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GONADAL PHYSIOLOGY AND DISEASE

TBHP-induced oxidative stress alters microRNAs expression in mouse testis

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Abstract
Purpose Reactive oxygen species (ROS) and oxidative stress is one of the main reasons of male infertility. MicroRNAs (miRNAs) regulate multiple intracellular processes. Alterations in miRNAs expression may occur in different conditions and diseases. In this study, the effect of oxidative stress induced by tertiary-butyl hydroperoxide (TBHP) on the expression of candidate miRNAs in mouse testis was investigated.

Methods After determining median lethal dose (LD_{50}), TBHP was intraperitoneally (ip) injected at the dilution of 1:10 LD_{50} into the adult male mice for 2weeks, and then testis tissues were removed in order to assay the ROS level. Total RNA was extracted and the expression of five miRNAs was quantified by reverse transcription-real time polymerase chain reaction (RT-qPCR).
Results The flow cytometry analysis showed a significant increase in ROS level in testis. The expression of three out of five selected miRNAs, including miR-34a, miR-181b and miR-122a, showed some degrees of changes following exposure to oxidative stress. These miRNAs are involved in antioxidant responses, inflammation pathway and spermatogenesis arrest.
Conclusions In conclusion, TBHP alters the miRNA expression profile of testis which might play a potential role in oxidative and antioxidative responses and spermatogenesis.

Keywords Male infertility · Oxidative stress · Reactive oxygen species · MicroRNA (miRNA) · Tertiary-butyl hydroperoxide (TBHP)

Capsule Oxidative stress in animal model could change the expression of microRNAs which may lead to spermatogenesis failure.

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Introduction

Infertility is a major worldwide reproductive problem that affects approximately 15 % of young couples. Male infertility is responsible for approximately 50 % of these cases [34]. Oxidative stress (OS), which is described as an imbalance between the production of reactive oxygen species (ROS) and the body antioxidant levels, is a real concern for the health of male reproductive system [20, 33]. According to the previous studies, the increased levels of ROS are detected in the semen of 25- 40 % of infertile men [12, 36].

Mammalian germ cells are highly sensitive to the oxidative damages caused by ROS because they closely associate with

the free radical generating phagocytic Sertoli cells [5]. Differentiation of spermatogonia cells during spermatogenesis within testis is particularly controlled by posttranscriptional modifications [41].

One of the important regulatory mechanisms at the epigenetic level is applied through microRNAs (miRNAs) [11]. MiRNAs are short (~22 nt in length) non-coding RNA molecules that bind specifically to mRNA molecules to post-transcriptional control of gene expression [52]. They regulate multiple intracellular processes, such as differentiation, growth, apoptosis [40, 42] and the response to cellular stress. Alterations in miRNAs expression may occur following exposure to stress-generating agents. Simone's in vitro study on human fibroblast indicated that miRNAs may play a role in cellular defense against oxidative stress [48]. Nonetheless, there are limited studies to demonstrate the role and function of miRNAs in testis tissue and their relationship with male infertility.

Tertiary-butyl hydroperoxide (TBHP) is an organic hydroperoxides which has been usually employed to induce oxidative stress in various biological systems [28, 1]. Previously, we have reported the effect of TBHP on sperm and testicular tissue in mouse [16].

In this study, we examined oxidative stress induced via TBHP through expression of candidate miRNAs in mature mouse testis. To achieve this goal, five miRNAs (miR-181b, miR-34a, miR-449, miR-122a and let-7e) were selected to examine their expression levels, according to the study of Yan et al. [57]. The selected miRNAs are involved in cellular processes such as cell cycle control and apoptosis.

Materials and methods

Animals and care

Ethics committee of Royan Institute has approved the use of animals in this study and the declaration of Helsinki and the Guiding Principles in the Care and Use of animals (DHEW publication, NIH, 80–23) were followed. Adult male mice strain Balb/c (8–10 weeks) were randomly drawn from the stock colony and housed under standard conditions (controlled atmosphere with 12:12 h light/dark cycles, and temperature of 20–25 °C). They had free access to water and food.

