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Effect of Incubation with Dimethyl Sulfoxide on the Mitotic Cycle of Cell Culture of Rabbit Dermal Papilla

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Effect of Incubation with Dimethyl Sulfoxide on the Mitotic Cycle of Cell Culture of Rabbit Dermal Papilla

Oksana Novikova*, Galina Bozhok, Tatyana Bondarenko
Department of Cryoendocrinology, Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences, Kharkov, Ukraine.

***Correspondence:** oyunovikova@biolik.com.ua

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Abstract
At cryopreservation, the issue of preserving the integrity and cytogenetic stability of dermal papilla (DP) cells of the hair follicle is topical. The study examined the effect of various concentrations of the cryoprotectant dimethyl sulfoxide (DMSO) (5-15%), as well as its combination with 5% bovine serum albumin (BSA), on the mitotic index (MI) and the number of pathological mitoses in the cell culture of DP on the first and second passages. It has been established that the treatment of cells with DMSO in concentrations of 10% and above leads to a significant increase in the number of pathological mitosis. This is accompanied by the decrease in MI (when processing concentrations of DMSO below 10%) at the first passage or a complete cessation of proliferation at the second passage (when processing concentrations of DMSO above 10%). The combination of 5% DMSO with BSA is optimal for minimizing toxic effects on the genetic apparatus of DP cells and can be used as part of cryopreservation media.

Keywords: Dermal papilla; Cryopreservation; Mutagenesis; Mitotic activity.

1. INTRODUCTION

Dermal papilla (DP) cells of the hair follicle represent a population of pluripotent cells derived from the neural crest. The possibility of cultivation and directed induction of this type of cells in vitro was shown [1], which is promising for use in regenerative medicine.

It is believed that abnormal cell mitosis is a source of malignant tumors and pathologies of human development [2]. Thus, according to modern concepts, a change in the normal course of mitosis and its arrest is one of the options of programmed cell death initiated in mitochondria by a cascade of reactions in response to various stimuli [3, 4]. However, even in the absence of stimulation of mutagenesis, aneuploidy may occur in an in vitro culture, especially with multiple passages of cells [5]. For example, it has been shown that in long-term cultivation, pluripotent stem cells acquire quantitative and qualitative chromosomal aberrations [6], which is a serious limitation for their therapeutic use.

Cryopreservation is a generally accepted way to keep plant or animal cell cultures frozen to avoid multiple passages. The viability and proliferative activity of cell cultures is maintained at a high level after thawing due to the use of special cryoprotectants [7]. Most modern protocols for cryopreservation of mammalian cells use cryoprotective media based on endocellular cryoprotectant dimethyl sulfoxide (DMSO) in concentrations of 5-10% and protein supplements (fetal calf serum or bovine serum albumin) [7]. As DMSO is an agent that has a significant effect on the state of proteins and nucleic acids in a cell, pathological changes can be induced in the division apparatus. Although the possibility of obtaining pluripotent DP cells from cryopreserved follicles has now been shown [8], the changes that occur in these cells under the influence of DMSO are still not well understood.

The purpose of the work is to evaluate the effect of incubation of rabbit dermal papilla cells in media containing different concentrations of DMSO and bovine serum albumin on the mitotic index and the number of pathological mitoses.

2. METHOD(S)

Obtaining a culture of DP cells was carried out according to the method of [9]. When confluent monolayer was reached, the cells were detached from the surface with a mixture of 0.05% trypsin in Versen solution. Next, cells were incubated in solutions with different concentrations of DMSO and placed at a concentration of 4×10^4 cells/cm^3 on coverslips into six-well plates.

Cultivation was performed for 48 h in DMEM/F12 nutrient medium with the addition of 10% fetal calf serum and antibiotic/antimycotic, after which cultures were fixed and cytological preparations were prepared according to the method [10].

For incubation of cells, DMSO was used at concentrations of 5, 7.5, 10, 12.5, and 15%. A number of solutions contained 5% bovine serum albumin. Cells were kept in a cryoprotectant solution for 25 min at room temperature, after which they were washed by centrifugation. Cells that were incubated in a nutrient medium without DMSO and bovine serum albumin (BSA) were used as a control.

Mitotic activity and the presence of pathological divisions were studied on fixed preparations of DP cell cultures of first and second passages using an inverted microscope Leika 2000. The mitotic index (MI) was determined as the ratio of the number of cells in mitosis to the total number of cells counted, and expressed in ppm (‰) [11]. The relative number of cells with mitosis pathology (centromere defects and chromosome misaggregation, multipolar, asymmetric, monocentric mitosis, mitosis with tripod metaphase formation, circular metaphase, chromosome bridges, and lagging chromosomes) was defined as the ratio of the number of cells with mitosis pathology to the total number of dividing cells, and expressed as a percentage.

