Epigenotoxic Effect of Dimethyl Sulfoxide on Buffalo Somatic Cells and Buffalo-Bovine Interspecies Somatic Cell Nuclear Transfer Embryos

Husamaldeen Alsaim, M.Sc.1, 2, Farzoooh Jafarpour, Ph.D.1, Faezeh Ghazvini Zadegan, M.Sc.3, Mohammad Hossein Nasr-Esfahani, Ph.D.3*, Amir Niasari-Naslaji, Ph.D.1*

1. Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
2. Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran
3. Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

*Corresponding Addresses: P.O. Box: 81593-58686, Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran
P.O.Box: 14155-6453, Department of Theriogenology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
Email: mh.nasr-esfahani@royaninstitute.org, niasari@ut.ac.ir

Received: 20/Aug/2017, Accepted: 14/Jan/2018

Abstract

**Objective:** In the present study, we investigated the possible epigenotoxic effect of dimethyl sulfoxide (DMSO) on buffalo fibroblast cells and on reconstructed oocytes during buffalo-bovine interspecies somatic cell nuclear transfer (iSCNT) procedure and its effect on rate and quality of blastocyst which derived from these reconstructed oocytes.

**Materials and Methods:** In this experimental study, cell viability of buffalo fibroblasts was assessed after exposure to various concentration (0.5, 1, 2 and 4%) of DMSO using MTS assay. The epigenetic effect of DMSO was also assessed in terms of DNA methylation in treated cells by flowcytometry. Reconstructed oocytes of buffalo-bovine iSCNT exposed for 16 hours after activation to non-toxic concentration of DMSO (0.5%) to investigate the respective level of 5-methylcytosine, cleavage and blastocyst rates and gene expression (pluripotent genes: OCT4, NANOG, SOX2, and trophectodermal genes: CDX2 and TEAD4) of produced blastocysts.

**Results:** Supplementation of culture medium with 4% DMSO had substantial adverse effect on the cell viability after 24 hours. DMSO, at 2% concentration, affected cell viability after 48 hours and increased DNA methylation and mRNA expression of DNMT3A in fibroblast cells. Exposure of reconstructed oocytes to 0.5% DMSO for 16 hours post-activation did not have significant effect on DNA methylation, nor on the developmental competency of reconstructed oocyte, however, it decreased the mRNA expression of NANOG in iSCNT blastocysts.

**Conclusion:** Depending on the dose, DMSO might have epigenotoxic effect on buffalo fibroblast cells and reconstructed oocytes and perturb the mRNA expression of NANOG in iSCNT blastocysts.

**Keywords:** Cloning, Dimethyl Sulfoxide, DNA Methylation, Embryo, Epigenetic

Citation: Alsalim H, Jafarpour F, Ghazvini Zadegan F, Nasr-Esfahani MH, Niasari-Naslaji A. Epigenotoxic effect of dimethyl sulfoxide on buffalo somatic cells and buffalo-bovine interspecies somatic cell nuclear transfer embryos. Cell J. 2019; 20(4): 544-551. doi: 10.22074/cellj.2019.5446.

Introduction

Embryonic development and differentiation processes in mammalians are precisely controlled by epigenetic mechanisms such as histone modifications and DNA methylation (1-3). Epigenetic reprogramming has a crucial role during embryonic and fetal development in mammals (2, 3). Any perturbation in epigenetic modifications during early and late development has negative consequences on offspring survival and health.

Dimethyl sulfoxide (DMSO) is an organosulfur and amphiphatic compound that has various applications in biomedical sciences. DMSO is used widely as a solvent, for water-insoluble compounds, (4) and cryoprotectant (5). It is also used to arrest human lymphoid cells at G1 phase of cell cycle in a reversible manner (6, 7). Furthermore treatment of P19 embryonic carcinoma cells with DMSO can differentiate them into cardiomyocytes and skeletal muscle cells (8). In addition, a significant improvement in terms of blastocyst formation and full term development was observed in mouse somatic cell nuclear transfer (SCNT), following addition of 1% DMSO, as a cytokinesis inhibitor, to the activation medium of reconstructed oocytes (9).

