ORIGINAL ARTICLE

Effect of troxerutin on the expression of genes regulating mitochondrial biogenesis and microRNA-140 in doxorubicin-induced testicular toxicity

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Background: Application of doxorubicin (DOX) in cancer patients is limited due to its dose-dependent toxicity to nontarget tissues such as testis and subsequent infertility. Due to limitation of our knowledge about the mechanisms of DOX toxicity in the reproductive system, reduction of DOX-induced testicular toxicity remains an actual and primary clinical challenge. Considering the potentials of troxerutin (TXR) in generating a protective phenotype in many tissues, we aimed to examine the effect of TXR on DOX-induced testicular toxicity by evaluating the histological changes and the expression of mitochondrial biogenesis genes and microRNA-140 (miR-140). **Materials and Methods:** Twenty-four adult male Wistar rats (250–300 g) were divided in groups with/without DOX and/or TXR. DOX was injected intraperitoneally at 6 consecutive doses over 12 days (cumulative dose: 12 mg/kg). TXR (150 mg/kg/day; orally) was administered for 4 weeks before DOX challenge. One week after the last injection of DOX, testicular histopathological changes, spermatogenesis activity, and expression of mitochondrial biogenesis genes and miR-140 were determined. **Results:** DOX challenge significantly increased testicular histopathological changes, decreased testicular expression profiles of sirtuin 1 (SIRT-1) and nuclear respiratory factor-2 (NRF-2), and increased expression of miR-140 ($P < 0.05$ to $P < 0.01$). Pretreatment of DOX-received rats with TXR significantly reversed testicular histopathological changes, spermatogenesis activity index, and the expression levels of SIRT-1, peroxisome proliferator-activated receptor-γ coactivator 1-alpha (PGC-1α), NRF-2, and miR-140 ($P < 0.05$ to $P < 0.01$). **Conclusion:** Reduction of DOX-induced testicular toxicity following TXR pretreatment was associated with upregulation of SIRT-1/PGC-1α/NRF-2 profiles and better regulation of miR-140 expression. It seems that improving microRNA-mitochondrial biogenesis network can play a role in the beneficial effect of TXR on DOX-induced testicular toxicity.

Key words: Doxorubicin, microRNA-140, mitochondrial biogenesis, testicular toxicity, troxerutin

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INTRODUCTION

Doxorubicin (DOX) is a widely employed anticancer drug in clinical use. However, the risk of serious dose-dependent toxicities has restricted its application in clinical chemotherapy. Until recently, most of the studies have been performed on DOX-related cardiotoxicity, hepatotoxicity, and nephrotoxicity. However, there are gaps in our knowledge about DOX adversity on the reproductive system. Experimental studies have revealed that DOX leads to infertility via altering the production, development, and structural integrity of sperm and depletion of sperm motion and count.[1,2] Although the pathophysiology of DOX-induced testicular damage is not yet completely clarified, the

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main mechanisms appear to involve lipid peroxidation, reactive oxygen species (ROS) generation, oxidative stress, inflammation, apoptosis, and mitochondrial dysfunction. Developing appropriate adjuvant therapies to target each of these mechanisms may counteract DOX-induced testicular toxicity.[3-5]