ANOVA was used to assess the significant differences between samples. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

The study of the mitotic stage showed a relatively equal distribution of cells in the stages of the cell cycle in both control and experimental samples. MI of intact DP cells (control) on the 2nd day was 23 ± 4.30% at the first passage and 41 ± 5.92% at the second passage (see Table 1).

No significant differences in MI in cultures treated with DMSO solo or in the presence of BSA were observed at the first passage (see Table 1). At the second passage, MI decreased by about two times compared to the control ($p < 0.05$) in the samples incubated with DMSO in concentrations from 5 to 10%. In the case of treatment with high concentrations of DMSO (12.5-15%), cessation of cell division was observed. Simultaneously, the addition of BSA to the DMSO solution had a protective effect, as the MI of the cells incubated in the medium with DMSO at concentrations from 5 to 10% did not significantly differ from the control, and the MI of the cells treated with 12.5 and 15% DMSO remained in the range of 25-35%.

The main types of pathological mitosis that occurred in the studied cell cultures are presented in Figure 1.

Cytomorphological analysis of control culture showed an insignificant part of cells with mitosis pathology. At the first passage, this indicator was 4.45 ± 0.49%, and at the second passage 7.14 ± 0.79% (Figure 2). Chromosome bridges and chromosome lag in anaphase were the most common anomalies of mitosis in control.

At the first passage, the number of pathologies of mitosis in cultures treated with DMSO at concentrations from 5 to 10% tended to increase compared with the control (Figure 2). When incubated with DMSO at concentrations of 12.5 and 15%, the level of pathologies of mitosis significantly ($p < 0.05$) increased to 15.0 ± 1.2% and 14.3 ± 1.2%, respectively. K-mitosis prevailed among the pathological forms.

At the second passage, in cultures treated with DMSO at a concentration of 10% and higher, there was a significant increase in the number of pathologies both in comparison with the control and with the first passage (Figure 2). Incubation with higher concentrations (12.5 and 15%) led to the cessation of cell division and degradation of culture; therefore, the data in the figure are not given.

Table 1: MI (%) on the second day of the cultivation of DP cells treated with different concentrations of DMSO and BSA.

Solution	MI (First passage)	MI (Second passage)
Control	23 ± 4.30	41 ± 5.92
5% DMSO	35 ± 3.65	19 ± 2.22[a]
7.5% DMSO	25 ± 4.65	19 ± 3.11[a]
10% DMSO	28 ± 4.5	25 ± 5.35
12.5% DMSO	23 ± 1.26	0[a]
15% DMSO	29 ± 2.89	0[a]
5% DMSO + BSA	20 ± 3.56	25 ± 4.2
7.5% DMSO + BSA	20 ± 3.77	30 ± 3.11
10% DMSO + BSA	19 ± 2.06	36 ± 4.65
12.5% DMSO + BSA	19 ± 3.5	35 ± 3.1
15% DMSO + BSA	26 ± 2.5	25 ± 3.77

[a] The differences are statistically significant compared with the control of the corresponding passage, $p < 0.05$.

Figure 1: Pathologies of mitosis in DP cell culture (first passage, 2 days of growth): a, lagging chromosomes telophase and lagging chromosomes; b, chromosome misaggregation; c, lagging chromosomes in anaphase; d, mitosis with tripod metaphase formation; e, circular metaphase; f, mitosis with tripod anaphase formation.

