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Research Article

The Antioxidant Effects of *Calligonum* Extract on Oxidative Stress in Spermatogonial Stem Cells Culture

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Abstract

Background: Spermatogenesis is a programmed route for germ cell proliferation and differentiation that can produce abundant numbers of spermatozoa. The antioxidants play a vital role in decreasing oxidative stress production in cells; therefore, the extraction of plants with antioxidant property can prevent cell damage. In the present study, antioxidant effects of *Calligonum* extract on proliferation and colonization rate of spermatogonial cells were assessed.

Methods: After isolation and culturing of spermatogonial stem cells (SSCs) on neonatal mice (4-5 days old) and identification by PLZF and Oct4 markers, the therapeutic effect of *Calligonum comosum* extract on cells treated with H_2O_2 was measured. The cultured cells were divided into four groups: Control, *Calligonum*, H_2O_2 and *Calligonum* + H_2O_2 groups. Induced oxidative stress cells were treated with 10 µg/ml extract for 3 weeks. Reactive oxygen species (ROS) levels were assessed by the flow cytometry, and proliferation and total antioxidant capacity (TAC) were evaluated by cell count and ferric reducing ability of plasma (FRAP) assay, respectively. Also, the apoptosis rate was measured with P53 and Bax genes by the real- time PCR method.

Results: After three-week treatment, ROS level was significantly lower in the *Calligonum* group than in the H_2O_2 group. Antioxidants levels were significantly higher in *Calligonum* group than in the H_2O_2 group ($P \leq 0.05$). There was also a strong inverse relationship between the two groups. Proliferation and colonization rate were significantly higher in *Calligonum* + H_2O_2 group than in H_2O_2 group ($P \leq 0.05$). Finally, the results suggested that P53 and Bax expression decreased in *Calligonum* + H_2O_2 group compared to H_2O_2 group.

Conclusion: The results of present study revealed that 30 µM doses of H_2O_2 increased oxidative stress and apoptosis on the one hand and decreased proliferation of SSCs on the other hand. As a plant with antioxidant effect, *Calligonum* could reduce the level of ROS and apoptosis, and increase proliferation, colonization rate and TAC.

Introduction

Spermatogenesis process is vital for the conduction of genetic data and maintaining sustainable generations.[1] Through the intricate route, spermatogonial stem cells (SSCs) reproduce themselves in order to preserve the reserves of stem cells, and differentiate then into various types of male germ cells.[2] Thus, it is essential to provide suitable culture conditions. A high concentration of oxygen and lack of defensive factors in culture can increase oxidative stress.[3]

Oxidative stress can lead to cellular injury and death, which arises from the oxidation of fundamental cellular ingredients such as DNA, proteins and lipids.[4] Reactive oxygen species (ROS) is produced through numerous interactions, which contain inadequate diminution of O_2, and finally the creation of superoxide ($O_2\bullet$), hydroxyperoxide (H_2O_2) and hydroxyl radicals ($\bullet OH$).[5,6] Balance creation between ROS and enzymatic or non-enzymatic antioxidants are essential for viability and proliferation of stem cells.[7]

Research shows that antioxidants play a vital role in cutting ROS production in cells, and prevent DNA mutation.[8] Antioxidant factors such as herbs can prevent some of these deleterious effects.[9] One of the plants known for its antioxidant properties is *Calligonum comosum*. It belongs to the Polygonaceae family, which is dispersed in tropical areas and develops in deserts.[10] Overall, 12 phenolic acids and 12 flavonoids were observed in the *Calligonum comosum* extract,[11] which chiefly contained flavonols (quercetin) and Flavanols (epicatechin and catechin (+)).[12] The *Calligonum comosum* is often used in traditional medicine to treat several diseases such as polycystic ovary,[13] hepatotoxicities,[14] diabetes[15] and osteoporosis.[16] Because of its antioxidant capacities, *Calligonum* extract is used as anti-ulcer[17] and anti- inflammatory drug.[18]

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However, there is a paucity of studies on the effects of *Calligonum comosum* extract on stem cells. Hence, this paper assesses therapeutic effects of *Calligonum* on SSCs and infertility reduction. The ultimate goal is to answer the question whether *Calligonum comosum* extract can increase proliferation and colonization rate of SSC treated with H_2O_2 along with antioxidant upregulation and ROS reduction in culture.

