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TROXERUTIN IMPROVES INSULIN SIGNALING IN TNFα TREATED 3T3-L1 ADIPOCYTES

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Keywords:

Insulin signaling, Insulin resistance, Troxerutin, TNFα, Caveolin, 3T3-L1 cells

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ABSTRACT: Chronic low-grade inflammation of adipose tissue is associated with obesity and insulin resistance (IR). The aim of this study is to determine the effect of troxerutin (TX) on insulin signaling in tumor necrosis factor α (TNFα) treated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were pre-treated with TX, elastatinal (ELAS) or sodium salicylate (SAL) before exposure to recombinant human TNFα (20 ng/mL) for 24 h. Glucose uptake was measured by 2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG) assay. The protein expression of insulin receptor-β (IR-β), insulin receptor substrate-1 (IRS-1), phosphatidyl inositol 3 kinase (PI3K) association with IRS-1, protein kinase B (Akt) and glucose transporter 4 (GLUT4) were studied. The mRNA and protein expression of caveolin-1 and suppressor of cytokine signaling 3 (SOCS3) were also analyzed. TNFα treated 3T3-L1 adipocytes displayed decreased tyrosine phosphorylation of IR-β and IRS-1. Further, the association of p85 subunit of PI3K with IRS-1, subsequent Akt phosphorylation and GLUT4 activation were also reduced. TX pre-treatment enhanced tyrosine phosphorylation of IR-β, IRS-1, IRS1-PI3K association and Akt and GLUT4 expression. These changes were higher as compared to ELAS and SAL. The expression of SOCS3 was downregulated, while that of caveolin-1 was upregulated by TX. These findings suggest that TX activates the IRS-PI3K-Akt pathway of insulin signaling, enhances caveolin-1 expression, suppresses SOCS3, promotes glucose uptake and improves insulin sensitivity in TNFα treated 3T3-L1 cells. Thus, TX could be a potential therapeutic agent for the management of IR.

INTRODUCTION: Insulin is a major anabolic hormone whose action plays a pivotal role in the maintenance of glucose homeostasis. The inability of target cells to respond to circulating levels of insulin results in a state of insulin resistance (IR) which is the underlying factor in the development of type 2 diabetes (T2D) [1]. Globally, the prevalence of T2D has increased dramatically and now reached epidemic levels [2].

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Insulin binding to the receptor activates the intrinsic tyrosine kinase activity of the receptor's β-subunit. This leads to autophosphorylation of the receptor and tyrosine phosphorylation of insulin receptor substrates (IRS) 1 to 4. These substrates, in turn interact with phosphatidylinositol 3-kinase (PI3K) and activate protein kinase B (Akt), a downstream ser/thr kinase. Subsequent activation of Akt leads to stimulation of glucose transporter 4 (GLUT4) translocation and glucose uptake.

Aberrations in any of the above events lead to IR. Serine phosphorylation of IRS-1 blocks tyrosine phosphorylation by causing either dissociation of IR and IRS-1, proteasome-mediated degradation of IRS proteins or dissociation of the p85 subunit of PI3K.

Increased activity of phosphatase-mediated dephosphorylation (phosphotyrosine phosphatase 1B) could decrease the activation of downstream signaling molecules like Akt.

Suppressors of cytokine signaling (SOCS) proteins are members of the SOCS protein family which exert negative effects on cytokine signaling. A member of this family, SOCS3 is a negative mediator of insulin signaling. SOCS3 can bind to insulin receptors, impair the phosphorylation of the IRS-1 and -2, and downregulate insulin signaling. Studies have shown that higher expression of SOCS3 aggravates IR [3-5].

Caveolae are a specialized type of lipid rafts that appear in the plasma membrane and are stabilized by oligomers of caveolin protein. Caveolin-1, the main structural and functional protein of caveolae has emerged as a key mediator of insulin transduction pathway [6]. Caveolin-1 interacts with the insulin receptor and enhances insulin-mediated phosphorylation of IRS-1 and the subsequent translocation of GLUT4 to the plasma membrane. A role for caveolin-1 in insulin signaling and its potential involvement in diabetes has been proposed [7]. Caveolae are particularly enriched in adipocytes where caveolin-1 isoform is responsible for caveolae formation [8]. Several genetic and environmental factors have been documented to promote IR through downregulation of IRS-PI3K-Akt pathway [9-12]. Among them, TNFα has received special attention owing to its inflammatory actions. 3T3-L1 cells are widely used to study IR in adipocytes [13].

TX, a trihydroxyethylated derivative of the natural bioflavonoid rutin possesses potent vasoprotective, antioxidative, anti-inflammatory, and anti-hyper-lipidemic activities [14, 15]. Our recent study has demonstrated that TX lowers lipid accumulation and regulates lipid metabolism, oxidative stress and suppresses inflammation in TNFα induced 3T3-L1 cells [16]. However, the possible mechanisms underlying the insulin sensitivity effects of TX in this model have not been explored.

The present study used TNFα, an IR-associated inflammatory cytokine to induce IR in 3T3-L1 cells and tested the insulin sensitivity effect of TX on insulin signal proteins and the expression of caveolin-1 and SOCS3 in cells. Elastatinal (ELAS), an elastase inhibitor [17] and sodium salicylate (SAL), a non-steroidal anti-inflammatory drug [18] were included in the study for comparison.

MATERIALS:

Chemicals, Antibodies, Primers and Kits: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and penicillin-streptomycin antibiotics were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. TX, insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT), 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]- 2-deoxyglucose (2-NBDG) and primer sets were purchased from Sigma – Aldrich, St. Louis, MO, USA. SYBR green master mix was purchased from Kapabio systems, Wilmington, MA, USA. Anti-Akt (Catalog #4685), anti-phospho Akt (Catalog #5106), anti-caveolin-1 (Catalog #3267T) and horse radish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse, anti-rat, anti-rabbit and anti-goat) were from Cell Signaling Technologies, Danvers, MA, USA. Anti-phospho IRS-1 (Tyr 632) (sc-17196), IRS-1 (sc-7200), anti-PI3-kinase p85α (3H2838), anti-IR-β (sc-57342) and anti-phospho IR-β (sc-81500) and anti-glucose transporter4 (GLUT4) (sc-53566) were obtained from Santa Cruz Biotech, Santa Cruz, CA, USA. Anti-SOCS3 (MAB5696-SP), anti-β-actin (MAB8929) antibodies and recombinant human insulin were procured from R&D Systems, Minneapolis, MN, USA. Immobilon polyvinylidene fluoride (PVDF) membrane from Millipore, Billerica, MA, USA, enhanced chemiluminescence Super Signal West Pico Chemiluminescent Substrate from Thermo Scientific, Rockford, IL, USA, and M-MuLV-reverse transcriptase from Thermo Scientific, Pittsburgh, PA, USA was used in the study. cDNA conversion kit, TRIzol reagent, and oligodT primers were purchased from GeNei, Bangalore, India. Fine chemicals and reagents were acquired from Himedia Laboratories Pvt. Ltd., Mumbai, India and Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

3T3-L1 Cell Culture and Differentiation: 3T3-L1 cells were obtained from the National Centre for Cell Science (NCCS), Pune, India and cultured following the procedure of Drira and Sakamoto *et al*., 2011 [19] with some modifications. 3T3-L1 preadipocytes were cultured in high-glucose DMEM

supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C under a 5% CO_2 atmosphere. Confluent 3T3-L1 cells were maintained in culture medium for 2 days to induce differentiation. Cells were exposed to differentiation medium 2 (DMEM containing 1.7 µmol/L insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 µmol/L dexamethasone, 0.2 mmol/L indomethacin (for promoting adipogenesis) and 10% FBS for 3 days. The medium was then changed with differentiation medium 1 (DMEM containing 10 µg/ mL insulin and 10% FBS) for an additional 3 days. Subsequently, the cells were maintained in DMEM containing 10% FBS for 2 more days to achieve mature adipocyte morphology. Matured adipocytes were used for further experiments.

Cell Viability: The cell viability of adipocytes in presence of TX, ELAS and SAL was assessed by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay [16].

Treatment of Cells: Matured 3T3-L1 cells were preincubated with 8 µM TX, 12.5 µM ELAS, 5 mM SAL for 1 h and then with 20 ng/mL recombinant human TNFα for 24 h.

Immunoprecipitation: Tyr phosphorylation of IR-β, tyr phosphorylation of IRS-1 and IRS-1 association with PI3K were assessed by immunoprecipitation and immunoblotting. For this, the cells were treated with 10 nM insulin for 30 min prior to assay. Samples containing an equal amount of protein were subjected to immunoprecipitation at 4 °C using antibodies specific to anti-IR-β and anti-IRS-1. The immune complexes were precipitated by the addition of 50 µL protein-A agarose slurry to each sample and then incubated at 4 °C for 2 h. Immune complexes were pelleted at 4000 × g for 10 min at 4 °C and washed thrice with homogenization buffer. The pellets were suspended in Laemmli sample buffer and boiled for 5 min. Proteins were resolved by 8-10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto PVDF membranes. The membranes were then blocked in Tris-buffered saline -Tween 20 (TBST) containing 3% bovine serum albumin (pH 7.4) for 2 h at room temperature. Proteins were immunoblotted with anti-phospho tyr antibody (for IR-β and IRS-1) and PI3K antibody for IRS-1. The membranes were washed with TBST and incubated with their respective secondary antibodies for 2 h at room temperature. Blots were subsequently stripped and reprobed with the same antibody used for immunoprecipitation. Protein band detection was performed by enhanced chemiluminescence assay. The band densities of phosphorylated proteins were normalized with the band density of corresponding total protein. For PI3K-IRS1 association, normalisation was done with the band density of IRS1 protein. Quantitative comparison of protein levels between various groups were performed using Image J software (NIH, Bethesda, MD, USA).

Quantitative Real-Time RT-PCR (qRT-PCR) for mRNA Expression Analysis: The cells were washed with ice-cold PBS. RNA was extracted using TRIzol reagent. The concentration of isolated RNA was determined spectrophotometrically at 260 nm (Biophotometer plus, Eppendorf, Hamburg, Germany) and the purity of RNA preparation was checked by calculating the absorbance ratio at 260/280 nm. The extracted RNA (2 µg) was reverse transcribed by the standardized procedure and the cDNA obtained was quantified (Biophotometer Plus, Eppendorf, Hamburg, Germany). After quantification, qRT-PCR amplification was performed in a 20 µL reaction mixture containing cDNA (100 µg), 1 µL each of 0.3 µM of reverse and forward primers, 10 µL Maxima SYBR Green qPCR Master Mix and DEPC water. The nucleotide sequences of primer sets used are provided in Table 1. PCR program was conducted using real-time PCR system Mastercycler realplex (Eppendorf, Hamburg, Germany) in universal cycling conditions (10 min at 95 °C, 40 cycles of 2 min at 95 °C, 30 s at 60 °C, and 20 s at 72 °C). The transcription levels of all the genes were normalized with that of an endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by $2^{-\Delta\Delta CT}$ method [20]. The relative quantity was expressed as fold change with respect to the control group.

TABLE 1: PRIMER SEQUENCE SETS USED FOR qRT-PCR

S. no.	Gene name	Accession number	Forward primer 5'<-sequence->3'	Reverse primer 5'<-sequence->3'	Product size
1	Caveolin-1	M32599.1	GTGTTCCTACCCCCAATGTG	AGGAGACAACCTGGTCCTCA	150
2	SOCS3	NM_007707.3	CCTTTGACAAGCGGACTCTC	GCCAGCATAAAAACCCTTCA	150

Western Blot Analysis: 3T3-L1 cells were washed thrice with PBS and lysed with cold extraction buffer containing 20mM Tris-HCl at pH 7.4, 150 mM sodium chloride, 1mM EDTA, 0.5% Triton X-100, 0.1% SDS, one mM PMSF (phenyl methosulphonyl fluoride) and 10µL protease inhibitor cocktail and centrifuged at 1500 × g for 10 min at 4 °C. The supernatant obtained was again centrifuged at 8000 × g for 15 min at 4°C. The resulting supernatant was retained as the total cell lysate. The protein content of the cell lysate was quantified using the method of Lowry et al., 1951 [21]. Cell lysate containing an equal amount of proteins were resolved by 8-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were electro-transferred onto PVDF membrane and then blocked with Tris buffered saline Tween 20 (TBS-T) solution, pH 7.4 containing 3% BSA for 2 h at room temperature. The membranes were rinsed with TBS-T and then incubated overnight at 4 °C with primary antibodies against IR-β, IRS-1, PI3K, Akt, GLUT4, caveolin-1 or SOCS3 in TBS-T buffer. After incubation, blots were washed with TBS-T and incubated with either HRP-conjugated anti-rat, anti-goat, anti-rabbit or anti-mouse secondary antibody for 2 h at room temperature. Protein bands were visualized by enhanced chemiluminescence (ECL) detection system. Densitometry was performed and quantitated using Image J software (NIH, Bethesda, MD, USA).

Glucose Uptake Assay: Glucose uptake was analyzed using 2-NBDG. 3T3-L1 cells were seeded in 96 well plates and incubated in a CO_2 incubator for 24 h. The cells were washed with PBS and then treated for 1 h with TX, ELAS or SAL and then treated with TNFα for 24h. After that the treatment medium was removed and 250 µmol/L of 2-NBDG (dissolved in PBS with 1% BSA) was added to the cells and incubated for further 30 min. After incubation, cells were washed two times with PBS to remove excess fluorescence in the wells. Then, fluorescence retained by the cells was measured using the fluorescence microplate reader, Perkin Elmer Victor3 V 1420 Multilabel Plate Counter (Perkin Elmer, USA) at an excitation and emission wavelength of 460 nm and 540 nm respectively [11].

Statistical Analysis: Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons of groups (SPSS 20.0 Chicago, IL, USA) software. A probability value of less than 0.05 was considered significant for all the cases.

RESULTS:

Effect of TX on 3T3-L1 Adipocytes: To investigate cell viability, assays were performed using MTT and the results were published in our previous report [16]. TX, ELAS and SAL had no significant cytotoxicity up to a concentration of 8 µM, 12.5 µM, and 5 mM, respectively. The concentration of TX, ELAS and SAL were therefore fixed at 8 µM, 12.5 µM and 5 mM, respectively and used for further experiments.

TX Enhances Glucose Uptake: TNFα treated cells showed reduced glucose uptake as compared to untreated cells. As shown in **Fig. 1**, TX pre-treatment significantly enhanced glucose uptake as compared to ELAS and SAL.

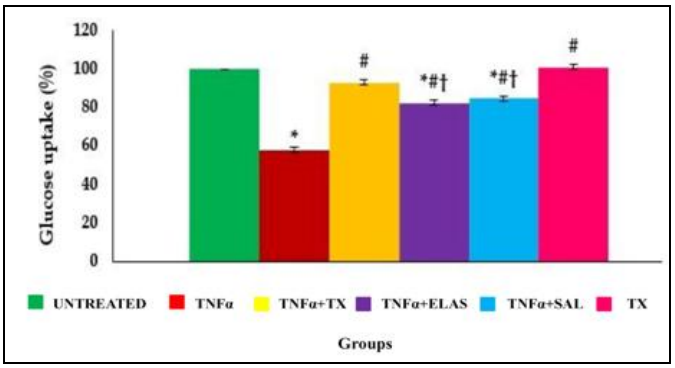

FIG. 1: EFFECT OF TX ON GLUCOSE UPTAKE IN 3T3-L1 ADIPOCYTES. Values are given as means ± SD (n=3). Differences in mean values between groups were evaluated by one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$. * Significant as compared to untreated cells; # Significant as compared to TNFα: † Significant as compared to TNFα + TX

Effect of TX on Insulin Signaling Pathway: Insulin signaling proteins were examined by immunoblotting (IB) and immunoprecipitation (IP) techniques. TNFα treated cells showed reduced insulin-stimulated tyrosine phosphorylation of IR-β and IRS-1 and IRS-1-PI3K association as compared to untreated cells **Fig. 2A** and **2B**. TX pre-treatment significantly improved the tyrosine phosphorylation of IR-β and IRS-1 as compared to ELAS and SAL. Similarly, the IRS-1-PI3K association was improved to a greater extent in TX pre-treated cells than those treated with ELAS or SAL.

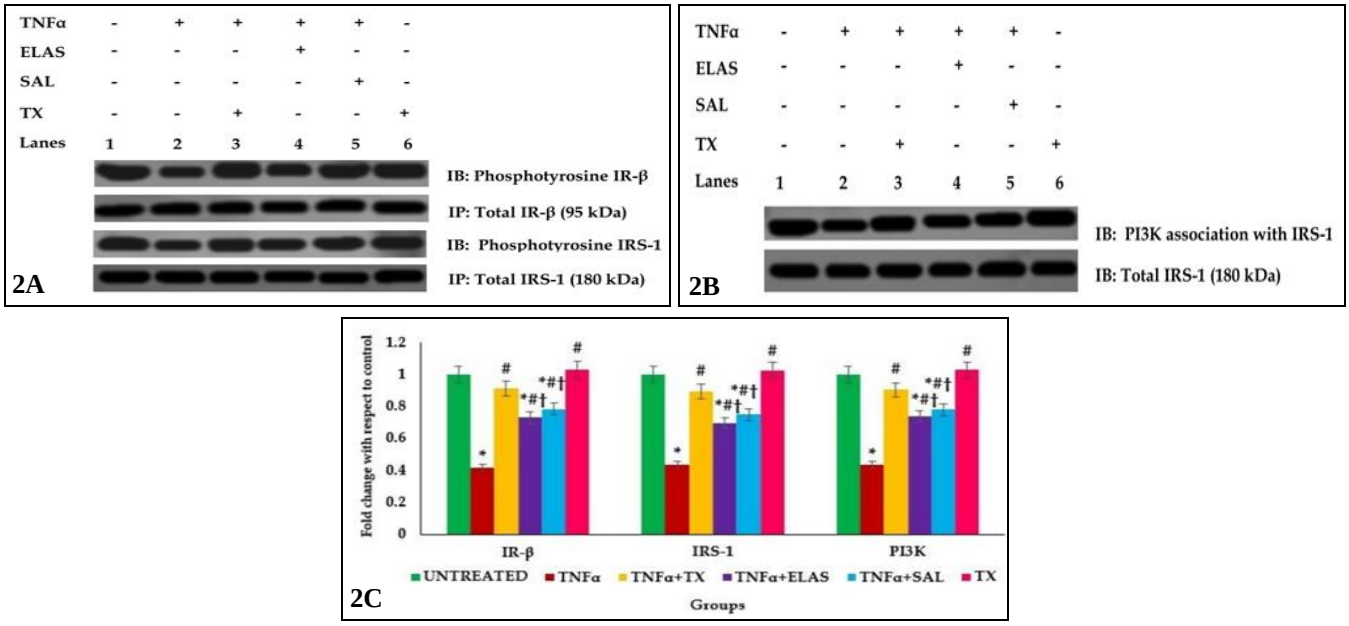

FIG. 2: EFFECT OF TX ON TYROSINE PHOSPHORYLATION OF IR-β AND IRS-1 AND PI3K ASSOCIATION WITH IRS-1 IN 3T3-L1 ADIPOCYTES. Cells were treated with 8 µM TX, 12.5 µM ELAS and 5 mM SAL and then were exposed to TNFα for 24 h. Lane 1- UNTREATED; Lane 2- TNFα; Lane 3- TNFα+TX; Lane 4- TNFα+ELAS; Lane 5- TNFα+SAL; Lane 6- TX. Tyrosine phosphorylation of IR-β, IRS-1 (A), PI3K association with IRS-1 (B) in matured adipocytes were analyzed by Western blotting, quantified by densitometric analysis (C). The fold change was calculated with respect to control after normalization with its total form. Values are given as means ± SD (n=4) and differences between groups were evaluated by one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$. *Significant as compared to untreated cells; #Significant as compared to TNFα; †Significant as compared to TNFα + TX

Downstream Signaling Proteins: Decrease in insulin-activated Akt phosphorylation **Fig. 3A** and GLUT4 abundance **Fig. 3B** was observed in TNFα treated cells as compared to untreated cells. **Fig. 3C** is the densitometry data for Akt and GLUT4, respectively. Pre-treatment with TX significantly refurbished these levels as compared to ELAS and SAL. There were no significant differences between normal cells treated with TX and untreated cells.

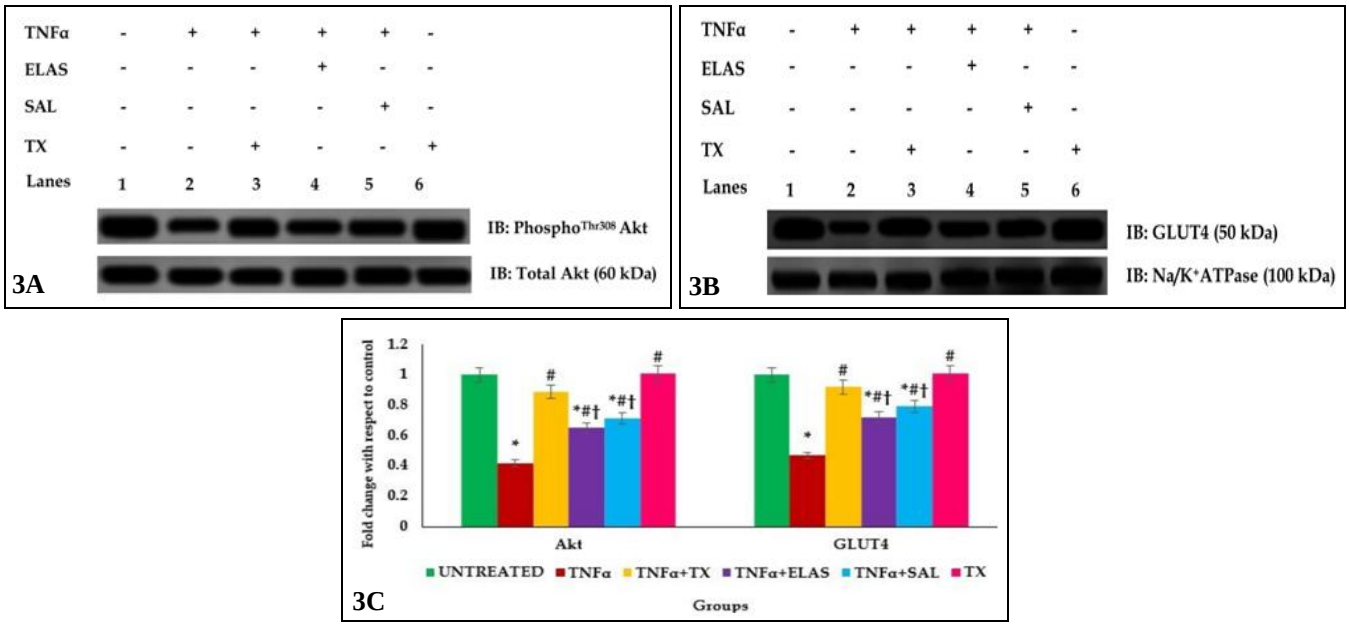

FIG. 3: EFFECT OF TX ON AKT AND GLUT4 ACTIVATION IN 3T3-L1 ADIPOCYTES. Cells were treated with 8 µM TX, 12.5 µM ELAS and 5 mM SAL and then were exposed to TNFα for 24 h. Phosphorylation status of Akt (A) GLUT4 levels (B)) in matured adipocytes were analyzed by Western blotting, quantified by densitometric analysis (C). The fold change was calculated with respect to control after normalization with total Akt and Na/K^+ATPase respectively. Values are given as means ± SD (n=4) and differences between groups were evaluated by one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$. *Significant as compared to untreated cells; #Significant as compared to TNFα; †Significant as compared to TNFα + TX

TX Downregulates Expression of Caveolin-1 in 3T3-L1 Adipocytes: The mRNA expression and protein abundance of caveolin-1, a key regulator of insulin signaling pathway were lower in TNFα treated cells as compared to untreated cells **Fig. 4A** and **4B**. TX pre-treatment increased caveolin-1 expression to a greater extent as compared to ELAS and SAL.

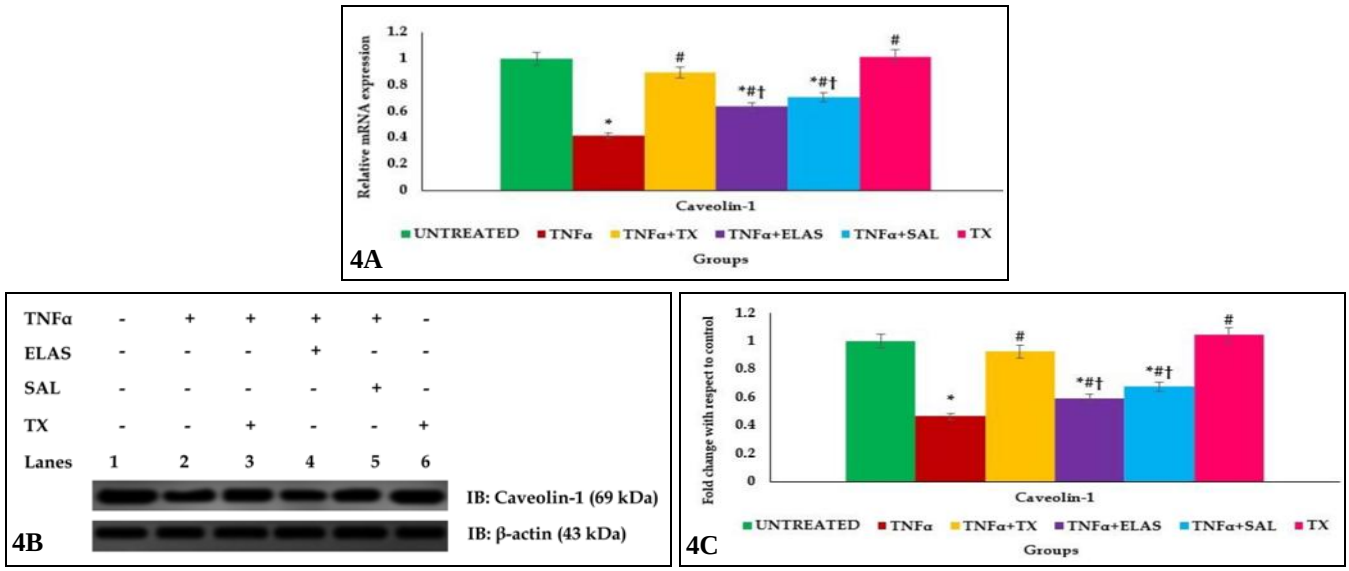

FIG. 4: EFFECT OF TX ON mRNA AND PROTEIN EXPRESSION OF CAVEOLIN-1 IN 3T3-L1 ADIPOCYTES. Cells were treated with 8 µM TX, 12.5 µM ELAS and 5 mM SAL and then were exposed to TNFα for 24 h. Total RNA was isolated and mRNA levels of caveolin-1 was measured by q-RT PCR and normalized with respect to GAPDH expression level (A). The protein levels of caveolin-1 in matured adipocytes were analyzed by Western blotting (B), quantified by densitometric analysis (C). The fold change was calculated with respect to control after normalization with β-actin. Values are given as means ± SD (n=3 for PCR, n=4 for blots) and differences between groups were evaluated by one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$. *Significant as compared to untreated cells; #Significant as compared to TNFα; †Significant as compared to TNFα + TX

TX Suppresses SOCS3 in 3T3-L1 Adipocytes: The mRNA and protein expression of SOCS3 was significantly increased in TNFα treated cells while cells pre-treated with TX, ELAS and SAL showed decreased expression **Fig. 5A** and **5B**. Among them, TX had profound negative effect on SOCS3. Treatment of cells with TX alone did not affect the mRNA or protein expression.

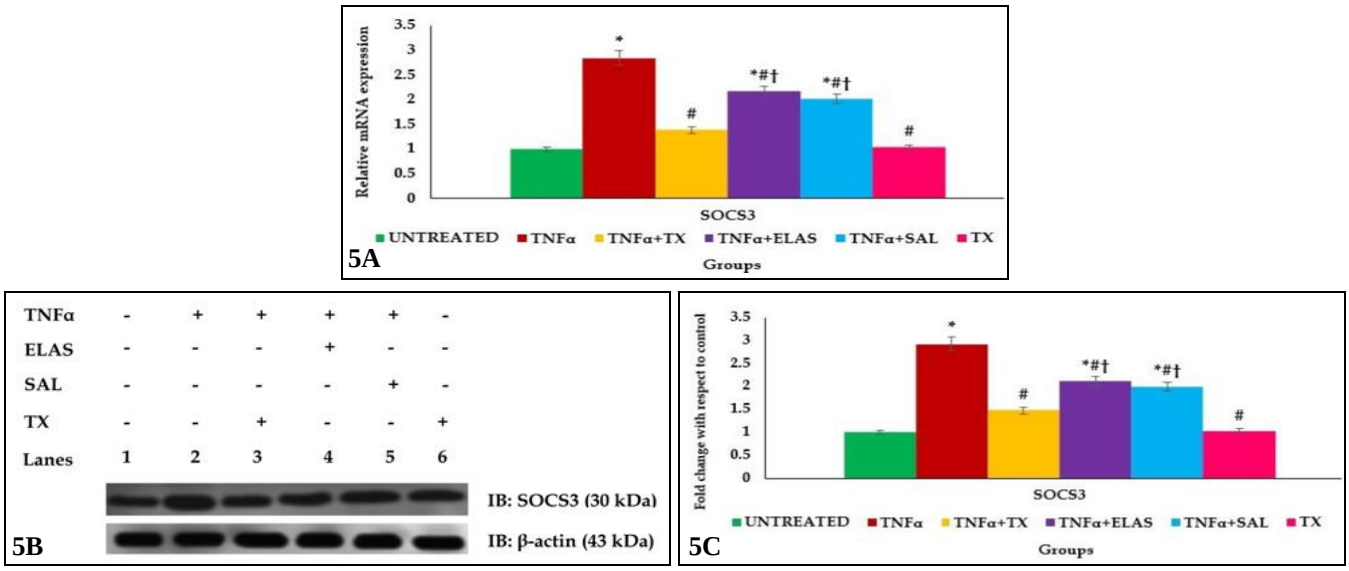

FIG. 5: EFFECT OF TX ON mRNA EXPRESSION AND PROTEIN ABUNDANCE OF SOCS3 IN 3T3-L1 ADIPOCYTES. Cells were treated with 8 µM TX, 12.5 µM ELAS and 5 mM SAL and then were exposed to TNFα for 24 h. Total RNA was isolated and mRNA levels of SOCS3 was measured by q-RT PCR and normalized with respect to GAPDH expression level (A). The protein levels of SOCS3 in matured adipocytes were analyzed by Western blotting (B), quantified by densitometric analysis (C). The fold change was calculated with respect to control after normalization with β-actin. Values are given as means ± SD (n=3 for PCR, n=4 for blots) and differences between groups were evaluated by one-way ANOVA followed by Tukey's post hoc test, $P < 0.05$. *Significant as compared to untreated cells; #Significant as compared to TNFα; †Significant as compared to TNFα + TX

DISCUSSION: Adipose tissue is the primary contributor for the development of obesity and IR by secreting a large number of inflammatory cytokines and chemokines which can modulate inflammation, lipid and glucose metabolism [22]. Among the adipokines, TNFα plays a major role in obesity-related IR and inflammation. TNFα is abundant in adipose tissue of obese people with IR and T2D. Mice which lack the expression of TNFα or its receptor exhibit protection against the development of IR. Administration or overexpression of TNFα cause reduced insulin sensitivity in a cell line or animal models [23, 24]. In the present study, TNFα induced IR in 3T3-L1 adipocytes.

TNFα suppresses the tyrosine kinase activity of the insulin receptor in mature adipocytes by inhibiting its autophosphorylation and by preventing the activation of IRS-1 and the downstream effectors. TNFα suppresses many adipocyte-abundant genes that encode proteins essential for insulin responsiveness. Among them, IRS-1 and Akt are essential for insulin signal transduction and GLUT4 is required for insulin-stimulated glucose uptake.

The negative modulators of insulin signaling primarily act by decreasing tyrosine phosphorylation and activating serine phosphorylation of insulin receptor and IRS-1. Phosphorylation of IRS proteins at particular serine residues Ser^{307} and Ser^{612} inhibits their interaction with the insulin receptor which in turn reduces tyrosine phosphorylation of IRS and subsequent activation of PI3K [25]. Interestingly, exposure of TNFα to fat cells has been shown to inhibit insulin signaling by affecting IRS proteins *via* proteasome-mediated degradation, phosphatase mediated dephosphorylation, and Ser phosphorylation of IRS-1 at Ser307 [26, 27].

In addition, many studies report that TNFα activates several serine kinases like ERK, p38MAPKs, JNK and IκB complex and thereby inhibit insulin signalling at the levels of IRS-1 and IRS-2 in 3T3-L1 adipocytes, human primary adipocytes and primary brown adipocytes [26-29]. The Ser307 residue has been identified as the site for TNFα induced phosphorylation of IRS-1 [25-29]. Consistent with these reports, our results show reduced tyrosine phosphorylation of both insulin receptor-β and IRS-1 in TNFα-treated 3T3-L1 adipocytes.

Akt/protein kinase B, a ser/thr kinase, is a down-stream target of insulin signaling that activates glucose uptake through GLUT4. Significant reductions in Akt phosphorylation at Thr308 and GLUT4 after TNFα treatment were observed in the present study. Defects in the phosphorylation of insulin receptor and IRS-1 and reduced IRS-1/PI3K association could contribute to Akt inactivation in TNFα-induced 3T3-L1 adipocytes.

Ando *et al.*, 2015 reported that pre-treatment with TNFα impaired both insulin-stimulated glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes [30]. A study demonstrated that 24 h exposure of 3T3-L1 adipocytes to TNFα resulted in reduced protein levels of GLUT4 and several insulin signaling proteins, including the IR-β, IRS-1, and Akt [31]. Insulin-stimulated IRS-1 association with phosphatidylinositol 4-phosphate 5-kinase 1α enhances GLUT4 translocation in 3T3-L1 cells [32]. Reduced levels of GLUT4 indicate diminished glucose uptake.

A number of plant compounds like quercetin, isoorientin and luteolin have been shown to induce glucose uptake in adipocytes and to improve insulin sensitivity in other cell lineages [33, 34, 35]. The activation of the IRS-1-PI3K-Akt pathway by TX leads to efficient glucose uptake. TX-treated cells showed improved tyrosine phosphorylation of IR-β, IRS-1 and IRS-1/PI3K association. TX also enhanced Akt phosphorylation and restored GLUT4, thereby facilitated glucose uptake. The favorable action of TX on insulin signal transduction can be accredited to its lipid-lowering, antioxidant and anti-inflammatory activities [16]. These properties of TX may downregulate the serine kinases like JNK, ERK and p38 MAPK. Our data substantiate the reports of other investigators who found that TX improves insulin signaling in the hippocampus of mice-fed high cholesterol diet [36] and in gastrocnemius muscle of high fat plus sucrose-induced T2D adult male rat [37].

Caveolin-1 has been proposed to be a key mediator of the insulin transduction pathway directly interacting with the insulin receptor-β and is important for the integrity of GLUT4. Further, caveolin-1 is necessary for the maintenance of an active and insulin-sensitive glucose uptake. Caveolin-1 knockout mice show IR and defective

receptor protein expression in adipose tissue [38]. In mature adipocytes, depletion of caveolin-1 causes insulin receptor and GLUT4 degradation [39]. Ortega *et al*., 2015 documented that TNFα induction downregulated the expression of the insulin signaling intermediates in 3T3-L1 adipocytes, totally blocking insulin-mediated glucose uptake and this is correlated with the reduced expression caveolin-1 [40]. Consistent with this data, the current study observed reduced expression of caveolin-1 in TNFα induced 3T3-L1 cells. The increased expression of caveolin-1 by TX, is indicative of GLUT4 and receptor protein stability, which then aids glucose uptake and insulin sensitivity.

SOCS3 is known to be a negative regulator of insulin signaling by activating serine phosphorylation of IRS. Also, SOCS3 can increase the ubiquitination of IRS-1 leading to its degradation. The degradation of IRS-1 by TNFα is found to be enhanced with increased expression of SOCS3 in 3T3-L1 cells [41]. In SOCS3 deficient adipocyte model, glucose uptake is increased *via* the phosphorylation of IRS-2 [42]. SOCS3 overexpression impairs leptin-stimulated AMPK activation, reduces tyrosine phosphorylation of IRS-1, PI3K activity, and Akt phosphorylation [8]. Hence SOCS3 is considered to be a target for IR and T2D therapy. The present investigation evidenced increased expression of SOCS3 in TNFα induced 3T3-L1 cells. It can be concluded that TX downregulates SOCS3 in TNFα treated 3T3-L1 cells and thus improves insulin sensitivity. The present study suggests that TNFα induced insulin signaling defects could be modified by TX and adds substantial evidence towards the insulin-sensitizing action of TX.

In our previous study, we reported that TX inhibits elastase activity, reduces mRNA expression and protein abundance of elastase and curtails the inflammatory state induced by TNFα in 3T3-L1 adipocytes. Therefore we used ELAS, an elastase inhibitor [17] and SAL, a non-steroidal anti-inflammatory drug [18] in this study. SAL has been found to prevent (RAW264.7 cells conditioned medium) RAW-CM-induced inflammatory reactions by reversing the inflammation-mediated increase of TNFα, IL-6 and resistin [18] and also useful to control inflammation in metabolic diseases including diabetes [43]. However, clinically SAL has some side effects associated with metabolic disorders [44] and therefore cannot be recommended for human treatment long-term. In conclusion, exposure of 3T3-L1 adipocytes to TNFα leads to impaired insulin signaling, while TX pretreatment improves insulin signaling by augmenting insulin-stimulated IRS-PI3K-Akt path-way. Further TX increases the expression of caveolin-1 and down-regulates SOCS3. The data strongly suggest that TX has potent insulin sensitizing effect in TNFα treated cells. The new findings on the modulatory role of TX on certain proteins of insulin signaling are significant and TX could be recommended as a possible candidate for enhancing insulin sensitivity in T2D patients.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

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