Determination of toxicity and experimental protocol

The median lethal dose (LD_{50}) was determined by intraperitoneally (ip) administration of the various dosages of TBHP (Sigma) ranging from 100 to 600 μmol/100 g body weight (bw) of mice (6 mice per dose).

The obtained mortality data was subjected to probit regression analysis [17] to compute the LD_{50} value. The statistically computed LD_{50} value was 508 μmol/100 g bw. The test group (n=5) was treated by daily injection of TBHP at doses equivalent to 1:10 LD_{50} for 14 consecutive days. The control group (n=5) received the same volume of distilled water. After completion of the treatment schedule, the test and control mice were killed by cervical dislocation, and then testes were immediately removed for further analysis.

Assessment of reactive oxygen species in testis

After enzymatic digestion of testicular tissue as mentioned before [14] with minor modifications, the reactive oxygen species (ROS) level was assayed by flow cytometry using 2′, 7′-dichlorofluorescin diacetate (DCFH-DA; Sigma, USA) according to previously mentioned method [31, 13]. Briefly, appropriate amounts of testis homogenate (1–3 million cells) were incubated with DCFH-DA (10 mmol) for 15 min at 37 °C with rotation in the dark to allow the probe to be incorporated into any membrane-bound vesicles. After washing with phosphate buffer saline (PBS), the conversion of DCFH to dichlorofluorescein (DCF) was measured using a flow cytometer (BD FACS Calibur, Becton-Dickinson, San Jose, CA, USA). Green fluorescence (DCF) was evaluated between 500 and 530 nm in the FL-1 channels. Data were expressed as the percentage of fluorescent cells.

Histological analysis

Number of Leydig cells, germ layer thickness and seminiferous tubules diameter were measured using a light microscope. For this purpose, the longitudinal sections of testis were fixed in Bouin's solution, and then dehydrated in an ethanol series. The fixed biopsies were embedded in paraffin block, cut into 5-μm-thick sections and stained with haematoxylene and eosin (H&E) using standard procedures. Seminiferous tubules diameter and germ layer thickness were measured using an eyepiece at 40X magnification.

RNA extraction

Immediately after retrieval, testicular tissues were frozen in liquid nitrogen and stored at −80 °C. Total RNA including miRNAs was isolated using miRNeasy Mini Kit and RNase-DNase free set (Qiagen, Germany). The extracted RNA was quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific), and then stored at −80 °C until use.

Real-time RT-PCR

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) was performed for determining the expression levels of candidate miRNAs. Five miRNAs,

Table 1 Overview of miRNAs

Gene	Function	References
MiR-449	Tumor suppressor, induction of cell cycle arrest and apoptosis; regulation of male germ cell development in mouse testis.	[56, 7, 4]
Let-7	Key regulator of cell proliferation, like altering cell cycle progression through its over-expression; reduction of cell division in cancer cells.	[24]
MiR-34a	Negatively involvement in the regulation of cell cycle, including induction of cell cycle arrest and apoptosis (target genes: Bcl-2 and Cyclin D2).	[49, 10, 39, 54, 55, 8]
miR-181b	Function as tumor suppressors; triggering growth inhibition; inducing apoptosis in glioma cells (target genes: PTEN and Rsbn1).	[45]
MiR-122a	Highly expressed in late-stage male germ cells; its restoration induces apoptosis and cell cycle arrest in cancer cells (target genes: Tnp2 and TSN).	[58, 30]