Materials and Methods

Study design

The neonatal NMRI mice (3-5 days old) were used in this study. The testes and cultured cells were isolated. There were four groups in the study:

- Control group (no treatment).
- *Calligonum* group treated with 10 µg/ml extract for three weeks.
- H_2O_2 group receiving 30 µM H_2O_2.
- *Calligonum* + H_2O_2 group receiving both 30 µM H_2O_2 + 10 µg/ml *Calligonum* extract.

In the first step, SSCs were isolated and cultured. After 24-h culture, the cells were preserved with 30 µM dose of H_2O_2 and then 10 µg/ml dose of *Calligonum* extract for 3 weeks. Proliferation rate and colonization, intracellular ROS of SSCs, total antioxidant capacity (TAC) and Bax and P53 genes expression were estimated in all experimental groups.

Spermotogonial stem cells culture

The testes were separated from 3-5 day-old neonate mice. Firstly, they were washed in PBS (Phosphate Buffer Serum) (Gibco, Germany) and then transferred to the culture medium. The waste tissues were then removed after being washed twice. Then, samples in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Germany) containing 0.5mg/ml of collagenase were separated into minor slices. The suspension was placed in an incubator at 32°C with 5% CO_2 and then mixed every 5 minutes by pipetting. Afterwards, cells were centrifuged at a speed of 1500 rpm at 4° C for 5 min. Then, the medium above the plaque was replaced with PBS and centrifuged 2 times. In the next step, 0.1% trypsin enzyme was added to cells and centrifuged afresh. When begin washed twice, the cell suspension containing SSCs and sertoli cells was added to the plates including DMEM+5% FBS.

Identification of SSCs

The PLZF and Oct4 proteins, as SSCs markers, were identified by immunocytochemistry. In this method, SSC colonies were developed on slides and after being washed with PBS twice, 4% paraformaldehyde was added to them at room temperature for 30 min. In order to improve the penetration of antibodies into cells, 0.2% Triton X-100 (Biomedicals, California, United State) was used for 1 h at room temperature after washing. Then, 10% goat serum was used for 30 min to block non-specific antigens. Afterwards, SSCs were covered overnight at 4°C with a mouse monoclonal anti-PLZF and Oct-4 1%, before being washed with PBS 3 times (each time for 5 min). The secondary antibodies conjugated by FITC (Oct4, Sigma, USA) and Texas Red (PLZF, Sigma, USA) were added to cells for 1 h in the dark at room temperature. Then, slides were washed with PBS twice, and the nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) for 1 min. Finally, slides were assessed by fluorescent microscope (Olympus, Tokyo, Japan) and images were captured.

Calligonum extraction method

For the extraction of *Calligonum*, the fruit of *Calligonum comosum* was collected by a botanist from the Natural Plants Research Center in Kashan at the end of May. Any additional plants or grass were then removed and the fruit was dried in at room temperature without sunlight. The dried fruit was ground into a soft powder. Then, 20 g of fruit powder was poured into a jar followed by the addition of 200 ml of petroleum ether and 200 ml of methanol and the mixture was stirred for 48 h almost every half hour. The extract was poured into another container without the fruit powder and placed in a refrigerator so that the cold can separate the oily and non-oily phases. In the next step, using a Pasteur pipette, the oil phase was completely separated and kept in a separate bottle. Once more, 200 ml of methanol and 200 ml of dichloromethane were added and the mixture was stirred for 48 h approximately every half hour. In the end, the extract was separated. The alcohol in this extract was largely removed using a rotary apparatus.

