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in vitro DEVELOPMENT OF CLONED EMBRYO IN CATTLE IN RELATION WITH FUSION AND ACTIVATION PARAMETERS

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Abstract

Embryo production through somatic cloning technology has the perspectives for application in reproductive biotechnologies in cattle in order to multiply the most productive and unique genotypes in livestock breeding and create new genotypes using genome editing. Success of somatic cloning depends on the ability of donor somatic cell nucleus (karioplast) to be reprogrammed to totipotent state. Relevant transformations of donor nucleus are mediated by oocyte cytoplasmic factors (cytoplasts) and start from the moment of their association (fusion). Effects of oocyte cytoplasm are direct and depend on many factors. The objective of the present study was to evaluate the cloning efficiency in terms of time of oocyte cytoplasm exposure to donor nucleus before activation, the time of oocyte maturation before their activation in the fused complexes (cytohybrids), and repeated electrofusion of the cytoplast and karyoplast. The effects of these factors on formation of cloned embryos and development to blastocyst stage were studied. Isolated oocyte-cumulus complexes (OCCs) were in vitro matured in TC-199 medium supplemented with 10 % fetal bovine serum, 10 μg/ml of FSH and 10 μg/ml of LH. After 20-24 h of maturation, OCCs were treated with a 0.1% hyaluronidase, then cumulus cells were mechanically removed and the oocytes with the first polar body were selected. Long-time conserved fetal fibroblasts were in vitro cultured up to monolayer and maintained in contact inhibition during 2 days. Then, cell suspension was prepared for transferring into enucleated oocyte. Somatic cell was transferred to perivitelline space of the oocyte, and two consecutive rectangular 20 μs pulses at constant current with a voltage of 35 V were performed (once or twice if there were no signs of cell-oocyte fusion). The obtained cytohybrids were activated with the ionomycin 1 or 2 hours after fusion (recipient oocytes were matured either 23-25 hours or 26-28 hours). Activated cytohybrides were then cultured up to blastocyst stage. Oocyte cleavage rate were similar in all experimental groups (60.7 to 70.4 %). Blastocyst development rate did not differ between the groups where single or double fusions were performed (29.4±4.4 and 22.8±3.5 %, respectively). Blastocyst rate was 17.4±2.6% at 1-hour interval between fusion and activation. Two-hour interval increased blastocyst rate to 31.1±3.8% ($p < 0.05$). In the case of early activation (23-25 hours of maturation), 29.4±4.8% of fused complexes developed to the blastocyst stage. With an increase of oocyte maturation time to 26-28 hours, blastocyst rate decreased to 14.6±2.2% ($p < 0.05$). Therefore, cloning efficiency depends on the interval between cytohybrid fusion and activation, and the age of MII oocytes at the time of activation of the fused complexes; 2 hours and 23-25 hours, respectively, were the optimal parameters. In addition, the repeated electrofusion of the enucleated oocytes and somatic cells did not affect cytohybrid quality, and, therefore, this procedure can be used for somatic embryo cloning in cattle.

Keywords: cattle, somatic cell nuclear transfer, fusion, activation, embryo development

Modern reproductive cell technologies, in particular generation of Bovine cloned embryos, have broad prospects in multiplication of the most productive

and unique genotypes in pedigree animal husbandry [1-4], as well as in creation of new genotypes by genomic editing methods [5-9]. However, efficiency of high-quality embryo production through somatic cloning remains low, including in cattle, while abortion, perinatal mortality, and birth of low viable offspring are high, which inhibits practical application of this technology [10-12].

In somatic cloning, a female reproductive cell (oocyte), instead of its own chromosomal material, contains the injected nucleus of a somatic cell derived from an animal selected for genetic copying. In this, the epigenetic pattern of differentiated somatic cells is erased, and the embryonic epigenetic characteristics and gene expression patterns are restored to a totipotent embryonic state. The resulting cloned embryos with totipotent status are again able to differentiate into various types of somatic cells. This process involves various molecular and epigenetic modifications, on which the cloning efficiency ultimately depends, and is called nuclear reprogramming [13]. It is believed that changes of somatic cell nucleus (karyoplast) are mediated by the cytoplasm of oocyte (cytoplast) and start upon the karyoplast—cytoplast fusion [14]. The effects of cytoplasmic environment on nuclear reprogramming depend on many factors and, therefore, can be altered purposefully [15].

