Research paper

Standardization of a common protocol for establishment and cryopreservation of fibroblast cell lines from different indigenous livestock species

Himani Sharma[1], Rekha Sharma[1]*, RAK Aggarwal[1], PK Vij[1], Sonika Ahlawat[1], Th Randhir Singh[2], NV Patil[3] and MS Tantia[1]

[1]*ICAR-National Bureau of Animal Genetic Resources, Karnal (Haryana) India*
[2]*Central Agricultural University, Imphal, (Manipur) India*
[3]*ICAR-National Research Center on Camel, Bikaner, (Rajasthan) India*

ABSTRACT

The majority of precious livestock genetic resources remain exposed to the vagaries of genetic erosion due to limitations of *in situ* conservation programs. Fibroblast bank offers a practical approach to preserve germplasm due to advancement of cell culture techniques. The objective of current study was to biologically develop, evaluate and cryopreserve the skin fibroblast cells of different Indian livestock species obtained using an efficient, common protocol. Ear marginal tissues from Manipuri and Marwari horses (n=8) and Kutchi camel (n=6) were collected for this purpose. Primary culture and first passage was done using fibroblast specific media (HiFibroXL™). Epithelial-like and fibroblast-like cells emerged from the tissue explant margins within 10-14 days of culture in both the species. Subsequent passaging of cells for both horse and camel was continued using DMEM+Ham's F12 (1:1) media with 10% FBS. Fibroblast cells showed typical fusiform morphology with centrally located oval nuclei. Cells exhibited radiating, flame like or whirlpool like migrating patterns and density dependent inhibition during cell proliferation. The growth curve at passage-4 represented typical S-shape as the cell population passed through a lag phase, a logarithmic phase and a plateau phase. Population doubling time varied between 27.9 to 31.37 hrs with multiplication rate of 0.76- 0.86 population doubling/24 hrs. The cells were cryopreserved from 3rd to 6th passage stocking at least 75 cryogenically-preserved vials (1×10^{6} cells/ml) per animal. The protocol was further validated in six more species, mithun, yak, buffalo, goat, sheep and donkey. It can be concluded that fibroblast cell culture can be established from eight different livestock species at a faster rate and in a cost effective manner following a single common protocol described in this paper.

Key Words: Cryopreservation, epithelial cell, fibroblast, Kutchi camel, Manipuri horse, Somatic cell culture
***Corresponding author**: rekvik@gmail.com
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INTRODUCTION

Biodiversity is facing unprecedented challenges worldwide and vulnerable animals are threatened by the introduction of foreign species, massive destruction of their natural habitats and industrial pollution, as also found in India. It is crucial to protect the genetic resources of indigenous livestock. The primary emphasis in conserving livestock animal genetic resources in India has been on *in-situ* conservation, which no doubt has several positive attributes. However, given the global progress, actions have proceeded at a relatively conservative pace in our country (Goswami et al., 2016). This is not surprising considering the number of technical and socio-economic factors and most importantly finances involved in governing the maintenance of live animals.

Modern scientific methodologies can aid in accomplishing this feat. Semen from cattle has been viably cryobanked and stored since the late 1950's. In the interim, cryobanking techniques have been enhanced which has extended our capacity to store a

variety of other tissues (e.g., embryos, blood cells, fibroblast cells, primordial germ cells). The role of genetic resource banks, which provide collection, processing and storage of biomaterial, in the management and conservation of livestock species is particularly noticeable in the last decade. Under correct usage these recourses are enough to keep up current genetic diversity in the populations and allow their reproduction in the future. A core problem in creating such banks is to determine the quantity and type of preserved genetic material (Goswami et al., 2016). Most cryobanks focus on cryopreservation of the gametes (primarily sperm) and the embryos, being targeted to offspring reproduction by means of assisted reproductive technologies, including artificial insemination, *in vitro* fertilization and embryo transfer. Cryobanking of somatic cells as the nuclei donors for cloning is recently considered the additional approach for preservation and improvement of agricultural animals and poultry gene pools. Establishing somatic cell lines has been proposed as a practical approach to facilitate the conservation and utilization of genetic resource from various species (Li et al., 2009; Liu et al., 2011; Lin et al., 2013).

