integrity Research Journals

Journal of Animal Science and Veterinary Medicine
Volume 8(3), pages 52-58, June 2023
Article Number: 1E8A88721
ISSN: 2536-7099
https://doi.org/10.31248/JASVM2023.361
https://integrityresjournals.org/journal/JASVM

Full Length Research

Development of buffalo ear skin fibroblast cell line for somatic cell nuclear transfer

M. F. Afroz[1]*, G. K. Deb[2], K. T. Tahira[2], Z. C. Das[3], T. N. Nahar[4] and S. M. J. Hossain[4]

[1]Animal Production Research Division, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh.
[2]Buffalo Production Research Division, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh.
[3]Department of Gynecology, Obstetrics & Reproductive health, Faculty of Veterinary Medicine & Animal Science at Bangabandhu Sheikh Mujibur Raman Agricultural University.
[4]Biotechnology Division, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh.

*Corresponding author. Email: famukta@yahoo.com

Received 6th February 2023; Accepted 9th March 2023

ABSTRACT: Fibroblast is the principal active cell of connective tissue. It can be used as a useful tool for reproductive and therapeutic studies for its ability to easily grow in culture, create a preferable environment to growth and can be used as both for primary and permanent cell lines. Somatic cell nuclear transfer (SCNT) is commonly used for complete genetic reprogramming of a fully differentiated cell (e.g., fibroblast). SCNT is influential means for studying genomic imprinting, nuclear-cytoplasmic interaction, totipotency, and the contribution of paternal and maternal genomes to developing embryos. For reproductive cloning, SCNT is a fundamental step. So, the objective of this study was to adapt buffalo fibroblast cell culture protocol in order to develop fibroblast cell line for SCNT. River type buffalo ear skin tissues were used for developing primary cell culture to establish fibroblast cell line. Cells were grown *in vitro*. The general morphology and growth of cell population and presence of any microbial contaminants were checked regularly under an inverted microscope in phase contrast. Cells were counted by using hemocytometers. Sliced tissue was sown in 15 culture dishes to create primary and subcultures, and cells were cultivated in 6 of those dishes. Cell confluence varied from 70 to 80%. There were performed subcultures. Cell confluence in passages 1 through 10 varied from 73 to 90%. Cell concentration was 2.72 x 105, 2.68 x 105, and 2.65 x 105 per ml in passages 1, 5, and 10, respectively. Viable cells ranged from 84-93% of the total cells in passages 1 through 10. Different passages of cultivated fibroblast cells showed significantly varying viability ($p< 0.05$). Cultured fibroblast cell lines could now be preserved using cryotechnology.

Keywords: Buffalo, cryopreservation, ear skin, fibroblast cell line, primary culture.

INTRODUCTION

Cell culture is the process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions. But in practice, it refers to the culturing of cells derived from animal cells. In cell culture, the cells are no longer organized into tissues. When cells are surgically removed from an organism and placed into a suitable culture environment; they will attach, divide and grow. This is called as primary culture. The genetic diversity of livestock and poultry plays an important role in overall biological diversity, as well as forms the basis for the survival and sustainable development of the human beings. Therefore, the preservation of genetic resources from endangered species is of important scientific significance. The preservation of individual animals, semen, embryos, genomic libraries and DNA libraries are all practical approaches. Nevertheless, the establishment of cell line using cryopreservation technique is another effective approach for preservation of individuals. Most cell banks prioritize conservation and utilization of animal resources, especially animal producing cells and embryos (Ho *et al*., 1997; Oishi, 1997; Simon, 1999; Park *et al*., 2009). In addition to these methods, modern somatic cell cloning

technique has made somatic cells become fascinating resource in the conservation of animal genetic materials (Wu 1999; Hong *et al*., 2005; Lee *et al*., 2007; Park *et al*., 2007; Yun *et al.*, 2008).

Cell culture is a general idiom used for the removal of cells or tissues from an animal and their next placement into an artificial environment conducive to growth. It is also known as techniques of keeping tissues alive and growing in an appropriate culture medium. Growing tissues of living organism outside the body is made possible in an appropriate culture medium, containing mixture of nutrient either in solid or liquid form. In cell culture, the cells are no longer organized into tissues. Cell culture was first successfully undertaken by Harrison in 1907. Harrison cultivated frog nerve cells in a lymph clot held by the 'hanging drop' method and observed the growth of nerve fibers *in vitro* for several weeks. However, Wilhelm Roux in 1885 for the first time maintained embryonic chick cells in a saline culture. The first liquid media consisted of sea water, serum, embryo extract, salts and peptones for cell culture experiment. This media supported growth of monolayer in limited scale. Later several techniques were developed for successful cell culture (Marquis, 2016). Development of use of trypsin to allow subculture of attached cells from one flask to another, development in cell culture vessels and bioreactors, cell cryopreservation methods and different media formulations were taken places during 1950s to 1960s. Vaccine production from cultured cell was also developed by this time. The technology for the production of different antibiotics from cultured cell lines was established by the continuous effort of researchers during the period of 1970 to 1980. In 1992, rat intestinal cells primary culture was developed by Evans *et al* (1992). Skin fibroblast cells are valuable for being the most frequently used somatic cells in somatic cell cloning, and thus, fibroblasts preservation is important to fulfill the future demands for animal cloning and other projects (Moro *et al*., 2015a, b). So far, culture of skin fibroblast cell lines using adherent culture has been achieved for many animal species (Webb *et al.* 2014; Santos *et al.* 2016; Daneshvar *et al.* 2017; Siengdee *et al.* 2018; Wang *et al.* 2020).