DMSO can regulate epigenetic mechanisms and alter CpG methylation patterns in various cells and tissues (10-15). It was proposed that any remnant of DMSO in embryo preservation media may affect the epigenetic status of cells, oocytes and embryos (15-18). Supplementation of culture medium with DMSO increased an expression of mRNA and DNA methyl transferase 3A (DNMT3A) in embryonic bodies. It also induced hypermethylation as well as hypomethylation on genomic loci of embryonic bodies (10). Exposure of MC3T3-E1 cells for 24 hours to DMSO, increased the mRNA expression of Tet family which are responsible for hydroxylation of DNA methylation and also decreased the mRNA expression of Dnmt family which are responsible for DNA methylation (2). MII oocytes exhibited lower DNA methylation when
treated with DMSO compared to glycerol (15). Activity of DNMT3A could be stimulated by the addition of DMSO. Although further enzymatic analysis suggested that the DMSO stimulation effect may depend on the interaction between DMSO and the reaction substrates (DNA and AdoMet) and not on the enzyme itself (19).

With regard to aforementioned literature and the presumptive effect of DMSO on epigenetic characteristics of treated somatic cells and embryos, we designed this study to investigate the epigenetic effect of non-toxic dose of DMSO on buffalo fibroblast cells and reconstructed oocytes of buffalo-bovine interspecies SCNT (iSCNT) as well as the quality and rate of blastocyst derived from these reconstructed oocytes.

Materials and Methods

In this experimental study, unless otherwise specified, all media and chemicals were obtained from Gibco (Invitrogen Corporation, Grand Island, NY, USA) and Sigma Aldrich Chemicals (St. Louis, MO, USA), respectively. This study received an approval from Ethical Committee of Royan Institute (www.royaninstitute.org).

Somatic donor cell preparation

Somatic donor cells from buffalo were prepared as described previously (20). Briefly, a skin biopsy was taken from a 3-month-old female buffalo. The biopsy was cut into very tiny pieces (1-2 mm²) and cultured as an explant in Dulbecco’s modified Eagle medium F-12 (DMEM/F-12, Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and antibiotic (1% penicillin-streptomycin) at 37°C under a humidified atmosphere of 5% CO₂ until 80% confluency. Fibroblast outgrowths were passaged and stored in liquid nitrogen as described previously (21). For iSCNT, frozen fibroblasts were thawed and cultured in DMEM/F-12 plus 10% FBS. Synchronization of donor cells in G0 were achieved by culture in DMEM/F-12 supplemented with 0.5% FBS for 3 days. Cells from passage 2-3 were used for iSCNT experiments.

Cytotoxicity assessment

Toxicity of different concentrations of DMSO on fibroblast cells were determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. In brief, 5000 buffalo cells were cultured in DMEM/F-12 supplemented with 10% FBS in 96 well dish. After 24 hours, DMEM/F-12+10% FBS containing varying concentrations of DMSO (0, 0.5, 1, 2 and 4%) were added to cultured cells and incubated for 24, 48 and 72 hours. Then MTS was added to each well and incubated for 4 hours at 37°C. Absorbance ratio of various concentrations of DMSO relative to control was measured at 492 nm by using multi-well spectrophotometer. All analyses were measured in three independent replication and each replication consisted of triplicate samples.

Semi-quantitative assessment of global DNA methylation

The respective effects of nontoxic doses of DMSO on global DNA methylation levels of buffalo treated cells were assessed using flow cytometry through measuring fluorescence intensity of the complexes between DNA and 5-methyl cytosine (5-mC) conjugated 4',6-diamidino-2-phenylindole (DAPI). For each treatment, a total of 10,000 cells were collected and analyzed using CELL QUEST_ 3.1 software (Becton Dickinson, USA). Appropriate negative controls were conducted to eliminate the possible effects of autofluorescence and nonspecific binding by the secondary antibody.

Gene expression analysis in fibroblasts

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) in cells treated with 0.5, 1 and 2% DMSO or considered as control. Extracted RNA from various groups was treated with DNase I (Fermentas, Germany) to remove any contaminating genomic DNA. Synthesis of cDNA was carried out according to previous recommendation (22). Briefly, 1 μg of total RNA was used for cDNA synthesis using random hexamer primer and RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). Real-time PCR was carried out with SYBR green (TaKaRa, Japan) in a thermal Cycler Rotor-Gene 6000 (Corbett, Australia). For each reaction, PCR mixture contained 5 μl Rotor-Gene SYBR Green PCR Master Mix (TaKaRa, Japan), 12.5 ng cDNA and 1.5 pmol of each primer in a final volume of 10 μl. Analysis of gene expressions was carried out by the ΔΔCT method and the relative levels of expression were normalized to GAPDH gene expression level. Primer sequences, annealing temperature and product size are listed in Table 1.
Table 1: Primers used for the quantitative real-time polymerase chain reaction (RT-PCR) experiments