Somatic cell gene-banks for farm animals are more common in developed countries, but in developing countries including India, where much of the worlds dwindling genetic variation currently resides, it is in its infancy. Given the national depletion of animal genetic resources, the introduction of somatic cells in national gene-banks is required urgently (Singina et al., 2014). To date cryobanking of somatic cells has been an underutilized tool in national conservation programs. The approach is considered to be cumbersome and costly and moreover it is assumed that protocols need to be standardized for each livestock species to obtain somatic cells (Sharma et al., 2018).

Hence, there is a need for designing scientific interventions to minimize the material, time and cost involved in generation of somatic cell lines for cryobanking. Standardization of a common protocol will lead to acquiring the genetic material relatively quickly and will be a source to provide an important reserve of genetic resources that can be used for a wide variety of conservation, and research interests. In the present study, an attempt has been made to design a somatic cell culture protocol that is cost effective and is applicable across the livestock species. Cost often been cited as a limitation of gene banking will reduce substantially as the need for different cell culture consumables will be minimized.

MATERIALS AND METHODS

Sample collection

Ear pinna was selected for collection of skin biopsies as it offers advantages of easy accessibility, non-invasiveness and no limitations of sex or age of animal. Animal were selected only from the Government livestock farms so that all the government guidelines of animal ethics were followed. Cell culture media and chemicals were purchased from Sigma, USA unless otherwise indicated. Human fibroblast specific media-HifibroXL was from HiMedia, India. Skin biopsies (approximately 0.5 cm^2 tissue) of 8 healthy adult animals each of Marwari and Manipuri horse and 6 Kutchi camels were collected in DMEM/Ham's F-12 (Dulbecco's Modified Eagle's Medium (DME) and Ham's F-12 Nutrient Mixture 1:1 ratio) with antibiotic solution (100 IU/mL penicillin and 100 µg/mL streptomycin) after cleaning the marginal ear. Samples were brought to laboratory at 4^0C in the shortest possible time.

Tissue explant culture

Tissue was chopped into 1-2 mm^2 pieces with sterile

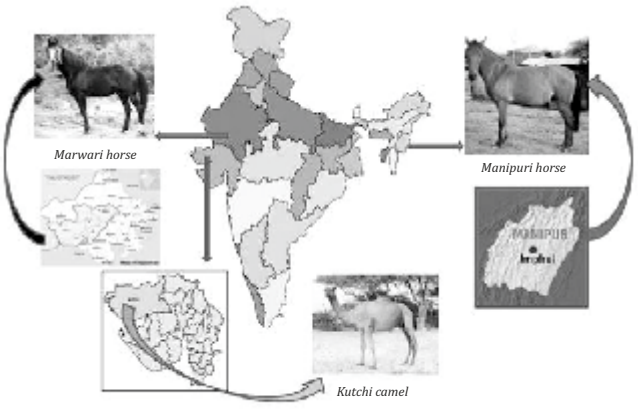

Figure.1 Distribution of horse and camel populations under study

forceps and scalpel after removing hair, epidermis and fat layer. Tissue explants were placed in a 35mm sterile petri dish. Pre-warmed media having 10% FBS (Fetal Bovine Serum) and antibiotics (100 units/mL penicillin and 100µg/mL streptomycin) was added and dishes were incubated at 37°C with 5% CO_2 in a humidified incubator. After 24 hrs, additional media was added to the tissue explants and these were left undisturbed for 6-7 days (Singh and Sharma 2011). Media was replaced every fourth day to remove cell debris. Dishes were observed for any sort of bacterial and fungal contamination. Explants were observed for migration of cells under inverted microscope every alternate day after one week.

