EFFECTS OF LOCATION OF SOMATIC CELL NUCLEAR TRANSFER ON EPIGENETIC MODIFICATION AND PREIMPLANTATION DEVELOPMENT OF CLONED BOVINE EMBRYOS

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SUMMARY

Somatic cell nuclear transfer (SCNT) is a high-potential technique in biological science. However, it struggles with low successful rates and errors in somatic cell reprogramming, which is suggested by different densities of cellular components such as protein regulation and growth factors in the cytoplasm of oocytes. This study aimed to investigate the effect of different somatic cell nucleus injection sites on reprogramming mechanisms of cloned bovine zygotes during the first cell cycle and embryonic development during preimplantation. The research examined the injection angles of 180° and 90° compared to the 0° group, which is located near the enucleation site. Results showed that the blastocyst rates were 27.08% for the 0° group, 22.33% for the 90° group, and 23.67% for the 180° group ($P>0.05$), with no significant differences in development rates. Immunofluorescent staining showed intensity levels of H3 lysine 9 acetylation (acH3K9) on pronucleus formation were 23.69, 17.32, and 19.3 for the 0°, 90°, and 180° groups, respectively ($P>0.05$), and insignificant differences in acH3K9 intensity between injection sites. Notably, although the pronuclei were established after nuclear transfer in all groups, some groups contain the second pronuclei, which might come from the appearance of a metaphase-like structure or premature chromosome condensation. In conclusion, changing the injection location of SCNT had a tiny effect on development during the preimplantation of a cloned bovine embryo as well as on histone acetylation expression of the cloned bovine zygote at the first cell cycle.

Keywords: Bovine, epigenetic modification, injection location, preimplantation, Somatic Cell Nuclear Transfer, SCNT.

INTRODUCTION

The first success of somatic cell nuclear transfer (SCNT) on Dolly the sheep in 1996 (Wilmut *et al*., 1996) marked a significant breakthrough in cell reprogramming and mammalian cloning. Even though numerous successful cloned mammalian species were born, SCNT still contains several challenges, relating to low success around 30% (So *et al*., 2020). This low development rate in nuclear transfer (NT) embryos is caused by many factors, such as aberrant epigenetic reprogramming, abnormal spindle formation, etc. (Srirattana *et al*., 2022). Although cells can correct errors and reprogram themselves by epigenetic modifications (Morgan *et al*., 2005), the nucleus might be not located in optimal positions for reprogramming, which leads to a decrease in blastocyst formation rate.

The density of cellular components varies across different cytoplasmic locations (Li *et al*., 2014). Major cell cycle regulators, such as maturation-promoting factor (MPF), are concentrated around the spindle poles, and mitogen-activated protein kinase (MAP kinase) is associated with the spindle microtubules (Tani *et al*., 2017). These substances play critical roles in chromosome formation and the reprogramming of the somatic cell nucleus when transferred into oocytes. However, there is limited information about the impact of them, on genetic alterations and growth during preimplantation in cattle.

Therefore, this study aims to unravel the mechanisms underlying reprogramming during cloning, with an emphasis on significant factors that affect the development of cloned bovine embryos, and to determine whether injecting the somatic cell nucleus into different sites could improve embryonic development. Moreover, the research seeks to provide insights that could improve SCNT efficiency and outcomes in agriculture.

MATERIALS AND METHODS

Nuclear transfer of bovine fibroblast cell nuclear into different locations of the enucleated bovine oocyte

Donor bovine fibroblast cells were isolated from lung and ear tissues, which were cleaned with ethanol and phosphate-buffered saline (PBS) and then cut into small pieces. These tissues were cultured in a medium

containing high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS) at 38.5°C with 5% CO2 for 8 to 10 days. At 80% confluence of fibroblast, they were passaged to new dishes with DMEM high glucose, 10% FBS, 0.07 mg/mL penicillin, and 0.05 mg/mL streptomycin.

