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Effect of Mitomycin-C Inactivation on Expression Pattern of Pluripotency Related Transcriptional Factors in Buffalo Fetal Fibroblasts and Wharton's Jelly

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ABSTRACT

Present study examined the effect of mitomycin-C (MMC) treatment on expression profile of pluripotency genes (Oct-4, Sox-2 and Nanog) in buffalo fetal fibroblasts (BFFs) and Wharton's jelly (BWJ) stem cells, generally used as a feeder cell support to grow the pluripotent stem cells. In a time dependent study, a variable response in relative mRNA expression of pluripotency genes was observed, expression of Oct-4 in BFF declined immediately post MMC inactivation but a significant elevation ($P<0.05$) was noticed later on. The relative mRNA expression remained unchanged in BWJ, up to 24 h post MMC treatment and thereafter, it increased significantly ($P<0.05$). A similar trend for the expression pattern of Sox-2 and Nanog was observed in both the cell types. MMC inactivation caused an upregulation in the expression of Sox-2 and Nanog in BFF while it remained unchanged ($P>0.05$) in BWJ. Results of this study suggests that MMC inactivation of the cells used as feeder support modulates the expression profile of pluripotency genes and this alteration in gene expression pattern is variable in different cell types.

Keywords: Buffalo, feeder layer, Fetal fibroblasts; Wharton's jelly, mitomycin-C, Pluripotency genes

Embryonic stem cells (ESCs), derived from the inner cell masses (ICM) of blastocyst stage embryos in many species and possess the potential to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm (Verma *et al.,* 2012). *In vitro* culture and propagation of ESCs require a feeder support, biological or synthetic, that offers suitable culture milieu for these cells. Under *in vitro* culture conditions, feeder support provide a more suitable microenvironment by serving as an attachment matrix, in addition it also secretes various cytokines/growth factors, such as fibroblast growth factor, bone morphogenetic protein-4, transforming growth factor-β1, activin A (Eiselleova *et al.,* 2008; Sharma *et al.,* 2012), etc. In addition, feeder layers also enhance the growth of ESCs and remove toxins from the culture medium (Li *et al.,* 2005). Conventionally, ESCs derived from humans and most of other species were cultured and propagated over inactivated mouse embryonic fibroblast feeder layer, but certain major shortcomings such as high batch to batch variation/s, possibility to introduce retro-viruses in cultures, limited proliferating abilities and loss of their ability to support ESCs with subsequent passages (Richards *et al.,* 2002; Park *et al.,* 2003) have deemed the heterologous feeder approach less appropriate for ESC culture. Previous studies have documented the use of homologous or mixed feeders for propagation of ESCs (Amit *et al.,* 2003; Fong and Bongso, 2006; Cho *et al.,* 2010; Kumar *et al.,* 2011; Sharma *et al.,* 2013; Cong *et al.,* 2014).

Since the feeder cells grow swiftly, they must be halted from overtaking the slower-growing ESCs; however it must still be viable to serve the function. Generally, two most common methods are used for inactivation of feeder cells are: irradiation and treatment of feeder monolayers

with a sub-lethal dose of mitomycin-C. Irradiation is time consuming and expensive. Presently, mitomycin-C offers an efficient, readily available, cost-effective and easy to use substitute for inactivation of feeder monolayers. However, the expression of growth factors/cytokines is reported to be modified in feeder-layers after treatment with mitomycin-C (Eiselleova *et al.*, 2008; Sharma *et al.*, 2012).

With this background, the present study was undertaken to evaluate the alteration in expression profile of key regulators of pluripotency in buffalo fetal fibroblasts and Wharton's jelly feeder layers before and after mitomycin-C treatment so as to further optimize the culture conditions for ES cell propagation.

MATERIALS AND METHODS

All the chemicals and media used in this study were procured from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise indicated.

