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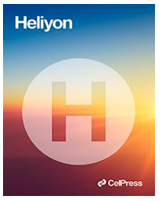

Research article

Protective impact of Betanin against noise and scrotal hyperthermia on testicular toxicity in Wistar rat: Role of apoptosis, oxidative stress and inflammation

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ABSTRACT

The heat exposure and white noise can induce damage on reproductive organs. The main objective of this study is to observe, if betanin administration could ameliorate oxidative stress, apoptosis and inflammation in testis of rodents following noise and scrotal hyperthermia exposure. Wistar rats were divided into 6 groups; control, betanin, noise, hyperthermia and two treatment groups. Scrotal hyperthermia model was performed by heat exposure of rat testicular (43 °C) for 15 min and 3 times per weeks for 14 days. Noise induction model was done following exposure of rats with 100-dB noise level for 14 days and 8 h daily similar to real exposure condition in human. Betanin was administrated at the sub-effective dose (15 mg/kg) by gavage route for 4 weeks (5 times a week) to male rats. The animals were euthanized and testis were dissected and stored at −80 °C. Then, the oxidative stress biomarkers (MDA and GSH), apoptosis (cytochrome *c* & Annexin V), and inflammatory cytokines (TNF-α & IL-6) were measured by the real time polymerase chain reaction (RT-PCR) of testis collected samples. The data output demonstrates the impact of noise and hyperthermia in testicular toxicity induction by mitigating oxidative damage, apoptosis and inflammatory mediators. Following treatment with 15 mg/kg per day of betanin, lipid peroxidation and GSH content have been modulated, and TNF-α and IL-6 gene expression has been declined. Our results revealed that in Wistar rats, betanin displays protective effects against noise and scrotal hyperthermia-induced acute testicular toxicity through the inhibition of oxidative stress, apoptosis, and inflammation.

1. Introduction

While males are reported to be the 50 % contributing in couples infertility, it is common in 20–30 % of cases male to be the sole cause [1]. The normal condition of spermatogenesis needs to lower than the body core temperature (2–8 °C) in males [2]. As numerous physicochemical factors can affect spermatogenesis [3], environmental exposure such as noise and high temperature seems inseparable from mankind daily life, and may cause harmful effects on spermatogenesis [2,4]. Urban traffic, aviation and industrial sites are

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the main source of noise production in nowadays life which could cause pathological and biochemical alterations in neural tissue, and increase the risk of neural disorder like depression and anxiety, leading malfunction of the reproductive system [5,6]. Noise is also reportedly associated with abnormalities in reproductive organ morphology, sex hormone hemostasis, and decline in reproductive performance and gestational ratio in animal studies without non-linear dose-response relationship [4,7]. The malfunction in HPA-axis performance and DNA damage in germ cells are probable factors causing alternation in sex hormones and inflammation in testicular tissue [8]. Previous data reported residents of cities with high levels of noise are more susceptible to infertility via decreased in seminiferous tubule diameter and germinal epithelium thickness, fibrotic changes and vacuolization following exposure to noise (115 dB) compared to the control group [9]. The similar data and variation in testis was reported by other studies in male rodent groups following exposure to traffic noise (100 dB) in testicular histology, morphology and amelioration in male steroidal hormones compared to control rats [10,11]. Noise exposure also reported to increase level of corticosteroids in body and increase risk of oxidative stress [12].

Hot water is frequently used for relaxation and to ease pain in hot springs or Jacuzzis. In such places, the temperature of the scrotum can reach 37–45 °C, which is above the optimum spermatogenesis temperature [13–16]. Therefore, it is supposed that scrotal hyperthermia following hot spring exposure can lead to spermatogenic malfunction and higher risk factors for male subfertility [4,6]. The previous studies revealed that scrotal hyperthermia caused to sharply inhibit the spermatogenesis process in rats, mice, monkeys and humans [2,17].

Various natural compounds (e.g. betanin) have been investigated for their potency to suppress cancer, inflammation and oxidative stress related disorders [18]. Betanin as a glycoside compound used frequently utilized as a dye reagent, and frequently found in pears, cactus and red beets [19,20]. The high antioxidant potency of betanin caused to its usage in foods to prevention of lipid oxidation and extend shelf life of pharmacological agents [21]. Some research indicated potency of betanin against the surge of oxidative stress in neurodegenerative and traumatic disorders [19,22,23]. The probable spermatogenesis improvement effect could be expected from compounds capable of reversing oxidative stress and antioxidant levels [24].

Unfortunately, there is limited data on impact of environmental pollutants (noise and heat) on spermatogenesis via cell death signaling pattern. Furthermore, there is no complementary evidence of anti-oxidant effect of betanin against testicular oxidative stress induced by noise and hyperthermia, in this case, this study tries to present more perspective in this context.

Betanin has shown potency in suppressing the oxidative stress in many studies regarding to malfunction of intrinsic cellular recovery markers, in this regard the effect on betanin in improving mechanical damage induced by heat and noise into Wistar rat's testes is highly probable.

2. Materials and methods

Materials: All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) in analytical grade (≥98 %) including: Betanin, Collagenase, trypsin, Glutamine, HEPES, Sodium pyruvate, Sodium chloride, Potassium Chloride, Calcium Chloride, Magesium Sulfate Heptahydrate, Sodium acetate, Magnesium Chloride Hexahydrate, Sodium Phosphate Dibasic Dihydrate, Potassium Phosphate Monobasic, D-Glucose, Sodium bicarbonate, D-mannitol, thiobarbutiric acid (TBA), Trichloroacetic acid (TCA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dithiobis-2-nitrobenzoic acid, (DTNB), reduced glutathione (GSH), GSSG (Oxidized glutathione),2′,7′-dichlorofluores-cein diacetate (DCFH-DA), Malondialdehyde (MDA), Tris–HCl, sodium succinate, sulfuric acid, n-butanol, tetramethoxypropane (TMP), Sodium borohydride ($NaBH_4$), Dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle Medium (DMEM), sucrose, KCl, Na_2HPO_4, $MgCl_2$, $MnCl_2$, Coomassie blue, ethylene glycol-bis(2-aminoethylether)-N,N,N', N'-tetra-acetic acid (EGTA), bovine serum albumin (BSA), and ethylenediaminetetraacetic acid (EDTA). Cytochrome *c* level was determined using the Quantikine Rat/Mouse Cytochrome *c* Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn.). Determination of cell apoptosis by flow cytometry was done by fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) detection kit (BD Biosciences, San Jose, CA, USA). Also, TRIzol was provided from Life Biolab (Heidelberg, Germany). Prime Script RT Reagent Kit was purchased from Takara Bio (Kusatsu, Japan). Master Mix green high ROX and low ROX were purchased from Ampliqon (Odense, Denmark). Revert Aid™ First Strand cDNA Synthesis Kit was purchased from Thermo Fisher Scientific (Waltham, United States). DNA ladder was purchased from SMOBIO (Hsinchu City, Taiwan), TBE from DNAbiotech Co. (Tehran, Iran) and DEPC water from Sinaclon Co. (Tehran, Iran). Agarose powder and Loading buffer were purchased from Yekta Tajhiz Azma Co. (Tehran, Iran). Primers were designed by primer III software (NCBI) and synthesized.