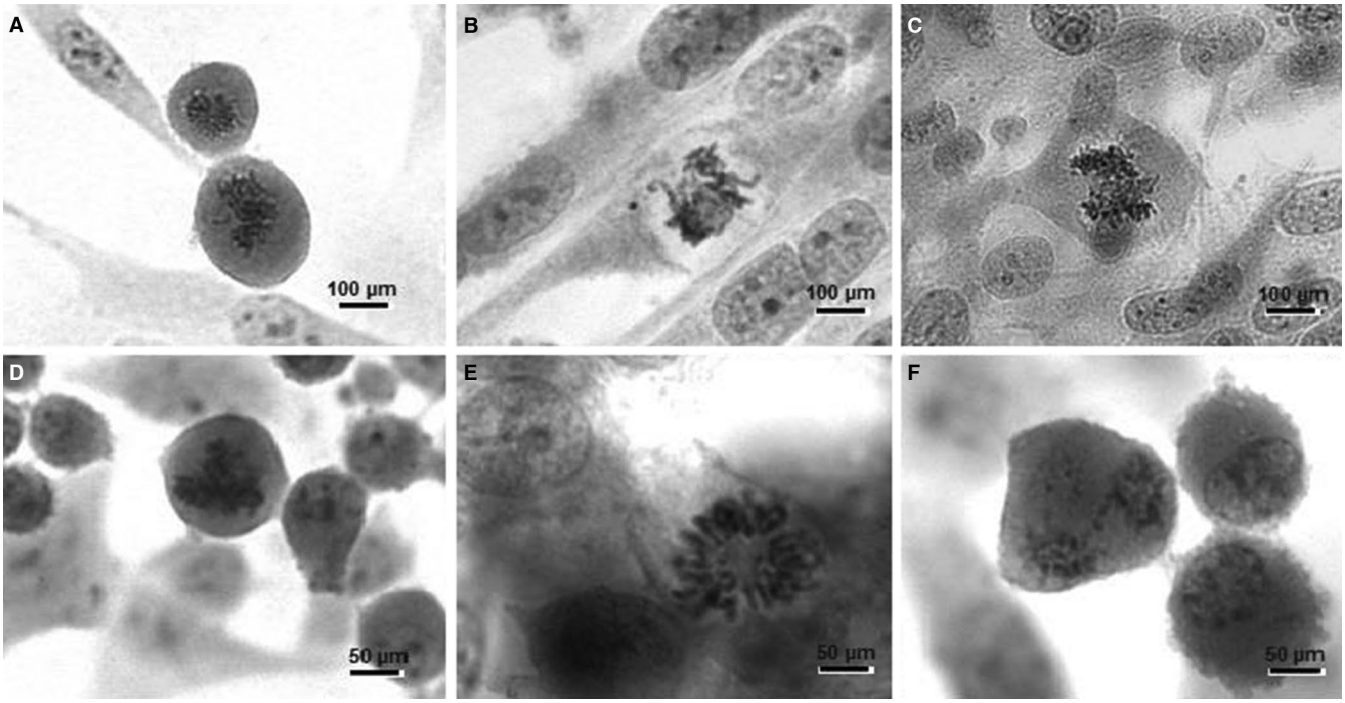
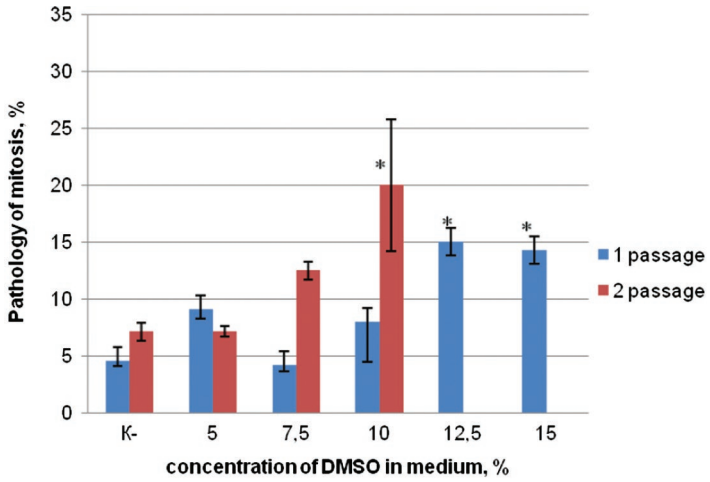


Figure 2: The relative number of pathological mitoses in cell cultures of the first and second passages treated with DMSO in various concentrations. * Differences are statistically significant compared with control, $p < 0.05$.



When cells with different concentrations of DMSO in combination with 5% BSA were incubated, at the first passage, there was no significant increase in the number of pathologies of mitosis compared with the control (Figure 3). At the second passage, the preservation of a pool of dividing cells treated with high concentrations of DMSO (12.5 and 15%) was observed in the presence of BSA, in contrast to the treatment of DMSO solo (Figure 2). At the same time, in all cultures treated with a combination of BSA with DMSO at concentrations of 10% and above, the number of cells with division pathologies increased both in comparison with the control (Figure 3) and in comparison with the treatment of DMSO without BSA (Figure 2).

DMSO is known to have a number of effects on the cell, including modification of the plasma membrane and cytoskeleton rearrangement [12]. The effect of DMSO on microtubule polymerization is widely known [13]. The mechanism is to reduce

the effective water concentration around the tubulin monomers, which contributes to the formation of nucleation centers. However, the dynamics of assembly and disassembly of microtubules in the presence of this substance is different from normal. Algaier J. *et al.* showed that when processed with 10% DMSO, the length of microtubules decreases [14]. Incubation of mouse and rabbit oocytes with 1.5 M DMSO for 30 min leads to the polymerization of microtubules in the form of star-shaped structures associated with pericentriolar material [15]. As a result, the configuration of the meiotic spindle of division changes [16]. Under hypothermia (4°C), DMSO has a stabilizing effect on microtubules if it is used in concentrations not lower than 0.75 M [17]. The effect of DMSO was described on the nematode embryo in which excessive duplication of the centrosome with the appearance of several daughter centrioles was observed [18].