Decreased mitochondrial biogenesis plays a central role in DOX-induced testicular toxicity. DOX reduces mitochondrial content, disrupts mitochondrial electron transport system, energy metabolism, and redox balance, produces ROS, and alters testicular antioxidant defense system. These findings indicate that DOX potentially causes disruption of mitochondrial biogenesis.[5,6] A key regulator of mitochondrial biogenesis is sirtuin 1 (SIRT-1), which activates peroxisome proliferator-activated receptor-γ coactivator 1-alpha (PGC-1α) through its direct deacetylation to form a regulatory axis for controlling mitochondrial performance.[7] PGC-1α activates nuclear respiratory factor-2 (NRF-2), which is a key transcriptional regulator of antioxidant gene expression. NRF-2 protects the cell against oxidative stress through modulation of mitochondrial integration and biogenesis.[8] Studies have reported that activation of NRF-2 can be considered as potential target to counteract DOX-induced cardiotoxicity through inhibition of oxidative stress.[9,10] Besides, microRNAs (miRs) that are short non-coding RNAs regulating the expression of gene at the post-transcriptional level may contribute to mitochondrial biogenesis.[11,12] Recent studies have shown a clear association between dysregulation of miRs and problems in testicular development, epididymal maturation, spermatogenesis, and sperm maturation or migration, demonstrating that targeting specific miRs involved in the regulation of mitochondrial biogenesis genes may increase testicular tolerance to DOX toxicity.[13] Some evidence suggests that microRNA-140 (miR-140) suppresses mitofusin 1 (Mfn1) expression and exerts its effect on mitochondrial fission and apoptosis through Mfn1 targeting.[14] Also, miR-140-3p is responsible for regulating the expression of anti-apoptotic target genes and increasing cell survival. Based on previous works, it seems that targeting miR-140 along with SIRT-1, PGC-1α, and NRF-2 may serve as a coordinated network for improving mitochondrial function and metabolic homeostasis in fundamental cellular processes under normal and diseased conditions.[15,16]

A number of adjuvant therapies have been developed to diminish DOX-related toxicity during cancer treatment. But application of most of these treatments was not very satisfactory because of their side effects or low preventive impacts on DOX-induced toxicity. One of the therapeutic interventions to reduce DOX-related toxicities is the use of natural compounds with less side effects.[17] Troxerutin (TXR), known as vitamin P4, has gained significant interest over the past years due to anti-inflammatory, anti-apoptotic, anti-tumor, and anti-diabetic activities.[18] Also, the potential of TXR in modulating the expression of specific miRs, as well as expression profiles of genes involved in mitochondrial biogenesis in cardiomyocytes has been shown in recent studies.[19,20] However, to our knowledge, the exact mechanisms by which TXR may affect DOX-induced testicular toxicity and its related molecular mechanisms have not been investigated yet.

Minimizing DOX-induced testicular toxicity and associated mitochondrial damage while exploiting its anti-tumor effect is a debating issue in cancer treatment. Considering the key role of mitochondrial dysfunction and specific miRs dysregulation in DOX-induced toxicity and the therapeutic potentials of TXR, it seems that targeting mitochondrial biogenesis and specific miRs by TXR is a beneficial strategy to counteract DOX-induced testicular toxicity. Therefore, this study aimed to examine the impact of TXR on testicular histopathological changes, spermatogenesis activity index, and the expression levels of mitochondrial biogenesis genes and miR-140 in DOX-treated rats.

MATERIALS AND METHODS

Animals

In this study, 24 adult male Wistar rats (12 weeks old, weighing 250–300 g) were kept in and obtained from the animal center of Tabriz University of Medical Sciences. Prior to the experiments, animals were allowed to adapt under laboratory condition for 2 weeks. Animals were placed in individual cages and housed under controlled temperature (25°C ± 2°C) and humidity (55% ±10%), and exposed to 12-h light/dark cycle. Standard rat chow diet and tap water were given freely to animals. Experimental procedures of this work were carried out in strict compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th Edition, NRC 2011), based on the principles of the Ethics Committee of Tabriz University of Medical Sciences (Ethical code: IR. TBZMED. REC.1395.957).

Study design

The rats were randomly assigned into four groups, each contained 6 rats as follows: Control (non-treated healthy rats receiving normal saline during the study), TXR (healthy rats receiving TXR), DOX (rats receiving cumulative dose of DOX hydrochloride), and DOX + TXR (rats receiving TXR before DOX challenge).