Animals: The present investigation included male Wistar rats, approximately weighing 180–200 g. They were housed in standard condition (rodents had free access to food and water, 12 h' day/night cycles, temperature of 21–23 °C and 65 % humidity and all experiments were performed between 10:00 a.m. and 2:00 p.m.). This study evaluated and approved by the ethics committees of the Zanjan University of Medical Sciences (IR.ZUMS.REC.1400.006), and all study procedures were performed following the National Institutes of Health (NIH) laboratory animal care guidelines (National Institutes of Health Publications No. 80-23, revised 1978).

Noise induction: The rodents which passed the criteria underwent the exposure to white noise 100 dB (100 dB) 8h per day for 14 days, so the same exposure level in workplace in a daily basis. A signal Software (Model CAT-WAVE, R Tokyo, Japan) as made to produce white noise and delivered to a loudspeaker fitted at 30 cm distance from the explored animal's cage [25,26]. The noise intensity was regularly monitored by a sound level meter [calibrated B & K noise meter device (MODEL 2238, Tokyo, Japan)]. The selected noise level was based on previously published data on rodents [5,25,26]. Then, the animals were randomly divided into 4 groups: (1) Control group, (2) Betanin group, (3) Animals exposure to noise and (4) treated scrotal noise rats with betanin.

Induction of transient heat stress: Induction of scrotal hyperthermia was done in hot water bath. The lower part of rat body consisting of the scrotum, hind legs, tail was submerged at the temperature of 43 °C for 20 min every other day for five consecutive

weeks following anesthetized by injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) via intraperitoneal route [27,28]. For control rats, water bath temperature was set to 22 °C in order to simulate water exposure effect. The rats were dried after each experiment and checked for any damage or harm on scrotal and next returned to its cage. Our Present data have shown that the hyperthermia periods did not cause any mortality among rodents.

After noise and hyperthermia induction rats were divided into 6 groups (1) Control group, (2) Betanin group, (3.4) animals exposed to noise and hyperthermia and (5.6) treated noise and hyperthermia rats with betanin.

Experimental Design: 48 rats were divided into 6 groups without specific pattern: (1) Control group (n = 6), received only vehicle (saline) via orally administration based on scrotal hyperthermia (n = 3) or noise induction models (n = 3); (2) Betanin group (n = 6) and received 15 mg kg^{-1} betanin dissolved in saline orally for 30 days every other day based on scrotal hyperthermia (n = 3) or noise induction models (n = 3). The pilot study revealed that non-significant difference between control or/and betanin treated rats in normal condition. Therefore, the results of two different control or/and betanin groups were used as one similar group in two different animal models. The dosage and duration of betanin administration were selected based on pilot and other previous studies in anti-oxidant enzyme activity test in noise or scrotal hyperthermia rat models. Our data showed the similar response in antioxidant enzyme activity in betanin treated between 15 and 20 mg/kg in noise or scrotal hyperthermia rats and the lowest dose was selected in present study [23,29]. Betanin in 15 mg/kg per day was administered by intra-gastric gavage method (i.g., saline as vehicle) for five consecutive weeks following; (3) Animals exposure with scrotal hyperthermia induction model (H, n = 6), every other day for five consecutive weeks; (4) Animals exposure with noise (100 dB) 8h per day for 14 days (N, n = 6); (5) Treated of scrotal hyperthermia model animals with betanin (H + B, n = 6); (6) Treated of noise model animals with betanin (H + B, n = 6).

It noted that exposure of animals with noise and hyperthermia induction caused morbidity before the completion of induction period (Data none shown). Also, control groups in two present models mixed together and shown as on the group due to no significant difference in response after checking of three biochemical and sperm analysis tests.

Anesthesia and euthanasia: It was performed using ketamine and xylazine (80 and 10 mg/kg).

Necropsy: Following completion of treatment, rats in all groups were euthanized, and the tactical tissue was isolated for tissue examination, and, in addition, the epididymis sperm was collected for sperm analysis and biochemical assays. Tissues were stored in −80 °C freezer in order to assess biochemical parameters such as total antioxidant capacity (FRAP)level, non-enzymatic antioxidant Glutathione (GSH) level and lipid peroxidation (MDA) level. For assessment of histological analysis of testicular tissue, the collected tissue samples were fixed in 10 % formalin. Finally, total protein content was determined in testis by the Bradford method [30].

Sperm Analysis: The sperm samples were collected by cutting the epididymis, and then incubating samples in saline solution (15 min; 37 °C; 5 % CO_2). Then, 10 μl of semen was transferred to a hemocytometer and the epididymal sperm count was done by optical microscope at 40× magnification [31,32]. Besides, percentage of morphological abnormal sperms were determined by staining 10 μl of the sperm by Eosin, and then examined under a light microscope at 40 magnification and then, the percentage of morphological normal sperm was obtained by subtracting the morphologically abnormal sperms from 100 as the percentage [32]. In addition, Computer Assisted Sperm Analysis (CASA) was done in the present study to determine the sperm concentration (10^6/mL) and sperm of class motility [PR: Class A + B or Grade 3 + 4: progressive ratio; Grade 2 or Class C: NP: non-progressive ratio; IM: Grade 1 or Class D: Non-motile].

