Research Paper: Ameliorating Effect of β-Carotene on Gene Expression Alteration in Spermatozoon Cultured With Titanium Oxide

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ABSTRACT

Introduction: In the present study, we assessed the effects of β-carotene on Titanium oxide Nanoparticle (TNP) induced mouse Spermatozoon Stem Cells (SSCs) apoptosis, at molecular level.

Methods: After isolation from cryptorchid mouse testis and characterization, spermatogonial stem cells were divided into four groups. In the control group, spermatogonial cells were cultured in α-MEM supplemented with 2% BSA (Bovine Serum Albumin). β-Carotene (BC) group was composed of control culture condition supplemented with 1 µg/ml β-carotene. TNP group comprised control culture condition supplemented with 1 µg/ml titanium oxide (TiO_2). Ultimately the last group contained control culture condition supplemented with both 1 µg/ml BC and 1 µg/ml TiO_2 for three days. After that, spermatozoon viability was evaluated by MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) assay, apoptotic and necrotic indices with Annexin V/PI kit and gene expression of CASP3 and MAPK14 using qRT-PCR method.

Results: TiO_2 could significantly decrease viability of the cultured spermatozoon in TNP group compared to the control group. In BC group, we determined increased frequency of live spermatozoon compared to TNP or control group. Expression of apoptotic related genes significantly increased in TNP group. Spermatozoon induced by titanium oxide might be useful in clinical procedures. Measurement of apoptosis index using Annexin V/PI method also showed significant increase in apoptotic index of germ cells in TiO_2 treated spermatozoon ($P<0.05$).

Conclusion: Expression of apoptotic related genes in cultured spermatozoon could efficiently be decreased by β-carotene treatment. Application of BC had a potential protective effect in preventing apoptosis in germ cells and might be useful in clinic.

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1. Introduction

Spermatogenesis is an elaborately regulated process devoted to continuous production of spermatozoa via creation of spermatozoon. This process is highly sensitive to environmental fluctuations [1]. Thanks to development of nanotechnology, nanoparticles (NPs) based on their physical and chemical properties are used in drug delivery systems, antibacterial materials, cosmetics, sunscreens, and electronics [2]. Many recent reports indicated that most NPs could harm fertility through a negative impact on male germ cells [3]. Male fertility could be affected by decline in sperm production or epigenetic alterations, causing negative consequences on the development of the offspring [4].

Since little is known about direct effects of NPs on spermatogonial stem cells, this area will likely be the subject of intensive research within the next decade. NPs, indeed, offer the most effective distributions to certain tissues by penetrating into very small capillaries throughout the body and passing through epithelia and biological membrane [5, 6]. Besides passing the blood-brain barrier, NPs can penetrate the blood-testis barrier and could have a cytotoxic effect on male germ cells [5]. Among the various metal nanomaterials, titanium oxide (TiO_2) is a fine, white, crystalline, odorless, and low-soluble powder. With regard to its optical, electrical, and catalytic properties, this nanomaterial has considerably important applications [7]. Many in vivo reports suggested that TiO_2 nanoparticles are toxic to many organs, including testis.

Male mice, which intraperitoneally received these NPs, showed significantly lower sperm quantity and motility, also many abnormal sperms and apoptotic germ cells are seen in the testis without significant signs of testicular and/or epididymis lesions [8]. In this regard, Ema et al. reported altered spermatogenesis and histopathological changes in male offspring testes of pregnant mice that received TiO_2 subcutaneously [9]. Guo et al. demonstrated that intraperitoneal treatment of mice with TiO_2 would reduce sperm density and motility, while sperm abnormality and germ cell apoptosis increase [10]. Takeda et al. also studied the migration of TiO_2 NPs towards the spermatids, Sertoli and Leydig cells in the male offspring of pregnant mice, resulting in disorganization in seminiferous tubules as well as reductions in daily sperm production, sperm motility, and Sertoli cell numbers [11].