| Gene    | Primer sequence (5'-3') | Tm (°C) | Accession number |
|---------|-------------------------|---------|-----------------|
| OCT4    | F: TAAGAAAGGAATTGGGAAC  | 50      | NW_005784454.1  |
|         | R: AGAACAAAGTGATGAGTG   |         |                 |
| NANO2   | F: TGGACTGGTTGGCTCTTATC | 62      | NW_005785373.1  |
|         | R: GCTGAGTTGAAGGAGAAGG  |         |                 |
| SOX2    | F: CCAAGAGAACCTTAAGATG  | 54      | N/A             |
|         | R: TGTGTACTTATCTTCTTCA  |         |                 |
| CDX2    | F: CACTACAGTCGCATACATC  | 56      | NW_005785289.1  |
|         | R: TTTCTTTGCTTACCGTTC   |         |                 |
| TEAD4   | F: AAGTGGAAGCCGAGATG    | 55      | NW_005785334.1  |
|         | R: GCTTGTGGAAGTGAAGATG  |         |                 |
| DNMT1   | F: GAAGCAGAATAAAGAATCGG  | 54      | NW_005783607.1  |
|         | R: TTTGAAGAGTCGTCTGGAA  |         |                 |
| DNMT3A  | F: TGGTCCTGGGCGTTAG     | 57      | NW_005784665.1  |
|         | R: CCGCCTTTAGGGAGTTCG   |         |                 |
| DNMT3B  | F: CGTCATCGCCACGTGTT    | 54      | NW_005785131.1  |
|         | R: CCTTCTCCTCGCATCT     |         |                 |
| β-ACTIN | F: CCATCGGCAATGAGAGATG  | 58      | NW_005783599.1  |
|         | R: CGTGTGGCGTAGAGTAG    |         |                 |
| GAPDH   | F: GTTCAACCGCACCAGCTCAG | 60      | NW_005785176.1  |
|         | R: TACTCAGCACCAGCATC    |         |                 |

Tm; Melting temperature.

Recovery and in vitro maturation of bovine oocytes

Bovine cumulus oocyte complexes (COCs) were recovered from slaughterhouse ovaries with 2–8 mm through 18 gauge needle attached with vacuum pump inside HEPES-buffered tissue culture medium 199 (H-TCM199, Sigma, USA) supplemented with 10% FBS. COCs with homogenous cytoplasm and with multiple layer of cumulus cells were selected for maturation, and incubated for 20 hours in TCM199 supplemented with 10% FBS, 2.5 mM sodium pyruvate (Sigma, USA), 10 ng/ml luteinizing hormone (LH, Sigma, USA), 10 ng/ml follicle-stimulating hormone (FSH, Sigma, USA), 1 µg/ml estradiol-17β, 0.1 mM cysteamine, 100 ng/ml epidermal growth factor (EGF, Sigma, USA) and 100 ng/ml insulin-like growth factor (IGF, R&D, USA) at 38.5°C, 6% CO₂, and maximum humidity.

Interspecies somatic cell nuclear transfer procedure

Procedure of iSCNT was carried out using manual oocyte enucleation using a pulled Pasteur pipette. In brief, matured oocytes were denuded by vortexing inside H-TCM199 supplemented with 300 IU/ml hyaluronidase for 3 minutes. For removing zona pellucida, denuded oocytes were exposed to 5 mg/ml pronase for 45 seconds followed by deactivated with H-TCM199+20% FBS for 20 minutes. The method of manual oocyte enucleation was used as described previously (23). Briefly, zona free oocytes were incubated in TCM199 supplemented with 4 µg/ml demecolcine for 1 hour in 38.5°C. Then, cytoplasmic protrusion containing MII spindle, was removed by hand-held manual oocyte enucleation pipette. For nuclear transfer, nucleus-free bovine oocytes that have been successfully enucleated were transferred to dishes containing a droplets of H-TCM199 supplemented with 10 mg/ml phytohemagglutinin, and a well-rounded buffalo fibroblast cells were attached to membrane of enucleated oocytes. Subsequently couplets in fusion buffer free of Ca²⁺ and Mg²⁺ (290 mOsm) were electrofused using sinusoidal electric current (7 V/cm) for 10 sec followed by two direct currents (1.75 kV/cm for 30 µ seconds and 1 second delay). After 30 minutes, oocyte activation induced by incubation of reconstructed oocytes with 5 µM ca-ionophore for 5 minutes followed by 4 hours incubation with 2 mM 6-dimethylaminopurine (6-DAMP). Subsequently, activated reconstructed
oocytes were cultured primarily in modified synthetic oviductal fluid (mSOF) for 12 hours (24). Thereafter, reconstructed oocytes (in a group of six) were cultured inside well containing 20 μl mSOF under mineral oil without epi-drugs at 38.5˚C, 5% CO₂, 5% O₂ and humidified air for 6.5 days.

