Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(4): 54-58

Received: 09-05-2018
Accepted: 15-06-2018

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Significant improvement in *in vitro* development of sheep hand-made cloned embryos using epigenetic modifiers in culture following nuclear tranfer

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Abstract
The present study was carried with the use of reprogramming enhancers trichostatin A (TSA), a histone deacetylase inhibitor (HDACi) and 5-aza-2-deoxycytidine (5-aza-dc), a DNA methyl transferase inhibitor on hand-made cloned (HMC) embryos. It was found that TSA treatment (50 nM) and 5-aza-dc (25nM) for 24 hours following oocyte activation, improved *in vitro* HMC embryo development. Cleavage percentage observed on Day 2, in case of TSA treated HMC embryos was found to be significantly ($p<0.05$) higher than non-treated controls (57.46%±2.02 v/s 48.02% ± 3.76). On day 7, the percentage of morulae was found to be non-significantly ($p>0.05$) higher in case of TSA treated HMC embryos than non-treated controls (42.98%±4.76 v/s 37.04%±7.18) while as the blastocyst percentage in case of treated group was found to be significantly ($p<0.05$) higher than non-treated group (16.00%±3.91 v/s 3.33%±3.00). Cleavage percentage observed on Day 2, in case of 5-aza-dc treated group was found to be non-significantly ($p>0.05$) higher than non-treated controls (52.93%±2.58 v/s 48.02% ± 3.76). On day 7, the percentage of morulae was found to be non-significantly ($p>0.05$) higher in case of 5-aza-dc treated HMC embryos than non-treated controls (43.23%±5.23 v/s 37.04%±7.18). The percentage of blastocysts was found to be non-significantly ($p>0.05$) higher in case of 5-aza-dc treated HMC embryos than non-treated controls (6.55%±4.36 v/s 3.33%±3.00). In conclusion, the study revealed that both trichostatin A and 5-aza-2-deoxycytidine have a positive effect as reprogramming enhancers but trichostatin A was found to be more potent reprogramming enhancer than 5-aza-2-deoxycytidine.

Keywords: TSA, 5-aza-dc, dnmt1, SCNT, HMC

Introduction
Reprogramming enhancers have been used either on the developing cloned embryos or on donor cells to check the epigenetic errors by altering histone acetylation levels or methylation pattern of DNA and increase the developmental competance of cloned embryos. Trichostatin A is a histone deacetylase inhibitor (HDACi) [1], which is known to be a critical factor for successful reprogramming during SCNT [2]. Histone acetylation decreases the affinity of histone proteins to DNA sequences by neutralizing the positive charge of the histone tails [3] which in turn, facilitates transcriptional processes [4], because a more extended and open chromosomal structure provides more access to transcriptional regulatory proteins to their target sequences [5]. Histone acetylation also facilitates better genome imprinting and there by increases the developmental competence of embryos [1]. DNA methylation is another well-described epigenetic mechanism that plays a key role in several biological processes such as transcriptional regulation, chromosomal organization, X-chromosomal inactivation and genomic imprinting [6-9]. DNA methylation in mammals is an essential process in the regulation of transcription during embryonic development [10]. However, epigenetic modification of DNA methylation in mammals occurs aberrantly in most cloned embryos [11, 12]. In particular, DNA methylation errors and incomplete reprogramming following nuclear transfer are thought to contribute significantly to the low efficiency of somatic cell cloning [13, 14]. 5-aza-2-deoxycytidine inhibits DNA methyltransferase1 (*dnmt1*) enzyme as a result of which DNA hypo-methylation occurs [15]. Hypo-methylated DNA allows transcription factors to bind to gene promoter regions and regulate gene expression [16]. Reactivation of silenced pluripotent genes improves nuclear reprogramming efficiency [17].

Reactivation of genes related to early embryo development enhances the developmental competence of cloned embryos [18]. When 5-aza-2-deoxycytidine was used on cloned embryos in different animals it lead to increase in cleavage, morula and blastocyst rate as reported in bovine SCNT embryos [19], porcine SCNT embryos [20], porcine SCNT embryos [21], buffalo HMC embryos [22].

The present study was carried with the aim of investigating the developmental competence of HMC embryos with TSA treatment (50 nM) and 5-aza-dc (25nM) for 24 hours following oocyte activation.