For invitro maturation of bovine oocytes, the cumulus-oocyte complexes (COCs) were cultured in the incubator with 38.5°C and 5% of CO2 in in-vitro maturation droplets, including TCM 199 medium supplement with 10% fetal bovine serum (FBS), 50 μg/mL of sodium pyruvate (Napy), 50 ng/mL of epidermal growth factor (EGF), 0.0065 IU/mL of luteinizing hormone (LH), 0.006 IU/mL of follicle-stimulating hormone (FSH), and 1 μg/mL of estradiol (ES) and covered by mineral oil for 19 to 20 hours. Matured bovine oocytes which contain the first polar body appearance were removed cumulus cells, by using hyaluronidase which was used for the enucleation step.

For enucleation, the matured bovine oocytes were placed in HEPES buffer with 5 μg/mL Cytochalasin B, and the metaphase II spindle was identified by the "metaphase corn" extrusion (Figure 1A). Then by using an XYClone Laser (Hamilton Thorne), a small area on zona pellucida was cut, and the cytoplasm containing chromosomes was removed (Figure 1B-D).

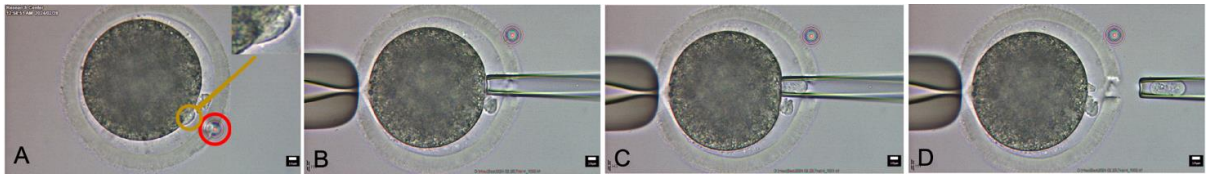

Figure 1. Enucleation process of matured bovine oocytes

A: Cutting zona pellucida by XY-laser. B, C: Removing nuclear inside oocyte by enucleation pipette. D: Enucleation of oocyte and cytoplasm which contain metaphase II spindle (metaphase corn). The red cycle is the location of the XYClone Laser. The yellow cycle is the location containing metaphase corn. Bar = 10 μm.

Following the enucleation step, the matured bovine oocytes were divided into three groups: the 0° group had fibroblast nuclei injected through the enucleation entrance and marked as the control group (Figure 2A), the other two groups had new holes drilled at 90° (Figure 2B) and 180° (Figure 2C) from the enucleation site for nuclei injection. After nuclear transfer, the reconstructed bovine oocytes were activated with 5 μm Ionomycin and treated with 5 mM 6-Dimethylaminopurine (6-DMAP), before being cultured in modified synthetic oviduct fluid (mSOF) at 38.5°C and 5% CO_2 until reaching the blastocyst stage.

