Optimizing Collagenase, Fetal Bovine Serum, and Insulin to Isolate, Proliferate, and Differentiate Rat Preadipocyte *in vitro*

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KEYWORDS *obesity; culture media; implant tissue; adipocyte precursor; proliferation*

ABSTRACT *Background The aim of this study is to develop a method for optimal in vitro proliferation and differentiation of rat adipocyte.*
Methods Preadipocyte were isolated from omentum of Rattus norvegicus Wistar using Collagenase type I dan II (Sigma). Cells were cultured in M199 culture containing either 0%, 8% or 10% Fetal Bovine Serum (FBS). Insulin were added into the media once the cells attached to the culture plate Proliferating and differentiated cells were counted and analysed by using Oil Red O dan Hematoxylen (HE) staining.
Results This study demonstrated that adipocyte could be isolated using Collagenase type I but not type II. The addition of 10% FBS significantly increased the number of preadipocyte and differentiation of adipocyte more than those of 8% FBS and without FBS. The timing of FBS addition was best performed on day 8 using 10% FBS. Specific adipocyte staining using Oil Red O revealed that there were core lipids in mature adipocyte.
Conclusions Collagenase tipe I could be used to isolate preadipocyte cells. Supplementation of culture media with 8-10% FBS could enhance the in vitro proliferation of preadipocyte. Standard media M199 containing 10% FBS and insulin may provide an environment to differentiate preadipocyte specifically into adipocyte in vitro.

Obesity is the most common wide world metabolic disorder which leads to, or significantly increases the risk of comorbidities involving various body systems including cardiovascular, respiratory, musculoskeletal, genito-urinary, and endocrine. Many efforts have not successfully controlled the increasing prevalence of obesity. With regard to this wide range of health implications, the need to develop new and effective strategies in controlling obesity has become more urgent.

The management of obesity problem have been extensively studied through in vitro adipocyte research using preadipocyte culture systems. Culture media composition should be established for achieving optimal growth and development of adipocyte or preadipocyte in cell culture technique. Using proliferation and differentiation protocol, it is possible to use rat adipocyte as a model in laboratory for study of the metabolic syndrome.

Preadipocyte cellular models have been proven to be useful in plastic surgery as a source of implant tissues (Smahel, 1989). In primary culture, the fibroblast like adipocytes are actually an adipose tissue precursor cells which be able to proliferate and differentiate to become mature adipocyte when being transplanted. (Bjorntorp *et al*., 1980; von Heimburg *et al*., 2001, 2003). As has been reported, precursor cells can be isolated from several depot of various species, including human.

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Adipocyte culture needs an appropriate number and proper proliferation and differentiation of preadipocyte. Proliferation and differentiation of preadipocyte to be a mature adipocyte depend not only on the process itself, but also the interaction among cells (Fajas, 2003). However, cellular mechanisms and gene expression involved in adipocyte proliferation and differentiation have not been completely elucidated (Hemmrich *et al*., 2005).

Many reports have shown controversy in using collagenase type I and II as an enzyme for preadipocyte isolation either from rat or human. A long proliferation period in fetal calf serum (FCS) media can inhibit adipogenic changing to become mature adipocyte (Hauner *et al*., 1989). It was revealed that the presence of serum almost completely prevents adipose differentiation. Hemmrich *et al*. (2005) also concluded that one of the possible cause of decreasing differentiation ability of preadipocyte is a long period of culture. Only a few studies have been reported evaluating physiological aspects of adipocyte in Dulbecco's Modified Eagle Media (DMEM) / Ham's F12 (F12) and 5% or 10% FCS as a culture media for proliferation. Serum free DMEM / F12 media with dexamethasone, insulin, isobutylmethylxanthine (IBMX) and other substances have been used to induce differentiation (Kawada *et al.,* 2000).