Skin cells provide the benefits of easy accessibility and non-invasiveness, without sex or age limitations (Kubota *et al*., 2000). Moreover, skin cells give an extra interest because of animal's phenotype is known (Forsberg *et al*., 2002). Therefore, ear fibroblasts can obtain without risk to the donor animal's health and is more convenient and practical to harvest and sub-culture than other cells (Lu *et al*., 2005; Hosseini *et al*., 2008). Fibroblast cell line is a good source of donor cells in SCNT for cloning purpose. Moreover, Tasripoo *et al*. (2007) showed that there was no difference in blastocyst formation among buffalo donor skin fibroblasts of a 6-month old calf, a 2-year old and a 4-year old buffalo. Establishment of pregnancy and birth of the first cloned Thai swamp buffalo obtained from adult ear skin fibroblast cells reported by Tasripoo *et al.* (2014). Fibroblasts from different anatomical sites in the body also express many genes that code for immune mediators and proteins (Krausgruber *et al.,* 2020). The technology for the production of cloned calf from bovine fibroblast cell line was successfully adopted, where a bovine fetal fibroblast culture was established and used as nucleus donor, slaughterhouse oocytes were matured and enucleated oocytes were fused with fibroblast. Reconstructed embryos were cultured and developed blastocysts were transferred to recipient cows which eventually produced a cloned calf.

A Texel sheep ear marginal tissue fibroblast cell line (named TSF19) was successfully established by using a primary explant technique and cell cryo-conservation technology by Li *et al*. (2009b). This newly established cell line will not only preserve the genetic resources of the important Texel sheep at the cell level, but will also provide a valuable resource for genomic, post-genomic, somatic cloning research. Establishment and characterization of the fibroblast cell line from Silkie Bantam was successfully adopted by Li *et al.* (2009a).

Somatic cell nuclear transfer (SCNT) is the procedure by which a nucleus from a fully differentiated cell (e.g., fibroblast) undergoes complete genetic reprogramming when it is introduced into an enucleated oocyte. Nuclear transfer of adult somatic cells from farm animals is the most efficient biotechnological means for obtaining large numbers of genetically identical animals (Kato *et al*., 1998) and could greatly increase agricultural production (Lu *et al*., 2011). The prime advantage of SCNT is the possibility of creating multiple genetically identical animals which is known as cloning.

The first successfully cloned mammal, Dolly (sheep) was created using this process in 1996 (Wilmut*et al*., 1997). The somatic cell nuclear transfer (SCNT) is a technique where nucleus from donor somatic (body) cell was implanted into an enucleated oocyte (egg cell). SCNT technique encompasses several approaches including- i) preparation of donor cell, ii) preparation of recipient oocyte, iii) replacement of nucleus of recipient with donor nucleus, iv) fusion of donor nucleus with recipient oocytes and v) culture of reconstructed oocytes until blastocyst development (Tian *et al*., 2003). The nuclear donor cells are an important component for determining success rate of SCNT (Powell *et al*., 2004).

Biotechnology division of Bangladesh Livestock Research Institute is working on *in vitro* embryo production, ovum pick up (OPU), embryo cryopreservation and embryo transfer (ET) protocols for the last three years. The *in vitro* embryo production system was already developed in BLRI. Researches on OPU, embryo cryopreservation and ET are carrying out for successful adoption of these techniques (Deb *et al*., 2014). The division has planned to adopt somatic cell nuclear transfer (SCNT) technique for cloning, transgenic animal, therapeutic and conservation of endangered farm animal species of Bangladesh, as this was suggested by the

experts of annual research review workshop, 2015 to improve the livestock sector to fulfill the vision 2041. Different somatic cell lines were successfully used for SCNT in different species. So, the objective of the study was to estimate the fibroblast cell line protocol of river type buffalo for reproductive cloning.

