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EFFECT OF GLUCOSE FEED ON CHOLESTEROL, TRIGLYCERIDES AND LIPOPROTEIN CONCENTRATIONS IN PREADIPOCYTES AND ADIPOCYTES: AN *IN-VITRO* STUDY

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ABSTRACT

Adipose tissue is one of the largest cholesterol depots in the body. Adipose cells specialized in energy storage, contain large intracellular triglyceride-rich lipid droplets, are enriched with free cholesterol, and express sterol-regulated transcription factors. In this study, human *in vitro* cultured human subcutaneous preadipocytes and adipocytes were employed to study the level of cholesterol and lipoproteins. Wharton's Jelly Mesenchymal Stem Cells were cultured till the accumulation of lipid droplets (Adipocytes). These adipocytes were visualized by Oil Red O staining under phase contrast inverted microscope. Cholesterol and lipoprotein levels were measured by spectrophotometric analysis in both preadipocytes and adipocytes cultured in variable glucose concentration (5-20 mM). The values were plotted using Microsoft excel. Student t-test was used for statistical analysis. The concentration of TC, TGL, LDL and VLDL increase as the glucose concentration increase both in preadipocytes and adipocytes. However concentration of HDL decreases when the glucose concentration increases. All values in each glucose concentration showed highly significant difference except HDL in 5mM and 10mM glucose concentration. The present study examines the aptitude of adipocytes to release their cholesterol, triglycerides and lipoproteins. We show here for the first time that the cholesterol, triglycerides and lipoproteins concentration has been increased due to increase in glucose concentration as the preadipocyte differentiates into adipocytes and showed linear correlation. In conclusion, this study provides new information on cholesterol, triglycerides and lipoproteins release from adipocytes indicates that the post obese complication in *in vivo* will increase the disease associated with lipid metabolism.

INTRODUCTION

Adipose tissue is one of the largest cholesterol depots in the body [1]. Cholesterol is an essential molecule in animals, serving as a principal component of plasma membranes, an obligatory precursor for the biosynthesis of steroid hormones, bile acids, and bioactive oxysterols and

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also exerting regulatory functions. In whole body energy metabolism, two types of adipose tissue (white, WAT and brown, BAT) plays crucial role. WAT are specialized for energy storage due to their largest energy reserve triglyceride constitutes while BAT have a high capacity for energy dissipation by brown adipocyte-specific uncoupling protein 1 (UCP1) in the inner membrane of the abundant mitochondria through adaptive thermogenesis [2].

Obesity is characterized by increased mass of adipose tissue (WAT), large adipocytes and increased basal (spontaneous) lipolysis in visceral fat and is associated

with inappropriate regulation of adipocyte lipolysis [3] and its metabolic complications such as cardiovascular diseases (CVD), insulin resistance and type 2 diabetes becomes a major health problems increasing throughout the world [4].

Adipose cells specialized in energy storage, contain large intracellular triglyceride-rich lipid droplets, are enriched with free cholesterol, and express sterol-regulated transcription factors. It has been reported that adipose tissue cholesterol increases during cholesterol feeding but its mechanism is not well understood [5, 6]. However, it is suggestive for the presence of a regulatory feedback mechanism [7] or simple exchange [8].

High-density lipoprotein (HDL) levels inversely correlate with atherosclerotic CVD. HDL plays a key role in reverse cholesterol transport by promoting cholesterol efflux from peripheral cells, including cholesterol-laden macrophages, and delivering acquired cholesterol to liver for excretion, a process that is believed to be atheroprotective [9].

As per cholesterol feeding previous reports the production of a cholesterol ester enriched very low density lipoprotein (VLDL) is of hepatic origin [10-12]. This abnormal VLDL and VLDL found in type III hyperlipoproteinemia patients are constitutively similar and found to suppress 3-hyroxy-3-methylglutaryl CoA reductase activity in fibroblasts (*in-vitro*) unlike normal VLDL [13]. This indicates that the uptake of type III VLDL was mediated by specific cellular receptor mechanism.

Objective: In this study, human *in vitro* cultured human subcutaneous preadipocytes and adipocytes were employed to study the level of lipoproteins.

