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Original article

In vitro antiapoptotic effects of the calligonum extract on spermatogonial stem cells

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Abstract

Background: Spermatogonial stem cells are the foundation of spermatogenesis and male fertility. So, their maintenance and culture are very important**.**

Objective: In this study, we assessed protective effects of the Calligonum on in vitro viability and apoptotic and antiapoptotic genes expression of spermatogonial stem cells.

Materials and Methods: After 24 hr of culture, the spermatogonial stem cells were treated with 30 µM dose of H_2O_2 and then 10 µg/ml the Calligonum extract was added for 3 wks. Viability was assessed by Trypan blue, apoptosis using PI-Annexin and finally Bax, Bcl-2 and P53 genes expression by Real-Time Polymerase chain reaction.

Results: After 3 wk of treatment, viability in the Calligonum extract+H_2O_2 group was significantly higher than H_2O_2 group alone (p=0.001). In the Calligonum extract+H_2O_2 group, apoptosis, as well as expression of apoptotic genes (Bax and P53), was significantly lower than the group treated with H_2O_2 alone.

Conclusion: The results of this study showed that 30 µM H_2O_2 increased apoptosis but decreased viability in spermatogonial stem cells. Calligonum has antioxidant properties that can reduce apoptosis, Bax and P53 expression and increase the viability and Bcl-2 expression.

Key words: *Stem cell, Apoptosis, Calligonum, Viability.*

Introduction

Spermatogonial stem cells (SSCs) that employed as the base of spermatogenesis to preserve fertility throughout a male's lifetime (1). Studies on SSCs are very difficult but important due to the scarcity of these cells (2, 3). Free radicals are highly unstable molecules that quickly and nonspecifically interact with biological molecules and lead to the development of a variety of injuries, including peroxidation of cell membranes, oxidation of amino acids and nucleic acids, apoptosis and necrosis, which result in damage to DNA and cellular structures as well as decreased viability and growth of cells (4-6). H_2O_2 could create oxidative stress in sensitive SSCs and cause serious damage and cell death (7).

Researchers have shown that increased infertility in men is closely related to decreased use of antioxidant agents, which could contribute to the treatment of male infertility (8). Antioxidants are compounds that protect the cells against damage caused by free radicals. Reduced consumption of antioxidants such as vitamin C, vitamin E, vitamin A, zinc, and selenium can increase infertility in males (9). Over the past decade, numerous studies have presented that antioxidants can attenuate oxidative stress and improve stem cell survival. Positive effects of antioxidants include increasing genomic stability, improving the connection of stem cells to culture media (10-12). Plants are the main source to supply the antioxidants, including the Calligonum comosum L` from Polygonaceae family (13, 14). The Calligonum has the important antioxidants like Catechin, Epicatechin, Quercetin, kamefrol, and Genistin (15).

Several studies showed that Catechin, Quercetin interact with glutathione-peroxidase enzyme and have inflammatory, antiulcer and anticancer properties that can change the expression of Tumor necrosis factor alpha (TNFα) in vitro (16). Studies have shown that the cytotoxic activity of Calligonum comosum might be through modulation of apoptosis (17).

Therefore, in this study, we attempted to assess the protective effect of the Calligonum

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comosum on viability, apoptosis as well as Bax, Bcl-2 and P53 genes expression on SSCs treated with H_2O_2 in vitro after 3 wks.

Materials and methods

Experimental design

The testes were isolated from 3 to 5 days male mice (5 mice per groups) and cultured according to previous study (18).

Experimental groups include:

1. The control group without treatment.
2. The Calligonum group treated with 10 µg/ml dose of the Calligonum extract.
3. The H_2O_2 group treated with 30 µM dose of H_2O_2.
4. The combined group treated with 30 µM dose of H_2O_2+10 µg/ml dose of the Calligonum extract

Assessment of DNA fragmentation or apoptosis by flow cytometry assay

Apoptosis was evaluated by flow cytometry and Annexin-PI kit [Detection Kit (ab14085)]. During programmed cell death or apoptosis, phosphatidylserine is transferred from inside to the outside of cell membrane, annexin is bound to extracellular phosphatidylserine and detected by flow cytometry. PI also binds to DNA of fragmented nuclei of dead cells, which are identified by flow cytometry (19). The cells (500,000 cells) were first separated and prepared according to instructions in the kit and apoptosis was determined by flow cytometry (Becton Dickinson apparatus).