By migration of these NPs, disorganized and disrupted seminiferous tubules, tubule lumens with few mature sperms, as well as decline in daily sperm production, epididymis sperm motility and number of Sertoli cells were also observed [9, 11]. Komatsu et al. suggested that TiO_2 NPs were taken up by Leydig cells causing increase in cell viability and proliferation, levels of hemeoxygenase-1 (a sensitive marker for oxidative stress) and steroidogenic acute regulatory protein, which tunes mitochondrial cholesterol transport [12]. However, there are still little evidence implicating toxicological effects of nanoparticles on the biology of adult stem cells, including Spermatozoon Stem Cells (SSCs) in the testis.

In the current study, we investigated the effects of TiO_2 on SSCs at cellular and molecular levels, in vitro. Since NPs affect testis homeostasis, it is essential to find a suitable medication for neutralizing or decreasing their side-effects. Carotenoids are yellow or orange, containing organic pigments found in the chloroplasts of plants, some bacteria, and fungi [13]. β-Carotene (BC) is the most widely studied type of carotenoid. Due to its unique structure and cleavage efficacy, BC is the most efficient pro-vitamin A carotenoid which acts as an antioxidant to protect the cells against free radicals [14]. Antioxidation, cardiovascular protection, radio protection and anti-epilepsy are some pharmacological and biological activities of BC [15]. Reportedly, BC supplements contribute in improvement of sperm quality [16].

Therefore, another purpose of the present study was to evaluate the ameliorating effect of BC on spermatogonial stem cell line, as a model in vitro, for assessment of TiO_2 cytotoxicity in the male germ cells. It has been indicated that toxicants might cause negative effects on the development of offspring, by damaging DNA [4]. Thus, several investigations are currently performed to determine the effect of NPs on gene and protein expressions in testis and/or germ cells. Accordingly, we examined the expression of caspase-3 and p38 Mitogen-Activated Protein Kinases (p38-MAPK) after induction of TiO_2 and BC to the medium of the cultured spermatozoon.

2. Materials and Methods

Animals

In this experimental study, 36 healthy adult male NMRI (Naval Medical Research Institute) mice (6–8 weeks old, 25–30 g weight) were used. This study was carried out in an ethically proper way by following the Ahvaz University Ethic Committee guidelines. The animals were housed under standard conditions (12:12 hours dark/light cycles, relative humidity of 50±5% and 22±3°C) from at least one week before the experiment until the end of it. Animal models were maintained in suitable

clean cages, supplied by ad libitum food and water. All experimental procedures on the animals were approved by the Institutional Animal Care and Use Committee of Ahvaz Jundishapur University (Ahvaz, Iran).

Donor mice and cell collection

Testicle cryptorchidism were performed by making two horizontal flank incisions 2 mm from caudal to the last rib in the abdominal wall skin and peritoneum, followed by suturing the fat pad of testis to the peritoneum. Cryptorchid testes were used to isolate the required cells, two months after surgery. They were placed on ice and transferred into the laboratory within 10 minutes. After decapsulation, the testes were minced into small pieces and suspended in minimal essential medium (α-MEM; Gibco, Paisley, UK) supplementing with 14 mM $NaHCO_3$ (Sigma Chemical Co., St. Louis, USA), 0.2% Bovine Serum Albumin (BSA; Sigma Chemical Co., St. Louis, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin (both from Gibco, Paisley, UK), 0.1% insulin-transferrin selenium, and 2 mM glutamine (both from Sigma Chemical Co., St. Louis, USA).

The minced pieces of testis were suspended in α-MEM, containing 0.5 mg/ml collagenase/dispase, 0.5 mg/ml trypsin, and 0.08 mg/ml DNase, for 30 minutes (with shaking and slight pipetting) at 37°C (all enzymes were purchased from Sigma). After three washes in α-MEM medium and removing most interstitial cells, spermatozoa, and some spermatid cells, the second digestion step (45 minutes incubation at 32°C) was performed in α-MEM by adding fresh enzymes to the seminiferous cord fragments. Following this treatment, most persisted cell aggregates were sheared gently by repeated pipetting with a Pasteur pipette for 5 minutes. The cells were separated from the remaining tubule fragments by centrifugation at 30g for 2 minutes at 37°C. The cells were subsequently washed twice followed by adding fresh α-MEM medium.

