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Isolation of Mature Adipocytes and Stromal Vascular Cells under Adverse Sampling Conditions

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Abstract

Methods are described to obtain adipose tissue for cell isolation, under adverse isolation conditions whereby no scientific controls were in place. Such methods could be used by laboratories of institutions where controlled environments (surgery rooms, abattoirs) are not available. While not ideal, we show that a variety of adipocytes and adipocyte-like cells may be isolated from such methods. These types of procedures may facilitate a greater number of persons entering into the research arena with adipocytes, and are easily adaptable to other animal models.

Keywords: Beef; Custom pack; Adipose tissue; Stromal vascular cells; Mature adipocytes; Isolation; Limited facilities; Adipocyte; Cell culture; Mature adipocytes; Stromal vascular

Introduction

Adipocytes are cells that comprise a portion of any adipose depot. While they are not the only cells present in the adipose depot [1], discernible adipocytes function to assimilate moderate and long-chain fatty acids, store them as triacylglycerol, and release the fatty acids in times of negative energy balance, or in response to a repartitioning agent [1,2]. Adipocytes of some adipose depots also synthesize and release regulatory cytokines into the blood, which have been postulated to regulate a variety of whole animal and local non-adipose tissue physiology [1,3,4]. As such, in addition to moderating aspects of energy balance, adipocytes also may play a role in altering the body's blood pressure [3,4], ability to respond to insulin, control the rate of cellular apoptosis, fight inflammation and maintain normal levels of connective tissue surrounding tissues [5,6]. Adipocytes have been directly linked to obesity, metabolic syndrome, hypertrophy of the heart, and numerous other dynamic pathologies [7-9].

Stromal Vascular (SV) cells in adipose depots are present and active in the presence of fully differentiated adipocytes [10], and rodent-derived cells have largely been used as a model for defining aspects of adipogenesis [1]. Are there other animal models that may help in defining aspects of adipocyte physiology, whereby adipocytes from all adipose depots are abundantly available? Research with large animals (such as cattle) provides such an animal model. These types of animals form the foundation of the National Institute of Health/United States Department of Agriculture directive towards use of "dual purpose animals" for both human and animal production-related research, because these large animals are good models/donors for studying molecular/cellular mechanisms of adipocyte physiology. For example, cattle are sufficiently large that all adipose depots may be easily sampled at the same time for comparative adipogenesis studies (depot vs depot), or individually (intramuscular depot) for adipogenesis and lipid metabolism studies [11,12]. In addition, the expanding marker databases of cattle promote the usage of this model.

Previous research with beef-derived adipocyte isolation utilized existing animal facilities in controlled environments such as either a local surgical suite or a meat laboratory (abattoir). This allows one to be capable of controlling the environment to which tissues are initially prepped/isolated/handled and packaged for transport. Moreover, tissue obtained for cell isolation could be extracted from the donor animal fast, efficiently transferred to a sterile (buffer) environment, and (all) promptly returned to the cell culture laboratory for processing. What about those without such facilities such as urban medical schools, researchers at (very) small colleges or other countries whereby facilities are quite limited? Can tissues be isolated from beef cattle if none of the environmentally controlled facilities are available? The focus of this paper was to obtain skeletal muscle from beef animals, which were being terminated and initially processed by a custom-packing/processing company with on-the-farm service. If successful, such tissue procurement results may immediately suggest that research personnel with few facilities might be in a position to conduct research in this area.

Material and Methods

Animals and pre-slaughter material preparation

Animals used for tissue sampling were slaughtered in a beef farm located in Viola, Idaho-USA by using a mobile slaughter unit (Figure 1). No attempt was made to influence their normal procedures. Instead, representatives of the commercial entity physically provided samples to laboratory personnel. Immediately after receipt of the samples, laboratory individuals returned to the cell culture laboratory expediently. However, the locale and personnel responsible for handling of the live animals/slaughter of the same/obtaining the muscle samples, and providing the samples to the laboratory personnel was new and never used for any work like this previously. Thus, we were not able to control any of the steps in tissue isolation-other than to request the specific muscle that should be sampled. Cattle were simultaneously slaughtered by cerebral concussion followed by jugular venesection

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Figure 1: Mobile unit used for cattle slaughter and transit to processing facilities. The picture also shows the cold weather conditions of the day that the samples were collected. The snow on the ground contributed for a muddy yard where the cattle were harvested and the muscle samples were collected.