including miR-181b, miR-34a, miR-449, miR-122a and let-7e were selected based on the pathways in which they were involved (Table 1). cDNA was produced using miScript Reverse Transcription Kit (Qiagen, Germany). RT reaction was prepared at final volume of 20 μl containing 5× Buffer (4 μl), RT Mix (1 μl), RNA (1 μg) and dH_2O (up to 20 μl) and then performed as follow: (i) 37 °C for 60 min (ii) 95 °C for 5 min. Real-time PCR was conducted in duplicate using an ABI 7500 system (Applied Biosystems, Germany). Each reaction was prepared at total volume of 20 μl containing 2×QuantiTect SYBR Green PCR Master Mix, 10×miScript Universal Primer, 10×miScript Primer Assay (Qiagen, Germany), and 50 ng cDNA. PCR was performed at 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 34 s. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The results were normalized to mouse U6 small nuclear RNA (snRNA). Data analysis was performed using the ΔΔCt method [29].

Statistical analysis

Data were expressed as the mean ± SD, and the differences between the control and test groups were analyzed by the student *t*-test (Levene's Test for Equality of Variances and unpaired, two-tailed) and Mann–Whitney using SPSS software (SPSS V.16, Inc, Chicago, IL, USA).

Results

DCFH processing by testis cells

The induction of intracellular ROS was assayed by determining intracellular H_2O_2 level using DCFH-DA in flow cytometry. With repeated doses of TBHP, a significant increase ($p<0.05$) in H_2O_2 level was observed in the testis after 2 weeks injection (Fig. 1). H_2O_2 levels increased from 57.96±8.74 in the control to 86.43±14.98 in treated samples.

Pathological and histological findings

The testicular biopsies from the control and treatment groups were examined by a light microscopy. For each sample, 100 round seminiferous tubules were counted in sagittal section. Leydig cell numbers were quantified in the interstitial space between these tubules.

The result of microscopic analysis revealed that no histopathological changes were seen in the control group unlike the treated group. The mean number of Leydig cells significantly decreased (6.5±1) compared to untreated mice (12.8±0.57) (Figs. 2 and 3).

A significant decrease in germ cells, thickness of germinal layer, and seminiferous tubules diameter was noticed. In addition, we recorded that spermatozoids in the tubules were rarely observable and the spermatogenesis process was

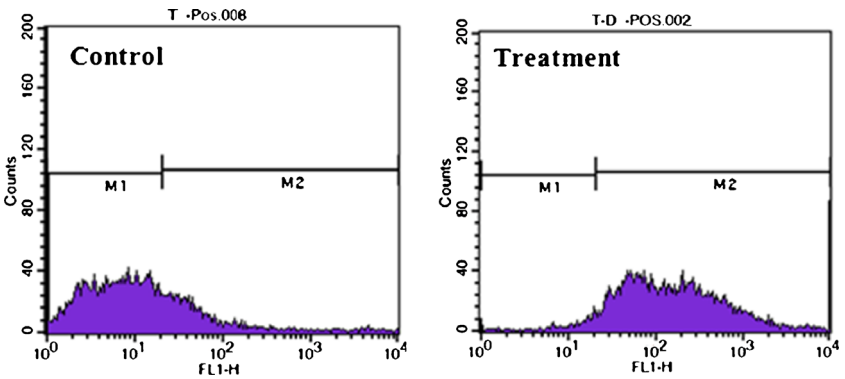

Fig. 1 Histogram graphs of flow cytometry of mouse testis cells stained with DCFH. Figure shows the level of H_2O_2 from one mouse of each group. A significant increase in H_2O_2 level was observed in the TBHP-treated mouse testis unlike control. (M2: development of fluorescent dye, the fluorescence of DCF was collected in fluorescence detectors 1 (FL1))

