

Postmortem Recovery of Proliferative Fibroblasts up to Three Days in Livestock Ear Skin Stored at 35°C

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Abstract

Effect of elevated temperature (35°C) on postmortem survival of cells in goat, sheep and bovine tissues was studied. Skin explants (n = 30; 2-3 mm²) were cultured in petri dishes after 2, 24, 48, 72, 96 and 120 hours of postmortem interval in DMEM media supplemented with 10% FBS, 50 units/mL of penicillin, 50 µg/mL of streptomycin and 2.5 µg/mL of fungizone. Outgrowth of cells around the explants was observed up to 72 h in sheep, 48 h in bovine and 24 h in goats. In general, the number of explants exhibiting outgrowth as well as the level of confluence decreased with increasing postmortem time interval. Secondary cultures established from primary cells for 72 h postmortem interval show cytogenetically stable chromosomes with 54XX[17] normal female sheep karyotype, comparable cell morphology, and growth curve to that of fresh tissue derived cells. Till date these cells have been passaged 21 times and show normal growth. These results suggest that normal, proliferative cells can be recovered from skin tissues stored at an elevated temperature of 35°C in livestock from 24-72 h of postmortem interval. Reprogramming of these cells to clone the dead animals or their use for cell therapies remain to be seen in future.

Keywords: bovine, sheep, goat, fibroblast, skin, postmortem cell recovery, animal cloning, cell survival, cell therapy

1. Introduction

Advances in somatic cell nuclear transfer (SCNT) *aka* cloning has allowed for the production of live animals from frozen postmortem tissues (Hoshino et al., 2009, Wakayama et al., 2008). Therefore, cryopreservation of mammalian tissues including livestock has been viewed as an important step in conservation of germplasm to meet the challenges originating from climate change and accidental death of elite animals. These preserved resources could be used to revive the lost genetics, to meet the global demand for food, specially protein source, to feed the increasing world population which is expected to be 9 billion by year 2050 (Alexandratos & Bruinsma, 2012). Animal tissues useful for cloning could be preserved directly by small biopsies from live animals or by first culturing cells from these biopsies followed by cryopreservation. An alternate and easier approach is by procuring tissue samples from animals postmortem and either cryopreserving directly or first culturing these tissues followed by cryopreservation of live proliferative cells. However, how long the live and proliferative cells can be recovered by culturing them from mammalian tissues after their death and, how different these cells are compared to fresh tissue derived cells, is not precisely known. Here we show *in vitro* culture of fibroblasts up to 3 days in livestock skin tissues incubated at 35°C after the animal death, simulating the summer field environment. Additionally, we show that these cells are cytogenetically stable with normal karyotype and have comparable growth profile.

2. Materials and Methods

Ears of slaughtered animals were procured from the university slaughter house. Ear skin was cleaned, first with tape water and subsequently with 70% alcohol swabs, wrapped in clean sterile paper towels, and stored in the laboratory in an incubator set at 35°C. Small slices were excised aseptically from the ear skin, chopped into small explants (2-3 mm²), and adhered onto 35 mm diameter dishes (Falcon, BD Biosciences, Oxnard, CA). Explants were cultured in DMEM media supplemented with 10% FBS, 50 units/mL of penicillin, 50 µg/mL of streptomycin and 2.5 µg/mL of fungizone (Gibco) at 37°C, 5% CO₂ incubator in a humidified environment. Media was changed weekly and the outgrowth of cells around explants was observed under an inverted microscope.

Primary outgrowing cells were trypsinized on day 10-12 of culture to recover outgrowing cells as described (Singh et al. 2011). Briefly, the cells in dishes were washed twice with 1.0 mL of the balanced salt solution without calcium and magnesium (Gibco, Carlsbad, CA) and incubated with 1.0 mL of 0.125% trypsin for 5-7 min at 37°C followed by neutralization with 5 vol. of growth media with serum. The cells were pelleted at 200 x g for 7 min and dissolved in 1.0 mL of growth media. An aliquot was taken to count the cells using Trypan Blue Dye Exclusion assay (Strober 2001). The remaining cells were plated in 35mm dishes to establish the secondary cultures from selected time intervals and purified by serial passaging. The purified cells were used for determination of cell morphology, growth curve, and for cytogenetic analysis.

Growth curve was generated by using a 24-well microtiter plate format. Briefly, 20,000 cells were cultured in each well in 500 µl of media and the growing cells were harvested and counted in triplicate wells after different time intervals using trypan blue dye exclusion assay (Strober, 2001). Mean of 3 replicates was plotted against time to generate the growth curve.