2. Materials and Methods: It includes the following

2.1 *In vitro* maturation of oocytes: Oocytes of usable quality were selected (Figure 1) and after washing thrice with IVM medium (Hepes buffered M-199 supplemented with 15% FBS, 5 µgmL¯1 pFSH, 1 µgmL¯1 17β-estradiol, 0.8 mM sodium pyruvate and 50 µgmL¯1 gentamicin and epidermal growth factor (EGF)). Groups of 15–20 COCs were cultured in 100-µL droplets of IVM medium, overlaid with sterile mineral oil in 35-mm diameter Petri dishes, and cultured for 24 h in 5% CO2 with maximum humidity at 38.5 ºC.

2.2 Preparation of Donor Cells for Nuclear Transfer: Ear tissue samples from adult sheep were collected and transported to the laboratory in Dulbecco's Phosphate Buffer Saline (DPBS) containing 10% foetal bovine serum (FBS),ampicillin (100 U/mL) and streptomycin (100µg/mL). The tissue samples were shaved in DPBS to remove the upper skin and hair follicles completely. The biopsies were then transferred into another dish containing drops of cell-culture medium, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. Tissue samples were washed three to four times in the medium and later on cut into 1mm pieces. These pieces were then placed in a tissue culture flask and kept in the CO_2 Incubator till attachment. After the attachment of the tissues fresh medium (DMEM + 10% FBS), was poured gently into the flask along the sides in order to prevent displacement of the tissues. The flask was then placed in the CO_2 Incubator for incubation at 37˚C and 5% CO_2. The medium was changed as needed and the cultures were observed daily for substantial outgrowth of cells from the tissue pieces.

The cells were passaged at 80% - 90% confluence (Figure 2). The cells at 5th to 9th passage were used for handmade cloning experiments. The confluent cells (adult sheep fibroblasts) at 5th to 9th passage were allowed to grow further 3 days in order to achieve over-confluence. As a result majority of the cells were expected to reach G1 stage of cell cycle. Culture medium of the cells was removed by aspiration and the cells were overlaid with calcium and magnesium free DPBS for 5 min. After removing DPBS the cells were subjected to trypsinization using Trypsin-EDTA solution. The dissociating cells were then harvested in T20 medium and centrifuged to get a loose cell pellet. The pellet was re-suspended with T20 and mixed by pipetting to get single cell suspension in a 1.5 ml tubes. The cells were ready for use as nucleus donors.

2.3 Production of Reconstructs by Hand-made Cloning Technique: *In-vitro* matured COC's with expanded cumulus were stripped of their cumulus investment and zona pellucida using hyaluronidase ($0.5mgmL^{-1}$) in T2 (where T denotes HEPES modified M-199 supplemented with 2.0mM glutamine, 0.2mM sodium pyruvate, $50\mu gmL^{-1}$ gentamicin and the following number denotes 2% FBS) and pronase ($2.0mgmL^{-1}$ in T containing 10% FBS) respectively. Oocytes with completely digested zona pellucida were transferred to T20 (T containing 20% FBS) and incubated at 38.5˚C for 10–15 minutes or until a prominent protrusion cone was easily visible. Protrusion cone guided ennucleation by manual bisection was done with the help of a micro blade followed by Hoechst 33342 staining to confirm removal of metaphase II plate. The larger demi-cytoplasts without protrusion cone were transferred to T20 (where T denotes HEPES modified M-199 supplemented with 2.0mM glutamine, 0.2mM sodium pyruvate, $50\mu gmL^{-1}$ gentamicin and the following number denotes 20% FBS) and incubated at 38.5˚C for 10 min to enable them to regain their spherical shape. The enucleated demi-cytoplasts were immersed in phytohemagglutinin (0.5mgmL−1 in T2) for 3–4 s and then transferred to T2 containing donor cells at a low cell density. Each demi-cytoplast was then allowed to attach to a single, rounded, medium sized cell by gently rolling the demi-cytoplast over it. The couplets (demicytoplast-donor cell pairs) were transferred to fusion medium (0.3M d- mannitol, 0.1mM $MgCl_2$, and 0.05mM $CaCl_2$ and 1mg/ml polyvinyl alcohol) for 5 min equilibration (figure 3). A single step fusion protocol was followed wherein a demi-cytoplast and a couplet were picked using a fine pulled capillary pipette (Unopette® IMV Technologies,) having an internal diameter of 100–150µm. Initially, the couplets were expelled and aligned with an A.C. pulse (6 V) using BLS CF-150B (BLS Budapest Hungary), so that the somatic cell faced the positive electrode [23] and immediately after alignment, another demi-cytoplast were introduced into the fusion chamber (BLS microslide 0.5mm gap, model GSS-500) close to the somatic cell. As soon as the somatic cell was sandwiched between the demi-cytoplasts (Figure 3), single D.C. pulse (2.96 kVcm−1 for 10µs) were applied. The triplets were then incubated in for rounding up and subsequent reprogramming for 2 h at 38.5 ^{0}C.

