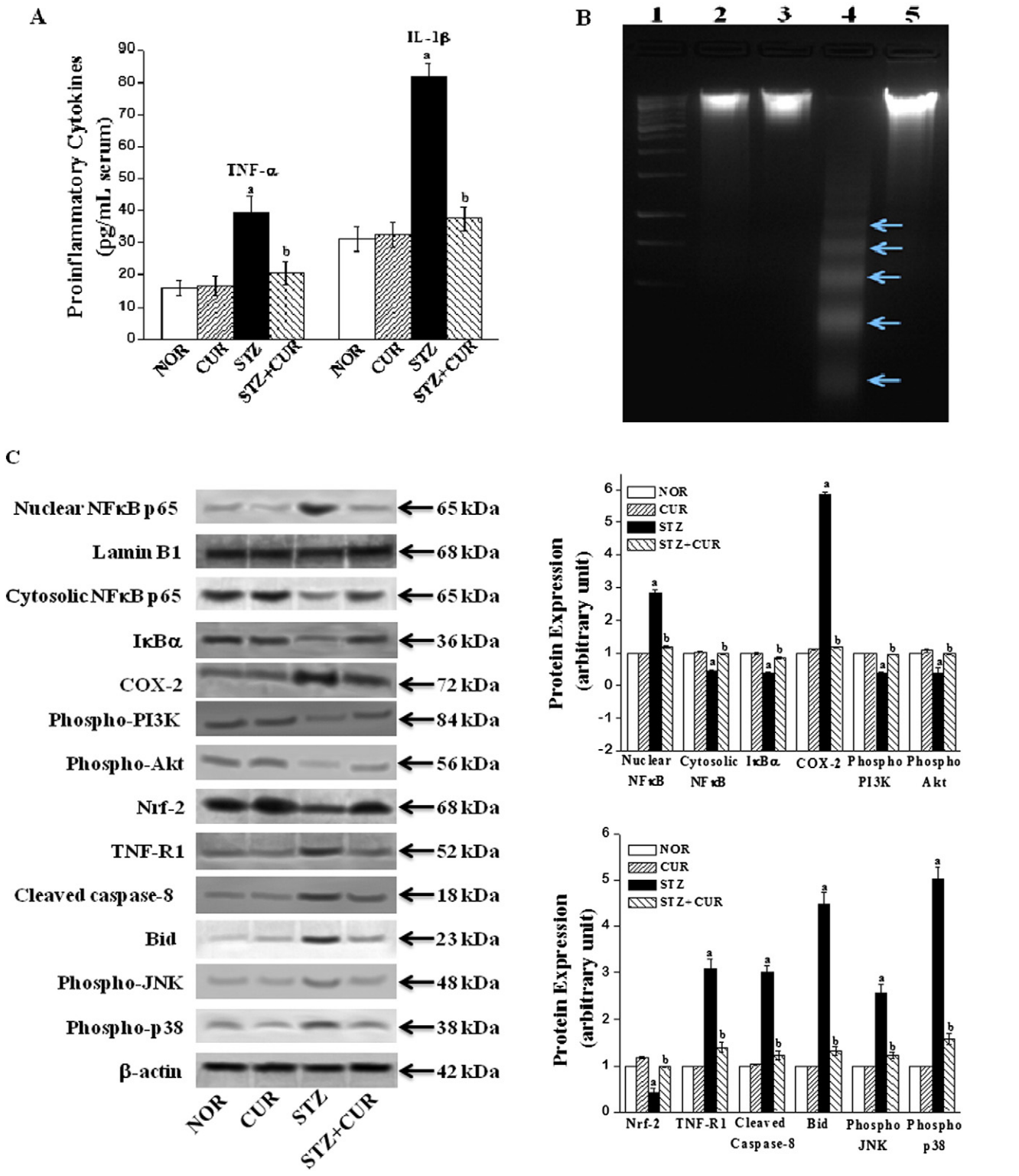

Fig. 6. Effect of CUR (CUR-100) on proinflammatory cytokines and determination of mode of cell death. (A) TNF-α and IL-1β in the serum of the experimental rats. NOR: normal rats; CUR: normal rats treated with CUR (CUR-100) only; STZ: diabetic control; STZ + CUR: diabetic rats treated with CUR. Each column represents mean ± SEM, $n = 6$. "a" indicates the significant difference between the normal control and diabetic control groups, and "b" indicates the significant difference between the diabetic control and CUR-treated diabetic groups ($^{a}P < 0.05$, $^{b}P < 0.05$). (B) DNA fragmentation on agarose/ethidium bromide gel. DNA isolated from experimental testicular tissues was loaded onto 1.8% (w/v) agarose gels. Lane 1: Marker (1 kb DNA ladder); Lane 2: DNA isolated from normal testis; Lane 3: DNA isolated from the testicular tissue of animals treated with CUR-100 only; Lane 4: DNA isolated from the testicular tissue of the diabetic animals (arrows indicate the DNA fragments); and Lane 5: DNA isolated from the testicular tissue of the animals treated with CUR after diabetic induction. (C) Western blots analysis of NFκB, IκBα, phospho-PI3K, phospho-Akt , COX-2, Nrf-2, TNF-R1, cleaved caspase-8, Bid, phospho-JNK and phospho-p38 in the testicular tissue of experimental animals. The relative intensities of bands were determined using NIH image software, and the control band was given an arbitrary value of 1. Data represent mean ± SEM, $n = 6$. "a" indicates the significant difference between the normal control and diabetic control groups, and "b" indicates the significant difference between the diabetic control and CUR-treated diabetic groups ($^{a}P < 0.05$, $^{b}P < 0.05$).

