ABSTRACT

The study attempted to mimic the state of obesity *in vitro* using 3T3-L1 adipocytes, since they represent white adipose tissue which is responsible for obesity and related disorders. 3T3-L1 adipocytes were exposed to various conditions occurring during obesity *in vivo* such as (a) hyperglycemia (using high glucose), (b) hypoxia (using cobalt chloride ($CoCl_2$)) and (c) macrophage infiltration (using RAW 264.7 conditioned medium (RCM). The study also attempted to assess the effect of a potential anti-adipogenic phytochemical (chlorogenic acid (CGA) - isolated from *Cichorium intybus*) and plant extract (*Gymnema sylvestre* methanol extract (GSM)) for their activity on the above conditions mimicking obesity.

A state of 25 mM glucose has been known to induce insulin resistance and hypertrophy in 3T3-L1 cells. To probe the role of glucose during hyperglycemia in adipocytes, the effect of various concentrations of glucose (45 mM, 65 mM, 85 mM and 105 mM glucose) were analyzed on 3T3-L1 adipocytes. Differentiation of preadipocytes in the presence of 105 mM glucose (high glucose) exhibited a significant decrease in adipogenesis through inhibition of CCAAT/enhancer-binding proteins (C/EBPα), sterol regulatory element binding protein 1c (SREBP1c) and peroxisome proliferator-activated receptor (PPARγ). Cells in the presence of high glucose (105 mM) showed increased cell proliferation through phosphotidyl inositol 3 kinase (PI3K) / protein kinase B (Akt) dependent pathway. Furthermore, inhibition of p27 validated the induction of hyperplasia of activated preadipocytes under the state of high glucose. Nitric oxide and reactive oxygen species (ROS) production was induced at this condition leading to an increase in the expression of toll-like receptor 4 (TLR4), tumor necrosis factor

α (TNFα) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), thereby decreasing adiponectin production. This condition also showed insulin resistance through decrease in insulin receptor β (IRβ), whereas an increase in glucose uptake was observed through activation of glucose transporter 1 (GLUT1). To summarize, 3T3-L1 adipocytes treated with 105 mM glucose mimicked the state of hyperplasia during obesity.

In this study, hypoxia condition was mimicked by treating differentiated adipocytes with 150 µM $CoCl_2$ for 6, 12 and 24 h wherein, $CoCl_2$ when treated for 12 h showed maximum expression of HIF1α. In comparison to control, cells under the state of hypoxia were found to inhibit lipid accumulation via C/EBPα, leading to a decrease in adiponectin levels. Although, cytotoxicity was observed in cells treated with $CoCl_2$, the expression of ROS was reduced in response to metabolic alteration during hypoxia. However, the levels of stress targets such as phosphorylated c-Jun N-terminal kinases (pJNK) and NFκB were found to increase. Also, $CoCl_2$ treatment showed no significant change in GLUT1 levels however, glycogen synthesis was found to increase showing signs of hypertrophy. To recapitulate, 3T3-L1 adipocytes treated with 150 µM $CoCl_2$ exhibited conditions imitating hypoxia during obesity.

To mimic the condition of macrophage infiltration in adipocytes during obesity, the effect of RCM on differentiated adipocytes was investigated for 6, 12 and 24 h. Maximum NO and ROS release along with an increase in inflammatory markers such as TNFα, pJNK and phosphorylated extracellular regulated kinase (pERK1/2) was observed at 24 h of RCM treatment. Further, RCM treatment induced cytotoxicity in adipocytes and the increase in lipid content due to significant increase in lipofuscin content

indicated the state of senescence. This was validated by an increase in senescence markers such as p53, p21, retinoblastoma protein (pRb), p27 and cyclin D1. The cells treated with RCM showed an inhibition of insulin signalling, since expression of insulin receptor substrate 1(IRS1) and PI3K were found to decrease. The study clearly shows that RCM induced inflammation and senescence in adipocytes, thereby mimicking the state of macrophage infiltration induced senescence.

CDK5 is a protein kinase specifically expressed in the adipose tissue during obesity and is known to be activated by TNFα and various cytokines. Cells differentiated in the presence of 105 mM glucose and adipocytes treated with RCM for 24 h increased CDK5 levels significantly in comparison to control cells. But differentiated adipocytes when treated with 150 µM $CoCl_2$ for 12 h showed reduced levels of ROS and TNFα with no significant change in CDK5 levels, signifying the role of ROS and TNFα in the regulation of CDK5 expression.

As obesity is a complex disorder, it would be advisable to use treatments possessing multiple effects, thereby minimizing side effects. Bioactive molecules have an advantage of optimisation of the dosage for consumption. In contrast, the plant extracts are expected to show strong potential as activities are exhibited from a mixture of active molecules. Hence the study attempts to study the bioactivity exhibited by a CGA and GSM.

CGA (100 ng/ml) exhibited significant down regulation of PPARγ, C/EBPα, phosphorylated hormone sensitive lipase (pHSL) and CDK5 along with an increase in adiponectin level. CGA (100 ng/ml) showed effective regulation of targets involved during obesity, and hence was selected for understanding its role on the three *in vitro* conditions mimicking obesity. The

cells differentiated with 105 mM glucose (hyperplasia model) when treated with CGA (100 ng/ml) showed significant decrease in GLUT1 expression and induced glycogen synthesis through increase in phosphorylated glycogen synthase kinase (pGSK3β - inactive form). CGA also inhibited CDK5 protein which was induced during hyperplasia. Treatment of CGA (100 ng/ml) in cells induced with hypoxia, showed an inhibition of HIF1α, pJNK and NFκB leading to an increase in adiponectin levels. Adipocytes treated with RCM in the presence of CGA (100 ng/ml) reduced ROS production and lipofuscin levels along with decrease in p53, p21 and pRb levels, showing reversal of senescence. The increased expression of CDK5 in RCM treated adipocytes was also reduced during CGA treatment.

GSM (10 µg/ml) exhibited optimum bioactivity by inhibition of adipogenesis through down regulation of PPARγ, SREBP1c and CDK5; however, an increase in C/EBPα and adiponectin was observed. GSM also inhibited the lipid protecting protein, perilipin with no significant change in pHSL levels. GSM was also investigated for its role on the three developed conditions mimicking obesity and results from the study clearly showed the potential of GSM in alleviating the various targets of obesity.

In conclusion, the present study has established *in vitro* models mimicking the conditions of obesity, characterized by hypertrophy/ hyperplasia, hypoxia and macrophage infiltration using 3T3-L1 adipocytes. The molecular programming involved during these conditions has also been elucidated through gene and protein expression studies. The therapeutic options for the developed conditions of obesity have been evaluated wherein CGA (100 ng/ml) and GSM (10 µg/ml) have been shown to be effective in alleviating various cellular targets involved during obesity.