MATERIALS AND METHODS

Development of primary cell line

Developments of primary culture of cell lines using ear tissue of buffalo were used to establish fibroblast cell line. Ear of River type buffalo was collected from slaughter house. Collected ear was kept into saline solution immediately after slaughtering of buffalo. Cells were growing *in vitro*. A piece of ear tissue cultured in the medium to develop primary fibroblast cell line. At first, tissue sample was washed with Phosphate Buffer Saline solution (PBS) followed by clipped, saved and washed again with PBS. After washing, the tissue samples were sliced with scalpel and blade. The sliced samples were washed again with PBS and incubated in PBS containing trypsin- EDTA (~3-4ml) for 5 min. After 5 min, DMEM working solution (DMEM+10% Fetal Bovine Serum) was added to inactivate enzymatic activity and then centrifuged at 800 rpm for 5 min. The supernatant was discarded and tissue slice was washed in PBS before seedling in Petridis and flask. The media was poured so that the slice was not completely submerged into the media. Then the culture flasks/Petri dishes were placed into a CO_2 incubator (5% CO_2 with 37°C temperature) for 24 h. After attachment of seeded tissues in dish, DMEM working media was added to submerge the tissue slice into media. The culture was continuing until 80-90% confluent. Culture medium was replaced after two days and then replaces it three times a week. After the seedling, the fibroblast outgrowth began to develop two to three days later

Passaging of cultured cell lines

A portion of the cultivated primary cells used in maintaining a cell line were cryopreserved, and the remainder was constantly sub-cultured. Under an inverted microscope in phase contrast, the general appearance, growth, and existence of the cell population as well as any microbial contamination would be monitored on a regular basis. Flasks with cells at about 70% confluences were treated with trypsin, the cells that were then harvested and either frozen are divided for further proliferation. Dishes with non-confluent cells medium was discarded and replaced with fresh medium: 7 ml for 100 mm Petri dishes, 5 ml for 60 mm Petri dishes and 5 ml for 25 cm^2 flasks. Cells were thoroughly washed with PBS and were detached by 0.25% Trypsin–0.02% EDT A (Gibco , 10X) solution at 37°C incubator for 3 min. After trypsinization, the cell suspension was centrifuged at 250 g for 7 min and supernatant was discarded. The cells were split into new culture flasks containing DMEM with 10% fetal bovine serum, 1% L-glutamine (2 mM) and cultured at 37°C with 5% CO_2. The medium was changed 3 times in a week to maintain proper proliferation and growth of cells.

Confirmation of fibroblast cell

DNA from ear tissue and cell culture was extracted using Wizard genomic DNA purification kit (Promega, USA). PCR final mixer contained GoTaq Green Master mix (USA), forward and reverse primers, 2µl DNA and nuclease free water. PCR result were validated under the following conditions: denaturation at 95°C for 5 min, followed by 95°C for 30 sec, 58°C for 30 sec for 20 cycles and final extension at 72°C for 5 min. PCR products were segregated on 2% agarose gel and visualized by ethidium bromide staining.

Cell viability and quality evaluation of developed cell lines

The quality of cell lines was evaluated by cell morphology and apoptotic status following appropriate procedure with little modification. Cells were counted using hemocytometers as described elsewhere (Louis and Siegel, 2011). Viable cells were detected following staining cells with Trypan Blue (Gibco, Grand Island, NY). Cells viability was observed using a traditional cell-counting method (Jauregui *et al*., 1981; Louis and Siegel, 2011). Briefly, at first cultured cells were treated with trypsin with a routine subculture. Cell suspension was mixed 1:1 with 0.4% trypan blue solution and kept for 5 min at room temperature. Then 20 µL of the cell suspension was taken and put between the cover slip and the edge of the hemocytometer chamber and examined immediately under a microscope according the method of Louis and Siegel (2011).

Cryopreservation of cultured cell lines

Suitable protocols for cryopreservation of cultured fibroblast cell lines were developed. Base medium containing 10% DMSO (Di-methyl-sulfoxide) were used for cryopreservation of fibroblast cells in the liquid nitrogen in this study.

Statistical analysis

Data were analyzed in one-way ANOVA using SPSS 25.0 version and for mean comparison, Duncan Multiple Range