Available data on improving efficiency of cloning embryos are methodologically contradictory and require additional elaboration to select the optimal parameters for manipulations. So, there is no consensus on the time of donor nucleus exposure to oocyte cytoplasm. Whereas in some works, the development of cloned embryos in cattle requires a long exposure before activation [16], in others it is reported that excessive exposure is unfavorable [17]. It was shown that the percentage of development of cloned blastocysts decreases as the period between fusion and activation increases from 1 to 5 hours [18]. A number of authors regard the 2-2.5-hour interval optimal [19, 20].

Cytoplasmic maturation of the oocyte which integrates donor nucleus is no less important for generation of viable cloned embryos. A mature oocyte in metaphase II is regarded the most suitable recipient cell [21]. However, aging of mature mammalian oocytes, which negatively affects their quality and competence to further embryonic development, occurs in the absence of activating stimuli [22, 23]. Prolonged culture of a mature oocyte due to its late activation during donor nucleus transfer is probably also accompanied by a complex of intracellular processes called “oocyte aging” [22, 24]. However, this aspect of oocyte quality deterioration, unfortunately, has not yet been regarded when improving in vitro culture systems, the optimal age of mature oocytes for cloning has also not yet been determined [25].

Commonly, to generate cloned cattle embryos, oocyte in metaphase II (MII) stage of meiotic division is fully enucleated, and somatic donor cell is injected into perivitelline space of the cytoplast [21]. Upon completion of enucleation, the cytoplast and karyoplast are fused in a pulsed electric field with the breakdown of their membranes at the contact point. The in vitro development of cloned embryos directly depends on how efficiently the recipient and donor cells fuse and whether they remain viable during such aggressive manipulations. [26]. The reconstructed oocytes failed to fuse are again subjected to electrofusion to increase the yield of cytohybrids [27]. This may have an adverse effect on a cloned embryo development that is not yet elucidated.

This paper reports optimized parameters of cattle embryo cloning protocol

(i.e. the time during which the donor nucleus should be exposed to the cytoplasm of reconstructed oocyte prior to activation, and the timing of the oocyte maturation until activation in the cytohybrid). Also, for the first time, we showed no negative effects of a re-fusion commonly used to increase the yield of cytoplast-karyoplast complexes on in vitro development of cloned cattle embryos.

Our objective was to assess effects of fusion-activation interval, the age of MII oocytes at activation, and re-fusion on the efficiency of cloning in cattle, i.e. on embryo formation and development to blastocyst stage.

Materials and methods. In all experiments, except for specially indicated, reagents from Sigma-Aldrich (USA) were used. Oocytes and embryos were cultured at 38.5 °C, 90% humidity and a 5% CO_2 atmosphere; outside the incubator, all manipulations were carried out at 37 °C.

Preparation of donor cells. Cattle fetal fibroblasts were donor cells. Uteri of cows on day 55 of pregnancy was delivered to the laboratory, the uterine horn containing the fetus was treated with 70% ethanol, the extracted fetus was released from head, limbs, and internal organs. The resulting fetal tissue was washed repeatedly in phosphate-buffered saline (PBS) with antibiotics and an antimycotic (100 IU/ml penicillin, 100 μg/ml streptomycin, 100 ng/ml amphotericin), mechanically fragmented and treated with 0.25% trypsin solution for 30 min at 37 °C. Trypsin was neutralized with an equivalent volume of DMEM manipulation medium (Gibco, USA, Cat. No. 31966021) containing 5% fetal bovine serum (FBS) and gentamicin (50 μg/ml) (DMEM-M). Cell suspension was filtered through a sieve with a pore of 100 μm diameter and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed, the cell pellet was resuspended in DMEM-M medium, centrifuged again, after cultured to form a monolayer on 100-mm-diameter Petri dishes with DMEM growth medium (Gibco, USA, Cat. No. 31966021), supplemented with 15 % FBS, 1% non-essential amino acids (Gibco, USA) and 50 μg/ml gentamicin (DMEM-P).

