UNDERSTANDING CYTOKINE MEDIATED INSULIN RESISTANCE IN FATE OF HADSC AND AMELIORATION USING INSULIN SENSITIZERS

CHAPTER 5

5. Understanding cytokine mediated insulin resistance in fate of hADSC and amelioration using insulin sensitizers

5.1. INTRODUCTION

Obesity is directly associated with inflammation which alters metabolism and induces insulin resistance. Excess fat starts getting accumulated in adipocytes which become hypertrophic with concomitant augmentation of adipogenesis (Gustafson et al., 2009). This phenomenon continues as a viscous cycle which alters the endocrine function of adipose tissue where, leptin, resistin and TNFα are profoundly secreted with concomitant reduction in adiponectin, omentin, IL-10, IL-4 etc. Thus modern scientific research is being focused on understanding the role of inflammatory mediators in obesity (Reilly & Saltiel, 2017).

TNFα is 26kDa pro-cytokine, wherein 76 amino acids are conserved which help to anchor this protein to the membrane, and then it is enzymatically cleaved by metalloprotease enzyme TNFα converting enzyme (TACE) to soluble 17kDa biologically active form. Both membrane bound and soluble form of this cytokine execute their response through autocrine, paracrine or endocrine mode. TNFα is mainly formed by macrophages of different tissues. It acts through two receptors TNF-R1 and TNF-R2 which are present on different cells, loci and regulate several crucial cellular processes like propagation, differentiation, survival and apoptosis. TNF-R1 are expressed ubiquitously on all mammalian cells whereas TNF-R2 are restricted to immune cells (Parameswaran et al., 2010).

The trimer molecules of TNFα bind to TNF-R1's extracellular domain and adaptor protein complex is released from the receptor which activate the NFkβ pathway through IKKβ. LPS, endotoxins, FFA evoke the activation and binding of TNFα to TNF-R1 which exert maximum responses towards immune stimuli and exerts inflammation by activation of JNK, ERK and p38 MAPKs pathways. These pathways induce the expression of serine-threonine kinases which phosphorylate serine residue of IRS-1, which inhibit auto-dimerization of IR and reduction in tyrosine kinase activity. This leads to insulin resistance by downregulating insulin signaling and insulin mediated glucose uptake and activated lipolysis in adipocytes, inducing

hyperglycaemia. It also downregulates PPARγ and thus, inhibits adipocyte differentiation (Tzanavari et al., 2010).

Resistin is known as adipose tissue specific factor (ADSF) that belongs to the family of (resistin like molecules) RELM/ FIZZ (found in inflammatory zone), is cysteine rich protein that is secreted during adipogenesis. Twelve % amino acids are cysteines which are required for binding of resistin to the receptors and intramolecular signaling in inflammatory conditions. During normal states, resistin is produced at basal levels in pre-adipocytes for maturation and lipid synthesis in adipocytes. Its expression is regulated by PPARγ, C/EBPα and SREBP-1c, the main transcriptional factors that govern conversion of pre-adipocyte to mature adipocyte during adipogenesis (A. Park et al., 2014; Seo et al., 2003). It is presumed that lack of PPARγ binding site on the promoter of resistin in human adipocytes might be the reason for their lack in production of resistin, whereas it is present in mouse adipocytes (Acquarone et al., 2019).

High fat diet, FFA and LPS bind to TLR4 and stimulate over production of resistin which propagate inflammation and adipogenesis. Resistin can also bind to TLR4, adenosine receptors and decorin to activate the inflammatory cascades which in turn stimulate production of pro-inflammatory cytokines like TNFα, IL6, and other chemokines and thus activate inflammatory pathways like NFκβ mediated inflammasome complex (Daquinag et al., 2011; Jang et al., 2017). Elevated resistin exaggerates lipid and leptin synthesis in adipocyte which leads to hypertrophic adipocyte. Enhanced resistin level stammers adipocyte maturation which causes leaky adipocyte phenotype.

In humans' macrophages embark deleterious effects by increasing inflammation. As a consequence, chronic stage of inflammation is established that drives adipocytes towards apoptosis and thus M1 macrophages form a crownet like structure surrounding the dying adipocyte. Thus, in the advanced stage of obesity, elevated inflammation leads to lipolysis in adipocytes (H. S. Jung et al., 2006). Increment in resistin levels also leads to leptin resistance. Moreover, circulating levels of resistin are associated with patients suffering from diabetes, CVD, rheumatoid and osteoarthritis (Benomar et al., 2019). Insulin solely stimulate release of resistin

(McTernan et al., 2003). Thus, hyperglycemia and hyperinsulinemia increases the levels of resistin which worsens the conditions.

A comprehensive study conducted in four different phenotypes of Indians have deliberated that non-obese metabolically obese, metabolically obese obese and obese metabolically healthy had persistently high circulating levels of resistin compared to those of TNFα levels. Thus, it can be deduced that resistin is more associated to obesity and metabolic diseases (Indulekha et al., 2015). Further, in chapter 4, we reported that obesity diminished stemness, metabolism, angiogenesis and differentiation potentials of hADSC from obese Indians. Reports demonstrated that TNFα influences proliferation and differentiation potentials of ADSC in concentration dependent manner (Lu et al., 2013; Qin et al., 2015; Salamon et al., 2014; Zubkova et al., 2016). Resistin embarks insulin resistance both in adipocytes and adipose tissue thus, altering their metabolic functions. Although, it is known that resistin induces insulin resistance and metabolic dysregulation in adipocytes, its repercussion on hADSC have not been elucidated except understanding of isoform of decorin being a receptor of resistin on ADSC (Daquinag et al., 2011). Moreover, there are no insulin resistance hADSC models as per best of our knowledge.

Calorie restriction and exercise were reported to reduce pro-inflammatory cytokine levels in obese subjects (Oh et al., 2018). Several class of drugs are used to treat obesity, diabetes and associated complications. Met is a gold standard drug which ameliorates insulin resistance by activating adiponectin, AMPK thus rendering glucose lowering effects. It reduces inflammation, effects of glucagon and hyperlipidemia which ameliorate diabetes and metabolic complications (Pernicova & Korbonits, 2014). However, all the classes of synthetic drugs have demonstrated minor to severe side effects like nausea, hypertenstion, pancreatitis and heart failures (Wilding, 2018).

Herbal medicines have been used as alternative treatment regime for several diseases like diabetes, arthritis, obesity etc. *EL* is a perennial herb found in some parts of India. It is a folk lore herb that has been used since decades for treatment of obesity, diabetes, arthritis, gastroenteritis etc. It is rich in phyto-compounds like flavonoids, biguanides etc. and has broad range of therapeutic activities which have been extensively explored in our lab. EL comprises 7% of SM, a secoiridoid glycoside

which embarks anti-hyperglycemic, anti-hyperlipidemic and anti-malarial activity (Patel et al., 2013; Soni et al., 2009).

