CHAPTER 5: OBJECTIVE 3

ASSESSMENT OF THE EFFECT OF INTRAMUSCULAR INJECTION OF hADSCs IN THREE DIFFERENT FORMS IN DIET INDUCED OBESITY MODEL OF MICE

5.1. INTRODUCTION

The physiological condition where cells fail to respond to normal levels of insulin is called insulin resistance (IR). Although the supply of insulin from the β cells of pancreas is adequate, cells are unable to use it effectively leading to a variety of disorders called the metabolic disorder. Primarily, organs like adipose, muscle and liver respond to insulin significantly but in IR condition they fail to do so. As a result, body needs higher levels of insulin for the uptake of glucose by the cells. At first pancreas makes enough insulin as required but later it is not able to produce insulin to keep the blood glucose to normal levels resulting in Type 2 Diabetes Mellitus (T2DM) (Wilcox, 2005). The epidemic of diabetes is likely to rise due to the diet containing high amount of fat and sugar supplemented by sedentary lifestyle (Paek et al., 2014).

Stem cells have a great potential for basic research and also hold the future for clinical applications. These cells have characteristic features which are quite unique from somatic cells (Ranganath et al., 2012). The area of mesenchymal stem cell (MSC) research has bloomed recently. It is certain that MSC have the capacity to repair several damaged tissues. MSCs are known to be multipotent and have the ability of self-renewal and differentiation into several mesodermal cell lineages (Pileggi, 2012). Adipose-derived stem cells (ADSCs) is emerging as a source of stem cells that can be obtained by a less invasive method and in larger quantities than from BM. These cells can be isolated from human lipoaspirates and, like MSCs, can differentiate toward osteogenic, adipogenic, myogenic, chondrogenic, and
neurogenic lineages. ADSCs, readily isolated from fat tissue after liposuction and easily expanded in culture in large numbers, have developed a striking source for cell therapy (Badimon et al., 2015; Cao et al., 2015). Si et al demonstrated a novel role of MSC administration in improving insulin sensitivity and showed improved GLUT4 expression and the member translocation in peripheral insulin target tissue through an insulin independent manner (Si et al., 2012). High fat diet (HFD) induced obesity is improved and corrects metabolic imbalance by restoring systemic MSC transplantation through niche dependent multisystemic regulations (Ji et al., 2015). Multiple infusions of MSCs promotes prolonged decrease in hyperglycemia and apoptosis in pancreatic islets and increases insulin sensitivity in HFD fed mice (Bueno et al., 2015b).

Based on previous findings we hypothesized that multiple *i.m* injections of ADSCs may help in normalizing glycemic status and lipid profile of DIO animals by controlling inflammation. We show here for the first time the importance of human ADSCs therapy in ameliorating IR in DIO mice by decreasing organ specific inflammation especially in liver and adipose tissue without any change in the body weight.

![Figure 22: Experimental design for Chapter 5](image)

5.2. RESULTS

**DIO model development:** A significant 2-fold increase in the body weight of the DIO mice was observed as compared to the lean control. Serum Triglyceride was significantly high
compared to the lean control. Glucose tolerance was impaired in the mice fed with HFD for 10 weeks. Mice were randomized into six different groups for treatment based on the bodyweight, serum triglyceride levels and OGTT.

**Figure 23:** Different parameters for randomisation A. LC vs DIO mice B. Body Weight C. Serum triglyceride D. Oral glucose tolerance test E. Area under the curve inversely proportional to tolerance.

**Effect of the treatment on body weight:** At the end of 8th week of treatment, metformin showed 16.4% decrease in the body weight as compared to the untreated control. However,
there was no significant change in any of the other treatments as compared to the untreated DIO control (Figure 24).

![Body weight measurement](image)

**Figure 24: Body weight at the end of the study**

**Restoration of the normoglycemic status:** There was no significant change observed during the initial weeks of the treatment (Figure 25A). At the end of 8th week of the treatment; there was a significant decrease in the fasting glucose levels (Figure 25B) The positive control metformin showed substantial decrease compared to the untreated DIO control (p<0.001). Treatment with CS, CM and CL also exhibited significant glucose lowering capacity CS (p<0.01), CM (p<0.05) and CL (p<0.05).

![Fasting glucose levels](image)

**Figure 25: Glucose levels in all the groups of mice.** A. Fasting glucose levels from Week 0 of treatment until the end of the study (Week 8). B. Bar graph showing fasting glucose levels at the end of the study (Week 8).