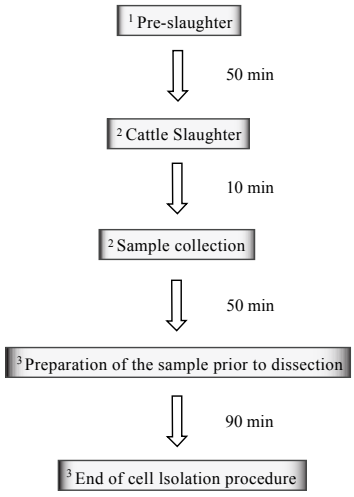

Figure 2: Approximate time spent at different steps of sampling and cell isolation. [1]Set-up of cell culture laboratory prior the sample collection including media and phosphate buffer saline (PBS) preparation and sterilization of material used for cell isolation procedure. The PBS solution used for sample collection were warmed up to 37 °C and place in a thermal box before leave the cell culture laboratory in order to keep it as warm as possible; [2]At the farm, samples were collected within 10 min after the animals were slaughtered and placed immediately in a beaker containing warm PBS (approximately 37 °C) and kept in a thermal box until arriving at the cell culture laboratory; [3]At the cell culture laboratory samples were rinsed with PBS (37 °C) several times before the sample dissection and cell isolation.

and the muscle samples were collectively collected immediately after bleeding.

The cell culture laboratory was prepared for cell isolation procedure one day before the tissue sampling. All the supplies needed including plastic and glassware, and buffer solution (PBS) were sterilized prior the tissue collection in order to optimize the cell isolation procedure. The buffer solution was warmed in a water bath to 37°C and kept in a thermal box during the pre-slaughter until the samples collection in order to keep it as warm as possible.

Tissue samples

At the farm, samples of sternomandibularis muscle from four Angus cattle were collected within 10 min after slaughter and immediately placed in a sterile beaker containing warm phosphate buffered saline (PBS) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 ng/ml Fungizone B and 50 µg/ml Gentamicin. The beakers containing the tissue were placed in a thermal box as soon as the samples were collected in order to keep the samples warm, and then taken to the cell culture laboratory. As soon as the samples arrived at the cell culture laboratory they were immediately processed 50 min after collection and delays were avoided on the way back to the cell culture laboratory in order to short the time between sampling and cell isolation. A timeline of the sample collection and cell isolation procedure is presented in Figure 2.

Cell isolation procedure

Mature Adipocytes (MAs) and Stromal Vascular (SV) cells from Intramuscular Fat (IMF) depot were isolated as described by Fernyhough et al. [13] with minor modifications as it follows (Figure 3). Preparation of reagents and media used for cell isolation is described in Table 1.

1. Rinse the muscle sample with PBS (37°C, pH = 7.08) supplemented with antibiotics/antimycotics and trim it prior to muscle dissection in order to reduce chances of contamination.
2. In a laminar flow hood, place the sample in a sterile dish, bath in PBS. Using sterile forceps and scissor dissect the muscle tissue in order to access the intramuscular fat depots. Once beginning the sample dissection, do not turn the tissue upside down since to keep the interior of the sample as clean as possible.