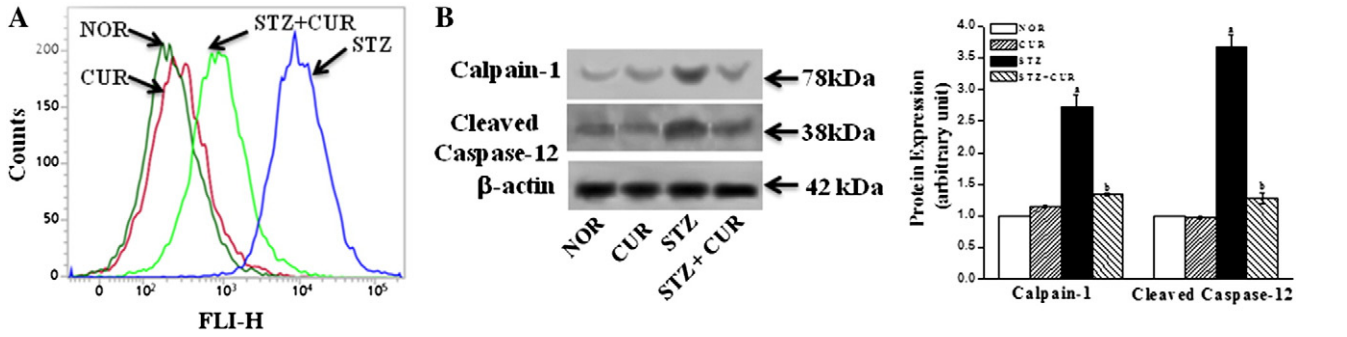

Fig. 7. Involvement of ER/SR-dependent apoptosis and role of curcumin in diabetic animals. (A) Intracellular Ca^{2+}, NOR: normal rats; CUR: normal rats treated with CUR (CUR-100) only; STZ: diabetic control; STZ + CUR: diabetic rats treated with CUR. Results represent one of the six independent experiments. (B) Western blots analysis of calpain-1 and cleaved caspase-12 in the testicular tissue of experimental animals. The relative intensities of bands were determined using NIH- image software, and the control band was given an arbitrary value of 1. Data represent mean ± SEM, $n = 6$. "a" indicates the significant difference between the normal control and diabetic control groups, and "b" indicates the significant difference between the diabetic control and CUR-treated diabetic groups ($^{a}P < 0.05$, $^{b}P < 0.05$).

excess production of free radicals. These free radicals are the key player in the stress-mediated damage of various tissues including testis [55,56]. In the present study, the beneficial role of curcumin was investigated to combat diabetes-induced adverse effect on testicular tissues [16,57,58]. Exposure to STZ caused elevation in blood glucose, reduction in serum insulin and loss in body weight of the experimental rats. However, oral administration of curcumin ameliorates all the changes suggesting its antidiabetic property. Decreased testis weight, testis-to-