Our lab and other groups have demonstrated antiglycemic, antilipidemic and antidiabetic efficacy of SM (Vaidya et al., 2013; Vaidya et al., 2012; Vaidya et al., 2009). We reported that SM modulated carbohydrate and lipid metabolic genes which resolved insulin resistance, dyslipidemia in adipose tissue, HGP and thus ameliorated NA-STZ induced NIDDM model (Patel et al., 2013). We further exhibited that SM targets PPARγ and dependent lipogenic genes *in vivo* and *in vitro*. It also regulated PPARα, LDH and insulin signaling, thus ameliorating oleic acid induced hepatic steatosis in HepG2 cells (Patel et al., 2016). SM demonstrated anti-adipogenic properties by inhibiting adipogenesis in 3T3-L1 preadipocytes (Tushar Patel, Ph.D. thesis, 2014). However, till date insulin sensitizing potentials of SM have not been explored on hADSC. Thus, the present study was conducted to explore the repercussions of pro-inflammatory adipokines TNFα and resistin independently on metabolic and stemness of hADSC. Also it was aimed to assess therapeutic potentials of SM on TNFα and resistin mediated alterations in stemness and metabolic profile of hADSC. Met, known antidiabetic drug was used as positive control in the study.

5.2. PLAN OF WORK

The study in this chapter is divided into three different sections. First, hADSC isolated from a healthy control were treated independently with 10ng/ml TNFα and 50ng/ml resistin upto 96H and proliferation was observed after 48H and 96H by MTT assay. To create insulin resistance model, hADSC were treated with both TNFα and resistin independently for 48H and expressions of key insulin signaling proteins were monitored by western blot. Once we observed that both the adipokines diminished proliferation and induced insulin resistance after 48H in hADSC, SM was then evaluated for its insulin sensitizing activity. hADSC were treated with TNFα and resistin independently for 24H and 72H followed by treatment with 25µg/ml SM and 64.58µg/ml Met for 24H and then cell cycle was analysed after total of 48H treatment and 96H treatment. As 96H of treatment induced apoptosis in cells, 48H was selected as treatment time for TNFα and resistin followed by 24H of SM and Met treatment in further experiments where gene expression of inflammatory mediators, pluripotency markers, adipogenic genes and insulin mediated glucose uptake were monitored. hADSC has two important fates either adipogenesis or osteogenesis hence, effects of

SM and Met were observed on TNFα and resistin mediated differentiation potential. TNFα and resistin treated hADSC were subjected to adipogenesis for 22D and osteogenesis for 14D in continuous stimulus of both the adipokines and insulin sensitizers. The adipocytes were phenotypically observed in all the groups by ORO staining on 22ndD. Similarly, osteocytes were observed in all the groups by alizarin red stain on 14thD of osteogenesis. The overall plan of work in depicted in the Figure 5.1.

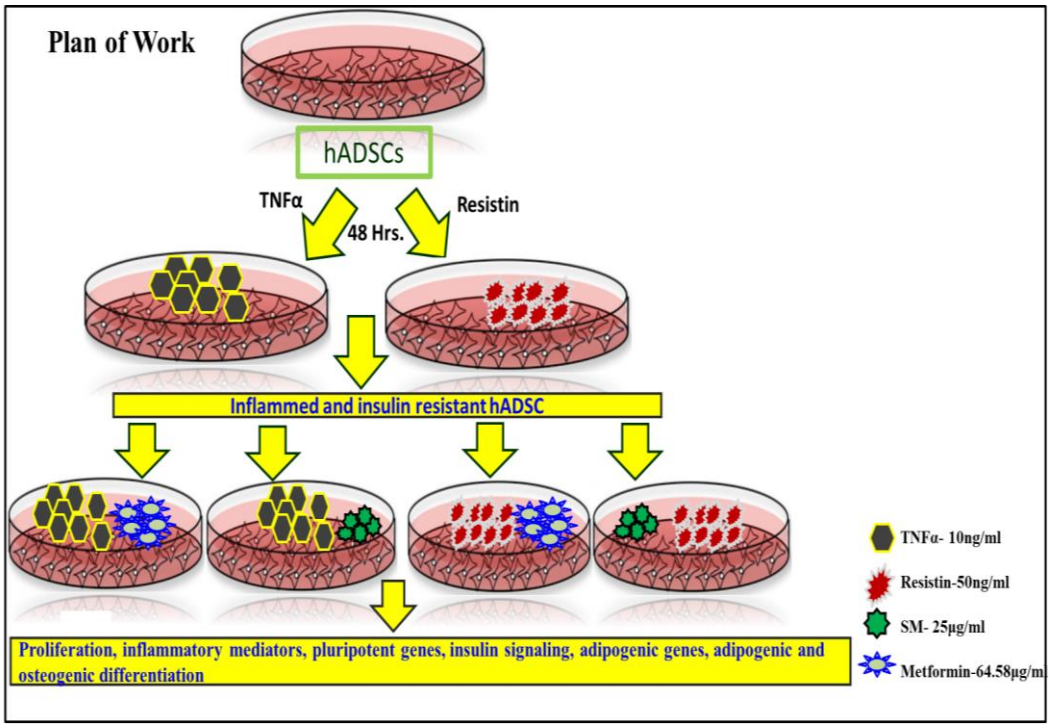

Figure 5. 1: Plan of Work

5.3. RESULTS

5.3.1 TNFα and Resistin mediated hADSC insulin resistance model

TNFα and resistin embarks insulin resistance in adipocytes. However, as there are no insulin resistance models in hADSC, treatments of TNFα and resistin were used for creating insulin resistance model and monitored for proliferation by MTT assay. TNFα reduced proliferation of hADSC after 48H of incubation while there was no significant difference after 96H of treatment whereas, resistin significantly diminished proliferation of the cells after 96H of treatment (Figure 5.2. A.). These results denote that resistin persistently hampered proliferation compared to TNFα. Based on MTT assay, 48H was considered as treatment time of TNFα and resistin independently for further experiments. Expressions of key insulin signaling proteins IR, total AKT, pAKT and pAKT/ total AKT (activated AKT) were monitored by western blot.

Densitometric analysis revealed that TNFα did not alter IR but increased total AKT with significant downregulation of pAKT and pAKT/ total AKT which embarked insulin resistance in hADSC. On the other hand, resistin upregulated expressions of total AKT but downregulated the key proteins like IR, pAKT and activated AKT, thus inducing insulin resistance in hADSC (Figure 5.2. B and C.). These results demonstrated that both TNFα and resistin independently downregulated insulin signaling and induced insulin resistance in hADSC. Thus, hADSC insulin resistance model was generated and further, efficacy of SM was monitored on proliferation, stemness, insulin resistance and adipogenic and osteogenic differentiation potentials of these insulin resistant hADSC.