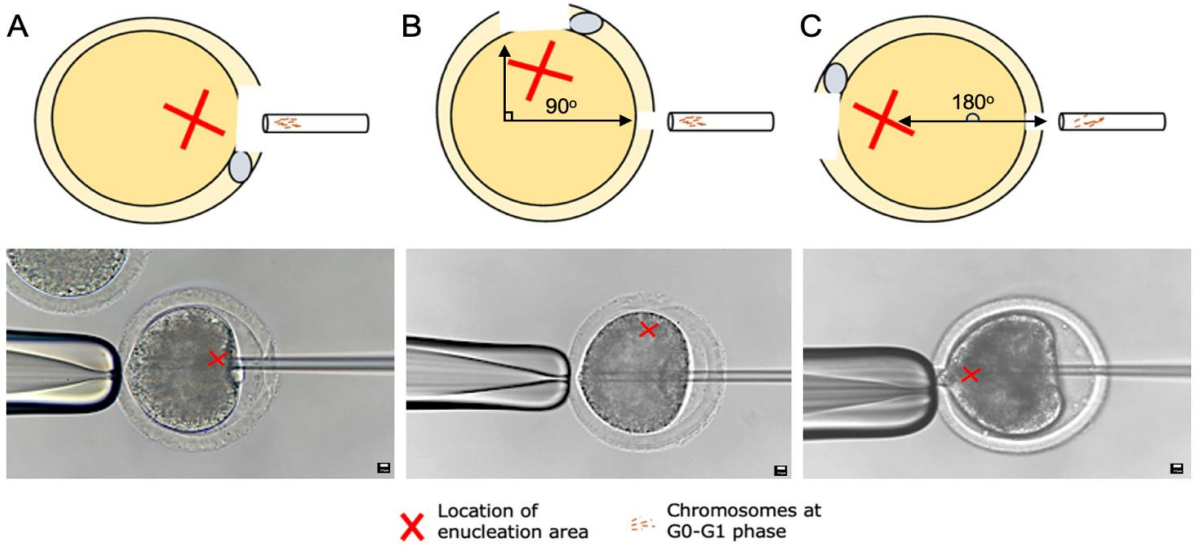

Figure 2. Nuclear transfer process in different locations into the enucleated bovine oocyte.

A: Injecting fibroblast nucleus into enucleation oocyte of 0° group, enucleated site. B: Injecting fibroblast at 90° group, compared to enucleated site. C: Injecting fibroblast at 180° group, compared to enucleated site. Red cross: Location of enucleation area. Scalebar = 10 μm.

Evaluation of preimplantation development of cloned bovine embryo

After the nuclear transfer process, all samples were classified into 3 groups which were marked as "0°", "90°", and "180°". After several days of culturing, in each cell cycle stage, the blastocyst formation rate of this stage was estimated and compared with the number of reconstructed oocytes developed from the beginning. After reaching the blastocyst stage, the number of completed blastocysts in each group was harvested and analyzed.

Evaluation of expression of histone acetylation during the first cell cycle of cloned bovine pronuclei

After the nuclear transfer process, reconstructed oocytes were placed in each mSOF droplet, and tagged based on the injection location, and then, a few from inside each droplet were selected randomly for immunofluorescent staining. At 10 hours post-activation, cloned bovine zygotes were transferred to individual wells, stained by anti-histone H3 acetyl-lysine 9 antibody (anti-H3K9ac, rabbit) with Alexa Fluor 488-labeled chicken anti-rabbit IgG (green color), as first and second antibody, and 4′,6-diamidino-2-phenylindole (DAPI, blue color) for immunostaining. An inverted microscope Nikon (Nikon Eclipse Ti) with UV light was then used to observe dye removal from each pronucleus, and histone modification levels were analyzed based on acH3K9 intensity level.

Data analysis

The experiment was conducted 3 times for reliable results. Besides, the number of blastocysts in each group was counted and presented as a percentage. Statistics were analyzed by using one-way ANOVA in IBM SPSS Statistical Software (version 20), with a P-value less than 0.05 identifying a statistically significant difference. Fluorescence data was detected from analytical results using the BR analysis NIS-Element BR Analysis 4.500.000 64-bit Program and calculated based on the average pixel values of the area within the nucleoplasm, excluding nucleoli. Fluorescence intensity in nuclear-transferred embryos was deliberated in comparison to the average intensity of control embryos.

RESULTS AND DISCUSSION

Effect of altering injection position on the development of cloned bovine embryo at blastocyst stage

The development rate of the 2-cell stage was about 81.69% at the 0°group, 74.67% at 90°, and 76.36% at 180°. These percentages gradually decreased through the 4-cell stage (76.64%, 65.27%, and 67.36%), the 8-cell stage (62.09%, 56.59%, and 55.64%), the morula stage was 39.6% (0°), 35.67% (90°), and 38.12% (180°). After seven days, the blastocyst formation rate dropped down in two-thirds, compared with the first stage, which was 27.08% for the 0° group, 22.33% for the 90° group, and 23.67% for the 180° group (Table 1). However, statistical analysis showed no significant differences between each experimental group (P>0.05).