Harvesting of cells

Once the minimum cell outgrowth of approximately 20 mm was observed around the tissue pieces, explants were removed from the petri dish. Cell monolayer was harvested using 0.25% trypsin-EDTA (Ethylenediaminetetraacetic acid) (Singh and Sharma 2011). Cell pellet was re-suspended in 1 ml of fibroblast specific growth medium. Cell count and viability was established with trypan blue dye exclusion method (Freshney, 2010). Viability of cells was determined by relative cell count of live cells to total number of cells under 10X magnification. All the cells were transferred to the 25cm^2 surface area culture flask and incubated at 37°C with 5% CO_2 in a humidified atmosphere. Flasks were kept undisturbed till 80% flask surface was confluent with the cells. Harvesting of the cells was done as described earlier. Cell pellet was re-suspended in 1 ml of DMEM/Ham's F-12 (1:1) complete growth media and sub-cultured at a ratio of 1:2. During subsequent serial passaging, the procedure was same as described above except for cell seeding density of 80,000 cells/flask.

Growth characteristics

Fibroblast cells of the 4^{th} passage were studied in DMEM/Ham's F-12 supplemented with 10% FBS and antibiotics. Thirty 25cm^2 cell culture flasks were set up per breed at a density of 8×10^4 cells per flask and were cultured for 10 days. Three flasks were harvested every day and cell numbers was recorded until the cell growth became negative. The multiplication rate (r) and population doubling time (PDT) was determined along the exponential phase of the cell growth as $r = 3.32(\log N_H - \log N_i)/ (t_2 - t_1)$, $PDT = 1/r$; N_H is number of cells harvested, N_i is number of cells seeded, t_2 is hours during harvest and t_1 is hours at initial time.

Cryopreservation and re-culture

Fibroblast cells in exponential phase of growth (70-80% confluency), were harvested using 0.25% trypsin-EDTA. The cell suspension ($1x10^6$ viable cells/ ml) was freezed in DMEM/Ham's F-12 having 10% DMSO. Cryogenic vials were labeled with the animal name, passage number, freezing serial number and date. Gradual freezing was followed with vials first placed overnight in 1ºC cooler at -80 ºC and then transferred to liquid nitrogen for long term storage. To recover cells, cryovials were quickly thawed at 37ºC, contents were transferred into flask containing DMEM/Ham's F-12 media with 10% FBS and cultured at 37ºC with 5% CO_2 in a humidified incubator. Subsequent culture conditions and harvesting followed by cell viability estimation was as described in the preceding section.

RESULTS AND DISCUSSION

This study represents first report of fibroblast cell line establishment from ear marginal tissues of different livestock species using a common cell culture protocol. Manipuri and Marwari horses and Kutchi camel were selected for the fibroblast cell line generation due to the urgent need for their conservation.

Marwari and Manipuri horses are of utmost historical and practical importance. These are found in different geographical regions of India (Fig. 1) and have unique features of adaptability to extreme climatic conditions, subsistence on poor feed and fodder and better resistance capabilities to withstand environmental stress and tropical diseases. Marwari horse is the desert horse breed of Marwar region of Rajasthan and is capable of covering long distances with good speed by virtue of its strong limbs and hooves. This breed is known for its elegance, viguor, endurance, intelligence, animated gait, beauty, alertness and peculiar ear tips