TXR (Sigma-Aldrich, USA) was dissolved in normal saline and administered by gavage at a dose of 150 mg/kg/day for

28 days in TXR-receiving groups before DOX challenge.[20] DOX challenge was induced by intraperitoneal injection of doxorubicin hydrochloride (Sigma-Aldrich, USA) dissolved in normal saline at a dose of 2 mg/kg every 48 h for 12 days (cumulative dose: 12 mg/kg). This protocol of DOX administration is similar to those employed in clinical chemotherapy. Seven days after the final injection of DOX, all rats underwent general anesthesia via an intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). Next, the animals were sacrificed and their testicular tissue samples were taken rapidly. One part of the testicular tissue in each sample was placed in formalin 10% for histopathological assessments, and the second part was placed in liquid nitrogen and stored at −80°C for later biochemical studies.

Assessment of testicular histopathological changes and spermatogenesis activity index

After placing testicular tissues in formalin 10% for 24 h, they were embedded in paraffin blocks. Next, preparation of transverse sections with 5 µm thickness was carried out by a microtome. Following staining the slices with hematoxylin-eosin (Sigma-Aldrich, USA), they were assessed under light microscopy by a histologists in a blinded manner. Testicular damage and spermatogenesis were evaluated based on Johnsen's criteria according to the previous studies.[4] In this scoring system, evaluation of all tubular sections in each section of the testicular tissue was conducted systematically, and a score from 1 to 10 was given to each of them. Score 10 was given to complete spermatogenesis with many spermatozoa present [Table 1].

Table 1: Scores for assessing spermatogenesis (mean Johnsen's score)

Score	Level of spermatogenesis
10	Full spermatogenesis
9	Slightly impaired spermatogenesis
8	Less than five spermatozoa per tubule
7	No late spermatids; many early spermatids
6	Few early spermatids; arrest of spermatogenesis at the spermatid stage
5	Many spermatocytes
4	Few spermatocytes; arrest of spermatogenesis at the primary spermatocyte stage
3	Spermatogonia only
2	No germ cells; sertoli cells only
1	No seminiferous epithelial cells; tubular sclerosis

Real-time polymerase chain reaction

The expression levels of SIRT-1, PGC-1α, NRF-2, and miR-140 were determined by real-time polymerase chain reaction. Total RNA was extracted from testicular samples using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) on ice, following the manufacturer's recommendations. The purity and quantity of RNA were determined with NanoDrop spectrophotometer (NanoDrop ND-2000C, Thermo Fisher Scientific, USA) at a wavelength of 260/280 nm. The conversion of RNA series to cDNA was conducted by an Exiqon cDNA Synthesis Kit. Using a LightCycler-96 Roche device, the assessment of the expression levels of SIRT-1, PGC-1α, NRF-2, and miR-140 was performed. Glyceraldehyde 3-phosphate dehydrogenase was employed as a normalization control for mRNA expression analysis, and U6 small nuclear RNA was used as an internal control for miR-140 expression analysis. The sequences of primers used in this study are summarized in Table 2. For analyzing the relative gene expression, $2^{-\Delta\Delta CT}$ method was used, and the results were expressed as fold-change differences to the relevant controls.[21]

Table 2: Sequences of the primers used in real-time polymerase chain reaction

Gene name	Primer sequence
SIRT-1	Forward: 5'-ACTGGAGCTGGGGTTTCTGTT-3' Reverse: 5'-GGAAGTCCACAGCAAGGCGAG-3'
PGC-1α	Forward: 5'-CTAGCGGTCCTCACAGAGACA-3' Reverse: 5'-GTCAGGCATGGAGGAAGGAC-3'
NRF-2	Forward: 5'-GCTGTGTGTTCTGAGTATCGT-3' Reverse: 5'-TCATAATCCTTCTGTCGCTGA-3'
GAPDH	Forward: 5'-CAAGATCATCAGCAATGCCTCC-3' Reverse: 5'-GCCATCACGCCAGTTTCC-3'

SIRT-1=Sirtuin 1; PGC-1α=Peroxisome proliferator-activated receptor-γ coactivator 1-alpha; NRF-2=Nuclear respiratory factor-2; GAPDH=Glyceraldehyde 3-phosphate dehydrogenase

Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software (San Diego, CA, USA) and reported as mean ± standard errors of the mean. The histopathological changes were analyzed by Kruskal–Wallis test, and the expression of genes between different groups were analyzed by one-way analysis of variance followed by Tukey *post hoc* test. The levels of significance were set at $P < 0.05$.