Spermatocyte cell isolation by PNA binding

The spermatocyte cells were isolated using a procedure described by van Pelt et al. [17] with some modifications [18]. Briefly, Petri dishes with a diameter of 60 mm were coated with 5 ml PNA (100 µg/ml) diluted in phosphate-buffered saline positive (PBS+) for at least 1 hour at 37°C. Next, the dishes were washed three times with α-MEM containing 0.5% BSA. They were stored with α-MEM containing 5 µg/ml DNase for at least 1 hour at 37°C. About 5-25 million cells per dish were incubated for 90 minutes at 32°C and 5% CO_2 atmosphere. After cell binding to PNA, the residual non-bound cells were collected by repeated washing of the dishes with pipette. Consequently, the suspended cells contained 30%-45% type A spermatogonia. The collected cells were used for further cell separation on coated plastic dishes by DSA-lectin.

Sertoli cell isolation by DSA-lectin binding

Sertoli cells were isolated using Scarpino et al. protocol [19] with some modifications [20]. Petri dishes with a diameter of 60 mm were coated with 5 µg/ml datura stramonium agglutinin (DSA; Sigma, Germany) soluble in PBS and incubated at 37°C for 1 hour. The coated plastic dishes were washed three times with α-MEM containing 0.5% BSA (Sigma, Germany). The mixed cell populations, obtained by enzymatic digestion, was placed on lectin-coated dishes at a concentration of 1.5×105 cells/cm^2 and incubated for 1 hour at 32°C in a humidified atmosphere of 5% CO_2. After incubation, the non-adherent cells were collected by twice washing with medium. Alternatively, 48 hours after seeding on the lectin-coated dishes, Sertoli cells were detached by ethylenediaminetetraacetic acid (EDTA)–trypsin treatment (0.02% EDTA, 0.1% trypsin in Ca- and Mg-free PBS) for 5 minutes at 37°C. The cells were counted, adjusted to desired densities and subsequently transferred into the individual wells of a 24 multi-wells dish (1.5×105 cells/cm^2) for the secondary culture in α-MEM at 32°C in the presence of 10% Fetal Bovine Serum (FBS; Gibco, Paisley, UK). This method helped us isolate Sertoli cells with more than 95% purity. The collected cells were then used for culture.

Cell quantity and viability

The number of Sertoli and spermatogonial cells was determined with a hemocytometer. Cell viability was also evaluated by using means of the dye exclusion test (0.04% trypan blue solution).

Cell culture

After isolation of spermatocyte and Sertoli cells, the residual suspended spermatogonial cells were collected and incubated at 32°C with humidified atmosphere, in the presence of 10% FCS. At this stage, more than 35% to 45% of the cells were spermatogonia. In addition, testicular cells quantity was determined with a hemocytometer, before culture. Cell viability was ultimately evaluated by means of the dye exclusion test (0.04% trypan blue solution).

Spermatogonial cells co-cultured with Sertoli cells

Four to seven days after indicated procedure, Sertoli cells formed a confluent layer. The collected testicular cells

(1×104 cells/cm^2) were co-cultured in four-well plate on top of the Sertoli cell layer in 4 different groups, including spermatogonial control group cultured in α-MEM and supplemented with 14 mM $NaHCO_3$, 0.2% BSA, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.1% insulin-transferrin selenium as well as 2 mM glutamine; BC group having spermatogonial control group supplemented with 1 µg/ml β-carotene; TNP group having spermatogonial control group supplemented with 1 µg/ml TiO_2; BC+TNP group having spermatogonial control group supplemented with 1 mg/ml BC and 1 mg/ml TiO_2 for 3 days.

Immunohistochemical identification of spermatogonial and Sertoli cells

The cells were fixed for 20 minutes in 4% paraformaldehyde at room temperature before they were rinsed with PBS. Following permeabilization by 0.2% Triton X-100, to facilitate antibody penetration, the cells were washed with PBS. Extraneous antibodies were blocked with 10% goat serum (Vector, USA). The slides were then incubated for 1 hour at 37°C with a mouse monoclonal anti-vimentin antibody (diluted 1:200; Sigma Chemical Co., USA), as a marker for Sertoli cells [14] or mouse anti-Oct-3/4 (R&D system, USA) polyclonal antibody diluted in PBS (1:50), as a marker for undifferentiated cells [20, 21].

