SHORT REPORT

In vitro culture of an accidentally deceased one month old goat kid skin up to 14 days of postmortem storage at 4°C

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Recovery of proliferative stem cells from postmortem tissues is an important area of research due to its potential applications in cellular therapies in human and veterinary medicine, and preservation of germplasm for future revival of lost genetics. The objective of this study was to assess the postmortem time interval (PMI) within which proliferative cells could be recovered from the tissues of newborn goat kids after their death. Skin explants (3-4 mm^2; n = 20) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) after 0, 7, 14, 21, and 28 days of postmortem (dpm) storage at 4°C. Outgrowth of cells was seen only up to 14 days of PMI in all (100%) of explants but not beyond that. Outgrowth started on day 3, 5, and 8 in 0-dpm, 7-dpm, and 14-dpm time intervals, respectively. The level of confluence was inversely corelated with increasing PMI. The average number of cells recovered from each explant was 26.11 x 10^4, 7.10 x 10^4, and 2.40 x 10^4 cells/mL for 0-dpm, 7-dpm, and 14-dpm cultures, respectively. Subcultures of these primary cells, when plated in equal numbers, showed comparable growth profile to that of fresh tissue derived cell populations. This study showed that postmortem tissues retained proliferative cells for much longer time than it was previously thought, and thus, could be preserved even after several days of animal death to recover the lost genetics in future.

Keywords: goat; neonate; fibroblast; skin; postmortem; stem cells; biobanking; animal cloning; cell therapy; cryopreservation.

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Introduction

Somatic cell nuclear transfer (cloning) facilitates production of live animals from postmortem tissues [1-3]. Recovery of live proliferative cells from tissues is a crucial step to ensure nuclear integrity of the somatic cells to be used for cloning. Most of the literatures showed that cloning of animals is performed from live and proliferative somatic cells recovered from biopsies of live animals within 48 h of animal death or from tissues/cells frozen within 48 h of animal death. Subsequent *in vitro* culturing of these tissues/cells was performed to ensure proliferative potential and thus nuclear integrity. Cryopreservation of tissues is important to meet the challenges originating from climate change and accidental death of animals. Small tissue samples from live or dead animals could be preserved for long term storage. Live proliferative cells can be recovered from these postmortem tissues [4-10]. However, how long the live and proliferative cells can be recovered from tissues after animal death is not precisely known. Therefore, the purpose of this study was to assess the postmortem time interval (PMI) within which proliferative cells could be recovered from newborn goat kids after their

death. Here we report the recovery of usable normal fibroblast cells as nuclear donors from the skin tissues of accidentally deceased newborn goat kids, which had been stored in refrigerator (4°C) for 14 days after death.

Materials and methods

Animal

An eight-pound singlet pure black female goat (*Capra hircus*) kid was born on April 2, 2022 at university pastures (Fort Valley State University, Fort Valley, GA, USA). It was produced after 152 days of breeding of two breeds of goats, a mixed Kiko doe and a pure Spanish buck, in October 2022. The kid was killed on May 2, 2022 exactly one month after its birth by an attack of black vultures in the field (Figure 1B). This accidental loss was discovered during the regular feeding time by the staff, and we believe that the kid might have been died about 10-20 h before we discovered it. However, we brought the tissues to the lab within one hour of our discovery in the field. Except the head area, rest of the body parts were missing as they were eaten by vultures leaving the bone of fore and hind limbs naked.

Ethics, tissue collection, and explant cultures

The research protocol of this study was approved by university Agricultural and Laboratory Animals Care and Use Committee (ALACUC) (Approval number: SR032021) (Fort Valley State University, Fort Valley, GA, USA). Both ears of the kid carcass were excised from the head and brought to the laboratory on ice within an hour of carcass detection. The ears were cleaned with water followed by 70% ethanol swabs and stored wrapped in a paper towel in the laboratory refrigerator set at 4°C until the tissues cultured. Small explants (3-4 mm^2, n = 20) were cultured weekly on 4 petri dishes (35 mm in diameter) (Falcon, BD Biosciences, Oxnard, CA, USA) with 5 explants per dish for each tested postmortem time interval (PMI) (0, 7, 14, 21, and 28 days of storage) in Dulbecco's modified eagle media (DMEM) supplemented with 10% Fetal bovine serum (FBS), 50 units/mL of penicillin, 50 µg/mL of streptomycin, and 2.5 µg/mL of fungizone (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The petri dishes were incubated at 37°C in a humidified 5% CO_2 incubator. The dishes were scored for presence or absence of outgrowth around the explants after 10-12 days of *in vitro* culture.