RESULTS

Histopathological findings

As shown in Figure 1, normal testicular tissue was observed in the Control as well as the group receiving TXR. DOX challenge resulted in severe degeneration of testicular tissue, disarrangement of seminiferous tubule basal membrane and seminiferous epithelium, degeneration and vacuolization of the germinal epithelium, and destruction and/or depletion of the germinal cells. Pretreatment with TXR diminished the intensity of the above-mentioned histopathological alterations in comparison with those in the DOX group. In addition, the mean Johnsen's score indicated that complete spermatogenesis was observed in the Control group (9.00 ± 0.258). In the group receiving TXR, the spermatogenesis was also complete but closer

to the ideal level (9.16 ± 0.307) as compared to the Control group. DOX challenge significantly decreased this score to 5.16 ± 0.307 as compared to the Control group ($P < 0.001$). Pretreatment with TXR significantly rescued this score to 6.50 ± 0.428 as compared to the DOX group ($P < 0.05$) [Table 3].

Real-time polymerase chain reaction findings

The expression of sirtuin 1 gene

As shown in Figure 2, a slight increase in the level of SIRT-1 mRNA was observed in the TXR group compared to the Control group, but this increase was not statistically significant. DOX challenge significantly decreased the mRNA expression level of SIRT-1 as compared to the Control group ($P < 0.05$). However, the expression level of SIRT-1 mRNA was significantly rescued by TXR (1.19 ± 0.08 vs. 0.83 ± 0.05; $P < 0.05$).

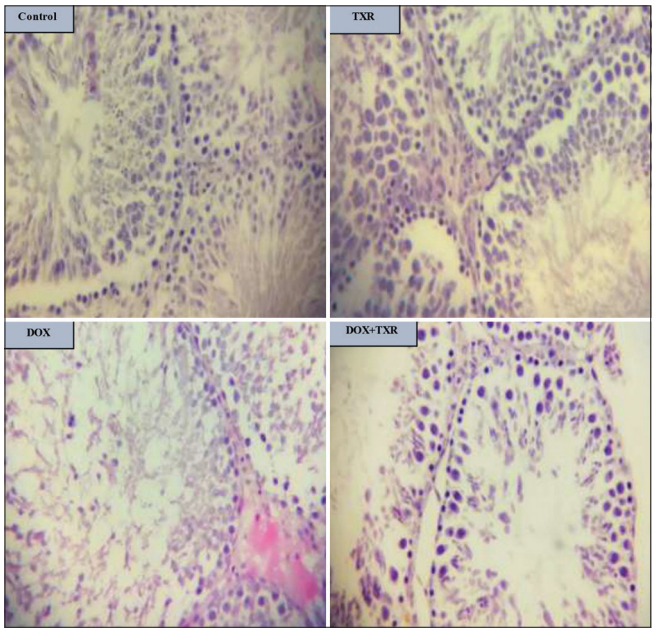

Figure 1: Evaluation of histopathological changes of testicular tissues in the experimental groups. Severe degeneration of testicular tissue was observed in the DOX group. Pretreatment with TXR diminished the intensity of tissue injuries in comparison with those in the DOX group. TXR = Troxerutin; DOX = Doxorubicin; DOX + TXR = Doxorubicin plus troxerutin