After extensively washing with PBS, goat anti-mouse IgM, labeled with fluorescein isothiocyanate, was added to the cells and incubated for 45 minutes. Nuclei were ultimately stained with ethidium bromide (diluted to 5 µg/ml; Sigma Chemical Co., USA). The control slides were processed under similar conditions except using the first antibody. All incubations were in a moist chamber at 37°C. The slides were then mounted with 90% glycerol in PBS. Evaluation was carried out using a fluorescence microscope with appropriate filters.

MTT assay

In this experiment, 20 µl of MTT solution (5 mg/ml) was added to each well and the plate was incubated at 37°C for 4 hours. Thereafter, the medium was aspirated and the wells were washed with PBS. The wells were then left to dry for approximately 2 hours, then 200 µl dimethyl sulfoxide was added to each well. Next, the plate was placed on a shaker to dissolve the formazan crystals dye, followed by determining the absorbance at 570 nm using a reference wavelength of 630 nm on an ELX800 UV universal microplate reader (BioTek Instruments Inc., Vermont, USA).

Annexin V/PI staining

Phosphatidyl Serine (PS) is normally located in the inner leaflet of the plasma membrane bilayer. Apoptosis causes membrane phospholipid asymmetry and translocation of PS into the outer leaflet of the membrane. Thus, detection of PS exposure has been well established as an early apoptotic marker. In the present study, PS externalization was detected in spermatogonia using Annexin V/PI staining (Bioscience, U.S.A) followed by modification of the manufacturer's protocol.

Briefly, the cultured cells were initially washed twice with calcium buffer for 10 minutes at 4°C. Next, the cells were suspended in the Annexin V labeling solution, containing recombinant Annexin V protein and Ca^{2+}. After incubation at room temperature for 15 minutes, the cells were washed twice with calcium buffer and stained by propidium iodide for 10 minutes. Annexin V was detected by the green fluorescence detector 1, after passing through a 530/30 nm band pass filter.

Quantitative reverse transcriptase PCR

Using the RNeasy Mini kit (Qiagen, USA), total RNA was isolated from spermatogonia according to manufacturer's instructions. We used 1×106 spermatogonia, isolated from Sertoli cells by DSA-lectin, for RNA extraction. Complementary DNA was produced from the extracted total RNAs using the cDNA synthesis kit, based on the manufacturer's instructions (Fermentas, Canada).

In this experiment, we quantitatively evaluated caspase3 (CASP3) and p38-MAPK (MAPK14) gene expressions using appropriate primer sequences (shown below). Approximately 2 µl of cDNA was amplified in 25 µl final volume of each quantitative reverse transcriptase PCR (qRT-PCR) reaction, containing 12.5 µl of 2× SYBR Green Master Mix (Fermentas, Canada), 0.2 µl of 10 pM forward and reverse primers (Qiagen, USA), and 10.1 µl DEPC water. After 10 minutes initial incubation at 95°C, qRT-PCR amplification was performed in 40 cycles using the following program: 95°C for 15 seconds, 5°C for 30 seconds and 60° C for 34 seconds. Data in all cases were compared to the GAPDH housekeeping gene. Expression levels were normalized to individual GAPDH, as an internal control. Relative quantification (RQ) profile was obtained by plotting the relative gene expression levels compared to the control, using $2^{-\Delta\Delta Ct}$ equation [21].

Gene sequences

GAPDH

Forward: TGCAGTGCCAGCCTCGTG

Reverse: TTGATGGCAACAATCTCCACTT

Caspase3

Forward: ATGGGAGCAAGTCAGTGGAC

Reverse: CGTACCAGAGCGAGATGACA

MAPK14

Forward: GCTCACCCCTTCTTTGAACC

Reverse: TTCGTCCACGCTGAGTTTCT

Statistical analysis

The results are expressed as mean(SD). Statistical significance between mean values was determined by 2-way analysis of variance and Tukey test. $P \leq 0.05$ was considered statistically significant.

