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Short communication

Technical Note: A comparison among adipogenic induction protocols for dedifferentiated fat (DFAT) cells obtained from subcutaneous fat of pigs

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ABSTRACT

In the current study we have performed two experiments to evaluate the effects of adipogenic induction media on dedifferentiated fat (DFAT) cells redifferentiation into mature adipocytes. In experiment 1, we aimed to evaluate whether it is necessary to use insulin in the induction media to allow DFAT cells differentiation into mature adipocytes by establishing two experimental treatments where insulin was either withdrawn from the culture medium, after 72 h of a normal induction period (Treatment 1: Insulin$^-$) or kept in culture media (Treatment 2: Insulin$^+$) for 16 d. In experiment 2, we aimed to evaluate if the lack of 3-isobutyl-1-methylxanthine (IBMX) in the induction medium would affect the differentiation of DFAT cells into mature adipocytes. For that, DFAT cells were induced to differentiate into lipid assimilating adipocytes using an induction medium containing IBMX (Treatment 1: IBMX$^+$) or without IBMX (Treatment 2: IBMX$^-$) during the first 72 h of induction. In both experiments we have evaluated the mRNA expression of lipid metabolism markers and cell morphology through Oil-Red staining as indicators of differentiation of DFAT cells into lipid-assimilating cells. The results of Experiment 1 revealed no differences in mRNA expression for any of the lipid metabolism markers with exception of *GLUT4* ($P=0.02$), which was greater in Insulin$^-$ compared to Insulin$^+$ treatment. Similarly, no differences were observed for mRNA expression of adipogenic markers between IBMX$^+$ and IBMX$^-$ treatments with exception of *FABP4* ($P=0.01$), which was greater for the IBMX$^-$ compared to IBMX$^+$ treatment. In both experiments we did not observed any differences in cell morphology among treatments. Our results suggest that neither insulin nor IBMX are required to accelerate redifferentiation process of pig-derived DFAT cells.

1. Introduction

Dedifferentiated fat (DFAT) cells are characterized as multipotent cells, obtained after a ceiling culture of mature adipocytes submitted to a dedifferentiation process that leads to a proliferative-competent progeny cells with potential to differentiate into mature adipocytes or transdifferentiate into other cells cell types (Sugihara et al., 1989; Fernyhough et al., 2004, 2005; Matsumoto et al., 2008).

Although many studies have used DFAT cells for adipogenesis studies, the induction efficiency varies according to the type of adipogenic medium and protocol adopted (Fernyhough et al., 2008; Matsumoto et al., 2008; Chen et al., 2010). A standard adipogenic medium consists of serum, insulin, a glucocorticoid agonist, mainly dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) (Cornelius et al., 1994). However, the protocols currently used to induce DFAT into mature adipocytes are not commonly optimized and it is not known whether all the components used in adipogenic medium are really necessary. For instance, several studies (Rubin et al., 1978; Hauner, 1990) highlighted the importance of insulin in the adipogenesis process but the use of insulin during the entire induction process has not been shown. Likewise, the real need of IBMX, which is a nonselective phosphodiesterase inhibitor that raises levels of intracellular cAMP and protein kinase A (PKA) required for transcriptional activation of PPARγ and thus adipogenic gene expression, in adipogenic medium of DFAT cells remains unknown. Eliminating the need of IBMX from the induction medium would be desired as IBMX is a foreign

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compound which activates and inhibits many cell processes (Brindle and Montminy, 1992).

Our long term goal with this research is to understand if cultured DFAT cells require an artificial induction medium to convert from proliferative-competent cells into lipid assimilating adipocytes. In this study we have tested two hypotheses by performing two independent experiments. In the first trial we hypothesized that the maintenance of insulin in adipogenic medium of DFAT cells throughout the period of differentiation is no longer necessary. In the second trial we hypothesized that addition of IBMX in adipogenic induction medium is no longer necessary to induce DFAT cells to adipogenesis differentiation. Therefore, in this study we aimed to establish a straightforward protocol for adipogenesis induction that requires only the essential compounds resulting in cellular responses closer to those observed *in vivo*.

2. Material and methods

2.1. Cell isolation and culture conditions

Mature pig-derived adipocytes were obtained from one adult female and were isolated from subcutaneous adipose depot. The ceiling method for lipid-laden cells was used as described by Sugihara et al. (1989), with some modifications to promote culture purification (Fernyhough et al., 2004, 2008), and obtain a purified porcine DFAT cell line. This protocol has been previously validated (Matsumoto et al., 2008) extensively used (Jumabay et al., 2009; Wei et al., 2012, 2013) as an efficient method to obtain a pure culture of mature adipocytes.