MATERIALS AND METHODS
Cell culture

Wharton's Jelly Mesenchymal Stem Cells (Himedia) were cultured for 48 h in Dulbecco's modified Eagle's medium/ Ham's F12 (1:1) supplemented with 10% fetal calf serum containing antibiotics and normal glucose levels (5 mM). These cells are isolated from human umbilical cords collected post-partum. Wharton's jelly is the gelatinous connective tissue from umbilical cord and is a rich source of multipotent stem cells. Cells isolated from Wharton's Jelly are known to differentiate in to adipogenic, osteogenic, chondrogenic, cardiomyogenic lineages and dopaminergic neurons. Proliferatium medium is composed of DMEM/F-12 medium (1:1, v/v), HEPES, FBS and antibiotics. Adipocyte differentiation medium is composed of DMEM/F-12 medium (1:1, v/v), HEPES, FBS, Insulin Transferrin selenium (ITS), Sodium bicarbonate, biotin, pantothenate and antibiotics. Adipocyte differentiation medium was used in differentiation phase which is optimized for adipogenic differentiation of actively proliferating human mesenchymal stem cells *in vitro*. Factors *viz.* Insulin Transferrin selenium (ITS), biotin and pantothenate that

potentiate adipogenic differentiation through activation of factors/regulatory enzymes like C/EBP and peroxisome proliferator-activated receptor- γ (PPAR- γ) can be invoked by supplementing the medium with growth factors. During maintenance of adipocytes biotin and panthothenate were added. Adipocytes were maintained till the accumulation of lipid droplets. At 7 days, a vast majority of cells (more than 90%) had accumulated lipid droplets.

Oil Red O staining

Cells were stained with Oil Red O. Oil Red O belongs to a family of lipophilic or fatty acid soluble dyes. These dyes are used to demonstrate triglycerides, lipids and lipoproteins. Oil-Red-O is generally used to detect presence of fat globules i.e. identification of adipocytes within tissue or adipocytic differentiation of cells. Cultured cells were fixed in a 10% solution of formaldehyde in phosphate-buffered saline for 5min at room temperature, washed with 60% isopropanol and stained with Oil Red O solution (in 60% isopropranol) for 10 min followed by repeated washing with water (4 times with 10 ml). Stained cells were immediately viewed under phase contrast inverted microscope and images captured using a mounted digital camera.

Biochemical assays

Preadipocytes and Adipocytes were centrifuged at 300g for 2 min. Supernatant was used for biochemical analysis. Total Cholesterol (TC) was measured in adipocyte cells using Ferric chloride-acetic acid reagent, mixed gently and centrifuged at 3000 rpm (3354xg) for 10 min. at 4^0C. Supernatant was poured in conc. H_2SO_4 and incubated at 50-60^{0}C for 10 min. The solution was cooled to room temperature and absorbance was read at 560 nm to estimate the TC concentration. High Density Lipoprotein (HDL) was measured using Phosphotungustate reagent and Magnesium Chloride ($MgCl_2$). The mixture was centrifuged at 3000 rpm (3354xg) for 30 min at 4^0C. Supernatant was mixed with Ferric chloride-acetic acid reagent followed by sulphuric acid (0.033N H_2SO_4) and incubated at 50-60^{0}C for 10 min. The solution was cooled to room temperature and absorbance was read at 560 nm to estimate the HDL concentration. Similarly Triglycerides (TGL) was measured in adipocyte cells using n-heptane, Iso-propanol, sulphuric acid (0.08N H_2SO_4), potassium hydroxide (KOH, 6.25 mol/L), sodium metaperiodate and acetyl acetone. Incubated the mixture at 70^{0}C for 20 min. and OD was taken at 425 nm to estimate the TGL concentration. Low Density Lipoprotein-Cholesterol (LDL) and Very Low Density Lipoprotein-Cholesterol (VLDL) were calculated by known formulae [LDL=TC – (TGL / 5 + HDL); VLDL=TC – (LDL + HDL)]. The readings were randomly checked by commercially available *Ecoline/Inoline* kits (Merck) in a double-beam UV-vis spectrophotometer. All readings were recorded in triplicates.

Statistical analysis

The values were plotted using Microsoft excel. Student t-test was used for statistical analysis. Differences were considered significant when P values were ≤0.05. Results were expressed as means standard deviations.