Evaluation of SSCs colonization

The number and diameter of colonies derived from SSCs was evaluated using an Invert-phase microscope (Zeiss, Germany) equipped with the ocular grid.

Oxidative stress evaluation in SSC culture

In order to measure the produced ROS levels, DCFH-DA (2 '7'-dichlorofluorescein diacetate) and fluorescent probes were used. Produced intracellular ROS oxidized DCFH-DA (Sigma-Germany) and enhanced its fluorescence properties. Firstly, the cells were treated with 30 µM doses of H_2O_2 for 3 h as described in a previous study.[19] After 24 h, cells were removed from the bottom of the culture plate, the cell suspension was centrifuged at a speed of 2000 rpm at 4°C for 5 min. Then, the medium above the plaque was replaced with PBS and centrifuged twice (each time for 3 min) at a speed of 1000 rpm. In the darkness, 20µM of DCFH-DA was added to cells and pipetted. It was then incubated at 37°C for 45 min. After 10-min incubation in the dark, 900 microliter of PBS was added and centrifuged at 2500 rpm at 4°C for 5 min. They were finally analysed using the flow cytometry (BD Biosciences, America).

Measurement of TAC by FRAP assay

At present, there are various methods used to measure the antioxidant capacity. FRAP method, developed by Benzie

and Strain in 1996, is a highly sensitive and accurate technique.[20] In the FRAP method, the water soluble antioxidants in the sample could reduce TpTz-Fe^{3+} to TpTz-Fe^{2+}, which turns into blue in the acidic environment. Standard solutions were prepared in 125, 250, 500, 1000 µM concentrations. Afterwards, 1.5 ml FRAP solution was added to 50 µl of sample and vortexed completely. They were then incubated at 37 °C for 10 min and the absorbance of all samples was read in 593 nm in front of the blank (zero concentration standard). The FRAP of samples was calculated based on the standard curve.

Real-Time PCR

After three weeks of SSCs culture and Calligonum extract treatment, RNA was extracted by Trizole. In the next step, cDNA synthesis was performed with cDNA synthesis kit (Fermentase, Lithuania) for all samples and 100 ng of cDNA was enhanced by Power SYBR Green Master mix (Applied Biosystems, California, USA). All of the samples were evaluated in triplicate and analyzed by the StepOne Software (Thermo Fisher Scientific). The expression of Bax and P53 genes was compared to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as the reference gene.

Statistical analysis of data

The study data was analyzed by SPSS software (IBM SPSS statistics 20). The one-way ANOVA and Tukey post hoc tests were used to calculate the statistical significance in the groups. Data is expressed as mean ± standard error of mean (SEM). Each data indicates the average of three repetitions of each experiment. A P-value<0.05 was considered statistically significant.

Results

Culture and identification of SSC

The colonies of SSCs were identified in different groups after 4-day SSCs culture. They were relatively rounded with a regular border. The spindle-shaped Sertoli cells are illustrated as a supportive layer in the bottom of SSCs (Figure 1). These colonies were identified by Oct-4 and PLZF proteins as specific markers after one week (Figure 2).

Evaluation of SSCs proliferation rate after three-week treatment

The highest (4.76 ± 0.05) and lowest (2.99 ± 0.11) proliferation rates were detected in the *Calligonum* and H_2O_2 groups, respectively. There was no significant difference between control and *Calligonum* groups. The proliferation rate increased in *Calligonum* + H_2O_2 group (3.83 ± 0.05) compared to the H_2O_2 group but it was lower than the control (4.36 ± 0.07) and *Calligonum* extract groups (Figure 3). According to the findings, the antioxidant effects of extract increased the *in vitro* proliferation of SSCs.