2.4 Activation of reconstructs and supplementation of epigenetic modifiers: The reconstructed oocytes of sheep were activated by incubating in T2 media containing 5µM calcium ionophore for 5min at 38.5 ^{0}C. After washing thrice with T20 the reconstructed oocytes were individually transferred to droplets of T20 containing 2mM of 6-dimethylaminopurine (6- DMAP). These drops were additionally supplemented with 50nM Trichostatin A [24] and 25nM 5-aza-2-deoxycytidine [25] separately and then were covered with mineral oil and incubated for 4 hours in a CO_2 incubator at 38.5 ^{0}C. After that the reconstructs were cultured in research vitro cleave media (RVCL) media which was separately supplemented with TSA 50nM [24] and 5-aza-dc 25nM [21] for 20 hours. The reconstructs were washed twice in RVCL media and then were proceeded for culture.

2.5 Culture of reconstructs: The reconstructed, activated and supplemented embryos were then transferred to 400µL of Research Vitro Cleave medium (RVCL) supplemented with fatty acid free bovine serum albumin (BSA) in 4-well dishes covered with mineral oil and kept undisturbed in a CO_2 incubator for 7 days. On day 2, cleavage rate was observed and on day 5, morula rates and subsequently on day 7, blastocyst rate was observed, as shown in (Figure 3). Non-treatment controls were kept separately for these supplementations.

2.6 Experimental Design: To study the effect of trichostatin A, six replications of treated HMC embryos were performed

and compared with the six replications of control non-treated HMC embryos. To study the effect 5-aza-dc, six replications of treated HMC embryos were performed and compared with the 6 replications of control non-treated HMC embryos.

3. Results and Discussion

Results pertaining to the study are presented in Table 1 and Table 2 & shown in Figure 4 and Figure 5.

Table 1: Effect of trichostatin A on the Developmental Competance of HMC Sheep Embryos

Group	No. of activated embryos (%)	No. of cleaved embryos (%)	No. of Morulae (%)	No. of Blastocyst
TSA Treated	75	57.46 ± 2.206^{a} **(43)**	42.98 ± 4.76^{a} **(18)**	16 ± 3.9^{a} **(7)**
Control	59	48.02 ± 3.76^{b} **(28)**	37.04 ± 7.18^{a} **(10)**	3.33 ± 3.00^{b} **(1)**

Figures quoted as percent Mean ± S.E.M.
Morulae and Blastocyst percentages calculated out of total number of cleaved embryos.
Values having different superscripts along columns, differ significantly (P<0.05).

Table 2: Effect of 5-aza-2-deoxycytidineon the Developmental Competance of HMC Sheep Embryos

Group	No. of activated embryos (%)	No. of cleaved embryos (%)	No. of Morulae (%)	No. of Blastocyst
5-azadc Treated	76	52.93%±2.58^{a} **(43)**	43.23±5.23^{a} **(18)**	6.55±4.36^{a} **(3)**
Control	59	48.02±3.76^{a} **(28)**	37.04±7.18^{a} **(10)**	3.33±3.00^{a} **(1)**

Figures quoted as percent Mean ± S.E.M.
Morulae and Blastocyst percentages calculated out of total number of cleaved embryos.
Values having different superscripts along columns, differ significantly (P<0.05).

A significantly higher cleavage rate was observed in treatment group as compared to control group (57.46 vs 48.02%). Similar increase of cleavage rate (72.4 vs 68.9%) was reported in SCNT sheep embryos [24], and in SCNT pig embryos (44.2 vs 34.5 % and 93.2 vs 66.3%, respectively) [25, 26].

Similarly, the Blastocyst percentage also was significantly higher in treatment group as compared to control group (16.00 vs 3.33%). Likewise, an improved blastocyst rate with TSA supplementation was also earlier reported in IVC of SCNT cloned sheep embryos (23.3 vs 16.7 %) [24], pig embryos (22.0 vs 8.9%) [26] and cloned pig embryos (70.7 vs 48.9%) [25].