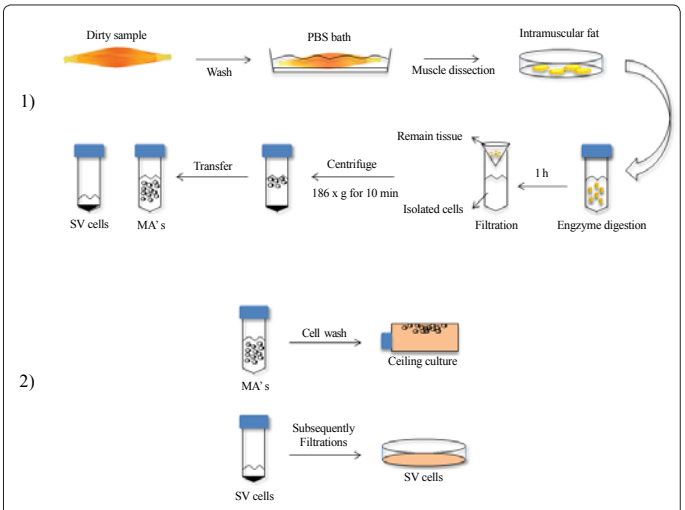

Figure 3: Flow diagram with the steps of the cell isolation procedure within the cell culture laboratory: 1) Preparation of muscle sample, muscle dissection and mature adipocytes (MA's) and stromal vascular (SV) cells isolation – muscle samples were rinsed several times with PBS containing antibiotics and antimycotics in order to clean the samples as much as possible before taking it to the lamina flow hood. Once washed, samples were bath in sterile PBS placed in a dish inside of the lamina flow hood for muscle dissection. Fragments of intramuscular fat (5 g) was isolated from the muscle using sterile forceps and placed in a 10cm dish containing PBS and minced into small pieces in order to increase the surface area to optimize the enzyme digestion. Minced tissue was digested by collagenase type I for 1h at 37 °C. After digestion, the solution was filtered through 1000 μm plastic mesh to separate the digested tissue from the cells. The solution containing isolated cells was centrifuged at 186 x *g* for 10 min and the two types of cells were segregated. MA's were found at the top layer of the solution and SV cells were found at the pellet on the bottom of the tube. 2) MA's were transferred into a new tube and washed by centrifugation at 186 x *g* twice with DMEM/F12 + 10% FBS before plating in a 12.5 cm^2 flask completely filled with DMEM/F12 + 10% HS. The SV cells were resuspended with DMEM/F12 +10% FBS and filtered subsequently through a 100 μm and 40 μm mesh filter before plating with the same media in a 10cm dish.

Solution	Chemical	Amount	Comments
PBS (pH = 7.08)			
	NaCl	10.0 g	PBS must be autoclaved at 121°C at 15 psi for 30 min in a glass bottle. Before the addition of antibiotics and antimycotics the solution must be cooled. After add the antibiotics and antimycotic the solution must be store at 4°C.
	KCl	0.25 g	
	Na_2HPO_4	1.44 g	
	Distilled H_2O	1 l (final volume)	
DMEM/F12 (pH = 7.08)			The solution must be filter sterilized in the lamina flow hood passing the media through a 0.22 µm pore filter into a sterile 1-l bottle. Store at 4°C.
	DMEM/F12 mixture	1-l envelope	
	$NaHCO_3$	2.438 g	
	Distilled H_2O	1 l (final volume)	
Enzyme solution			Collagenase must be dissolve in 100 ml of PBS (37°C) and subsequently filter sterilized by passing the solution through a 0.22 µm pore vacuum filter. The solution must be prepared just before use.
	Collagenase type I	0.25 g	
	PBS	100 ml	
DMEM/F12 + 10% serum (FBS or HS)			Basal medium must be prepared in a lamina flow hood by adding serum and antibiotics to the final volume of 500 ml. Store at 4°C.
	DMEM/F12 solution	442.5 ml	
	Serum (FBS or HS)	50 ml	
	Penicillin/ streptomycin	5.0 ml	
	Gentamicin	2.5 ml	

Table 1: Preparation of reagents and media used for cell isolation.