Isolation of testicular cells: After transferring the testicular tissue in 40 ml of Hanks buffer (pH = 7.4 and T = 34 °C) and was dispersed by adding of collagenase (0.02 w/v) for 10–15 min, gently shaking and allowed to settle. The testicular cells were washed (2–3 time) and incubated in 25 ml of Hanks and trypsin (0.02 w/v), shaking (5–10 min) and then washed again within 20 ml Hanks containing 0.3 mg trypsin inhibitor and allowed to settle. The prepared cells were centrifuged at 500 rpm for 4 min to split pellet testicular cells (washed three times again). Finally, the pellets were re-suspended in 10 ml of DMEM/F-12 medium which is a non-acidic medium for the culture of a variety of mammalian cells [27].

Histopathological assessment: The testis samples were isolated then immersed in 10 % formalin. Serial dehydration by utilizing step by step higher ethanol concentration was performed, and samples were dyed using hematoxylin and eosin (H&E), prepared slides were observed under light microscope (Olympus BX51) at 400X magnitude. Five photos were collected from each group.

3. Biochemical assays

Reactive oxygen species (ROS) assay: The amount of ROS in homogenates of testis tissue was measured by adding dichlorodihydro-fluorescein diacetate (DCFH-DA) reagent. A fluorescence spectrophotometry method was utilized, and samples observed under 485 nm excitation and 520 nm emission wavelength [33]. There is a linear pattern between fluorescence intensity and ROS content [34].

MDA assay: Malondialdehyde (MDA) as a marker of lipid peroxidation, which can be used as a biomarker for oxidative stress and the increase of free radical, was measured by thiobarbituric acid (TBA) method using absorbance at 532 nm. The calibration curve was done by tetramethoxypropane (TMP) as the standard of MDA content (μmol/g tissue) [35].

Reduced and Oxidized glutathione content: The GSH (reduced glutathione) level was obtained using 5,5′ -dithiobis-2-nitro-benzoic acid (DTNB) reagent in testis tissue homogenates. The developed yellow color was measured, which are expressed as μmol/g tissue [36]. Then, the total glutathione (GSSG + GSH) level was measured in the supernatant after mixing with 1 ml of reducing agent (5 % sodium borohydride; $NaBH_4$) and then incubated at 37 °C for 30 min. The mixture was neutralized with 0.5 ml of 2.7 N HCl and total amount of GSH level were determined by spectrophotometric assay at 412 nm. GSSG(oxidized glutathione) level was expressed as μmol/g tissue following subtracting total glutathione level from reduced glutathione level [37].

Cell Viability assay (CVA): Briefly, 20 μL of MTT (5 mg/ml) was added to 100 μl of homogenate suspensions (1 mg protein/ml) in

DMEM and then incubated at 30 °C for 30 min. The formation of purple formazan following from yellow indicator is related to mitochondrial succinate dehydrogenase function and cell viability. The product of formazan crystals was dissolved in 50 μl of dimethyl sulfoxide (DMSO), and the wavelength of 570 nm was set for samples and 690 nm for reference [36].

Computer-aided sperm analysis (CASA): CASA was performed a routine test in pathobiology and medical diagnosis laboratories for sperm concentration and motility. After passing of liquefaction stage (37 °C), the sperm sample is placed directly on a slide, and is placed under a microscope and the images are transferred from the camera to the computer and analyzed by powerful software by frequency 50 Hz. Motility sperm classification was done based on other definitive characterization such as: parameters: VCL (Velocity of Curved Line), VAP (Velocity of Average Path), VSL (Velocity of Straight line); STR (Straightness) = VSL/VAP x 100, LIN (Linearity) = VSL/VCL x 100, ALH (Lateral amplitude) and BCF (Beat frequency) (1) PR (Progressive movement) or A + B class; (2) NP (Non progressive movement) or C class; (3) IM (Non motile) or D class.

Release of cytochrome *c* assay: The Quantikine Rat/Mouse Cytochrome *c* Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn.) was utilized, for the test solution the combination of 75 μL conjugated (consisting of monoclonal antibody specific for cytochrome *c* conjugated to horseradish peroxidase) 50 μL of standard and 10 μg of supernatant protein content of samples was added to the sample wells. After incubation for 2 h, substrate solution was added to the wells and incubation continued for 30 min, then the stop solution was added, and samples underwent an optical density process on spectrophotometer set to 450 nm.

Determination of apoptosis: The main marker of cell entering early stages of apoptosis is the transfer of phosphatidylserine (PS) from inner to outer side of plasma membrane. Cells were separated using Krebs–Henseleit buffer and collagenase (pH = 7.4), and the outer PS was detected by double-staining and using Fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI). Three factors separated cells into apoptotic and non-apoptotic; type 1 nor Annexin V and PI dyes were observed (non-apoptotic), type 2 first apoptotic stained only by annexin V- FITC and type 3 were the necrotic cells dyed by Annexin V and PI. The measurement was performed using channels FL1 and FL3 of the flowcytometry [38].

cDNA synthesis and real time PCR analysis: TRIzol® reagent used to extract RNA content from testicular tissue. For reverse transcription process, 1 μg of the extracted RNA was used for cDNA synthesis. Then, for gene expression analysis, real time PCR was performed using SYBR Premix Ex *Taq* Kit in a light cycler instrument (Mannheim, Germany). The specific primers for *Rattus norvegicus* species were designed based on NCBI Primer3 software. (Table .1).

Thermal pattern for PCR is as follows: activation phase consisted of 30-s activation at 95 °C, then the denaturation and simultaneous extension/annealing in 45 cycles of 5 s at 95 °C and 20 s at 60 °C. The threshold cycle of the target genes was normalized to GAPDH was set as the reference gene (this reference gene's expression does not alter by stress inducing factor and other genes' expression were divided by its expression level), and analyzed following $2^{-\Delta\Delta ct}$ method, which used to calculate related expression.

4. Analysis of statistical results

The results were illustrated as mean ± SD and statistical significance of the tests was done by one-way analysis of variance (ANOVA), for comparison between the groups. The degrees of freedom and variance Between groups were also added in results. $P < 0.05$ was selected as the minimal significance level of comparison.