Techniques that allow stable cultivation of adipocyte precursor cells may provide a valuable tool for better understanding of several parameters which induce or as a risk factors of obesity, included adipocytokines (Interleukine 6 (IL-6), interleukine 10 (IL-10), Tumor Necrosis Factor-α (TNF-α), hormones (Leptin, Adiponectin, Resistin), protein, gene and others. Using TNF-α as adipocyte proliferation stimulant in human stromal vascular cell culture Krystyna *et.al.,* (2000) revealed that TNFα could inhibit adipocyte differentiation through decreasing preadipocyte specific antigen (AD3: the initial marker of cell differentiation) and glycerol 3 phosphate dehydrogenase (GDPH) activity.

Therefore, it is necessary to improve adipocyte culture by optimizing isolation, proliferation dan differentiation methods. The aim of this study is to optimize isolation, proliferation and differentiation Wistar rat preadipocyte by determining the suitable type of collagenase and concentration of FBS and insulin supplementation in culture media. The experiments were divided into two steps. In the first step, preadipocyte proliferation was examined after supplemented with FBS. The second step, cell differentiation were evaluated in the culture media following the addition of insulin.

MATERIALS AND METHODS

Materials

Rat (2-5 month old age) strain Wistar, Collagenase CLS type I and type II, TCM199, and Mayer's hematoxylin were from Sigma (Sigma Chemical Co., St. Louis, MO); DMEM/F12 culture media were from Biochrom (Berlin, Germany); HEPES, 2 mM L-Glutamin and penicillin/streptomycin from PAA Laboratories (Colbe, Germany), and HBSS culture media, bovine serum albumin (BSA) and fetal bovine serum (FBS) were from Gibco Laboratories. Tissue culture dishes (25 cm2) were from Greiner (Solingen, Germany) and Millex GV syringe driven serum units were from Millipore (Eschborn, Germany).

Methods

Cell isolation and culture

Adipose tissue was dissected from Wistar rat's omentum under sterile condition and then washed with PBS pH 7.4 to eliminate erythrocyte. After being minced in the TCM199 media, adipose tissue was divided into two groups. The first group was suspended in TCM199 containing collagenase type I and the second group in TCM199 containing collagenase type II. The tissues were incubated in 37^{o}C for 40 minutes with gentle shaking in a water bath shaker. Cell

suspensions were filtered through 250 µm nylon filter and then centrifuged at 1,500 g for 7 minutes. The supernatant was removed and the pellets were resuspended in TCM199. Cell suspensions were divided into three fresh tubes, centrifuged at 1,500 g for 7 minutes, and then washed three times with TCM199. Pellets were resuspended in TCM199 media and FBS was added into each tube in a concentration of either 0%, 8% or 10%. Each cell suspension was plated on 35 mm culture dishes and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2 for 12–24 hours.

Morphological cell analysis and determination of total cell number

Monolayer cultures of cells were analyzed using inverted microscope at 100X magnification. Cells were photographed prior to detachment. To examine the rate of cell proliferation, cultured cells were observed at day 3, and 8. To determine the total number of cells and the number of differentiated versus non-differentiated preadipocytes, cells were counted at 5 different spots under the microscope at 100 X magni-fication. Main morphological criteria for adipocytes differentiation were an increasing number and size of visible lipid droplets in cells as well as a change in cell morphology from elongated to a round contours. Such morphological charac-teristics as well as the number and size of visible droplets were used to distinguish between the differentiated and undifferentiated cells.

Oil Red O staining

Orbital preadipocyte fibroblast cultures were plated in one-well culture chamber slides (Nalge Nunc International, Rochester, NY) in medium TCM199 containing 10% FBS, grown to confluence, and subjected to the differentiation protocol or an control conditions. Cells were washed twice with PBS, fixed in 10% formalin overnight at room temperature, and rinsed in 60% isopropanol prior staining with filtered 0.21% Oil Red O in isopropanol-water for 1 h (Phillipe *et al*., 1983). Washed cells were exposed to Mayer's hematoxylin solution for 5 min and rinsed with tap water before being visualized and photographed at 340 and 3100 using an Olympus Corp. BX60 light microscope (Olympus Corp., Melville, NY).