Experimental design

To find out the effect of mitomycin-C (MMC) treatment on expression pattern of pluripotency markers (Oct-4, Sox-2 and Nanog) in buffalo fetal fibroblasts (BFF) and Wharton's jelly (BWJ). The BFF and BWJ feeder layers were treated with MMC (10 µg/ml) for 3 h. In the first group, the monolayers after MMC treatment were trypsinized immediately (at 0 h) and processed for real time PCR study. In second and third group, the monolayers after treatment were further cultured in media containing DMEM + 15% FBS + gentamycin for 24 h and 48 h respectively, afterwards the cells were trypsinized and further processed for real time PCR studies. Untreated monolayers were used as controls. Three replicates for each experiment were performed.

Isolation and expansion of buffalo fetal fibroblasts and Wharton's jelly feeder layers

Preparation of buffalo fetal fibroblast and Wharton's jelly monolayer

Pregnant uteri (approx. 45-50 days old) were procured from local abattoir immediately after slaughter and transported in normal saline solution (NSS) at 25°C - 30°C to the laboratory at the earliest. Fetuses were thoroughly washed with sterile NSS. For isolation of BFF, ear tissue biopsies were taken and skin was removed by gently scratching the outer surface and minced thoroughly. For preparation of BWJ feeder layer, umbilical cords were separated aseptically from fetus. The cord was cut open using Bard-Parker (BP) blade. Jelly explants were harvested aseptically. Both types of explants were washed in sterile phosphate-buffered saline (Ambion, Life Technologies) and cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 50 µg/ml gentamycin sulphate in CO_2 incubator (5% CO_2) at 38.5 °C. The explants were removed after 24-48 h and both types of monolayers were allowed to grow till confluency. Medium was replaced after every 24 h. Upon 80% confluency, BFF cells were treated with accutase and further sub-cultured for subsequent passages.

Characterization of buffalo fetal Fibroblasts and Wharton's jelly

Immunocytochemical localization of pluripotency markers

Cells derived from BWJ and BFF were expanded in medium to 60-80% confluency. The cells were washed with sterile PBS (pH 7.4) thrice and fixed in 4% formalin solution for 20 min at 37°C and permeabilized with 0.25% triton-X 100 for 15 min at 37°C. Permeabilized cells were washed with pre-warmed PBST (PBS-0.01% Tween 20), followed by blocking with 5% bovine serum albumin for 1 h. Cells were probed with goat polyclonal antibodies (Santa Cruz Biotechnology, Texas, USA) at a dilution of 1:100 and kept at 4°C for 12 h. Respective antigens were localized with donkey anti-goat Fluorescein isothiocyanate (FITC) or Texas Red (TR) conjugates at a dilution of 1: 500 and kept at 37°C for 2 h in dark. DAPI (0.4 mg/ml in PBS) was used as a counterstain. Negative controls, excluded from the exposure of primary antibody, were run parallel. Images were captured using fluorescent microscope (IX 71, Olympus, Shinjuku, Tokyo, Japan). Primary antibodies used in this experiment were against Oct-4 (#sc-8628, N-19), Sox-2 (#sc-54517, S-15), Nanog (#sc- 30328, W-18).

RNA isolation and cDNA synthesis

Total RNA was isolated from BFF and BWJ cells using Trizol reagent (Ambion, USA) by following standard

protocol. The extracted total RNA was quantified using Nanodrop Spectrophotometer (Thermo Scientific, USA) and integrity was accessed by agarose gel electrophoresis. Samples with $A_{260/280}$ values between 1.8-2.0 were taken for further study. For synthesis of cDNA, 1 µg of total RNA were reverse transcribed to cDNA using Verso cDNA synthesis kit (Thermo Scientific, USA). Briefly, a master mix (20 µl) of following components was prepared: 11 µl RNA + nuclease free water (NFW), 4 µl of 5X cDNA synthesis buffer, 2 µl dNTP mix (5 mM), 1 µl each of random hexamer, RT enhancer and Verso enzyme mix. The mixture was incubated at 42°C for 60 min followed by inactivation at 95°C for 2 min. The cDNA thus synthesized was stored at -20°C until further use. Primer details for pluripotency associated genes have been summarized in table 1. No template control (NTC) containing NFW instead of cDNA was used as negative control. The PCR products were stained with LabSafe nucleic acid stain (G-Biosciences), separated on 2% agarose gel electrophoresis and images were captured using gel documentation system (Alpha Imager Pvt. Ltd.).

