



Thoughts on the source of tissue on subsequent cell culture success

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Abstract. This paper describes attempts to initiate equine adipocyte cultures from necropsy cases with varying intervals from time of death to isolation and culture. Equine adipocytes were isolated from 21 necropsy cases, regardless of the interval from time after death to establishment in primary ceiling cultures. However, while all cultures produced adipocytes, only 2 attempts to produce long-term equine adipocyte cultures from the subcutaneous rump fat depots were successful and not contami-

nated. Findings from these experiments indicate that it is possible to collect and culture equine adipocytes from necropsy cases with varying intervals of time of death to culturing provided that the issue of contamination is addressed. Viable cells were produced from tissue with an interval of 38.5 hours as well as 45 minutes. This result encourages the continuation of research using equine necropsy cases as a source of adipose tissue.

Key words: Adipose, Contamination, Culturing, Equine

Abbreviations: BSA = Bovine Serum Albumin; CS = Calf Serum; DMEM = Dulbecco's Modified Eagle's Medium; FBS = Fetal Bovine Serum

1. Introduction

Ceiling cell culture [2, 4] has been used to establish intermuscular and intramuscular ovine adipocyte cultures [5]. Equine adipocytes, however, have not been isolated and propagated in cell culture. Due to the relatively high economic cost of horses, the potential loss in performance due to tissue removal and non-recovery (depending on the location of tissue collection), the ethical use of live animals for research, and the facilities required to maintain research animals, we sought an alternative approach to traditional tissue collection techniques. Herein, we report the design of an adipose tissue collection system involving necropsy cases. The focus of this report is the identification of the successful harvest of equine adipocytes from animals at varying intervals after death.

The use of equine necropsy cases as the source of adipose tissue has several implications. First, a method has not been reported for the collection of the adipose tissue from live horses. Second, the availability of necropsy cases on a continual basis leads to a good supply of adipose tissue. Third, the use of adipose tissue from necropsy cases may lead to qualitative and quantitative experiments on adipocytes that may be harvested from animals that

have been dead for a few minutes compared to those dead for up to 38.5 hours. Since this research area is new to our laboratory, the unavailability of appropriate equipment and facilities within our laboratory forced preparation of media and isolation and preparation of tissue at various locations within the department. Conceivably, this may have contributed to an initial problem of contamination in our cultures of equine adipocytes.

The concept of harvesting living tissue from necropsy cases, which, until now has not been used as a tissue source, is the point of this presentation. The potential ability to produce viable, clean cell cultures from necropsy samples is worth pursuing as animals are not sacrificed and the entire process is less costly than obtaining tissue from live animals. In addition, this option may allow experimental designs to define aspects of adipocyte subpopulation viability after death as it is possible to produce adipocytes from necropsy-specimens. What are the mechanisms that allow these cells to remain viable for up to 38.5 hours after death of the animal? This latter type of study might have significance towards defining regulatory aspects of living fat cells. Thus, the goal of this paper is to fill a void that exists between tissue isolation and cell culture.

2. Materials

A. Equipment

1. Incubator, CO₂, Model No. 3326.¹
2. Laminar flow hood, Model No. NU-408FM-600.²
3. Microscope, inverted, Model No. Redyx.³
4. Eppendorf Easypet, Model No. 4420.⁴
5. Chemical balance (Ohaus Scale Corp.), Model No. B 300.⁵
6. Stirrer/hotplate (Corning), Model No. PC-320.⁶
7. Shaking water bath (Laboratory Line), Model No. R3545.⁷

B. Cell culture medium

1. DMEM/F12, Cat. No. 11330-032.⁸
2. 0.9% Saline (Baxter), Cat. No. 2F7124.¹⁰

C. Cell culture reagents

1. Fetal bovine serum, Cat. No. 16000-036.⁸
2. Calf serum, Cat. No. 16170-086.⁸
3. EDTA, Cat. No. ED2SS.⁹
4. BSA, Cat. No. A-7888.⁹
5. Collagenase, Type I, Cat. No. 17100-017.⁸
6. D-glucose, Cat. No. G-7528.⁹
7. NaH₂PO₄ (monobasic), Cat. No. S-369.⁴
8. Na₂HPO₄ (dibasic), Cat. No. S-0876.⁹
9. NaCl, Cat. No. S271-1.⁴
10. Antibiotic-antimycotic, Cat. No. 15240-062.⁸