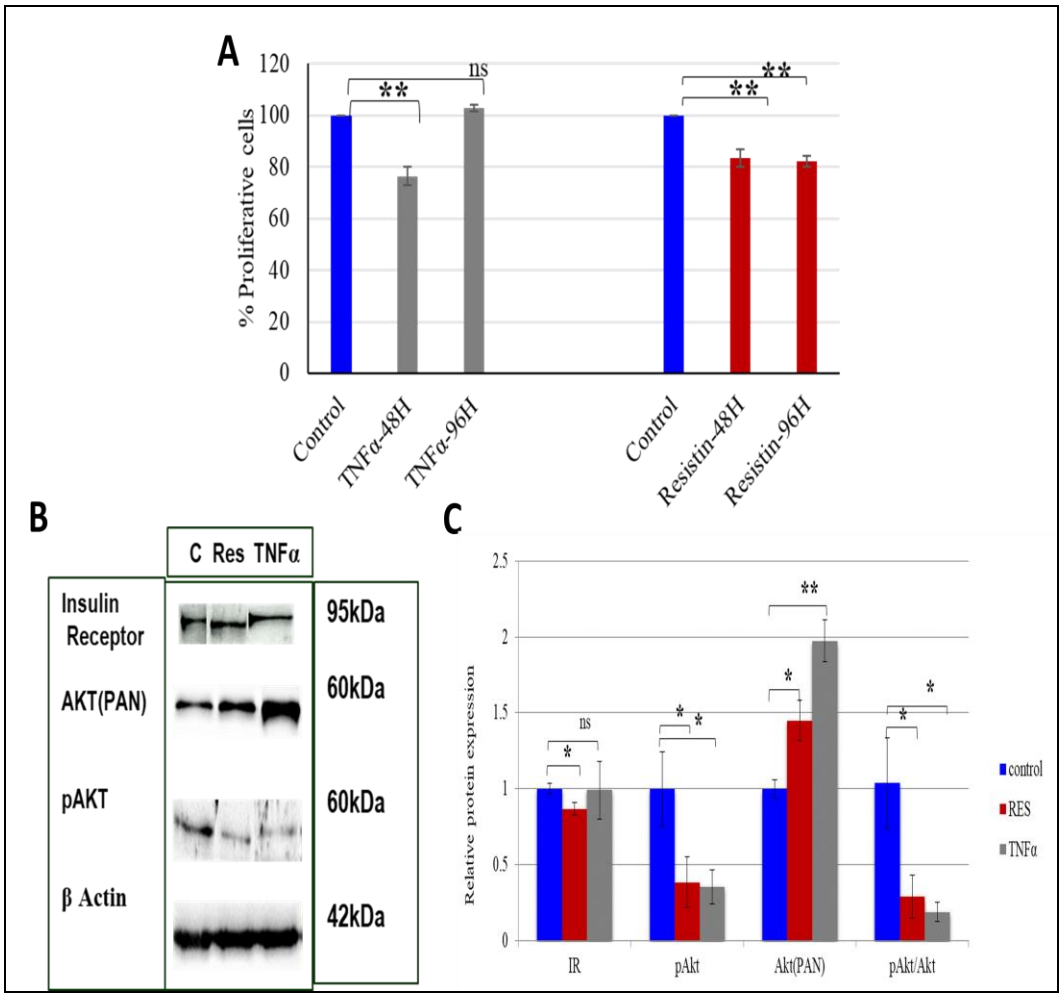

Figure 5. 2: TNFα and Resistin mediated hADSC insulin resistance model, A. Effects of TNFα and resistin on cell proliferation were monitored by MTT assay done after the treatment of TNFα and resistin independently for 48H and 96H. n=3, p Value *<0.05, ns-non significant. B. Western blot of insulin signaling proteins. C. Densitometric analysis of insulin signaling proteins. p Value *<0.05, **<0.01, ns-non significant.

5.3.2. Effect of SM on TNFα and resistin mediated cell cycle of hADSC

hADSC are highly proliferative which make them potential stem cells for regenerative therapy. However, several insults like obesity, age and cytokines hamper their proliferation. Thus, to monitor the effects of SM on TNFα and resistin mediated alterations in proliferation of hADSC, different phases of cell cycle were monitored. Cell cycle experiment revealed that there was no effect observed in any of the groups after 48H of treatment (Figure 5.3.A-D.). 96H treatment demonstrated that TNFα induced apoptosis in the cells as approximately 50% cells were found in Sub G0/G1 phase and 40% in G0/G1 phase. SM demonstrated 75% cells in Sub G0/G1 phase and 10% cells in G0/G1 phase. Met rescued the cells by almost 18% as only 22% cells were observed in Sub G0/G1 phase and around 65% were restored in G0/G1 phase (Figure 5.3. E and F.). When effects of resistin were observed after 96H, more than 92% cells were in Sub G0/G1 phase while less than 5% in G0/G1 phase. SM rescued 10% of cells while Met drastically reduced resistin mediated apoptosis as only 22% cells were in Sub G0/G1 phase while approximately 72% cells regained G0/G1 phase of cell cycle (Figure 5.3. G. and H.). The results of cell cycle clearly demonstrate that resistin induced apoptosis more potently then TNFα, while Met rendered better protection to the cells compared to SM.