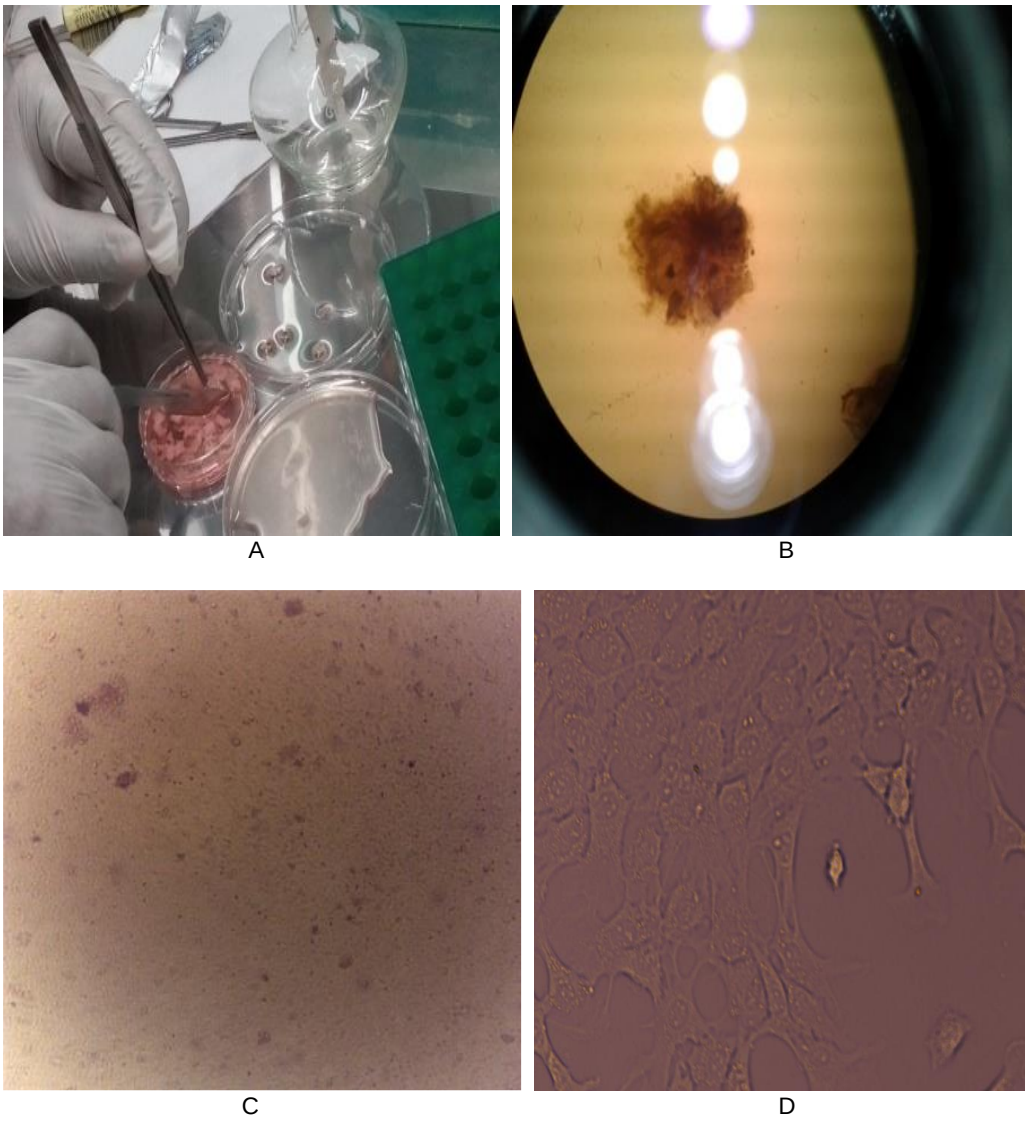

Figure 1. Different steps of cell culture: A) Preparation of ear tissue for cell culture, B) Adhering of the tissue on the culture flask, C) and D) Visualization of cultured fibroblast cell.

Test (DMRT) was used. P-value of ≤ 0.05 was considered statistically significant. All data were presented as the mean **±** SE (Standard Error).

RESULTS AND DISCUSSION

Now days, many technologies are taken to conserve animal genetic resources in many countries. With the research report from Ian Wilmut of the cloning of Dolly, the application of cloning to conservation efforts for endangered species seems appropriate (Ryder and Benirschke, 1997). Moreover, rapid development and broad application of nuclear transfer using somatic cells increases the choice of making transgenic domestic animals (Lee and Piedrahita, 2003).

Results of the current study revealed that animal ear tissue was suitable to achieve an animal fibroblast cell line which was in accordance with other studies conducted by Zhou *et al*., (2004), Groeneveld (2007) and Wang *et al*., (2020). Ear sample collection, skin fibroblasts isolation, ear tissue preparation for fibroblast cell were done under a modified protocol, as described in previous reports (Li *et al*., 2009a; Vangipuram *et al*., 2013; Mestre-Citrinovitz *et al*., 2016; Siengdee *et al.,* 2018) (Figure 1A). Prepared sample were cultured for 5–10 days (Figure 1B and 1C). Confluence of cultured cells ranged from 70 to 80%. Cells were continued to proliferate and were sub-cultured when they reached about 80–90% confluence (Figure 1D). Confluence of cells in passaged 1- 10 were ranged from 73 to 90%. It was observed that fibroblast cells could attach to the plates more easily and readily than epithelial