3. Results

Isolation and characterization of spermatogonial and Sertoli cells

We demonstrated that DSA-lectin isolated cells were able to be enriched and grown in monolayer culture conditions. These cells had an irregular outline with a granular appearance. In contrast, the other cell types with spherical outline and two or three eccentrically placed nucleoli, could be observed within the first week or immediately after passage, creating a colony after proliferation. Findings showed expression of Oct-4 protein, as a molecular marker for spermatogonial stem cells, in the respective colonies (Figure 1).

TNP effects on survival rate

MTT assay was performed 24, 48, and 72 hours after cell culture. Findings did not show any significant change of spermatogonial cells survival rate in the BC group after 24, 48, and 72 hours compared to control group. In the TNP group, survival rate significantly decreased at all experimented times. Spermatogonia in both BC treated groups after 24, 48, and 72 hours culture significantly increased, compared to TNP group, but reduced compared to control group (Figure 2).

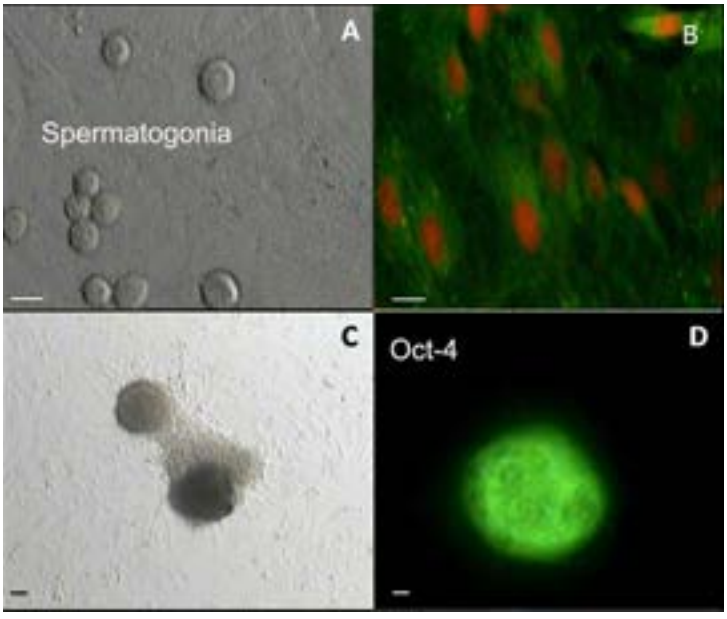

Figure 1. Images represents (A) Co-culture of Sertoli and spermatogonial stem cells, (B) Vimentin (green color) localization in the cytoplasm of Sertoli cells and ethidium boromide (red color) in the nucleus, (C) Colony formation in culture medium, and (D) Oct-4 localization for spermatogonial stem cells. bar for A and B × 400, C and D: ×200.

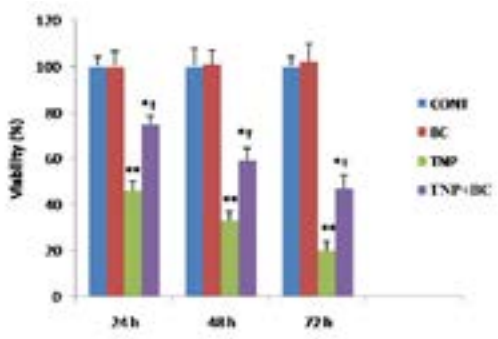

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Figure 2. Comparing survival rate of spermatozoon in different groups, CONT: Control, BC: β-carotene, TNP: TiO2, TNP+BC: β-carotene and TiO2 groups 24, 48 and 72 hours after culture. (Mean±SD). **P<0.01 *†P<0.001 compared to the control group.

The effects of TNP on apoptotic rate

After Annexin V staining, spermatogonia were classified into four groups, including 1) Necrotic cells with PI-positive red nuclei and cytoplasm, 2) Early apoptotic cells with homogeneous Annexin V-positive signals in membrane, 3) Late apoptotic cells with PI-positive nuclei, and 4) Normal cells which could not be stained by Annexin V-FITC or PI (Figure 3). We determined few cells in the control group with necrotic and apoptotic (early and late) features (Figure 4 and 5).