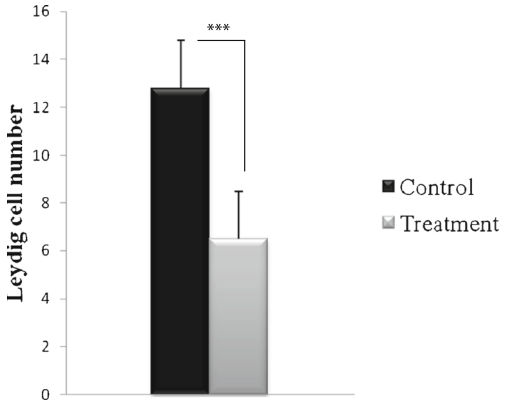

Fig. 2 Number of Leydig cell in control and treatment mice. Values are expressed as mean±SD; $n=5$; data was analyzed by Mann–whitney. *** There is a significant difference in the number of cells between control and treatment groups ($P<0.05$)

incompletely arrested. Also, the treated testes showed progressive degeneration, as well as association between Sertoli and germ cells was disrupted (Fig. 3).

The average thickness of germinal layers in control and treatment testes was 81.6±5.8 and 56.12±11.7 μm ($p<0.05$), respectively. The average of seminiferous tubules diameter in control and treated testes were 200.58±18.21 and 170.52±2.36 μm ($p<0.05$), respectively (Table 2).

Analysis of miRNAs expression

QRT-PCR analysis of the control and treated testicular samples was performed for miR-122a, miR-181b, miR-449, miR-34a and let7e. The results showed that the expression of miR-122a was significantly increased, but the expression of miR-181b and miR-34a was down-regulated in treated mice. Also, no