Quantitative real-time PCR of pluripotency markers

The expression profile of pluripotency markers was performed with DyNAmo SYBR green (Thermo Scientific, USA) and Real-Time qPCR machine (Bio-rad, USA). Briefly, a master mix of following components was prepared- 2X SYBR green mix, forward primer, reverse primer (0.5 µM each) and NFW. The master mix was added to strip tubes and equal volume of cDNA template was added. Thermal cycling conditions consisted of initial denaturation at 95°C for 420 sec followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 15 s varied with respect to primers used (Table 1) and extension at 72°C for 10 s followed by melting curve. β-actin and RPS15A were used as housekeeping genes. No template control (NTC) was placed with each reaction set up for checking any contamination in reaction components. At the end of the reaction, cycle threshold (C_t) values and amplification plot were acquired and relative expression of PCR product was determined by the equation suggested by Pfaffl (2001).

STATISTICAL ANALYSIS

All experimental data are expressed as the mean ± SEM. Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey HSD and/ or Duncan's multiple range tests using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Immunostaining of buffalo fetal fibroblasts and Wharton's jelly

The cellular localization by immunocytochemistry revealed the presence of Oct-4, Sox-2 and Nanog protein in both BWJ and BFF (Figure 1).

Table 1. Gene transcripts, primer sequence and resulting fragment size.

Target gene	Primer sequence (5'-3')	Annealing Temperature (°C)	Predicted Size (bp)	References
Oct-4	GATATACCCAGGCCGATGTG	60	232	XM006052299.1
	TCGATACTCGTCCGCTTTCT			
Sox-2	CTATGACCAGCTCGCAGAC	60	111	Sreekumar *et al.*, 2014
	ACTTCACCACCGAGCCCA			
Nanog	GCAGGTGAA GACCTG GTTC	60	175	Sreekumar *et al.*, 2014
	CCACATGGGCAGGTT TCCA			
RPS 15A	AATGGTGCGCATGAATGTC	60	100	Dangi *et al.*, 2014
	GACTTTGGAGCACGGCCTAA			
β-Actin	AGTTCGCCATGGATGATGA	60	54	Dangi *et al.*, 2014
	TGCCGGAGCCGTTGT			