Expression of Bax, Bcl2, and P53 genes with Real-Time PCR

RNA extraction and cDNA synthesis were done 3 wks after cell culture and treatment. RNA was isolated by chloroform and isopropanol and washed with 75% ethanol. Finally, the contaminations were destroyed by RNase-free DNase. After cDNA synthesis of samples [cDNA synthesis kit (Fermentase, Lithuania)], the expression of P53, Bax, and Bcl-2 genes was compared to Glyceraldehyde 3-phosphate dehydrogenase as the housekeeping gene. The mRNA expression of Bax, Bcl2, and P53 genes in four groups was quantified using the ABI 7500 Sequence Detector (Applied Biosystems, UK) according to the manufacturer's instructions.

Ethical consideration

All actions on these animals were accepted by the Medical Ethics Committee of Tarbiat Modares University. The animals were housed under a 12 hr light/dark cycle in a room with controlled temperature (23±2^{o}C) and free access to food and water. All research and animal maintenance procedures were done according to international guidelines on the use of laboratory animals. It was attempted to minimize the number of animals and their suffering. First, the isolation, culture and identification of cells were done, then the SSCs (300,000 cell per plate of 6 well plate) were treated with 30 µM dose of H_2O_2 for 24 hr and then 10 µg/ml dose of the Calligonum extract for 3 wk.

The culture, evaluation of the viability of SSCs was done according to a previous study (18). Furthermore, apoptosis and the expression of Bax, Bcl2, and P53 genes were evaluated in all the experimental groups.

Statistical analysis

The data of all stages of this study was calculated by mean±standard error. Each data point represents the average of three repeats of each experiment. Using SPSS software (Statistical Package for the Social Sciences, version 20.0, SPSS Inc, Chicago, Illinois, USA), one-way ANOVA and Tukey post hoc tests were applied to determine the statistical significance of observed differences in the mean values among the groups. A $p<0.05$ was considered statistically significant.

Results

Isolation and culture of SSCs

After culture and identification of spermatogonial stem cells, the colonies were recognized in different groups after four days. They were relatively rounded with a regular border. Sertoli cells (fibroblast-like cells) were distinguished as a supportive layer in the bottom of spermatogonial stem cells (Figure 1).

Viability assessment of SSCs after 3 wk of treatment

Results indicated the lowest level of viability (54.93±2.35%) in the group treated with H_2O_2 and the highest viability (95.86±0.29%) in the control group. There was a significant difference the between two groups ($p=0.001$). The survival rate in the

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group treated with H_2O_2+Calligonum extract (71.13 ±1.05%) was higher than the group treated with H_2O_2 alone and was also lower than the group treated with the Calligonum extract alone (91.6±0.45%). There was no significant difference between control and the Calligonum extract groups (Figure 2). So, it can be concluded that the antioxidant properties of plant extract increase the survival rate of cells under oxidative stress.

Evaluation of apoptosis in SSCs after 3 wk of treatment

The results of this study showed that the highest rate of apoptosis was in the group treated with H_2O_2 (47.6±0.17) and the lowest in the control group (1.18±0.103). There was a significant difference between the two groups (p=0.001). Apoptosis rate in the group treated with H_2O_2+Calligonum (13.15±0.08) was lower than the group treated with H_2O_2 alone and was higher than the group treated with the Calligonum extract (2.01±0.51) (Figure 3).

