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Recovery of Fibroblast-Like Cells after 160 Days of Postmortem Storage of Goat Skin Tissues in Refrigerated Media

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Abstract

Animals have been cloned from frozen decade old postmortem tissues preserved within few hours of animal death. Delay in tissue preservation may reduce cloning success due to compromised nuclear DNA integrity. *In vitro* culture of cells ensures nuclear integrity and is a preferred method of preparing somatic cells for cloning. However, the time limits of postmortem recovery of cells capable of *in vitro* culture are not precisely known. Here we show recovery of fibroblast-like cells after 160 days of postmortem storage of goat skin in culture media at 4°C. Forty skin explants were cultured at 10 days interval up to 160 days and the outgrowing fibroblast-like cells around them were observed under inverted microscope. Explants with a cluster of more than 50 cells, after 10-12 days of culture initiation, were considered positive. We observed the outgrowth in all the time points, however, the confluence level reduced with increasing postmortem time interval. Secondary cultures established from primary outgrowth of 0-, 90- and 160-dpm tissues exhibited similar fibroblast-like morphology. Growth curves of 0- and 90-dpm cells were similar but 160-dpm cells grow slightly slower. Cytogenetic analysis performed on twenty G-banded metaphase cells of 160-dpm cell line revealed an apparently normal male goat karyotype with 60 chromosomes and their post-freezing cell-viability was >67%. Potential of using these cells to clone the goats remains to be seen in future. These findings may be useful in decisions for preservation of tissues for future cloning of animals and cell therapy.

Keywords: Goat skin; Fibroblast outgrowth; Explants; Postmortem; Cryopreservation; Animal cloning; Cell therapy

Introduction

One of the important challenges that come along with the high demographic growth is the decrease in diversity of livestock and poultry genetic resources. Therefore, preservation of tissues from various species/breeds or animals with desired traits has been suggested to conserve genetic diversity, before it is too late [1,2]. These preserved genetic resources could be utilized globally to overcome the climatic and/or other challenges to meet the increasing global demand for food, especially the protein sources. Some of the practical options for conservation are the preservation of semen, ovarian tissues, embryos, genomic and cDNA libraries [3]. However, preservation of small tissue samples, or cells cultured from them, seems to be ideal after the success of somatic cell nuclear transfer (SCNT) aka cloning technique. SCNT was developed first in Scotland [4] and subsequently practiced in many laboratories to clone almost every livestock species [5].

Different types of somatic cells have been used successfully to perform SCNT in mammals [2,6-8]. However, fibroblasts are the most commonly used cells because these cells are characterized as unspecialized mesenchymal cells with a high proliferation potential and their ability to differentiate into a variety of other connectives tissues, such as bone, cartilage, and muscle [9]. In order to obtain a healthy clone with full term development, the integrity of the nuclear genome of donor cells is essential [10]. It has been shown that cells that have a normal behavior in culture including their viability, proliferation rate, and longevity ensure the genomic integrity and enhance the success rate of animal cloning [11]. Recent studies have shown that the postmortem tissues can be preserved for many years at sub-zero temperatures before producing live animals by SCNT [12]. However, these tissues are preserved within few hours or days of animal death. Sometimes, it is not possible at farm level or even desired, for example, cloning of superior meat animals is possible only after post slaughter carcass evaluation (for meat quality) of animal herd. Delay in tissue preservation from slaughtered animals reduces success rate due to inability of retrieving live cells and/or intact nuclear DNA for cloning [10]. *In vitro* culture of cells is one way of ensuring nuclear integrity [11]. *In vitro* culture of cells from live as well as dead animal tissues preserved at sub-zero temperatures has been reported in several studies [10,13-16]. The nucleus from these cultured cells has been used to clone the animals even after many years of their death [10,15].