To get enough fibroblasts, the primary cell culture was propagated by passaging the formed monolayer (1:4). Petri dishes, after the growth medium replacement with trypsin/EDTA solution (Gibco, USA), were incubated at 37 °C, the cell suspension was transferred to centrifuge tubes with DMEM-M to neutralize trypsin and centrifuged at 1500 rpm for 5 minutes The supernatant was removed, the pellet was resuspended and re-cultured on 100-mm-diameter Petri dishes with DMEM-P, as described above. After the second passage, the cells were frozen in DMEM (Gibco, USA, Cat. No. 31966021) with 40% FBS and 10% dimethyl sulfoxide (DMSO) and stored in 1 ml cryovials (Corning, USA) at 196 °C until use (for 8 years).

Seven days before somatic cloning, the frozen fibroblasts were thawed in cryovials in a water bath at 37 °C, centrifuged at 1500 rpm in tubes with 10 ml of DMEM-M, cultured in DMEM-P until complete monolayer formation, and allowed for 2-day contact inhibition to synchronize the cell cycle. The cells were suspended in TC199 medium containing 10% FBS and 50 μg/ml gentamicin (TC199-M) at most 30 minutes before transfer to an enucleated oocyte.

Preparation of recipient oocytes. Cows' ovaries delivered at 30-35 °C within 3-5 hours were released from surrounding tissues and washed many times in sterile saline with antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin). Cumulus-oocyte complexes (COC) were isolated from follicles, washed 3 times in TC199-M medium with heparin (10 μg/ml), and morphologically examined. Round shaped oocytes with a homogeneous cytoplasm, uniformly wide

zone pellucida and multilayered compact cumulus were selected and cultured for 19-23 hours, 20-30 OCCs per 500 μl of TC-199 medium containing 10% PBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 10 μg/ml follicle-stimulating hormone (FSH) and 10 μg/ml luteinizing hormone (LH).

Reconstruction of mature oocytes. Matured oocytes were released freed from cumulus cells in a 0.1% hyaluronidase solution in TC199-M medium) for 1 min at 37 °C, followed by disaggregation of the complexes by pipetting. Only oocytes with a first polar body (FPB) were selected for cloning.

Fifteen to twenty oocytes were microsurgically manipulated at once in 20 μl drops of TC199-M medium which were put on a Petri dish bottom pre-covered with light mineral oil. The procedure was performed with an inverted microscope Diafort (Nikon Corporation, Japan) equipped with a Narishige micromanipulation system (Japan). During the reconstruction, the oocytes were focused with a holding pipette in the field of view of the microscope in a position that allows clear visualization of a first polar body (PB1) in the perivitelline space in the position to 1 or 5 hours of the conditional dial. A biopsy micropipette (13-15 μm internal diameter) was brought close to oocyte membrane, the zone pellucida was punctured at the site of PB1 localization, chromosomes were removed blindly by aspiration of the PB1 and 10-20% of adjacent cytoplasm. A somatic cell was introduced into the perivitelline space of a fixed oocyte with the micropipette, previously used for PB1 biopsy, through an opening formed during enucleation.

To obtain a cloned cytohybrid, the enucleated oocyte and somatic cell were electro-fused with an Eppendorf multiporator (Great Britain). The oocyte/somatic cell complexes were placed in a microchamber pre-filled with buffer (270 mM mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$), with a 0.2 mm distance between the electrodes and were first exposed to an alternating current electric field (5 V, 5 s) to drive cell complexes apart towards the electrodes, then two consecutive rectangular pulses of direct current (35 V, 20 μs) were applied. Treated cell complexes were short-cultured in 50 μl TC199-M drops covered with light mineral oil. After 1 h incubation, morphologically normal cloned cytohybrids formed from oocyte/somatic cell complexes were selected. The complexes with no signs of the oocyte and somatic cell fusion were repeatedly subjected to electrofusion procedure as described above.