5. Results

Betanin treatment has remarkably improved sperm parameters following scrotal hyperthermia and noise: As shown in Table .2 total number and morphology of sperm units was significantly decreased in scrotal hyperthermia and noise groups, when compared to control ($p < 0.05$). Furthermore, treatment with betanin could significantly increase two proposed parameters in treating scrotal hyperthermia and noise rats. Although, the highest level of decrease in sperm number and morphology was observed in scrotal hyperthermia exposure rats compared to noise exposure rats.

As shown in Table .2, a lowered motility factor in sperm sample was observed in scrotal hyperthermia and noise treated groups when compared to control (***$p < 0.001$). But, betanin has significantly increased the sperm motility class percentage compared to scrotal hyperthermia and noise treated groups. As shown in (Table .2), the sperm concentration level in H + B treated groups increase to normal level as compared to the hyperthermia groups. The betanin administration did not cause any noticeable alternation in motility factors compared to the control group ($p > 0.05$).

Betanin ameliorated the negative impact of scrotal hyperthermia and noise on cell viability: As shown in Fig. 1a, there is a significantly decline in cell viability in the testis of rats treated with noise and scrotal hyperthermia compared with control groups [Fig. 1a; $F(5,30) = 65.289$; ***$p < 0.001$]. Also, betanin administration showed significantly rise in cell viability compared to rats treated only with noise or scrotal hyperthermia (Fig. 1a; ###$p < 0.001$). Besides, no significant changing pattern noticed in betanin administrated rats compared to control ($p > 0.05$).

Table 1
Primer sequences used for real-time PCR assay.

Name	Forward sequence (3′–5′)	Reverse sequence (5′–3′)	GenBank accession number
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTG	NM_012589.2
TNF-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA	NM_012675.3
GAPDH	GGACTCATGACCACAGTCCA	TCAGCTCAGGGATGACCTTG	NM_017008.4

Table 2
Effect of Betanin on sperm parameters based on the CASA result in scrotal hyperthermia and noise induced rats Values are presented as the mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Treatment groups were compared to control in case is of significances (*p < 0.05, **p < 0.01, ***p < 0.001), and this comparison was also carried out between treatment groups and hyperthermia-noise induced rats (#p < 0.05, ##p < 0.01, ###p < 0.001) (n = 3).

Groups	Sperm count (*10^6/ 1.5 ml)	Normal Sperm Morphology (%)	Concentration (million/ml)	Motility Class			
				A + B(PR) %	C(NP) %	D(IM) %	A + B(PR) + C %
Control	25.07 ± 2.18	25.16 ± 2.18	55.2 ± 1.4	62.1 ± 4.6	25.2 ± 4.7	9.2 ± 3.1	87.3 ± 2.8
Betanin (B)	25.38 ± 1.09	25.41 ± 1.09	54.9 ± 2.1	60.4 ± 0.6.2	21.4 ± 5.3	10.3 ± 1.4	81.8 ± 3.4
Hyperthermia (H)	10.34 ± 2.79***	10.73 ± 2.81***	30.1 ± 1.59***	20.5 ± 3.1***	6.5 ± 1.5***	80.8 ± 4.3***	27 ± 2.6***
H + B	18.99 ± 1.54**###	18.56 ± 1.69**###	47.2 ± 1.34***###	37.5 ± 1.9*** ###	15.6 ± 2.3**#	46.5 ± 3.9*** ###	53.1 ± 4.3***###
Noise (N)	14.18 ± 2.81***	14.78 ± 2.38***	34.6 ± 3.1***	28.3 ± 4.4***	10.2 ± 1.21***	40.1 ± 3.5***	38.5 ± 3.7***
N + B	20.87 ± 1.23*** ###	20.96 ± 1.23***##	54.4 ± 1.21###	58.8 ± 5.7###	28.4 ± 2.9###	14.9 ± 2.2###	87.2 ± 5.1###
One Way ANOVA	F(5,18) = 33.71	F(5,18) = 33.95	F(5,18) = 136.40	F(5,18) = 63.69	F(5,18) = 26.29	F(5,18) = 302.07	F(5,18) = 200.43

Betanin ameliorated the negative effect of scrotal hyperthermia and noise on ROS level: As shown in Fig. 1b, noise and scrotal hyperthermia induced significant boost in ROS level in comparison to control groups (F(5,30) = 43.367; ***p < 0.001]. However, betanin did not significantly increase ROS accumulation compared with control mitochondria (P > 0.05). However, betanin treatment managed to decline ROS level when compared to exposure with noise (N) and scrotal hyperthermia exposure in animal treated groups (###p < 0.001; Fig. 1b) A more substantial plummet in ROS formation was observed after betanin treatment in scrotal hyperthermia was observed compared with control rats (*p < 0.05).

Betanin ameliorated the negative effect of scrotal hyperthermia and noise on MDA level: The difference between the MDA level of normal rats and control rats exposure with noise and scrotal hyperthermia group was significant [Fig. 1c; F(5,30) = 52.266; ***p < 0.001]. Treatment of the animal's noise and scrotal hyperthermia exposure with betanin significantly decreased MDA level compared to normal exposed rats with noise and scrotal hyperthermia (Fig. 1c; ###p < 0.001). Moreover, the effect of treated rats with betanin was not significant compared to control animals (p > 0.05).

Betanin ameliorated the negative effect of scrotal hyperthermia and noise on reduced and oxidized glutathione level: As shown in Fig. 2a, there is a significant decrease in GSH content in the testis tissue of rats under influence of noise and scrotal hyperthermia compared with control group [Fig. 2a; F(5,30) = 1/890; ***p < 0.001]. Also, betanin group showed significantly enhanced the GSH compared to rats treated only with noise or scrotal hyperthermia (Fig. 2a; ###p < 0.001).

As depicted in Fig. 2b, there is a significant rise in the GSSG level, which correlated with the oxidative stress condition in the testis of rats, treated with noise and scrotal hyperthermia comparing to normal rats [Fig. 2b; F(5,30) = 131.042; ***p < 0.001]. Also, betanin group showed significantly decline in oxidized glutathione form compared to the rats exposed with noise or scrotal hyperthermia (Fig. 2b; ###p < 0.001). Also, normal rats treated with betanin showed none significantly difference in GSH and GSSG compared to control animals (Fig. 2a and b; p > 0.05).

