CHAPTER 3: OBJECTIVE 1

EXAMINING THE ROLE OF HUMAN ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS (hADSCs) IN THE REVERSAL OF INSULIN RESISTANCE IN VITRO

3.1. INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stem cells which can differentiate into various cell types. Multiple mechanisms are likely to contribute in co-ordination, for the therapeutic effect of MSCs (Wei et al., 2013). MSCs are immunomodulatory and after infusion they are efficient in homing to the injured tissue sites with inflammation (Horwitz et al., 2002; Mahmood et al., 2003). Although MSCs show great potential in treating different disease, there are major concerns for the usage of MSC as a cell therapy viz. the optimal dosage of MSCs to be injected, best route of administration, best engraftment time and the fate of the cells after infusion (Karp and Leng Teo, 2009). The key mechanism through which MSCs work is paracrine secretion which is rich in growth factors and cytokines for the repair and regeneration of damaged tissue. The secreted factors are the collection of proteins containing a single peptide consisting of various enzymes, growth factors, cytokines and other soluble factors that can be set up in the medium where stem cells are cultured and are collectively referred to as Secretome/ conditioned medium (CM) (Dowling and Clynes, 2011). The usage of secretome has lots of advantages over cellular therapy. CM from stem cells has good potential to be produced as pharmaceuticals for regenerative medicine. CM can be collected, freeze-dried, boxed and transported more easily. CM from various MSCs are extensively worked on for their application as therapeutics viz. Bone marrow, umbilical cord, amnion and adipose derived (ADSCs). There are several reports showing the beneficial
effect of CM. It has been demonstrated that ADSCs-CM treatment is equally effective as ADSCs in the treatment of pulmonary hypertension and pulmonary fibrosis which may provide a cutting-edge approach to treat cardiopulmonary disorders (Rathinasabapathy et al., 2016). It has also been established that hypoxic-conditioned media of adipose-derived stem cells increases the viability of hepatotoxic hepatocytes and enhances liver regeneration in partially hepatectomized mice (Lee et al., 2016). Although ADSCs-CM has various application in wound healing, skin injury, brain injury, liver injury and many more the role of ADSCs CM in insulin resistance has not been established so far (Pawitan, 2014). There are many insulin sensitizers available until now for the management of type 2 diabetes. Metformin, a biguanide class of drug is routinely being used worldwide as one of the most effective oral treatment available for the management of type 2 diabetes targeting insulin resistance (IR) (Rojas and Gomes, 2013). Inspite of several beneficial properties metformin carries, there are many side effects associated with the long term usage of metformin (Bray and Greenway, 2007; DeFronzo, 1999). Metformin may cause serious condition called lactic acidosis with the subsequent symptoms like severe drowsiness, dizziness, tiredness, muscle pain, chills, nausea or vomiting, blue or cold skin, fast or difficult breathing, slow or irregular heartbeat and stomach pain with diarrhea (Nasri and Rafieian-Kopaei, 2014). Hence there is a need to look for an alternative which has a better therapeutic value to overcome the limitations of metformin.

In the present study, we investigated the effect of ADSCs CM on palmitate and TNFα induced insulin resistant adipose and muscle cells in vitro specifically on 3T3L1 cells, a murine preadipocyte cell line with sodium palmitate and TNFα alone or in combination. C2C12, a murine myoblast cell line was also treated with sodium palmitate and TNFα in order to achieve the insulin resistant state.
3.2. RESULTS

Characterization of ADSCs

ADSCs were cultured (Figure 7A) and characterized for the mesenchymal stem cell markers by flow cytometry analysis. ADSCs were positive for CD 105 and CD 90. Markers negative for ADSCs were HLA DR and CD 34. (Figure 7B) The ability of the ADSCs to differentiate into trilineage viz. Adipocytes, Osteocytes and Chondrocytes was confirmed by differentiation and staining of the cells. Oil O red staining showed adipogenic differentiation, Alizarin Red staining for osteogenic differentiation and Alcian blue staining for chondrogenesis (Figure 7C)
ADSCs CM effective in enhancing glucose uptake

The key source of energy for skeletal muscle is glucose. The rate determining step in glucose dependent intracellular energy production is the glucose transport (Balasubramanian et al., 2014)(Alvim et al., 2015). It has also been suggested that in early stages of obesity, adipocyte hypertrophy is sufficient to provoke insulin resistance (Kim et al., 2015). Hypertrophied 3T3L1 model was developed as shown in Figure 8. We examined the effect of ADSCs-CM on palmitate and TNFα induced insulin resistant 3T3L1 (hypertrophied as well as diseased) and C2C12 cells. Treatment with ADSCs CM significantly enhanced glucose uptake in
hypertrophied 3T3L1 as well as diseased (Figure 9A and 9B) and C2C12 (Figure 10) cells as revealed by 2-NBDG uptake.

Figure 8: Hypertrophy in 3T3L1 cells.