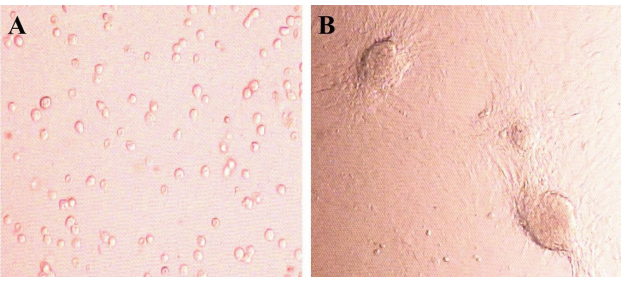

Figure 1. Morphology of SSCs after culture. **A:** Spermotogonial stem cells in the first day of culture (×200) **B:** SSCs colonies in the sixth day of culture (×400).

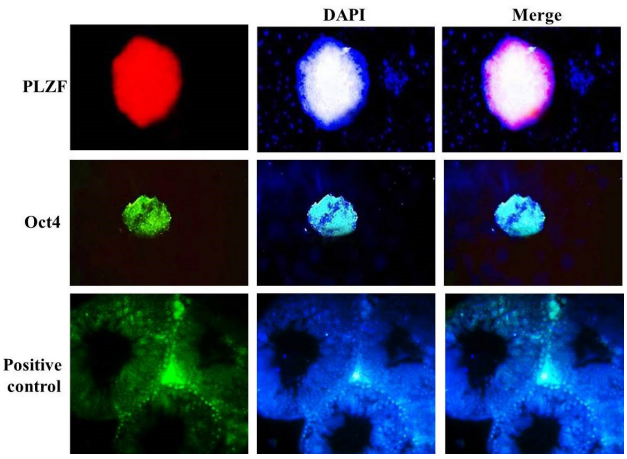

Figure 2. Identification of SSCs colonies after the first week of culture. The upper row shows the immunofluorescent image of the PLZF positive cells in the colony (red), nuclei (blue) and their combination. The middle row shows immunofluorescent image of the Oct4 positive cells in the colony (green), nuclei (blue) and their combination (×200). The lower row shows Oct4 positive cells in the testis tissue as positive control, nucleus staining by DAPI and the merged image (×400).

Assessment of SSC colonization after three-week treatment

According to the study data, the lowest and highest colony number and diameter were observed in H_2O_2 [(14.12 ± 0.83), (23.67 ± 0.33mm)] and *Calligonum* [(27.87 ± 0.87), (45.89±0.31mm)] groups, respectively. The number and diameter of colonies in the group treated with *Calligonum*

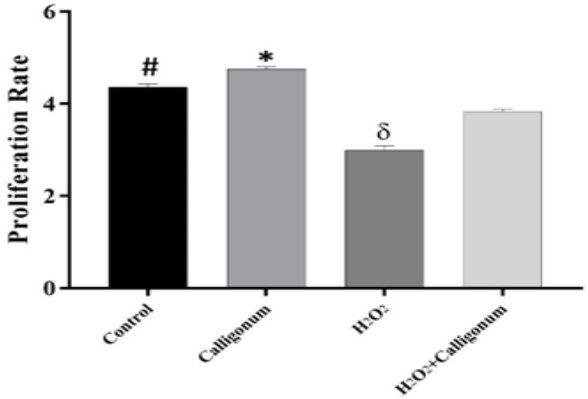

Figure 3. Assessment of SSCs proliferation rate in all groups after three-week treatment. According to the results, the proliferation rate decreased in H_2O_2 group but it was raised by the *Calligonum* treatment. Data are expressed as the mean±SEM (n=3). The significance is indicated by P≤0.05 as opposed to the H_2O_2 and H_2O_2+Calligonum groups. *P≤0.05 vs. H_2O_2 and H_2O_2+Calligonum. δP≤0.05 vs. H_2O_2+*Calligonum*.