Semi-quantitative assessment of DNA methylation in reconstructed embryos

Reconstructed oocytes (16 hours after activation) were washed in PBS containing 0.1 mg/ml polyvinyl alcohol (PBS-PVA) and fixed for 20 minutes in 4% paraformaldehyde (Sigma, USA). Then permeabilization occurred with 1% Triton X-100 in PBS-PVA for 20 minutes at RT. For incorporation of 5-methylcytidine antibody into DNA, reconstructed oocytes were treated with 4 N HCl for 30 minutes at RT and then neutralized for 20 minutes with Tris-HCl buffer (100 mM in pH=8.0). For blocking non-specific binding sites, reconstructed oocytes were incubated in blocking solution [PBS-PVA containing 1% BSA (Sigma, USA) and 10% goat serum] for 2 hours at RT. Incubation of reconstructed oocytes with primary and secondary antibodies was conducted according to the protocol explained earlier. Finally, reconstructed oocytes were exposed to Hoechst and pixel intensity of pseudo-pronucleus was evaluated using Image J. software [National Institute of Mental Health, Bethesda, Maryland, USA] (25). Appropriate controls were included to check the autofluorescence of the first and second antibodies.

Gene expression analysis in interspecies somatic cell nuclear transfer blastocysts

RNeasy Micro Kit was used for RNA extraction from blastocyst embryos as described previously (26) (Qiagen, Germany). Reverse transcription was immediately performed using a QuantiTect Reverse Transcription (RT) Kit (Qiagen, Germany). The cDNA was stored at -70˚C and analysed by quantitative RT-PCR (qRT-PCR) using standard conditions. Relative expression was calculated using Ct values which were normalized against β-actin (reference gene). Three replicates were done for each PCR reactions. ΔΔCT method was used to estimate fold changes between genes of target following RT-qPCR. The value comparative threshold cycle (CT) denotes the threshold cycle, and ΔCT was calculated as CT of the target gene -CT of reference gene. Fold change in gene expression was calculated using 2-ΔΔCT, where ΔΔCT was calculated as ΔCT. Primer sequences, annealing temperature and product size are listed in Table 1.

Experimental design

A non-toxic and non-effective concentration of DMSO (0.5, 1, 2 and 4%) for treatment of buffalo- and bovine reconstructed oocytes, were determined using the tests for cell viability and intensity of methylation as well as the expression levels of DNMTs family on fibroblast cells. Next, the effects of exposing reconstructed oocytes, for 16 hours after activation, to DMSO (0.5%) on the respective level of 5-methylcytosine, cleavage rates and blastocyst rates and gene expression (pluripotency genes: OCT4, NANOG, SOX2, and trophectodermal genes: CDX2 and TEAD4) of produced blastocysts were investigated.

Statistical analysis

The response variables had a discrete nature with a binomial distribution; therefore, all percentage data were subjected to ArcSin transformation. Cell viability, epigenetic level of treated fibroblasts were analyzed using one-way ANOVA followed by Tukey multiple comparison post hoc test in SPSS (SPSS, Version 20, IBM, USA). Epigenetic level of reconstructed oocytes and gene expression in fibroblast cells and blastocyst and developmental rates of experimental groups were compared using independent samples t test. Data were presented as mean ± SEM. P<0.05 were considered as statistically significant.

Results

Cell viability

The possible toxicity effect of DMSO on the viability of buffalo fibroblast cells, was determined using MTS assay following exposure of buffalo fibroblast cells to 0, 0.5, 1, 2 and 4% DMSO for 24, 48 and 72 hours. Exposure of fibroblasts to 0.5% DMSO for 24, 48 and 72 hours did not reveal any adverse effect on the cell viability. However, cell viability started to decline following exposure to 1 (86.51 ± 3.57%) after 72 hours, 2 (89.80 ± 2.71%) after 48 hours and 4% (70.86 ± 3.17%) DMSO after 24 hours compared to control (Fig.1A, P<0.05).