Activation and post-activation culture of cloned cytohybrids. The cytohybrids were activated 1 or 2 hours after fusion (i.e. 23-25 or 26-28 hours from the start of maturation of recipient oocytes) by incubation for 5 min in a 5 mM ionomycin-containing Tyrode solution [28], followed by culture of reconstructed oocytes in CR1aa medium [29] with 2 mM 6-dimethylaminopurine and 10 μg ml cyclohexedine. After 4 hours, putative zygotes were transferred to CR1aa medium and cultured for 4 days, after which the developing embryos were transferred into the same medium with 5% FBS. On day 2 after activation of cytohybrids, morphologically of the cleaved zygotes were evaluated; on day 7, the number of embryos developed to the blastocyst stage was determined. Evaluation was performed with a SMZ stereo microscope (Nikon, Japan, magnification ×40).

One-way analysis of variance was performed with a SigmaStat software (Systat Software, Inc., USA). The data are presented as mean values (*M*) and standard errors of the mean (±SEM). The Tukey's test ($p \leq 0.05$) was used to assess the significance of differences between the compared means.

Results. Figure 1 illustrates the stages of preparation of fetal fibroblasts and cattle oocytes for the cloning procedure (a — a monolayer of cultured donor

cells, b — a suspension of fetal fibroblasts, c — selection of oocytes with the first polar body).

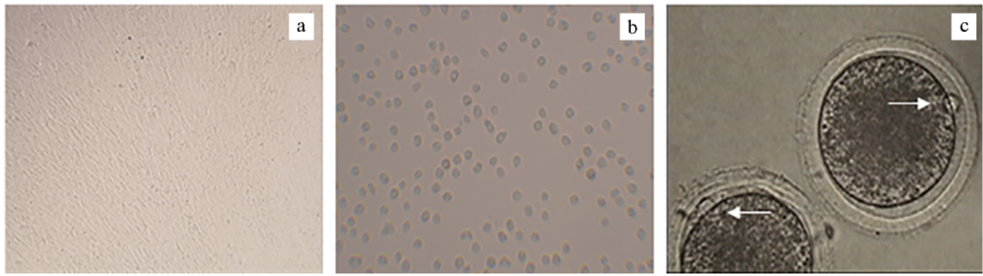

Fig. 1. Cattle fetal fibroblasts and oocytes during somatic cell nuclear transfer (SCNT) procedure: a — culture of fetal fibroblasts after 2 days of contact inhibition; b — fetal fibroblasts suspension (magnification ×200); c — oocytes at metaphase II stage (white arrow indicates the first polar body, magnification ×400) (an Eclipse Ti-U microscope, Nikon, Japan).

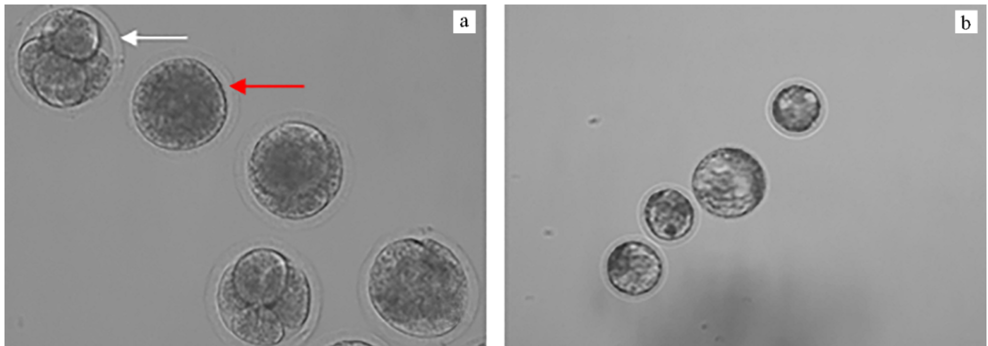

Fig. 2. Cloned cattle embryos derived from fused enucleated MII oocytes and somatic cells (fetal fibroblasts) after activation: a — fused (red arrow) and cleaved (white arrow) cytohybrids (magnification ×200); b — cytogybrids developed to the blastocyst stage (magnification ×100) (an Eclipse Ti-U microscope, Nikon, Japan).