There is not enough literature to show as for how long the live and culturable cells can be recovered from postmortem tissues in mammalian species. There are reports of successful autograft skin transplantation up to 140 days and allograft transplantation up to 185 days of skin storage in refrigerated media [17]. However, since structural integrity and viability of refrigerated skin does not survive so long, it was suggested that these reports represent repopulation of cells from recipient in the lattices of grafts [17]. Fibroblast cells in postmortem skin tissues stored at 4°C were cultured up to 12-14 days after animal death in rabbits, pigs, goats and sheep [18,19], up to 41 days postmortem in goats [20], up to 24 days in human foreskin when stored in refrigerated media [21]. Muscle stem cells have been shown to survive for 17 days postmortem in human beings and 16 days in mice [22]. Here we show, recovery of fibroblast-like cells 160 days postmortem from goat skin tissues stored in refrigerated media, and demonstrate that these cells are cytogenetically stable with normal

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karyotype, were successfully cryopreserved, and exhibit normal growth profile.

Material and Methods

Sample collection and storage in refrigerated media

Goat skin samples were procured from the university slaughter house. Ears were excised from the animal head, cleaned with 70% alcohol swabs and brought to the laboratory in Ziploc bags. Tissues were processed within an hour of collection. About 2.0 cm^2 slices of skin were excised from the inner side of the ears using scalpel and a sharp blade. The slices were washed twice in DMEM media containing 50 I.U./mL penicillin and 50 µg/mL streptomycin. The tissue slices were stored in DMEM media with 10% fetal calf serum in 15 ml tubes in a laboratory refrigerator set at 4°C until used for *in vitro* culture.

Explants preparation and primary cultures

Skin slices from each tube were cultured, one at a time, after different days of postmortem storage. The excised slices from each tube were chopped into 4-5 mm 2 pieces (explants) and adhered onto two 60 mm diameter dishes (Falcon, BD Biosciences, Oxnard, CA) for each time point. Each dish contained 5 explants. These explants were cultured in P116 fibroblast culture media (Cell Applications Inc., San Diego, CA) at 37°C, 5% CO2 in a humidified environment. On Mondays, Wednesdays and Fridays the media was changed and the dishes were observed for any microbial or fungal contamination, explant dislodging, and for outgrowth of fibroblast-like cells under an inverted microscope. Contaminated dishes were removed from the study as soon as observed. On day 10-12 of culture, the presence or absence of the outgrowth around each explant was recorded. Any outgrowth containing a cluster of more than 50 cells was considered positive.

Secondary cultures, cell viability determination and cryopreservation

Primary outgrowing cells around the explants were trypsinized at 70-90% confluence and secondary cultures established as described [23]. Briefly, the outgrowing cells in dishes were washed twice with 2.0 mL of the balanced salt solution without calcium and magnesium (Gibco, Carlsbad, CA) and incubated with 2.0 mL of 0.125% trypsin for 5-7 min at 37°C. The trypsinized cells were neutralized with 5 vol. of P116 complete growth media, counted to assess cell viability using Trypan Blue Dye Exclusion Method [24], and pelleted at 200 x g for 7 min. The cells were re-suspended in Synth-a-Freeze® (Life Technologies Corp., Carlsbad, CA) media, aliquoted into cryogenic storage vials (1.0 × 105 cells/vial) and frozen at -80°C o/n using Nalgene™ Cryo 1°C Freezing Container (Nalgene, Rochester, NY). Next day the vials were transferred to liquid nitrogen tank and stored till used for further experiments. Representative cryovials were thawed and the post-freezing cell-viability percentages determined. To establish secondary cultures and/or to expand cell cultures, the frozen vials were quickly thawed at 37°C, mixed slowly with 10 vol. of the media, pelleted at 200 x g for 7 min, dissolved in complete growth media, and cultured in appropriate (T25 or T75) culture flasks.