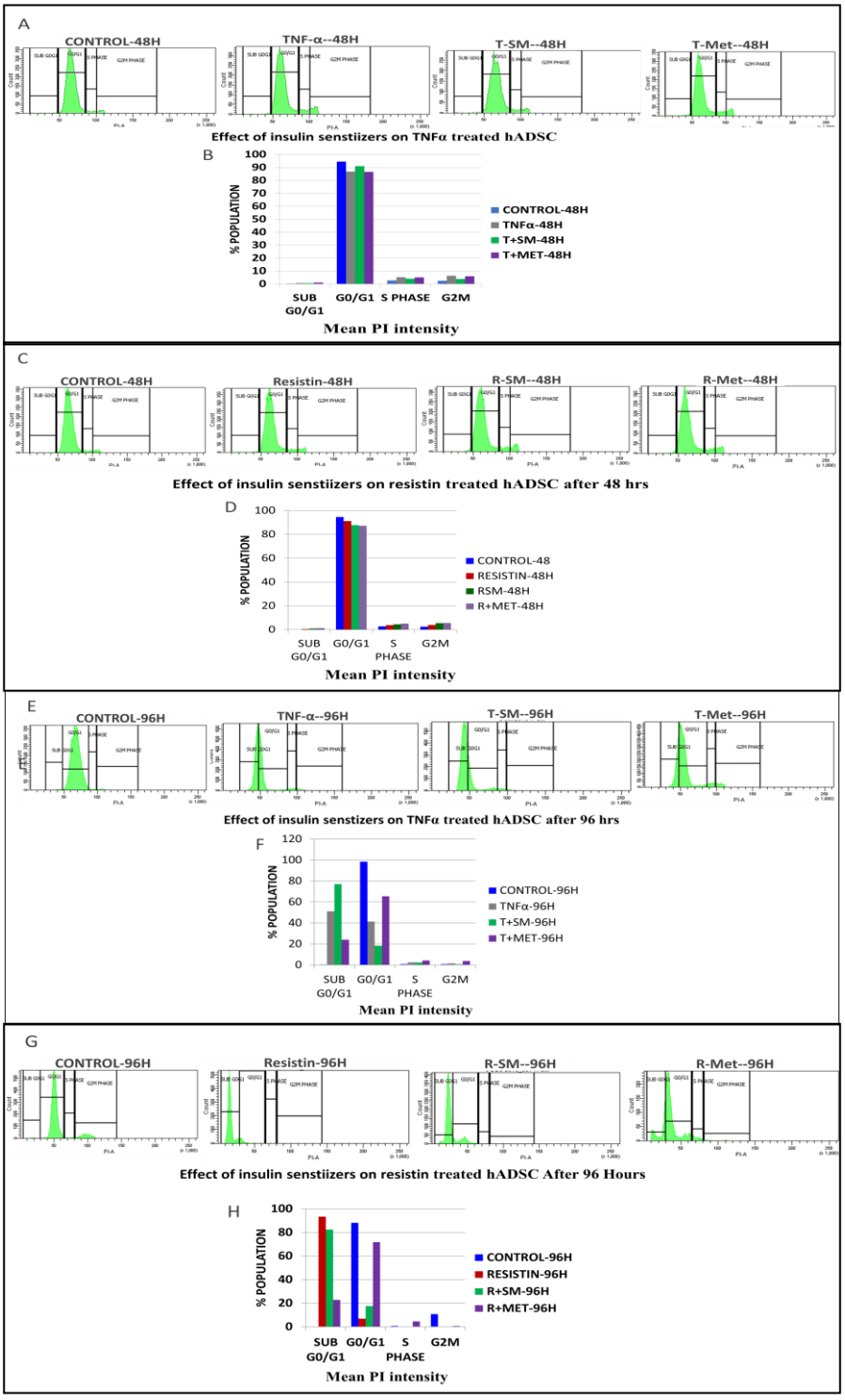

Figure 5. 3: Effect of SM on TNFα and resistin mediated cell cycle of hADSC A. Mean intensity of PI demonstrate different phases of cell cycle analysed after 48H of TNFα treatment to hADSC. B. Results are depicted as Bar graph. C. Mean intensity of PI demonstrate different phases of cell cycle analysed after 48H of Resistin treatment to hADSC. D. Results are depicted as Bar graph. E. Mean intensity of PI demonstrate different phases of cell cycle analysed after 96H of TNFα treatment to hADSC. F. Results depicted as Bar graph. G. Mean intensity of PI demonstrate different phases of cell cycle analysed after 96H of Resistin treatment to hADSC. H. Results are depicted as Bar graph. Results are pooled values of three replicates of each group.

5.3.3 SM ameliorated TNFα and resistin mediated inflammation and altered pluripotency in hADSC.

TNFα and resistin both are pro-inflammatory in nature which plays central role in establishing inflammation. However, their effects on downstream inflammatory mediators have not been observed on hADSC till date. Therefore, gene expressions of these mediators were monitored in presence of TNFα and resistin, also the effects of SM and Met were monitored on these cells. TNFα significantly upregulated gene expressions of all the three inflammatory mediators MCP-1, IKKβ and NLRP3 compared to control cells. SM significantly reduced expressions of MCP-1, while expressions of IKKβ were below detection level and no significant difference was observed in expression of NLRP3. Met also significantly downregulated expressions of IKKβ but no significant difference was observed in MCP-1 and NLRP3 genes (Figure.5.4. A.). Resistin also upregulated all the three inflammatory mediators and SM did not affect MCP-1 but significantly downregulated IKKβ and NLRP3 expressions. Met significantly downregulated all the three inflammatory mediators in resistin treated cells (Figure.5.4. B.). Therefore, these results exemplify that both TNFα and resistin induce inflammation in hADSC through inflammasome. Both SM and Met diminished inflammation with greatest effect on IKKβ gene expression. hADSC are pluripotent stem cells which are efficiently implicated in regenerative therapy. These cells express pluripotent genes which enable them to differentiate into all the three germ line *in vitro*. As observed in chapter no. 4. obesity alters pluripotency, inflates inflammation and induces insulin resistance in hADSC, therefore, it was instigated to scrutinize the repercussion of TNFα and resistin on pluripotent genes. Also the effects of SM and Met were monitored on these inflamed cells. TNFα significantly upregulated gene expressions of OCT4 with concomitant reduction in SOX2 and NANOG but no alterations in c-MYC levels. SM significantly upregulated SOX2 but no significant difference was observed in expressions of OCT4, NANOG and c-MYC. Met significantly upregulated SOX2 and NANOG but did not alter gene expressions of OCT4 and c-MYC (Figure 5.4.C.).

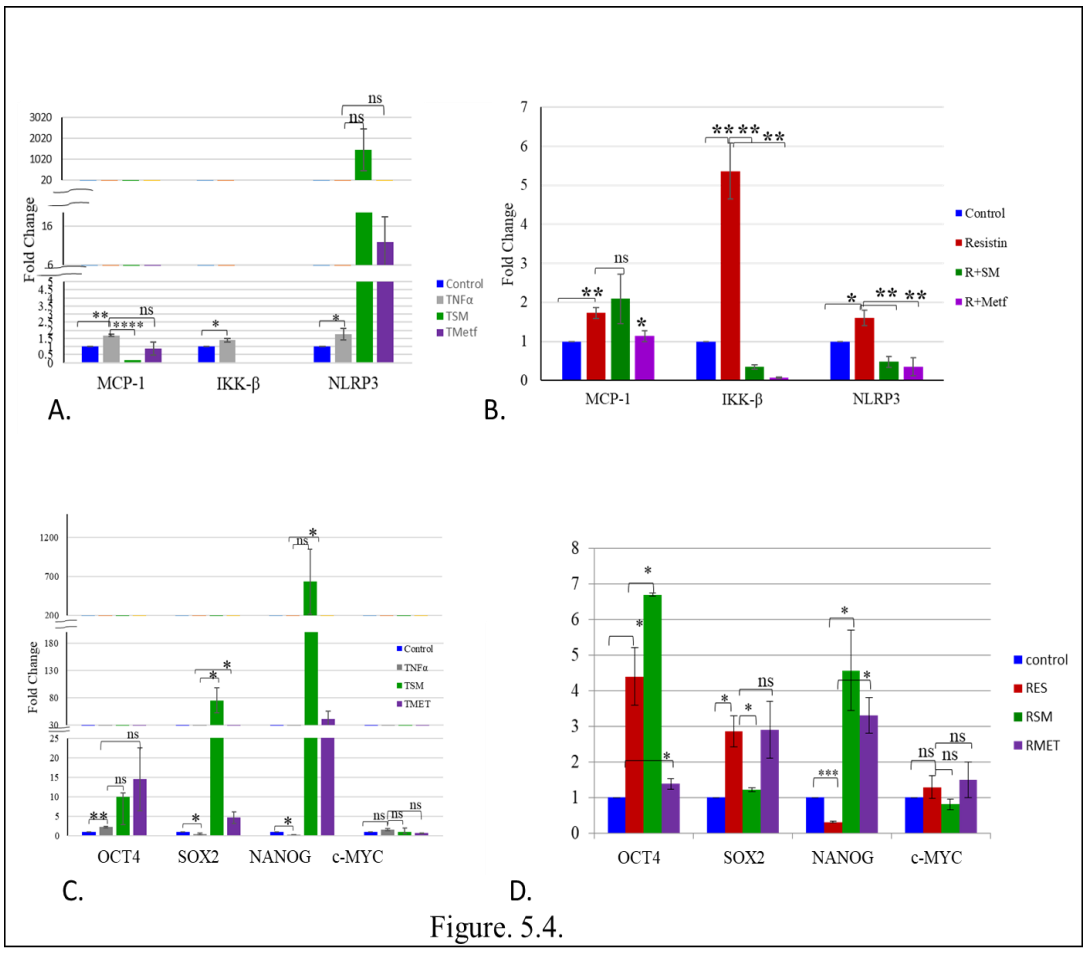

Figure. 5.4.