Betanin ameliorated the negative effect of scrotal hyperthermia and noise on Cytochrome *c* level: Cytochrome *c* release amount from the control rats exposed to noise and scrotal hyperthermia group was increased in comparison with the control group [Fig. 3; F(5,30) = 16/828; ***p < 0.001]. Betanin reversed cytochrome *c* level in the testis of rats treated with noise and scrotal hyperthermia (Fig. 3; ###p < 0.001). Besides, the administration of betanin on the aforementioned factor was not significantly different compared to control animals (p > 0.05).

Betanin ameliorated the negative effect of scrotal hyperthermia and noise on apoptosis determination: The flow cytometry histograms obtained from testis tissue of rats are illustrated in Fig. 4. Staining control group by with two indicators of FITC-conjugated annexin V and PI showed that the majority of cells remain intact (Q_3). Also, the proportion of early apoptotic cells (Q_4), and late apoptotic or necrotic cells (Q_2) were significantly raised in rat exposure with noise and scrotal hyperthermia. Furthermore, betanin could decrease the proportion of apoptotic (Q_4), and necrotic cells (Q_2) compared to the rats exposed to noise or scrotal hyperthermia (Fig. 4). Also, there was no noticeable alternation in cells remains intact (Q_3) amongst normal testis rat and betanin treatment. As Fig. 4 depicts, these proportions were similar in all the intervention groups.

Betanin down-regulated the gene expression of pro-inflammatory genes following scrotal hyperthermia and noise exposure: The analyzed data confirmed, that the expression of TNF-α [Fig. 5a; F(5,12) = 116.551; ***p < 0.001] and IL-6 genes [Fig. 5b; F(5,12) = 32.080; ***p < 0.001], which are pro-inflammatory mediators, in the rats exposed to noise and scrotal hyperthermia were upregulated compared to the control or normal rats. Also, post treatment of the noise and scrotal hyperthermia rats with betanin caused down-regulation of TNF-α and IL-6 expression values compared to the noise and scrotal hyperthermia rats, respectively (Fig. 5a and b; ###p < 0.001). Besides, betanin treatment in normal rats demonstrated insignificant alternation in the expression values compared to control groups.

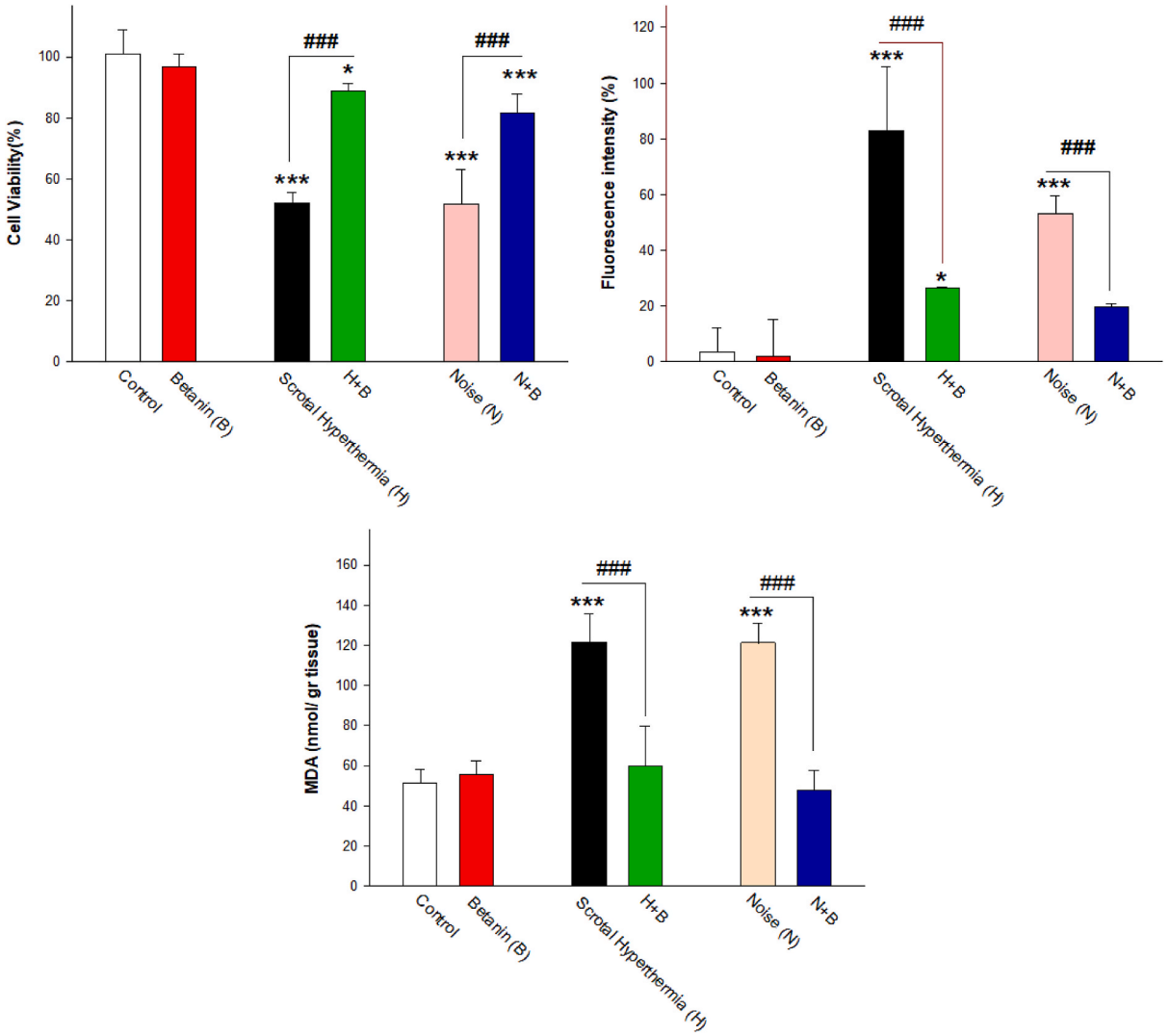

Fig. 1. Effect of Betanin on (a) cell viability, (b) ROS level and (c) MDA level in scrotal hyperthermia and noise induced rats in testicular tissue Values are presented as the mean ± SD and were analyzed using one-way ANOVA followed by Tukey's post hoc test. Significant difference compared to control group (*p < 0.05, **p < 0.01, ***p < 0.001), and this comparison was also carried out between treatment groups and hyperthermia-noise rats (#p < 0.05, ##p < 0.01, ###p < 0.001; n = 6).