Table 1. Effect of changing different injection locations on development of cloned bovine embryos during preimplantation

Group	Percentage of the embryo development at each stage of preimplantation (%)				
	2-Cell	4-Cell	8-Cell	Morula	Blastocyst
0°	(81.69%)[a]	(76.64%)[a]	(62.09%)[a]	(39.60%)[a]	(27.08%)[a]
90°	(74.67%)[a]	(65.27%)[a]	(56.59%)[a]	(35.67%)[a]	(22.33%)[a]
180°	(76.36%)[a]	(67.36%)[a]	(55.64%)[a]	(38.12%)[a]	(23.70%)[a]

It can be observed that the blastocyst rate of 90° and 180° groups was around 23%, and 27.08% at 0° group as well as insignificantly different in each. Notably, this rate dropped by about 70% compared to the 2-cell stage, like previous blastocyst development rates in our laboratory. Moreover, there was a study, by Tani T and Kato Y (2017), focused on the relationship between the metaphase spindle apparatus MPF and MAP kinase, and they found that MPF concentrated around spindle poles and MAP kinase associated with spindle microtubules, indicating the importance of spindle structure. However, MPF and MAP kinase did not regulate reprogramming directly (Tani T, Kato Y, 2017). This means, that although the loss of MPF and MAP kinase caused the drop-down in the successful rate of SCNT, it only caused the minimal impact on the development of cloned embryos. Besides, even though the oocyte's growth factor was different in each area inside cytoplasm and tended to decrease from the cell nucleolus to cytoplasm in quantity, the experiment in treating porcine oocyte enucleation with demecolcine showed that after cyclin B1 levels increased and distributed evenly throughout the cytoplasm, the developmental rates of embryos from 2-cell stage to blastocysts stage were similar (Li *et al.*, 2014). This aligns with research in duplicate amounts of cytoplasm inside donor oocytes before nuclear transfer, indicating higher cytoplasm and growth factors do not significantly affect cloned embryo development (Sayaka *et al*., 2008). For those, this study suggests that although there was a difference in the density of material in the cytoplasm of enucleated oocyte, changing injection location on SCNT has a small impact on the development of cloned bovine embryos during preimplantation.

Effect of altering injection position on the expression of histone acetylation on pronuclei at first cell cycle

The intensity level of histone acetylation expression on pronuclei on average was 23.69 for the 0° group, 17.32 for the 90° group, and 19.3 for the 180° groups (Figure 3E). However, the acetylation expression levels of histone H3-Lysine 9 were insignificant across all experimental groups (P>0.05).

Immunofluorescent staining during the first cell cycle, by using acH3K9 based on the function it in an expression of histone acetylation on cloned bovine decondensed zygote at 10 hours post-activation, to assess how different areas in enucleated oocytes' cytoplasm affect histone acetylation. Immunofluorescent staining intensity levels were analyzed the expression level of histone acetylation on cloned samples. Notably, the formation of 1-pronuclei and 2-pronuclei was observed in all experimental groups, including the 0° group (Figure 3A-D). The

intensity levels in our pronuclei ranged from 15 to 26, like previous research on bovine embryos which persuaded the normal development of cloned bovine embryos after SCNT, indicating a consistent cytoplasmic impact on histone modification (Wu *et al*., 2010), then, it might be estimate that there was not abnormal appearance in our formation of 1 pronuclei after changing injection location. In the case of 2-pronuclei formation, on the other hand, there was a study, by Van Thuan N and others (2006), found that abnormal donor chromosome separation caused monopolar and bipolar spindle formation in mouse SCNT because of the long waiting time from the nuclear-transferred process to the activation process. Monopolar spindles fail to orient and separate chromosomes properly, leading to chromosomal abnormalities and reduced embryo survival (Thuan *et al*., 2006). Additionally, as the appearance of premature chromosome condensation (PCC), which was unnecessary for reprograming, at 4 to 6 hours after activation, the generation of scattered chromosomal clusters was completed and led to the formation of two or more nuclear structures after 12 hours post-activation (Sung *et al*, 2007). Based on those, our staining results suggest that some injected oocytes might have reached metaphase-like structures or PCC in post-nuclear transfer, causing visible abnormalities in the appearance of pronuclei after 10 hours of culturing, indicated by particles binding with anti-acetyl histone H3 lysine 9 (Figure 3D).