Figure 5. 4: SM ameliorated TNFα and resistin mediated inflammation and altered pluripotency in hADSC. A. Gene expressions of Inflammatory mediators in TNFα, SM and Met treated cells expressed as Fold Change ± S.E.M. with β actin as internal control. B. Gene expressions of Inflammatory mediators in resistin, SM and Met treated cells expressed as Fold Change ± S.E.M. with β actin as internal control. C. Expressions of pluripotent genes in TNFα, SM and Met treated cells expressed as Fold Change ± S.E.M. with β actin as internal control. D. Expressions of pluripotent genes in resistin, SM and Met treated cells expressed as Fold Change ± S.E.M. with β actin as internal control. n=3, p Value- *<0.05, **<0.01, ***<0.0001, ns- non-significant.

On the other hand, resistin increased expressions of OCT4, SOX2 with concomitant reduction in NANOG expressions but did not alter c-MYC expressions. SM significantly upregulated OCT4 and NANOG with reduction in SOX2 expressions but no difference in c-MYC. Met significantly downregulated OCT4 with increase in NANOG but no significant differences were observed in SOX2 and c-MYC expressions (Figure 5.4.D.).

These results depict that both TNFα and resistin alter pluripotency in different manner and SM and Met upregulated stemness. No differences in the expressions of c-MYC in

any of the treatment group, is in concordance with no difference observed in cell cycle after 48H of treatment.

5.3.4. SM ameliorated TNFα and resistin mediated dysregulated metabolic profile of hADSC

Insulin plays an important role in metabolism of the cells. As observed, TNFα and resistin embarked insulin resistance and inflammation in hADSC, to further confirm these results insulin mediated glucose uptake demarcated as hallmark of insulin resistance was monitored in these cells by observing the mean intensity of 2-NBDG labelled with FITC at 530nm. Analysed data revealed that TNFα and resistin significantly downregulated uptake of fluorescent glucose in hADSC compared to control. 10μg/ml to 50ug/ml SM significantly upregulated insulin mediated glucose uptake in both TNFα and resistin group. Similarly, Met significantly upregulated insulin mediated glucose uptake at 1.3μg/ml to 129.2μg/ml in TNFα group and at 2.6μg/ml to 129.2μg/ml in resistin group (Figure 5.5. A-D).

These results thus signify that both TNFα and resistin independently perturbed insulin mediated glucose uptake and caused insulin resistance. SM and Met independently ameliorated insulin resistance in wide range of concentrations and protected cells from the deleterious effects of these pro-inflammatory adipokines. It is interesting to note that SM proved its insulin sensitizing effects at very low dose compared to Met a standard drug used in clinical practice. As insulin signaling regulates metabolism of adipocytes, further study was conducted to monitor effects of SM and Met on adipogenic genes of TNFα and resistin treated hADSC. TNFα and resistin down-regulated C/EBPβ and C/EBPα but upregulated PPARγ2 in hADSC compared to control cells. SM and Met reduced C/EBPβ and PPARγ2 with concomitant rise in C/EBPα compared to TNFα treated cells whereas, in resistin treated group, SM significantly upregulated C/EBPα expressions. However, metformin did not demonstrate any significant differences in all the three genes. These results depicted that both TNFα and resistin altered adipogenic genes towards lipogenesis and SM upregulated C/EBPα which upregulated insulin sensitivity of hADSC (Figure 5.5.E. and F.).

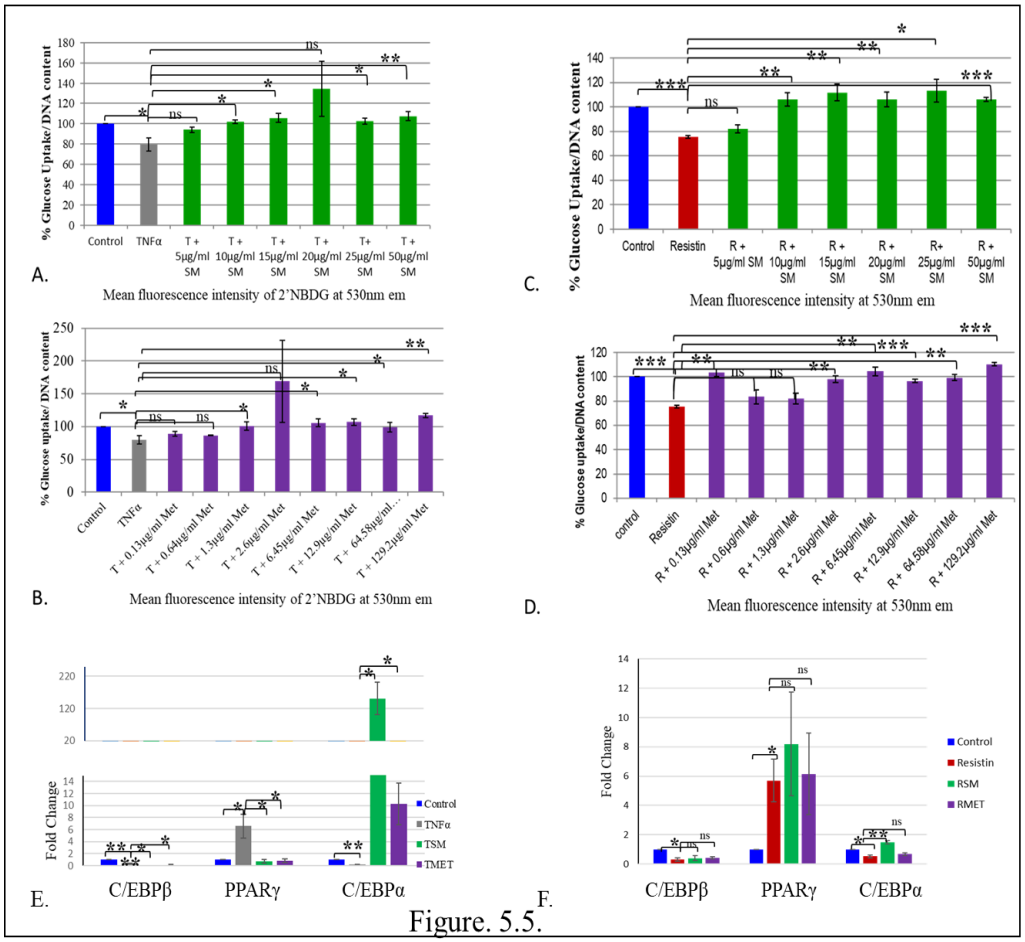

Figure. 5.5.