DNA methylation in buffalo fibroblasts

To investigate the possible epigenetic effect of DMSO on global DNA methylation, buffalo fibroblast cells were treated for 24 hours with nontoxic doses of DMSO (0.5, 1 and 2%), according to the cytotoxicity results elaborated in the cell viability experiment of the present study. The relative intensity of 5-methylcytosine increased in a dose dependent manner after treating buffalo fibroblast cells with DMSO. The level of 5-methylcytosine in 0.5 and 1% DMSO (115.24 ± 13.05 and 148.46 ± 15.68% respectively, Fig.1B) was not significantly higher than control group (P>0.05). However, this increase reached a significant level after treating the fibroblast cells with 2% DMSO (184.46 ± 10.07%, P<0.05, Fig.1B).

Gene expression of DNA methyl-transferase family in buffalo fibroblasts

In order to understand the reason of elevated level of
5-methylcytosine in 1 and 2% DMSO treated cells, we designed an experiment to investigate the effect of nontoxic doses of DMSO (0.5, 1, and 2%) on the expression of DNMTs family (DNMT1, DNMT3A and DNMT3B). Relative mRNA expression of DNMT1 and DNMT3B were similar in control and various concentrations of DMSO (Fig.2, P>0.05). However, mRNA expression of DNMT3A was greater in 2% DMSO treated cells compared to other groups (Fig.2, P<0.05).

**In vitro development of buffalo-bovine interspecies somatic cell nuclear transfer**

In order to investigate the possible effect of DMSO (0.5%, the safe concentration of DMSO on buffalo fibroblast cells achieved in the previous experiment of the present study) on cleavage and blastocyst rates of buffalo-bovine iSCNT embryos, reconstructed oocytes were treated with 0.5% DMSO for 16 hours after activation. There was no difference between experimental groups in cleavage (control: 87.2 ± 1.59% and treatment: 86.9 ± 1.34%) and blastocyst rates (control: 4.8 ± 0.91% and treatment: 4.6 ± 0.74%, P>0.05, Table 2).

**DNA methylation in buffalo-bovine reconstructed oocytes**

The exposure of buffalo-bovine reconstructed oocytes to DMSO (0.5%) for 16 hours post activation did not affect DNA methylation, assessed by the intensity of 5-methylcytosine in pseudo-pronucleus of 1-cell iSCNT embryos (134.55 ± 9.15%) compared to control (Fig.3, P>0.05).

**Expression of developmental genes in blastocysts**

In order to evaluate the quality of derived iSCNT blastocyst after exposure of reconstructed oocytes to 0.5% DMSO, the mRNA expression of pluripotent genes (OCT4, SOX2 and NANOG) and trophectodermal genes (CDX2 and TEAD4) were assessed in both control and treated groups. The relative expression of OCT4, SOX2, CDX2 and TEAD4 genes in blastocyst stage was not different between DMSO and control groups (Fig.4, P>0.05). However, expression of NANOG was significantly lower in DMSO treated group compared to control (Fig.4, P<0.05).

### Table 2: Development of buffalo-bovine iSCNT embryos after exposing reconstructed oocytes to 0.5 % DMSO

| Group            | Reconstructed oocytes | Cleaved oocytes | Blastocyst       |
|------------------|-----------------------|----------------|------------------|
| Control-iSCNT    | 583                   | 456 (87.2 ± 1.59) | 22 (4.8 ± 0.91)  |
| DMSO-iSCNT       | 679                   | 525 (86.9 ± 1.34) | 24 (4.6 ± 0.74)  |

Values with the same superscripts within column did not have significant differences (P>0.05).

iSCNT; Interspecies somatic cell nuclear transfer and DMSO; Dimethyl sulfoxide. Data were presented as number (% ± SEM).
Epigenotoxic Effect of DMSO on iSCNT Embryos

Fig. 3: Semi-quantitative analysis of fluorescence intensity of 5-methylcytosine in buffalo-bovine reconstructed oocytes. A. Relative intensity of 5-methylcytosine in buffalo-bovine reconstructed oocytes after exposure to 0.5% DMSO for 16 hours post activation in compare to control. B. Immunofluorescence images of 5-methylcytosine in buffalo-bovine reconstructed oocytes exposed to 0.5% DMSO for 16 hours post activation in compare to control. Control (scale bar: 50 μm). DMSO; Dimethyl sulfoxide. Similar letters indicates non-significant differences.