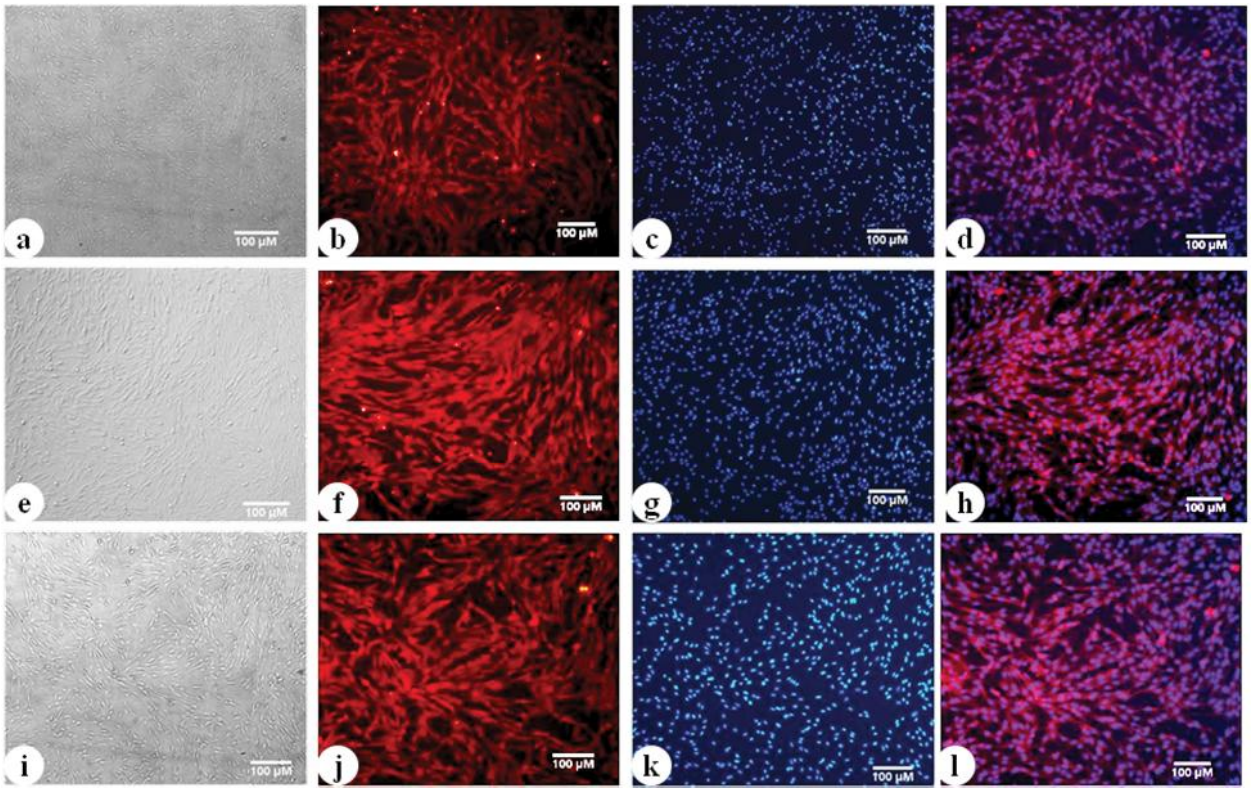

Figure 1. Immunocytochemical localization of pluripotency markers in buffalo fetal fibroblasts. Cells were stained with primary antibodies directed against Oct-4 (b), Sox-2 (f) and Nanog (j) stained by TR conjugated secondary antibodies. In panels, (a), (e) & (i) representative fields as observed under bright field. In next panel, (c), (g) & (k) representative fields of DAPI. In next panels (d), (h) & (l) are merger photographs of DAPI and TR.

Effect of mitomycin-C treatment on proliferation of buffalo fetal fibroblasts and Wharton's jelly

Morphology of BFF was altered as cells became slightly slender immediately after MMC treatment with reduced density (Figure 3 B), while no major difference was registered in case of BWJ (Figure 3 F).

Effect of mitomycin-C inactivation on expression profile of pluripotency genes

The expression of Oct-4 decreased significantly ($P<0.05$) after MMC inactivation in comparison to control; thereafter, elevated significantly at 24 h and 48 h. In BWJ, the relative mRNA abundance remained unchanged ($P>0.05$) at 0 h and 24 h post MMC treatment, however it increased significantly ($P<0.05$) at 48 h. (Figure 4 A). A similar expression pattern was observed in Sox-2 and Nanog in both types of cells. MMC inactivation dramatically upregulated the expression level of Sox-2 and Nanog ($P<0.05$) in BFF at 0 h, then declined ($P<0.05$) at 24 h but escalated ($P<0.05$) at 48 h after MMC exposure. Interestingly, MMC treatment did not amend the expression profile of aforesaid genes in BWJ ($P>0.05$) (Fig 4. B-C)

MMC is a DNA cross-linking agent obtained from *Streptomyces caespitosus*. It represses cell division by getting inserted between the DNA strands. Once mitomycin-C inserts into the genetic material, the cell has a restricted lifetime that ranges between 1 to 3 weeks (Ponchio *et al.*, 2000). Recently, growing evidences have indicated that MMC treatment alters the expression and secretory profile of feeder layers (Eiselleova *et al.*, 2008; Sharma *et al.*, 2012). A better understanding of the MMC