Figure 5. 5: SM ameliorated TNFα and resistin mediated dysregulated metabolic profile of hADSC. A. Dose dependent effects of SM on insulin mediated glucose uptake in TNFα treated hADSC depicted by mean fluorescence intensity of 2'NBDG-FITC± S.E.M. B. Dose dependent effects of Met on insulin mediated glucose uptake in TNFα treated hADSC C. Dose dependent effects of SM on insulin mediated glucose uptake in resistin treated hADSC. D. Dose dependent effects of Met on insulin mediated glucose uptake in resistin treated hADSC n=3, p Value-*<0.05, **<0.01, ***<0.0001, ns-non significant. E. Expressions of adipogenic genes in TNFα, SM and Met treated cells expressed as Fold Change ± S.E.M. with β actin as internal control. F. Expressions of adipogenic genes in resistin, SM and Met treated cells expressed as Fold Change ± S.E.M. with β actin as internal control. n=3, p Value- *<0.05, **<0.01, ***<0.0001, ns- non-significant.

5.3.5. Assessment of SM on differentiation potentials of hADSC under the influence of TNFα and resistin.

hADSC can differentiate efficiently into adipocytes, osteocytes, chondrocytes, myocytes, islets, neurons etc. among them the former two are main cell types in which the cells differentiate. As we monitored efficacy of SM and Met on stemness and metabolism of TNFα and resistin treated hADSC, it was inevitable to examine the modulatory roles of these adipokines and insulin sensitizers on adipogenic and

osteogenic potentials of hADSC. (Figure 5.6. A and B). denote anti-adipogenic activity of SM and Met on hADSC and (Figure 5.6. C and D.) denote anti-osteogenic activity of SM and Met on hADSC. hADSC were treated with TNFα and resistin independently for 48H to induce insulin resistance. SM and Met were treated to TNFα and resistin treated cells undergoing adipogenesis and osteogenesis.

The cells were allowed to differentiate till 22ndD and adipocytes were phenotypically confirmed by ORO staining. AC demonstrated lipid laden adipocytes. ATNFα reduced adipocyte formation compared to AC as evident by ORO. ATSM and ATMet group demonstrated elevated adipogenesis compared to ATNFα group. These results demonstrate that TNFα diminishes adipogenesis while SM and Met rendered protection to adipocytes against deleterious effects of TNFα (Figure 5.6. E. and F.).

Secondly SM and Met were then monitored for adipogenesis and osteogenesis in resistin treated cells. hADSC subjected to adipogenesis in presence of resistin embarked marked elevation in adipogenesis with formation of huge lipid laden hypertrophic adipocytes. Adipogenesis was augmented in resistin treated cell since its induction. ARSM and ARMet significantly downregulated adipogenesis compared to resistin group, respectively (Figure 5.6. G. and H.).

OC demonstrated calcium forming osteocytes as observed by Alizarin Red staining. TNFα reduced osteogenesis and SM, Met also depicted reduced osteogenesis compared to OTNFα group (Figure 5.6. I. and J.). hADSC when subjected to osteogenesis in presence of resistin, it enhanced osteogenesis compared to OC but SM and Met reduced osteocyte formation compared to resistin group (Figure 5.6. K. and L.). These results depict that resistin predominantly enhanced the adipogenic and osteogenic differentiation potentials which led to the formation of hypertrophic adipocytes and over produced osteocytes and these effects of resistin were reversed by SM and Met.

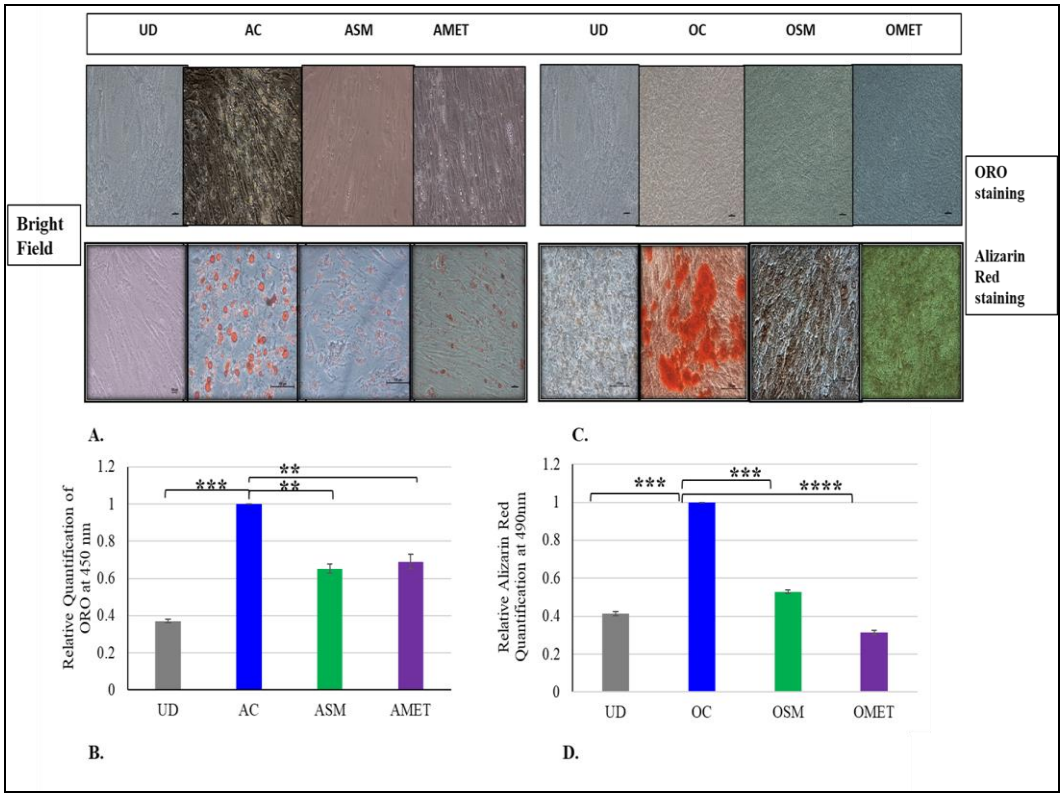

Figure 5. 6: Assessment of SM on differentiation potentials of hADSC under the influence of TNFα and resistin A. Effects of SM and Met on adipogenesis, bright field and ORO staining. B. Graph representing ORO quantification C. Effects of SM and Met on osteogenesis, bright field and Alizarin red staining. D. Graph representing relative Alizarin red quantification. n=3, p value, *<0.05, **<0.01, ***<0.0001, ns-non significant.