Figure 9: Glucose uptake in 3T3L1 cells. ‘^’ depicts control vs no insulin, ‘$’ no insulin vs insulin, ‘*’ signifies insulin vs metformin and ADSCs CM. A. 2-NBDG uptake in hypertrophied 3T3L1 cells (chronic condition): Metformin shows significant enhancement of glucose uptake, p value < 0.05. ADSCs CM shows remarkable enhancement in glucose uptake with a p value <0.001. B. 2-NBDG uptake in diseased 3T3L1 cells (acute condition): In acute condition, metformin and ADSCs CM treatment showed dramatic enhancement of glucose uptake with a common p value <0.001.
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Figure 10: 2-NBDG uptake in C2C12 cells: Depicts the enhancement of glucose uptake in muscle cells by metformin and ADSCs CM (p value < 0.01).

CM effectively reduces adipogenesis in differentiating 3T3L1 cells

Treatment with ADSCs CM resulted in reduction of adipogenesis in 3T3L1 cells as depicted in Figure 11A. Figure 11B and 11C shows the downregulation of the genes involved in adipogenesis when treated with ADSCs CM.
Figure 11: Adipogenesis in 3T3L1 cells. A: Triglyceride estimation of differentiated 3T3L1 cells showing inhibition of adipogenesis by gemfibrozil and ADSCs CM treatment (p value <0.001). B. Cebpα gene expression: Gene expression of Cebpα gene expression shown to be downregulated by ADSCs CM treatment (<0.05). C. Pparγ gene expression: Gene expression of Pparγ gene expression shown to be decreased by ADSCs CM treatment (<0.01).

Reduction in IMTG accumulation in C2C12 cells by ADSCs CM

The possibility of the specific lipid metabolites accumulation that in turn may negatively affect insulin sensitivity is represented by intramuscular triglyceride(IMTG) accumulation in skeletal muscle of obese and Type 2 Diabetes Mellitus (T2DM) (Corcoran et al., 2007). We observed a statistically significant reduction in the IMTG levels in ADSCs CM treatment as shown in Figure 12.
**Figure 12:** Intramuscular triglyceride accumulation in C2C12 cells. Accumulation of triglycerides in muscle is inhibited by ADSCs CM treatment (p value < 0.05).

**Regulation of the key genes by ADSCs CM**

We performed gene expression studies for the genes involved in glucose uptake and inflammation in both 3T3L1 and C2C12 cells. A change in mRNA level of GLUT4 is observed in physiologic states of the altered glucose homeostasis which vary in tissue specific manner and occur much more rapidly in adipose tissue and skeletal muscle. Treatment with palmitate and TNFα significantly reduced the expression of GLUT4 gene and ADSCs CM treatment restored the levels of GLUT4 mRNA in 3T3L1 (Figure 13A) and C2C12 cells (Figure 14A). Genes involved in inflammation viz. IL6 and PAI1 were significantly reduced in the treated cells as compared to the untreated control in 3T3L1 cells (Figure 13B and 13C) and C2C12 cells (Figure 14B and 14C).
**Figure 13: Gene expression pattern in 3T3L1 cells.**

A. Upregulation of Glut4 gene expression which is involved in glucose uptake by ADSCs-CM treatment ($p$ value < 0.001). Decreased expression of the genes involved in inflammation viz. B. IL-6 ($p$ value < 0.01) and C. Pai-1 ($p$ value < 0.001) upon ADSCs-CM treatment.
Figure 14: Gene expression pattern in C2C12 cells. A. Upregulation of GLUT4 gene expression which is involved in glucose uptake by ADSCs-CM treatment (p value < 0.01). Decreased expression of the genes involved in inflammation viz. B. Il6 (p value < 0.01) and C. Pai1 (p value < 0.001) upon ADSCs-CM treatment.

GLUT4 translocation mediated by ADSCs CM treatment

As revealed by the western blotting of C2C12 cells depicted in Figure 15A, GLUT4 protein (50KDa) showed enhanced protein expression in the membrane fraction when normalised to the total GLUT4 protein in the whole cell lysate. Enhancement of GLUT4 protein in the membrane fraction indicates elevated glucose uptake.
Enhancement of insulin signalling through the phosphorylation of Akt

It is widely known that insulin stimulates the Akt canonical pathway under physiological conditions (Li et al., 2015a). Phosphorylation of Akt at Ser 473 indicates the increment in insulin signalling by the ADSCs-CM when compared to the diseases conditioned in C2C12 cells (Figure 15B). Densitometry for Glut4 and pAkt protein expression is shown in Figure 15C and 15D respectively.