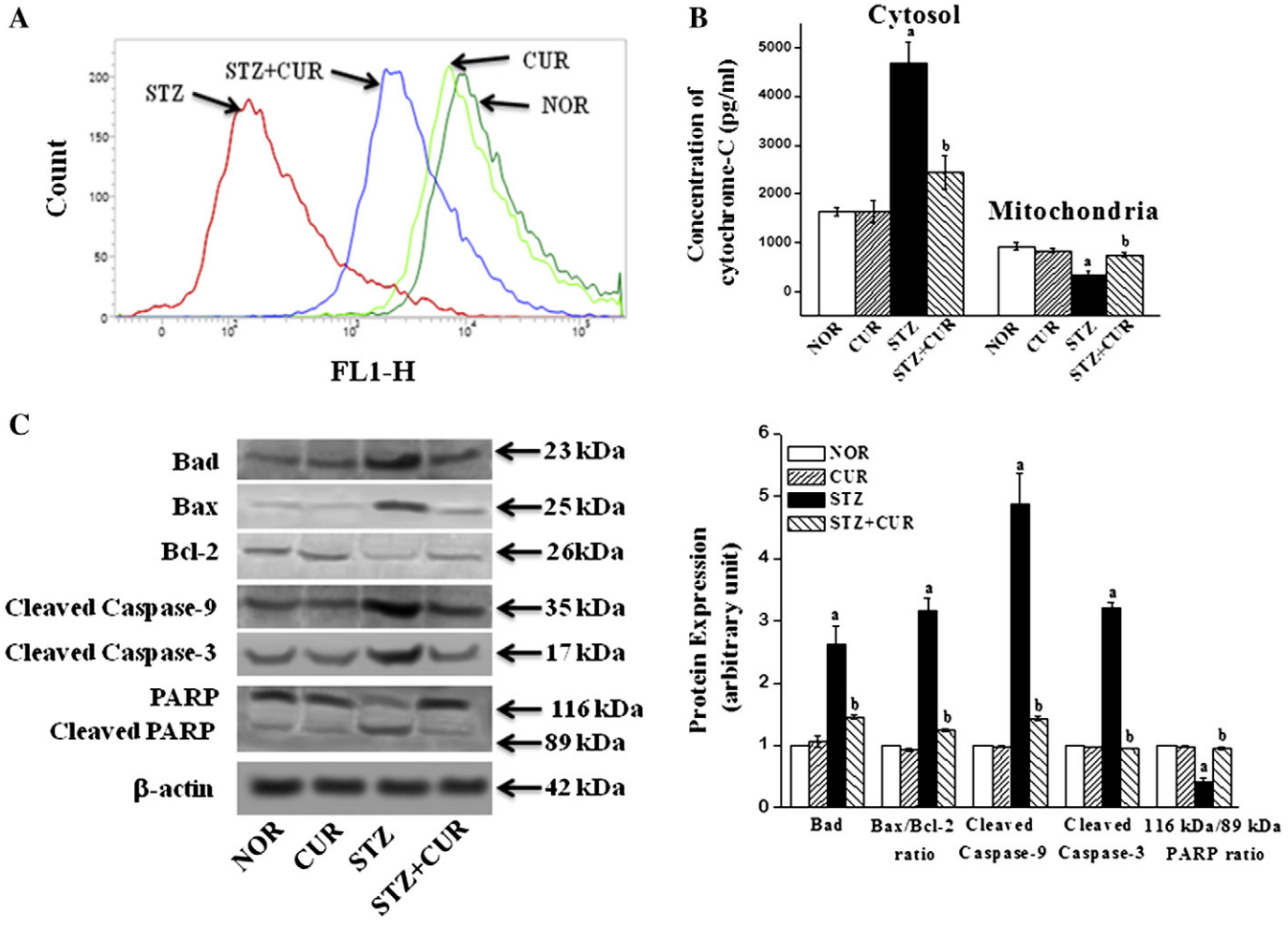

Fig. 8. Involvement of Mitochondria-dependent apoptosis and role of curcumin in diabetic animals. (A) Study on the mitochondrial membrane potential (MMP) by flow cytometry analysis. Mitochondria were isolated from testicular tissue. NOR: mitochondria isolated from the testicular tissue of normal animals; CUR: mitochondria isolated from the testicular tissue of animals treated with CUR only; STZ: mitochondria isolated from the testicular tissue of the diabetic animals; STZ + CUR: mitochondria isolated from the testicular tissue of the animals treated with CUR after diabetic induction. The measurements were made in six times. (B) Measurement of the concentration of cytosolic and mitochondrial cytochrome *c* in testicular tissue. NOR: normal rats; CUR: normal rats treated with CUR-100 only; STZ: diabetic control; and STZ + CUR: diabetic rats treated with CUR. (C) Western blot analysis of Bad, Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3 and PARP in the testicular tissue of animals. The relative intensities of bands were determined using NIH- image software and the control band was given an arbitrary value of 1. Data represent mean ± SEM, $n = 6$. "a" indicates the significant difference between the normal control and diabetic control groups, and "b" indicates the significant difference between the diabetic control and CUR-treated diabetic groups ($^{a}P < 0.05$, $^{b}P < 0.05$).