Determination of growth pattern

The growth curves were generated from passage 4 cultures of 0-dpm (0-day postmortem), 90-dpm, and 160-dpm tissue derived cell-lines, using a 24-well micro titer plate format, as described earlier [25]. Briefly, 20,000 cells per well were inoculated in 0.5 mL of growth media to initiate the culture. Triplicate wells were trypsinized after 2, 4, 7, 9, 11, 14 and 16 days of culture. The viable cells were counted in each well using a Countess™ Automated Cell Counter (Life Technologies Inc.). Mean ± standard error of the means (SEM) of cell counts/mL in triplicate were plotted against time to generate growth curves using Microsoft Excel program.

Cytogenetic analysis

Cell cultures from 160-dpm cell line at p3 level were analyzed for cytogenetic stability using previously established methodologies [26] at Cell Line Genetics (Madison, WI; www.clgenetics.com). GTL banding technique was used. Chromosome assignments were made as per the Atlas of Mammalian Cytogenetics [27]. Cytogenetic analysis was performed at least on 20 G-banded metaphase cells.

Results

Effect of postmortem time interval on *in vitro* culture of cells from tissues stored in refrigerated media

In order to determine the postmortem time-limits, within which live cells can be cultured from tissues stored in refrigerated media, we analyzed 393 skin explants from 4 different goats for *in vitro* culture. Forty skin explants were cultured after 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150 and 160 days of postmortem tissue storage for each time point. The outgrowing cells around the explants were allowed to grow until the confluence was reached at around 70-90%. Subsequently, the dishes were trypsinized to recover the primary cells and stored in liquid nitrogen till used for further analysis. To compare the primary outgrowth around the tissues of various time points, we recorded the data for presence or absence of cell outgrowth on day 10-12 of culture (Table 1). Explants exhibiting clusters of>50 cells around them were considered positive. Outgrowth of fibroblast-like cells was visible as early as day 4 of culture in controls (0-dpm, fresh tissue

Days postmortem interval	# of outgrowth positive / total explants adhered [$]					
	Goat 1 ID:5025	Goat 2 ID:1614	Goat 3 ID:1629	Goat 4 ID:1636	Total	%outgrowth
00	10/10	10/10	10/10	10/10	40/40	100
10	06/06	10/10	09/09	09/09	34/34	100
20	10/10	09/09	10/10	08/08	37/37	100
30	10/10	09/09	09/09	08/08	36/36	100
40	10/10	09/09	10/10	05/05	34/34	100
50	10/10	10/10	05/05	08/10	33/35	94
60	10/10	09/09	10/10	05/05	34/34	100
70	09/09	10/10	10/10	10/10	39/39	100
80	*	10/10	10/10	05/05	25/25	100
90	10/10	10/10	*	*	20/20	100
100	n/d	09/09	*	*	09/09	100
110	n/d	10/10	*	*	10/10	100
120	n/d	04/10	*	*	04/10	40
130	n/d	03/05	*	*	03/05	60
140	n/d	n/d	*	05/05	05/05	100
150	n/d	n/d	n/d	10/10	10/10	100
160	n/d	n/d	n/d	10/10	10/10	100
Total	85/85	122/130	83/83	83/95	383/393	97.46

Note: n/d, not done; *Both dishes were fungal contaminated during culture and thus removed from the study; [$]In our experience we never observed outgrowth around tissues that did not adhere to the dish surface.

Table 1: Outgrowth of fibroblast-like cells around skin explants after different days of postmortem storage in refrigerated media.

derived cells) but increased slightly with increasing postmortem time interval. However, the outgrowth was visible around tissues within first 10-12 days of culture in all the time points studied. Out of 393 explants that adhered to dish surface (for at least first 3 days of culture), 383 (97.46%) exhibited fibroblast-like cell outgrowth. Table 1 summarizes the *in vitro* culture results obtained from 4 different goats for various postmortem tissue storage time intervals. As can be seen in Table 1, the outgrowth of cells was observed even after 160 days of postmortem tissue storage, at least in one goat.