Fig. 4: Real-time reverse-transcriptase polymerase chain reaction (PCR) gene expression analysis in blastocysts derived from DMSO (0.05%) compared to control. DMSO; Dimethyl sulfoxide and "a"; Different letters indicates significant differences (P<0.05).

Discussion

The main objective of the present study was to examine the effect of DMSO on epigenetic status of treated somatic cells, buffalo-bovine iSCNT reconstructed oocytes as well as the cleavage and blastocyst rates of these reconstructed oocytes. Initial attempts to achieve such objectives was to elaborate the safest dose of DMSO for treating the reconstructed oocytes. Supplementation of culture medium with DMSO could have substantial adverse effect on the cell viability depending on the amount and exposure time. Accordingly, significant decrease in cell viability was noticed following exposure of fibroblast cells to 2 and 4% DMSO. This is in agreement with previous studies in which DMSO had toxic effect at these concentrations (27, 28). However, cell viability was not affected by 0.5% DMSO concentration. This is consistent with the report investigated in rat (28).

In the present study, DNA methylation in fibroblast treated cells amplified by increasing the concentration of DMSO. The highest level of DNA methylation was observed at 2% concentration of DMSO, which was associated with a significant increase in the expression of DNMT3A. However, the lower concentration of DMSO (0.5%) did not affect the methylation nor the gene expression of DNMTs family. Consistent with our results, Iwatani and colleagues (10) demonstrated the upregulation of mRNA and protein of DNMT3A by DMSO in embryonic bodies derived from embryonic stem cells. Furthermore, they showed that "DMSO affected DNA methylation status at multiple loci, inducing hypomethylation as well as hypermethylation using restriction landmark genomic scanning" (10). Moreover, Yokochi and Robertson have shown that DMSO could increase the activity of DNMT3A and DNMT1 enzymes in in vitro condition (19). This is in agreement with the result of the present study when 2% DMSO increased the activity of DNMT3A. Thaler’s report (12) showed that DMSO increased global and gene-specific DNA hydroxymethylation levels and expression of TET and GADD45A genes in pre-osteoblastic MC3T3-E1 cells. In addition, their results revealed a loss of 5-methylcytosine on Fas (pro-apoptotic gene) and Dlx5 (early osteoblastic factor) promoters as well as an increase in 5-hmC.

In the current study, there was a slight, but not significant, increase in level of DNA methylation in treated buffalo-bovine reconstructed oocytes (0.5% DMSO) compared to control group. However, in the embryonic bodies of mice, any concentrations of DMSO, between 0.02 and 1%, could alter the level of methylation significantly (10).

There was no adverse effect of 0.5% DMSO on cleavage and blastocyst rates. This confirms that the safe concentration of DMSO was selected throughout the dose-response study conducted on buffalo fibroblast cells. Interestingly, Wakayama has shown that “addition of 1% DMSO to the activation medium during SCNT procedure significantly improved the frequency of development to the blastocyst stage and full term” (9).

The effect of DMSO (0.5%) on mRNA expression of some developmentally important genes (OCT4, NANOG, SOX2, CDX2 and TEAD4) in buffalo-bovine iSCNT blastocysts was assessed using real time RT-PCR. The expression of NANOG decreased in DMSO treated reconstructed oocytes compared to control. This reduction in expression of NANOG in reconstructed oocytes may be
related to the slight global hyper-methylation of genome. The level of methylation is very important throughout embryonic development. In mice, before implantation the embryos undergoes a wave of DNA demethylation, which erases the inherited parental methylation pattern, while after implantation the embryos undergo a wave of de novo DNA methylation that establishes a new DNA methylation pattern (29, 30). In the present study the slight global hyper-methylation in reconstructed oocytes may be related to the expression of DNMT3A (based on the effect of 2% DMSO on buffalo fibroblast). The expression of DNMT3A significantly expressed after day 10 in mouse embryo (31), but not for the DNMT3B, and any error in the expression of these genes could affect the fate of embryonic development (32).

