Effect of TiO2 Nanoparticles and Curcumin on Sperm Parameters in Response to Temperature-Induced Stress in Scrotal Hyperthermia Rats: Role of miR455

Maryam Mollaei 1, Mehrdad Hashemi 2, 3, *, Elham Siasi 4, Sayeh Jafari Marndi 4 and Malihe Entezari 2, 3

1 Department of Genetics, Faculty of Sciences, North Tehran Branch, Islamic Azad University, Tehran, Iran
2 Department of Genetics, Faculty of Advanced Science and Technology, Islamic Azad University, Tehran, Iran
3 Farhikhtegan Medical Convergence Sciences Research Center, Farhikhtegan Hospital, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
4 Faculty of Biological Sciences, North Tehran Azad University, Tehran, Iran

* Corresponding author: Department of Genetics, Faculty of Advanced Science and Technology, Islamic Azad University, Tehran, Iran. Email: drmehashemi@gmail.com

Received 2021 December 28; Accepted 2022 January 01.

Abstract

Background: Temperature changes cause testicular dysfunction. It has been observed that testicular hyperthermia leads to oxidative stress and as a result a severe reduction in testicular parameters. Causes a severe reduction in sperm parameters to become oxidative due to stress. Recently, natural plant materials and magnetic nanoparticles have been considered. In the internal mitochondrial apoptosis pathway, gen bcl2 is a target of miR455.

Objectives: The present study aimed to investigate the effects of titanium dioxide nanoparticles and improve their impacts by using the antioxidant curcumin on sperm parameters by investigating changes in expression miR455 in response to temperature-induced stress in scrotal hyperthermia rats.

Methods: After preparation, the rats were placed in a hot water bath at 43°C for 30 minutes for six consecutive days. The rats were then divided into eight groups. We used TiO2 nanoparticles at a concentration of 0.03 mg/kg of body weight and curcumin at a concentration of 0.02 mg/kg of body weight. After killing the animals, such parameters of sperm as viability, concentration, motility, and morphology of spermatozoa were studied. RNA extraction and cDNA synthesis were performed using appropriate kits. A gene primer was designed and RT-PCR was used to assess gene expression. The t-test and ANOVA were used to examine differences between different groups. Data analysis was performed using Prism8 software and SPSS version 26.

Results: The results showed that miR455 expression was lower in the treatment groups and was associated with curcumin (P < 0.05). A positive effect of curcumin on improving sperm parameters in rats with scrotal hyperthermia and a negative and toxic effect of TiO2 nanoparticles were shown. However, a significant improvement in sperm parameters was observed when rats were given TiO2 nanoparticles along with curcumin.

Conclusions: The changes in the expression miR455, shown in curcumin have controlled the damage to TiO2 nanoparticles. It seems that miRNA455 can be used as a marker to predict sperm health status. So Curcumin can play a protective role in reducing the toxic effects of testicular hyperthermia as well as titanium dioxide nanoparticles.

Keywords: Rats, Curcumin, Nanoparticle, TiO2, Gene Expression miR455

1. Background

Infertility is a complex disorder with serious medical, psychological, and economic implications. In the vicinity of 20% of couples do not experience pregnancy within a year, 15% of them seek medical treatment, and less than 5% of these couples never have children. Infertility affects both men and women. 50% of couples’ infertility causes are related to men (1).

Stress is inevitable in this world. Stressful situations can lead to many physiological and psychological changes. The side effects of stress on the male reproductive system are described. and it has been shown that heat stress in humans can impact sperm concentration, motility, and morphology (2). In different mammalian species, relatively low temperatures are required for the normal process of spermatogenesis and sperm fertility (3). Studies have shown that the temperature of the scrotum in most mammals is 4°C to 5°C lower than the temperature inside the ventricular area (4). Disruption of the spermatogenesis process following occultation of the testicles is caused by the testicles being exposed to high temperatures in the ventricular-
lar area. It has been reported that normal spermatogenesis is achieved by cooling the testicles inside the ventricle (5). Heat damage to the testicles is one of the infertility causes in men. Even for a short time, exposure to heat leads to a cessation of spermatogenesis, which takes 40 or even 60 days to heal. Testicular weight has not been shown to return to normal even 60 days after heat exposure (6).