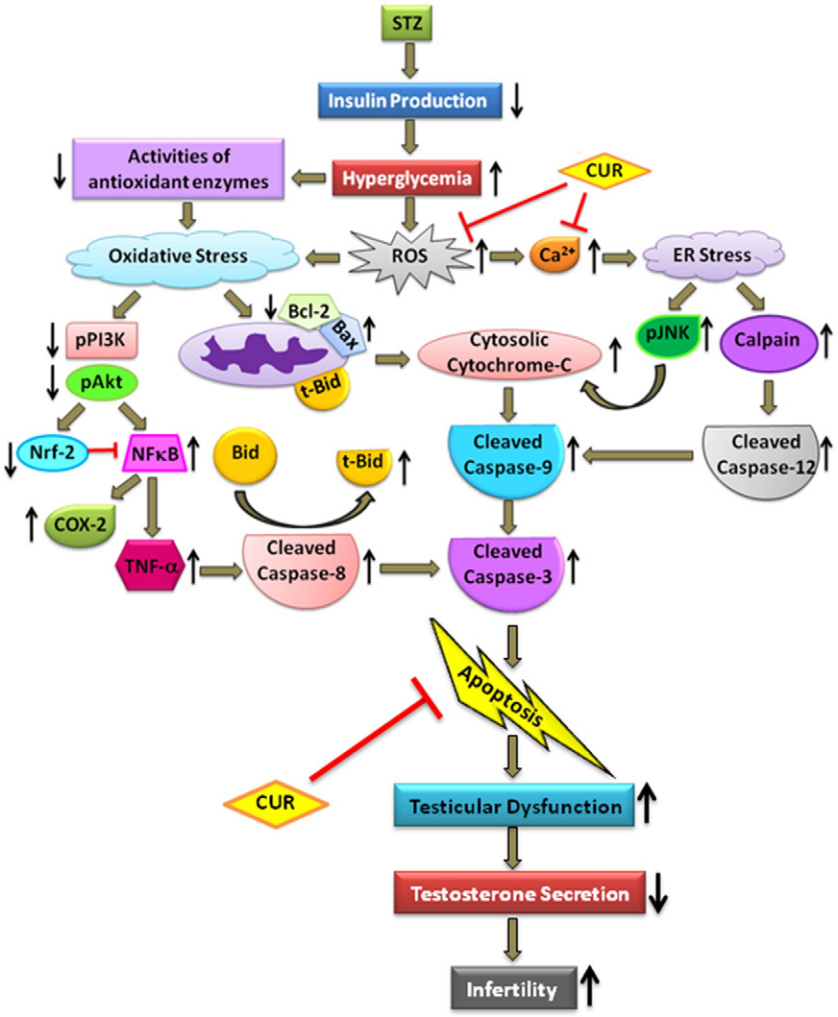

Fig. 9. Schematic diagram of the STZ-induced testicular damage in diabetes and its protection by CUR.

body weight ratio; diminution in the activity of SDH, G6PD; and reduction in serum testosterone level provided the indication of testicular damage in diabetic animals. Along with these alterations, reduced sperm count, motility and increased sperm abnormality were also observed in the diabetic group. Decreased testis-to-body weight ratio is associated with the degradation of the structural proteins [59]. SDH catalyses conversion of sorbitol to fructose and provide energy to the sperm cells to carry out normal metabolism, whereas G6PD is involved in the glutathione metabolism and helps in the hydroxylation of steroids by providing reducing equivalents [60]. Reduced activity of these enzymes promotes oxidative stress and hampers the normal function of testicular cells. High level of testosterone is necessary for regulating spermatogenesis and maintaining the normal physiological state of seminiferous tubules [61]. The enzymes 3β-HSD and 17β-HSD are known to catalyze testicular androgenesis [62]. Therefore, to find out the possible reason for the reduction of serum testosterone in the diabetic group, activities of these enzymes were measured and a significant fall was observed. Besides, histological studies showed disruption of the seminiferous tubule, loss of sertoli cells, diminution of leydig cells and absence of centrally located spermatozoa in STZ administered group. Treatment with curcumin, however, effectively restored all these adverse changes in the diabetic subjects and provided sufficient evidence to attenuate the initiation and progression of diabetes-induced testicular dysfunction. We also found the reduced activities of antioxidant enzymes, GSH/GSSG ratio and the elevated level of ROS, MDA (Malondialdehyde), protein carbonyl as well as that of proinflammatory cytokines (TNF-α and IL-1β), which further accelerates the production of ROS in the testes of the diabetic animals. Curcumin showed its potent antioxidant and anti inflammatory activities by inhibiting all the changes and providing protection from these detrimental effects.