Data analysis showed a decrease in necrotic and apoptotic cells in BC group in contrast to control, although this difference was not statistically significant. Comparing BC group with controls did not show any statistically significant difference, while this level significantly reduced (P≤0.001) compared to TNP group. Further analyses showed a significant decrease in the apoptotic level of BC treated group, in comparison with control or TNP group (Figure 5).

mRNA expression of Caspase3 and p38-MAPK in spermatogonia cells

qRT-PCR analysis revealed no significant difference of CASP3 and MAPK14 mRNA expression levels between BC and control groups (Figure 6). Although, these gene expression levels were significantly up-regulated in TNP group compared to the control. Spermatogonia in BC group showed lower mRNA expression levels of CASP3 and MAPK14 in comparison with TNP group.

4. Discussion

Despite several benefits of nanotechnology, unique properties of NPs can cause harmful effects on biological systems [22]. In this regard, some NPs negatively influence the public health by impairing the normal function of reproductive system. Meta-analysis showed changes in male reproductive system, including sperm shape and count, in industrial countries, leading to alteration in sexual behaviors and low fertility level [23].

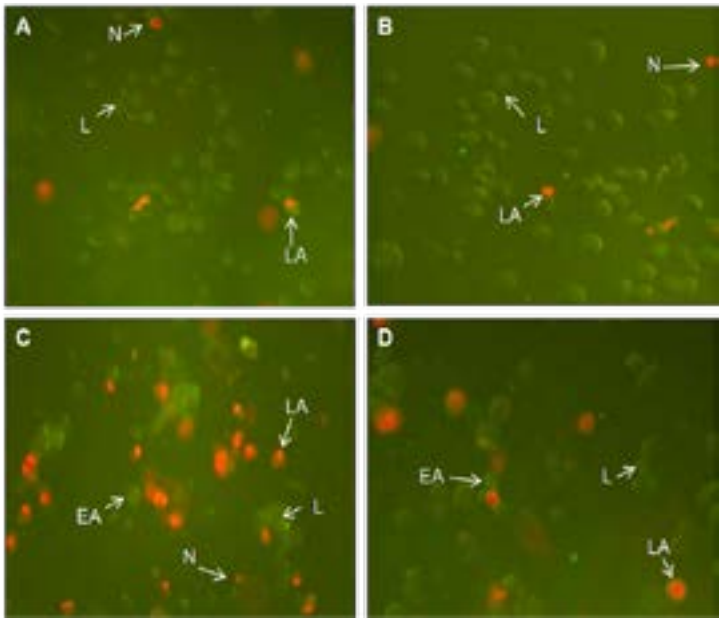

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Figure 3. Annexin V/PI immunofluorescent staining of spermatozoon in different groups. Images represent (A) control, (B) BC, (C) TNP, and (D) BC+TNP treated groups. Images were captured with 400× magnitude. N: Necrosis, L: Live cells, LA: Late apoptosis, EA: Early apoptosis.

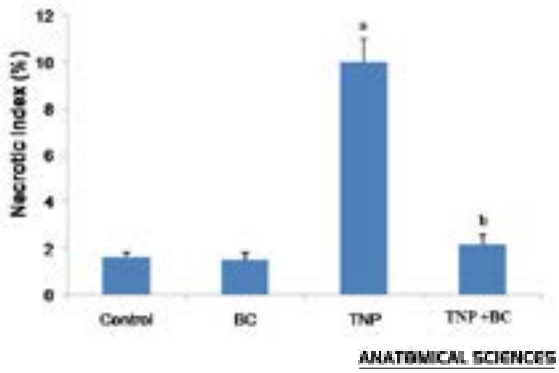

Figure 4. Necrotic index in different groups of treatment. Error bar: Mean±SD, aSignificant difference (P≤0.001) compared to control group, bSignificant difference (P≤0.001) compared to TNP group.