Testicular germ cell apoptosis is an essential mechanism in testicular development as well as the destruction of germ cells under physiological and pathological conditions (7). Previous studies have shown that heat induces cell death in the testis so that the death of primary spermatocytes and spermatagonia is quite evident (8). miRNAs (micro RNAs) cause gene silencing by degrading the target mRNA or inhibiting its translation and are involved in developmental processes, apoptosis, cell cycle, and metabolism (9). Impaired biogenesis and miRNA function cause infertility.

Different drugs with different mechanisms have been used to reduce the effects of heat stress on spermatogenesis cells in testicular tissue, and among these, the use of antioxidants to overcome these side effects has been considered (10).

Curcumin is the main and active compound of the turmeric plant, a yellow phenolic compound, that has a wide spectrum of biological and pharmacological activity (11). The most important biological effects of the substance are its anti-inflammatory, antioxidant and antitumor properties (12, 13). In addition, curcumin is a popular antioxidant and one of the most powerful free radical scavengers capable of preventing the production of reactive oxygen species (ROS) in biological and extracellular environments (14). Many studies have reported the antioxidant properties of curcumin and its protective effects on the male reproductive system against environmental pollutants and inducers of oxidative stress (15). Therefore, curcumin may be useful and effective as a powerful antioxidant in preventing major abnormalities caused by oxidative stress due to hyperthermia in the testes and sperm.

With the development of nanotechnology and materials engineering, various nanoparticles with new properties have been made, and although the potential for their toxic effects is unknown in many cases, these materials have found increasing applications (16). Studies have shown nanoparticles’ negative and destructive effects on male germ cells (17). Among nanoparticles, ZnO2 and TiO2 are significant, that are used on an industrial scale in many countries (18). Numerous studies have shown the accumulation of nanoparticles in various tissues as well as the destruction of the blood-testicle barrier and the blood-brain barrier (19). In addition to the beneficial effects of nanoparticles, according to research, titanium nanoparticles can cause harmful effects in various tissues in humans and mice (20). The death of dendritic cancer cells, treated with marked curcumin is due to increased Bax/bcl2 expression ratio resulting in apoptosis. This results strengthens therapeutic potentials of the new nano-curcumin compound (21).

Therefore, the present study was aimed to study the effect of TiO2 nanoparticles with curcumin on the parameters of sperm in rats with scrotal hyperthermia.

2. Objectives

Curcumin neutralized the detrimental effect of heat stress on sperm parameters in rats with testicular hyperthermia. Also, Curcumin reduced the toxic effects of titanium oxide nanoparticles on sperm parameters greatly. Therefore, in subsequent studies, the results of this study can be of great importance.

3. Methods

3.1. Materials

TiO2 nanoparticles (5 mg/mL) (Sigma Aldrich, German) were prepared, with a purity of 97% or higher and a particle size of 5 nm. Purchased curcumin (Merck Cop) with over 80% purity and molecular weight of 388.38. The dose of TiO2 nanoparticles was determined by LD50 (concentration causing the death of half of the rats). Therefore, concentrations (0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/kg body weight) were administered to rats, and LD50 was measured at 0.02 mg / kg body weight. The concentrations were used in subsequent experiments. Furthermore, miR455 was designed and examined in different groups.

3.2. Animals

Forty-eight adult male rats were purchased from the Pasteur Institute in Tehran-Iran. Animals were housed under standard conditions of 12h/12h photoperiod, 25 ± 2°C temperature, and 50% ± 10% RH. (Relative humidity). All animals were fed by corn, wheat, barley, and kernels in equal portion, and under equal nutritional conditions. Free access to water was available to all.

3.3. Induction of Scrotal Hyperthermia

Scrotal hyperthermia was induced by placing the testicles containing the scrotum in a hot water bath at 43°C, for 30 minutes in a 43°C hot tub once a day for 6 consecutive days. The control rats were placed in a water bath at 22°C. After introducing scrotal hyperthermia, the animals were dried, inspected for damage to the scrotum, and placed in.
cages. Studies have shown that not a single animal was harmed.