**Improvement in the glucose tolerance:** As stated earlier, OGTT represents the most physiological route of entry of glucose (Ayala et al., 2010). Glucose tolerance after an oral dose of 2g/kg body weight of glucose improved in metformin (p<0.01) as well as CS treated
group (p <0.05) as shown in Figure 26C. Area under curve (AUC) is inversely proportional to the tolerance derived using GraphPad prism software as depicted in Figure 26D.

**Figure 26: Oral glucose tolerance test (OGTT) in all the groups of mice.**

A. Glucose levels at different time points (0, 30, 60 and 120 minutes). B. Bar graph shows area under the curve which is directly proportional to the glucose tolerance.

**Effect of treatment on hyperinsulinemia:** One of the features of the IR is hyperinsulinemic condition. Mice fed on HFD attained hyperinsulinemia as compared to the lean control (p<0.001). On the other hand, all the treatment groups showed significant reduction in the serum insulin levels except for the CL treated group (Figure 27).

**Figure 27: Bar graph showing levels of insulin at the end of the study**
Effect on lipid profile

At the end of 8th week, measurement of serum triglycerides and oxidized LDL after overnight fasting showed significant increase in the DIO control mice as compared to the lean control (p< 0.001). The treatment of CS, CM and CL exhibited similar pattern in decreasing triglyceride which was comparable to the positive control metformin. Serum triglyceride at week 0, week 4 and week 8 is shown in Figure 28A. All the treatment groups shared the same p value being <0.01 in the reduction triglyceride at the end of the study as shown in Figure 28B. Decrease in oxidised LDL levels in all the treatment group was observed having a p value of <0.05 (Figure 28C)

Figure 28: Lipid profile in all the groups of mice. A. Fasting triglyceride levels from Week 0 of treatment until the end of the study (Week 8). B. Bar graph showing fasting triglyceride levels at the end of the study (Week 8). C. Bar graph showing levels of oxidised low density lipoprotein in the serum at the end of the study.
Pro-inflammatory cytokine measurement: To investigate the status of pro-inflammatory cytokine namely IL6, we measured IL6 in the serum and found a dramatic reduction in the secreted IL6 by CS (p<0.05) and CM (p<0.05). Metformin and CL did not show any significant change. (Figure 29).

![Serum interferin-6 levels](image)

**Figure 29: Bar graph showing IL6 levels in the serum at the end of the study**

Hepatic Triglyceride analysis: Hepatic triglyceride levels were high in the DIO control than lean control (p < 0.05). CS reduced liver triglycerides significantly as compared to untreated DIO control (p < 0.01) (Figure 30).

![Hepatic triglyceride levels](image)

**Figure 30: Bar graph showing triglyceride levels in the liver tissue at the end of the study**

Assessment of Insulin resistance: In accordance with the earlier reports which states that HOMA IR (Figure 31A) and TyG (Figure 31B) are the useful and reliable indicators of Insulin resistance our data shows a remarkable decrease in HOMA IR and TyG in all the
treatment groups as compared to DIO control. Metformin, CS, CM and CL showed decrement in both the parameters (p<0.05)

![Graph A: HOMA-IR](image1)
![Graph B: TyG](image2)

**Figure 31: Assessment of insulin resistance.** A. Homeostatic model assessment of insulin resistance (HOMA IR) B. Triglyceride Glucose index (TyG)

**Impact on tissue architecture:** In an attempt to understand the tissue architecture of the untreated and the treated mice, histopathological studies were carried out. Figure 32A depicts Hematoxylin and Eosin staining of the tissues demonstrating the reduction of fatty infiltration in liver and Figure 32B shows decreased macrophage infiltration in adipose tissues mainly by CS at 400X magnification. Figure 32C depicts reduced hypertrophy of the pancreatic islets by all the treatments at 200X magnification.
Figure 32: Representative images of histopathological examination different tissues after H&E staining. A. Liver tissues showing recovery of the fatty infiltrated liver due to HFD. B. Subcutaneous adipose tissue sections depict the reduction of macrophage infiltration in the adipose tissue especially in CS treated group. C. Hypertrophied islets in the pancreatic sections of untreated DIO control reduced in all the treatment groups.
GLUT4 translocation: Immunohistochemistry for the skeletal muscle section showed membrane staining in all the treatment groups whereas untreated control showed cytoplasmic staining. CS treated group showed the best membrane staining of all the treatment groups (Figure 33).

