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Improved *in vitro* developmental rates of cloned sheep embryos by Trichostatin A supplementation of embryo culture media

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Abstract
Animal cloning is one of the latest and most fascinating modern biotechnological technique with potential applications in biomedical and agricultural fields which include creation of disease models and bioreactors, propagation of elite germplasm of animals, conservation of endangered animal species and for studying early embryonic development. The efficiency of *in vitro* embryo development is usually low. Various modifications in cloning techniques and composition of embryo culture media have been made with an aim to increase *in vitro* developmental rates of cloned embryos. Certain supplementations and additions to *in vitro* maturation media and embryo culture media can potentially increase the efficiency of animal cloning, Trichostatin A being one of such supplements. This study was carried out to evaluate the effect of Trichostatin A supplementation (100nm/ml) in IVC media on *in vitro* development of cloned sheep embryos. It was observed that TSA significantly ($P<0.05$) improved cleavage percentage (79.25% v 68.18%). Morula/Blastocyst percentages also were significantly ($P<0.05$) higher in TSA supplemented IVC medium (56.25% v 48.21%).

Keywords: developmental rates, cloned sheep embryos, Trichostatin, upplementation, embryo culture media

1. Introduction
IVEP is one of the most powerful tools in controlling and manipulating mammalian reproduction (Cognie *et al*., 2003) [2]. *In vitro* embryo production is rendered important in sheep as it has shown the potential of producing sheep embryos, even during the non-breeding season (Pugh *et al*., 1991) [7]. The improved IVEP technologies of *in vitro* maturation (IVM) and *in vitro* culture (IVC) have further led to another generation of reproductive techniques, such as intra cytoplasmic sperm injection (ICSI), the production of transgenic animals and improved efficiency of animal cloning procedures.

Cloning of farm animals has not been applied on commercial scale as successfully as it could have been due to various factors like lower efficiencies in terms of low pregnancy rates and low live birth rates and also complicated by the use of costly equipment as well as high level of skill requirement. Notwithstanding the enormous potential of cloning by nuclear transfer, there are numerous bottlenecks in the actual implementation of these applications. These include low pregnancy rates and low efficiency (2 to 7%) in terms live offsprings born which could be due to inefficient reprogramming (Vajta and Gjerris, 2006) [12] and also due to higher incidences of fetal losses after embryo transfer and implantation (Paramio and Izquierdo, 2014) [6]. Cloning is still a technique which is evolving since last couple of decades and significant improvement has been brought about in processes involved in cloning like *in vitro* maturation, optimisation of activation and electrofusion techniques and *in vitro* culture techniques but the overall procedure is still far from being refined and optimised, so there is ample scope for investigation in the field of cloning in farm animals. The efficiency of *in vitro* embryo development is usually low. Various modifications in cloning techniques and composition of embryo culture media have been made with an aim to increase *in vitro* developmental rates of cloned embryos. Certain supplementations and additions to *in vitro* maturation media and embryo culture media can potentially increase the efficiency of animal cloning, Trichostatin A being one of such supplements. This study was carried out to evaluate the effect of Trichostatin A supplementation (100nm/ml) in IVC media on *in vitro* development of cloned sheep embryos.

2. Materials and Methods

2.1 Oocyte collection

Abattoir derived ovaries from different breeds of sheep slightly above three years of age were collected in warm isotonic saline (32-37 ˚C) and transported to the laboratory within 2-4 h. After removing the extra tissue around ovaries, they were washed 3-4 times with warm isotonic saline (32-37 ˚C) containing 400 IU/ml penicillin and 500 μg/ml streptomycin. Oocytes from follicles (2 to 8 mm in diameter) were harvested by puncturing with an 18-gauge needle. The collection medium consisted of calcium and magnesium-free Dulbecco's Phosphate Buffered Saline (DPBS) containing 0.3% bovine serum albumin (BSA). Oocytes were washed two to four times with the washing medium which consisted of Hepes buffered tissue culture medium (M-199) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Canada), 0.68 mM L-glutamine, 0.8 mM sodium pyruvate and 50 μg/ml gentamicin. The aspirated oocytes were graded according to the following criteria:

Usable quality: Grade-A and Grade-B compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having ≥2 layers of cumulus cells, and with homogenous, evenly granular ooplasm.

Unusable quality: Grade-C and Grade-D oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm.

Oocytes of only usable quality were used for *in vitro* maturation.