After induction of scrotal hyperthermia, the rats were randomly divided into 8 groups: (1) control group; (2) TiO2 nanoparticle (0.03 mg/kg body) recipient control group; (3) curcumin recipient control group (0.02 mg/kg body); (4) TiO2 nanoparticles (0.03 mg/kg body) and curcumin (0.02 mg/kg body) recipient control group simultaneously; (5) scrotal hyperthermia group; (6) TiO2 nanoparticle (0.03 mg/kg body) recipient scrotal hyperthermia group; (7) curcumin (0.02 mg/kg body) recipient scrotal hyperthermia group; (8) TiO2 nanoparticles (0.03 mg/kg body) and curcumin (0.02 mg/kg body) recipient scrotal hyperthermia group simultaneously.

After completion of treatment, all the animals were sacrificed by anesthetic overdose, and then the testicular tissue was removed for tissue examination, and the semen was collected from the epididymis for sperm analysis and cytological study parameters.

This study has been approved by Azad University, Tehran North Branch, Tehran, Iran by the ethics code of IR.IAU.TNB.REC.1399.001.

3.4. Sperm Analysis

The morphology, viability, concentration, viability, and motility of spermatozoa were assessed. So, first, we collected sperm from the tissue of the epididymis. Sperm (10 µL) was transferred to a hemocytometer and the number of spermatozoa was counted under an optical microscope at 40x magnification. Sperm motility was assessed under a microscope in ten fields of view in accordance with the recommendations of the World Health Organization.

MTT sperm viability assay presented by Nasr-Esfahani et al. (2002) was used to assess the viability of spermatozoa (22). Aniline blue staining was also used to study the morphology of spermatozoa. The preparations were evaluated for the presence of morphological abnormalities in the tail, neck, or head, and normal and abnormal forms of spermatozoa were examined under a microscope at 100X magnification.

3.5. Genetic Study

The expression of Bax, bcl2, tfam and miR455 genes was evaluated in collected tissues using the real-time RT PCR technique.

3.5.1. Design Primer

In this study, miR455 was selected as the target gene for expression and b-actin as the reference gene.

Gene runner version 6.4 (for DNA and cDNA) was used to design the primers. The primers were also blasted using the NCBI site and synthesized and sent by Takapozist.

The primers were diluted with the specified volume of sterile deionized water to the desired concentration according to the manufacturer’s instructions. The resulting solution is the main solution. Then dilutions of 10 micromoles were prepared from this solution, which was a working solution. Working solutions were stored at -20°C. Table 1 is shown primer sequences.

3.6. Statistical Analysis

Analysis of variance (ANOVA) was used to detect significant differences in the studied traits between groups of rats. We analyzed the data using SPSS software version 26. P < 0.05 was considered statistically significant.

4. Results

4.1. Sperms Viability

The results of the current study showed that scrotal hyperthermia reduces the rats’ sperm viability. In healthy rats, the co-administration of titanium oxide nanoparticles reduced sperm viability; however, the co-administration of titanium oxide nanoparticles with curcumin improved sperm viability, which is shown in Figure 1.

In rats with scrotal hyperthermia, a positive effect of curcumin was observed in increasing sperm viability, and the highest sperm viability was observed in rats given curcumin or titanium oxide nanoparticles combined with curcumin. These results indicate the toxic effect of titanium oxide nanoparticles on sperm viability. However, curcumin was able to reduce these effects.

4.2. Sperm Concentration

In the present study, a strong effect of testicular scrotal hyperthermia was observed in reducing sperm concentration in rats. Also, titanium oxide nanoparticles decreased sperm concentration in healthy rats and rats with scrotal hyperthermia. However, curcumin increased sperm concentration in healthy rats and rats with testicular scrotal hyperthermia. Curcumin appears to have a positive effect on increasing sperm concentration, which is shown in Figure 2.