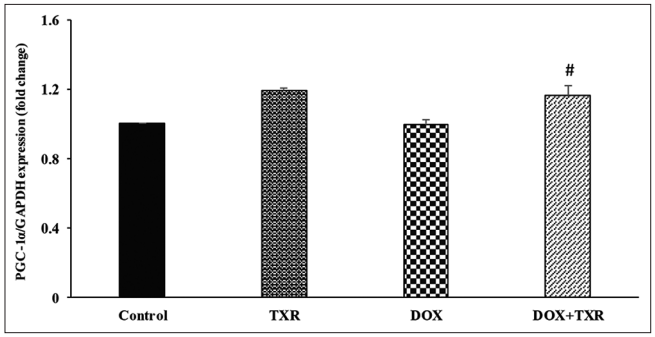

Figure 3: Testicular mRNA levels of PGC-1α in the experimental groups ($n = 6$ per group). The data were expressed as mean ± SEM. ($^{\#}P < 0.05$ vs. DOX group). TXR = Troxerutin; DOX = Doxorubicin; DOX + TXR = Doxorubicin plus troxerutin

The expression of peroxisome proliferator-activated receptor-γ coactivator 1-alpha gene

Figure 3 shows that application of TXR caused a slight but not significant increase in the mRNA expression level of PGC-1α in comparison with the Control group. DOX challenge tended to decrease the mRNA expression level of PGC-1α as compared to the Control group, but this effect was not statistically significant. Pretreatment with TXR significantly increased the expression of PGC-1α mRNA as compared to the DOX group (1.16 ± 0.05 vs. 0.99 ± 0.02; $P < 0.05$).

The expression of nuclear respiratory factor-2 gene

As shown in Figure 4, the mRNA level of NRF-2 did not differ between TXR-receiving and those of non-treated healthy rats. The mRNA expression level of NRF-2 was significantly decreased by DOX ($P < 0.05$), however, pretreatment with TXR significantly enhanced the expression of this gene in

Table 3: The effects of doxorubicin and troxerutin on mean Johnsen's score (spermatogenesis activity index)

Groups	Control	TXR	DOX	DOX + TXR
Mean Johnsen's score	9.00±0.258	9.16±0.307	5.16±0.307***	6.50±0.428#

The data were expressed as mean±SEM. ***P<0.001 versus Control group, #P<0.05 versus DOX group. TXR=Troxerutin; DOX=Doxorubicin; DOX + TXR=Doxorubicin plus troxerutin; SEM=Standard error of the mean

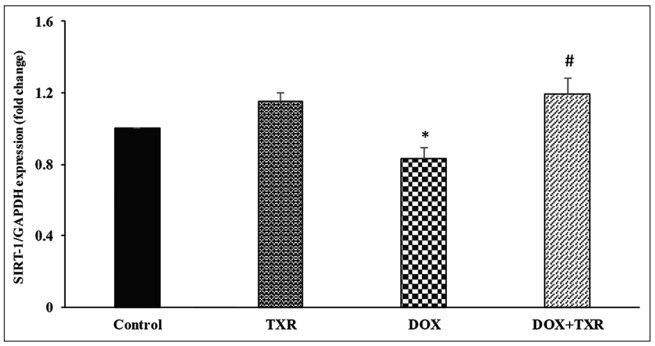

Figure 2: Testicular mRNA levels of SIRT-1 in the experimental groups ($n = 6$ per group). The data were expressed as mean ± SEM. ($^{*}P < 0.05$ vs. Control group, $^{\#}P < 0.05$ vs. DOX group). TXR = Troxerutin; DOX = Doxorubicin; DOX + TXR = Doxorubicin plus troxerutin