The time of exposure of the oocyte cytoplasm to the donor nucleus before activation is known to be critical for the development of cloned embryos [18, 20]. Reprogramming processes that are necessary for a cell to return to the totipotency [21] are initiated under the influence of cytoplasmic factors in nucleus of somatic cell from the moment of its integration into the cytoplast via fusion.

The influence of the interval between fusion and activation (1 or 2 hours) on somatic nucleus reprogramming efficiency was assessed by the ability of activated cytohybrids (n = 142) to enter the first cleavage division (Fig. 2, a) and to reach the blastocyst stage (see Fig. 2, b). The cleavage rate of activated oocytes on day 2 did not differ for 1- and 2-hour pre-activation exposure of donor nucleus to oocyte cytoplasm (66.6±4.9 and 70.0±5.8%, respectively). However, 1- and 2-hour intervals resulted in different blastocyst yields, i.e. 17.4±2.6% for 1.0 hour vs. a significant increase to 31.1±3.8% ($p < 0.05$) for 2 hours (Fig. 3).

These data are partially consistent with the results of other researchers [19, 20, 30] and suggest that the 2-hour interval between karioplast-cytoplats fusion and activation of resultant cytohybrids provides donor nuclei with enough exposure to the MII oocyte cytoplasm to initiate reprogramming events, while the 1-hour interval reduces blastocyst-stage embryo production. K.I. Aston et al. [20] also reported a positive effect of 2-hour exposure on donor nuclei, but at the same time, they found similar effect for 1-hour exposure. Perhaps such differences are due to peculiarities of somatic cells used for cloning. In our experiments, we used long-stored fetal fibroblasts.

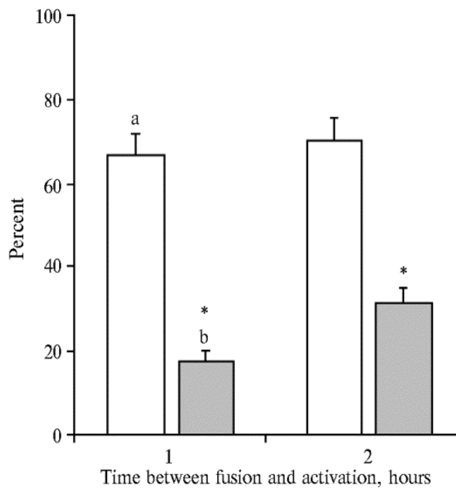

Fig. 3. In vitro development of Bovine cloned embryos as influenced by intervals between karyoplast-cytoplast fusion and activation: a — cleavage rate, b — blastocyst rate. Standard errors of the mean (±SEM) are indicated for $n = 6$ (independent experiments). An asterisk (*) indicates statistically significant differences between the compared groups at $p < 0.05$.

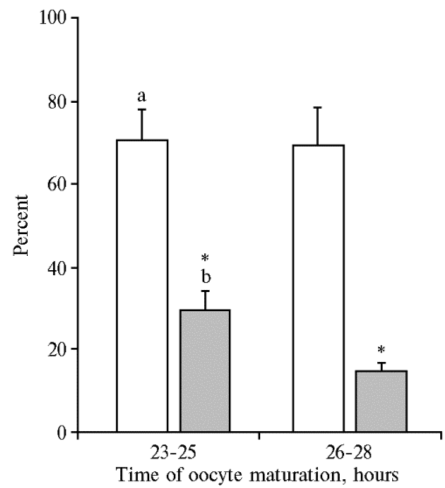

Fig. 4. In vitro development of cytohybrids to the blastocyst stage as influenced by the age of MII oocyte at activation: a — cleavage rate, b — blastocyst rate. Standard errors of the mean (±SEM) are indicated for $n = 9$ (independent experiments). An asterisk (*) indicates statistically significant differences between the compared groups at $p < 0.05$.