Cell passaging and counting

The recovered outgrowing cells were plated in 35 mm (diameter) dishes and purified by serial passaging as described before [9]. Briefly, the cells in dishes were washed twice with 3.0 mL of the balanced salt solution without calcium and magnesium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cleaned cells around explants were incubated with 1.0 mL of 0.125% trypsin for 5–10 min at room temperature. These cells were then neutralized with five volumes of serum containing complete growth media and were counted to assess their viable number using trypan blue dye exclusion method [11]. The remaining cells were pelleted by centrifugation at 200 × g for 7 min and the pellet was resuspended in media. These purified cells were then used to compare growth profiles.

Results and discussion

Black vultures pose a risk to livestock kid yield. Recently, 5 of the 8 (62.5%) newborn goat kids perished in our pastures due to vulture attacks. Research has shown that vultures often kill vulnerable kids by sharp blows to the head and pecking out the eyes. We were unsure as to what motivated the attacks since all kids were in perfect health and moving well. The increase in livestock attacks is thought to be occurring due to increased population of black vultures and increased competition between them for whatever food is available [12]. Due to the black vulture species being a migratory bird, which is protected by federal and state law, it makes it impossible to act in getting rid of these birds. Therefore, precaution such as higher fencing and less bird accessible facilities should be implemented to protect livestock from this

Table 1. Skin explant cultures after different days of postmortem tissue storage at 4°C.

Days of postmortem	# of outgrowth explants[$]	# of days when cells began to outgrow	# of days to reach ~90% confluence	# of recovered cells/mL (x10^5) [*]	# of cells/mL/explant (x10^4)
0-dpm	9	3	10	23.50	26.1
7-dpm	10	5	12	7.10	7.10
14-dpm	5	8	18	1.20	2.40

Notes: #: mean value. $: twenty explants (5 explants/dish) were initially used for each time point. *: numbers represented the mean values in at least two *in vitro* culture dishes containing 5 explants for each after reaching ~90% confluence. Contaminated dishes as well as floating explants were removed from the study. In our experience floating explants never exhibited outgrowth.

growing issue. Accidental deaths at livestock farms or in forests and during transportation are not uncommon. However, technology exists to bring back these lost genetic resources by providing the live and proliferative cells collected from these dead animals. The facilities for processing tissues for cryopreservation or tissue culture may not be available within a reasonable time frame, especially at small farms. Therefore, one of the objectives of this study was to determine the time limits within which proliferative cells can be recovered from animal tissues after their death.

The outgrowth of cells around explants up to 14 days of postmortem (dpm) interval in all the explants (100%) that adhered to dish surface was observed (Table 1). None of the explants exhibited outgrowth in 21 or 28 PMI. Cells started outgrowing from the explants on day 3, 5, and 8 in 0-dpm, 7-dpm, and 14-dpm time intervals, respectively. The level of confluence, as recorded on day 10-12 of culture initiation, was inversely corelated with increasing postmortem time interval (Figure 1). The cells from 14-dpm stored tissues, as seen on day 10, also reached high confluence as in other time points but took longer time (Figure 1, 14-dpm, day 16). A reasonable explanation for this could be that the number of functional skin stem cells, from which the outgrowth had come, was reduced after longer time storage of tissues at 4°C comparing to that of fresh tissue due to tissue decomposition. The support for this argument comes from an earlier study by Birdsill, *et al*., where they have shown that RNA degrades progressively with increasing postmortem time interval and thus reducing the proportion of genes involved in cell survival and proliferation with increasing PMI [13].

We also recovered the outgrowing cells by trypsinization of the primary cultures upon reaching around 90% confluence in each PMI and counted their numbers. The average number of cells recovered from each explant was 26.11 x 10^4, 7.10 x 10^4, and 2.40 x 10^4 cells/mL for 0-dpm, 7-dpm, and 14-dpm cultures, respectively (Table 1). This result also supported the observed progressive reduction in confluence levels of primary cultures (Figure 1). The recovered cells were also plated in 35 mm dishes and purified by serial passaging [9]. When equal number of these primary cells for each PMI were plated, we observed comparable growth profile and morphology to that of fresh tissue derived cell populations, suggesting the similarity of cell populations.

In conclusion, these results suggested that live, proliferative, and usable cells could be recovered from newborn goat kid skin tissues up to 14 days of postmortem interval, if the skin tissues from accidentally dead animals were stored at 4°C. Reprogramming of these cells to clone the dead goat genetics remains to be seen in future studies. This study showed that postmortem tissues retained proliferative cells for much longer time than it was previously thought, and thus, could be preserved even after several days of animal death to recover the lost genetics in future.