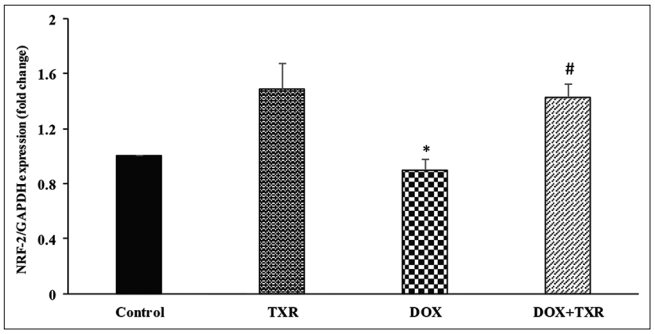

Figure 4: Testicular mRNA levels of NRF-2 in the experimental groups ($n = 6$ per group). The data were expressed as mean ± SEM. ($^{*}P < 0.05$ vs. Control group, $^{\#}P < 0.05$ vs. DOX group). TXR = Troxerutin; DOX = Doxorubicin; DOX + TXR = Doxorubicin plus troxerutin

comparison with those in the DOX group (1.43 ± 0.09 vs. 0.89 ± 0.07; $P < 0.05$).

The expression of microRNA-140

Figure 5 shows miR-140 expression level in experimental groups. As shown, there was no significant difference between TXR and Control groups. Administration of DOX caused significant increase in the expression level of miR-140 comparing to the Control group ($P < 0.01$). However, pretreatment of rats with TXR significantly prevented DOX-induced miR-140 overexpression (3.5 ± 0.48 vs. 7.18 ± 0.38; $P < 0.01$).

DISCUSSION

In the present work, we found that DOX challenge diminished spermatogenesis activity index and the expression levels of genes involved in mitochondrial biogenesis (SIRT-1, PGC-1α, and NRF-2) in the testicular tissue of rats. In addition, DOX challenge overexpressed miR-140 level. Interestingly, administration of TXR for 4 weeks before the DOX challenge improved spermatogenesis activity index, increased the expression levels of genes regulating mitochondrial biogenesis, and modulated the expression level of miR-140 in the testicular tissue of DOX-received rats. These findings documented the positive impact of TXR preconditioning against DOX-induced testicular toxicity.

One of the most important reasons behind DOX-induced toxicity is disruption of mitochondrial biogenesis. A key regulator of mitochondrial biogenesis is SIRT-1, which causes activation of PGC-1α and subsequent promotion of mitochondrial gene transcription and biogenesis. SIRT-1 stimulates NRF-2, a negative regulator of oxidative stress, which binds to the promoter of antioxidant genes and leads to the upregulation of antioxidant genes expression and augmentation of tissue resistance to oxidative stress. In addition, NRF-2 binds with its co-activator PGC-1α to maintain mitochondrial biogenesis.[22,23] Following exposure to DOX, it binds to the phospholipid cardiolipin in mitochondria and forms an irreversible complex that disrupts mitochondrial biogenesis, increases mitochondrial ROS generation, and reduces mitochondrial ATP levels and membrane potential.[3,24,25]

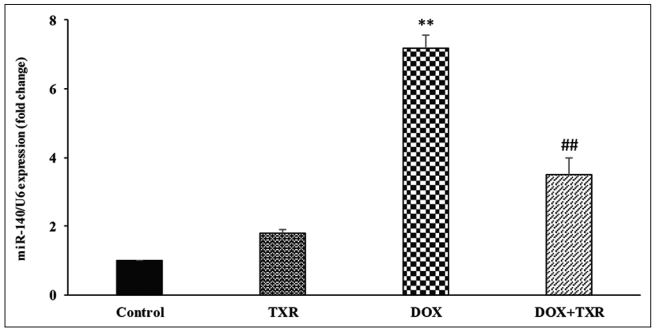

Figure 5: Testicular expression levels of miR-140 in the experimental groups ($n = 6$ per group). The data were expressed as mean ± SEM. (**$P < 0.01$ vs. Control group, ##$P < 0.01$ vs. DOX group). TXR = Troxerutin; DOX = Doxorubicin; DOX + TXR = Doxorubicin plus troxerutin