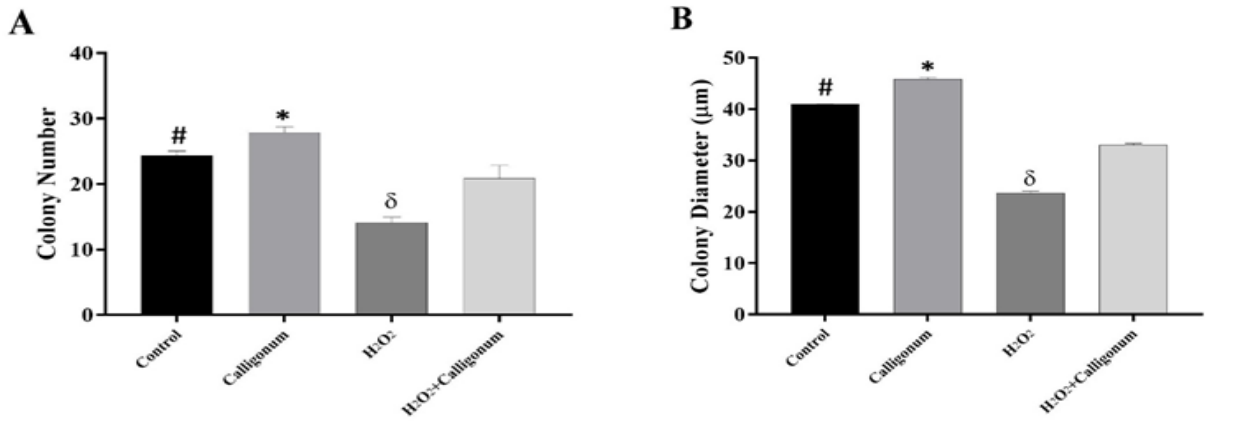

Figure 4. Evaluation of SSCs colonization in all groups after three-week treatment. The results showed that **(A)** colony number and **(B)** colony diameter decreased in H_2O_2 group but *Calligonum* treatment could increase colonization. Data are expressed as the mean±SEM (n=3). The significance is indicated by P≤0.05 compared with H_2O_2 and H_2O_2+*Calligonum* groups. *P≤0.05 vs. H_2O_2 and H_2O_2+*Calligonum*. δP≤0.05 vs. H_2O_2+*Calligonum*.

+ H_2O_2 [(20.87 ± 1.98), (33.03 ± 0.33mm)] was higher than the group treated with H_2O_2 alone, but it was lower than the control group [(24.37 ± 0.67), (41 ±0.02 mm). Therefore, plant extract increased the number and diameter of colonies after 3 weeks (Figure 4A, B).

Assessment of intracellular ROS levels in SSCs after three-week treatment

The highest ROS level was observed in H_2O_2 group (21.37±1.67) and the lowest in the control group (1.87±0.3). A significant difference was observed between the two groups (P≤0.05). ROS level in *Calligonum* + H_2O_2 group (10.43±0.76) was lower than the H_2O_2 group and higher than the *Calligonum* group (2.71±0.37) (Figure 5). Hence, the antioxidant activities of the extract decreased the oxidative stress induced by H_2O_2.

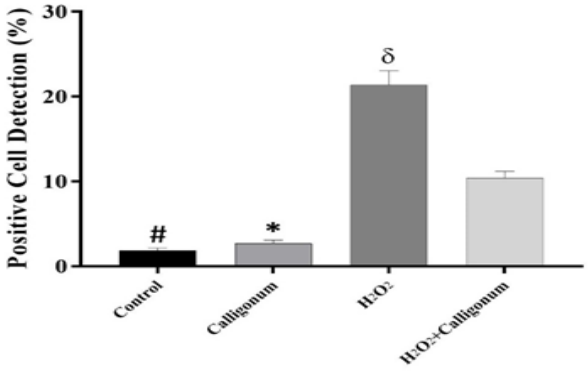

Figure 5. Evaluation of intracellular ROS of SSCs in all groups after three-week treatment. The results suggested that the intracellular ROS upregulated H_2O_2 group but *Calligonum* extract reduced ROS. Data are expressed as the mean±SEM, n=3. The significance is indicated by P≤0.05 compared with H_2O_2 and H_2O_2+Calligonum groups. *P≤0.05 vs. H_2O_2 and H_2O_2+*Calligonum*. δP≤0.05 vs. H_2O_2+*Calligonum*.