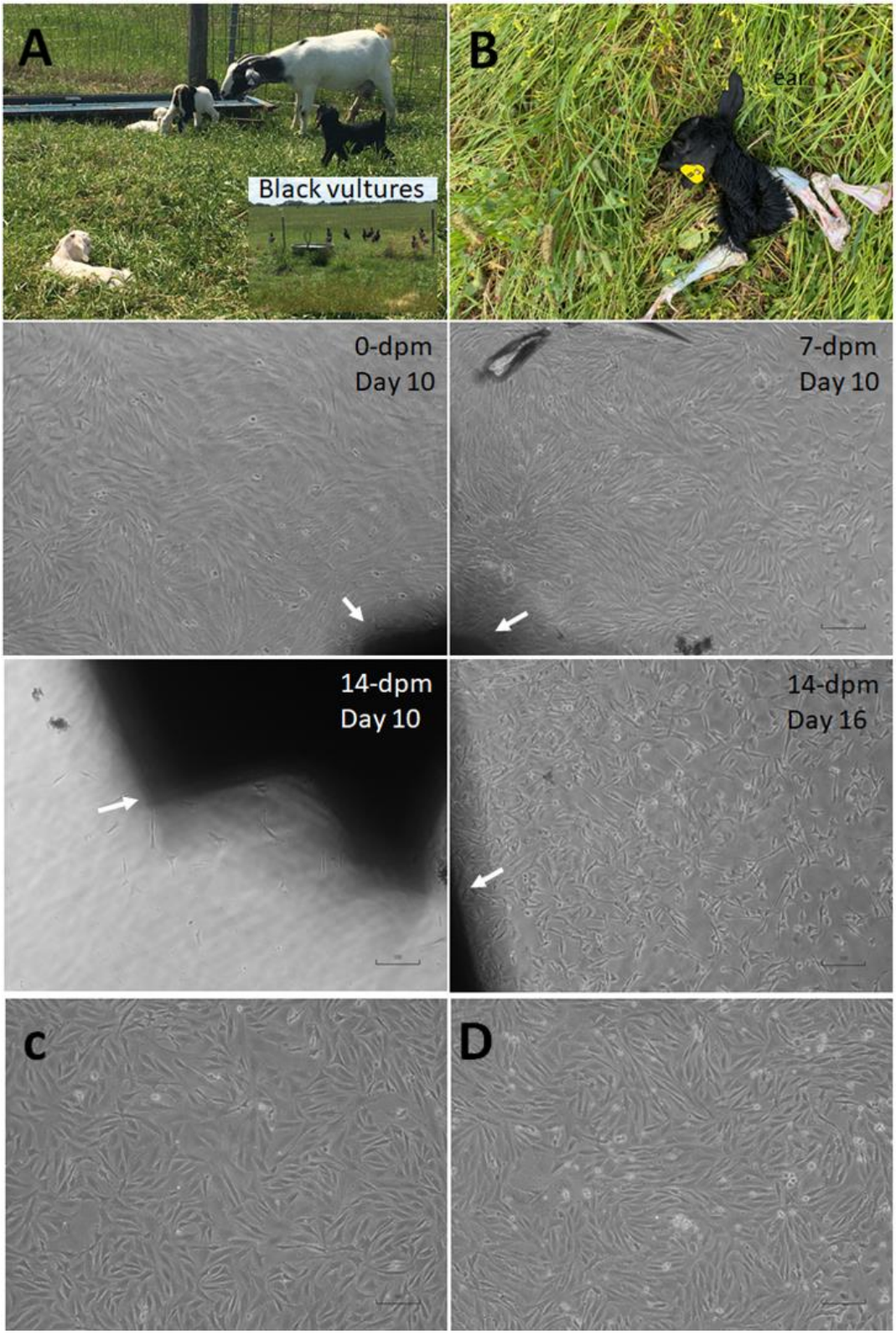

Figure 1. Comparative confluence of outgrowing cells around skin explants after different days of postmortem storage. Images taken by Nikon TS100 inverted microscope with 100x magnification (scale bar = 100 µM). dpm: days postmortem. Arrow marked black shaded areas are skin explants. **A:** newborn kids along with their mothers in the pasture. Also seen is a bunch of black vultures observed in the field. **B:** remains of a newborn kid killed and eaten by vultures. **C & D:** secondary cultures of 0-dpm and 14-dpm time intervals plated in equal amount (day 3 cultures).

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