4.3. Sperm Motility

There were statistically significant differences in sperm motility between different groups of rats (P < 0.001). A sharp decrease in sperm motility was observed in rats with scrotal hyperthermia. Also, titanium dioxide nanoparticles harmed sperm motility percentage. However, curcumin increased the percentage of sperm motility, which is shown in Figure 3.
Table 1. Sequence of Gene Primers

<table>
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<th>Reverse Primer</th>
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<tr>
<td>B-actin</td>
<td>5’ CGGTTCGAGGCCCTGGACGCTCTT 3’</td>
<td>5’ CTTACACTTCATGAAGGAAATIGA 3’</td>
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<tr>
<td>MiR455</td>
<td>5’ UAUGUGCCUUUGGACUACAUGC 3’</td>
<td>miRbase Acc No.MIMAT0022957</td>
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**Figure 1.** Effect of curcumin and TiO2 nanoparticles on the percentage of viability of spermatozoa in different groups of rats (*, ** and *** represents a significant difference in probabilities P < 0.05, P < 0.01, and P < 0.0001; Ch, healthy control; Ch+Cur, healthy curcumin intake control; Ch+TiO2, healthy TiO2 nanoparticles intake control; Ch+Cur+TiO2, healthy curcumin and TiO2 nanoparticles intake control; Ct, rats with scrotal hyperthermia; Ct+Cur, rats with scrotal hyperthermia treated with curcumin; Ct+TiO2, rats with scrotal hyperthermia treated with TiO2 nanoparticles; Ct+Cur+TiO2, rats with scrotal hyperthermia treated with receiving curcumin and TiO2 nanoparticles).

**Figure 2.** Effect of curcumin and TiO2 nanoparticles on sperm concentration in different groups of rats (*, ** and *** represents a significant difference in probabilities P < 0.05, P < 0.01, and P < 0.0001; Ch, healthy control; Ch+Cur, healthy curcumin intake control; Ch+TiO2, healthy TiO2 nanoparticles intake control; Ch+Cur+TiO2, healthy curcumin and TiO2 nanoparticles intake control; Ct, rats with scrotal hyperthermia; Ct+Cur, rats with scrotal hyperthermia treated with curcumin; Ct+TiO2, rats with scrotal hyperthermia treated with TiO2 nanoparticles; Ct+Cur+TiO2, rats with scrotal hyperthermia treated with receiving curcumin and TiO2 nanoparticles).

4.4. Sperm Morphology

The percentage of sperm with normal morphology was significantly reduced in rats with scrotal hyperthermia. Also, in healthy rats treated with titanium oxide nanoparticles, a significant decrease in spermatozoa with normal morphology was observed compared to the control, which is shown in Figure 4.

However, curcumin was able to significantly increase the percentage of normal sperm in healthy rats and scrotal hyperthermia rats. Changes in seminiferous tubule morphology in rats were studied, and the results showed that its structure was abnormal in rats with testicular hyper-
thermia compared with the control group, which is shown in Figure 5.

### 4.5. Flow Cytometry Results

Flow cytometry results show that cells treated with titanium dioxide nanoparticles become necrotic and increase cell apoptosis when treated simultaneously with titanium dioxide nanoparticles and the antioxidant curcumin. It is shown in Figure 6.

### 4.6. Analysis of the mir455

In the above analysis, considering that the P-value is less than 0.05, it was found that hyperthermia and treatment have a significant effect on mir455 expression, which is shown in Figures 7 and 8, and Table 2.

### 5. Discussion

To date, many studies have been conducted on the destructive effect of heat stress on testicular tissue and fertility in mammals. In this study, we studied the protective ef-
Figure 5. A, Seminiferous tubule morphology in healthy control; B, Scrotal hyperemia rats receiving curcumin; and Control scrotal hyperemia rats. D, Spermatozoa stained with Aniline Blue.

Table 2. Statistical Analysis of the Effect of Hyperthermia and Treatments on miR455 Expression, P = 0.00000

<table>
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<th>Variables</th>
<th>DF</th>
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<th>Mean.Sq</th>
<th>F.value</th>
<th>P</th>
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<td>Hyperthermia</td>
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<td>9.71890</td>
<td>1605.00000</td>
<td>0.00000</td>
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<tr>
<td>Treatment</td>
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<td>38.44100</td>
<td>6.40680</td>
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<td>Interaction</td>
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<td>3.92400</td>
<td>0.65400</td>
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The effect of curcumin on the destructive effect of titanium oxide nanoparticles on sperm parameters in rats with scrotal hyperthermia. The results of this study showed that titanium oxide nanoparticles can reduce the sperms’ viability, concentration, motility, and normal morphology. However, oral administration of curcumin to the rats affected by scrotal hyperthermia, resulted in significant obstruction of testicular damage.

