Establishment of Oxidative Stress Modeling during Spermatogonial Stem Cells Cultivation Treated with Different Doses of H_2O_2

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ABSTRACT

Introduction: Nowadays, spermatogonial stem cells (SSCs) cultivation has been used by many researchers as an effective tool for infertility treatments. Oxidative conditions can be effective on cell proliferation and differentiation of these cells. So, the aim of this study was to establish oxidative stress model for antioxidant activity of some drugs investigation during SSCs in vitro culture.

Methods: Neonatal NMRI male mice (3-5 day) were used for isolation of SSCs. The cell suspension was prepared by twice enzymatic digestion. The cell suspension contents were spermatogonial and sertoli cells and treated by different doses of H_2O_2 logarithmic concentrations from 0-100 µM after 24 hours. To access the optimal dose, extra doses from 10-100µM was evaluated. After 2 hours of H_2O_2 treatment, viability was determined by Trypan blue assay. The data were analyzed using SPSS software and One-way ANOVA test.

Results: Our data showed that spermatogonial stem cells colonies appeared after 4 days of isolation. These cells expressed OCT4 and PLZF proteins. Many of spermatogonial stem cells were removed after using higher doses of H_2O_2. The results showed that 30 µM concentration of H_2O_2 could induce oxidative stress in spermatogonial stem cell during in vitro culture.

Conclusion: According to this study, 30 µM concentration of H_2O_2 can cause cell death lower than 50% of total number of cells and can increase oxidative stress in cultivation of SSCs. This model is a suitable tool for studying some new antioxidant drugs.

1. Introduction

Studies on spermatogonial stem cell (SSC) are very complicated because few studies have been carried out in this regard [1]. SSCs provide a valuable source of germ cells that transfer genetic information from one generation to the next. As a result it was considered as a valuable source for biological and medical research [2, 3].

Isolation, proliferation and differentiation of cells in culture is very important. Factors such as nutritional and

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hormonal balance, heat and oxidative stress effect on germ cells in in vitro. Also, the concentration of oxygen in culture media is high, thus production of oxygen free radicals increases in the culture conditions compared to the body [4]. Moreover, there is no defense factor against these factors in *in vitro*.

The germ cells are constantly influenced by factors such as ROS (reactive oxygen specious) that lead to DNA damage and cells structures [5].

Free radicals including superoxide anions (O_2), hydroxyl radical (OH) and non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2) are highly unstable and interact quickly and nonspecifically with biological molecules. They produce and develop a variety of injuries including peroxidation of cell membranes, oxidation of amino acids and nucleic acids, apoptosis and necrosis, which leads to a decrease in viability and growth of cells in the laboratory [6, 7].

It is possible for H_2O_2 to create ROS insensitive SSC, primary spermatid, spermatozoa and also interstitial cells and sertoli cells, it could be caused serious damage and loss of these cells. The aim of this study was to establish oxidative stress model in *in vitro*. This model is suggested as a suitable tool for studying some new antioxidant drugs.

2. Materials & Methods

Isolation and culture of SSC

The testes were removed from 3 to 5 days male mice and washed in PBS (Phosphate Buffer Serum) and transferred to DMEM (Dulbecco's Modified Eagle Medium) culture medium. After twice washing, capsule and epididymis of the testes were removed. Then samples in DMEM containing enzyme (0.5mg/ml of collagenase) were divided into smaller parts. The mixture of cells and enzymes was put inside incubator 32°C and 5% CO_2; they were mixed every 5 minutes by pipetting. Then cells were centrifuged for 5 min with 1500 rpm speed at 4°C.

Then the above medium on plaque was exchanged with PBS and centrifuged 2 times and each time 3 min with 1000 rpm speed. Then 0.1% trypsin enzyme was added to cells, centrifuged for 5 min with 1500 rpm speed at 4°C. After twice washing, cell suspension that included sertoli cells and spermatogonial cells was divided into plates containing DMEM+5% FBS [8-10]. All materials used in the cell culture were obtained from the Gibco company in Germany.