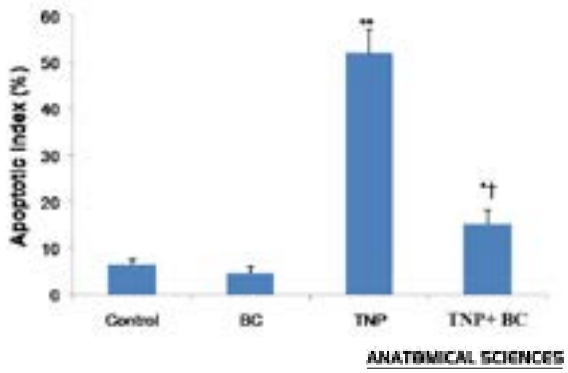

Figure 5. Apoptotic index in different groups of treatment. Mean±SD.*P≤0.01 in comparison with control group, **P≤0.001 in comparison with control group, †P≤0.001compared to TNP group.

Therefore, studying the effects of NPs on particular germ cell populations might ultimately help find a therapeutic solution for some reproductive disorders. Thus far, investigations on animal models have determined the testicular cytotoxicity due to induction of some NPs, although the molecular mechanism of this behavior has not been appropriately elucidated yet. In addition to in vivo models, some in vitro ones have attempted to reproduce the complex cell-cell interactions, taking place between germ cells and Sertoli cells [23].

In the present study, after isolation and characterization of spermatogonial stem cells, MTT assay was performed to determine vitality, apoptotic, and necrotic indices. In addition, we evaluated CASP3 and MAPK14 gene expression levels, using qRT-PCR method. Analysis of MTT assay demonstrated significant decrease in vitality level of spermatozoon in TNP group after 24, 48, and 72 hours, while this level significantly increased in the BC-treated group compared to control.

We have previously reported cytotoxic effects of TiO_2 on different germ cells and suggested that these effects would be due to Sertoli cell damage or defects in testosterone synthesis, culminating in germ cell damage or apoptotic induction [24]. Subsequently, we determined that TiO_2, possibly by passing through blood-testis barrier, can directly affect spermatozoon, in vitro. As indicated earlier, spermatozoon is a stem cell, contributing in the spermatogenesis. Thus, defects in the number of these cells can reduce sperm production. In line with that, we have previously demonstrated that decrease in the spermatozoon cells could negatively alter various sperm production parameters [24].

Moreover, Braydich-Stolle et al. reported that mammalian spermatogonial stem cells are sensitive to TiO_2 NPs [3]. Asare et al. also demonstrated genotoxicity and cytotoxicity of silver NPs on primary cultured mouse testicular cells and NT2 cell line (human embryonic carcinoma cell line). They reported increased toxic effects of silver NPs associated with decrease in their size [25].

Previous studies have shown NPs induced germ cell apoptosis and reduced male fertility in animal models [3, 15, 23, 26]. By evaluating treated spermatogonial cells,

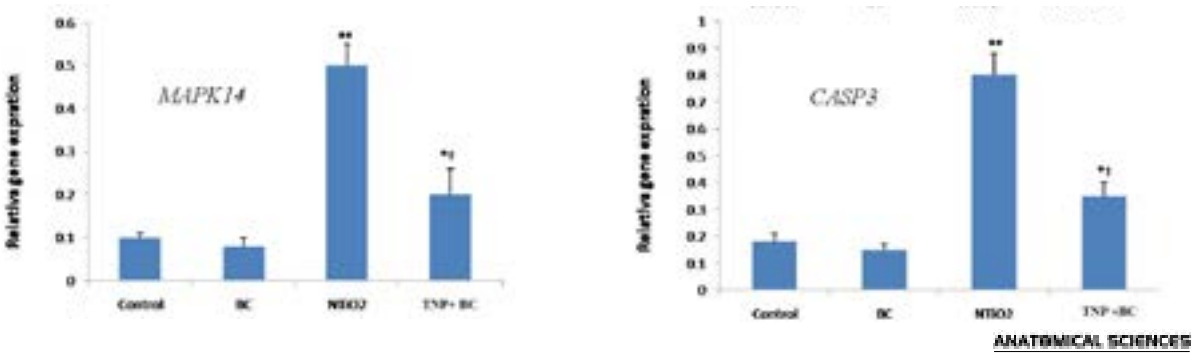

Figure 6. mRNA expression level analysis of CASP3 (caspase3) and MAPK14 (p38-MAPK) in the treated groups. Mean±SD, *Significant difference (P<0.01) compared to control group, **Significant difference (P<0.001) compared to control group, †Significant difference (P<0.001) compared to TNP group.