Effect of postmortem tissue storage time interval on confluence of primary outgrowth

We compared confluence level of outgrowth, reached on day 10-12 of culture, for different postmortem tissue storage time intervals. As can be seen in a representative Figure 1, the confluence of outgrowing cells near explants was observed to be in decreasing order with increased postmortem storage time interval. For example, the explants cultured prior to storage (i.e. 0-dpm) reached to 100 % confluence in about 10 days; while during the same time period about 30% confluence was reached in 90-dpm tissues, and about 5% in 160-dpm tissues (Figure 1). The outgrowth in all the explants studied, including 160-dpm, reached to 70-100% confluence, however, it took about 5-10 days longer to reach the same level of confluence as that of fresh tissue-derived outgrowth.

Comparative morphology, growth curve, and freezing cell-viability of postmortem stored tissue derived cell lines

To determine the differences between cell populations derived from tissues stored for different time points, we established secondary cultures from primary outgrowth of 0-dpm (control), 90-dpm (middle level) and 160-dpm (last time point) tissue derived cells by serial passaging as described earlier [28]. Secondary cultures of these cells grow much faster, as compared to the primary outgrowth, and reach 70-90% confluence in 5-7 days in subsequent passages. As shown in Figure 2, morphology of fibroblast-like cell populations from three cell-lines look similar i.e. elongated, fibrous and bipolar, which is typical characteristics of fibroblast cells. Their growth curves, although show similarity for 0- and 90-dpm cell lines, differed slightly from 160-dpm cell line which seems to grow slower (Figure 3). To see if the postmortem tissue derived cells can be cryopreserved for future use, without losing their cell-viability significantly, we determined their post-freezing cell-viability. As can be seen in Figure 4, we observed>67% post-freezing cell-viability in these cell populations.

Cytogenetic stability of postmortem tissue derived cell-line

To determine any genetic change in the cells cultured from postmortem tissues, a cytogenetic analysis was performed on twenty G-banded metaphase cells of 160-dpm cell-line. We observed a normal male goat karyotype (Figure 5). The diploid number of the chromosomes was 60. It consisted of 58 autosomes and two (X & Y) sex chromosomes (Figure 5). These results are consistent with earlier studies on goat cytogenetics [29-32]. Twenty metaphase spreads were analyzed for any genetic aberration, of which 19 displayed normal 60 XY chromosomes, while one had a Y chromosome deletion. This aberration was thought to be most likely a technical artifact and thus these results are apparently consistent with a normal male goat karyotype.

Discussion

Reduction of metabolic activities upon refrigeration is well known and makes the basis of food and organ storage at low temperatures, to increase shelf life. Storage in buffered solutions such as culture media further assists retaining cellular integrity in biological tissues. However, little is known about the time limits of cell survival in mammalian tissues procured postmortem. Knowing as how long the individual cells in postmortem biological tissues remain alive will help design strategies to recover, preserve, and utilize the cells for cell-therapy and cloning of livestock to meet the increasing demand of food especially protein sources in future. In a recent report, fibroblast-like cells were recovered up to 41 days postmortem from goat tissues stored at 4°C [20]. These

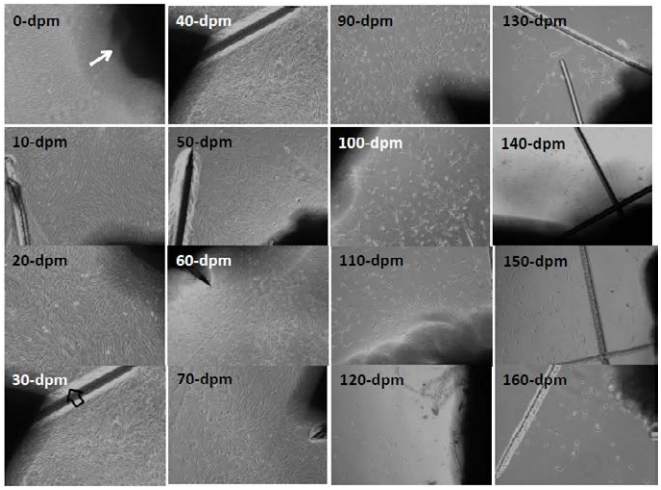

Figure 1: Comparative confluence of primary outgrowing cells on day 10-12 of culture for different time points: Skin explants are arrow marked dark shaded areas. Open arrow mark show scratches on dish surface under explants made to facilitate adherence of skin explants. Light microscopy; 100x magnification. dpm, days postmortem.