Figure 33: Illustrative image of skeletal muscle tissue immunohistochemistry: Images showing plasma membrane staining for GLUT4 in all the treatment groups. On the other hand, the untreated DIO control shows cytoplasmic staining.

Upregulation of miRNA-206 and increment in total protein content of the skeletal muscle: In an effort to investigate the role of miRNA-206 in DIO model and the effect of the treatments which can plausibly dictate the mechanism of actions of ADSCs and the effect of i.m. injection we performed the gene expression studies of miRNA-206 in all the treatment groups. The expression levels of miRNA-206 decreases remarkably in HFD fed mice and the levels of relative expression is brought up by CS treatment (p< 0.05) as shown in Figure 34A. Figure 34B shows the dramatic increase in the protein content of the skeletal muscle only in CS treated group.
Improvement in gene expression pattern: Gene expression analysis was carried out for liver and muscle tissue samples. Figure 35A shows significant increase in the GLUT4 gene expression levels of the muscle tissue indicating increase in the insulin sensitivity and an outstanding upregulation of MyoD gene clearly defines the regeneration of muscle increasing the muscle mass through miRNA-206 as mentioned earlier. Gene expression analysis for the liver tissues showed remarkable decrease in Il-6, Pai-1 and ApoB depicted in Figure 35B. Downregulation of these genes in all the treatments explain the reduction in the extent of inflammation in the liver.
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5.3. DISCUSSION

In the present investigation we compared the effect of human ADSCs, their CM and their CL on glycemic status, insulin resistance, triglycerides, pro-inflammatory cytokines viz. IL6 and Oxd LDL using metformin as a positive control. We found that amongst the three treatments tested ADSCs suspension (CS) was the best as it restored not only the organ function but also the tissue architecture as revealed by serum and histopathological analyses. Earlier reports showed the beneficial effect of MSC injection via i.v. route supporting our present study (Badimon et al., 2015; Cao et al., 2015). However, this study is unique wherein we have shown the effect of human ADSCs via i.m. route in ameliorating the ill effects of HFD. The logic behind using i.m route was a reported cross talk between skeletal muscle and pancreatic beta cell (Bouzakri et al., 2011; Gopurappilly and Bhonde, 2012; Pedersen and Hojman, 2012). It is shown that a healthy muscle secretes myokines which positively impact the pancreatic beta cell whereas a diabetic muscle secrete cytokines which negatively impacts the beta cell function. Moreover, in Indian population the muscle mass is always shown to be less than the fat mass as compared to the European community (Hardikar et al., 2015; Yajnik and Yudkin, 2004). Therefore, we were interested in examining the effect of i.m. injections of ADSCs on the metabolism of HFD fed mice.

Figure 35: Gene expression pattern in skeletal muscle and liver tissue. A. GLUT4 and MyoD in skeletal muscle. B. Expression levels of genes mediating inflammation in the liver viz. Il6, Pai1 and ApoB.
The DIO model was developed following the similar procedure as reported earlier with slight modifications (Wang and Liao, 2012). It is worth mentioning that there was a steady increase in the body weight over the period of ten weeks. The establishment of DIO was confirmed by and substantial increase in body weight accompanied by hyperglycemia, hyperinsulinemia, hypertriglyceridemia and abnormal OGTT. None of the treatments resulted in decrease in body weight as reported earlier with i.v. injection using rodent MSCs. The study by Cao et al concluded that treatment with mouse ADSCs was effective in lowering the blood glucose level and improving the glucose tolerance in mice along with decrease in body weight (Cao et al., 2015). The striking feature of our study is the management of inflammation which is the root cause of the metabolic syndrome (Roberts et al., 2013; Wannamethee et al., 2005) as evidenced by HOMA-IR, TyG and OGTT (Atabek and Pirgon, 2007; Katsuki et al., 2001; Unger et al., 2014). As described earlier, in severe insulin resistance, there is a reduction in muscle GLUT4 protein and mRNA expression similar to adipose tissue (Atkinson et al., 2013; Kampmann et al., 2011). The decrease in hyperglycemia and restoration of OGTT could be endorsed to higher expression of GLUT4 in the skeletal muscle supports our finding of decrease in IR leading to restoration of normoglycemia. Moreover, the levels of gene expression for IL6, PAI1 and ApoB in the liver were drastically reduced in all the treatment groups indicating the therapeutic value of ADSCs and their Secretome (Alessi et al., 2003; Auguet et al., 2014; Moon et al., 2012; Targher et al., 2007).