Assessment of the effect of Calligonum extract on oxidant and total antioxidant capacity in groups after three-week treatment

According to the data, the lowest concentration of antioxidants was observed in the group treated with H_2O_2 (52.63 ± 1.31) and the highest in the *Calligonum* extract group (163.75±2.34). The TAC in all four groups displayed a significant difference (P≤0.05) (Figure 6). Based on the results, TAC in *Calligonum* + H_2O_2 group (110.5 ± 1.84) was significantly higher than the control group (98.95 ± 2.18) and H_2O_2 group. The results of correlation test between the mean concentration of antioxidant and ROS average in the four experimental groups are shown in Table 1.

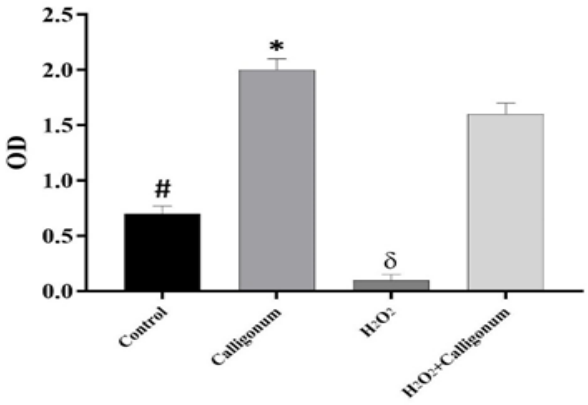

Figure 6. Assessment of TAC in SSCs culture after three weeks of *Calligonum* treatment. The results of TAC measurement revealed that *Calligonum* extract treatment increased antioxidant levels. Data are expressed as the mean±SEM, n=3. The significance is shown by P≤0.05 compared to the three other groups. *P≤0.05 vs. H_2O_2 and H_2O_2+*Calligonum*. δP≤0.05 vs. H_2O_2+*Calligonum*.

Assessment of P53 and Bax genes expression

According to the results of data analysis, the lowest and highest expression levels of Bax and P53 genes were observed in the control and H_2O_2 groups, respectively (P≤0.05). The gene expression decreased in the *Calligonum* group + H_2O_2 compared to the H_2O_2 group. The results showed that *Calligonum* extract could reduce apoptosis genes (Figure 7 A,B).

Table 1. Assessment of oxidants, antioxidants and their correlation. Data are expressed as the means ± SE (n=3). There is a significant difference between a, b, c and d (P≤0.05).

Group	ROS	Mean concentration of antioxidant	Correlation between ROS and TAC
Control	$1.8^{a} \pm 0.3$	$98.95^{a} \pm 2.18$	-0.433 (Weak inverse relationship)
***Calligonum* 10 µg/ml**	$2.7^{a} \pm 0.37$	$163.75^{b} \pm 2.34$	-0.808 (Strong inverse relationship)
H_2O_2 30 µM	$21.37^{b} \pm 1.67$	$52.63^{c} \pm 1.31$	-0.865 (Strong inverse relationship)
H_2O_2 30 µM + *Calligonum* 10 µg/ml	$10.43^{c} \pm 0.76$	$110.5^{d} \pm 1.84$	-0.554 (Mean inverse relationship)