A higher cleavage rate was observed in 5-aza-2-deoxycytidine treatment group as compared to control group (52.93% vs 48.02%). Similar increase of cleavage rate (91.32% vs 86.72%) was also published in earlier reports in case of SCNT porcine embryos [21] and in bovine SCNT embryos (74.04% v/s 77.7%) [19].

Similarly, the Blastocyst percentage was found to be higher in treatment group as compared to control group (6.55% vs 3.33%). Our findings are in agreement as reported by various researchers in porcine SCNT embryos (28.61% vs 19.99%) [21] and in bovine SCNT embryos (38.34 % vs 25.98%) [19].

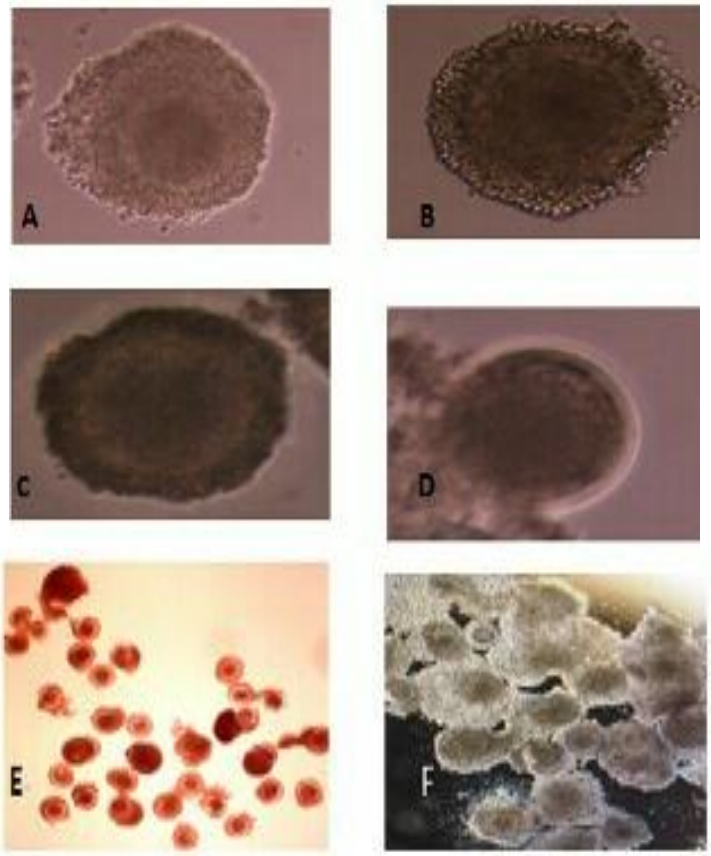

Fig 1: Different grades of Cumulus Oocyte Complexes (COCD) (A) Grade-A (B) Grade-B (C) Grade-D € Grade-A and Grade-B in 100 ul droop of *in vitro* maturation media (IVM) overlaid with sterile mineral oil (F) In vitro matured oocytes after 24 hours of incubation in IVM medium at 37C, showing cumulus expansion

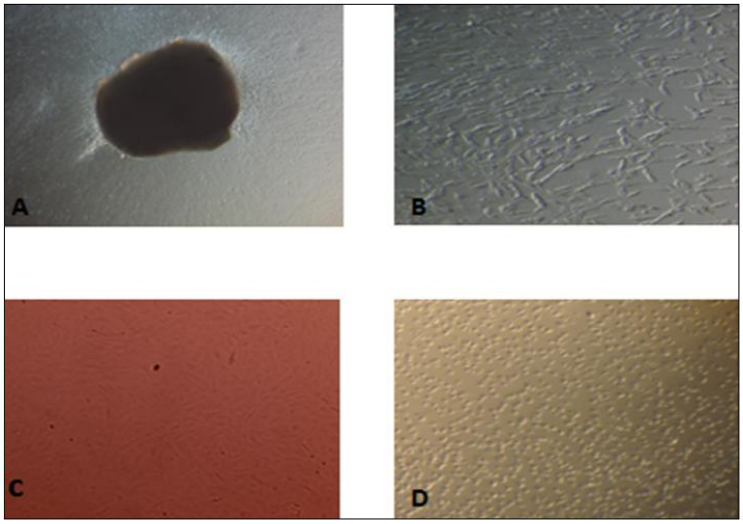

Fig 2: Prepration of donor cells (A) Tissue explant –ear cartilaginous tissue (B) cellular attachments with flask surface (C) 80-90 % Confluent culture (D) trypsinised cells