All the treatment also led to decrease in pro-inflammatory cytokine IL6 and lowering Oxidised LDL except metformin and CL which did not show any significant change in the reduction of secreted IL6 confirming the anti-inflammatory role of MSCs. The decrease in inflammation was further confirmed by histological examinations of adipose tissue, liver and pancreas. Adipose tissue exhibited decrease in macrophage infiltration indicating reduction in inflammation. It is known that HFD is one of the causes of fatty liver. Non-Alcoholic Fatty
Liver Disease (NAFLD) being one of the most common disease across the world. NAFLD is believed to be strongly associated with IR, visceral obesity and dyslipidemia. The main feature of the disease are triacylglycerol (TG) accumulation inside the liver which can lead to extremely serious complications. Rats fed with HFD develop IR, hypertriglyceridemia, hepatic steatosis and liver damage, which is the characteristics of NAFLD (Bravo et al., 2011; Ferolla et al., 2015). The treatment with CS as well as CM improved the fatty infiltration in the liver which was comparable to that of metformin treated mice liver. Hypertrophy of the beta cell is the normal physiological response of the body to compensate for the increased demand of insulin during obesity (Roat et al., 2014). Histological analysis of the pancreas from various groups exhibited a different picture. Hypertrophy of the islets was observed in HFD mice pancreas which was found to be reduced in CS treated mice.

The major organs affected in IR are liver, adipose tissue, pancreas and skeletal muscle. Association of type 2 diabetes with excessive loss of skeletal muscle and trunk fat mass in older adults is established (Park et al., 2009). If trunk fat is considered, accumulation of fat in the legs is likely to be protective against a dysregulated glucose metabolism, particularly in women (Snijder et al., 2004).

Regeneration of adult skeletal muscle can occur in response to exercise, injury and disease. A very small population of stem cells known as the satellite cell is responsible for the regeneration (Ma et al., 2015). Micro RNAs (miRNAs) have emerged lately and are known to be involved in many vital biological processes (Pillai, 2005). They are short non coding RNA known to negatively regulate the gene expression at post transcriptional levels either by inhibiting the translation or by degrading the target mRNA (Tanzer and Stadler, 2006; Williams and Mitchell, 2012; Ying et al., 2006). There are reports stating that miRNA 206 expression is elevated in regenerated muscles. One week after the injury, enhanced muscle regeneration accompanied by inhibition of muscle fibrosis was observed in a group of rats.
injected with miRNAs speculating that miR-206 plays a positive role in the treatment of skeletal muscle injury (Liu et al., 2012).

Since diabetes is widely known to be a muscle wasting disease we were interested in finding out the expression levels of miRNA 206 in skeletal muscle and we found a remarkable decrease in miR206 expression of DIO mice as compared to that of lean control which has not been reported so far. However, it is worth mentioning that miR206 was significantly upregulated in the CS treated mice as compared to DIO control. Muscle regeneration through miRNA 206 possibly enhances the muscle mass and hence the protein content of the muscle is proportionally elevated as shown by our protein quantification data in skeletal muscles. We also found that high fat diet decreased the protein content of the muscle which was restored by CS treated mice. On the contrary, the DIO mice injected with CS exhibited higher protein content demonstrating higher muscle mass and all treatment (CS, CM, CL) showed significant upregulation of MyoD indicating regeneration of the muscle. Ma et al reported that MiR-206 inhibits the expression of Pax7 and promotes the function of MyoD, thus establishing a positive regulatory feedback loop. Upregulation of miR-206 by MyoD further suppresses Pax7, thereby promoting muscle cell differentiation (Dey et al., 2011; Ma et al., 2015).

Based on our results we assumed that increase in inflammation of the target organ like adipose tissue, liver and muscle elevates IR which could be alleviated by i.m. injections of ADSCs.

5.4. CONCLUSION

Our results clearly demonstrates the role of ADSCs in reducing systemic inflammation as revealed by serum parameters and organ specific inflammation by histological analysis and gene expression. Taken together our data indicates for the first time the importance of ADSCs therapy in ameliorating IR in DIO mice.