Molecular studies

Assessment of quantitative Bax gene expression (as an apoptotic gene)

The lowest level of expression of this gene was seen in the control group and the highest in the group treated with H_2O_2. Bax gene expression was lower in the group treated with H_2O_2+Calligonum extract than the group treated with H_2O_2 alone.

Assessment of quantitative P53 gene expression (as an apoptotic gene)

The highest expression level of this gene was in the group treated with H_2O_2 and the lowest in the control group, which showed a significant difference (p=0.001). P53 expression was lower in the group treated with H_2O2+Calligonum extract than the group treated with H_2O_2 alone.

Assessment of quantitative Bcl-2 gene expression (as an anti-apoptotic gene)

The lowest level of Bcl-2 expression was observed in the group treated with H_2O_2 and highest in the control group, which had a significant difference (p=0.001). Bcl-2 expression was lower in the group treated with H_2O_2+Calligonum extract than the control group, while it was higher than the group treated with H_2O_2 alone. According to the results of genetic studies, we can say that the addition of H_2O_2 to SSC in vitro causes increased apoptosis.

The Calligonum extract with its antioxidant properties increased the expression of the Bcl-2 anti-apoptotic gene and decreased the expression of P53 and Bax apoptotic genes, so it could suppress apoptosis (Figure 4).

The ratio of Bax/Bcl-2 expression indicates the apoptosis rate in each group. This ratio was 23.21, 0.2 and 3.91 in the group treated with H_2O_2, the groups treated with the Calligonum extract and the group treated with H_2O_2+Calligonum extract, respectively. Bax and Bcl-2 are apoptotic and anti-apoptotic genes, respectively. Apoptosis in the groups treated with the Calligonum was significantly reduced (p=0.001) in comparison to the group treated with H_2O_2.

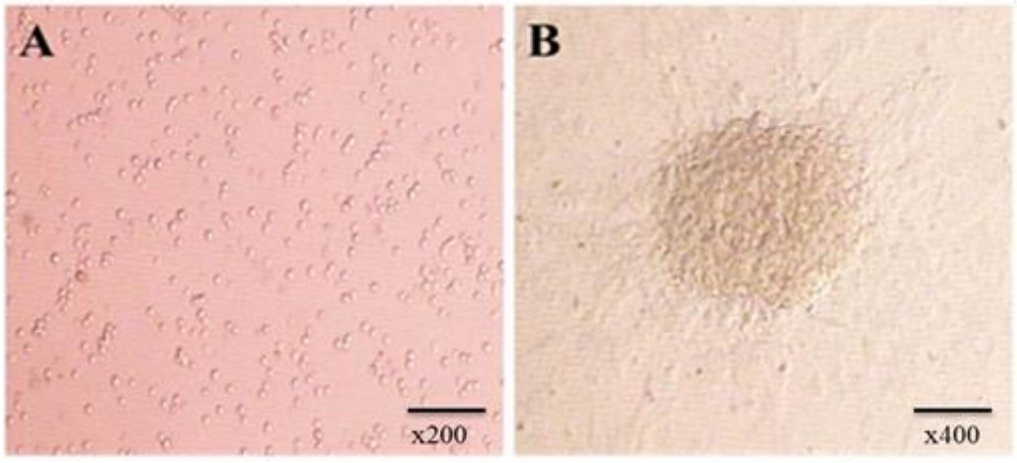

Figure 1. SSCs isolation and cultivation.
A: Morphology of SSCs in the first day of culture. B: SSCs colony in the sixth day of culture.