D. Plastic and glassware

1. 10 ml pipettes (Falcon), Cat. No. 13-675-20.⁴
2. Culture flask (Corning), 75 cm², Cat. No. 10-126-30.⁴
3. Centrifuge tubes (Corning), 50 ml, Cat. No. 05-538-55.⁴
4. Stericup 0.22 μm filter system, Cat No. SCGPUO5RE.⁴

3. Procedures

A. Preparation of materials and solutions

1. Wash medium

Prepare wash medium by adding 5 ml antibiotic-antimycotic to 495 ml DMEM/F12, aliquot 30 ml per centrifuge tube, and freeze at -4 °C.

2. Collagenase digestion preparation

Prepare 200 ml of collagenase preparation by adding 0.5 g collagenase (0.25%), 0.18 g glucose (5 mM), 3.0 g BSA (0.5%), and PBS to 200 ml, filter sterilize, aliquot 30 ml per centrifuge tube, and freeze at -4 °C.

3. Culture medium

Prepare 500 ml of culture medium by adding 25 ml FBS, 25 ml CS, and 5 ml antibiotic-antimycotic to 445 ml DMEM/F12, filter sterilize and use immediately.

4. PBS

Prepare 500 ml of medium by adding 4.1 g

NaCl, 0.131 g NaH₂PO₄, 0.575 g Na₂HPO₄ to 500 ml ddH₂O, filter sterilize.

5. EDTA/PBS

Prepare 1 mM EDTA by adding 0.208 g EDTA to 500 ml PBS, filter sterilize, aliquot 30 ml per centrifuge tube, and freeze at -4 °C.

B. Cell culture methods (Flow Diagram 1)

Wash glassware and rinse eight times with tap water and eight times with deionized water. Steam sterilize before use for 15 minutes at 121 °C. Filter sterilize medium using a 0.22 μm filter system.

1. Tissue collection (at Necropsy)

- a) Clip the rump with #30 and #40 blades, scrub the clipped area with chlorhexidine solution, and rinse with 70% isopropyl alcohol.
- b) Make 2 15–20 cm incisions into the skin in an '+' pattern and reflect the corners back with towel clamps.
- c) Remove a 20 g adipose sample (approximately 2.54 cm²).
- d) Place the sample into 1 L sterile saline containing 20 ml antibiotic-antimycotic.
- e) Transport the sample to the laboratory (approximately 10 km).

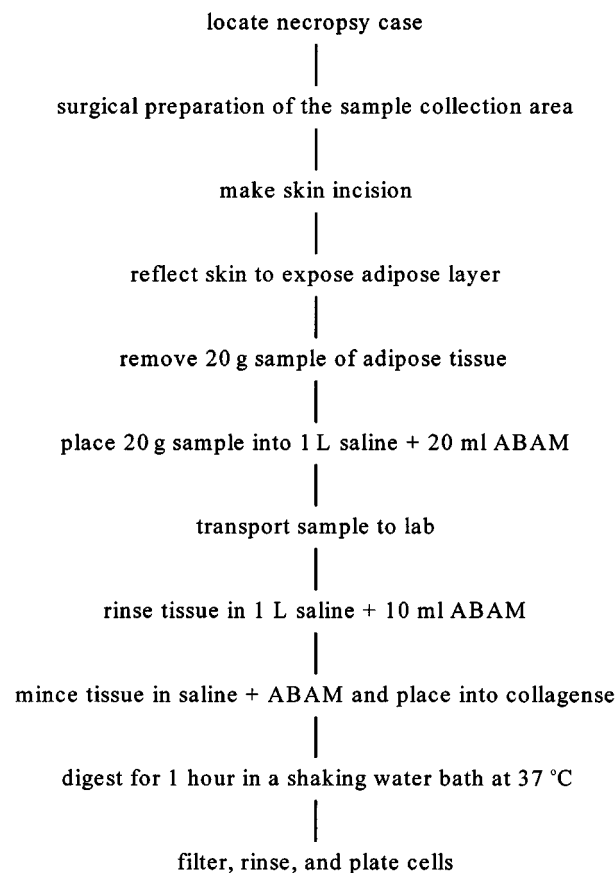


Figure 1. Flow diagram depicting the chain of events from specimen collection to culturing of the harvested adipocytes.