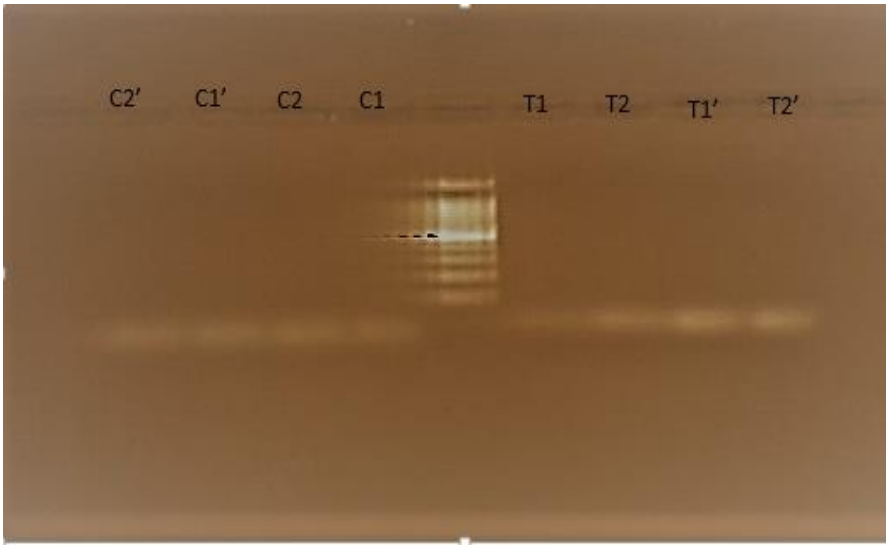

Figure 2. Confirmation of fibroblast cell. T1, T2 and T1',T2' represents samples collected from tissue using FSP1 and FIN _1 primers respectively; C1, C2 and C1',C2' represents samples collected from cell culture using FSP1 and FIN _1 primer respectively.

Table 1. Cell concentration and total viable cell from cell culture passage no.1, 5 and 10.

Replication	Passage					
	Passage 1		Passage 5		Passage 10	
	Concentration of cells/ml	Total viable cell /ml (%)	Concentration of cells/ml	Total viable cell /ml (%)	Concentration of cells/ml	Total viable cell/ml (%)
Replication 1	2.73 x10^5	2.56 x10^5(94%)	2.66 x10^5	2.47 x10^5(93%)	2.58 x10^5	2.12 x10^5(82%)
Replication 2	2.69 x10^5	2.48 x10^5(92%)	2.71 x10^5	2.46 x10^5(91%)	2.67 x10^5	2.32 x10^5(87%)
Replication 3	2.74 x10^5	2.54 x10^5(93%)	2.68 x10^5	2.30 x10^5(86%)	2.70 x10^5	2.24 x10^5(83%)

cells and showed distinctive tolerances to trypsin as fibroblasts more easily detach from the flasks that were similar with Li *et al*. (2009) and Wang *et al*. (2020). Moreover, fibroblast attach faster than epithelial cells, which barely attach or attach unstably by themselves, and require certain growth substrates such as collagen to sustain them (Zhou *et al*., 2004; Ren *et al*., 2002). To identify the fibroblast cell Fibroblast specific protein 1 (FSP1) and Fibronectin 1 (FIN _1) primers were used. The Fibroblast cell was taken both from ear pinna and primary cell culture of fibroblast (Figure 2).

Subculture was accomplished with the harvested cells split into new culture under the ratio of 1:3. Confluence of cells in passaged 1-10 ranged from 75 to 90%. This result was supported with others findings according to Zhou *et al*. (2004) (in case of cell culture from Debao Pony ear fibroblast) and Wang *et al*. (2020) (in case of fibroblast cell culture from Chinese muntjac). Xiong *et al.* (2014) also found 90% confluence of cell in passage 3 from Duroc pig's ear fibroblast. Table 1 showed concentration of cells in passage 1 were 2.73 x 10^5, 2.69 x 10^5 and 2.74 x 10^5 per ml; in passage 5 were 2.66 x10^5, 2.71 x 10^5 and 2.68 x 10^5 per ml and in passage 10 were 2.58 x10^5, 2.67 x 10^5 and 2.70 x 10^5 per ml for replication 1, 2 and 3 respectively. Table 1 also showed the viable cell per ml for each passage. Concentration of cells and total viable cell per ml (%) (Mean ± SE) were presented in Table 2. Concentration of cells per ml in different passage were not significantly differed but viability of cultured fibroblast cell of different passage differed significantly ($p<0.05$).

From the present study, the percentage of viable cells of Passage 1, Passage 5, and Passage10 before cryopreservation was 93, 89, and 84%, respectively (Table 2). Similar results were found 97.3 ± 4.3% by Siengdee *et al*. (2018). Wang *et al*., (2020) found that viable cells of Passage 3, Passage 5, and Passage 10 before cryopreservation were 97, 96, and 85%, respectively. According to Li *et al*. (2009), viability of cultured cell before freezing was 96.54 ± 3.22% which also supported these results. Xiong *et al*. (2014) also found the viability of duroc fibroblasts cell culture before freezing and after recovery were 99.2 and 98.7%, respectively.