While expression of OCT4 is highly regulated by the methylation status of its promoter, the mRNA expression of this gene in the present study remained unchanged in DMSO group compared to control. In this notion, Iwatan and colleagues have shown that thousands of loci remained unchanged in EBs after treatment with DMSO (10), which can explain the unchanged expression of OCT4 in DMSO group compared to control.

Conclusion

The results of this study revealed the epigenotoxic effect of DMSO in buffalo fibroblast cells and reconstructed oocytes derived from buffalo-bovine iSCNT procedure. DMSO at the concentration of 2% could induce a global DNA hyper-methylation, possibly through high expression of DNMT3A in treated fibroblast cells. However, there was slight global DNA hyper-methylation in reconstructed oocytes after treatment with 0.5% DMSO. This phenomenon may account for lower expression of NANOG in iSCNT derived blastocysts. Collectively, these results may have some implications and precaution for using DMSO as a solvent or cryoprotectant in biomedical sciences.

Acknowledgments

The authors would like to express their gratitude to the Government of Iraq, Ministry of Higher Education and Scientific Research for financial support and Royan Institute and Deputy for Research at University of Tehran for their full support. The authors have no competing interests to disclose.

Authors’ contributions

F.J., M.H.N.-E.; Perceived and designed the study. H.A., F.J., F.G.Z; Performed the experiments. H.A., F.J., A.N.-N., M.H.N.-E.; Wrote the manuscript. A.N.-N.; Analyzed the data. All writers read and approved the final manuscript.

References

1. Meissner A. Epigenetic modifications in pluripotent and differentiated cells. Nat Biotechnol. 2010; 28(10): 1079-1088.
2. Santos F, Dean W. Epigenetic reprogramming during early develop-

ment in mammals. Reproduction. 2004; 127(6): 643-651.
3. Reik W, Dean W, Walter J. Epigenetic reprogramming in mamma-
lian development. Science. 2001; 293 (5532): 1088-1093.
4. Balakin KV, Ivanenkoy YA, Skorenko AV, Nikolsky YY, Savchuk NP, Ivashchenko AA. In silico estimation of DMSO solubility of organic compounds for bioscreening. J Biomol Screen. 2004; 9 (1): 22-31.
5. Hey JM, MacFarlane DR. Crystallization of ice in aqueous solu-

tions of glycerol and dimethyl sulfoxide: 2. Ice crystal growth kinet-

ics. Cryobiology. 1996; 37(2): 119-130.
6. Sawal M, Takake K, Teraoka H, Tsukada K. Reversible G1 arrest in the cell cycle of human lymphoid cell lines by dimethyl sulfoxide. Exp Cell Res. 1990; 187 (1): 4-10.
7. Teraoka H, Mikoshiba M, Takake K, Yamamoto K, Tsukada K. Re-

versible G1 arrest induced by dimethyl sulfoxide in human lymph-

cell lines: dimethyl sulfoxide inhibits IL-6-induced differentiation of SKW6-CL4 into IgM-secreting plasma cells. Exp Cell Res. 1996; 222(1): 218-224.
8. Skerjanc IS. Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. Trends Cardiovasc Med. 1999; 9(5): 139-143.
9. Wakayama T, Yanagimachi R. Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. Reproduction. 2001; 122(1): 49-60.
10. Iwatan M, Ikemage K, Yuta K, Nishimura S, Yagi S, et al. Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. Stem Cells. 2006; 24(11): 2549-2556.
11. Kawai K, Li YS, Song MF, Kasai H. DNA methylation by dimethyl sulfoxide and methionine sulfoxide triggered by hydroxyl radical and implications for epigenetic modifications. Bioorg Med Chem Lett. 2010; 20(1): 260-265.
12. Thaler R, Spitzer S, Karlic H, Klaushofer K, Varga F. DMSO is a strong inducer of DNA hydroxymethylation in pre-osteoblastic MC3T3-E1 cells. Epigenetics. 2012; 7(6): 635-651.
13. De Mello F, Garcia JS, Godoy LC, Depinco A, Labbé C, Streit DP Jr. The effect of cryoprotectant agents on DNA methylation patterns and progeny development in the spermatogonia of Colossoma macropomum. Gen Comp Endocrinol. 2017; 245: 94-101.
14. De Geyter C, De Geyter M, Steimann S, Zhang H, Holzgreve W. Comparative birth weight of singletons born after assisted reproduction and natural conception in previously infertile women. Hum Reprod. 2006; 21(3): 705-712.
15. Chatterjee A, Saha D, Niemann H, Gryshko O, Glasmacher B, Hofmann N. Effects of cryopreservation on the epigenetic profile of cells. Cryobiology, 2017; 74: 1-7.
16. Matsumura K, Hayashi F, Nagashima T, Hyon SH. Long-term cryopres-