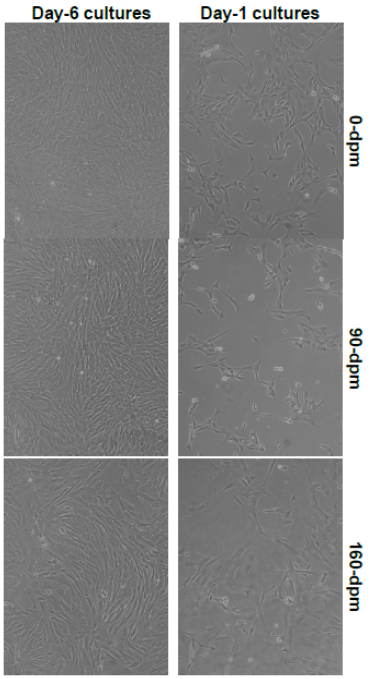

Figure 2: Comparative fibroblast cell morphology of 3 cell lines (passage 4): Light microscopy, x100 magnification. TS100 inverted microscope and DSL2 camera (Nikon) were used to capture images.

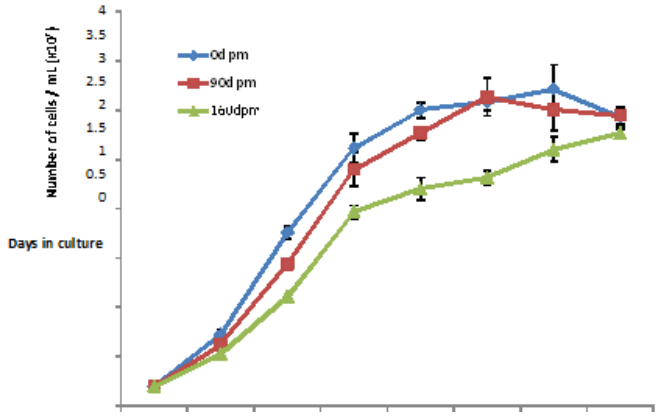

Figure 3: Growth curves of 0-dpm, 90-dpm and 160-dpm cell-lines, respectively: Each value represents mean ± SEM of cells in triplicate.

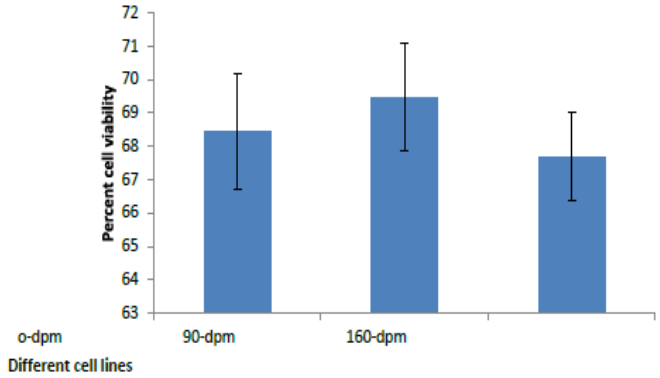

Figure 4: Post-freezing cell-viability estimations: Cell-viability values represent mean SEM of 3 independent experiments for each of the 3 cell lines.