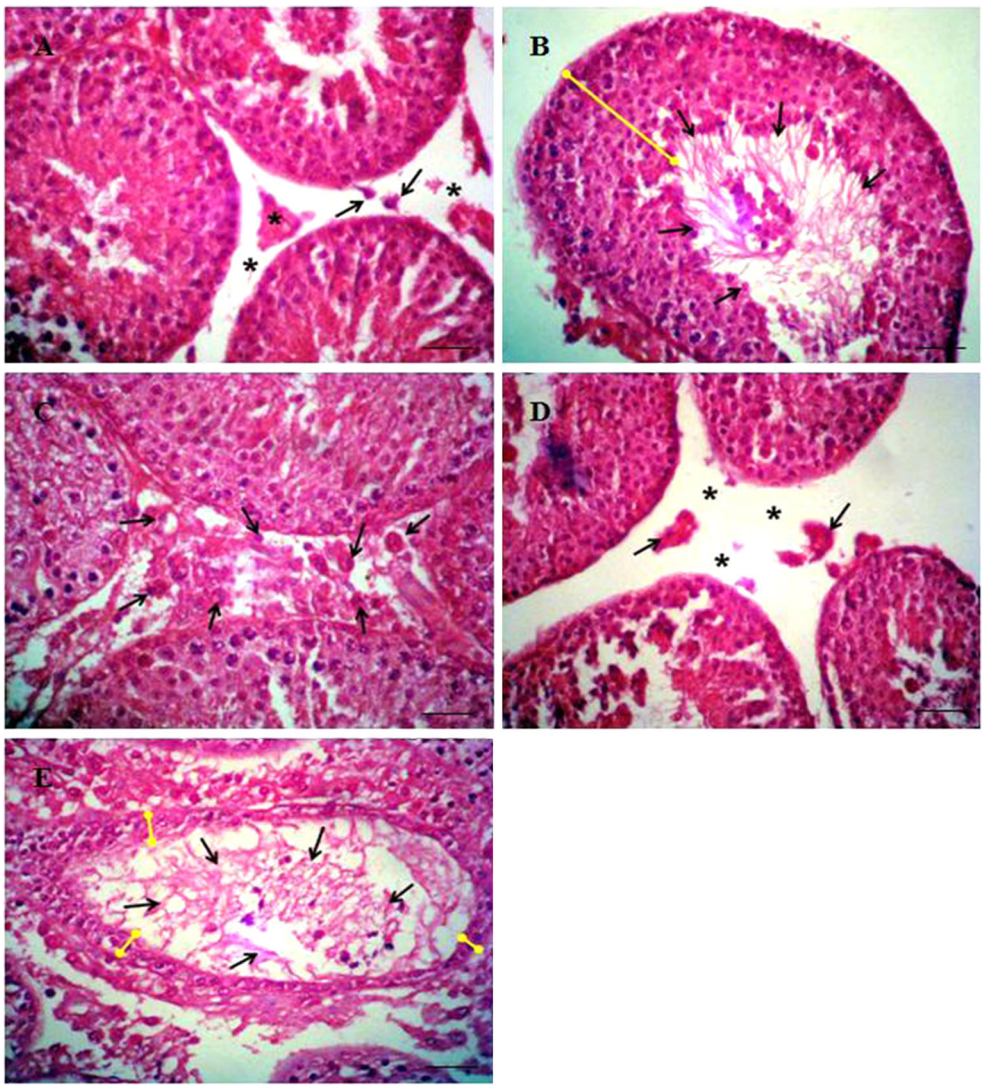

Fig. 3 Haematoxylin- and eosin-stained sections of testis showed: **a** Normal histological structure, normal seminiferous tubules with normal germinal layers. **b** Increasing number of Leydig cells. **c**, **d** The sharp decline in the number of Leydig cells. **e** Decreasing of thickness of germinal layer and progressive degeneration. Bar, 50 μm

Table 2 The histological changes in diameter of seminiferous tubules and thickness of germinal layer (in microns) in control and treated testicular samples (Mean ± SD)

Groups	Avg. diameter of seminiferous tubule (μ)[a]	Avg. thickness of germinal layer (μ)[a]
Control	200.58±18.21	81.6±5.8
Treatment	170.52±2.36	56.12±11.7

[a] Difference is statistically significant based on student's 't' test at 5 % level of significance

significant differences were detected in the expression of miR-449 and let7e between the control and treated mice (Fig. 4).

Discussion

Oxidative stress plays an important role in male infertility and defective sperm function [2, 38, 16]. In vivo model studies are necessary for studying the effects of oxidative stress on testis function and sperm quality. A number of chemical components have been identified to induce oxidative stress in animal models ([53, 3, 46, 46, 15]). TBHP is a well-known ROS inducer which has been used for induction of oxidative stress and damages in male reproductive system and spermatogenesis ([27, 26, 1]). Recently, we reported an increased level of ROS, H_2O_2 and $O_2^{\bullet}$ in mouse sperm and testis following TBHP treatment. Also, exposure to TBHP showed a significant decrease in the number of mature sperms due to mitotic arrest and a reduction in seminiferous tubules containing spermatozoa [16].

In this study, testicular histopathology following TBHP treatment revealed a significant decrease in Leydig cells, seminiferous tubule diameter and germ layer thickness. In addition, germ cells disorganization and progressive degeneration accompanied by spermatogenesis failure were detected. These results are similar to pervious findings in which oxidative stress was induced by formaldehyde [32, 51, 18, 30].

The roles of miRNAs in mammalian testis and spermatogenesis have been identified by the facts that a number of miRNAs are expressed preferentially in male germ cells (Ro et al. 2007) and a suite of novel miRNAs are expressed in spermatozoa [35].

The change of miRNAs expression in response to oxidative stress has been investigated in different cells and tissue samples such as human fibroblasts and vascular and adipose tissues [48, 23]. However, the effect of TBHP-induced oxidative stress on miRNA expression has not been investigated in mouse testis so far. Based on microarray information of miRNAs expression profile in mouse testis [57], five miRNAs were selected to examine this idea whether failure in spermatogenesis associated with oxidative stress is at least partly due to changes in testicular miRNAs profile.

Following exposure to TBHP and induction of oxidative stress, three out of five miRNAs, including miR-122a, miR-181b and miR-34a, showed significant changes in expression level. MiR-122a is predominately expressed in adult stage male germ cells and it degrades the Transition Protein 2 (TNP2) transcript which is a testis-specific gene involved in chromatin remodeling during mouse spermatogenesis [58]. Over-expression of miR-122a could lead to mitotic arrest and apoptosis of spermatocytes [30] which is in agreement with the histopathology results of this study.