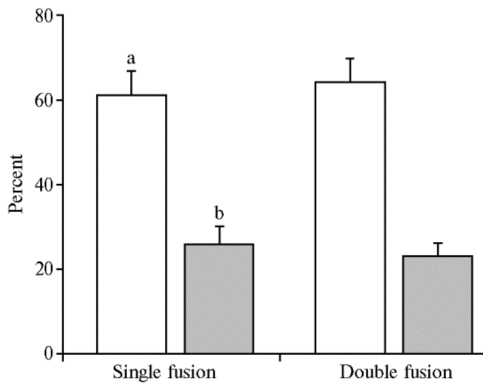

Fig. 5. In vitro development of cloned embryos to the blastocyst stage as influenced by re-fusion of enucleated oocyte and somatic cell: a — cleavage rate, b — blastocyst rate. Standard errors of the mean (±SEM) are indicated for $n = 9$.

Cytoplasmic maturation of the original germ cells is essential to generate cloned embryos capable of normal development [21]. MII oocytes are convenient recipient cells for cloning, since in their cytoplasm, due to specific changes, there are factors ensuring formation of embryonic competence in cloned cytohybrids [21, 24]. However, aging processes are shown to negatively affect quality of mature oocytes not subjected to activation [22, 23]. As capability of MII oocytes to acquire competence to activation in cytohybrids is age-dependent, the age of MII oocytes can also critically affect the development of cloned embryos [24, 25].

We compared developmental competence in cytohybrids activated in 23-25 hours ($n = 104$) and 26-28 hours ($n = 78$) after the maturation of recipient oocytes begins (Fig. 4). The number of cleaved oocytes did not differ between the variants and ranged from 69.4 to 70.4%. Upon early activation, cytohybrids developed to the blastocyst stage constituted 29.4±4.8%. Note, this parameter significantly decreased to 14.6±2.2% ($p < 0.05$) with an increase in the age of oocytes to 26-28 hours. Our data indicate the adverse effect of prolonged culture of mature oocytes on the development of cloned cattle embryos. Also, the age of MII oocytes subjected to activation in hybrid complexes should not exceed 26 hours. Other researchers also indicate the advantage of earlier activation to produce viable offspring [25, 31].

We also investigated the effects of re-fusion on in vitro embryonic development in complexes with no signs of combining an enucleated oocyte and a somatic cell 1 hour after the first fusion (Fig. 5). The electrofusion parameters were the same as in the first procedure. Analysis of in vitro development of the resultant embryos did not reveal adverse effects of the repeated manipulation on the percent of cleaved cytohybrids. Also, there was no decrease in the competence of cytohybrids to form blastocysts neither upon single nor double fusion (29.4±4.4 and 22.8±3.5%, respectively).

Practically, repeated electrofusion is used in somatic cloning to obtain a larger number of cell complexes with signs of combining an enucleated oocyte and a donor cell [27], and also as a method for activating cytohybrids [31]. At the same time, given possible negative consequences of repeated electrofusion, as a rule, more gentle electric pulse modes are used. In our study, these parameters were similar in both manipulations, however, we did not observe any deterioration in in vitro development of the embryos till blastocyst stage.

Thus, we have confirmed that the efficiency of producing cloned cattle embryos of preimplantation stages depends on the interval from fusion to activation, and also on the age of MII oocytes in activated cytohybrids. As per the in vitro cloning protocol we suggest, 2 hours and 23-25 hours, respectively, are the optimal. It is also obvious that the repeated electrofusion of an enucleated oocyte and a somatic cell does not adversely affect the quality of the resulting cytohybrid, and therefore can be used for obtaining cloned embryos in cattle.