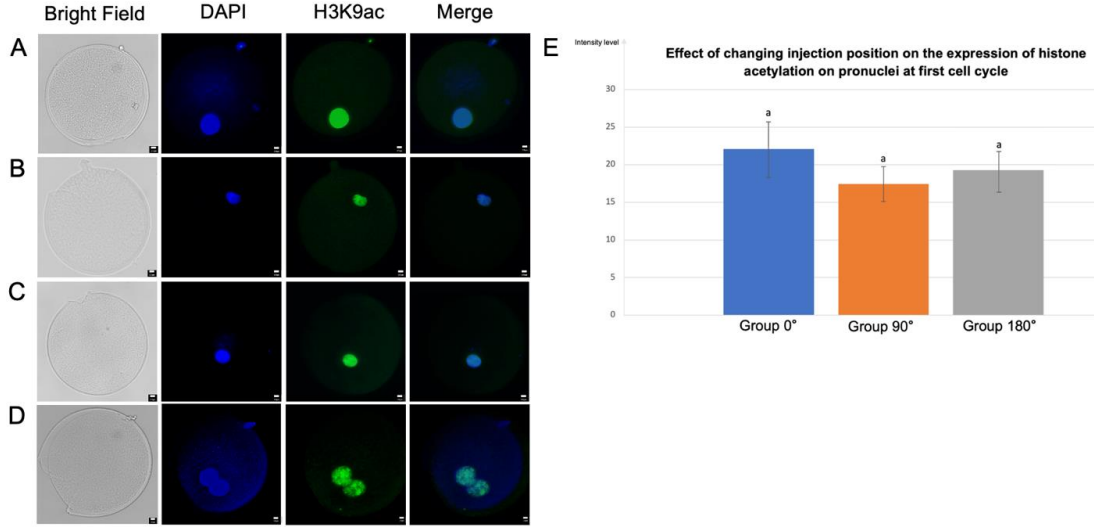

Figure 3. Effect of changing different injection locations on histone acetylation expression on cloned bovine zygote during the first cell cycle, at 10 hours post activation

A-C: Immunostaining of 1-pronuclei formation group. A: Immunostaining of group 0°. B: Immunostaining of group 90°. C: Immunostaining of group 180°. D: Immunostaining of 2-pronuclei formation group. E: Intensity level of histone acetylation expression on pronuclei formation of cloned bovine zygote at first cell cycle. Scalebar = 10 µm.

While this study showed the insight in effect of altering injection location on preimplantation, it was lacking by the limitation of clarifying the mechanisms by which the nuclear injection site affects somatic cell reprogramming, as well as the development of further stage during post-implantation. Hence, further research should extend the evaluation of embryo development beyond the blastocyst stage by monitoring growth and survival after embryo transfer into the bovine uterus, providing deeper insights into long-term viability. Additionally, assessing embryo quality through techniques such as fluorescent staining could help evaluate chromosome distribution, cell morphology, and apoptosis levels, offering a more comprehensive understanding of developmental potential. Furthermore, performing gene expression analysis by new generation sequencing would be crucial to determine how the nuclear injection site influences genes involved in somatic cell reprogramming and embryo development, potentially uncovering key molecular mechanisms that affect cloning success.