MiR-34 family, which is composed of miR-34a, miR-34b and miR-34c, modulates cell cycle progression, senescence and apoptosis [6, 21, 22]. MiR-34a is highly expressed in normal tissues, like testis, lung, adrenal gland and spleen. A number of mRNAs have been proved to be miR-34a targets, such as apoptotic (*p53*) and anti-apoptotic (*Bcl2* and *Sirt1*) genes [10]. MiR-34a promotes apoptosis via down-regulating

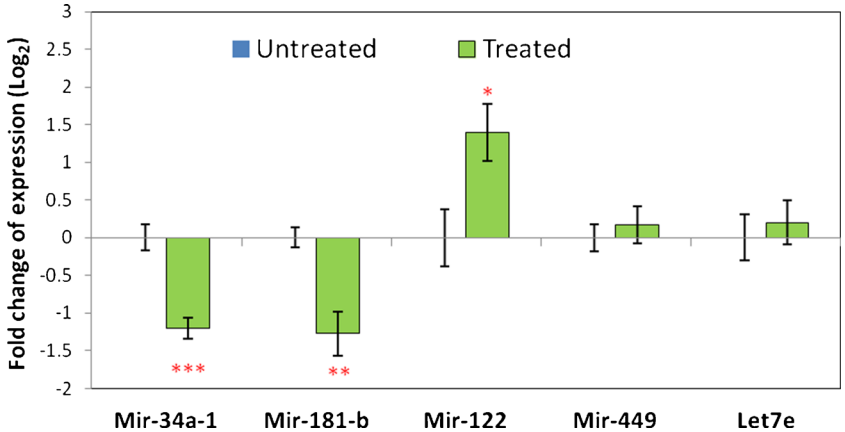

Fig. 4 Real time RT-PCR of miRNAs expression: The expression of Mir-34**a**-1 ($p<0.001$) and Mir-181-b ($p<0.01$) were significantly down-regulated, while Mir-122 ($p<0.05$) was significantly up-regulated compared to those in untreated mice. These results also showed that there are no significant differences in the expression of Mir-449 and Let7**e**. An unpaired, two-tailed *t*-test was used to assess statistically significant differences between two groups. (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. Error bar: SEM

anti-apoptotic proteins [10]. Down-regulation of miR-34a could lead to overexpression of *Sirt1* gene, which is a class III protein deacetylase, regulates several survival functions by deacetylating histones and many transcription factors such as those controlling ROS production. It seems that oxidative stress and increased levels of ROS, in turn, can control the activity of Sirt1 [43]. For instance, *Sirt1* gene stimulates the expression of antioxidants via the FoxO pathways [9, 44]. In this study, therefore, down-regulated miR-34a might provide a protective mechanism against detrimental effects of TBHP on testes.

The members of the miR-181 family of genes produce four mature miRNAs: miR-181a, miR-181b, miR-181c, and miR-181d [19]. The miR-181b inhibits importin-α3 expression, which is an important molecule involved in the NF-kB signaling pathway [50]. NF-kB is a stress responsive transcription factor which actively regulates apoptosis, inflammation [37] and antioxidant enzymes genes [25]. Therefore, down-regulation of miR-181b might exert its effect via up-regulation of NF-kB pathway. It is consistent with pervious study by Kaur et al. [25] that showed expression of testicular NF-kB in response to TBHP-induced oxidative stress.

In the present research, the change in expression levels of miRNAs after TBHP-induced oxidative stress was studied in mouse testis. These results demonstrated that oxidative stress alters the miRNA expression profile of testis, and deregulated miRNAs might play a potential role in the pathogenesis and protective mechanisms of testis.

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