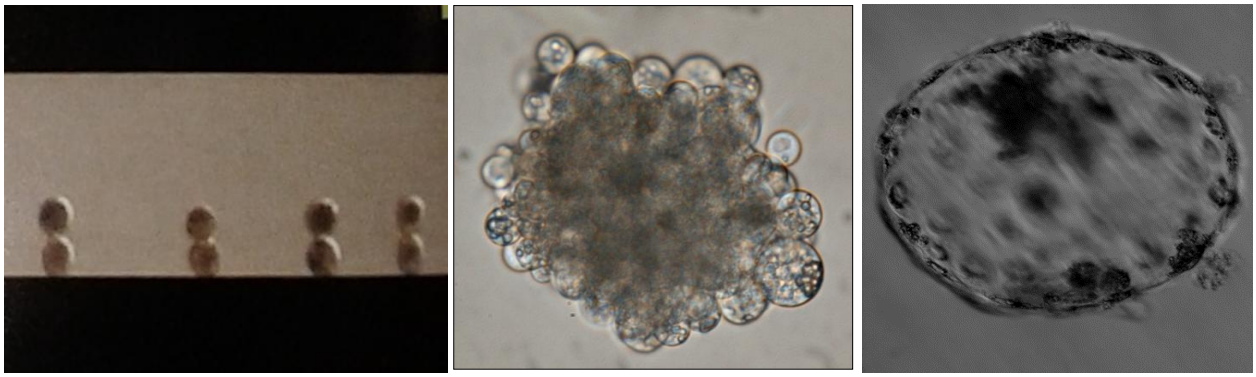

Fig 3: (A) Zona-free triplet fusion (somatic cell sandwiched between two zona-free oocyte cytoplasts (B) morula at day 5 (C) blastocyst at day 7

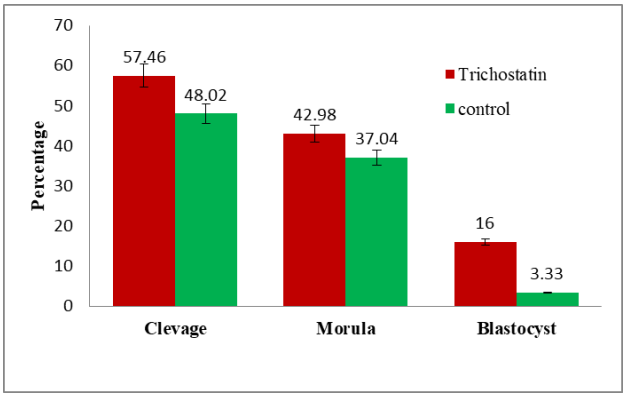

Fig 4: Differential Cleavage, morula and blastocyst rates in trichostatin A treated and control HMC embryo groups

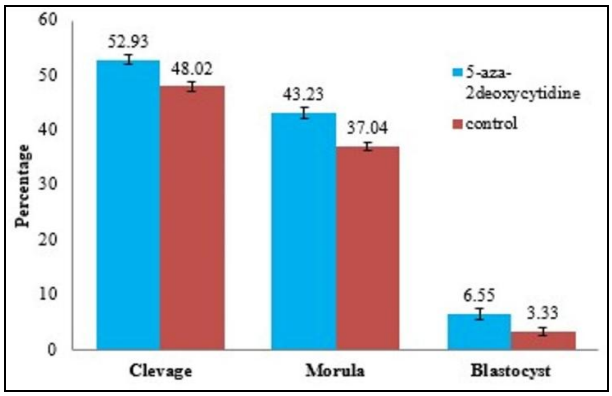

Fig 5: differential cleavage, morula and blastocyst rates in 5-aza-2 deoxycytidine

5. Conclusion

The overall study reveals that both trichostatin A and 5-aza-2-deoxycytidine are positive reprogramming enhancers with their ability to increase the mean cleavage, morulae rate and blastocyst rate. Trichostatin A was found to be more potent reprogramming enhancer than 5-aza-2-deoxycytidine.

6. Acknowledgements

This study was carried out with the funding support of DBT, Govt. of india. The authors acknowledge the partial support and facilities provided by the department of Biotechnology, Shere-Kashmir University of Agricultural Science and Technology, Shuhama, Alusteng, Srinagar, Jammu and Kashmir, India.

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