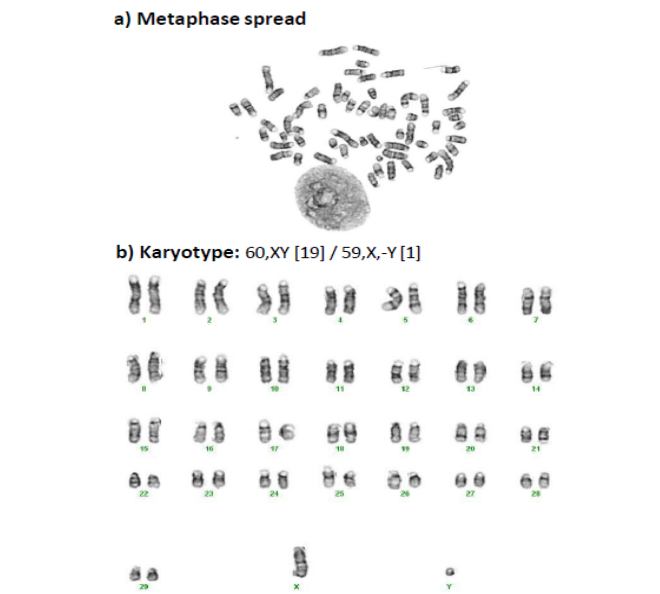

Figure 5: Cytogenetic analysis of 160-dpm fibroblast cell-line.

results prompted us to ask, if live cells can be recovered even beyond 41 days, if tissues are preserved in refrigerated media. To accomplish this, a simple *in vitro* culture procedure, previously established for goat skin tissue, was utilized [28]. Using this procedure, a comprehensive *in vitro* study was conducted on 393 skin explants from 4 different goats. Forty explants were cultured every 10th day up to 160 days of postmortem storage. Our results show outgrowth of cells around explants in all the time points including 160 days of postmortem tissue storage in refrigerated media. To our knowledge, this is the first report of *in vitro* culture of mammalian cells from postmortem tissues stored in refrigerated media for such a long time of 160 days. Although, reduced temperature reduces metabolic activity and consequently the pH fluctuations and metabolic toxic buildup, some cells might still be active. Storing tissues in media which is buffered might help neutralize the negative effect of pH and toxin fluctuations and thus help the cells to survive longer in tissues. Media also contain antioxidants e.g. Vitamin- E which might help cells to counteract effects of oxidative damage, if any.

Another interesting observation of this study was that the number of outgrowing cells around explants was in decreasing order, with increased postmortem tissue storage time interval, when compared on day 10-12 of culture. This could be possible due to reduced number of viable adult skin stem cells in tissues with increasing postmortem storage time interval. In similar studies, reduction in confluence of cells with increasing postmortem time interval has been reported in goats and sheep, when tissues were stored at room temperature [23,33] and in goat when stored at 4°C [20].

The morphology of cells derived from tissues of different storage time intervals was similar (Figure 2). However, the cell populations derived from 160-dpm tissues seem to grow slightly slow as compared to 0-dpm and 90-dpm cell populations (Fig. 3). Rudd et al. suggested that chromosomal imbalance may contribute to slower growth [34]. However, 160-dpm cells in this study retained normal karyotype, without any chromosomal anomalies, and thus could not be the likely cause of reduction in cell growth. Since the cells were embedded in tissues for longer time postmortem (and not involved in physiological processes at 4°C), it is possible that the activity of genes involved in growth was compromised. These assumptions are supported by earlier observations by Kaji and Matsuo, where they observed reduction of growth and population doubling times of the cell populations derived from primary outgrowth that was maintained from 6-73 days after the confluence was reached [35]. All cells retained high rate of post-freezing cell-viability ensuring long term storage for conservation of precious germplasm. It was also interesting to learn that such a long postmortem time lapse of 160 days did not affect their cytogenetic stability and thus their utility for animal cloning in future.

Conclusions

This study show that, a) skin tissue of goats stored in refrigerated media can be cultured up to 160-days of postmortem storage; b) the cells are genetically stable; c) the confluence of primary outgrowing cells decreases with increasing postmortem storage time interval and; d) the cell populations derived from 0-dpm, 90-dpm and 160-dpm tissues have comparative growth morphology but 160-dpm cells grow slightly slower. Future studies should reveal the potential of these cells for reprogramming and cloning of the animals.

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