Pure cell populations from 72 hours postmortem (hpm) time interval in sheep (p8 level) were used for karyotyping, using GTL Banding technique, as previously established (Meisner & Johnson, 2008) at Cell-line Genetics (Madison, WI; www.clgenetics.com). Chromosome assignments were made according to the Atlas of Mammalian Cytogenetics (O'Brien, et al., 2006).

3. Results and Discussion

To evaluate the effect of elevated temperatures on postmortem survival of cells in mammalian tissues, we procured ear skin samples from university slaughterhouse from 3 livestock species *i.e.* goat, sheep and bovine. Using an *in vitro* explant culture assay as described previously (Okonkwo & Singh, 2015), ten explants each (5 explants/35mm dish) were cultured after 2, 24, 48, 72, 96 and 120 h of postmortem storage of ear skin tissues at 35°C for each species, simulating a hot summer field environment. Outgrowth around skin explants was observed under inverted microscope and recorded as positive or negative for each explant. Outgrowth was seen in every explant cultured from tissues that were stored at 35°C up to 24 h of postmortem in all three species. However, only sheep and bovine exhibited outgrowth up to 48 h of storage. Interestingly, sheep exhibited outgrowth even up to 72 h (table 1). None of the species could show outgrowth beyond 72 h of postmortem storage at 35°C. These results show that sheep tissues remain alive for longer time followed by bovine and goat. Perhaps it may be due to less amount of fat in goat tissues as compared to bovine and sheep (Park & Humphrey, 1986).

The percentage of explants exhibiting outgrowth (when combined for all three species) decreased with increasing postmortem time interval (PMI) from 100% for 2 h, and 24 h of storage to 66.67% and 33.33% for 48 h and 72 h of PMI, respectively (table 1). The level of confluence of cell outgrowth around explants was also seen inversely correlated with increasing PMI. For example, in figure 1, the outgrowth of 2-hpm tissues (uppermost panel) exhibits almost 100% confluence on day 10 of culture, whereas 72-hpm storage tissue outgrowth exhibits only about 10% confluence within same duration of *in vitro* culture. These cells from 72-hpm stored tissues also reached same level of confluence as in 2-hpm but took about a week longer. A reasonable explanation for this could be that the number of functional skin stem progenitor cells, from which the outgrowth has come, was reduced after longer time storage of tissues at 35°C as compared to fresh tissue (2-hpm storage) derived outgrowth. This statement is supported by the studies of Birdsill and his colleagues where they have shown that RNA degrades progressively with increasing postmortem time interval and thus a greater proportion of genes had decreased rather than increased expression with increasing PMI (Birdsill et al., 2011).

Table 1. Outgrowth of cells around explants excised from skin tissues stored at 35°C postmortem

hours postmortem (hpm)	Number of outgrowth positive explants (%)			Total (%)
	Goat	Sheep	Bovine	
2	10 (100)	10 (100)	10 (100)	30 (100)
24	10 (100)	10 (100)	10 (100)	30 (100)
48	00 (00)*	10 (100)	10 (100)	20 (66.67)
72	00 (00)*	10 (100)	00 (00)	10 (33.33)
Total (%)	20 (50)	40 (100)	30 (75)	90 (75)

Note: *explants exhibited only few keratinocytes. Ten explants were cultured for each time point for each animal species.