ervation of human mesenchymal stem cells using carboxy-

bated-poly-l-lysine without the addition of proteins or dimethyl sulfox-

ide. J Biomater Science Polym Ed. 2013; 24(12): 1497-1517.
17. McEwen KR, Leitch HG, Amouroux R, Hajkova P. The impact of culture on epigenetic properties of pluripotent stem cells and pre-

implantation embryos. Biochem Soc Trans. 2013; 41(3): 711-719.
18. Liang Y, Fu XW, Li JJ, Yuan DS, Zhu SE. DNA methylation pattern in mouse oocytes and their in vitro fertilized early embryos: effect of cryopreservation. Reprod. 2014; 22(2): 138-145.
19. Yokochi T, Robertson KD. Dimethyl sulfoxide stimulates the catalytic

activity of de novo DNA methyltransferase 3a (Dnmt3a) in vitro. Bioorg Chem. 2004; 32(4): 234-243.
20. Hortaesini SM, Moulavi F, Forouzanfar M, Hajian M, Abedi P, Reza-

zade-Valojerdi M, et al. Effect of donor cell type and gender on the efficiency of in vitro sheep somatic cell cloning. Small Rum Res. 2008; 78(1-3): 162-168.
21. Jafarpoor F, Hosseini SM, Hajian M, Forouzanfar M, Ostadhosseini S, Abedi P, et al. Somatic cell-induced hyperacetylation, but not hypermethylation, positively and reversibly affects the efficiency of in vitro cloned blastocyst production in cattle. Cell Reprogram. 2011; 13(6): 483-493.
22. Rabiee F, Forouzanfar M, Ghazvini zadegan F, Tanhaei S, Ghaderi K, Motovali Bashi M, et al. Induced expression of Fndc5 significantly increased cardiomyocyte differentiation rate of mouse embryonic stem cells. Gene. 2014; 551(2): 127-137.
23. Hosseini SM, Hajian M, Moulavi F, Asgari V, Forouzanfar M, Nasr-

Esfahani MH. Cloned sheep blastocysts derived from oocytes enu-

clated manually using a pulled Pasteur pipette. Cell Reprogram. 2013; 15(1): 15-23.
24. Safari S, Hosseini MS, Hajian M, Forouzanfar M, Jafarpoor F, Abedi P, et al. Improved in vitro development of cloned bovine embryos using S-adenosylhomocysteine, a non-toxic epigenetic modifying reagent. Mol Reprod Dev. 2011; 78(6): 576-584.
25. Jafarpoor F, Hosseini SM, Ostadhosseini S, Abbasi H, Dalman A,
Nasr-Esfahani MH. Comparative dynamics of 5-methylcytosine reprogramming and TET family expression during preimplantation mammalian development in mouse and sheep. Theriogenology. 2017; 89: 86-96.

26. Pezhman M, Hosseini SM, Ostadhosseini S, Rouhollahi Varnosfaderani S, Sefid F, Nasr-Esfahani MH. Cathepsin B inhibitor improves developmental competency and cryo-tolerance of in vitro ovine embryos. BMC Dev Biol. 2017; 17(1): 10.

27. Qi W, Ding D, Salvi RJ. Cytotoxic effects of dimethyl sulphoxide (DMSO) on cochlear organotypic cultures. Hear Res. 2008; 236(1-2): 52-60.

28. Galvao J, Davis B, Tilley M, Normando E, Duchen MR, Cordeiro MF. Unexpected low-dose toxicity of the universal solvent DMSO. FASEB J. 2014; 28(3): 1317-1330.

29. Monk M, Boubelik M, Lehnert S. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development. 1987; 99(3): 371-382.

30. Sanford JP, Clark HJ, Chapman VM, Rossant J. Differences in DNA methylation during oogenesis and spermatogenesis and their persistence during early embryogenesis in the mouse. Genes Dev. 1987; 1(10): 1039-1046.

31. Watanabe D, Suetake I, Tada T, Tajima S. Stage-and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech Dev. 2002; 118(1-2): 187-190.

32. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential in de novo methylation and mammalian development. Cell. 1999; 99(3): 247-257.