Previous studies have highlighted the role of mitochondrial biogenesis in DOX-induced testicular toxicity. DOX downregulates PGC-1α expression in parallel to NRF-2 expression, which is associated with diminished expression of cellular antioxidant enzymes.[22,26] In agreement with previous works, our study showed that DOX challenge caused dysregulation of mitochondrial biogenesis, which was confirmed by decreased expression levels of SIRT-1, PGC-1α, and NRF-2. Interestingly, pretreatment with TXR significantly reversed DOX-related alterations in the expression of genes involved in mitochondrial biogenesis. In details, TXR preconditioning enhanced SIRT-1 expression, which in turn, it could subsequently increase PGC-1α transcriptional activity and NRF-2 expression, indicating that TXR acts as a potential activator of mitochondrial biogenesis. It can be said that TXR-induced NRF-2 over-activation could promote antioxidant genes and proteins activity. These findings show the ability of TXR to preserve mitochondrial biogenesis and counterbalance oxidative stress, which were accompanied with the reduction of DOX-induced testicular histopathological damages. In agreement with our observations, several previous works have revealed that restoration of mitochondrial biogenesis using pharmacological agents or strategies like high-intensity interval training (HIIT) could diminish DOX-induced toxicity in rats. For example, our previous works have revealed that TXR preconditioning had positive impact on DOX-induced cardiotoxicity in rats, which was mediated by restoring mitochondrial function, upregulating the expression of genes involved in mitochondrial biogenesis, and increasing antioxidant enzymes.[20] Also, prior training of rats with HIIT improved the expression of genes regulating mitochondrial biogenesis and modulated microRNA-149 in DOX-induced cardiotoxicity. Hence, we documented that modulation of specific miRs could exert beneficial impacts against DOX-induced toxicity.[27]

In the present work, TXR preconditioning partially increased the expression level of miR-140, which was associated with its impact on increasing the expression of genes involved in mitochondrial biogenesis. Interestingly, it was revealed that miR-140 expression level was very high under DOX challenge, which may represent a compensatory response to DOX-related pathological conditions; however, exploring the underlying mechanisms needs further evaluations. Pretreatment of rats with TXR significantly decreased miR-140 overexpression in DOX-received rats, suggesting that the beneficial impacts of TXR on the expression of SIRT-1/PGC-1α/NRF-2 network may be associated with its

regulatory impact on the activity of miR-140. In agreement with the present study, it has been shown that targeting specific miRs involved in the regulation of mitochondrial biogenesis genes counteracts DOX-related testicular toxicity.[13] Furthermore, previous studies demonstrated that miR-140 increases cell survival and regulates mitochondrial fission and the expression of anti-apoptotic target genes.[14-16] Generally, it seems that miR-140 along with SIRT-1, PGC-1α, and NRF-2 may serve as a coordinated network for improving mitochondrial performance in DOX-induced testicular toxicity and associated mitochondrial damage. These findings demonstrate the specific potential of TXR to provide potent protection against DOX-related testicular toxicity through modulating miR-140 expression and restoring mitochondrial biogenesis.

CONCLUSION

Our study provided the evidence that DOX-induced testicular toxicity could be, at least in part, due to decreased expression profiles of SIRT-1/PGC-1α/NRF-2 network as well as dysregulated miR-140 expression. Interestingly, TXR preconditioning had protective effect against DOX-related testicular toxicity, which was accompanied by reversing the expression of these genes and miR-140. The results of the current work appear to be promising due to documenting the potentials of TXR in decreasing DOX-related testicular toxicity in healthy rats, however, it is encouraged to perform comprehensive evaluations on rats with cancer for achieving more realistic results. Additionally, further investigation is needed to determine the anti-tumor activity of TXR as well as its effective dose to obtain promising clinical outcomes in cancer patients.

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Conflicts of interest

There are no conflicts of interest.

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