Table 2. Analysis of concentration of cells and total viable cell per ml (%) (Mean ± SE).

Parameter	Passage			Level of sig.
	Passage 1 (n=3)	Passage 5 (n=3)	Passage 10 (n=3)	
Concentration of cell/ml	2.72 x 10^5±0.02	2.68 x 10^5±0.01	2.65 x 10^5±0.04	**NS**
Total viable cell/ml(%)	2.53 x$10^{5\,a}$±0.03(93%)	2.41x$10^{5\,a}$±0.05(89%)	2.23x$10^{5\,b}$±0.06(84%)	*

[a,b]Means with different superscripts within the same row are differ significantly. Figure in the parenthesis indicate the number of replications. *=significant (p<0.05), NS= Non significant p>0.05).

Conclusion

In conclusion, cell culture protocol for River type buffalo fibroblast was developed and confirmed. Cryopreservation of cultured fibroblast cell line had also developed and could be better source of SCNT in reproductive cloning.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

The authors acknowledge Bangladesh Livestock Research Institute (BLRI) to assist in research work. This research was financially supported by the Buffalo Development Project (Component-B).

REFERENCES

Daneshvar, A. A., Mohebali, N., Farzaneh, P., Shahzadeh Fazeli, S. A., Nikfarjam, L., Ashouri Movasagh, S., Moradmand, Z., Ganjibakhsh, M., Nasimian, A., Izadpanah, M., Vakhshiteh, F., Sadat, G. N., Masoudi, N, S, Farghadan M, Mohamadi, M. S., Khalili, M., & Khaledi, K. J. (2017). Establishment and characterization of Caspian horse fibroblast cell bank in Iran. *In Vitro Cellular & Developmental Biology-Animal*, *53*, 337-343.

Deb, G. K., Kabir, M. A., Khan, M.Y., A, Nahar, T. N., Rahman, M. M., & Islam, M. R.(2014). Establishment of embryo culture system maintaining genetic identity of embryos. Proceeding of the *Annual Research Review Workshop of Bangladesh Livestock Research Institute, Savar, Dhaka*.

Evans, G. S., Flint, N., Somers, A. S., Eyden, B., & Potten, C. S. (1992). The development of a method for the preparation of rat intestinal epithelial cell primary cultures. *Journal of cell science*, *101*(1), 219-231.

Forsberg, E. J., Strelchenko, N. S., Augenstein, M. L., Betthauser, J. M., Childs, L. A., Eilertsen, K. J., Enos, J. M., Forsythe, T. M., Golueke, P. J., Koppang, R. W., Lange, G., Lesmeister, T. L., Mallon, K. S, Mell, G. D., Misica, P. M., Pace, M. M., Pfister-Genskow, M., Voelker, G. R., Watt, S. R. & Bishop, M. D. (2002). Production of cloned cattle from in vitro systems. *Biology of Reproduction*, *67*(1), 327-333.

Groeneveld, E. (2007). A worldwide emergency programme for the creation of national genebanks of endangered breeds in animal agriculture. *Stočarstvo,* 61(6), 427-434.

Harrison, R. G. (1907). Observations on the living developing nerve fiber. *Proceedings of the Society for Experimental Biology and Medicine*, *4*(1), 140-143.

Ho, S. K., Lister, E. E., &Touchbum, S. P. (1997). An overview of Canadian farm animal genetic resources conservation and its associated biotechnological approaches. *Proceedings of International Conference of Animal Biotechnology*, Beijing, China. Pp. 155-160.

Hong, S. B., Uhm, S. J., Lee, H. Y., Park, C. Y., Gupta, M. K., Chung, B. H., Chung, K.S., & Lee, H. T. (2005). Developmental ability of bovine embryos nuclear transferred with frozen-thawed or cooled donor cells. *Asian-Australasian Journal of Animal Sciences*, *18*(9), 1242-1248.

Hosseini, S. M., Moulavi, F., Foruzanfar, M., Hajian, M., Abedi, P., Rezazade-Valojerdi, M., Parivar, K., Shahverdi, A. H., & Nasr-Esfahani, M. H. (2008). Effect of donor cell type and gender on the efficiency of in vitro sheep somatic cell cloning. *Small Ruminant Research*, *78*(1-3), 162-168.

Jauregui, H. O., Hayner, N. T., Driscoll, J. L., Williams-Holland, R., Lipsky, M. H., & Galletti, P. M. (1981). Trypan blue dye uptake and lactate dehydrogenase in adult rat hepatocytes—freshly isolated cells, cell suspensions, and primary monolayer cultures. *In vitro*, *17*(12), 1100-1110.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J. Y., Doguchi, H., Yasue, H., & Tsunoda, Y. (1998). Eight calves cloned from somatic cells of a single adult. *Science*, *282*(5396), 2095-2098.