The groups under the study were UD, A adiogenic control C, ARes- only resistin treatment, ARSM- SM and resistin treatment during adipogenesis, ARMet- Met and resistin during adipogenesis.

UD, OC-osteogenic control, OTNFα- TNFα treatment during osteogenesis, OTSM- SM and TNFα treatment during osteogenesis, OTMet- Met and TNF, The groups involved in the study were UD, OC, ORes- Resistin treatment during osteogenesis, ORSM- SM and resistin treatment during osteogenesis, ORMet- Met and resistin.

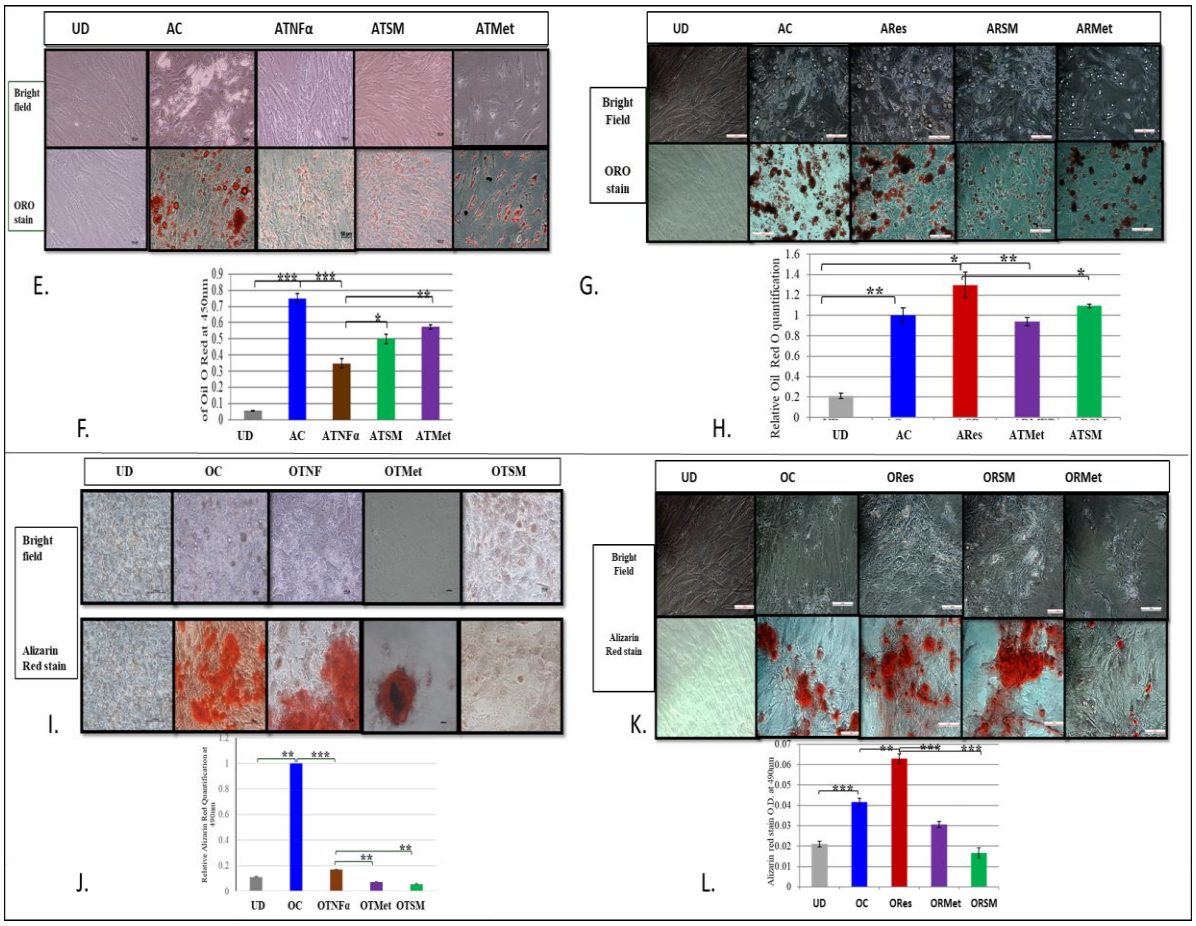

Figure 5.6 : Assessment of SM on differentiation potentials of hADSC under the influence of TNFα and resistin E. Effects of SM and Met on TNFα mediated adipogenesis in hADSC, bright field and ORO staining. F. Graph representing ORO quantification G. Effects of SM and Met on resistin mediated adipogenesis, bright field and ORO staining. H. Graph representing relative ORO quantification. I. Effects of SM and Met on TNFα mediated osteogenesis in hADSC, bright field and alizarin red staining. J. Graph representing relative alizarin red stain quantification. K. Effects of SM and Met on Resistin mediated osteogenesis in hADSC bright field and alizarin red staining. L. Graph representing relative alizarin red stain quantification, n=3, p value, *<0.05, **<0.01, ***<0.0001, ns-non significant

5.4. DISCUSSION

Obesity is a multifaceted clinical manifestation with altered adipokine secretion, increased inflammation, insulin resistance leading to expansion of adipose tissue, excessive of lipid deposition and dyslipidemia (Indulekha et al., 2015). Macrophage infiltration into adipose tissue is the major phenomena that leads to secretion of more pro-inflammatory adipokines like resistin, TNFα, IL-1β etc. with reduced adiponectin and IL-10 levels. It is well established that TNFα and resistin are profoundly found in circulation and dysregulates metabolism in adipocytes, but less is known about their

actions on hADSC (Indulekha et al., 2015). Thus, to extend our knowledge our prime focus in this chapter was to understand the effects of TNFα and resistin on insulin sensitivity and proliferation of hADSC. Further, their effects have been explored on cell cycle, inflammatory mediators and downstream insulin signaling pathway and potentials of SM, a secoiridoid a known insulin sensitizer have been monitored.

TNFα diminished cell cycle of hADSC and provoked them towards apoptosis after 96H of treatment. Reports demonstrated that TNFα treated 0.1ng/ml to 10ng/ml concentration also did not affect proliferation of bone marrow stem cells (Daniele et al., 2017). Moreover, TNFα demonstrate variable effects on proliferation which is time and concentration dependent (Qin et al., 2015). SM reduced proliferation of hADSC. Hairul-Islam et al, reported that SM enhanced proliferation of calvarial osteoblast obtained from neonatal mice (Hairul-Islam et al., 2017). In the present study, Met had protected some TNFα treated cells. In line of these, there are several reports which state that Met enhances proliferation of periodontal ligament stem cells into osteoblast and prevented from apoptosis (Zhang et al, 2019). However, both SM and Met inhibits proliferation of cancer cells and induce apoptosis in them (D. Chen et al., 2018; J. Lee et al., 2019; Saravanan et al., 2014).

In the present study, resistin after 96H rendered a profound impact on proliferation of hADSC as more than 90% cells underwent apoptosis compared to 75% in TNFα treated group. These results demonstrate that resistin has a strong deleterious effects on hADSC. SM and Met both rescued cells from entering into apoptosis with remarkably better effect of Met.