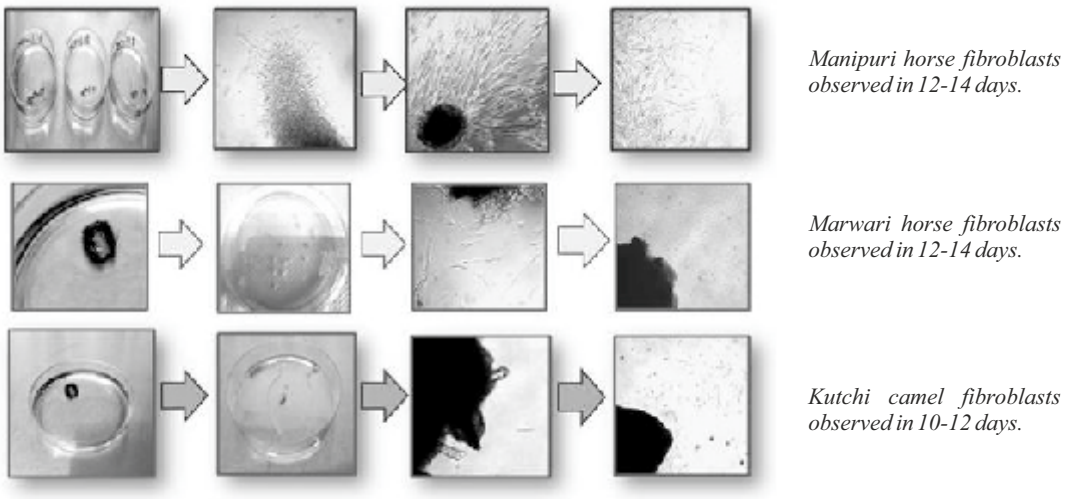

Figure 2. Cells emerging from ear tissue explant during primary culture in Human fibroblast specific medium (x40 magnification). a) tissue biopsy, b) tissue explants, c) primary cells coming out of tissue explants, d) cells ready for harvesting

touching each other when horse rotates its ears to 180 degree. These horses are now reared mainly for riding and sports (Chauhan et al., 2010). Manipuri breed is one of the purest and prestigious breed of equines of India. Manipuri ponies are loved for their stamina and speed and are globally famous for use in polo game. The Marwari population in India deteriorated in the early 1900s due to improper management of the breeding stock, and only a few thousand purebred Marwari horses remain (Jun et al., 2014). At present their population is 42,175 (Estimated livestock population breed wise based on breed survey, 2013). Manipuri horse is only 477 out of total 6,24,732 horse population of India. Breed numbers have dwindled to an extent that the Manipur state government officially declared the ponies as an endangered breed in 2013. Key reasons for this decline were loss of grazing land, urbanization and encroachment of wetlands that formed the natural habitat of this species. Exports of animals also contributed towards its population decline.

Kutchi breed of camel inhabits the *Ran of Kutch* in Gujarat state. Kutchi camel is facing seriously threatened situation due to more than 20 percent continuous decline in its population in the last two Livestock census of India. Major issues faced by camel breeders include rapid depletion in grazing land and water sources, increase in soil salinity, lack of health care services. Moreover, new generation is getting attracted to easier livelihoods options than the camel rearing. The population of Kutchi camel is only 20,653 out of 0.4 million camels in India.

A combination of two commercially available media, Human fibroblast specific media (HiFibroXL) and DMEM/Ham's F12 were selected for supporting outgrowth of fibroblasts from tissue explants and subsequent multiplication of the cells. Adherent method of primary cell generation from skin was selected over the tissue enzyme digestion method as it was simple and avoided injury to tissue explants (Hu et al., 2009; Anand et al., 2012). The tissue explants got adhered to the surface of petri-dish and cells started to emerge from these explants after 10-14 days in culture, irrespective of the species (Fig. 2). Cells were heterogeneous which included fibroblast, epithelial or keratinocytes. Fibroblast cells migrated from sides of the explants and started multiplying with increase in the incubation time. These findings were consistent with the time duration reported in literature for fibroblast emergence for various species (Pei et al., 2015). Similar to our observations, fibroblasts migrated from the tissue pieces upto 12 days after explanting for the Luxi cattle (Liu et al., 2008) and Fars native goat (Singh et al., 2011).

The principal concern during primary culture of skin tissue expalnts should be to maximize the fibroblast cells and to reduce other possible cell types such as

epithelial, keratinocytes or osteocytes. Keratinocytes as well as epithelial cells were observed to be very less in the current study, which can be attributed to the use of fibroblast specific media (HiFibroXL). Use of this media was continued for the first passage also to further reduce the unwanted cell types. This approach resulted in the establishment of epithelial free fibroblast cell culture in the second passage itself. Otherwise a culture of pure fibroblasts is obtained only after four to five passages (Mehrabani et al., 2016). With serial passaging fibroblasts gradually outnumber their epithelial counterparts (Wang et al., 2012). Compared to fibroblasts, epithelial cells take more time to detach from the tissue culture flask surface during trypsinization (Liu et al., 2014). Further, these cells take comparatively more time to attach after subculturing. Moreover, fibroblasts have higher growth potential. However, due to the use of fibroblast specific medium in explant culture followed by first subculture, the process of obtaining fibroblast cells was expedited substantially. It leads to almost pure fibroblasts just after the first passage itself. This saved not only the time but also the costly consumables which are required for execution of higher number of cell passages. More importantly, it also maintains the cell health. It is not uncommon for cells to cease growth and show changes in biological characteristics or lose their diploid properties with time in cultures (Li et al., 2009). It happens due to a variety of stimuli and factors, especially those determining hereditary traits might be undermined due to repeated trypsinization (Wang et al. 2012). Effective measures are thus required to ensure diploid stability in cultures of cells that are used for preserving valuable genetic resources (Liu et al. 2014). The genetic characteristics of the cells change by *in vitro* culture conditions after many passages, so a minimal number of passages are desirable to conserve them.