Betanin treatment has improved cellular structure of testis following scrotal hyperthermia and noise exposure in histopathological assessment: As illustrated in Fig. 6, following noise and hyperthermia exposure the frequency of spermatogonia (SP), Spermatocyte (SPR), Spermatid (SPT), Sertoli (SERT) and Leydig (LID) cells has decreased, and seminiferous tubes (ST.L) have become shorter compared to control. The treatment with betanin has increase the frequency of aforementioned cell types. In addition, ST. L have extended, and resembles to ones in control sample.

6. Discussion

In this study, rodents underwent testicular-like disorder using noise and heat. These rodents showed lesser level spermatogenesis, higher level of MDA and ROS level in germ cells and a dropped level of GSH. Treatment with 15 mg kg^{-1} betanin once per day via oral route for 30 days has improved testicular performance via revering GSH level and downregulating TNF-α/IL-6 pro-inflammatory pathways, which are the noble aspects of this study.

During the past two decades, many studies have tried to observe the effect of various stresses inducing factors as a byproduct of modern life like heat stress and noise on inhibition of testicular performance and the quality of semen [7,39,40]. Ample evidences support the role of these pollutants in affecting pathways such as oxidative stress, inflammation and cellular apoptosis in testicular function [2,5,6,9,27,41]. While noise induce alternation in DNA structure, heat may hinder the performance of male genital in sperm

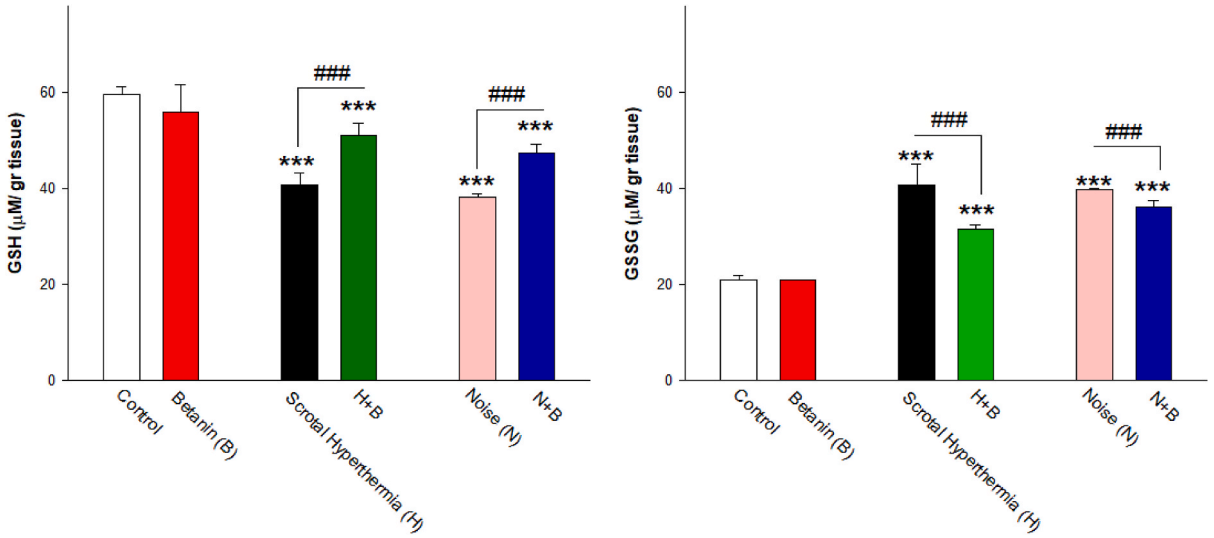

Fig. 2. Effect of Betanin on (a) GSH and (b) GSSG level in scrotal hyperthermia and noise induced rats in testicular tissue Values are presented as the mean ± SD and were analyzed using one-way ANOVA followed by Tukey's post hoc test. Significant difference compared to control group (*p < 0.05, **p < 0.01, ***p < 0.001) (n = 6).

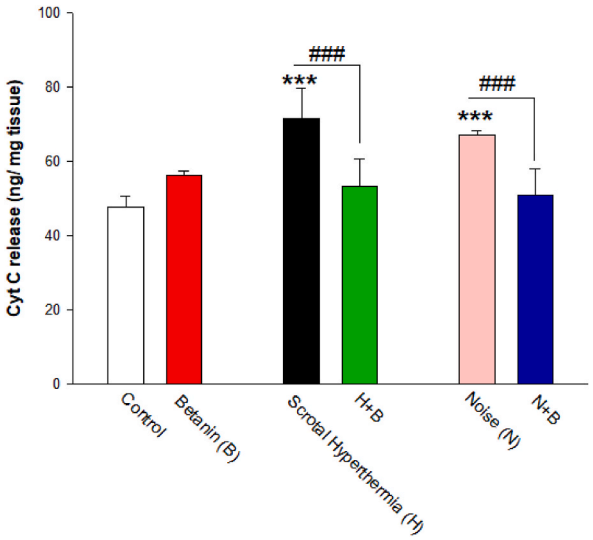

Fig. 3. Effect of Betanin on cytochrome C release in scrotal hyperthermia and noise induced rats in testicular tissue Values are presented as the mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Treatment groups were compared to control in case is of significances (*p < 0.05, **p < 0.01, ***p < 0.001; n = 6).

production, the final outcome may vary form decreased number of breeding to lack of impregnating potency.