**Figure 15: Western blotting.** 1: Control, 2: Diseased, 3: ADSCs-CM, 4: Metformin.

A. Western blot for GLUT4 protein showing enhanced membrane GLUT4 translocation ADSCs-CM treated group. B. Augmentation of insulin signalling revealed by phosphorylation of Akt at Ser 473. C. Densitometry for Glut4 protein expression. D. Densitometry for pAkt protein expression.
3.3. DISCUSSION

In the present study we examined the effect of ADSCs-CM treatment on *in vitro* models of IR. It has been reported that ADSCs have biological advantages in higher proliferative rate, secreted proteins and immunomodulatory effects compared to bone marrow-derived MSCs (Li et al., 2015b). It has been established that adipocyte-derived adipokines such as adiponectin, leptin, and vaspin exert hormone-like activities at the systemic level (Zvonic et al., 2007). The topical application of allogenic ADSCs-CM has been found to be effective method for enhancing wound healing and reducing transient unwanted adverse effects facilitating rejuvenation (Zhou et al., 2013). It has been well established that ADSC-CM reduces neural injury induced by glutamate excitotoxicity in a concentration-dependent manner. The optimal protective effect of the CM as reported is 50% diminished effect is observed when CM concentrations lower than 50 % and 70% is used (Hao et al., 2014). ADSCs are shown to be good for bone regeneration and the amount and quality of the bone regenerated show similar results when compared to the conditioned media (Urayama and Banks, 2008). However, there are no reports so far showing that ADSCs-CM can be used as a treatment of choice for IR. Our data demonstrates for the first time the improvement of glucose uptake in both 3T3L1 and C2C12 cells treated with ADSCs-CM indicating the insulin sensitizing role of the CM. It has been established that triglycerides respond biphasically to energy deprivation, decreasing with short-term fasting, increasing with starvation, and tend to be elevated with obesity (Linero and Chaparro, 2014). We found that the differentiation of 3T3L1 preadipocytes to mature adipocytes was inhibited by ADSCs-CM treatment indicating its possible therapeutic usage in prevention of obesity. Several studies described a strong correlation between plasma free fatty acid levels, intramuscular triglyceride accumulation and the development of insulin resistance (Timmermans et al., 2006). In this study, we observed a significant decrease in the accumulation of intramuscular
triglyceride in C2C12 cells by ADSCs-CM treatment thereby leading to decrease in IR. It has been reported that GLUT4, a glucose transporter is one of the key genes responsible for glucose uptake and the gene expression of GLUT4 is regulated by metabolic, hormonal and nutritional control (Im et al., 2007). Usually, the expression of GLUT4 gene is down regulated in the states of relative insulin deficiency (Berger et al., 1989; Sivitz et al., 1989). Our data revealed a significant upregulation of GLUT4 gene under the influence of ADSCs-CM in both 3T3L1 and C2C12 cells. It is worth mentioning that ADSCs-CM showed a better upregulation of GLUT4 gene in both 3T3L1 and C2C12 cells compared to metformin.

Human obesity and IR studies have revealed a clear association between the chronic activation of pro-inflammatory signaling pathways and decreased insulin sensitivity (Carl et al., 2008). Elevated levels of tumor necrosis factor-α (TNFα), interleukin-6 (IL-6) and interleukin-8 (IL-8) have all been reported in various diabetic and insulin-resistant states (Hotamisligil and Spiegelman, 1994; Hotamisligil et al., 1995; Roytblat et al., 2000; Sartipy and Loskutoff, 2003; Straczkowski et al., 2002). Our data demonstrate that inflammatory markers involved in inflammation and complications of metabolic disorder viz. IL6 and PAI1 were downregulated as evidenced by the gene expression profiling. Since inflammation is the root cause for all the problems, our data has a translational significance in the reduction of inflammatory milieu which is of significance in decreasing IR.

GLUT4 also plays a vital role in the regulation of glucose transport and is highly expressed in the cell types that exhibit regulated glucose uptake such as skeletal muscle, adipocytes and cardiomyocytes (Govers et al., 2004). The acute regulation of GLUT4 is determined majorly by the intracellular trafficking. In the basal non stimulated state, GLUT4 is present in an intracellular tubule vesicular compartment, from which it undergoes insulin-independent movement to the cell surface, resulting in a 10- to 20-fold increase in cell surface GLUT4 levels (Bryant et al., 2002). In an effort to understand the translocation of GLUT4
from the cytosol to the membrane, we fractionated the cells and found the enhancement of glucose uptake in the membrane fraction of ADSCs-CM treated cells documenting the glucose uptake by insulin mediated translocation of GLUT4 with concomitant augmentation of insulin signalling revealed by phosphorylation of Akt at Ser 473. The in vitro results obtained with these two chronic insulin resistant cell models could be attributed to the cumulative effect of all the components of the CM. However, limited amount of factors exhaust soon after being used up by the damaged tissue and hence repeated dose of CM is required for a longer period of time for better results.

3.4. CONCLUSION

Overall, our data demonstrates that the usage of ADSCs CM for reversal of IR will eliminate the need of cell infusion making it safer stem cell solution to IR.