Krausgruber, T., Fortelny, N., Fife-Gernedl, V., Senekowitsch, M., Schuster, L. C., Lercher, A., Nemc, A., Schmidl, C., Rendeiro, A.F., Bergthaler, A., & Bock, C. (2020). Structural cells are key regulators of organ-specific immune responses. *Nature*, *583*(7815), 296-302.

Kubota, C., Yamakuchi, H., Todoroki, J., Mizoshita, K., Tabara, N., Barber, M., & Yang, X. (2000). Six cloned calves produced from adult fibroblast cells after long-term culture. *Proceedings of the National Academy of Sciences*, *97*(3), 990-995.

Lee, C. K., & Piedrahita, J. A. (2003). Transgenesis and germ cell engineering in domestic animals. *Asian-Australasian Journal of Animal Sciences*, *16*(6), 910-927.

Lee, Y. S., Ock, S. A., Cho, S. K., Jeon, B. G., Kang, T. Y., Balasubramanian, S., Choe, S. Y., & Rho, G. J. (2007). Effect of donor cell types and passages on preimplantation development and apoptosis in porcine cloned embryos. *Asian-Australasian Journal of Animal Sciences*, *20*(5), 711-717.

Li, L. F., Guan, W. J., Li, H., Bai, X. J., & Ma, Y. H. (2009a). Establishment and characterization of the fibroblast line from Silkie Bantam. *Asian-Australasian Journal of Animal Sciences*, *22*(4), 492-499.

Li, X. C., Yue, H., Li, C. Y., He, X. H., Zhao, Q. J., Ma, Y. H., Guan, W. J., & Ma, J. Z. (2009). Establishment and characterization of a fibroblast cell line derived from Jining Black Grey goat for genetic conservation. *Small Ruminant Research*, *87*(1-3), 17-26.

Li, L. F., Guan, W. J., Li, H., Zhou, X. Z., Bai, X. J., & Ma, Y. H. (2009b). Establishment and characterization of a fibroblast cell line derived from Texel sheep. Biochemistry and Cell Biology, 87(3): 485–492.

Louis, K. S., & Siegel, A. C. (2011). Cell viability analysis using trypan blue: Manual and automated methods. In: Stoddart, M. (ed.). *Mammalian cell viability. Methods in Molecular Biology*, vol 740, pp. 7-12. Humana Press.

Lu, F., Jiang, J., Li, N., Zhang, S., Sun, H., Luo, C., Wei, Y. & Shi, D. (2011). Effects of recipient oocyte age and interval from fusion to activation on development of buffalo (*Bubalus bubalis*) nuclear transfer embryos derived from fetal fibroblasts. *Theriogenology*, *76*(5), 967-974.

Lu, F., Shi, D., Wei, J., Yang, S., & Wei, Y. (2005). Development of embryos reconstructed by interspecies nuclear transfer of adult fibroblasts between buffalo (*Bubalus bubalis*) and cattle (Bos indicus). *Theriogenology*, *64*(6), 1309-1319.

Marquis, C. P. (2016). Fundamentals in Biotechnology. In: Doelle, H. W., Roken, S., & Beroric, M. *Encylopedia of Life Support System (EOLSS), Mammalian cell culture*. Biotechnology, Volume-I.

Mestre-Citrinovitz, A. C., Sestelo, A. J., Ceballos, M. B., Barañao, J. L., & Saragüeta, P. (2016). Isolation of primary fibroblast culture from wildlife: the Panthera onca case to preserve a South American endangered species. *Current Protocols in Molecular Biology*, *116*(1), 128.117.111-128.117.114.

Moro, L. N., Hiriart, M. I., Buemo, C., Jarazo, J., Sestelo, A., Veraguas, D., Rodriguez-Alvarez, L., & Salamone, D. F. (2015a). Cheetah interspecific SCNT followed by embryo aggregation improves *in vitro* development but not pluripotent gene expression. *Reproduction*, *150*(1), 1-10.

Moro, L. N., Jarazo, J., Buemo, C., Hiriart, M. I., Sestelo, A., & Salamone, D. F. (2015b). Tiger, Bengal and domestic cat embryos produced by homospecific and interspecific zona-free nuclear transfer. *Reproduction in Domestic Animals*, *50*(5), 849-857.

Oishi, T. (1997). Conservation and evaluation of animal genetic resources. *Farming Jpn*., *6*,18-25.

Park, H. S., Jung, S. Y., Kim, T. S., Park, J. K., Moon, T. S., Hong, S. P., Jin, J. I.,Lee, J. S., Lee, J. H., Sohn, S. H., Lee, C. Y., & Moon, Y. S. (2007). Production of cloned Korean native goat (Capra hircus) by somatic cell nuclear transfer. *Asian-Australasian Journal of Animal Sciences*, *20*(4), 487-495.