REFERENCES

1. Tian X.C., Kubota C., Sakashita K., Izaike Y., Okano R., Tabara N., Curchoe C., Jacob L., Zhang Y., Smith S., Bormann C., Xu J., Sato M., Andrew S., Yang X. Meat and milk compositions of bovine clones. *Proceedings of the National Academy of Sciences*, 2005, 102(18): 6261-6266 (doi: 10.1073/pnas.0500140102).
2. Hoshino Y., Hayashi N., Taniguchi S., Kobayashi N., Sakai K., Otani T., Iritani A., Saeki K. Resurrection of a bull by cloning from organs frozen without cryoprotectant in a –80 °S freezer for a decade. *PLoS ONE*, 2009, 4(1): e4142 (doi: 10.1371/journal.pone.0004142).
3. Yonai M., Kaneyama K., Miyashita N., Kobayashi S., Goto Y., Bettpu T., Nagai T. Growth, reproduction, and lactation in somatic cell cloned cows with short telomeres. *Journal of Dairy Science*, 2005, 88(11): 4097-4110 (doi: 10.3168/jds.S0022-0302(05)73094-0).
4. Van Eenennaam A.L. Application of genome editing in farm animals: cattle. *Transgenic Research*, 2019, 28(2): 93-100 (doi: 10.1007/s11248-019-00141-6).
5. Brophy B., Smolenski G., Wheeler T., Wells D., L'Huillier P., Laible G. Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. *Nature Biotechnology*, 2003, 21(2): 157-162 (doi: 10.1038/nbt783).
6. Richt J.A., Kasinathan P., Hamir A.N., Castilla J., Sathiyaseelan T., Vargas F., Sathiyaseelan J., Wu H., Matsushita H., Koster J., Kato S., Ishida I., Soto C., Robl J.M., Kuroiwa Y. Production of cattle lacking prion protein. *Nature Biotechnology*, 2007, 25(1): 132-138 (doi: 10.1038/nbt1271).
7. Wu H., Wang Y., Zhang Y., Yang M., Lv J., Liu J., Zhang Y. TALE nickase-mediated SP110 knocking endows cattle with increased resistance to tuberculosis. *Proceedings of the National Academy of Sciences*, 2015, 112(13): E1530-E1539 (doi: 10.1073/pnas.1421587112).
8. Proudfoot C., Carlson D.F., Huddart R., Long C.R., Pryor J.H., King T.J., Lillico S.G., Mileham A.J., McLaren D.G., Whitelaw C.B., Fahrenkrug S.C. Genome edited sheep and cattle. *Transgenic Research*, 2015, 24(1): 147-153 (doi: 10.1007/s11248-014-9832-x).
9. Gao Y., Wu H, Wang Y, Liu X, Chen L, Li Q., Cui C., Liu X., Zhang J., Zhang Y. Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. *Genome Biology*, 2017, 18(1): 13 (doi: 10.1186/s13059-016-1144-4).
10. Farin P.W., Piedrahita J.A., Farin C.E. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology*, 2006, 65(1): 178-191 (doi: 10.1016/j.theriogenology.2005.09.022).
11. Bertolini M., Bertolini L.R., Gerger R.P.C., Batchelder C.A., Anderson G.B. Developmental problems during pregnancy after in vitro embryo manipulations. *Rev. Bras. Reprod. Anim.*, 2007,

31(3): 391-405.