Statistical analysis

The One Way Anova test was used to identify differences in cell proliferation and differentiation between treatment groups. Significance was set at P=0.05. All statistical computations were conducted using SPSS version 11 software.

RESULTS

The influence of Collagenase type I dan II on Fibroblast like Adipocyt Cells Isolation

The isolation of Wistar rat omental adipose precursor cell was performed enzymatically using collagenase. In this research, we chose collagenase type I and type II to determine which one more suitable for optimal adipose cell isolation. The results showed that adipocyte could be isolated successfully by using collagenase I. In our hands, isolation using collagenase II did not yield in any adipocyte cells. These results suggest that to isolate adipocyte cells using protocol described in this study collagenase type I was better than type II.

Preadipocyte Proliferation after Fetal Bovine Serum (FBS) Supplementation

To reveal the influence of FBS on preadipocyte proliferation, FBS was added in culture media when precursor cells (fibroblast like adipocyte) have become monolayer. Cell counting was conducted on day 3 and 8. The amount of preadipocyte was parallel with FBS concentration. The results showed that cell proliferation was highest in groups treated with 10% FBS (Table 1).

Our time–lapse observation on cells cultured in media containing FBS demonstrated that cell number was increasing overtime, highest on day-8 in groups with 0 and 8% FBS but decreasing in the group supplemented with 10% FBS

(Figure 1). Statistically, there was a significant difference between groups cultured without and with FBS supplementation, no significant difference between groups of 8 and 10% FBS (Table 1).

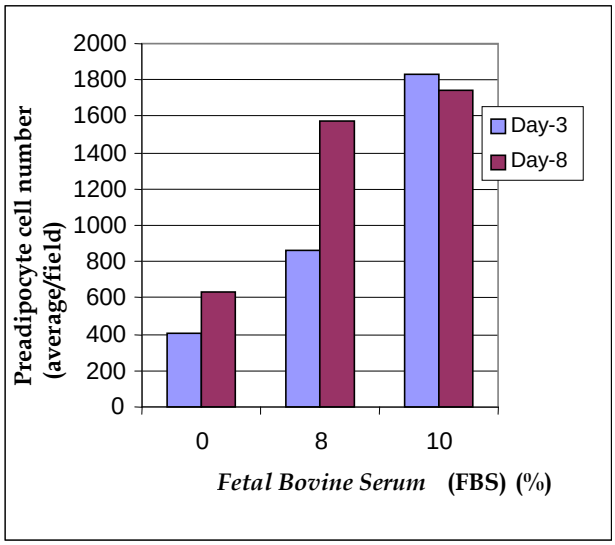

Figure 1. Proliferation of adipocyte cells following the addition 0, 8 and 10% FBS in culture media. As shown on the figure, cell numbers on particular days were higher in groups treated with FBS than in control group without FBS, indicating that the presence of FBS may help adipocyte cell proliferation in vitro.

Table 1. One way Anova test of the adipocyte cell number during proliferation after 0%, 8%, and 10% FBS supplementation

FBS (% v/v) Suppl.	N	Subset for alpha = 0,05	
		1	2
0	40	8.93	
8	40		39.63
10	40		55.65
Sig.		1.000	0.195

Differentiation of Preadipocyte after Human Insulin Supplementation

To evaluate the effect of insulin on preadipocytes differentiation, preadipocyte culture was supplemented with 17 nM insulin. Cells were cultured to allow preadipocytes differentiate into mature adipocytes. Cells were observed on day-8 and 20 and both the differentiated and undifferentiated cells were counted. Our result showed that number or percentage of differentiating cells increased over time in both groups treated with 0 nM and 17 nM insulin (Figure 2), however such differentiation was significantly improved in media containing insulin.