Park, M. C., Kim, J. Y., Kim, S. B., Park, Y.S., Park, H.D., Lee, J. H., Oh, D. S., &Kim, J. M. (2009). The effect of cryopreservation on the mouse embryos at various-pronuclear Stages. *Asian-Australasian Journal of Animal Science,* 22(2), 174-180.

Powell, A. M., Talbot, N. C., Wells, K. D., Kerr, D. E., Pursel, V. G., & Wall, R. J. (2004). Cell donor influences success of producing cattle by somatic cell nuclear transfer. *Biology of Reproduction*, *71*(1), 210-216.

Ren, F. L., Li, Y., & Zhang, Y. (2002). *In vitro* cultivation and freezing of bovine skin fibroblast cells. *Scalper Magazine*, *28*, 8-10.

Roux, W. (1885). Beitragzur Entwicklungsmechanik des Embryo. *Zeitschrift fur Biologie, 21*, 411-526.

Ryder, O. A., & Benirschke, K. (1997). The potential use of “cloning” in the conservation effort. *Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association*, *16*(4), 295-300.

Santos, M. L., Borges, A. A., Neta, L. B. Q., Santos, M. V., Oliveira, M. F., Silva, A. R., & Pereira, A. F. (2016). *In vitro* culture of somatic cells derived from ear tissue of collared peccary (Pecari tajacu Linnaeus, 1758) in medium with different requirements. *Pesquisa Veterinária Brasileira*, *36*, 1194-1202.

Siengdee, P., Klinhom, S., Thitaram, C., Nganvongpanit, K. (2018). Isolation and culture of primary adult skin fibroblasts from the Asian elephant (*Elephas maximus*). *PeerJ6*: e4302.

Simon, D. L. (1999). Better decisions in conservation of farm animal genetic resources by use of international sources of information. *Quarterly bulletin of the International Association of Agricultural Information Specialists= Bulletin trimestriel de l'Association internationale des spécialistes de l'information agricole*.

Tasripoo, K., Srisakwattana, K., Sophon, S., Nualchuen, W., & Usawang, S. 2007. Cloning of buffalo fibroblast cell from donor of different ages. *Buffalo Journal*, *23* (2), 141-152.

Tasripoo, K., Suthikrai, K., Sophon, S., Jintana, A. R., Nualchuen, W., Usawang, S., Bintvihok, A., Techakumphu, M., &Srisakwattana, K. (2014). First cloned swamp buffalo produced from adult ear fibroblast cell. *Animal, 8*(7), 1139-1145.

Tian, X. C., Kubota, C., Enright, B., & Yang, X. (2003). Cloning animals by somatic cell nuclear transfer–biological factors. *Reproductive Biology and Endocrinology*, *1*, Article number 98.

Vangipuram, M., Ting, D., Kim, S., Diaz, R., & Schüle, B. (2013). Skin punch biopsy explant culture for derivation of primary human fibroblasts. *Journal of Visualized Experiments*, 77, e3779.

Wang, T., Li, Z., Zheng, D., Liu, W., Huang, P., Zeng, Z., Xu, C., Wang, B., & Wei, J. (2020). Establishment and characterization of a fibroblast cell line from postmortem skin of an adult Chinese muntjac (*Muntiacus reevesi*). *In Vitro Cellular & Developmental Biology-Animal*, *56*, 97-102.

Webb, S. J., Zychowski, G. V., Bauman, S. W., Higgins, B. M., Raudsepp, T., Gollahon, L. S., Wooten, K.J., Cole, J. M., & Godard-Codding, C. (2014). Establishment, characterization, and toxicological application of loggerhead sea turtle (*Caretta caretta*) primary skin fibroblast cell cultures. *Environmental Science and Technology*, *48*(24), 14728-14737.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., & Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature*, *385*(6619), 810-813.

Wu, C. X. (1999). Theory and technique of animal genetic resources conservation: idioplasmic basis for persistant development of animal agriculture in the 21st century. *Journal of Yun Nan University (Natural Sciences)*, *21*, 7-10.

Xiong, H., He, X., Zhang, W., Li, C., Li, M., Guan, W., & Ma, Y. (2014). Establishment and characterization of a fibroblast line from Duroc. *Pakistan Journal of Zoology*, *46*(2), 363-369.

Yun, J. I., Koo, B. S., Yun, S. W., & Lee, C. K. (2008). In vitro development of interspecies somatic cell nuclear transfer embryos derived from murine embryonic fibroblasts and bovine oocytes. *Asian-Australasian Journal of Animal Sciences*, *21*(11), 1665-1672.

Zhou, X. M., Ma, Y. H., Guan, W. J., &Zhao, D. M. (2004). Establishment and Identification of a Debao Pony Ear Marginal Tissue Fibroblast Cell Line. *Asian-Australasian Journal of Animal Science*,17(10), 1338-1343.