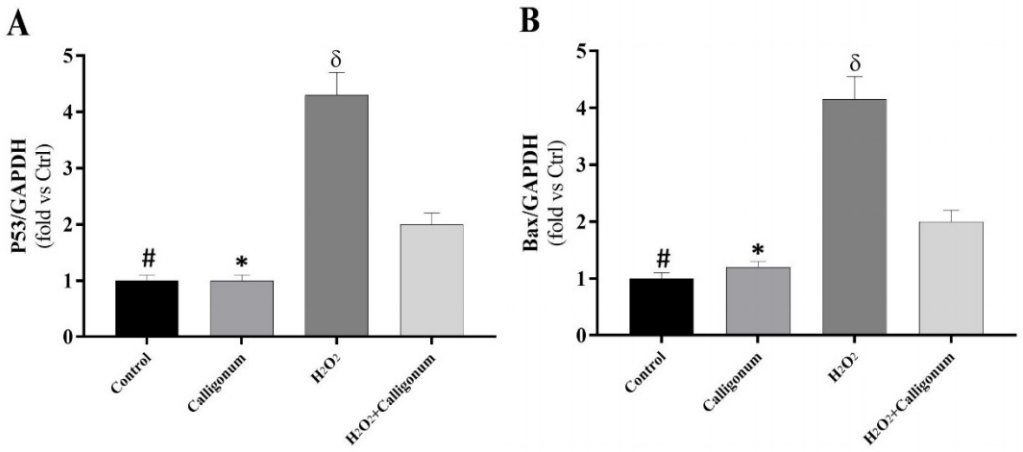

Figure 7. Assessment of apoptotic gene expression in SSCs after three-week *Calligonum* treatment. The results of qRT-PCR using specific primers for **(A)** P53 and **(B)** Bax, as apoptosis genes, revealed that H_2O_2 application increased apoptosis rate but it was raised by *Calligonum* extract treatment. Data are expressed as the mean±SEM (n=3). The significance is indicated by P≤0.05 compared to H_2O_2 and H_2O_2+Calligonum. *P≤0.05 vs. H_2O_2 and H_2O_2+Calligonum. δP≤0.05 vs. H_2O_2+Calligonum. GAPDH was used as an internal control for normalization.

Discussion

The SSCs are the basis for spermatogenesis during the reproductive life of testis.[21,22] The SSCs are capable of self-renewal or differentiation.[23] Therefore, ideal environments are essential for their culture and influence the proliferation and colonization of SSCs. As described in a previous study, SSCs were cultured and then 30 µM dose of H_2O_2 was added for ROS induction in the SSC culture for the assessment of antioxidant effects.[19] The objective of this study was to evaluate the effect of 10 µg/ml dose of *Calligonum* extract (as an antioxidant) on proliferation and colonization rate of the oxidative stress induced SSCs by 30 µM dose of H_2O_2. The results of proliferation and colonization after three-week treatment showed that the diameter and number of colonies were lower in the H_2O_2 groups than in the *Calligonum* and control groups. The results were aligned with those reported by Kang *et al.*[24] They assessed the impact of several plants on the maintenance of male fertility, they showed that plant extracts can enhance the proliferation of SSCs by a serum-free culture method. In this study, we assessed apoptotic genes such as P53 and Bax. The data showed that *Calligonum* treatment decreased the apoptotic rate.

The results showed that H_2O_2, acting as s a stress inducer, reduced proliferation and colonization accompany. Therefore, it can be argued that adding *Calligonum* extract to oxidative stress in the culture medium increased proliferation and colonization. In line with our study, Ha *et al.*[25] evaluated the effect of apoptosis inhibitors and antioxidants on SSCs, reporting that the presence of hypotaurine in the basal freezing media significantly improved the proliferation and mitochondrial activity of germ cells. Kiani *et al.*[26] investigated the effects of *Calligonum comosum* on the development of endometriotic lesions. They found that *Calligonum comosum* extract had a substantial anti-angiogenic effect. Also, *Calligonum* significantly inhibited the growth and cyst formation of developing murine endometriotic lesions. This was associated with reduced vascularization, cell proliferation and immune cell infiltration. Their results suggested that *Calligonum comosum* had a bearing on the pathogenesis of endometriosis, which may contribute to the treatment of this common gynecological disorder.