2.2 *In vitro* maturation of oocytes

Oocytes of usable quality were selected and after washing thrice with IVM medium (Hepes buffered M-199 supplemented with FBS, 5 μg/ml pFSH, 1 μg/ml17β-estradiol, 0.8 mM sodium pyruvate and 50 μg/mlgentamicin 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% CO_2 with maximum humidity at 38.5 ^{o}C.

3. Preparation of donor cell line

3.1 Establishment of somatic cell cultures

3.1.1 Primary fibroblast cell cultures from adult sheep

Ear tissue samples from adult sheep were collected and transported to the laboratory in Dulbecco's Phosphate Buffer Saline (DPBS) containing 10% 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 ^{o}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.

3.1.2 Sub-culturing the cells

When a monolayer of the cells (60-70% confluent) was formed, the cells were ready for subculture. The cells were washed with Ca^{++} and Mg^{++} free DPBS for 5 min. After decanting the supernatant, the cells were overlaid with Trypsin-EDTA (0.25%) and incubated for a few minutes, till the cell monolayer started dissociating from each other and the cell culture dish. In the primary cell cultures, the monolayer was trypsinized till the separation of different cells from each other. This was in view of the fact that in primary cell cultures, morphologically different cells had established. A selected population of the cells was required to be chosen at this stage. The passaged or sub cultured cells were observed for *in vitro* growth for about 12h. The dead cells were removed and anchored cells were allowed to grow further. The cells were passaged at 80%-90% confluence. The cells between 5-9 passage levels were used for cloning (both zona-free and zona-included) experiments.

3.2 Preparation of donor cells for nuclear transfer

The confluent cells (adult sheep fibroblasts) between 5-9 passage levels were allowed to grow further upto 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 wi 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 resuspended 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.

3.3. Production of reconstructs by zona-included (SCNT) technique

In-vitro matured COCs were cultured in 50-ml drops of maturation medium covered with an overlay of mineral oil and incubated at 38.5 ˚C-39 ˚C in 5% CO_2. After 23–24 h of maturation, the cumulus cells were removed from the matured oocytes by incubating the COC's in warmed 1 mg/ml hyaluronidase and then vortexing the COCs for 2–-3 min in 1 mg/ml hyaluronidase. The denuded oocytes were washed in T20 and were returned to maturation medium. Enucleation were done using micromanipulators (Narishige Japan) in 4mL T20 containing 2.5 µg/mL cytochalasin B, the enucleation process were initiated within 2 hours of oocyte denuding.

Following enucleation by micromanipulation, a somatic cell is taken up in the polar body biopsy pipette and inserted into the enucleated oocyte through the puncture made for enucleation. The reconstructs (enucleated oocyte-somatic cell complex) were transferred to fusion medium (0.3M d- mannitol, 0.1mM MgCl2, and 0.05mM CaCl2 and 1mgmL−1 polyvinyl alcohol) for 5 min equilibration. The reconstructs were subjected to electrofusion (BLS CF-150BBLS Budapest Hungary) with an A.C. pulse (6 V) and a single D.C. pulse (2.96kVcm−1 for 10µs) were applied. The reconstructs were then incubated in T20 (for rounding up and subsequent reprogramming) for 1 hour at 38.5 ˚C.

3.4 Activation and culture of reconstructs

The reconstructed oocytes of sheep were activated by incubating in T2 containing 5µM calcium ionophore for5min at 38.5 ˚C. After washing thrice with T20 the zona free reconstructed oocytes were individually transferred to droplets of T20 containing 2mM of 6-dimethylaminopurine (6- DMAP) and zona included reconstructs were transferred to 100µL drops of T20 containing 2mM of 6-dimethylaminopurine (6- DMAP) in groups of 20-25 and both