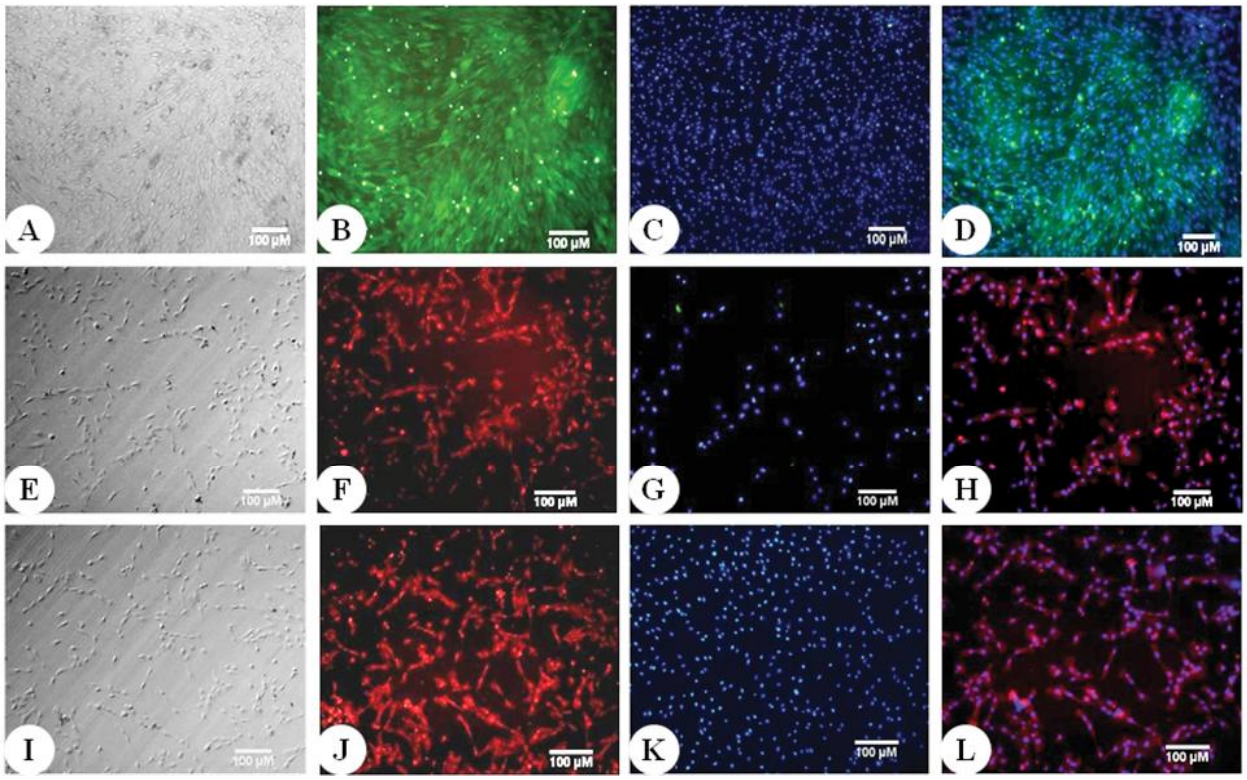

Figure 2. Immunocytochemical localization of pluripotency markers in buffalo Wharton's jelly. Cells were stained with primary antibodies directed against Oct-4 (B), Sox-2 (F) and Nanog (J) stained by TR conjugated secondary antibodies. In panels, (A), (E) & (I) representative fields as observed under bright field. In next panel, (C), (G) & (K) representative fields of DAPI. In next panels (D), (H) & (L) are merger photographs of DAPI and FITC/TR.