Briefly, fat tissue was minced and digested in 0.25% collagenase solution (Collagenase Type I – Gibco™, Whaltham, USA) at 37 °C for 1 h with gentle agitation. The digested cell suspension was then filtered through 1000 μm nylon meshes, allowing the free cells to pass and retaining the undigested tissue. The cells were separated by centrifugation, when the mature adipocytes (MA) float and the stromal vascular cells stay at the bottom of the conical tubes. Then, the MA in the top layer were collected and washed by centrifugation ($186 \times g$ −10 min) three more times, to promote culture purification. Washed cells were then transferred to a 12.5 cm^2 flask (Sarstedt™ - Newton, USA) fulfilled with Dulbecco's modified Eagle's medium nutrient mixture (DMEM/F12 - Gibco™, Whaltham, USA) supplemented with 10% Horse Serum (HS - Gibco™, Whaltham, USA) and antibiotics (1% Pen/Strep; 0.5% Gentamicin - Gibco™, Whaltham, USA). The flasks were maintained inverted on ceiling culture for 6 days incubated in a humidified 5% CO_2 atmosphere to promote dedifferentiation. After that, the flasks were reinverted and the culture was maintained to cells proliferate to reach confluence (proliferation medium: DMEM/F12 supplemented with 20% fetal bovine serum (FBS – Gibco™, Whaltham, USA) and antibiotics. The medium was changed every 2 days and cultures were checked for contamination with other cell types and a cell surgery was performed to remove any possible cell contamination. Then, the cells were harvested (Tryple selection 10x – Gibco™, Whaltham, USA), resuspended with cryopreservation medium (DMEM supplemented with 20% HS and 10% DMSO – Sigma-Aldrich™- St. Louis, USA) and cryopreserved in liquid nitrogen until induction protocols experiments.

2.2. Experimental treatments

Two independent experiments were performed in order to evaluate the need or not of insulin maintenance during the induction period (Experiment 1) and the need or not of IBMX to adipogenesis occurrence (Experiment 2). Although two experiments were performed separately, cells were initially treated similarly for both experiments, prior the beginning of the assays as it follows.

Cells were thawed (37 °C for 1 min), plated at 25-cm^2 tissue culture dish (Sarstedt™ - Newton, USA) provided with DMEM/F12 supplemented with 10% FBS + antibiotics (CCM - cell culture medium), and allowed to proliferate until semi-confluence. Then, cells were harvested, centrifuged and the total number of viable cells was counted by using the Cell Countess (Invitrogen™- Eugene, USA). A total of 2.5×10^4 cells was seeded in each well (9.6 cm^2) of a 6-well tissue culture plate (Sarstedt™- Newton, USA) and allowed to grow with CCM for 48 h until cells reach semi-confluence. Once reached semi-confluence, cells were submitted to 12 h serum starvation to allow the synchronization of cell cycle prior adipogenesis induction. After being synchronized cell cultures were tested for Experiment 1 or Experiment 2.

Table 1
Experimental treatments of Experiment 1.

Days	Adipogenesis induction protocol	
	Insulin$^+$	**Insulin$^-$**
0–3	CCM + 0.25 μm IBMX + 0.5 μm DEX + 1 μg/μl INS	CCM + 0.25 μm IBMX + 0.5 μm DEX + 1 μg/μl INS
4–16	CCM + 1 μg/μl INS	CCM

CCM: cell culture medium: DMEM/F12 + 10% FBS + antibiotics; IBMX: Isobutyl-1-methylxantine (Sigma-Aldrich-St. Louis, USA); INS: Insulin (Sigma-Aldrich™); DEX: Dexamethasone (Sigma-Aldrich™); 0–3d: 72 h without changing medium; 3–16d: medium was changed every 48 h.

In Experiment 1 we tested two medium conditions to determine if the lack of insulin throughout the 16d adipogenesis induction would slow down the cell differentiation process. Experimental treatments for Experiment 1 are shown in Table 1.

In Experiment 2 we tested two adipogenesis induction media to determine the essentiality of 3-Isobutyl-1-methylxantine (IBMX) to trigger adipogenesis differentiation. It is noteworthy that the IBMX is only needed during the first 3 days of induction and it is no longer necessary to be maintained during the entire differentiation process. The effect of the lack of IBMX was also evaluated throughout the 16d differentiation. In both experiments we considered a cell culture well as an experimental unit with a total of 6 well per treatment (n = 6). Experimental treatments for Experiment 2 are shown in Table 2.

2.3. Evaluation of lipid accumulation

In order to track lipid accumulation during the differentiation process, cell cultures from both experiments and medium conditions were collected at days 0, 2, 4, 8, 12, 16 of the experiments. At every time-point (0, 4, 8, 12, 16 days), two 6-well plates with both experimental treatments being 6 wells per treatment were evaluated for morphological characteristics and lipid accumulation capacity. Cells were stained with Oil Red-O technique to evidence intracytoplasmatic lipid content (Ramirez-Zacarias et al., 1992). Briefly, cells were washed three times with phosphate buffered saline (PBS) followed by fixation with 10% formalin in phosphate buffer for 1 h at room temperature. Then, fixed cells were washed once with PBS and stained with a filtered Oil Red-O (Sigma-Aldrich™, St. Louis, USA) stock solution (0.5 g of Oil Red-O in 100 mL of isopropyl alcohol) for 15 min at room temperature.

Table 2
Experimental treatments of Experiment 2.

Day	Adipogenesis induction protocol	
	IBMX$^+$	**IBMX$^-$**
0–3	CCM + **0.25 μm IBMX** + 0.5 μm DEX + 1 μg/μl INS	CCM + 0.5 μm DEX + 1 μg/μl INS
4–16	CCM + 1 μg/μl INS	CCM + 1 μg/μl INS

CCM: cell culture medium: DMEM/F12 + 10% FBS + antibiotics; IBMX: Isobutyl-1-methylxantine (Sigma-Aldrich-St. Louis, USA); INS: Insulin (Sigma-Aldrich™); DEX: Dexamethasone (Sigma-Aldrich™); 0-3d: 72 h without changing medium; 3–16d: medium was changed every 48 h.