Identification of SSC

PLZF and Oct4 protein markers for SSCs were detected in these cells by immunocytochemistry test, according previous study [11]. In this method, SSC colonies were grown on glass slides then washed twice with PBS for 5 minutes and cells were in 4% paraformaldehyde at room temperature for 20 minute. After washing twice in PBS for 5 minutes 0.2% Triton X-100 (MP Biomedicals, Irvine, California, and United State) was used for 1 hour to facilitate antibodies penetration into cells. 10% goat serum was used for 30 minutes in order to block non specific antigen. SSCs were stored overnight at 4°C with a mouse monoclonal anti-PLZF and Oct4 1%, then they were washed 3 times with PBS and each time for 5 minutes. Conjugated anti-mouse secondary antibody with Texas Red (PLZF) and FITC (Oct4) [Sigma, USA] 1% was added to cells for 2 hours in the dark at room temperature. Then they were washed three times with PBS for 5 minutes, the nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole) for 1 minute. Samples were cemented with glycerol phosphate.

At this stage, the cells were treated with 0, 1, 10, 30, 50, 70 and 100 µM doses of H_2O_2 for 3 hours. After 24 hours, viability, proliferation and ROS level were evaluated and the optimal dose was determined to continue working.

Evaluation of SSC viability

Viability was evaluated with 0.04% trypan blue solution. In this method, living cells maintain the integrity of plasma membrane and prevent from cell penetration, while dead cells were observed to be blue. 103 numbers of cells were cultured in 6 wells plates. After 24 hours cells were treated with 0, 1, 10, 30, 50, 70 and 100µM dose of H_2O_2 for 3 hours. After 24 hours of treatment, cells were separated from the plates. 10µl trypan blue and 10µl of cell suspension were mixed together. Then, cells were counted using a hemocytometer [12].

Evaluation of SSC proliferation rate

After 24 hours treatment of cells with different doses of H_2O_2, cells were separated and counted by a hemocytometer.

ROS induction in SSC culture

For this purpose, different doses (0, 1, 10, 30, 50, 70, 100µM) of H_2O_2 (Invitrogene, UK) were added for 3 hours. Viability, proliferation rate and intracellular ROS of SSC were evaluated after 24 hours, then optimal dose

of H_2O_2 which is not fatal was selected, but it produced mainly ROS.

The oxidative stress measurement in SSC

DCFH-DA was used for measuring the produced ROS levels (2 '7'-dichlorofluorescindiacetate) [13]. In this method, we used fluorescent probes for detection of intracellular ROS. Produced ROS in cells oxidized DCFH-DA (Sigma-Germany) and increased its fluorescence properties that can be used for measurement of the produced hydrogen peroxide. After 24 hours treatment of cells with 0, 1, 10, 30, 50, 70, 100 µM doses of H_2O_2 for 3 hours, the cells were separated and collected from the bottom of the plate, then cell suspension was centrifuged with 2500 rpm at 4°C for 5 minutes, and then above medium on plaque was exchanged with PBS and centrifuged 2 times and each time 3 min with 1000 rpm speed. In the darkness, 20µM of DCF-DA was added to cells and slowly was pipetting. After that it was incubated for 45 min at 37°C. After a period of incubation in the dark, 900 microliter of PBS was added and centrifuged with 2500 rpm at 4°C for 5 minutes, they were analyzed by flow cytometry (BD Biosciences, America).

3. Results

Isolation and culture of SSC

The spermatogonial stem cell suspension was obtained by two enzymatic digestions. Colonies were established in different groups after 4 days. The colonies were relatively rounded and had a certain extent. Sertoli cells (fibroblast-like cells) were detected as a protective layer in the bottom of spermatogonial cells (Figure 1).

Identification of SSCs colony

Oct-4 and PLZF proteins were detected as specific markers in these colonies (Figure 2).