Studies have shown an increased incidence of programmed cell death or apoptosis in testicular germ cells after exposure to high temperatures (23). With the increasing temperature in testicular tissue, the amount of fat peroxidation in germ cells increases. In addition, the activity of antioxidant enzymes is impaired (24). It has also been shown that oxidative stress may play a critical role in inducing apoptosis under thermal stress conditions (25). In the current study, the sperms’ survival, concentration, motility, and morphology of sperms were affected by heat stress, which led to a decrease in sperm parameters. It seems that due to heat stress, testicular tissue is damaged, and the process of spermatogenesis is disrupted. Studies have also shown that purified spermatocytes and primary spermatids in the testes are very sensitive to heat and are rapidly damaged by an increase in temperature in the testicular tissue (26). Therefore, the decrease in sperm parameters in rats with scrotal hyperthermia in the present study can be attributed to the induction of apoptosis due to oxidative stress in sperm germ cells. However, the administration of curcumin greatly improved sperm parameters.

Curcumin, the most important secondary metabolite of the turmeric plant, is a powerful antioxidant compound, that has important pharmacological properties. Curcumin has been reported to scavenge free radicals and ROS, thereby reducing fat peroxidation (27). Therefore, the improvement in sperm parameters in this study by administration of curcumin in rats with testicular hyperthermia...
Figure 6. A, Healthy testicular tissue cells treated with titanium dioxide nanoparticles; B, Hyperthermia cells of testicular tissue treated with curcumin; C, Healthy testicular cells treated with simultaneous treatment of curcumin and titanium dioxide nanoparticles; D, Testicular hyperthermia cells with simultaneous treatment of curcumin and titanium dioxide nanoparticles.

Figure 7. A separate comparison of each treatment on mir455 expression changes in hyperthermia and healthy groups $P = 0.00000$. 

can be attributed to the antioxidant effect of curcumin. Other studies on the impact of the curcumin on reproductive performance have reported similar results (28).

In recent years, the toxic impact of nanoparticles has been studied, but so far, little research has been done to reduce their toxic effects. In one study, it was stated that nanoparticles can cross the blood-testicular barrier and some of them have destructive and toxic effects on germ cells (29). In another study, it was shown that titanium dioxide nanoparticles reduce sperm concentration and motility (30). It has been suggested, that sperm motility can be an indicator of the effect of toxins on sperm (31). Therefore, in the present study, it is possible that titanium oxide nanoparticles entered the seminiferous tube by destroying the blood-testicular barrier and directly affecting germ cells or Sertoli cells. Other effects of nanoparticles such as silver and gold nanoparticles on the germ cells and sperms have been shown (32, 33). This is in line with current research findings. However, in the present study, the toxic effect of titanium oxide nanoparticles on sperm parameters was reduced by curcumin administration and reached to control level.

Due to the significant changes in the expression of miR 455 in comparison with different control and experimental groups, it was found, that in control groups that used nanoparticles without curcumin, the rate of necrosis due to sperm mortality increased compared to experimental groups that nanoparticles with Curcumin was used. Targeted apoptosis of damaged cells was observed, and no complications of necrosis were observed.

Acknowledgments

I would like to thank all those who helped me in the process of this study. This study is part of a Ph.D.

Footnotes

Authors' Contribution: Not declared by authors.

Conflict of Interests: One of the authors of this article is a member of the committee board of the Journal of Human Genetics and Genomics.

Data Reproducibility: The dataset presented in the study is available on request from the corresponding author during submission or after its publication.

Ethical Approval: This study was approved by Azad University Tehran Shomal Branch with the ethics code of IR.IAU.TNB.REC.1399.001 (ethics.research.ac.ir/EthicsProposalView.php?id = 123369).

Funding/Support: Azad University Tehran Shomal Branch.

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