Morphological characteristics of preadipocyte and adipocyte cells

Using Hematoxylin staining, we observed that preadipocytes appeared as spherical shaped cells with nuclei at a central position (Figure 3A), whereas mature adipocytes, the differentiated cells, were characterized by core lipid in the cytoplasm revealed after Oil Red O staining (Figure 3B).

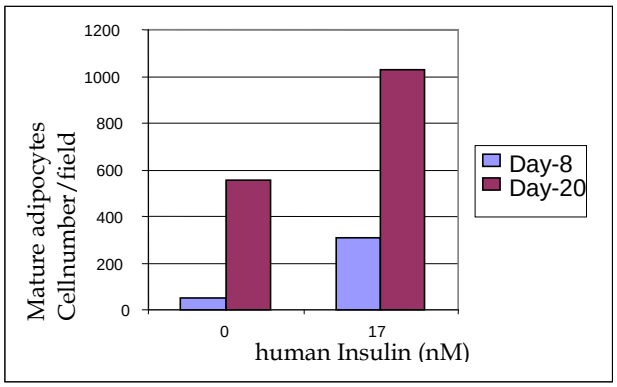

Figure 2. Differentiated adipocyte number observed on day 8 and 20 of cultivation. The-diagram showed that number of differentiated cells was higher in group cultured in TCM199 supplemented with insulin 17 nm.

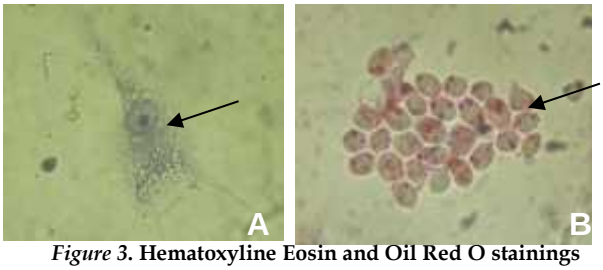

Figure 3. **Hematoxyline Eosin and Oil Red O stainings on preadipocyte and mature adipocyte cells**. A. Preadipocyte with nucleus at the centre (arrow). B. Mature adipocytes with core lipid in the cytoplasm. Following Oil Red O staining, lipid accumulation appeared as intracellular red granules. Images were of 400 x magnification.

DISCUSSIONS

Preadipocyte were isolated from rat omental for getting optimization of isolation, proliferation and differentiation pre-adipocyte *in vitro*.

The aim of this study was to evaluate the effect of collagen type I and II on isolation of fibroblast like preadipocye. We also investigated the influence of FBS on

preadipocyte proliferation and role of insulin in differentiation. Both collagenase type I and type II can be used as supplement in isolation of rat adipocyte precursor cells. Phillipe *et al*. (1983) utilized collagenase type II in their study on replication and differentiation of rat adipose precursor cells, Ailhaud (1997), chose collagenase type I as a supplement in cultivation of adipose precursor cells, while Vincent *et al*., (2000) isolated successfully pig adipose stromal vascular cell using collagenase tipe II and XI.

This study demonstrated that preadipocytes were able to proliferate and differentiate in standard culture media TCM199 (Figure 3), while many researchers recommended using DMEM/ F12 because of its higher glucose content (Donovan, 1994; Vincent *et al*., 2000; Mc Intosh *et al*., 2005) or Hank's balanced salt solution (HBSS) (Robin *et al*., 1976).