Determining the optimal dose of H_2O_2 for induction intracellular ROS

Assessment of viability of spermatogonial stem cells treated with different doses of H_2O_2

Viability of spermatogonial stem cells was assessed 24 hours after treatment with 0, 1, 10, 30, 50, 70 and 100µM doses of H_2O_2 for 3 hours by trypan blue staining. The result showed that increasing doses of H_2O_2 reduced viability. So the highest survival rate in control group (94.66±0.88) and the lowest survival rate at 100µM dose of H_2O_2 (7.66±1.45) was observed. There was significant difference between two groups ($P \leq 0.05$). Viability maintained about 50 percent at 30µM dose of H_2O_2 (49.66±1.45), so this dose was selected as optimal dose (Figure 3).

Assessment of proliferation rate of spermatogonial stem cells treated with different doses of H_2O_2

Results of this study showed that oxidative stress decreased proliferation rate. There are highest proliferation rate in control group (0.98±0), and the lowest in group treated with 100µM dose of H_2O_2 (0.76±0.1). There was significant difference between two groups ($P \leq 0.05$) (Figure 4).

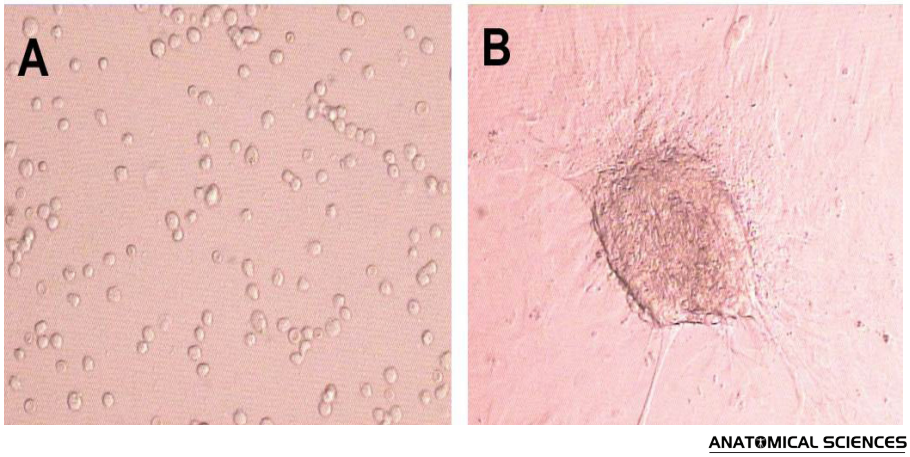

Figure 1. SSCs isolation and cultivation. A: Morphology of SSC in the first day of culture (×200) B: SSC colony in the sixth day of culture (×400).