RESULTS

After 48 h culture of mesenchymal stem cells in adipocyte differentiation medium, preadipocytes were formed and their differentiation phases towards adipocytes from day 0 to day 7 have been shown in Figure 1. Biochemical analysis including total cholesterol (TC), triglycerides (TGL), High Density Lipoprotein (HDL), Low Density Lipoprotein-Cholesterol (LDL) and Very Low Density Lipoprotein-Cholesterol (VLDL) were analyzed in both preadipocytes and adipocytes cultures in varying glucose concentrations (5, 10, 15 and 20 mM). Biochemical values with (mean ± standard deviation) have been shown in Figure 2. The data suggest that the concentration of TC,

TGL, LDL and VLDL increase as the glucose concentration increase both in preadipocytes and adipocytes. However concentration of HDL decreases when the glucose concentration increases. All values in each glucose concentration showed highly significant difference except HDL in 5mM and 10mM glucose concentration. One drastic observation was that the concentration of all biochemical parameters increases but the HDL concentration dicreases as the preadipocytes differentiated to adipocytes as shown in Figure 2.

Percent change in biochemical estimation in preadipocytes and adipocytes at 5, 10, 15 and 20 mM glucose concentration with P-values have been shown in Table 1. We observed 19.27% (± 4.58%), 21.85% (± 3.86%), 30.33% (± 5.65%) and 21.85% (± 3.86%) percent of increase in TC, TGL, LDL and VLDL respectively in adipocytes as compared to preadipocytes. However 10.41% (± 2.11%) decrease in HDL concentration was observed.

Table 1. Percent change in biochemical estimation in preadipocytes and adipocytes at 5, 10, 15 and 20 mM glucose concentration with P-values.

Glucose Concentration →	% increase	5mM (P -values)	% increase	10mM (P-values)	% increase	15mM (P-values)	% increase	20mM (P-values)	Mean (%) ± S.D. (%)
Preadipocyte vs Adipocyte TC	15.97	0.0005	17.31	0.0006	17.78	0.0000	26.04	0.0001	19.27± 4.58
Preadipocyte vs Adipocyte HDL	7.50	0.3658	10.53	0.1050	11.11	0.0080	12.50	0.0023	10.41± 2.11
Preadipocyte vs Adipocyte TGL	21.43	0.0004	26.53	0.0000	22.32	0.0001	17.12	0.0003	21.85± 3.86
Preadipocyte vs Adipocyte LDL	28.98	0.0000	27.33	0.0011	26.36	0.0000	38.64	0.0003	30.3 ± 5.65
Preadipocyte vs Adipocyte VLDL	21.43	0.0004	26.53	0.0000	22.32	0.0001	17.12	0.0003	21.85± 3.86

Figure 1. Adipogenesis phases of human subcutaneous preadipocytes. (A) Day 0: Wharton's Jelly Mesenchymal Stem Cells (B) Day 2: Prolifiration phase and appearance of preadipocytes (C) Day 4: Differentiation phase and accumulation of lipid droplets (D) Day 7: Mature adipocytes with accumulated lipidroplets

(A)

(B)

(C)