A recent report suggests that increased production of free radicals causes apoptotic death of the testicular cells under diabetic condition [16]. To investigate the effect of curcumin on testicular damage, we did DNA fragmentation assay and found that curcumin efficiently restored DNA from damage and protects the cells from apoptosis via its antiapoptotic activity.

Previous studies demonstrated that hyperglycemia-mediated testicular damage is associated with the elevated translocation of the transcription factor NFκB into the nucleus and the activation of inflammatory response [63]. We have also found decreased expression of IκBα and cytosolic NFκB; increased level of nuclear NFκB and its downstream protein COX-2 in the testes of STZ-exposed animals. However, treatment with curcumin restored all these alterations and protects cells from inflammation. Besides, our study showed that curcumin protected testicular cells by increasing the nuclear translocation of Nrf-2, a transcription factor responsible for the activation of the antioxidant defense system of the cell. In addition, the identification of the upstream signaling mechanism responsible for the increased nuclear translocation of Nrf-2 in curcumin-treated group revealed the activation of PI3K/Akt signaling cascade.

Initiation of the extrinsic pathway of apoptosis is known to involve the binding of TNF-α with its receptor TNF-R1 and caused activation

of caspase-8 [64]. We, therefore, measured the expression of these proteins and Bid in both STZ-exposed and curcumin-treated groups and found that curcumin could minimize diabetes-induced upregulation of these three proteins.

JNK is known to augment translocation of proapoptotic protein Bax to the mitochondria for the promotion of mitochondrial pore formation, and p38 helps in the activation of caspases to induce apoptosis [65,66]. We, therefore, measured the levels of these two proteins to determine their role in diabetes-induced testicular dysfunction and observed that STZ increased the phospho-level without affecting the total (data are not shown), although curcumin efficiently antagonize these alterations. Based on the earlier reports, next we investigated whether JNK activation is associated with ER stress, as the role of curcumin on ER stress-mediated apoptotic death of testicular cells under diabetic condition is not clearly known [67]. Here it is worth mentioning that caspase-12 is a key player of ER stress-induced apoptosis and calpain-1 helps in the cleavage as well as activation of caspase-12. We found elevated level of intracellular Ca^{2+}, increased expression of calpain-1 and cleaved caspase-12 in the testes of diabetic animals although these changes were attenuated by curcumin.

Enhanced level of Bid and phospho-JNK in our earlier experiments led us to investigate the involvement of mitochondrial-dependent intrinsic apoptotic pathway in testicular damage and its protection. We observed reduced MMP, increased proapoptotic proteins (Bad and Bax), decreased antiapoptotic protein Bcl-2, elevated cytosolic and diminished mitochondrial cytochrome *c*, augmented cleaved caspases (9 and 3) and PARP in STZ administered diabetic animals; curcumin, however, significantly restored these changes proposing its potential antiapoptotic property by suppressing intrinsic pathway of apoptosis.

In conclusion, the present study demonstrated that curcumin is able to provide protection to testes from oxidative and ER stress by ameliorating hyperglycemia and testicular damage markers, regulating intracellular redox balance, attenuating NFκB-mediated inflammation and activating PI3K/Akt-dependent signaling mechanism. Probably, this study first time illustrated the protective role of curcumin in testicular tissue under hyperglycemic condition by activating Nrf-2; antagonizing stress-induced proteins (JNK and p38) and inhibiting ER as well as mitochondrial-dependent apoptotic pathways, and crosstalk between them (Fig. 9). Curcumin, therefore, could be considered as a promising therapeutic approach for the treatment of cellular stress-mediated testicular dysfunction in diabetes.

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