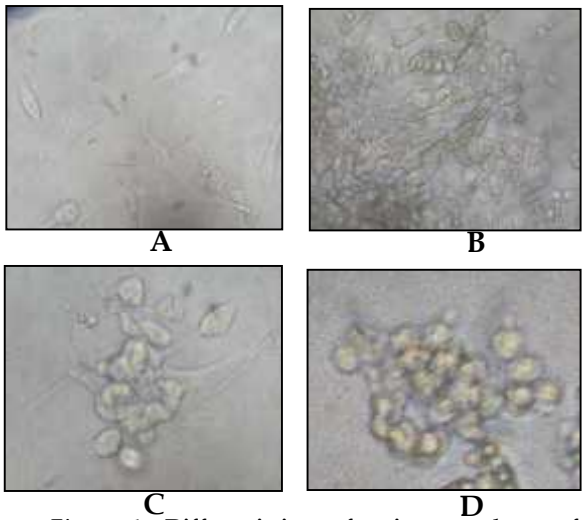

Figure 4. **Differentiation of primary culture of adipocyte isolated from rat omentum.** Morphological changes during differentiation in cell culture: Fibroblast like preadipocyte on day-2 (A), preadipocytes on day-5 (B), undifferentiated adipo-cytes on day-10 (C), and differentiated adipocyte on day-21(D). Images were taken at 400 x magnification.

FBS or FCS is commonly used as a supplement in cell culture due to its beneficial effect on cell proliferation (Freshney, 1987). There are some discrepancies on the use of FBS in human and animal cell culture. Some researchers used serum-free media to avoid contamination from bovine serum (Kawada *et al*., 1990; Tezel and Del Priore, 1998; Gstraunthaler, 2003), others found that serum-free media was not a good culture media (Ethier, 1986; Koller *et al*., 1998), and added either synthetic serum-like substances containing animal or plant derived proteins (Ultroser G or Prolifix S6, respectively) (Anselme *et al*., 2002), or 10% FBS (Phillippe *et al*., 1983) to facilitate cell proliferation and differentiation. In this study, to exploit whether FBS would be beneficial for adipocyte cell culture we used TCM199 culture media supplemented with FBS at 0, 8 or 10% concentrations. Our subsequent observations revealed that cells proliferated faster and the number of differentiated cells was more in media containing 10% FBS compared to those without or with 8% FBS. It was suggested that the growth of cells cultured in vitro was optimal in the presence 10% FBS.

To examine the relationship between culture duration and cell proliferation and differentiation, cell number were counted on day 0, 3 and 8. The results showed that cell numbers were increasing in relation with time in media containing 0% and 8% FBS (Figure 1). Cell numbers increased also overtime in media supplemented with 10% FBS but then slightly decreased on day-20. These suggests that excessive supplementation of FBS may disturb cell growth, further studies however are required to confirm this or to elucidate other possible causes underlying these phenomena.

Insulin and other growth factors such as fibroblast growth factor (FGF) and endothel growth factor (EGF) have been used as agents for facilitating cell differentiation (Bjorntorp, 1980). As reported in this study, the addition of 17 nM insulin increased the number of cells differentiated into mature adipocytes (Figure 2 and 4). The concentration of human insulin used in this study was lower compared to that of Philippe *et al*. (1983) recommended, 1 µg/mL bovine insulin as a supplement, yet the overall results in term of differentiation was comparable in both studies.

Mature adipocyte could be recognized by its morphological characteristics in culture. Adipocytes are derived from fibroblast like precursor cells, called preadipocyte. One of adipocyte distinct characteristics is based on the occurrence of lipid accumulation in cells once proliferation stopped (Albright and Stern, 1998). Lipid accumulation could be identified as a core lipid in cytoplasm *(*see Figure 3B*)*. Preadipocyte differentiation may yield osteoblast, chondrocyte, myoblast or neuron like cells instead of mature adipocyte. (Zulk *et al*., 2002). In this study, we did not detect cell type other than mature adipocyte (shown in Figure 4) suggesting that the culture system as described in this study could support specific diffentiation of preadipocytes cells into mature adipocytes.

CONCLUSIONS

As conclusions, it was shown in this study that collagenase I was better than type II as an enzyme for preadipocyte cells isolation, and the supplementation of TCM199 standard culture media with 8-10% FBS and 17 nM insulin were beneficial for proliferation preadipocyte cells and subsequent differentiation into mature adipocyte cells, respectively. The optimized culture of the preadipocyte *in vitro* resulted from this study may provide a useful method in further investigation of the molecular mechanisms of obesity and metabolic syndrome.

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