The 2–8 °C lower degree for testes is critical for spermatogenesis process, as a mild higher temperature may increase number of sperm count, the sperms form with inefficient morphology [40]. In higher temperature a longer time spans induced by water bath or cryptorchidism, has caused irreversible damage in germ and sertoli cells. Sperms which form in this condition, in addition to be lower in total numbers, do not carry the right structure to act functional. The morphemically functional sperms in this case have been separated and still lacked the potency to fertilize gamete [42]. The heating pattern utilized in this study (temperature of 43 °C for 20 min every other day for five consecutive weeks), has tried to mimic long-term exposure to high heat. The cell analyzes output indicated higher count of pre-apoptotic and necrotic cells in testes, and a higher level of cytochrome C indicates the cells taking apoptotic death pattern turn [43].

The biochemical markers under hyperthermia exposure have been altered in testis tissue, as ROS and MDA level surged, leading to the decline of GSH and surge of its oxidized form (GSSG). Inflammatory causing substances have demonstrated to rise GSSG level in a time-dependent manner by promoting expression of GSSG gene [44]. The performance of anti-oxidative enzymes namely, superoxide

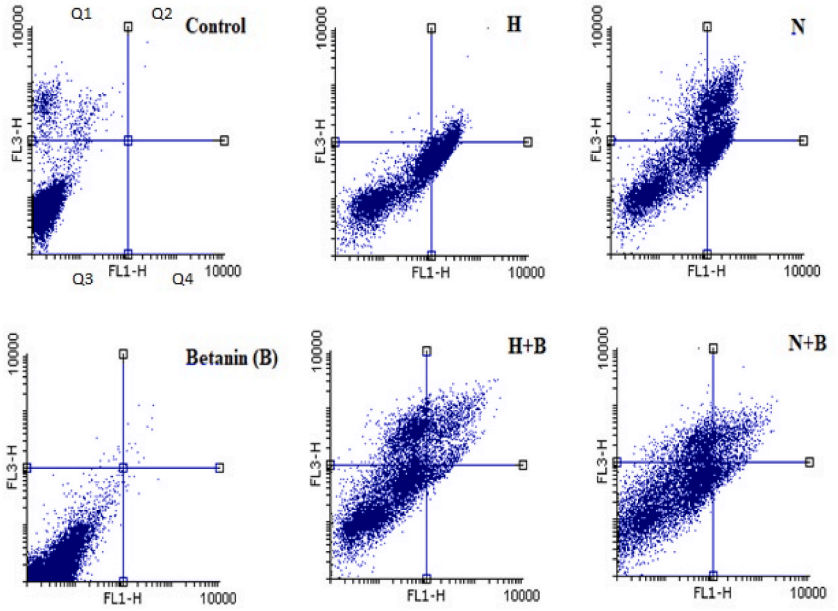

Fig. 4. Effect of Betanin on apoptosis in scrotal hyperthermia and noise induced rats in testicular tissue by Annexin V/Propidium iodide technique. The assay gives information about the numbers of vital (AV−/PI−) vs. apoptotic (AV+/PI−), and secondary necrotic cells (AV+/PI+).

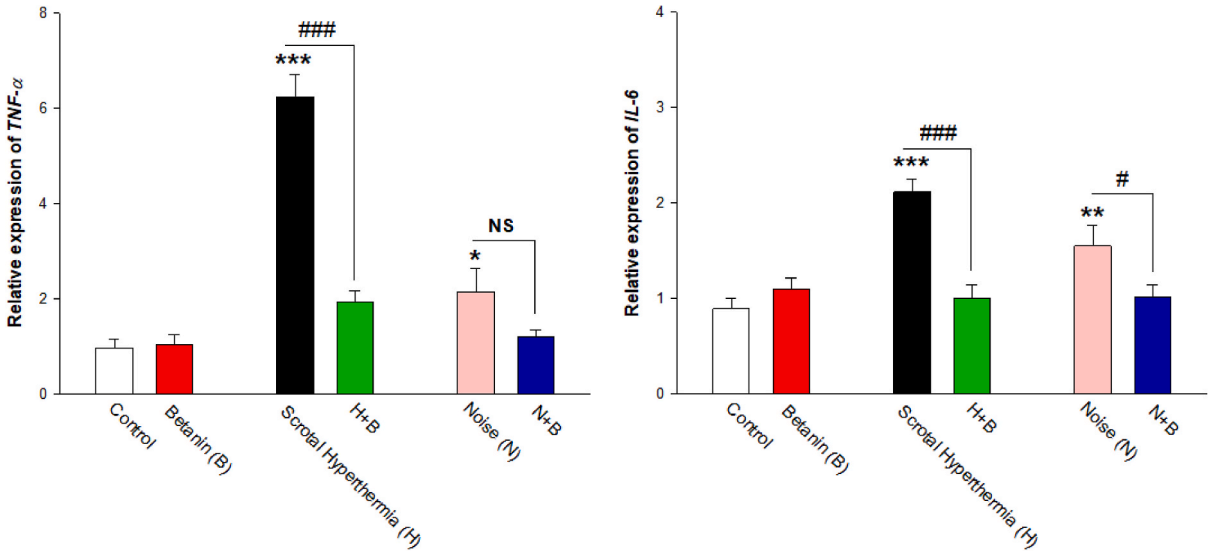

Fig. 5. Effect of Betanin on expression of pro-inflammatory genes (a) TNF-α; (b) IL-6 in scrotal hyperthermia and noise induced rats in testicular tissue. Values are presented as the mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Treatment groups were compared to control in case is of significances (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 3$).

dismutase and catalase have also reported to drop [45]. In this study, the relative expression of Tnf-α and Il-6 has surged by heat exposure meaning the activation of inflammatory markers are playing a role in testicular function, yet showing the reason of pre-apoptotic and necrotic cells rise in flow cytometry results.

In former study, long term exposure to heat has also caused down regulation of StAR and HspB10 genes, while the former one controls the transfer of cholesterol to sex hormones and its downregulation can attenuate the storing of cholesterol in testis, the later one controls the formation of sperm's tail and its proper motility [46]. In this study, the heat exposure has significantly dropped normal morphology count of sperms, it also hindered progressive and non-progressive movements. Under this situation the number of non-motile sperm increased, other studies reported noticeable increase of membrane damage [47].