(D)
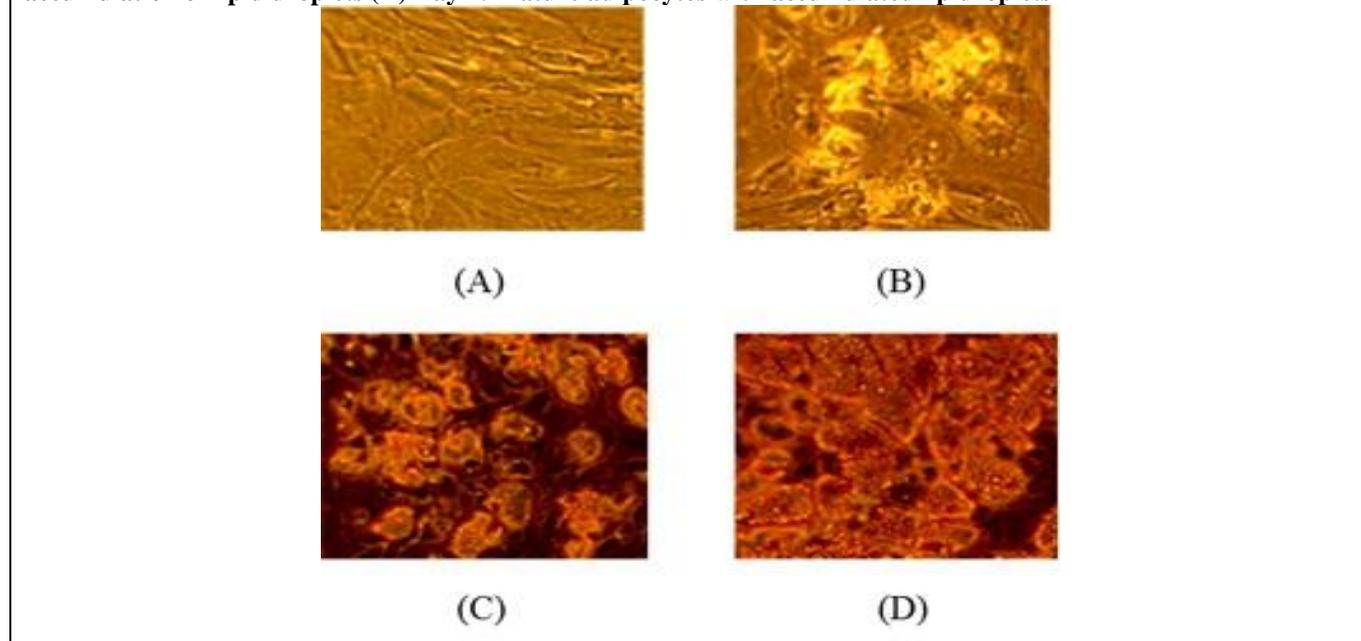
Figure 2. Biochemical estimation in preadipocytes and adipocytes at 5, 10, 15 and 20 mM glucose concentration. (A): Total Cholesterol; (B): Triglycerides; (C): High Density Lipoproteins; (D): Low Density Lipoproteins and (E): Very Low Density Lipoproteins.
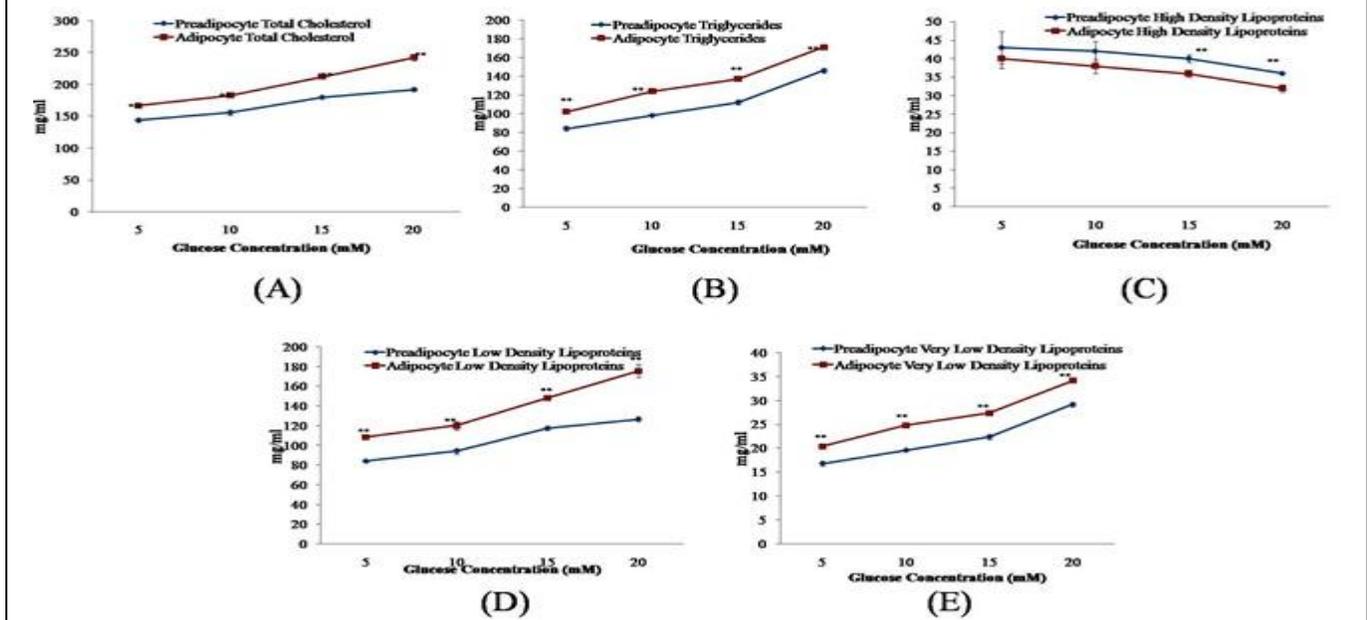
DISCUSSION