3. Place approximately 5 g of intramuscular fat tissue in a sterile 10 mm dish containing 10 ml of PBS. Cut the isolated fat tissue in small pieces (approximately 1-cm^2) and place the minced tissue in a sterile 50 ml centrifuge tube containing 25 ml of warm (37°C) sterile collagenase type I solution (0.25% collagenase in PBS). Place the tube in a rocker and incubate for 1 h at 37°C.

4. After the enzyme digestion, filter the solution through a sterile 1000 µm plastic mesh in a sterile funnel into a new 50 ml tube.

5. Centrifuge the filtrate for 10 min at 186 x *g*. After the centrifugation, an underlying pellet will be seeing at the bottom of the tube which contains the stromal vascular cells and the mature adipocytes will be floating in the top layer of the solution.

6. To isolate the mature adipocytes, gently pipette the top layer of the solution into a new tube. Add the same volume (1:1 v/v) of DMEM/F12 + 10% FBS into the tube and wash the cells twice by centrifugation at 186 x g. After wash the cells, place the isolated mature adipocytes into a 12.5 cm^2 cell culture flask filled with DMEM/F12 + 10% horse serum (HS). Invert the flask so the bottom of the flask is on top and incubate it at 37°C in a 5% CO_2 incubator [13].

7. To culture the stromal vascular cells, resuspend the underlying pellet of the digested solution with 10 ml of DMEM/F12 + 10% FBS and filtered once through a 100 µm and subsequently through 40 µm cell strainers into a new 50 ml centrifuge tube. After filtration vortex it vigorously and transfer the solution to a 10 cm dish. Rinse the tube with an additional 10 ml of DMEM/F12 + 10% FBS and transfer into a cell culture dish. Incubate the isolated stromal vascular cells at 37°C in a 5% CO_2 incubator.

Photomicrographs

All photomicrographs were taken with a Sony RGB digital camera (3/4–inch chip) coupled to a Nikon Diaphot phase contrast microscope and Image Pro Plus® image analysis software. The major equipment,

Equipments	Source	Model
Autoclave, gravity air remover type	American Sterilizer Company	P-89501-091
CO_2 water-jacket incubator	Beckman Instruments Inc.	TJ-6
Dry heat gravity oven	The Newell Group	1370 GM
Lamina flow, biological safety cabinet, Labguard Class II	Nu Aire Inc.	NU-425-4000
Peristaltic pump	Milipore Corp.	7015-72
Pipet aid	Drummond Scientific Co.	174
pH meter, digital	Corning Incorporated life Sciences	430
pH electrode	Corning Incorporated life Sciences	47636
Propane torch	Sigma Chemical Company	TS2000
Water bath	Precision Scientific	185
Microscope	Nikon	Diaphot-TMD phase inverted

Table 2: Major equipment used for cell isolation procedure.

Item	Source	Product Number
Dulbecco's modified eagle's medium (DMEM)/Ham's F12 (F12)	Invitrogen	12500-062
Fetal bovine serum	Invitrogen	26140-079
Horse serum	Invitrogen	16050-114
Collagenase Type I	Invitrogen	17100-017
Gentamicin solution, 10 mg/ml	Invitrogen	15710-064
Penicilin-streptomycin (pen/strep)	Invitrogen	15140-122
KCl	Sigma Chemical Company	P-5405
KH_2PO_4	Sigma Chemical Company	P-5655
NaCl	Sigma Chemical Company	S-5886
$NaHCO_3$	Sigma Chemical company	S-5761

Table 3: Media and chemicals used for solutions preparations.

Item	Source	Product Number
Media bottles		
125 ml with cap	Wheaton Scientific Products	219715
250 ml with cap	Wheaton Scientific Products	219717
500 ml with cap	Wheaton Scientific Products	219719
Pipettes		
5 ml glass disposable	VWR Scientific Products Corporation	53283-774
10 ml glass disposable	VWR Scientific Products Corporation	53283-776
Flask, dishes and centrifuge tube		
Tissue culture flask, 25 cm^2	Thermo Fisher Scientific, Inc.	163371
Tissue culture dish, 10 cm	Thermo Fisher Scientific, Inc.	172931
Tissue culture dish, 15 cm	Thermo Fisher Scientific, Inc.	168381
50 ml conical tube, plastic	Thermo Fisher Scientific, Inc.	339653

Table 4: Glassware and plastic supplies used for cell isolation procedure.