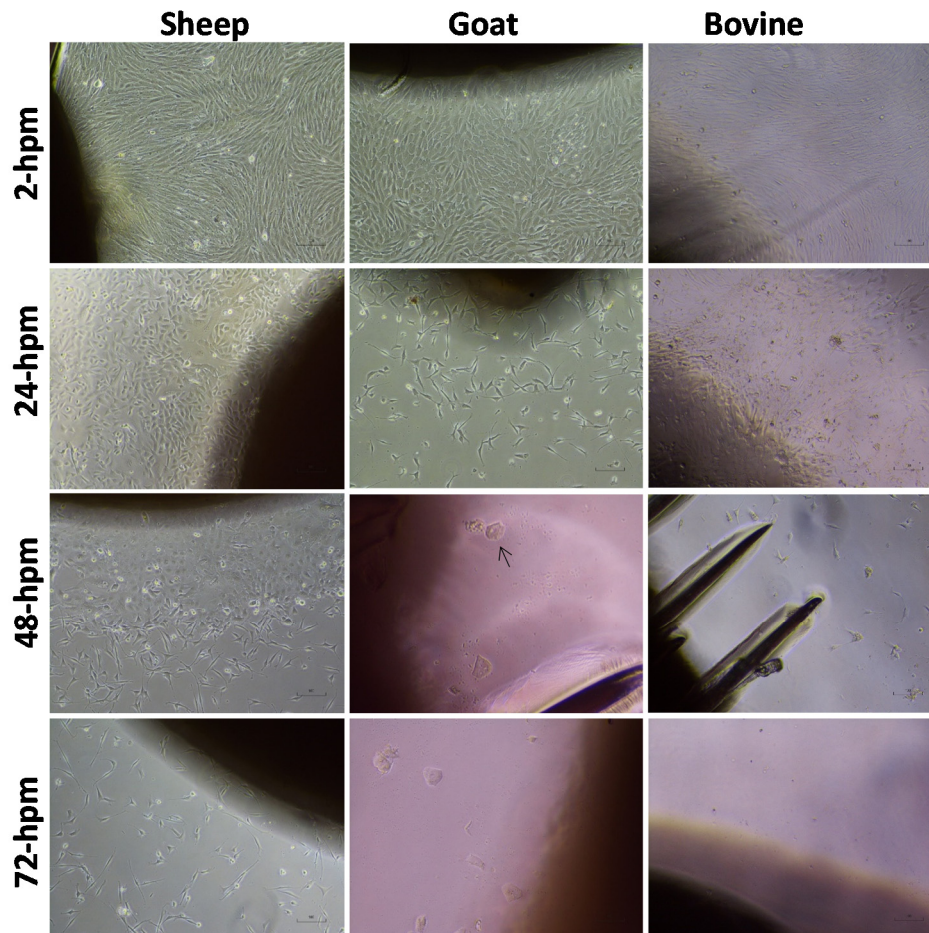


Figure 1. Comparative confluence of outgrowth around skin explants on day 10 of culture in tissues stored at 35°C for different PMI. Nikon TS100 inverted microscope was used to capture the images. Magnification, 100x; Black shaded areas are skin explants; hpm, hours postmortem. Arrow marked cell shows keratinocyte cells in goat

In order to have a quantitative assessment of the number of outgrowing cells around explants, in different postmortem time intervals, we harvested the cells from primary culture dishes after 10 days of *in vitro* culture and counted the number of cells using trypan blue assay (Strober, 2001). Primary cell count show a reduction pattern with increasing PMI in all the three animal species (Figure 2) which is consistent with confluence observed around explants in different postmortem time intervals (Figure 1).

In order to determine the differences between cell populations derived from fresh tissues (2-hpm) and the tissues stored at 35°C beyond 24 h, we established secondary cultures and purified them using serial passaging approach. Subsequently, we characterized these pure cell populations with respect to their growth profile and cytogenetic stability. The cell populations were morphologically very similar for both freshly cultured (2-hpm) and stored tissue derived (72-hpm) cells (Figure 3, panel B&C). Their growth curves also exhibited similar pattern, although 72-hpm derived cells seem to grow slightly slower (Figure 3, panel F). To determine, if there was any genetic change in cells derived from tissues stored at 35°C for 72-hpm, we analyzed 20 G-banded metaphase spreads of SSF35C-72HPM cell line (p8 level), which exhibited 54,XX[17] an apparently normal female sheep karyotype. Out of 20 metaphase cells counted, 17 demonstrated apparently normal karyotype while 3 cells (15%) demonstrated non-clonal chromosomal aberrations which are most likely artifacts of culture (Figure 3, panel D & E).