12. Su J., Wang Y., Liu Q., Yang B., Wu Y., Luo Y., Hu G., Zhang Y. Aberrant mRNA expression and DNA methylation levels of imprinted genes in cloned transgenic calves that died of large offspring syndrome. *Livestock Science*, 2011, 141(1): 24-35 (doi: 10.1016/j.livsci.2011.04.012).
13. Yang X.Z., Smith S.L., Tian X.C., Lewin H.A., Renard J.P., Wakayama T. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nature Genetics*, 2007, 39(3): 295-302 (doi: 10.1038/ng1973).
14. Latham K.E. Early and delayed aspects of nuclear reprogramming during cloning. *Biology of the Cell*, 2005, 97(2): 119-132 (doi: 10.1042/BC20040068).
15. Akagi S., Matsukawa K., Takahashi S. Factors affecting the development of somatic cell nuclear transfer embryos in cattle. *Journal of Reproduction and Development*, 2014, 60(5): 329-335 (doi: 10.1262/jrd.2014-057).
16. Wells D.N., Misica P.M., McMillan W.H., Tervit H.R. Production of cloned bovine fetuses following nuclear transfer using cells from a fetal fibroblast cell line. *Theriogenology*, 1998, 49(1): 330 (doi: 10.1016/S0093-691X(98)90683-5).
17. Akagi S., Yokota M., Neguebi T., Taniyama A., Fuebimoto D., Izaile Y. The timing of fusion and chemical activation in nuclear transfer affects development potential of bovine embryos. *Theriogenology*, 2001, 55(1): 252 (doi: 10.1016/S0093-691X(00)00457-X).
18. Choi J.Y., Kim C.I., Park C.K., Yang B.K., Cheong H.T. Effect of activation time on the nuclear remodeling and in vitro development of nuclear transfer embryos derived from bovine somatic cells. *Molecular Reproduction and Development*, 2004, 69(3): 289-295 (doi: 10.1002/mrd.20131).
19. Liu L., Shin T., Pryor J.H., Kraemer D., Westhusin M. Regenerated bovine fetal fibroblasts support high blastocyst development following nuclear transfer. *Cloning*, 2001, 3(2): 51-58 (10.1089/15204550152475554).
20. Aston K.I., Li G.P., Hicks B.A., Sessions B.R., Pate B.J., Hammon D., Bunch T.D., White K.L. Effect of the time interval between fusion and activation on nuclear state and development in vitro and in vivo of bovine somatic cell nuclear transfer embryos. *Reproduction*, 2006, 131(1): 45-51 (doi: 10.1530/rep.1.00714).
21. German S.D., Campbell K.H.S. Livestock somatic cell nuclear transfer. In: *Sustainable food production*. P. Christou, R. Savin, B.A. Costa-Pierce, I. Misztal, C.B.A. Whitelaw (eds.) Springer, New York, 2013: 1067-1095 (doi: 10.1007/978-1-4614-5797-8_2).
22. Miao Y.L., Kikuchi K., Sun G.Y., Schatten H. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Human Reproduction Update*, 2009, 15(5): 573-585 (doi: 10.1093/humupd/dmp014).
23. Lebedeva I.Yu., Singina G.N., Lopukhov A.V., Zinovieva N.A. Dynamics of morphofunctional changes in aging bovine ova during prolonged culture in vitro. *Cell and Tissue Biology*, 2014, 8(3): 258-266 (doi: 10.1134/S1990519X14030080).
24. Akagi S., Geshi M., Nagai T. Recent progress in bovine somatic cell nuclear transfer. *Animal Science Journal*, 2013, 84(3): 191-199 (doi: 10.1111/asj.12035).
25. Akagi S., Matsukawa K., Takahashi S. Factors affecting the development of somatic cell nuclear transfer embryos in cattle. *Journal of Reproduction and Development*, 2014, 60(5): 329-335 (doi: 10.1262/jrd.2014-057).
26. Singina G.N., Lopukhov A.V., Zinov'eva N.A., Shapkanova E.V., Puzik A.A. Optimization of parameters of the oocyte enucleation and fusion with the somatic cell during production of mammalian cloned embryos. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2013, 2: 46-51 (doi: 10.15389/agrobiology.2013.2.46eng).
27. Ross P.J., Cibelli J.B. Bovine somatic cell nuclear transfer. In: *Cellular programming and reprogramming. Methods in molecular biology (methods and protocols*). S. Ding (ed.). Humana Press, 2010, V. 636: 155-177 (doi: 10.1007/978-1-60761-691-7_10).
28. Bavister B.D., Liebfried M.L., Lieberman G. Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biology of Reproduction*, 1993, 28(1): 235-247 (doi: 10.1095/biolreprod28.1.235).
29. Rosenkrans C.F.Jr., First N.L. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. *Journal of Animal Science*, 1994, 72(2): 434-437 (doi: 10.2527/1994.722434x).
30. Liu J., Wang Y., Su J., Wang L., Li R., Li Q., Wu Y., Hua S., Quan F., Guo Z., Zhang Y. Effect of the time interval between fusion and activation on epigenetic reprogramming and development of bovine somatic cell nuclear transfer embryos. *Cellular Reprogramming*, 2013, 15(2): 134-142 (doi: 10.1089/cell.2012.0052).
31. Shen P.C., Lee S.N., Liu B.T., Chu F.H., Wang C.H., Wu J.S., Lin H.H., Cheng W.T.K. The effect of activation treatments on the development of reconstructed bovine oocytes. *Animal Reproduction Science*, 2008, 106(1-2): 1-12 (doi: 10.1016/j.anireprosci.2007.03.019).