Fibroblast specific media was not used further after the first passage. Reason being that the published literature stated that the number of cells harvested from this media were comparatively less than that of observed in the other commonly used cell culture media such as MEM, DMEM-Hi glucose or DMEM/Ham's F-12 under similar culture conditions (Sharma et al., 2018). It was reported that total cell count in the HiFibroXL media (11.85±0.74 x 10^5/ml) was less than half of that observed in the DMEM/Ham's F-12 (27.50 ± 1.84 x 10^5/ml) on day 6 of the culture This reduction was due to the synthesis of elongated and larger cytoplasmic cell extensions in HiFibroXL media, which cover the tissue culture surface much faster than the smaller cells and lead to phenomenon of contact inhibition. As a result, cell multiplication was inhibited and less number of cells was obtained for cryofreezing (Wang et al., 2012).

DMEM/Ham's F-12 media has been reported to support growth of a broad spectrum of cells that include epithelial, endothelial and granulosa cells (Yi et al., 2012). Since, it is devoid of growth factors and

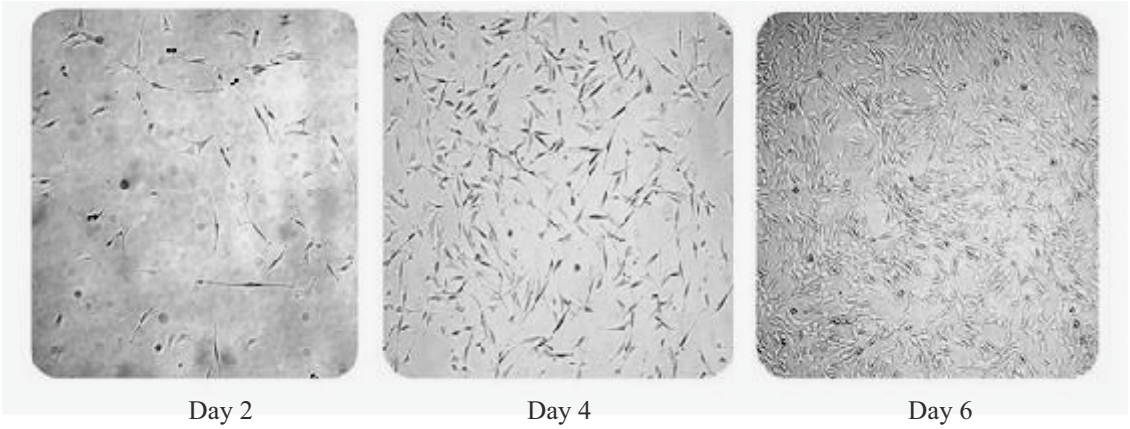

Figure 3. Morphology of the fibroblast cells in vitro. a) Subcultured cells on day 2, b) fiber like cells on day 4, c) near confluence cells on day 6 of culture.