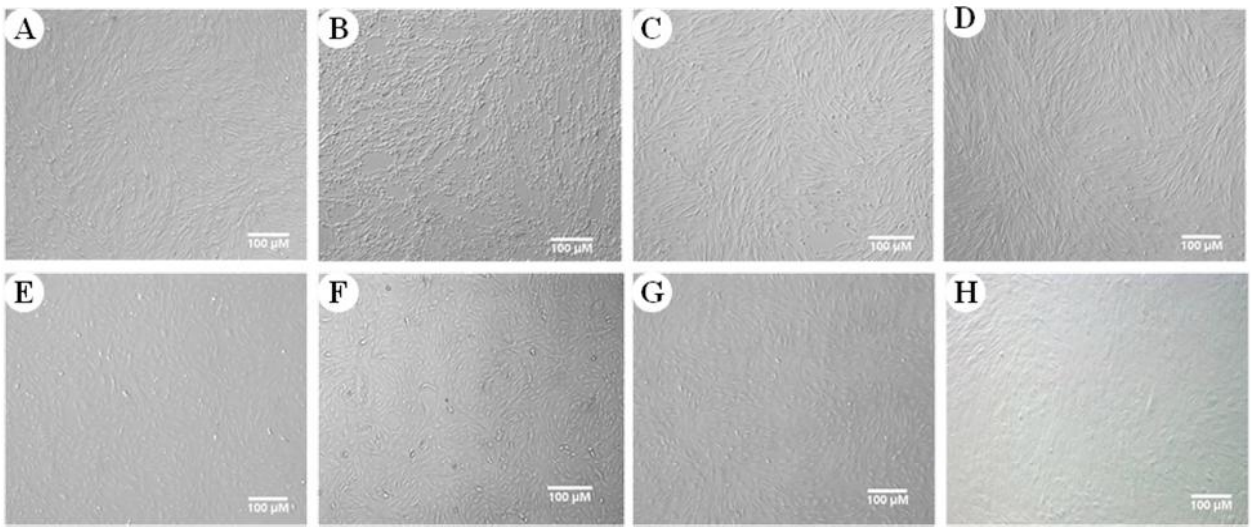

Figure 3. Effect of mitomycin-C on morphology on fetal fibroblasts and Wharton's jelly. The feeder layers were treated with mitomycin-C for 3 h. After mitomycin-C treatment the monolayers were further cultured in media for 24 & 48 h. In panels, (A) & (E) are monolayers of BFF and BWJ respectively before mitomycin-C treatment. (B) & (F) after 3 h, (C) & (G) after 24 h and (D) & (H) post 48 h treatment.