The increase in the number of pathological mitoses in the cell culture of DP, which we found, is probably due to the modifying effect of DMSO on microtubules. An important role is played by the concentration of the substance. In high concentrations of DMSO (12.5-15%), the number of pathological mitoses is observed to double. However, concentrations from 5 to 7.5% do not lead to its significant changes compared with the control. This corresponds to the results obtained by Gulevsky A. *et al.*, and the results did not reveal pathologies of mitosis in the culture of mesenchymal stromal cells after incubation with 5% DMSO [19].

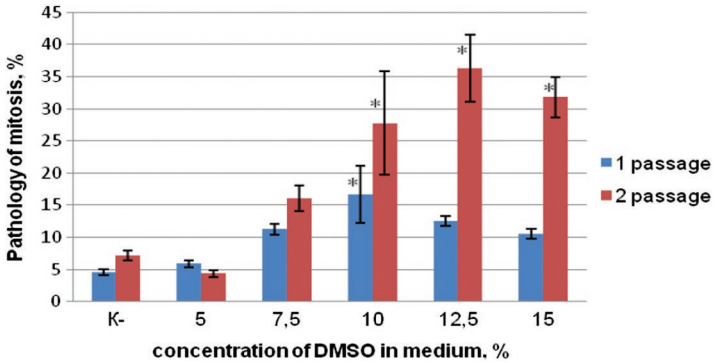
Interestingly, the number of pathological mitoses in the treated cells increases at the second passage compared with the first passage. Consequently, the effect of DMSO on the cell does not disappear after the substance is removed from the incubation medium and is prolonged. This result is consistent with the data obtained by Johnson M. *et al.*, who showed partial irreversibility of the effect of DMSO on the meiotic spindle in mouse oocytes [20]. Thus, examination of the culture immediately after exposure to DMSO and during the first passage does not fully reflect the actual degree of damage to the genetic apparatus of the cells. This must be considered when using them for transplantation.

It is known that serum or purified serum albumin contributes to maintaining cell viability when frozen in the presence of penetrating cryoprotectants, such as DMSO [21]. It is believed that high-molecular protein supplements are involved in stabilizing the cell membrane structure and reducing the damage caused by osmotic stress that develops with the introduction of DMSO. In addition, the protein components of the environment reduce the negative effects of DMSO on membrane fluidity and phase transitions of lipids in it, thereby maintaining normal cytoskeleton-membrane and receptor interactions in the cell [22].

In our experiments, the addition of BSA contributed to the survival of cells under conditions of treatment with high concentrations of DMSO and led to a decrease in the number of pathological mitosis. For example, processing 12.5-15% DMSO resulted in stopping cell division at the second passage (Figure 2). Under the same conditions, but in the presence of BSA, a sufficient number of dividing cells were observed in the culture (Figure 3). However, it must be emphasized that the pathology of mitoses among them was the overwhelming number (up to 35%). This can be explained by the fact that, in the presence of BSA, the changes in cell division apparatus induced by DMSO do not become lethal, but the accumulated negative effect of high concentrations of cryoprotectant manifests itself later on the second passage as an increase in abnormal mitoses. Thus, the use of protein supplements leads to a decrease in the toxic action of DMSO and contributes to the preservation of the DP cell culture after exposure to high concentrations of the cryoprotecnant. However, the surviving cells can be of both normal and transformed nature. So, there are several options for further development of the culture, including postmitotic cell death, their pathological transformation, or a return to the normal genotype by deploidization [23].

The best result was observed when treating DMSO at a concentration of 5% in the presence of BSA. In this case, both on the first and second passages, there were no significant differences between the MI (see Table 1) and the relative number of pathologies of mitoses (Figure 3) compared to the control, which indicated the absence of disturbances affecting the cell mitotic apparatus. Thus, when developing protocols for the cryopreservation of DP cells, a cryopreservation medium can be recommended, which includes DMSO at 5% concentration in combination with BSA.

Figure 3: The relative number of pathological mitoses in cell cultures of the first and second passages treated with DMSO in various concentrations in the presence of 5% BSA. * Differences are statistically significant compared with control, $p < 0.05$.



4. CONCLUSIONS

1. Treatment of DMSO cells in concentrations of 10% and above leads to a significant increase in the number of pathologies of mitosis. This is accompanied by a decrease in MI (when processing concentrations of DMSO to 10%) at the first passage or a complete cessation of proliferation at the second passage (when processing concentrations of DMSO above 10%).
2. 5% concentration of DMSO in combination with BSA is optimal from the point of view of minimizing toxic effects on the genetic apparatus of DP cells, and therefore this combination can be recommended for use as part of cryopreservation media.

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Author Contributions
All authors contributed equally to this study.

Conflict of Interest
None.

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