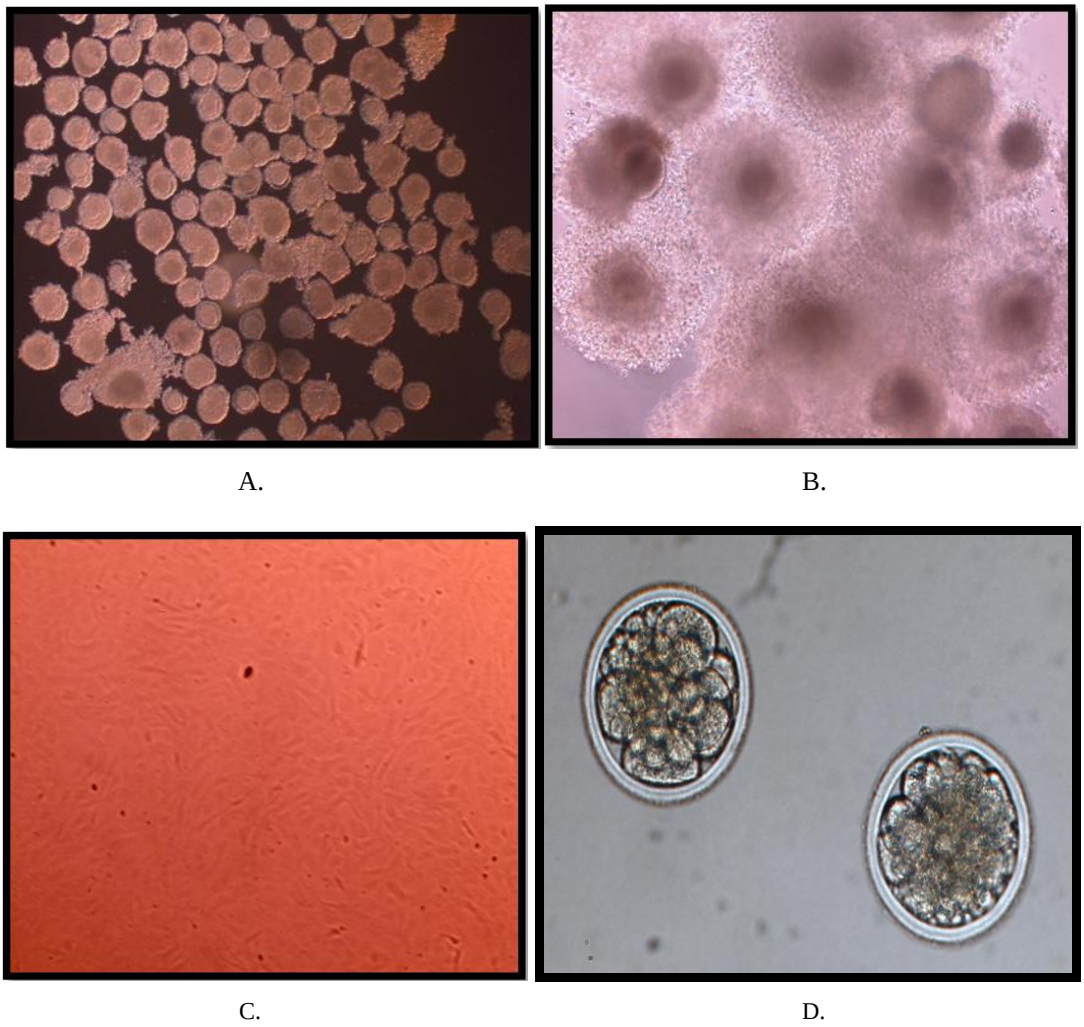
were then covered with mineral oil and incubated for 3-6 hours in a CO_2 incubator at 38.5 ˚C. The reconstructed, activated embryos were then transferred to 500 µL of Research Vitro Cleave medium (K-RVCL-50, Cook®, Australia) supplemented with fatty acid free bovine serum albumin (BSA), B27, EGF, cysteamine and100 nm/ml Trichostatin A(TSA) in 4-well dishes covered with mineral oil and kept undisturbed in a CO2 incubator for 7 days. TSA was not added to control IVC media to facilitate compariso

3.5 Statistical Analysis

The data obtained from the experiments was analysed using software package SPSS 20.0 (IBM SPSS Statistics). The differences between means were analysed by one way ANOVA followed by Fisher's LSD test and significance was determined at $P<0.05$. For analysing the effect of Trichostatin A, the data was analysed by unpaired t-test significance was determined at $P<0.05$.

4. Results

Eight (08) such cloned embryo production experiments were carried out. 103 presumed cloned reconstructed embryos were cultured in IVC supplemented with Trichostatin A and 72 embryos were cultured in same medium without the addition of Trichostatin A, which served as control.