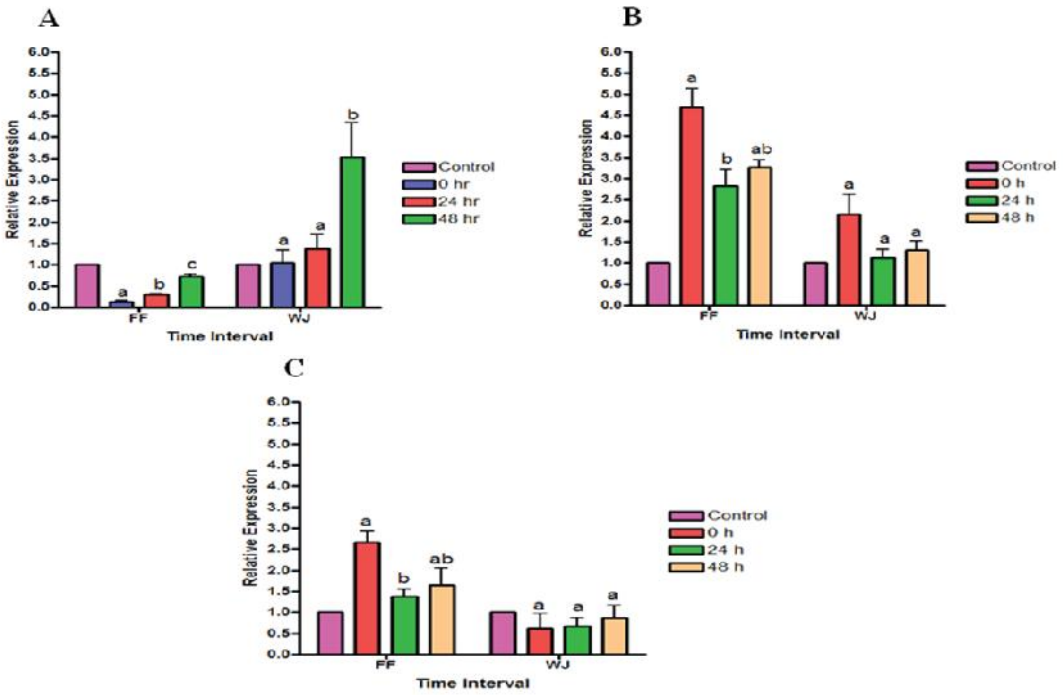

Figure 4. Expression profile of pluripotency related transcriptional factors. Data represents the mean±SEM. Means bearing different superscripts differ significantly (*P*<0.05) at different exposure hours. (A) Expression of Oct-4 mRNA in BFF and BWJ. (B) Expression of Sox-2 mRNA in BFF and BWJ. (C) Expression of Nanog mRNA in BFF and BWJ.

effects on the feeder layers is essential to optimize the culture conditions. In this study, we have reported the effect of MMC on relative mRNA expression of key pluripotency genes in BWJ and BFF derived mesenchymal stem cells. To access the long-lasting effect of MMC on BFF and BWJ monolayers the cells after MMC exposure were again cultured in media for 24 h and 48 h. To the best of our knowledge, this is the first report highlighting the impact of MMC treatment on expression of pluripotency genes (Oct-4, Sox-2 and Nanog). To permit dissection of the incidents, primary cultures were initiated using BFF and BWJ explants in DMEM containing serum. BFF and BWJ derived MSCs were successfully isolated by explant culture method as determined on the basis of their characteristic fibroblastic/spindle-shaped phenotype, positive expression of chief pluripotency related transcriptional factors. Our results are in agreement with previous reports (Azari *et al.*, 2011; Yadav *et al.*, 2012; Pratheesh *et al.*, 2013; Sreekumar *et al.*, 2014).

A sub-lethal concentration (10 µg/ml) of MMC for 3 h could effectively arrest the proliferation of BFF; however it appeared to be insufficient for BWJ. Our findings suggested that the cytotoxicity effect of MMC appear to be cell-specific. After MMC inactivation, the concentration of cells in suspension escalated with time for BFF, however it was found to be minimal in case of BWJ. MMC has been reported to cause apoptotic cell death in human cell culture (Kang *et al.*, 2001). Our results indicate that BFF may be more vulnerable to MMC exposure compared to BWJ; however, further investigations are needed to validate these facts. Oct-4 is a transcriptional factor required for the maintenance of pluripotency of cells within the ICM and used as a molecular marker for pluripotent ESCs (Pesce *et al.*, 1998). For Oct-4, both cell types responded differently to MMC treatment; it caused immediate down regulation in BFF while BWJ cells remained unaffected. Surprisingly, MMC inactivation immediately caused substantial rise in the expression of Sox-2 and Nanog in BFF, while it remained unaffected (*P*>0.05) at all time intervals in BWJ. Our results suggest that after MMC inactivation expression of pluripotency related transcriptional factors varies with cell type. Previous study has also reported the alteration in apoptosis gene expression after MMC treatment in human tendon capsule fibroblasts (Crowston

et al., 2002), changes in growth factors in feeder layer cells derived from mouse embryonic fibroblasts and human embryonic fibroblasts (Eiselleova *et al.*, 2008) and buffalo fetal fibroblasts (Sharma *et al.*, 2012) after MMC treatment.

It can be concluded from the present study that 1) Different cell types respond variably to MMC treatment. 2) MMC exposure modulates the expression profile of pluripotency genes. 3) Alteration in expression pattern is variable in different cell types.

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