Exposure to noise has suppressed the androgen secretion. In former studies, this affect was related to the suppression of pituitary

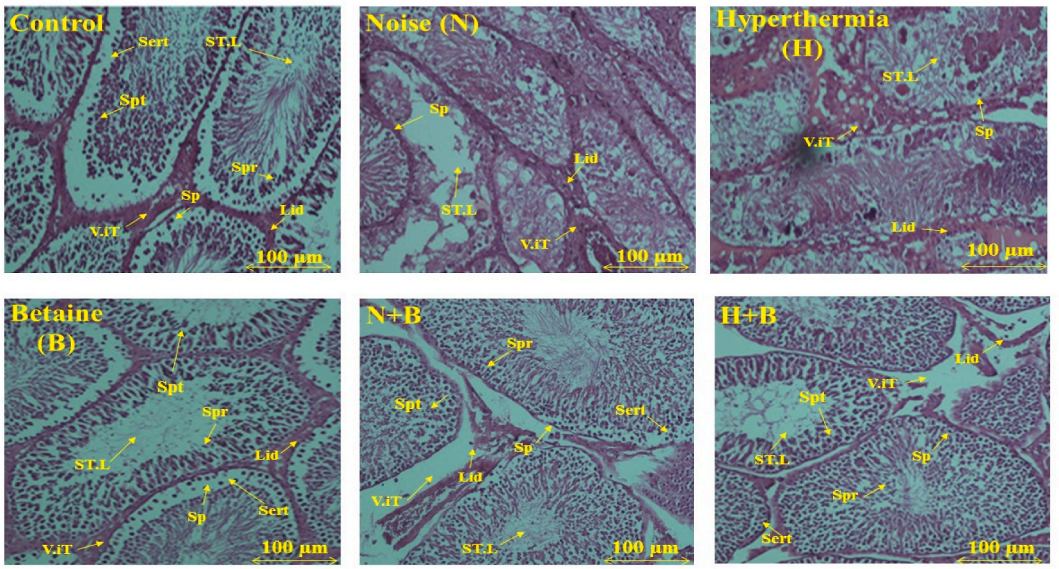

Fig. 6. Effect of Betanin on histopathological change in scrotal hyperthermia and noise induced rats in testicular tissue. H&E staining, *100. The frequency of spermatogonia (SP), Spermatocyte (SPR), Spermatid (SPT), Sertoli (SERT) and Leydig (LID) cells was evaluated in different groups following noise and hyperthermia exposure and treatment with betanin.

gland, and consecutive lowered level of FSH and LH have been observed. While FSH stimulates testosterone production, LH directly stimulates spermatogenesis [9]. In this study, the noise exposure has lowered the sperm count, the sperms became less motile and possessed abnormal morphology. The progressive and non-progressive movement of sperm dropped, while non motile sperms became more dominant. The suppression of hypothalamic–pituitary–gonadal axis played a key role in alternating GnRH secretion, and affecting the morphology of sperm and decreased weight of sex glands in other studies [48].

The 100 db noise has resulted the markers of oxidative stress namely, ROS and MDA to increase, and antioxidant capacity of the testes to fall. To make matters worse, the proportion of pre-apoptotic and necrotic cells have been escalated, leading to decline in germ and sertoli cells frequency in histopathological output [49]. The relative expression of Tnf-α and Il-6 has also surged, indicating white noise potency to cause inflammation.

In order to hinder the impact of environmental toxins, many potential candidates have been studied, but so far, minor research has been performed to investigate the potential impact of synthetic and natural antioxidants on aforementioned toxins, though herbal extracts applications have shown promising results [50–52]. Betanin is recognized as one of the greatest antioxidant compounds that has important pharmacological properties such as anti-inflammatory and anti-diabetes potentials. Phenolic and cyclic amine groups in the molecular structure of betanin make its potential for the high free-radical scavenging activity and thereby reducing the MDA level possible [23,29]. Many studies have indicated the improvement in mitochondrial performance followed by induction of toxicity and disorders in rodents both in anti-apoptotic effect and improvement in the electron transfer chain [53–55].

Based on data in the present investigation, the improvement in sperm parameters and reproductive organ structure following administration betanin in rats after undergoing testicular model for noise and hyperthermia could be attributed to the enhancement of antioxidant resources, and a decrease in ROS and MDA level by betanin treatment. It seems that the high electron-donor tendency of betanin is one of the key factors for its potent antioxidant capability of betanin and neutralizing free radicals, and modulation of the cell signaling pathways [56]. In a relative study, Beetroot juice (contains nearly 95 % betanin) applied at 8 ml//kg/day in cisplatin induced testicular dysfunction rodent model has improved sperm motility, count. In addition, this treatment recovered formed vacuoles, acidophilic and degenerated areas [57].

The over expression values of Tnf-α and Il-6 is highly associated with men infertility [58]. As an escalation in ROS content can boost the expression of Il-6 via impacting NF-kB signaling pathway [59]. The betanin potential in suppressing the values for Il-6 and Tnf-α expression, and acting as a free radical scavenger has been confirmed in this study.

In conclusion, the 15 mg kg^{-1} betanin treatment against testicular-like disorder followed by noise and hyperthermia induction, has improved the motility, morphology and sperm count. These effects accompanied by germ and sertoli cells becoming more functional, as oxidative related, apoptotic, inflammatory and antioxidant factors have been modulated.

Limitations: The alternation of various genetic contributing factors in the induction of infertility including assembly of the coagulating proteins (PLSCR1 and PLSCR2), energy production pathways (including AMPK and GAPDH) and motility specified factors (Catsper1 and protamine 2), and markers including PCO and NO level could be investigated to achieve a deeper understanding of betanin protective mechanism. Also, the simultaneous effect of noise and heat did not study in this research and could be investigated in further researches. Moreover, the steroids level alternation can also provide more long-term data on the effect of noise and heat on testicular performance, and changes physical factors including alternation of body, testicular weight and fertility potency of rodents

can also be measured in future studies.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethical Code: IR.ZUMS.REC.1400.006).

Informed consent

Informed consent was obtained from all individual participants included in the study.

CRediT authorship contribution statement

Soroush Bijani: Writing – review & editing, Writing – original draft, Validation, Software, Project administration, Investigation, Conceptualization. **Parvaneh Naserzadeh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Mir-Jamal Hosseini:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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