CONCLUSION

In conclusion, this research aims to address the effect of altering location in injection on SCNT. The result showed that changing injection locations during somatic cell nuclear transfer had insignificant impacts on both preimplantation development on cloned bovine embryo and histone modification during the first cell cycle, indicating by the percentage of blastocyst during the final stage in preimplantation and intensity level of histone expression during zygote stage. However, this research still provided limited details in mechanism of nuclei injection effect on development. Hence, the future research should explore the expression of each gene in development as well as the interaction between maternal cytoplasm and somatic cell nuclei after SCNT to show the process which impact on preprogramming and development after changing injection location. Besides, the research should apply this technique for post-implantation to evaluate the development of newborn being in the future.

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ẢNH HƯỞNG CỦA VỊ TRÍ CHUYỂN NHÂN TẾ BÀO SINH DƯỠNG LÊN QUÁ TRÌNH THAY ĐỔI BIỂU SINH VÀ PHÁT TRIỂN CỦA PHÔI BÒ NHÂN BẢN VÔ TÍNH Ở GIAI ĐOẠN TIỀN LÀM TỔ

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TÓM TẮT

Chuyển nhân tế bào soma (SCNT) tạo động vật nhân bản là một kỹ thuật tiên tiến trong lĩnh vực công nghệ sinh học của thế kỷ 21. Tuy nhiên, kỹ thuật này vẫn còn rất nhiều hạn chế, như các vấn đề trong quá trình tái lập trình tế bào soma, cũng như những khác biệt về mật độ của các thành phần tế bào và các yếu tố tăng trưởng trong bào tương của trứng, từ đó dẫn đến việc giảm tỷ lệ thành công tạo ra động vật nhân bản thông qua kỹ thuật SCNT. Nghiên cứu này đã kiểm tra các tác động do thay đổi vị trí tiêm của nhân tế bào soma lên sự hình thành tiền nhân cũng như mức độ acetyl hóa histone protein của hợp tử bò được nhân bản trong chu kỳ tế bào đầu tiên và sự phát triển phôi trong giai đoạn tiền làm tổ. Nghiên cứu đã xem xét các góc tiêm ở vị trí 180° và 90° so với nhóm ở gần vị trí lấy nhân (0°). Kết quả cho thấy tỷ lệ phôi nang là 27,08% đối với nhóm 0°, 22,33% đối với nhóm 90° và 23,67% đối với nhóm 180° (P>0,05), và không có sự khác biệt đáng kể nào về tỷ lệ phát triển tiền làm tổ của phôi bò nhân bản. Bên cạnh đó, kết quả nhuộm huỳnh quang miễn dịch cho thấy mức độ acetyl hóa lysine 9 trên histone H3 (acH3K9) trong quá trình hình thành tiền nhân lần lượt là 23,69, 17,32 và 19,3 đối với các nhóm 0°, 90° và 180° (P>0,05), và không có sự khác biệt đáng kể về cường độ acH3K9 giữa các nhóm SCNT với các vị trí khác nhau. Ngoài ra, mặc dù đa số các nhóm thí nghiệm đều xuất hiện một tiền nhân sau khi chuyển nhân, một số ít còn lại có hiện tượng xuất hiện tiền nhân thứ hai, nguyên nhân có thể xuất phát từ những trứng bò đã chuyển nhân sớm và DNA của tế bào soma đã ở trạng thái ngưng tụ thành nhiễm sắc thể. Kết luận, tiêm nhân của tế bào sinh dưỡng vào trứng bò đã lấy nhân tại những vị trí khác nhau so với vị trí MII của trứng không ảnh hưởng đến sự phát triển của phôi bò nhân bản trong giai đoạn tiền làm tổ cũng như biểu hiện acetyl hóa histone của hợp tử bò nhân bản ở chu kỳ tế phân bào đầu tiên.

Từ khóa: Bò, chuyển nhân tế bào sinh dưỡng, thay đổi biểu sinh, vị trí chuyển nhân, phát triển tiền làm tổ, SCNT.

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