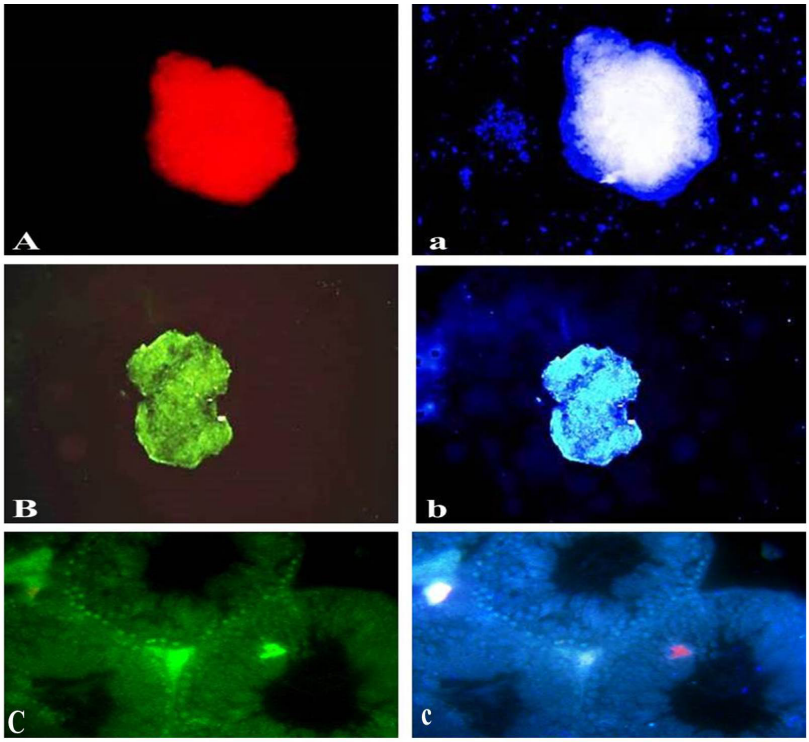

Figure 2. Identification of SSCs colony. A: PLZF, B: Oct4 (×200), C: PLZF in testis tissue as positive control (×400). a, b and c: DAPI staining (nucleus)

Evaluation of intracellular ROS levels in spermatogonial stem cells treated with different doses of H_2O_2

Highest average fluorescent reflectance DCF in group treated with 100µM dose of H_2O_2 (29.59±0.68) and lowest in control group (0.76±0.21) was observed. This increase reflected was indicating an increase of intracellular ROS level. There was significant difference between two groups (P≤0.05) (Figure 5).

4. Discussion

Spermatogenesis is an organized process of proliferation and differentiation germ cells that leads to production of an unlimited number of spermatozoa [14, 15]. This process is done through spermatogonial stem cells which are located on basement membrane of seminiferous tubules. Spermatogenesis was started from one or more small centers and each center is created as a result of a SSC proliferation [16]. For this reason optimal culture conditions can be affecting in survival, proliferation and cell colonization. In this study first step was achievement of an optimal culture system for isolation and proliferation SSC. In this study, SSC were isolated using two enzymatic digestion methods. This method was previously used by other researchers. Raph-Brinster and colleagues in 1994 for the first time isolated cells in *in vitro* and transplanted into Azoospermia model mice [17]. SSC has specific markers such as GFRα1, PLZF [11]. In this study, in order to identification SSC was used Oct-4 and PLZF markers. SSC markers lead to isolation and purification these cells from rest of cells.

For preservation and continuation of the species, it is necessary that germ cell's DNA remain without defect

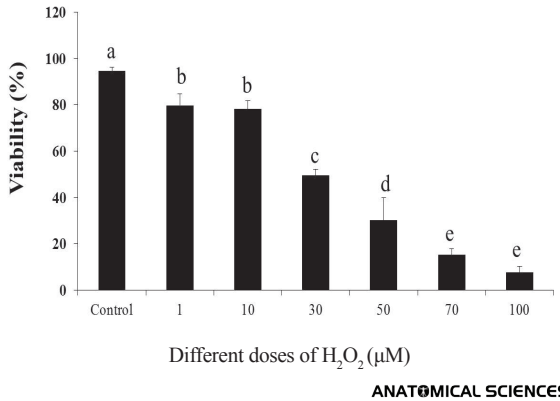

Figure 3. Viability rate assessment of SSCs with different doses of H_2O_2 after 3 hours treatment. Data are means±SE, n=3; a, b, c, d and e significant differences compared to other groups (P≤0.05).