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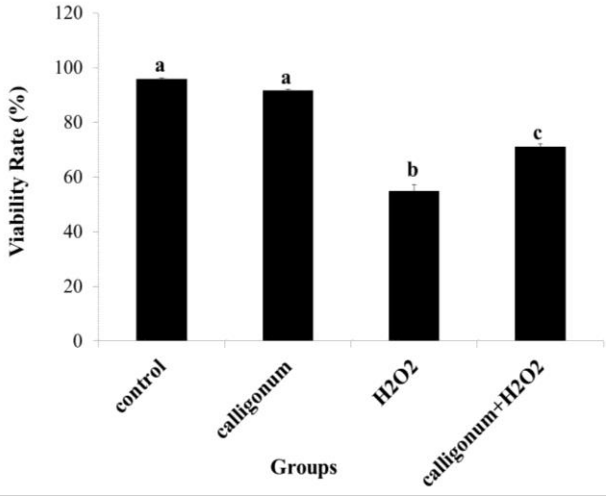

Figure 2. Assessment of viability rate of SSCs in four experimental groups after 3 wk of treatment. Data include means±SE, n=3; a, b and c had significant differences (p=0.001).

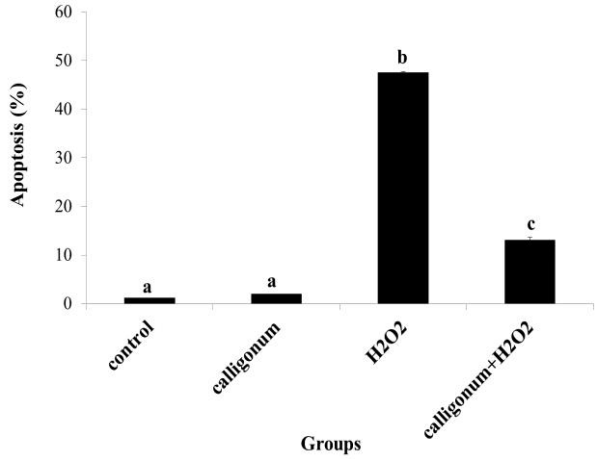

Figure 3. Assessment of SSCs apoptosis in the four experimental groups after 3 wk of treatment. Data are means±SE, n=3; a, b and c had a significant difference (p=0.001).

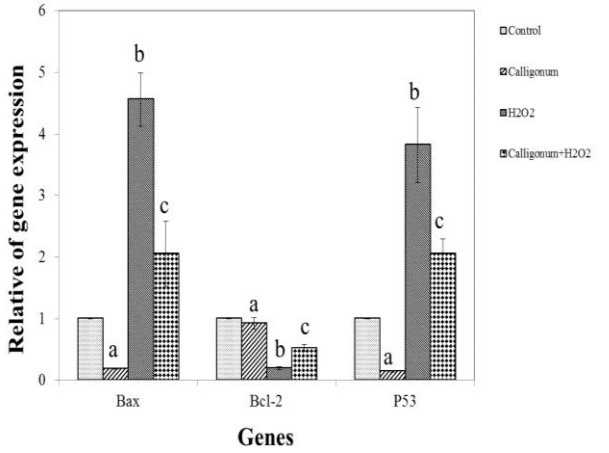

Figure 4. Relative gene expression in SSCs in the four experimental groups after 3 wk of treatment. Data are means±SE, n=3; a, b and c had a significant difference (p=0.001).

Discussion

Spermatogenesis is the procedure of proliferation and differentiation of germ cells, which leads to the creation of a limitless number of spermatozoa (20-22). For this reason, optimal culture conditions can be important and affect the viability and proliferation of SSCs. In this study, SSCs were isolated and cultured. Raph-Brinster and colleagues in 1994 for the first time isolated and cultured SSCs and transferred them into a mouse model of azoospermia (23). According to a previous study, 30 µM dose of H_2O_2 was selected as the optimal dose that could induce reactive oxygen species (ROS) and cause the lowest toxicity in SSCs culture (18). The induced model can be utilized by researchers who study the effect of drugs on the decrease of oxidative stress on SSC in vitro. The aim of this part of the study was the assessment of effects of a 10 µg/ml dose of the Calligonum extract on apoptotic effects of 30 µM dose of H_2O_2.

After 3 wk of treatment, viability in the group treated with the Calligonum and the control group was higher than the groups treated with H_2O_2, which showed that the addition of the Calligonum extract to culture medium with oxidative stress resulted in increased viability. Zhou and colleagues in 2006 showed that antioxidant properties of vitamin E reduced the devastating effects of formaldehyde on viability and proliferation of all cells in the testis (24).