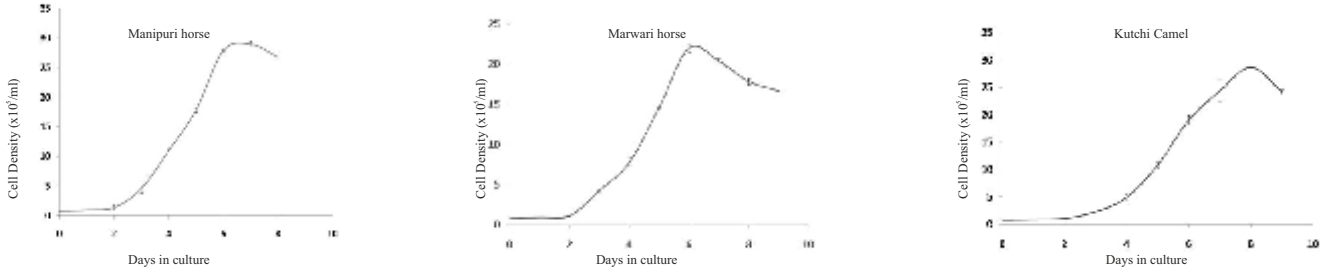

Figure 4. Growth curve of fibroblast cells (Mean ± SE of three observations) in different species in DMEM/Ham's F12 media (x 10 magnification).

other cell growth and multiplication stimulants as well as hormones that make any media specific and selective for a single type of cells. Moreover, this media is considered to be enormously nutritious for cells. It's simple constitution along with the widespread use makes it cheaper also. Thus a commonly used media for fibroblast culture (DMEM/Ham's) was selected to multiply the skin fibroblasts after second passage. Morphology, as the most important qualitative parameter of fibroblast cells was evaluated by light microscopy (Fig. 3). The cells had fibrous characteristics with turgor vitalis cytoplasm, and during growth, they showed typical fibroblast like morphology as radiating, flame-like or whirlpool migrating shapes. The selection of media for mass multiplication of the fibroblats was further supported by the ability of culture media to support cell growth. It was reflected in the pattern of cell growth depicted by a typical 'S shape' sigmoid mammalian growth curve in both the species (Fig. 4). The initial lag phase was approximately 48 h which correspond to the adaptation of fibroblasts as well as their recovery from trypsin damage occurred during harvesting of cells. It was followed by exponential phase of cell growth after which contact inhibition directed the cell multiplication to slow down at sixth day for horse and eighth day for camel. Stationary phase was finally culminated in the death phase of the cells.

Morphological observations were well supported by the observed values for population doubling time (PDT) and cell multiplication rate (r) in the two species. Within the tested samples, highest rate of proliferation was observed in Manipuri horse followed by Marwari horse and least in Kutchi camel and (PDT= 26.9h <30.5h <31.37h; r= 0.76> 0.79 > 0.86). Observed values corresponded to the optimum cell culture conditions including suitable culture medium in accordance with the reports of Singh et al. (2011) who established three fibroblast cell lines from lower edge ear skin samples of healthy dairy goats with a PDT of 25-30 h. Growth curve studies of Bactrian camel cell line revealed PDT of 26.13 h (Sharma et al., 2018). Liu et al. (2014) observed PDT of 30.2h for adherent fibroblasts of Wuzhishan miniature pig WPF22 cells. However, much higher PDT have been reported in some other species viz. 35.7 h for Sinihe horse (Cui et al., 2013) and 48 h for Jining Black Grey goat (Li et al., 2009) and Wenchang Chicken (Pei et al., 2014). On the basis of cell growth characteristics observed in the present study, it is rational to conclude that DMEM/Ham's F-12 media should be an appropriate choice for secondary fibroblast cell culture.

Cell line preservation was done by freezing of fibroblast cells in the freezing media. A minimum number of 75 vials per animal were cryopreserved. Cell viability before freezing varied between 95-98%, after thawing between 83-89% and after re-culture of thawed cells, it was between 95-99% with normal morphology. Standard behavior of cells in culture as presented in viability, proliferation rate and integrity of nuclear genome increase the chances of successful cloning (Ogura et al., 2013). Standardization of a common protocol for two different livestock species, horse (*Equus caballus*) and camel (*Camulus dromedaries*) in the current study, was further validated in six more species, mithun (*Bos frontalis*), yak (*Bos grunniens*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*), sheep (*Ovis*

aries) and donkey (*Equus asinus*). Cell health as well as a faster pace of cell multiplication was retained across these species. Morphology, confluency, cell count and viability of fibroblasts were also maintained.