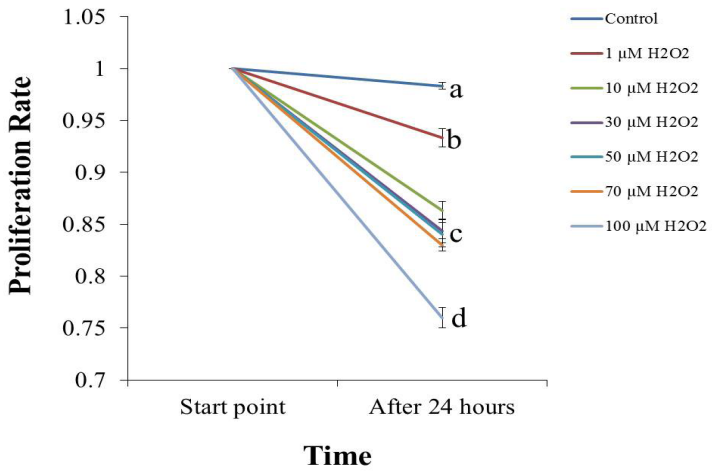

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Figure 4. Proliferation rate assessment of SSCs with different doses of H_2O_2 after 3 hours treatment. Data are means±SE, n=3; a, b, c and d significant differences compared to other groups (P≤0.05).

during proliferation and differentiation. These cells are constantly exposed to damaging agents such as ROS and these factors can lead to DNA and cell membrane damages. ROS, free radicals (such as hydroxyl, superoxide, nitric oxide), atoms or molecules damage to macromolecules into body because of having a free electron constantly circulate in living bodies, and its high reactivity, [5]. There is normally balance between production of free radicals in body and components of antioxidant defense. However, when body is exposed to environmental factors such as drugs and toxins and pollutants, increased productions of free radicals lead to imbalance between production radicals and antioxidant components and make a condition known as oxidative stress which leads to tissue damage [18].

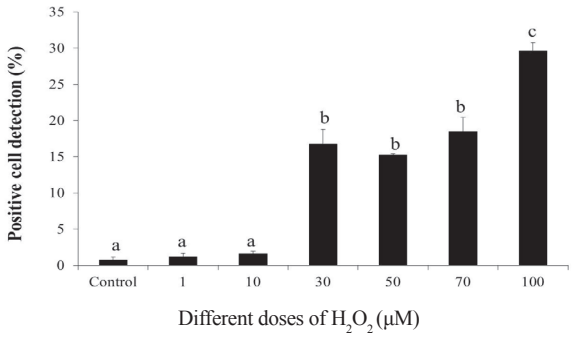

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Figure 5. Intracellular ROS assessment of SSCs with different doses of H_2O_2 after 3 hours treatment. Data are means±SE, n=3; a, b and c significant differences compared to other groups (P≤0.05).

In this study, we used different doses of H_2O_2 and increased intracellular ROS until we could create oxidative stress model in *in vitro* in order to investigate influence of drugs, and antioxidants on behavior of these cells. H_2O_2 is used for this purpose by Agarwal and Habas.

Agarwal et al. in 2009 evaluated the effect of 0 to 15 µM doses of H_2O_2 on sperm motility. They demonstrated that H_2O_2 increased ROS and NO levels and had influence on sperm parameters [19]. In present study, toxic effects of 0, 1, 10, 30, 50, 70 and 100 µM doses of H_2O_2 on SSC was investigated for 3 hours. At the first, it was shown that H_2O_2 as intracellular ROS factor caused oxidative stress, then effects of H_2O_2 on viability and proliferation rate of SSC in culture was studied.

Habas and colleagues in 2014 examined the toxicity effects of H_2O_2 on male reproductive system. They evaluated effects of 1 and 10 µM doses of H_2O_2 on all of male germ cells by the TUNEL method [20]. Increasing dose of H_2O_2 can harm DNA structure of cells and eliminate proliferation and colonization. So viability of SSC was assessed after treatment of these cells with various doses of H_2O_2. The results showed that oxidative stress levels in control group without H_2O_2 was minimal while it had highest viability and proliferation rate.

The highest level of ROS was in group treated with 100 µM dose of H_2O_2 that had the lowest viability and proliferation. Group treated by 30 µM dose of H_2O_2 had comparatively high ROS, but retaining 50% viability.

Collectively, based on obtained results, 30µM dose of H_2O_2, was selected as the optimal dose that with ROS

induction, made the lowest toxicity in SSC cultured. So, the induced model can be used by researchers which study effects of drugs on the improvement of stress on SSC in *in vitro*.

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