we demonstrated an increase in necrotic and particularly apoptotic indices. Finding molecular pathways of apoptosis in spermatogenic cells, under different conditions, is a fundamental step towards progression of novel therapeutic procedures on targeted treatment of germ cell tumors and infertility. It has been shown that nano-TiO_2 induces apoptosis in a variety of cell types, although the precise molecular mechanisms involved in this procedure has not been fully determined yet [27-29].

In this work, we reported a novel mechanism, whereby TiO_2 NP induces germ cell apoptosis mediated by activation of the CASP3 and MAPK14. CASP3 plays an important role in andrologic defects through spermatogenesis, decreased sperm motility, DNA fragmentation, and infertility. To date, 14 caspase types have been implicated in the human apoptotic pathway. Among these, CASP3, as a major executioner protease activated during the early stages of apoptosis, is involved in the stimulation of apoptosis in various cell types [26].

Recent studies have shown that male reproductive function is modulated through MAPK cascades. In the testis, MAPKs are involved in processes controlling gene expression, cell cycle progression, differentiation of germ cells and germ cell apoptosis [30]. At least three MAPK families have been characterized, including Extracellular signal-Regulated Kinase (ERK), Jun Kinase (JNK/SAPK) and MAPK14. JNK and MAPK14, in particular, are key regulators of the pro-apoptotic signal transmissions [31-33]. We determined that TiO_2 activ ates MAPK14 signaling pathway in spermatogonia. Function of these kinases is necessary for CASP3 activation with different efficiencies.

This data agree with Park's et al. finding indicating that MAPK14 had an essential role in mitochondrial dependent apoptosis induction, as a crucial cell death in male rats after hormonal deprivation [34]. Li et al. reported that MAPK14 signaling is important for induction of apoptosis during a variety of stress responses [30]. MAPKs are also implicated in activating the caspase cascades. As a case, a recent study has reported that MAPK14 signaling is linked to the activation of CASP8. The activated CASP8 in turn cleaves and activates CASP3, initiating subsequently a set of events that culminate in apoptosis [35].

On the other hand, we showed that BC treatment could significantly decrease the gene expression levels of CASP3 and MAPK14 in the cultured spermatozoon. The exact mechanism of anti-apoptotic effects of BC against $NTiO_2$ has not been elucidated yet. It has also been shown that BC protects cellular membranes and lipoprotein against oxidative damages, by easily passing through biological membranes and rapidly entering to the cells [36]. Antioxidant properties might most likely be a principle cause of the BC anti-apoptotic effects. It is well known that oxidative stress has direct effect on various types of cell death, including apoptosis. Therefore by suppressing oxidative stress, BC may reduce apoptosis induced by TiO_2 NPs.

It has also been reported that BC decreased oxidative stress and prevented ethanol-induced cell death by inhibiting caspase-9 and caspase-3 expression [37]. Liu et al. (2004) showed that β-carotene may prevent the smoke-induced activation of the MAPK14 in lungs of ferrets. With regard to these data and previous reports, NPs could affect animal spermatogenesis. Since several NPs exist in our environment, they could threaten human, particularly in societies who are largely exposed to these agents.

In conclusion, our results provided new insights into the mechanisms involved in the process of apoptosis induced by $NTiO_2$ in testicular tissue. Additionally, we showed that BC could effectively suppress the apoptotic pathways. BC has a potential protective effect against the testicular toxicity induced by NPs and might be clinically useful. Extrapolation of these data to the human situation is not appropriate. However, this information might pave the way to conduct better clinical investigations in future.

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Conflict of Interest

All authors certify that this manuscript has neither been published in whole nor in part nor being considered for publication elsewhere. The authors have no conflicts of interest to declare.

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