2. Digestion and washing (in the laminar flow hood)
 - a) Rinse the sample in 1 l sterile saline containing 10 ml antibiotic-antimycotic.
 - b) Mince the sample in a sterile petri dish filled with saline and antibiotic-antimycotic (taken from step a) prior to rinsing sample) using sterile scissors and transfer the tissue in 5 ml aliquots into 50 ml centrifuge tubes.
 - c) Add 5 ml collagenase digestion preparation to each tube.
 - d) Digest tissue for 1 hour in a shaking water bath at 170 rpm at 37 °C.
 - e) Filter tissue through 4 layers sterile gauze to remove the undigested tissue.
 - f) Allow cells to settle to the top of the filtrate (3–5 minutes).
 - g) Pipet the filtrate from the bottom, leaving the cell layer intact.
 - h) Pipet 10 ml wash into cell layer and repeat steps f) through h) 3 times.
3. Culturing
 - a) Place the cell layer (5–10 ml) into a 75 cm² flask that is completely filled (approximately 370 ml) with culture medium, tighten the cap securely and place the flask upside down into a humidified, 37 °C incubator with 5% CO₂.
 - b) Remove all medium after 4 d and replace with 20 ml fresh culture medium, and invert the flask from this point on, leaving the lid slightly loosened.
 - c) Observe cells microscopically (under 10× power) daily for several days to determine growth of cells and the presence of contamination and change medium every 2–3 days. If contamination is noted, the contaminated cells must be decontaminated or destroyed.

4. Results and discussion

Isolation of equine adipocytes

Cell culture systems provide many advantages to address research questions, since the carefully controlled *in vitro* environment allows monitoring of the exact response of the cells to different treatments [1]. In the present study, primary cultures of equine adipocytes were prepared from the subcutaneous rump fat depots collected from horses submitted for necropsy. Harvesting tissue immediately following death was not an option, therefore, adipocytes were harvested from horses between 45 minutes and 38.5 hours after death.

There were 13 samples harvested from horses that had been dead for 45 minutes to 3 hours prior to collection, 5 samples from horses dead 5 to 10 hours

prior to collection, and 3 samples from horses dead 15 to 38.5 hours prior to collection. Cells were harvested from all samples collected, therefore, the interval from time of death to isolation and culturing of the adipocytes did not appear to have an impact on the ability of a population of cells to survive. Based on visual observations, the growth pattern of cells collected from the one case that had an interval of 38.5 hours post-mortem appeared to be slower than that for all other cells collected. Cell growth in all other samples did not appear to be different based on visual observations.

Adipocytes were isolated and cultured by modifying the method of Vierck et al. [5]. Site preparation involved clipping then cleaning the site with chlorhexidine solution and 70% isopropyl alcohol. Approximately 20 g samples were removed from the incision site, placed into sterile saline containing 20 ml antibiotic-antimycotic, and transported to the laboratory. Samples were rinsed in another liter of sterile saline plus 10 ml antibiotic-antimycotic, minced with sterile scissors and digested in a collagenase preparation in a 37 °C shaking water bath at 170 rpm for 1 hour. After washing, approximately 5–10 ml of tissue suspension was placed into ceiling culture at 37 °C, 5% CO₂. Flasks were observed daily for cell growth, and the medium was replaced with 20 ml fresh culture medium on d 4–5 and subsequently every 2–3 days. Although adipocytes were established [2, 4, 5], as indicated by the inclusion of Oil Red O (Figure 2), contamination problems resulted that did not allow us to observe the differentiation of the adipocytes [5].