Somatic cell cryopreservation is an alternative *in vitro* option for genetic diversity maintenance which needs customized culture condition for different species. Differential ability of a culture media in supporting fibroblast cells of species has previously been documented in the literature (Gorji et al., 2016). Singh and Sharma (2011) tested three commercially available media, known to support human and porcine specific fibroblast cultures, for their growth potential on goat skin explants. They concluded that the best medium for goat cell line proliferation is porcine-specific P-116. On the similar lines, different media have been recommended for establishing cell lines in case of diverse species such as Minimal essential medium for buffalo explants and Wenchang Chicken (Pei et al., 2014; Tasripoo et al., 2014), Porcine fibroblast growth medium for goat ear explants (Mahipal et al., 2011), DMEM for Sinihe Horse (Cui et al., 2013) and Jining Black Grey goat (Li et al., 2009). The main objective here was to design a protocol that minimizes the cost of obtaining somatic cells for cryo-conservation and that is functional across the species so that gene banks in the developing countries can afford this mode of preserving livestock biodiversity for posterity. Viable cryopreserved cell lines from different livestock species can be obtained using the common protocol described here from very small amount of biopsy material, including that of dead animals and contain complete genome and proteome. Cryopreserved somatic cells after repeated thawing are capable to regenerate, and hence almost infinitely may serve as a source of biomaterial for use in assisted reproductive technologies and biological research, including retrospective reconstruction unlike the germ cells and embryos. Fibroblast cell lines are most commonly used, because they are differentiated adult cells and are considered as a good source for IPSC (Induced Pluripotent Stem Cells) that can also be used for drug testing and molecular studies.

The development of fibroblast cell banks for various livestock species can provide an excellent resource for biological research and preserve valuable genetic materials. These fibroblasts can be used for various applications such as feeder layer of embryonic stem cells, tissue engineering, and wound healing researches and are not restricted to their use as a donor cell for nuclear transfer in cloning. In summary, a single method has been described for skin fibroblast generation from different indigenous livestock species. This method will make fibroblast cell cryopreservation more efficient, faster, cost effective and can be a method of choice for the rapid creation of cell banks.

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REFERENCES

Anand V, Dogra N, Singh S, Kumar SN, Jena MK, Malakar D, Dang AK, Mishra BP, Mukhopadhyay TK, Kaushik JK and Mohanty AK. 2012. Establishment and characterization of a buffalo (*Bubalus bubalis*) mammary epithelial cell line. PLoS One 7(7):e40469.

Chauhan M, Gupta A and Dhillon S. 2010. Genetic diversity and population structure of three Indian horse breeds. Molecular biology reports 38:3505-11. Doi-10.1007/s11033-010-0461-z.

Cui P, Hu P, Li C, Guan W and Ma Y. 2013. Establishment and Characterization of a fibroblast cell line from Sinihe Horse. Life Science Journal 10(4).

Estimated livestock population breed wise based on breed survey. 2013. Department of Animal Husbandry, Dairying and Fisheries. Ministry of Agriculture and Farmers Welfare, Government of India, pp 41.

Freshney RI. 2010. Culture of animal cells: a manual of basic technique and specialized

applications. 6th ed. Hoboken NJ: Wiley-Blackwell 732.

Gorji ZE, Khaledi KJ, Amoli AD, Ganjibakhsh M, Nasimian A, Gohari NS, Izadpanah M, Vakhshiteh F, Farghadan M, Moghanjoghi SM, Rahmati H, Abolhassan S, Fazeli S and Farzaneh P. 2016. Establishment and characteristics of Iranian Sistani cattle fibroblast bank: a way to genetic conservation. Conservation Genetics Resources 9(28): 1-8.

Goswami M, Mishra A, Ninawe AS, Trudeau VL and Lakra WS. 2016. Bio-banking: an emerging approach for conservation of fish germplasm. Poultry, Fisheries and Wildlife Sciences 4:143. doi:10.4172/2375-446X.1000143.

Hu H, Wang J, Bu D, Wei H, Zhou L, Li F and Loor JJ. 2009. In vitro culture and characterization of a mammary epithelial cell line from Chinese Holstein dairy cow. Plos one 4(11):e7636. doi: 10.1371/journal.pone.0007636.