Adipose tissue or adipocyte or fat cells or lipocytes are found in stereotypical depots throughout the body and mixed with other cell types such as loose connective tissue and is characterized by a marked cellular heterogeneity: among its cellular components. Fat tissue consists of approximately one-third of mature adipocytes and remaining portion is a combination of small mesenchymal stem cells (MSCs), T-regulatory cells, endothelial precursor cells, macrophages and preadipocytes in various stages of development. Preadipocytes have the ability to proliferate and differentiate into mature adipocytes. Our main understanding about adipocyte differentiation and adipogenesis comes from *in vitro* studies of fibroblasts and preadipocytes [14]. In white adipocytes, single, large lipid droplets is present and occupies most of the of cell volume and remaining cellular components resides in the peripheral part of the cell. These preadipocytes that are similar to fibroblasts are used for most of the *in vitro* metabolic studies after their culture and differentiation. While in brown adipocytes, multilocular lipid droplets with high mitochondrial content are major characteristics and they are derived from distinct, highly vascular and innervated adipose tissue depots.

The present study examines the aptitude of adipocytes to release their cholesterol, triglycerides and lipoproteins. This release has not been examined so far. We show here for the first time that the cholesterol, triglycerides and lipoproteins concentration has been increased due to increase in glucose concentration as the preadipocyte differentiates into adipocytes and showed linear correlation. The adipocyte lipid droplet is mainly formed with a triacylglycerol core surrounded by a free cholesterol-containing phospholipid monolayer. The coupling of triacylglycerol and cholesterol during the lipolytic process that leads to the mobilization of energy from the adipocyte is still unclear. Under normal feeding conditions, *in vivo* studies have examined the contribution of adipose tissue to plasma cholesterol levels, and have concluded that adipose tissue does not significantly contribute to hypercholesterolemia [15]. Rather, it has been suggested that adipose tissue might be a sink for circulating cholesterol, which could help to buffer the high levels of circulating cholesterol frequently found in obese states. Adipose tissue cholesterol content is strongly correlated with fat cell size in humans or rodents [15, 16]. It has been suggested that the low ability of adipocytes for cholesterol efflux because cholesterol being a signaling molecule in adipocytes. Such studies have been performed with ABCA1 gene in 3T3-L1 preadipocytes and concludes that the regulation of ABCA1 mRNA in adipocytes closely resembles that described in monocyte-macrophage cell systems and also the phospholipid efflux was doubled between preadipose and fully differentiated states [17]. From previous reports it is suggested that cholesterol is redistributed toward a nonmembrane pool in differentiated cells, presumably the lipid droplet [18, 19].

CONCLUSIONS

We conclude that the preadipocytes and adipocytes were positively correlated with all serum biochemical concentrations in *in vitro*. Total cholesterol, triglycerides, low density lipoproteins and very low density lipoproteins showed significant association with the increasing feed of glucose concentration. However, high

density lipoproteins showed negative correlation with it. It was also observed that the release of TC, LDL and VLDL in adipocytes is about twenty percent more as compared with preadipocytes. Moreover, TGL increment was upto thirty percent in adipocytes as compared to preadipocytes. However, HDL concentration showed negative correlation of ten percent in adipocytes as compared with preadipocytes. In conclusion, this study provides new information on cholesterol, triglycerides and lipoproteins release from adipocytes indicates that the post obese complication in *in vivo* will increase the disease associated with lipid metabolism such as metabolic syndrome, type II diabetes, CVDs, hypercholesterolemia, etc.

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