A. B. C. D.

Fig 1: (A) Oocytes of different grades (B) *In-vitro* matured oocytes (C) Confluent Monolayer of sheep fibroblasts (D) Developed Morula on Day 5 of IVC supplemented with TSA

Cleavage rate of embryos cultured in Trichostatin A supplemented IVC was 79.75 ± 2.95 which was significantly higher ($P<0.05$) than that of embryos cultured in control medium (68.18 ± 2.80) (Table-7, Fig.6)

2-4 Cell and 8-16 Cell stage embryo percentages were numerically higher in control medium as compared to Trichostatin A supplemented medium (19.57 ± 1.15 v14.55 ± 1.77 and 32.21 ± 1.52 v 29.19 ± 2.94), although the difference was not significant ($P<0.05$).

Table 1: Effect of Trichostatin A in IVC media on *in vitro* development of cloned sheep embryo production

Medium	No. Activated	Cleaved	2-4 Cell	8-16 Cell	Morula + Blastocyst
Treatment	103	79.75 ± 2.95[a] (82)	14.55 ± 1.77 (12)	29.19 ± 2.94 (24)	56.25 ± 2.17[a] (46)
Control	72	68.18 ± 2.80[b] (50)	19.57 ± 1.15 (10)	32.21 ± 1.52 (16)	48.21 ± 1.78[b] (4)

Figures quoted as percent mean ± s.e.m (n).

Morulae and Blastocyst percentages calculated out of total number of cleaved embryos

Values having different superscripts along columns, differ significantly ($P<0.05$)

Morula + Blastocyst rate of embryos cultured in Trichostatin A supplemented IVC was 56.25 ± 2.17 and that of embryos cultured in control medium was 48.21 ± 1.78. The difference between two being statistically significant ($P<0.05$).

5. Discussion

The overall efficiency of animal cloning by nuclear transfer remains still low, one of the underlying causes being inefficient nuclear reprogramming (Zhao *et al*., 2010) [14]. The mechanisms underlying nuclear reprogramming are also not entirely clear, although various research studies on this topic are in progress. The reprogramming processes of the transferred somatic nucleus into enucleated oocytes include epigenetic events. One of these epigenetic modifications is the acetylation of histones (Turner, 2000) [11], which is known to be a critical factor for successful reprogramming during SCNT (Armstrong *et al*., 2006) [1]. Histone acetylation decreases the affinity of histone proteins to DNA sequences by neutralizing the positive charge of the histone tails (Hong *et al*., 1993) which in turn, facilitates transcriptional processes (Horn and Peterson, 2002) [3] because a more extended and open chromosomal structure provides more access to transcriptional regulatory proteins to their target sequences (Vettese-Dadey *et al*., 1996) [13]. Histone acetylation also facilitates better genome imprinting (Turner, 2000) [11]. Therefore, it appears that successful reprogramming of histone acetylation patterns could play crucial role in cloned embryo development.Histone acetylation is catalyzed by histone acetyl transferases (HATs), whereas the opposing reaction is controlled by histone deacetylases (HDACs). So one of the methods to increase acetylation is to inhibit HDACs which could be done by compounds such as Trichostatin A (TSA) and Valproic acid etc.

Keeping this in our vision, we conducted a few experiments to investigate the effect of Trichostatin A supplementation in IVC media on parthenogenetic sheep embryo production.

50 nM TSA was added to *in vitro* culture media of parthenogenetically produced embryos. A significantly higher cleavage rate was observed in treatment group as compared to control group (79.75 v 68.18%). Similar increase of cleavage rate (72.4 v 68.9%) was obtained by Hu *et al*., 2012 [4] in SCNT sheep embryos, Sameic and Skrzyszowska, 2012 [8] and Jeong *et al*., 2013 [5] in SCNT pig embryos (44.2 v 34.5% and 93.2 v 66.3%, respectively).

Similarly, the Morula/Blastocyst percentage also was significantly higher in treatment group as compared to control group (56.25 v 48.21). Hu *et al*., 2012 [4] also observed an improved blastocyst rate with TSA supplementation in IVC of SCNT cloned sheep embryos (23.3 v 16.7%). Jeong *et al*., 2013 [5] also reported a higher blastocyst rate in TSA supplemented IVC (22.0 V 8.9%) in pigs. Similarly, Sameic and Skrzyszowska, 2012 [8] were able to achieve higher Morula/Blastocyst percentage (70.7 v 48.9%) in TSA supplemented IVC for cloned porcine embryos.

6. Conclusion

Our findings confirm the positive effect TSA could possibly have on improving *in vitro* development of sheep embryos which were produced parthenogenetically, the effect of TSA on cloned sheep embryo production could not be investigated in current study due to constraints of time during which the current master's research was to be completed. Nevertheless, it could be done in future.

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