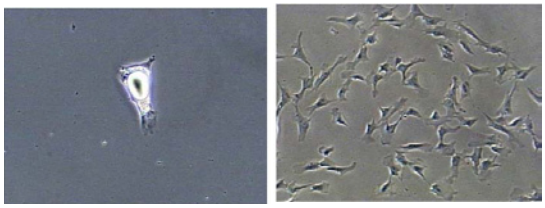

Figure 4: A) 48 h after cell isolation, mature adipocytes attached the ceiling of flask; B) 7 d after cell isolation, progeny cells derived from mature adipocytes reached confluence. [A, 200 x magnification; B, 100 x magnification].

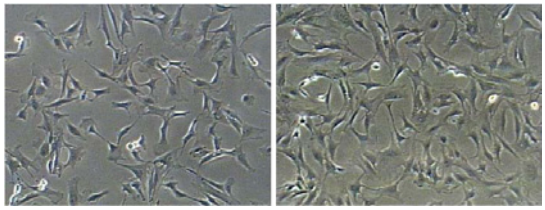

Figure 5: Photomicrographs (A and B) of stromal vascular cells cultures 72 h after cell isolation [100 x magnification].

media and chemicals, glassware and plastic supplies for cell isolation are presented in Table 2-4.

Results and Discussion

It is fairly clear that the environment in which tissue culture is carried out must be clean. This concept leads us to think that for a successful isolation procedure tissue handling must be done with extremely aseptic manner by trained individuals in order to avoid any source of contamination and transported to cell culture laboratory as soon as possible. As such, to isolate cells from a tissue in order to obtain primary cell culture more than an equipped cell culture laboratory is required, being necessary to be as close as possible to the place that the tissues are obtained. Additionally, one of the most important considerations that should be taken prior to cell isolation is the cooperation and collaboration of the clinical staff, which can be easily achieved if a person responsible for tissue isolation is also a member of the cell culture laboratory.

From the moment that a biological sample is removed from its natural environment within the animal, it is susceptible to deterioration from external factors such as moisture loss and temperature fluctuation and excessive passage of time. Another threat to the success of any cell isolation method is contamination from microbial sources including the host's own natural flora. All phases of the isolation process offer opportunities for contamination to occur unless strict adherence to proper aseptic techniques is consistently followed [14]. Additionally, the availability of the cells isolated from a tissue sample also depends on how fast the tissue sampling, dissection and digesting can be done, as in most cases contamination problems are related to the time [15].

In this study Mature Adipocytes (MA; Figure 4) and Stromal Vascular (SV; Figure 5) cells were successfully isolated from bovine muscle collected under non-asepsis conditions. No signs of contamination were detected by microscopy visualization such as cloudiness of media, deterioration of the cells and detachment of the cell monolayer, which would indicate mycoplasm contamination [16,17]. The photomicrographs results clearly shows that a great number of cells were successful isolated even though the tissue was collected under adverse conditions and was completely exposed to a non-aseptic environment prior to cell isolation procedures, which was never done in this laboratory before. Additionally, due to the distance between the local of tissue sampling and the cell culture laboratory, the samples were not promptly processed as suggested by several previously studies [14,18,19].

The results obtained in this study encourage new researchers in cell culture field by showing a protocol that allows the isolation of cells from muscle tissue without proper animal facilities. By using the cell isolation procedure developed in our laboratory additionally to the use of basic sterile techniques at the cell culture facility, we were able to obtain non-contaminated, healthy, primary cultures of cells. However, even though we were able to isolate cells in an adverse scenario, it is still recommended the use of sterile techniques during sample collection to avoid contaminations and cell death.

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