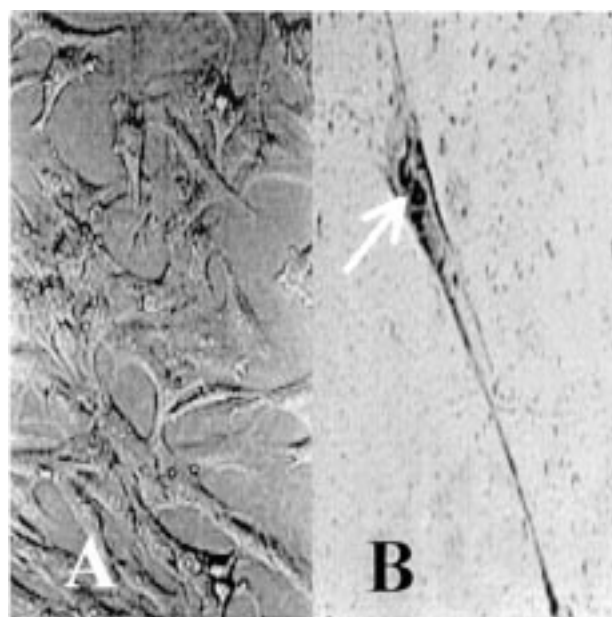


Figure 2. Photographs taken at 10× magnification of equine adipocytes growing in culture (A) and a stained adipocyte (B) showing oil red O inclusion. Photo A shows the lipid droplets around the nucleus.

Contamination

Although cells were produced in all cases, nineteen of twenty-one individual attempts to isolate and culture mature equine adipocytes were contaminated. Interestingly, the incidence of contamination appeared unrelated to the time of tissue collection relative to death. Furthermore, the identity of the contamination was variable, since yeast (*candida* sp.), bacteria (various environmental species) and mold were observed in different preparations. Samples were collected at three different facilities, and contamination was observed in cells harvested from horses at all three locations. These observations suggest that one particular collection environment was not critical in the development of contamination.

Time for the appearance of contamination varied from 3 days to 3 weeks, with an average of 10 days, and attempts to decontaminate were not successful. Since cell proliferation was observed, contamination appeared to have little impact on cell de-differentiation and initial proliferation of adipocytes. The sources of contamination were unknown. All precautions were used to ensure sterile plasticware, glassware, and media. Samples were collected in an aseptic manner, and bacteriological cultures taken from various media lots did not produce contaminants. In view of these considerations, it was believed that the most likely source of contamination was the tissue itself or the collection environment.

Applications

The focus of research by this laboratory is the hormonal control of seasonal reproduction in the mare. Although regarded as a long-day seasonal breeder, a small proportion of mares exhibit estrous cycles during the short days of the winter months. Ongoing studies by our laboratory have suggested that this phenomenon may be related to endogenous fat stores and associated leptin concentrations [3]. Therefore, understanding the mechanism controlling leptin secretion and the role that leptin plays in the control of reproductive function may provide new methods for the manipulation of reproduction in this commercially important species. Culturing adipocytes from horses may allow researchers to address some of the questions concerning leptin and its suggested function with reproduction. Therefore, the ability to isolate and culture adipocytes from horses would be of great benefit to this area of research.

In summary, this paper describes methods for isolation and initial culture of mature equine adipocytes from adipose tissue collected at necropsy. To date, mature adipocytes are reliably produced from necropsy samples with intervals from time of death to initiation of culture of 45 minutes to 38.5 hours. Once the problem of contamination is con-

trolled, long-term culture of adipocytes from equine adipose tissue of necropsy donors will be a viable experimental method. Once a dedicated laboratory can be made available, contamination problems should be minimal and equine adipocytes should be able to be produced in an easy and reliable manner.

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Notes on suppliers

1. Forma Scientific, Marietta, OH, USA
2. Nuair, Inc, Plymouth, MN, USA
3. Leitz, Germany
4. Fisher, Pittsburgh, PA, USA
5. Mettler-Toledo, Inc, Hightstown, NJ, USA
6. Corning, Corning, NY, USA
7. Laboratory Line instruments, Inc, Melrose Park, IL, USA
8. Gibco-BRL, Gaithersburgh, MD, USA
9. Sigma, St. Louis, MO, USA
10. Butler, Lexington, KY, USA

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