The study of apoptosis on SSC after 3 wk of treatment showed that cell death in the cells treated with H_2O_2 was significantly higher than the other groups. So, the addition of a 30 µM dose of H_2O_2 to SSC culture led to cell death. Free radicals are an important factor of cell death in necrotic and apoptotic processes (25). Jahromi *et al* assessed the effects of the Calligonum extract on sperm parameters and the rate of apoptosis in aged male mice testis. They have shown the Calligonum extract (30 mg/kg) can improve sperm parameters and decrease apoptosis in the testes of aging male mice (14). A number of agents can cause harmful effects through the production of free radicals or depletion of antioxidant defense and oxidative stress (26).

So, it can be stated that H_2O_2 created intracellular ROS and thus disrupted the balance between oxidants and antioxidants,

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increasing cell death and apoptosis (27). Lai and colleagues in 2011 studied the effect of propofol on oxidative stress and apoptosis induced by doxorubicin on cardiac cells of the rat. They found that propofol decreases apoptosis, inhibiting P53 signaling and toxicity of doxorubicin in cardiac cells (28). H_2O_2 is among the factors responsible for the increase in ROS, activating the expression of BAX and P53 but inhibiting Bcl-2 genes (7).

In this study, the effects of the Calligonum extract were assessed on the expression of genes involved in apoptosis after the induction of ROS. P53 and Bax are apoptotic genes but Bcl-2 is an anti-apoptotic gene (29). The expression of P53 and Bax genes in the group treated with H_2O_2 was highest compared to the other groups. Therefore, the addition of H_2O_2 to SSC in vitro increases apoptosis, although the results showed that the addition of the Calligonum as an antioxidant to induced ROS culture causes to reduced apoptosis. Based on the obtained results from Bcl-2 gene expression in the four groups, gene expression was significantly lower in the group treated with H_2O_2 than the other groups.

Bcl-2 expression was significantly declined in the group treated with Calligonum compared to the control group. It is an anti-apoptotic gene, so H_2O_2 reduced Bcl-2 expression and enhanced apoptosis. Bcl-2 gene expression in H_2O_2+Calligonum group increased compared to H_2O_2 alone, which indicated that addition of this plant as an antioxidant to SSC culture with oxidative stress caused decreased apoptosis. Shalabi and colleagues assessed the anticancer activity of Aloe vera and Calligonum comosum extracts separately on hepatocellular carcinoma cells. They found that both of extracts have antitumor effects against HepG2 cells. Gene and protein expressions of both p53 and Bcl2 were significantly altered in response to extracts (17).

The ratio of Bax to Bcl-2 gene expression was calculated. It seems that the ratio of Bax to Bcl-2 gene expression is very important in determining the stimulation of apoptosis. This ratio was ratio higher in the group treated with H_2O_2 than the other groups so that apoptosis was higher in this group compared to others groups. Therefore, the addition of Calligonum extract as an antioxidant reduced oxidative stress and apoptosis in SSC culture. Singh in 2007 evaluated the effects of H_2O_2 as a cause of oxidative stress on Hela cells. In their study, apoptosis was made by H_2O_2 and the expression of Bax, P53, and Bcl-2 genes was evaluated. The results of their research showed that addition of H_2O_2 to cell culture medium reasons increased expression of P53 and Bax genes (as apoptotic genes) by 32% and 71%, respectively and reduced Bcl-2 expression (as the anti-apoptotic gene) by 32% (7).

Conclusion

In this study, we tried to assess the protective effects of Calligonum comosum extract on viability, apoptosis, as well as Bax, Bcl-2 and P53 genes expression on SSCs, treated with H_2O_2 in vitro after 3 wk. In the end, we showed that Calligonum with antioxidant properties could reduce apoptotic effects in vitro.

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Conflict of interest

The authors approve that this article content has no conflict of interest.

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