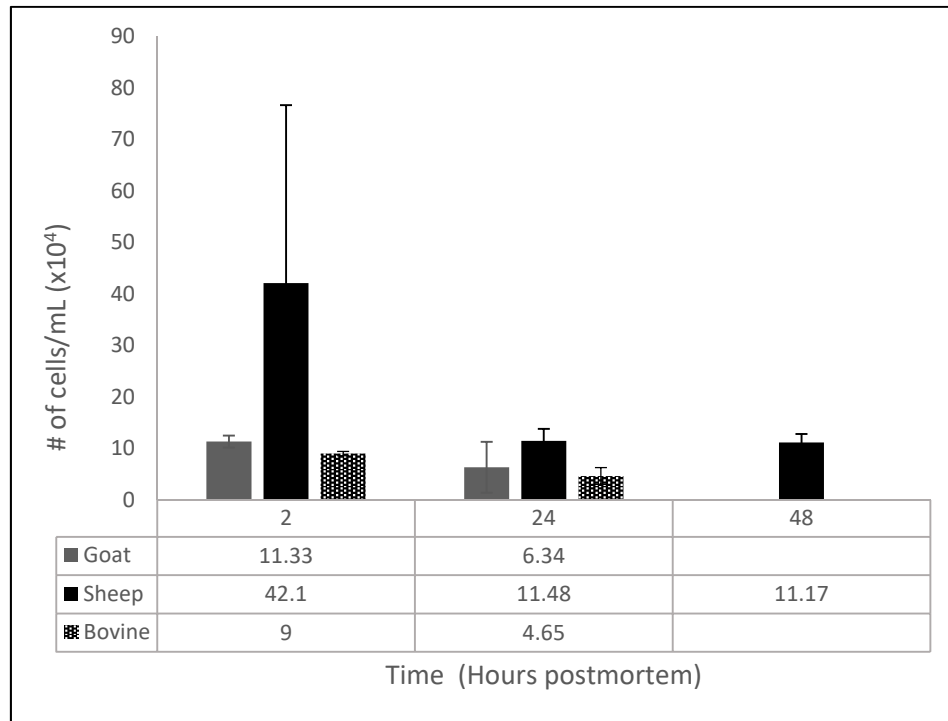


Figure 2. Number of cells obtained from primary outgrowth in each species after 2, 24 and 48 h of postmortem storage. Values represent mean \pm SD of counts in two replicate dishes, containing 5 explants each, after 10-12 days of *in vitro* culture

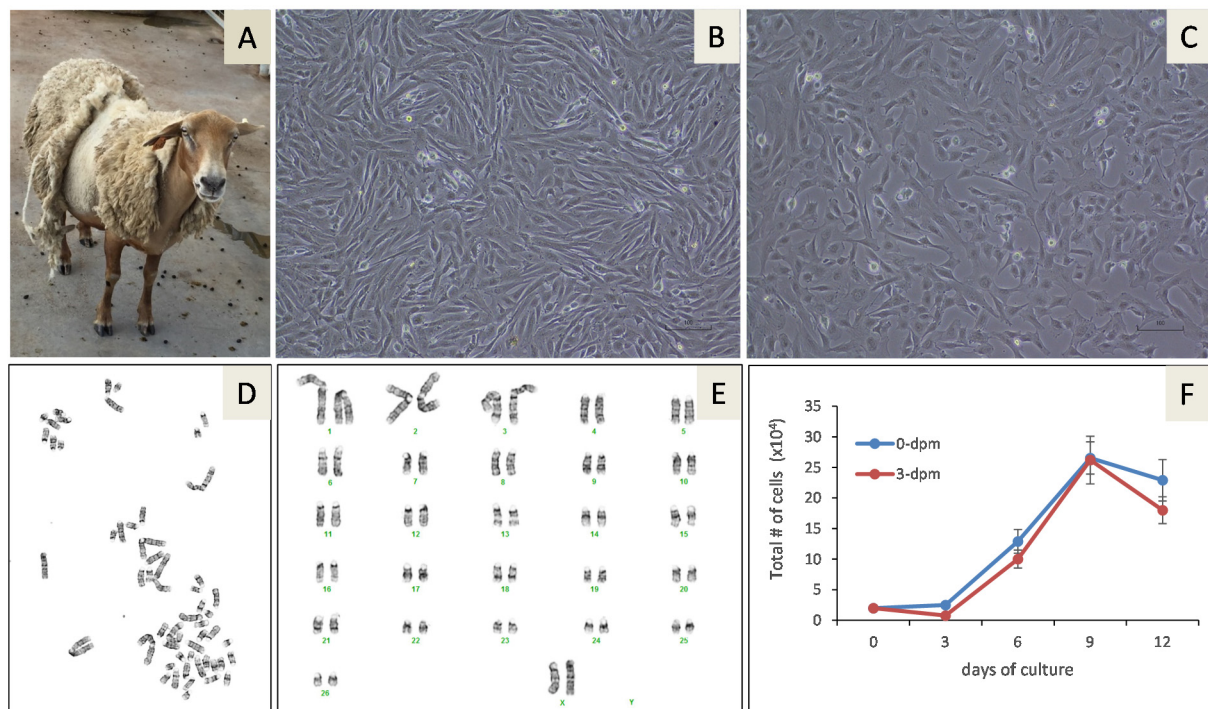


Figure 3. Morphology, growth curve, and cytogenetic profile of SSF 35C-72HPM cell line at passage 8 level: panel A, female sheep from which cell line was established, panel B & C, fibroblast cell morphology of 2-hpm (control) and 72-hpm cell cultures on day 3; Panel D&E, metaphase spread and karyotype of SSF 35C-72HPM cell line, respectively; panel F, growth curve of 2-hpm and 72-hpm cell cultures. Values in growth curve show mean \pm SD of total cell counts in triplicate on different days

In conclusion, this study shows that fibroblast-like cells can be recovered up to 3 days from mammalian tissues stored at the elevated temperatures of 35°C. The recovered cell populations can be normally frozen in LN₂ for later use, have normal growth profile, and are cytogenetically stable. Reprogramming of these cells to clone animals or for cellular therapies remain to be seen in future studies.

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