Jun J, Cho YS, Hu H, Kim HM, Jho S, Gadhvi P, Park KM, Lim J, Paek WK, Han K, Manica A, S Edwards JS and Bhak J. 2014. Whole genome sequence and analysis of the Marwari horse breed and its genetic origin. BMC Genomics 15(Suppl 9):S4. doi:10.1186/1471-2164-15-S9-S4.

Li LF, Yue H, Ma J, Guan WJ and Ma YH. 2009. Establishment and characterization of a fibroblast line from Simmental cattle. Cryobiology 59:63-68.

Li XC, Yuea H, Li CY, Heb XH, Zhaob QJ, Mab YH, Guanb WJ and Maa JZ. 2009. Establishment and characterization of a fibroblast cell line derived from Jining Black Grey goat for genetic conservation. Small Ruminant Research 87(1):17-26.

Liu C, Guo Y, Guan W, Yuehui Ma, Zhang HH and Tang X. 2008. Establishment and biological characteristics of Luxi cattle fibroblast bank. Tissue and Cell 40:417–424.

Liu C, Guo Y, Lu T, Li X, Guan W and Ma Y. 2014. Establishment and genetic characteristics analysis of in vitro culture a fibroblast cell line derived from Wuzhishan miniature pig. Cryobiology 68:281-287.

Liu CQ, Guo Y, Guan WJ and Ma YH. 2011. Establishment and characterization of a fibroblast cell line derived from Mongolian sheep. Animal Science Journal 82(2):215–222.

Lin PY, Yang YC, Hung SH, Lee SY, Lee MS, Chu IM and Hwang SM. 2013. Cryopreservation of human embryonic stem cells by a programmed freezer with an oscillating magnetic field. Cryobiology 66:256–260.

Mahipal S, Anil S and Pushpa Y. 2011. Characterization of GSF289: a fibroblast cell line derived from goat ear skin explants. Journal of Biotech Research 3(1):1-6.

Mehrabani D, Booyash N, Aqababa H, Tamadon A, Zare S and Dianatpour M. 2016. Growth kinetics, plasticity and characterization of hamster embryonic fibroblast cells. Italian Journal of Zoology 83(3): 306-311.

Ogura A, Inoue K and Wakayama T. 2013. Recent advancements in cloning by somatic cell nuclear transfer. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 368(1609):20110329.

Pei P, He X, Zhao Y, Zhu Z, Kan J, Guan W and Ma Y. 2015. Establishment and characterization of a fibroblast cell line derived from Gansu alpine fine-wool sheep. Biocell 39(1):8–13.

Pei P, Pu Y, Zhao Y, Zhu Z, Kan J, Guan W and Ma Y. 2014. Establishment and biological characterization of fibroblast cell line from the Wenchang Chicken. Life Science Journal 11(8): 85-89.

Sharma R, Sharma H, Ahlawat S, Aggarwal RAK, Vij PK and Tantia MS. 2018. First attempt on somatic cell cryopreservation of critically endangered Camelus bactrianus of India. Gene Reports 10:109-115.

Singh M, Sharma AK and Yadav P. 2011. Characterization of gsf289: a fibroblast cell line derived from goat ear skin explants. Journal of Biotech Research 3(1):1-6.

Singh M and Sharma AK. 2011. Outgrowth of fibroblast cells from goat explants in three different culture media and establishment of cell lines. In Vitro Cellular and Development Biology 47:83-88.

Singina GN, Volkova NA, Bagirov VA and Zinovieva NA. 2014. Cryobanking of somatic cells in conservation of animal genetic resources: prospects and successes (review). Agricultural Biology 6:3-14. doi: 10.15389/agrobiology.2014.6.3eng.

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

Wang H, Li X, Li C, Zhang W, Guan W and Ma Y. 2012. Establishment and Biological Characteristics of Qing Kedan Chicken Embryonic Fibroblast Line for Genetic Resource Conservation. Journal of Animal and Veterinary Advances 11:4374-4381.

Yi J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134–146.