The analysis of flowcytometric results for oxidative stress after three-week treatment showed that the intracellular ROS increased significantly in the H_2O_2 group compared to the other groups. These results suggest that the addition of the *Calligonum* extract to oxidative stressed culture medium reduced ROS level based on antioxidant properties of the plant. Research shows that antioxidants in green tea can significantly reduce the lipid peroxidation of cell membranes caused by cellular stress – which is induced by cadmium.[27] The protecting effect of quercetin on oxidative stress injury was studied by Yuling Mi Caiqiao Zhang *et al.*[28] through the stimulation of Aroclor 1254 on SSC. The destructive effects of Aroclor 1254 may stimulate

an increase in TBARS as an indicator of lipid peroxidation, a decrease in SOD activity as a cleaner superoxide and a reduction in intracellular GSH as an antioxidant. Quercetin, a flavonoid with antioxidant property, can mitigate the effects of oxidative stress on macromolecules such as DNA and lipids.

In the present study, for the evaluation of apoptosis rate, P53 and Bax as apoptotic genes, were assessed using the qRT-PCR method. According to the results, the apoptosis rate increased in H_2O_2 group and *Calligonum* administration for three weeks could downregulate apoptotic genes, which demonstrates apoptosis rate reduction. Alehaideb *et al.*[29] assessed the anti-proliferative and pro-apoptotic effects of *Calligonum comosum* extract on breast cancer cells. They found that the *Calligonum* inhibited the viability of breast cancer cells with low cytotoxic effects. In conclusion, they observed that *Calligonum* exerts anti-proliferative effects against breast cancer cells through the induction of apoptosis and cell growth, which involves the activation of the mitochondrial-dependent apoptotic pathway.[29]

The evaluation of TAC by FRAP test after three weeks of treatment showed that TAC surged significantly in the *Calligonum* group compared to the other three groups, which is indicative of antioxidant properties of *Calligonum* extract and consistent with other studies.[13] Dhief *et al.*[30] compared secondary metabolites in three species of *Calligonum* (C. arich, C. comosum C. azel) grown in deserts of Tunisia. They identified 110 different chemical compositions in the oil of these plants, some of which have antioxidant properties.

Tahmasebi *et al.*[13] studied antioxidant effects of *Calligonum* extract on ovarian tissues in the PCO model. They observed that estradiol valerate was able to increase oxidative stress within the ovary and cause ovarian cysts after two months. Also, the cyst formation decreased in *Calligonum* group compared to the PCO group. The percentage of pre-antral and antral follicles decreased significantly in the *Calligonum* group compared to PCO group. The oxidative stress dropped significantly in *Calligonum* group compared to PCO group. Moreover, *Calligonum* could significantly increase the TAC of ovarian tissues as well as the percentage of in vitro fertilization compared to the PCO group.

The relationship between oxidative stress and antioxidant was analyzed using the correlation test. In living organisms, there is a balance between the level of oxidants and antioxidants in cells. We observed a weak inverse relationship between oxidants and antioxidants in the control group. A strong inverse relationship was also found in H_2O_2 group, meaning that increased oxidants caused a sharp decline in antioxidants. In the *Calligonum* group, a strong inverse relationship was noted between oxidants and antioxidants. In *Calligonum* + H_2O_2 group, a moderate inverse relationship was documented. However, these changes did not show any significant differences.

In sum, the results of the present study suggest that *Calligonum* extract treatment could increase colonization and proliferation rate of SSCs in culture due to its antioxidant property. Also, *Calligonum* extract administration for three weeks decreases oxidative stress and apoptosis of SSCs. Hence, it could be used as an approach to the infertility improvement.

Conclusion

The present study assessed the antioxidant effect of *Calligonum* extract on proliferation and colonization rate in inducing oxidative stress in SSCs treated with H_2O_2 in culture for three weeks. Finally, these results showed that *Calligonum*, as an antioxidant, can decrease ROS and apoptosis of SSCs and increase *in vitro* proliferation and colonization rate of cells.

Ethical Issues

This experimental study was conducted in accordance with ethics code (52/6707) set by the Medical Ethics Committee of Tarbiat Modares University. The research procedure was in compliance with international guidelines on laboratory animals.

Author Contributions

Shirin Barati carried out the experiment, performed the analytic calculations and wrote the manuscript. Mansoureh Movahedin conceived the original idea and supervised the project. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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