As both TNFα and resistin embark inflammatory response, inflammation was monitored. Both the adipokines induced expressions of inflammatory mediators MCP-1, IKKβ and NLRP3 and hence enhanced inflammation in hADSC. These results are supported by the reports stating that both resistin and TNFα induce inflammation in adipocytes, adipose tissue and enhance infiltration of macrophages that leads to chronic inflammation (Tzanavari et al., 2010). SM and Met significantly downregulated inflammation as they targeted IKKβ which is the major regulator of inflammation. Both SM and Met did not have any effect on major inflammasome complex protein expressions in TNFα group but they demonstrated severe reduction in NLRP3 in resistin treated hADSC, thus rendering protection to the resistin treated

cells. These results are supported by the evidence that SM and Met render anti-inflammatory potentials (Cameron et al., 2016; Saravanan et al., 2014).

Inflammation hampers stemness and metabolic profile of hADSC. TNFα and resistin profoundly elevated OCT4 with reduced NANOG levels, however, the former downregulated SOX2 levels while latter upregulated it. These results signify that both TNFα and resistin hampered stemness and self-renewal of hADSC which are governed by NANOG. Elevated levels of OCT4 demonstrated propensity towards differentiation which was very profound in resistin treated cells as SOX2 was additionally activated by resistin. In line of these results, supporting evidence suggest that NANOG plays an important role in self-renewal and maintenance of stem cells, while OCT4 and SOX2 provoke stem cells towards trilineage differentiation (Han et al., 2014; Pitrone et al., 2017; Z. Wang et al., 2012). SM reversed the effects of TNFα and resistin mediated alterations in pluripotency of hADSC. There are no reports on SM with respect to stemness. However, report described that Met downregulated NANOG and restricted proliferation of cancer stem cells to inhibit cancer progression (He et al., 2018).

In the present study for the first time, we made an attempt to create insulin resistant hADSC with TNFα and resistin treatment independently and further studied downstream signalling pathway. Glucose uptake an important insulin dependent effect was reduced in both the groups. These results were supported by earlier reports which suggested that inhibition of insulin mediated glucose uptake leads to dyslipidemia (K.-H. Kim et al., 2004; Olefsky et al., 2010; Petersen & Shulman, 2018). SM and Met ameliorated glucose uptake in TNFα and resistin induced insulin resistance proving their insulin sensitizing abilities in hADSC. Earlier studies from our lab with 3T3 L1 and HepG2 cells also demonstrated SM as potent insulin sensitizer (Tushar Patel, Ph.D. Thesis, 2014, Patel et al, 2016).

Resistin mediated insulin resistance in hADSC, enhances a plausibility of dysregulated adipose tissue metabolism and dyslipidemia. Adipogenic genes of hADSC under the influence of pro-inflammatory adipokines, resistin and insulin sensitizers were monitored. Both TNFα and resistin upregulated PPARγ2 expressions with concomitant reduction in C/EBPβ and C/EBPα levels. Studies supporting the result state that insulin resistance diminishes C/EBPα levels in adipocytes (Matulewicz et al., 2017).These results determine that these adipokines hampered metabolic

functions and rendered insulin resistance in hADSC (Ikeda et al., 2013; Isakson et al., 2009). SM and Met significantly ameliorated dysregulated adipogenic genes and hence restoring metabolic profile and insulin sensitivity of hADSC. Our earlier reports suggested that SM targets lipogenic genes and rectified metabolism of both adipose tissue and adipocytes obtained from 3T3L1 cells (Patel et al, 2013, Tushar Patel, Ph.D. Thesis, 2014).

Serena et al, reported that hADSC of obese Caucasians have high inflammatory mediators which alter their differentiation fate (Serena et al., 2016). We observed the same phenomena in obese Indians with high inflammatory markers and altered differentiation potentials as mentioned in chapter 4. Therefore, the study was extended to understand the effects of SM and Met on differentiation potentials of hADSC under the influence of TNFα and resistin. TNFα downregulated adipogenesis and osteogenesis. These results comply with the reports which state that TNFα inhibited adipogenesis and osteogenesis (Cawthorn et al., 2008; L. Wang et al., 2018). As per our observation TNFα downregulated SOX2 which also denotes diminished differentiation potentials of hADSC. In contrast, resistin formed hypertrophic adipocytes with huge lipid droplets. This results could be justified by the notion that resistin embarks adipogenesis through ROR upregulation (Sánchez-Solana et al., 2012). Also it complies with its effect on pluripotent genes as resistin upregulated OCT4 and SOX2 which propagate stem cells towards differentiation. Resistin also over produced osteocytes. SM rectified adipogenic differentiation mitigated by TNFα. Thus, these results provide an evidence of intense insulin sensitizer effect of SM as it ameliorated TNFα mediated downregulation of adipogenesis supported by anti-adipogenic activity from our earlier work (Tushar Patel, Ph.D. thesis, 2014). SM and Met reduced resistin mediated elevation of adipocyte and osteocyte differentiation of hADSC, thus normalizing the corollary of resistin. Met at similar concentration induced osteogenesis in rat bone marrow stem cells (Smieszek et al., 2018). Chen et al, described that Met alters adipogenic differentiation potentials in dose dependent manner in 3T3-L1 cells (D. Chen et al., 2018).

In the present study it was observed that both TNFα and resistin showed similar effects on insulin mediated signaling pathways however, differential effects were observed in maintenance of stemness and differentiation. These results are supported by the facts that TNFα inhibits adipogenesis, induces inflammation which

phosphorylate serine of IRS-1 and downregulates downstream insulin signaling pathway while resistin provokes adipogenesis but inhibits adipocyte maturation, which also leads to insulin resistance through inflammation (Cawthorn & Sethi, 2008; Ikeda et al., 2013). It can be presumed that TNFα levels might be more in metabolically obese non-obese phenotype and resistin could be predominant in both in metabolically healthy but obese and metabolically obese obese phenotypes.

5.5. CONCLUSION

TNFα and resistin, the proinflammatory adipokines instilled inflammation and insulin resistance in hADSC. They altered pluripotency and fate of hADSC. This is the first report to our knowledge which demonstrate resistin as a profound proinflammatory adipokine which diminished stemness of hADSC by inducing insulin resistance and altering plasticity of hADSC. Also effects of TNFα and resistin were studied for the first time on pluripotent genes of hADSC. The study resembles the effects of resistin on hADSC of obese individuals and may be the link between resistin mediated precarious stemness of hADSC and adipose tissue dysregulation and degenerative diseases due to dysregulated osteocytes *in vivo*. SM effectively restored stemness and insulin sensitivity both in undifferentiated and differentiated hADSC into adipocytes and osteocytes. This study opens avenue to explore SM as potential target for metabolic alterations, anti-obesity and anti-diabetic treatment. It can also be used *in vitro* for increasing efficacy of compromised hADSC, which then can be used for beneficial regenerative therapy.

SUMMARY OF CHAPTER 5: