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Research Paper

Effect of styrene exposure on plasma parameters, molecular mechanisms and gene expression in rat model islet cells

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ABSTRACT

Styrene is an aromatic hydrocarbon compound present in the environment and have primary exposure through plastic industry. The current study was designed to evaluate styrene-induced toxicity parameters in rat plasma fasting blood glucose (FBG) level, oral glucose tolerance, insulin secretion, oxidative stress, and inflammatory cytokines in cellular and molecular levels. Styrene was dissolved in corn oil and administered at different doses (250, 500, 1000, 1500, 2000 mg/kg/day and control) to each rat, for 42 days. In treated groups, styrene significantly increased fasting blood glucose, plasma insulin ($p < 0.001$) and glucose tolerance. Glucose tolerance, insulin resistance and hyperglycemia were found to be the main consequences correlating gene expression of islet cells. Styrene caused a significant enhancement of oxidative stress markers ($p < 0.001$) and inflammatory cytokines in a dose and concentration-dependent manner in plasma ($p < 0.001$). Moreover, the activities of caspase-3 and −9 of the islet cells were significantly up-regulated by this compound at 1500 and 2000 mg/kg/day styrene administrated groups ($p < 0.001$). The relative fold change of GLUD1 was downregulated ($p < 0.05$) and upregulated at 1500 and 2000 mg/kg, respectively ($p < 0.01$). The relative fold changes of GLUT2 were down regulated at 250 and 1000 mg/kg and up regulated in 500, 1500 and 2000 mg/kg doses of styrene ($p < 0.01$). The expression level of GCK indicated a significant upregulation at 250 mg/kg and downregulation of relative fold changes in the remaining doses of styrene, except for no change at 2000 mg/kg of styrene for GCK. Targeting genes (GLUD1, GLUT2 and GCK) of the pancreatic islet cells in styrene exposed groups, disrupted gluconeogenesis, glycogenolysis pathways and insulin secretory functions. The present study illustrated that fasting blood glucose, insulin pathway, oxidative balance, inflammatory cytokines, cell viability and responsible genes of glucose metabolism are susceptible to styrene, which consequently lead to other abnormalities in various organs.

1. Introduction

Styrene is one of the volatile aromatic organic compounds (VAOC), used as a solvent in many industrial settings, which is derived from benzene. This compound is the precursor of polystyrene and other copolymers. Styrene is produced in large quantity in the United States, while a small amount is naturally produced by bacteria, fungi, and plants (Braun-Lüllemann et al., 1997; Burback and Perry, 1993;

Abbreviations: FBG, fasting blood glucose; VAOC, volatile aromatic organic compound; ROS, reactive oxygen species; MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin-8; NF-κB, nuclear factor-kappa-B; SO, styrene oxide; h, hours; MAPK, mitogen-activated protein kinase; ERKs-1/2, extracellular signal-regulated kinases-1/2; GSK-3, glycogen synthase kinases-3; MeHg, methyl mercury; NaCl, Sodium chloride; TPTZ, 2,4,6-tripyridyl-S-triazine; TBA, thiobarbituric acid; EDTA, ethylene diamine tetra acetic acid; IL-1β, interleukine-1β; ELISA, enzyme-linked immunosorbent assay; BSA, Bovine serum albumin; DCFH-DA, 2′,7′-Dichlorofluorescin diacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TUMS, Tehran University of Medical Sciences; OGTT, Oral glucose tolerance test; HOMA-IR, homeostasis assessment of insulin resistance; TBARS, thiobarbituric acid reactive substances; DCF-DA, 2′, 7′-dichlorofluorescin diacetate; TAP, total Antioxidant Power; TTM, total thiol molecules; PI, propidium iodide; RT-PCR, real time-reverse transcription polymerase chain reaction; GDH, glutamate dehydrogenase; GLUT2, glucose transporter-2; GCK, glucokinase; GAPDH, glyeraldehyde-3-dehydrogenase; GIT, gastrointestinal tract; IRS-1, insulin receptor substrate-1; NAC, N-acetyl cysteine

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Mooney et al., 2006). It also exists in the automobile drain and cigarette smoke along with plastic and rubber industries (Vainiotalo et al., 2008; Wang et al., 2007). Styrene is found in natural component of dried plants, roasted chicken, meat, beef, eggs, clams, nectarine, and Beaufort cheese (Fleming-Jones and Smith, 2003; Tang et al., 1983). So, the average intake of styrene in household exposure from food and packaging materials is almost 1–35 μg/day. The annual estimated consumption of styrene from food is 8.8–4.5 mg/person from food items, which disrupt the endocrine system and physiology (Tang et al., 2000; Vitrac and Leblanc, 2007).

Styrene exerts their toxic effects *via* dermal, inhalation and oral routes. Even accidental exposure may lead to genotoxicity, endocrine disruption, hematological, immunological, respiratory and carcinogenic effects (Ban et al., 2006; Cruzan et al., 2002; Cruzan et al., 2001; Laffon et al., 2002; Vodicka et al., 2004). There are several evidence showing that the lung epithelial cells are primarily involved in the lethal and inflammation reactions. These cells secrete pro-inflammatory chemokines, which act as immune effectors. The exposure of epithelium and liver hepatocytes to styrene and other VAOCs can be interrelated with the high concentrations of provoking chemokine like monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) along with oxidative stresses (Fischäder et al., 2008; Srivastava et al., 1983). The external stressor/environmental toxicants induce inflammatory responses *via* nuclear factor-kappa-B (NF-κB) signaling pathway, which acts an important function in the toxic effects of VAOCs (Röder-Stolinski et al., 2008). Several genes and various proteins involved in inflammation are upregulated through this pathway. So far, free radicals, reactive oxygen species (ROS) and inflammatory mediators have been thought to be the vital physiological mediators in glucose homeostasis and insulin signaling. It has been reported that hydrogen peroxide and styrene oxide motivate the glucose-stimulated insulin release from pancreatic cells, besides this, they alter molecular mechanisms of insulin receptor and insulin receptor substrate proteins (Mahadev et al., 2001; Pi et al., 2007). However, these alterations are under control of ROS. Sometime, the inappropriate yield of ROS leads to altered structure of macromolecules and successive disruption of biological activities and molecular mechanisms. It has been reported that the exposure of Clara cells to styrene oxide (SO) can significantly increase the activity of caspase-3 after 12, 24 and 48 h (h). Similar studies indicate SO in neuron cells induces caspase-3, which the activation of caspase-3 *via* intrinsic and/or extrinsic pathways play important role as executioner, as it induces apoptosis (Dare et al., 2004; Harvilchuck et al., 2009).

In a reproductive study, it had been revealed that styrene significantly enhanced the levels of lactate dehydrogenase, glucose-6-phosphate dehydrogenase, gamma-glutamyl transpeptidase and β-glucuronidase (Srivastava et al., 1989). This increased/abnormal level of glucose-6-phosphate dehydrogenase gives indication toward diabetes, which was not studied (Pinna et al., 2013). However, the appropriate process through which insulin resistance occurs is not yet known, still the variations in mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases-1/2 (ERKs-1/2) and glycogen synthase kinases-3 (GSK-3) along with certain oxidative stress and inflammatory chemokines acting crucially in insulin sensitivity pathways (Fröjdö et al., 2009; Styskal et al., 2012). It is well established that styrene induces hepatocytes toxicity in the mouse model (Carlson, 2012). Though, intermediate and chronic exposure of styrene in correlation to glucose metabolism, oxidative stress, inflammatory cytokines and molecular pathways of plasma and pancreatic islets is poorly explored. It is well documented that environmental toxicants such as benzene, methyl mercury (MeHg) and arsenic positively influence the diabetes induction and other chronic diseases (Bahadar et al., 2015b; Bahadar et al., 2014; Maqbool et al., 2016). Therefore, the aim of the present experimental study was to evaluate styrene-induced toxicity in rats regarding the plasma FBG level, oral glucose tolerance, insulin secretion, oxidative stress, inflammatory cytokines, and the rate of cell viability in pancreatic islets, along with related gene expression, which could alter glucose.

2. Materials and methods

2.1. Chemicals

Styrene (S7020079 816), sodium chloride (NaCl), 2,4,6-tripyridyl-*S*-triazine (TPTZ) were purchased from the Merck Schuchardt OHG Company (Germany). K_2HPO_4, thiobarbituric acid (TBA), 5,5-dithiobis (2-nitrobenzoic acid (DTNB)), ethylene diamine tetra acetic acid (EDTA) and trichlororacetic acid were purchased from Sigma-Aldrich Company (Dorset, England). *N*-butanol, rat TNF-α and interleukin-1β (IL-1β) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Bender MedSystems Inc. (Vienna, Austria). Bovine serum albumin (BSA), 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), RNase solution, iScript cDNA synthesis kit and propidium iodide were from Sigma-Aldrich (Munich, Germany). Glucose meter of Bionime (GM110) was purchased for fasting blood glucose and oral glucose tolerance evaluation from Validus Technology. Roche life science was selected to purchase tripure reagent and expand reverse transcriptase. SYBER® green real-time reverse transcription polymerase chain reaction (RT-PCR) master mix along with primers synthesized delivered from Taq DNA polymerase GenScript (Amplicon, Denmark) were utilized in the current study.

2.2. Animals and group design

All tests were performed using male Wistar rats (200–250 g), age 2–3 weeks, which were provided by animal house present in the Faculty of Pharmacy, Tehran University of Medical Sciences (TUMS). All animals were kept and fed under standard laboratory conditions, along with *ad libitum* watering and balanced diet in pellet form. Animals were adapted for one week prior to the trial for maintenance period. According to the ethical animal house keeping guidelines, during the trail, normal temperature of 25 ± 1 °C, 50% humidity and 12 h light/day were provided. All the procedures and protocols were under the ethical committee of TUMS with the code IR.TUMS.REC.1395.2493. Animals were distributed in six (06) groups, each group containing eight (08) and were adopted in polypropylene boxes. Groups were randomly allocated as below:

Group 1: Control: Received only 1 mL/day corn oil through gavage.

Group 2: Received styrene at 250 mg/kg/day in corn oil through gavage.

Group 3: Received styrene at 500 mg/kg/day in corn oil through gavage.

Group 4: Received styrene at 1000 mg/kg/day in corn oil through gavage.

Group 5: Received styrene at 1500 mg/kg/day in corn oil through gavage.

Group 6: Received styrene at 2000 mg/kg/day in corn oil through gavage.

Both corn oil as a carrier and styrene were administered *via* oral route at 1 mL/100 g of body weight for 42 days. Food in pellet form and water were available at ad libitum concentration/quantity. Moreover, the fresh styrene solution was prepared twice on the trail after 20 days interval for each group.

2.3. Choosing of doses and route of administration

The selections of the dosages in the present trial were done *via* previous described studies and pharmacokinetics of styrene in rats. In our trial, the highest concentration was used as about 1/3 of LD_{50}, which is 2000 mg/kg, according to the Safety Data Sheet of styrene version 1.6 and the remaining doses were selected based on 1/1.4, 1/2, 1/4 and 1/8 of the maximum dose that was utilized. The oral route has

been suggested as the most favorable way for systemic toxicity in such experiment regarding styrene.

2.4. Measurement of FBG

After 42 days of styrene administration, one day off in a week, the animals of all groups were fasted for 12 h. The tail of each rat was pricked with a sterile needle to obtain blood to assess FBG *via* glucometer according to manufacturer guidelines.

2.5. Oral glucose tolerance test (OGTT)

The purpose of this assay is to clarify the accumulation of adipose glucose after oral intake (Muniyappa et al., 2008; Ta, 2014). To measure glucose tolerance, glucose solution (65%) was prepared and 1 mL of this solution was gavaged to each rat. The tail was punctured with a sterile needle and the glucose level was measured at time 0, 30, 60 and 120 min (min) post glucose administration. To know about the glucose intolerance and type-2 diabetes mellitus, the OGTT is important and simplest laboratory method.

2.6. Collection of blood

For generalized anesthesia, all animals were sedated injecting sodium pentobarbital through intraperitoneal route. During the procedure, all rats were put in supine position and checked for breathing. After that, blood was collected in a heparinized micro-tube *via* cardiac puncture and plasma was separated through centrifugation and kept at −20 °C.

2.7. Evaluation of plasma insulin

This analysis was done using the company guidelines *via* specific rat insulin ELISA kit. Briefly, preparing the mixtures 10 μL of sample and 100 μL standard solutions were put in each well and incubated at 25 °C for 2 h. All wells were splashed thrice, after that 200 μL of TMB substrate was put in each well and ELISA plate was placed at 25 °C for 15 min in the dark. The 50 μL of stop solution was mixed and absorbance was observed at 450 nm.

2.8. Evaluation of homeostasis assessment of insulin resistance (HOMA-IR)

As previously described by Izquierdo-Vega et al. (2006), HOMA-IR was calculated through the following formula.

$$\mathrm{HOMA-IR} = \mathrm{Fastinginsulin}\left(\frac{u}{l}\right)\mathrm{xFBS}\left(\frac{\mathrm{mg}}{\mathrm{dl}}\right)/405$$

2.9. Measurement of oxidative stress parameters of plasma

2.9.1. LPO assay

Thiobarbituric acid reactive substances (TBARS) method is a well-established method to evaluate LPO. In brief, plasma was collected using centrifugation. The complex formation was evaluated spectrophotometrically, when the compounds react with lipid peroxides and form the complex of TBARS. Plasma was mixed with buffer saline at 1:5, and took 400 μL of homogenized this mixture, add with 800 μL of trichloroacetic acid, which was centrifuged for 30 min at 3000*g*. Later, 600 μL of supernatant after centrifugation was mixed together with 150 μL of TBA (1% w/v). Finally, the cocktail was allowed to incubate for 15 min in steaming water bath followed by the addition of 400 μL of *n*-butanol. After cooling down, the absorbance was recorded at 532 nm by an ELISA reader as previously set up (Pedram et al., 2015).

2.9.2. ROS assay

ROS in plasma was measured as formerly set up by Momtaz et al. (2010). In brief, plasma was taken, sonicated in 75 μL extraction buffer. Then 80 μL of assay buffer was mixed and kept for 30 min at 37 °C. The 2′, 7′-dichlorofluorescin diacetate (DCF-DA) (fluorogenic reagent) cleaved by intracellular esterase to DCFH. This transforms the high fluorescent DCF by cellular peroxides, which can measure ROS production. The absorbance was identified by ELISA fluorometer (Biotec, Tecan U.S.) at 488 and 525 nm with excitation and emission spectra individually for 1 h.

2.9.3. Total antioxidant power (TAP) assay

For the plasma, the ferric reducing power test was done to know about the antioxidant potential of biological fluids/tissues. In brief, 50 μL of plasma with mixed with 1 mL of Tris-EDTA buffer. In this assay, the conversion of Fe^{3+}-TPTZ (2, 4, 6-tris-(2-pyridyl)-*s*-triazin) compound to the ferrous form was recorded at 593 nm as previously set up (Ghayomi et al., 2015).

2.9.4. Determination of total thiol molecules (TTM)

Briefly, for the measurement of TTM, 1 mL Tris-EDTA buffer was added to 50 μL of plasma followed by measurement of absorbance at 412 nm as set up by Navaei-Nigjeh et al. (2015).

2.10. Estimation of inflammatory cytokines

2.10.1. Evaluation of plasma TNF-α

Quantitative measurement of TNF-α content of plasma was done using a rat specific TNF-α ELISA kit. Control and treated groups were added to the wells embedded with immobilized antibodies. Biotinylated anti-rat TNF-α antibody was added to the wells subsequent to the proper wash and later, HRP conjugated streptavidin was imparted to each well. The procedure was followed by the addition of TMB producing a color proportional to the amount of TNF-α present. Finally, the stop solution, which changes the color from blue to yellow, was introduced and the color intensity was recorded at 450 nm. The TNF-α level was expressed as pg/mg.

2.10.2. Determination of IL-1β

Determination of IL-1β was possible using a rat specific IL-1ß kit. As the manufacturer's instructions, all the reagent/solutions were made, so both sample and standards were added into the wells. The ELISA plate was kept at 37 °C for 90 min following by the addition of biotinylated antibody (0.1 mL) and again incubated for 60 min at the 37 °C. Wells were washed 3-times (with TBS) and ABC working solution (0.1 mL) was put in and ELISA plate was kept at 37 °C for 30 min. Afterwards, TMB coloring agent (90 μL) was added, and the strips were placed in dark for 30 min. At the end, the stop solution (0.1 mL) was added and the absorbance was recorded at 450 nm.

2.11. Assessment of styrene toxicity on pancreas

2.11.1. Islet of Langerhans isolation

The rats were administrated *via* intraperitoneal injection with a combination of ketamine-xylazine in a ratio of 10:1 to anesthetize. Next the laparotomy process, the pancreas was allotted and perfused with Hanks–Hepes ([g/L] 8 NaCl, 0.4 KCl, 0.2 $MgSO_4{\cdot}7H_2O$, 0.02 $Na_2HPO_4{\cdot}12H_2O$, 0.06 KH_2PO_4, 0.35 $NaHCO_3$, 2.32 HEPES, 0.4 glucose$\cdot 1H_2O$, 0.186 $CaCl_2{\cdot}2H_2O$, pH 7.2). Later, the pancreas was cut off and the isolated tissues were preserved in Krebs buffer ([g/L] 8 NaCl, 0.27 KCl, 0.42 $NaHCO_3$, 0.06 NaH_2PO4, 0.05 $MgCl_2$, 2.38 HEPES, 0.22 $CaCl_2{\cdot}2H_2O$, 0.5 glucose$\cdot 1H_2O$, pH 7.4) in order to remain healthy and prepared for the next step. The pancreas was further parted and homogenized, so after a proper washing process, analyzing samples were centrifuged at 4 °C for 1 min at 3000*g*. To make all the islets detached, collagenase enzyme was added and this mixture was shaken for 10 min in 37 °C. To halt the breakdown procedure, BSA was put and washed 2-time. Subsequently, with the use of stereomicroscope, same

number and size of islets were picked up as set up previously (Moeini-Nodeh et al., 2016). Lastly, islets were saved for further evaluations.

2.11.2. Evaluation of pancreatic apoptosis and necrosis

To know about the physiological activities of cells, it is important to assess cell viability, homogeneity, cell surface, cytoplasmic antigens and apoptosis and necrosis. ROS levels in viable cells are measured by high-quality flow cytometry, where laser beams pass through the samples. In this study, initial apoptosis, necrosis and cell viability were assessed using flow cytometer. In this manner, Annexin V-FITC/propidium iodide (PI) staining method was used to evaluate the percentage of the viable pancreatic cells treated with various concentrations of styrene along with the control group. Briefly, with the help of Annexin V-FITC antibody and PI islet of Langerhans were washed and stained. The fluorescence intensities of FITC and PI were scanned in FL-1 and FL-2 channels, respectively. Quadrant statistics were used to analyze the fraction of cell populations as previously described (Rahimifard et al., 2015).

Following the isolation of the islets of Langerhans of each group, they were aggregated and washed 2-times with PBS and added in 500 µL of 1X binding buffer. According to the instruction of ApoFlowEx® FITC kit, 5 µL Annexin V-FITC and 5 µL PI were supplemented at 25 °C for 15 min and the islet cells were examined by flow cytometry in FL-1 (FITC) and FL-2 (PI) channels (Shoae-Hagh et al., 2014).

2.11.3. Assay of pancreas islets caspase-3 and −9

Colorimetric assays were used to measure the caspase-3 and −9 activities by distinctive identification of specific amino acid sequences. In brief, the use of chromophore r-nitroaniline (qNA) on tetrapeptide substrates, as released by cleavage of caspase activity, produced a yellowish color that was observed by spectrophotometrically at 405 nm. The activities of caspases are directly proportional to production of yellow color. During the experiment, the islets of Langerhans of each group were degraded by a lysis buffer and incubated for 10 min on ice. Furthermore, the caspase buffer (100 mM HEPES, pH 7.4, 20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol) comprising 100 mM of caspase-3 and −9 specific substrate (Ac-DEVD-qNA and Ac-LEHD-qNA, respectively) along with total cell lysates were incubated at 37 °C for 4 h. The standard absorbance of caspase-3 and −9 was observed at 405 nm, as the activities were assumed 100% in the treatment groups as compared to the control described previously (Rahimifard et al., 2015).

2.12. Evaluation of RT-PCR for the islets of the pancreas

For the determination of genes expression *via* quantitative RT-PCR, vital gene of islets were evaluated; such glutamate dehydrogenase (GDH), glucose transporter-2 (GLUT2), glucokinase (GCK) and glyeraldehyde-3-dehydrogenase (GAPDH) as housekeeping gene (Table 1) along with symbols and forward and reverse primer pairs. In this regard, following 24 h incubation, islet cells were dissociated through trypsin and centrifuged for 5 min at 2300*g*. The micro-tubes were emptied gently *via* sampler and pellets were washed three times with sterile PBS to isolate RNA. With the help of trizol reagent, all 100-treated islets RNA were detached from cultured cells and the quantification of RNA was done spectrophotometrically. Through DNase-I and RNase-free kit, all genomic DNA was extracted. Beside, by the use of the iScript cDNA synthesis kit, cDNA was reverse copied. Computable RT-PCR was done in a LightCycler® 96 System (Roche) using the SYBR Green master mix. The relative gene expression or folded changes can be measured by an amplification curve with the help of relative cycle threshold way known as cycle number (Ct) of each reaction (Schmittgen and Livak, 2008). $2^{-\Delta\Delta Ct}$ was the standard for comparative gene expression change. All Ct values measured from targeted genes were adjusted as compared to untreated control cells.

3. Statistical analysis

In this study, data were presented as mean ± SD. StatsDirect version 3.0.194 was used with one way analysis of variance (ANOVA) followed by Tukey's multi-comparison tests to calculate the statistical difference ($p < 0.001$) between treated and control groups.

4. Results

4.1. General toxicity findings

During this trial, some pathological infections were found on necropsy finding. The visible lesions were pitchy hemorrhages on liver and laceration in the gastrointestinal tract (GIT) in dead rats of fifth and sixth groups (2 and 4 rats, respectively).

4.2. Effect of styrene on FBG

Styrene significantly enhanced the FBG level in all groups with comparison to control group (Fig. 1). Comparing treated with the control group (65.50 ± 2.50 mg/dL), styrene-administered animals at concentrations of 250, 500, 1000, 1500 and 2000 mg/kg showed 68.33 ± 3.10, 69.50 ± 2.20, 71.80 ± 3.20, 82.20 ± 2.20 and 89.00 ± 2.20 mg/dL increase in blood glucose levels, respectively. The 1500 and 2000 mg/kg groups showed significant elevation of blood glucose levels in comparison with control group ($p < 0.01$ and $p < 0.001$, respectively).

4.3. Styrene effect on OGTT

The curve of glucose absorbance and tolerance with time from 0 to 120 min in rats before and after administration of 1 mL glucose (65%) are shown in Fig. 2A. Fig. 2B indicates the glucose tolerance was significantly time-dependent except 250 mg/kg group which was even below from the control group after 30 min. Additionally, in styrene-treated rats, the area under the curve (AUC) of glucose was shown to be

Table 1
Primers of genes GLUD1, GLUT2, GCK and GAPDH for performing RT-PCR.

Gene name	Gene symbol	Accession no.	Primer sequence (5′–3′)
Glutamate dehydrogenase 1	GLUD1	NM_008133.4	F: TCAGTTGGAATCAGCCCCTT R: GTGACTGACTGCTCCTGACT
Solute carrier family 2 (facilitated glucose transporter), member 2	SLC2A2/GLUT2	NM_031197.2	F: GCCCAGCAGTTCTCAGGAAT R: ACATGCCAATCATCCCGGTT
Glucokinase	GCK	NM_001287386.1	F: CTGTGAAAGCGTGTCCACTC R: GTGATTTCGCAGTTGGGTGT
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_008084.3	F: ACTGAGCAAGAGAGGCCCTA R: TATGGGGGTCTGGGATGGAA

GLUD1: Glutamate dehydrogenase, SLC2A2/GLUT2: Solute carrier family-2/Glucose transporter-2, GCK: Glucokinase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase (housekeeping agent).

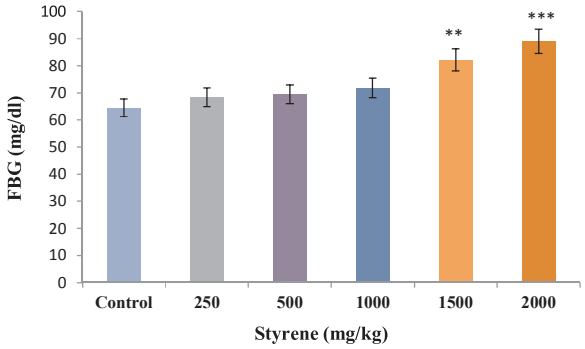


Fig. 1. Effects of styrene on FBS in rats.
One as control and the rest, styrene was orally administered at different doses of 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group (n = 8). **Significantly different from control at $p < 0.01$. ***Significantly different from control at $p < 0.001$.

higher than the control group in all concentrations with significant alteration of $p < 0.01$ (Fig. 2A).

4.4. Effect of styrene on plasma insulin

Styrene significantly increased plasma insulin levels at all doses tested (250, 500, 1000, 1500 and 2000 mg/kg) as 5.94 ± 0.32, 7.09 ± 0.82, 8.86 ± 0.82, 8.66 ± 0.46 and 12.06 ± 0.48 mU/mL, respectively comparing to control (1.99 ± 0.29 mU/mL) ($p < 0.001$) (Fig. 3).

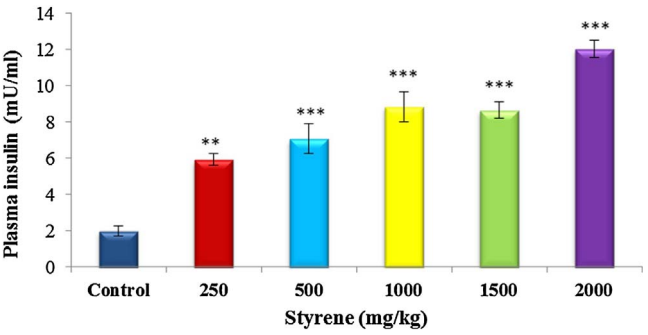
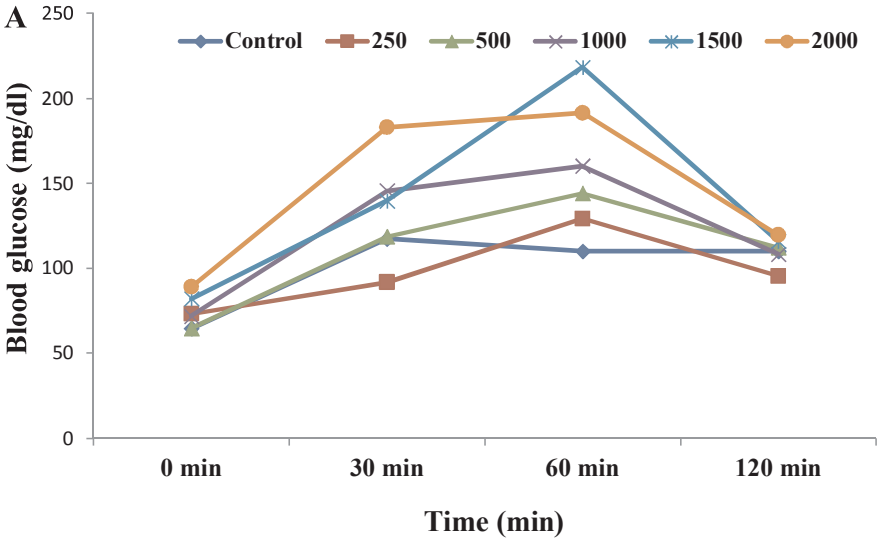


Fig. 3. Effects of styrene on plasma insulin in rats.
One as control and the rest, styrene was orally administered at different doses of 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group. **Significantly different from control at $p < 0.01$. ***Significantly different from control at $p < 0.001$.



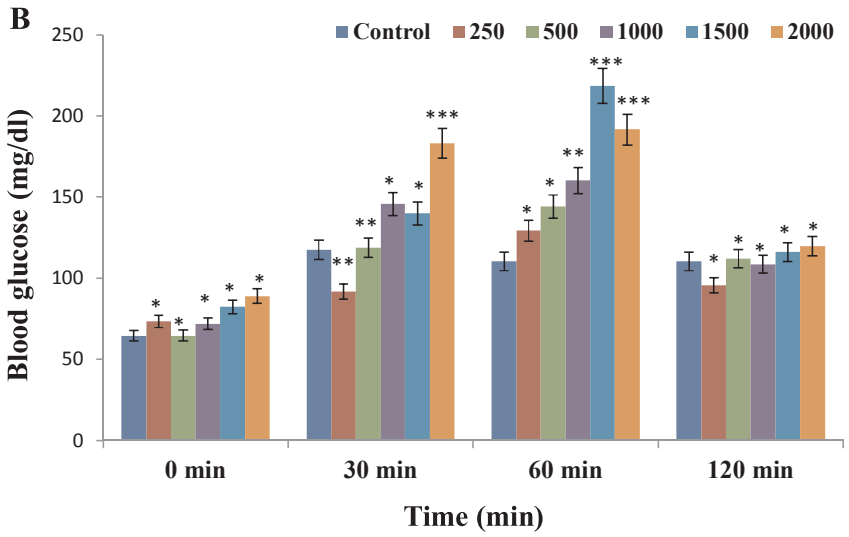


Fig. 2. (A) Effects of styrene on glucose tolerance in rats (B) Effects of styrene on glucose tolerance in rats.
One as control and the rest, styrene was orally administered at different doses of 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group.
One as control and the rest, styrene was orally administered at different doses of 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group. *Significantly different from control at $P < 0.05$. **Significantly different from control at $p < 0.01$. ***Significantly different from control at $p < 0.001$.

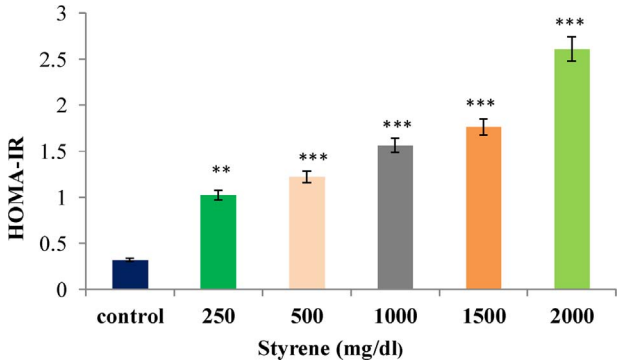


Fig. 4. Effects of styrene on insulin resistance in rats. One as control and the rest, styrene was orally administered at different doses of 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group. **Significantly different from control at $p < 0.01$. ***Significantly different from control at $p < 0.001$.

4.5. Calculating insulin resistance

As shown in Fig. 4, with the help of HOMA-IR formula, insulin resistance was calculated. A significant increase of HOMA-IR value was observed in styrene-exposed rats ($p < 0.001$).

4.6. Measuring plasma oxidative stress parameters

4.6.1. LPO assay

Measurement of TBARS concentration gives a clue of LPO. Styrene at all concentrations was able to raise TBARS levels in plasma significantly in comparison to the control group (94.675 ± 4.575 μM/g protein) (Fig. 5A). TBARS values were found to be 105.61 ± 5.13, 107.63 ± 6.00, 145.38 ± 5.42, 159.53 ± 5.30, and 180.32 ± 3.52 μM/g protein, while styrene was used at dose concentrations of 250, 500, 1000, 1500, and 2000 mg/kg. The significant enhancement in LPO was observed in styrene concentrations of 1000, 1500, and 2000 mg/kg with $p < 0.001$.

4.6.2. ROS assay

The animals administered with styrene in all groups caused an elevation in ROS levels dose dependently (at concentrations of 1000, 1500 and 2000 mg/kg with $p < 0.001$ values) in comparison with the control group in plasma (Fig. 5B).

4.6.3. FRAP assay

There was significant reduction in FRAP values in all styrene treated groups as compared to control one ($p < 0.001$). A steady reduction of FRAP levels was obtained along with the enhancement of doses of styrene (Fig. 5C).

4.6.4. TTM assay

Statistical data displayed in Fig. 5D indicated styrene effect on blood plasma TTM. The quantity of TTM was significantly reduced in 1500 and 2000 mg/kg as compared to control one ($p < 0.001$). Styrene at doses 500 and 1000 mg/kg disrupts thiols quantity as associated with control group ($p < 0.05$).

4.7. Inflammatory cytokines

4.7.1. Plasma TNF-α assay

The level of plasma TNF-α was increased dose-dependently in

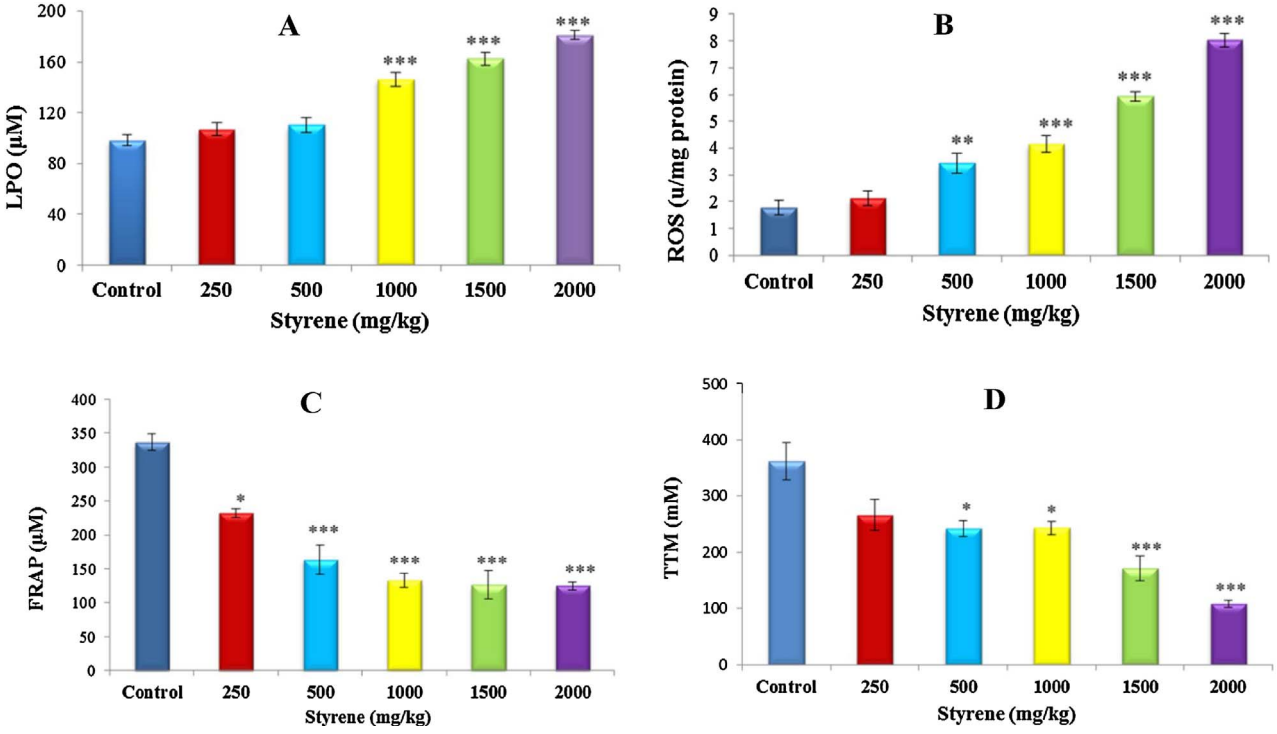


Fig. 5. Oxidative stress biomarkers of blood plasma.

A: Effects of styrene on LPO in plasma of rats. One control and in rest, styrene was orally administered at different doses of 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group. ***Significantly different from control at $p < 0.001$. **B**: Effects of styrene on ROS in plasma of rats. One control and in rest, styrene was orally administered at doses 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group. **Significantly different from control at $p < 0.01$. ***Significantly different from control at $p < 0.001$. **C**: Effects of styrene on FRAP in plasma of rats. One control and in rest, styrene was orally administered at doses 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group. *Significantly different from control at $P < 0.05$. ***Significantly different from control at $p < 0.001$. **D**: Effects of styrene on TTM in plasma of rats. One control and in rest, styrene was orally administered at doses 250, 500, 1000, 1500 and 2000 mg/kg for 42 days. Values are expressed as mean ± SEM for eight animals in each group. *Significantly different from control at $P < 0.05$. ***Significantly different from control at $p < 0.001$.

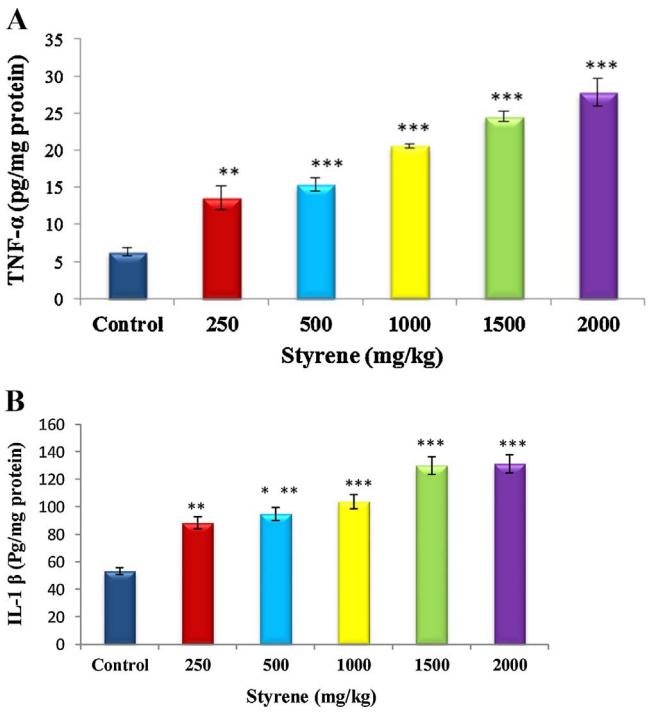


Fig. 6. A: The effect of styrene on inflammatory cytokines (TNF-α). **B:** The effect of styrene on inflammatory cytokines (IL-1β).
In this Figure, the effect of styrene on TNF-α level is shown. Values are expressed as mean ± SEM for eight animals in each group. ***Significant difference from the control group at $p < 0.001$. **Significant difference from the control group at $p < 0.01$.
In this Figure, the effect of styrene on IL-1β levels is shown. Values are expressed as mean ± SEM for eight animals in each group. ***Significant difference from control group at $p < 0.001$, **Significant difference from control group at $p < 0.01$.

styrene administrated groups as shown in Fig. 6A. There was a significant rise in TNF-α amount at concentrations of 500, 1000, 1500, and 2000 mg/kg in styrene groups as compared to the control animals ($p < 0.001$).

4.7.2. IL-1β level in plasma

Styrene-exposed groups at 500, 1000, 1500, and 2000 mg/kg significantly upraised the quantity of IL-1β in plasma ($p < 0.001$) (Fig. 6B).

4.8. In vivo model for evaluating styrene toxicity on the pancreas

4.8.1. Flow cytometry evaluation of pancreatic apoptosis and necrosis

The percentage of viable, apoptotic and necrotic cells in various groups are illustrated in Fig. 7. To clarify, Q1 represents necrosis (FLTC $^-$, PI $^+$), Q2 shows late-apoptosis cells (FLTC $^+$, PI $^+$), Q3 expresses apoptosis (FLTC $^-$, PI $^-$) and Q4 describes alive cells (FLTC $^+$, PI $^-$). A high level of cell viability was observed in the control group (64.0%) in comparison to the other groups (Q3). The rate of late apoptotic cells in Q2 exhibited a positive enhancement at 250, 500, 1000, 1500, and 2000 mg/kg comparing to those of controls. Styrene successfully decreased the number of viable cells at all concentrations tested ($p < 0.001$).

4.8.2. Effect of styrene on caspases-3 and −9 activities

As presented in Fig. 8, exposure to styrene caused a significant enhancement in the caspase-3 and −9 activities in islets of Langerhans ($p < 0.001$) in both 1500 and 2000 mg/kg groups, as compared to the control group.

4.9. The influence of styrene toxicity on expression of GDH, GLUT1 and GCK in isolated rat pancreatic islet of Langerhans through RT-PCR

The details of forward and reverse primer of genes GLUD1, GLUT2, GCK and GAPDH (housekeeping gene) with accession numbers have been provided in Table 1. The investigation of statistical data illustrated that styrene disrupt and/or mutate gene expression significantly different as compared to control one. As presented in Table 2, GDH/GLUD1 were downregulated in 250, 500 and 1000 mg/kg groups, while these genes were upregulated at 1500 and 2000 mg/kg (Table 2). The relative fold changes of GLUT2 were down regulated at 250 and 1000 mg/kg and up regulated in the remaining doses of styrene ($p < 0.01$). The expression level of GCK indicated a significant upregulation at 250 mg/kg and downregulation of relative fold changes at 500, 1000 and 1500 mg/kg of styrene, while there was no change fold seen at 2000 mg/kg.

5. Discussion

Styrene increased the FBG level in comparison to the control group, which was significant at higher doses. Serum glucose levels in OGTT were significantly increased with time and dose dependent manner by styrene while the plasma insulin levels, as well as insulin resistance were significantly increased dose dependently.

The effect of low and long term exposure of monocyclic aromatic hydrocarbons, including styrene on insulin resistance was explored by (Won et al., 2011). A significant increase in fasting glucose and insulin resistance were obtained, which may be due to the elevation of cytokines and oxidative stress levels. In a similar investigation, styrene lifted up the oxidative stress markers which are supposed to be associated with insulin resistance, β cell dysfunction, and impaired glucose tolerance (Rains and Jain, 2011). The most evident factor of such enhancement of plasma insulin is believed to be insulin resistance or declined sensitivity to the insulin in a subchronic *in vivo* study. Therefore, it is suggested that the induction of oxidative stresses may also damage pancreatic islets, which might affect the physiological function of β-cells along with the release of plasma insulin. The insulin resistance was also assessed *via* HOMA and it was found that styrene-treated groups showed a gradual increase in insulin resistance. The increases in the production of ROS and LPO levels observed in this study believed to be linked to the insulin resistance (Tangvarasittichai, 2015). It is also well established that with the increase of the dose of styrene will induce toxicity leading to the subsequent effect on insulin resistance. Styrene and its metabolites have been associated with different organ damages (liver, brain, and lungs) as a result of oxidative damage *via* the enhancement and reduction in oxidants and antioxidant levels, respectively (Katoh et al., 1989; McCague et al., 2015; Sisto et al., 2016). In our study, the amount of TBARS level drew a slew of LPO, as all doses of styrene raised TBARS concentration in plasma significantly in a dose-dependent manner. Styrene increased LPO indirectly by lessening the antioxidant glutathione (Katoh et al., 1989), which through enzymatic processes, reduced hydrogen and lipid peroxides to their corresponding alcohols (Flohe, 1988). Lower doses of styrene and its oxide (≤600 mg/kg) have been reported to enhance LPO in the liver by depleting glutathione to the critical levels (Katoh et al., 1989; Srivastava et al., 1983). One more study supported this claim where styrene (600 mg/kg) and its metabolites (300 mg/kg) significantly reduced glutathione levels in bronchioalveolar lavage fluid and plasma (Carlson, 2010). El-Ziney et al. (2016) used TBARS assay and showed very low doses of styrene (monostyrene) could increase LPO in female rats more than males. This may be due to the high conversion of this compound to its oxide forms in females than males (Leibman, 1975).

Our findings indicated a dose-dependent escalation of ROS levels, which were observed in styrene treated groups as compared to the control. High levels of ROS, which results from an imbalance in its production and removal have been implicated in the pathogenesis of

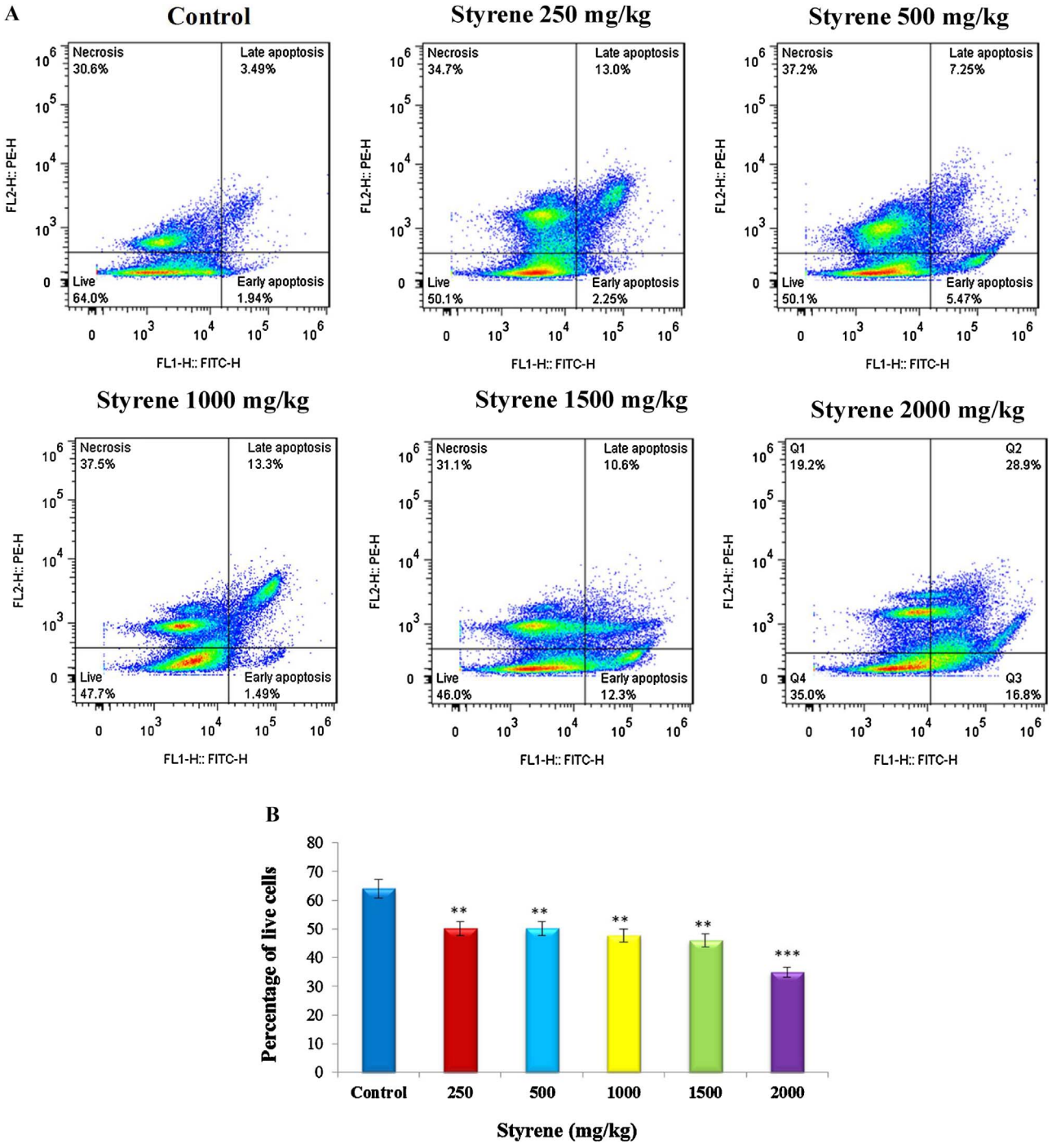


Fig. 7. A: Flow cytometry assessment of styrene-induced apoptosis and necrosis at different concentrations on islet cells.
Changes in percent of live, apoptotic and necrotic populations of the islet cells are exhibited. Furthermore, variations in the percent of live and late apoptosis rate are shown in the graph. left down square shows live cells with FITC- and PI-, right down square indicates early apoptotic cells with FITC + and PI-, above the right square display late apoptotic cells with FITC + and PI + and above left square expresses necrotic cells with FITC- and PI +. **B:** Percentage of live cells. Values are expressed as mean ± SEM for eight animals in each group. **Significant difference from control group at $p < 0.01$. ***Significant difference from control group at $p < 0.001$.

many diseases (Held, 2012). Lower doses of styrene (≤500 mg/kg) have been reported to enhance ROS production dose-dependently, which affected sperm counts and motility (Chun et al., 2005). Another study demonstrated increased ROS production following the exposure of styrene and styrene oxide *in vivo* (whole lung) and *in vitro* (Clara cells) (Harvilchuck et al., 2009).

Besides the increase of styrene concentration, a steady reduction in FRAP levels was gained in our experiment. The FRAP assay was first explored by Benzie and Strain, (1996) and to measure the antioxidant activity of plasma constituents on its use. This technique provides more relevant information compared to individual antioxidant measurement, which may describe dynamic equilibrium between pro-oxidants and antioxidants in plasma. Reduction in FRAP as seen in this study, has also been reported by Sati et al. (2011), where it was significantly lower in workers exposed to styrene compared to the control.

Thiols constitute major part of total body antioxidants, which play a major role in defense against free radicals, detoxification, signal transduction, and apoptosis. Decrease in the serum levels of thiols may imply chronic disorders related to kidney, cardiovascular and neurological disorders (Mungli et al., 2009). Styrene in the present study

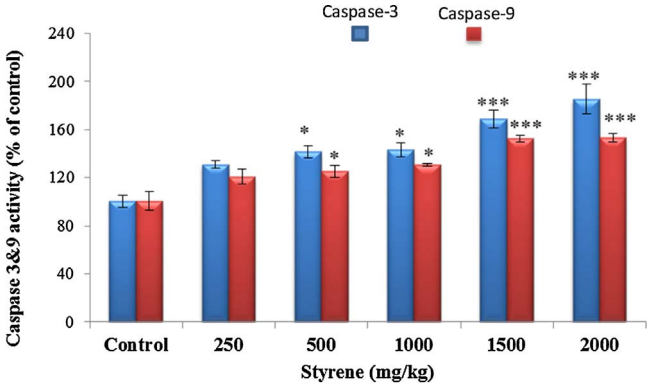


Fig. 8. The effect of styrene on caspase-3 and −9 in islet cells.
Values are expressed as mean ± SEM for eight animals in each group.*Significant difference from the control group at $p < 0.05$. ***Significant difference from the control group at $p < 0.001$.

decreased serum TTM with significant increase in intensity based on dosage.

Styrene exposure has also been reported to cause elevation in serum pro-inflammatory cytokines (Strafella et al., 2013), such as TNF-α which inhibits insulin signaling by inhibiting serine phosphorylation of insulin receptor substrate 1(IRS-1) function (Hotamisligil et al., 1993). Likewise, it can decrease insulin by elevating blood levels of fatty acids that promotes lipolysis in adipose tissues (Ruan and Lodish, 2003). IL-1β also was lifted in serum by styrene, which can cause dysregulation in insulin signaling by suppressing IRS-1 expression both dependent and independent on ERKs pathway during transcription and post transcription respectively (Jager et al., 2007). Elevation of the circulating levels of pro-inflammatory cytokines TNF- α and IL-1β has been allied with cell damage which results in the activation of NF-κB pathway involved in several disease states. IL-1β is an important mediator of inflammatory responses affecting cell differentiation, proliferation, and programmed cell death (Mandrup-Poulsen, 1996). TNF-α has also been associated with apoptotic cell death, as well as inhibition of tumorigenesis and viral replication (Scheringa and Marquet, 1990). Involvement of cytokines has been proposed as a possible mechanism of apoptosis in pancreatic β cells in humans and animals (Donath et al., 2003; Mandrup-Poulsen, 1996). In our study, styrene significantly raised the plasma levels of both TNF-α and IL-1β. This is in agreement with (Strafella et al., 2013) who reported an up regulation of genes encoding stress responsive proteins such as IL-1β and TNF-α, which were further confirmed in serum following the low level of styrene exposure (Röder-Stolinski et al., 2008) suggested the possible activation of NF-κB signaling pathway *via* styrene induced oxidative stress in human lung epithelial cells.

It is well-known that caspase-3 and −9 are cell-death proteases, which are not only responsible to play vital roles in apoptosis, but their activation can damage cellular morphologies such as DNA breakup, chromatin shortening, caspase-like cleavage activity and reduction of cell size (Jänicke et al., 1998). Several studies reported that environmental toxicants such as benzene and methyl mercury initiate apoptosis *via* the activation of caspase-3 and −9 (Bahadar et al., 2015a; Kondoh et al., 2002; Maqbool et al., 2016). Our data showed positive agreement of other findings as styrene presented a significant increase of apoptosis, activities of caspase-3 and −9 in islets of Langerhans's cells.

Several studies have reported the ability of styrene and its metabolites to cause cell death *via* apoptotic and necrotic pathways in a time and dose dependent way. The major metabolite of styrene (such as SO) has been shown to cause neuronal PC12 cell apoptosis through loss of cytoskeletal organization, caspase-3 initiation, and variation of Bcl-2 family proteins (Boccellino et al., 2003). Human astrocytoma cells have been shown to undergo apoptosis post exposure to styrene oxide dose dependently, similar to that of neuronal cells *via* a caspase dependent pathway by increasing the activities of caspases-3 and −8 (Coccini et al., 2003). Since the formation of styrene metabolite (such as SO) has been shown to be dependent on erythrocytes and oxyhemoglobin, its deleterious effect on human cord blood cells was determined *via* the evaluation of apoptosis and gene expression (Diodovich et al., 2004). In the cord blood cells, necrosis was occurring not apoptosis, which may be as a result of overexpression of the Bcl-2 and decreased level of Bax in which the former allows chemically induced DNA damaged cells to survive and proliferate. Acute exposure of styrene and SO on Clara cells also revealed limited apoptosis as a result of the increased Bax/Bcl-2 ratio after 24 h and 240 h with a mild increase in caspase-3 activity (Harvilchuck et al., 2009). Chen et al., 2007 stated that styrene at a dose of 800 mg/kg induced ototoxicity and cell death, mainly through apoptotic pathway, while further histochemical staining revealed the activation of caspases. This indicated the possible involvement of mitochondrial and death receptor dependent pathways. (Yang et al., 2009) confirmed the participation of both apoptotic and necrotic pathways in styrene (400 mg/kg) induced ototoxicity and hair cell loss which may be reduced by a potent antioxidant such as *N*-acetyl cysteine (NAC).

Styrene induced relative folded change in gene expression and was significantly different as compared to control. Relative fold change of GDH/GLUD1 was up regulated in all groups, including 1500 and 2000 mg/kg. GLUT2 and GCK expression indicated significant up regulation and down regulation of gene expression, respectively in all doses of styrene. GDH/GLUD 1 gene encodes glutamate dehydrogenase (an enzyme in the mitochondrial matrix) which catalyzes the deamination of glutamate to alpha keto-glutarate and ammonia (Nissim, 1999). This gene regulates amino acid induced insulin secretion; hence mutations in this gene can lead to the congenital hyperinsulinism (De Leon and Stanley, 2007). GDH/GLUD 1 is expressed at high levels in the liver, brain, kidney and pancreas; where in the pancreatic cells, it is thought to be involved in insulin secretion mechanisms (De Leon and Stanley, 2007). Overexpression of GDH in rat islets have been shown to significantly potentiate high glucose stimulated insulin secretion, though the overexpression does not affect secretion at low glucose (Carobbio et al., 2004). GDH activity is regulated by both GTP and ADP,

Table 2
Description of relative fold changes of genes GDH, GLUT2 and GCK after RT-PCR.

	Experimental groups (Relative fold change ± SEM)				
	Concentration of styrene (mg/kg)				
Gene symbol	250	500	1000	1500	2000
GLUD1	92 ± 5*	80 ± 4*	79 ± 4*	140 ± 7***	309 ± 15***
SLC2A2/GLUT-2	4.26 ± 0.21**	6.11 ± 0.31***	5.21 ± 0.26**	7.01 ± 0.35***	9 ± < 1***
GCK	298 ± 15***	199 ± 10**	144 ± 7**	54 ± 2*	39 ± 2

Values are expressed as mean ± SEM in each group.
* Significantly different from control at $P < 0.05$ (Down regulated).
** Significantly different from control at $p < 0.001$ (Down regulated).
*** Significantly different from control at $p < 0.01$ (Up regulated).

which both are negative and positive allosteric effectors, respectively (Stanley, 2009). In this study, styrene induced a dose dependent increase in blood glucose and plasma insulin that may be correlated with its effect on up regulation of GDH/GLUD 1 at higher doses.

GLUT 2 serves as the primary glucose transporter and sensor in rodent islets, hence mutations may cause elevation in fasting plasma glucose (McCulloch et al., 2011). GLUT 2 gene expression was more pronounced in a high glucose culture medium (Tal et al., 1992) which is in agreement with our findings on styrene with elevated glucose levels and up regulation of this gene. It is important to sustain normal bodily glucose level, physiological activities of the pancreas, where mice deficient in GLUT 2 transporter had abnormal glucose tolerance *in vivo* but impaired glucose stimulated insulin secretion and loss of glucose control of insulin gene expression (Guillam et al., 1997).

GCK/glucokinase plays important role in glucose metabolism by catalyzing the conversion of glucose to glucose-6-phosphate. It has been reported that inhibition of hepatocyte protein formation and gluconeogenesis due to cytokines and nitric oxide have been endorsed to increase the level of cGMP. So, some phosphodiesterase inhibitors may increase the susceptibility of the hepatocytes to the toxicity of cyanide and other environmental toxicants (Abdollahi et al., 2003). Mutations in this gene have been linked to many forms of diabetes, such as non-insulin dependent diabetes, maturity onset diabetes and persistent hyperinsulinemia hypoglycemia of infancy (Gloyn et al., 2008). In our trial, styrene administration led to the higher amount of glucose and insulin, which supported the up regulation of GCK gene at low doses. Up regulation of GCK in response to high glucose level is mediated by insulin, while pathways involved in its transcription include insulin receptor B type, PI3K class 1a and p70 s6 kinase (Bae et al., 2010; Leibiger et al., 2001). The results of the present data explored that GLUD1, GCK and GLUT2 are the main responsible genes related to glycogenolysis and glycolysis. GLUT2 plays an active role in the transportation of glucose inside the cells where various metabolites are produced and used for energy purposes. The conversion of glucose to glucose-6phasphate is under the enzymatic activity of GCK enzyme, which assists in phosphorylation. Hyperglycemia is a direct indication of the present study that correlates gene suppression and styrene-induce toxicity. In fact, styrene down regulated GCK and GLUT2 at 1000 mg/kg. GCK and GLUT2 relative folded changes indicated that styrene targets glucose metabolism, which leads to hyperglycemia and glucose intolerance. Thus, GLUD1, GCK and GLUT2 enzymatic mechanisms and/or activities would play an important role in glucose intolerance, as these genes have shown significant relative fold changes at high doses. For the future, study should be done to evaluate hepatocytes cAMP trigger glycogenolysis and gluconeogenesis. However, cGMP may have negative correlation with cAMP in gluconeogenesis.

6. Conclusion

The present study concluded that styrene is responsible for potential disruption of gluconeogenesis, glycogenolysis pathways, insulin secretory functions, oxidative balance, inflammatory responses, cell viability and gene expression (GLUD1, GLUT2 and GCK). In fact, oxidative stress/ROS formation constituted the first block of styrene induced hyperglycemia in pancreatic islets. The intermediate exposure to styrene triggered the abrupt secretion of insulin and increased FBG level, which is the primary reason of insulin resistance and type-2 diabetes. Glucose intolerance, insulin resistance and hyperglycemia are counted as the foremost consequences of styrene exposure, in association with relative gene expression changes of islet cells. Therefore, styrene disrupts insulin release pathways and β-cells were mutated on molecular levels. Our results re-confirmed that styrene strongly manipulated the parameters related to oxidative stress and inflammatory cytokines in blood plasma along with the direct influence on responsible genes for glycogenolysis and glycolysis in islets of Langerhans in rat models. Further experimental studies are compulsory to explore the epigenetic alteration of styrene on diabetes and interconnected abnormalities in other organs.

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Author contributions

All authors have directly participated in the planning, conducting, analyzing, and drafting of the manuscript. MA conceived the study. All authors have read and approved the final version.

Conflict of interest

The authors declare no conflict of interest with respect to the contents of this article.

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Effect of styrene exposure on plasma parameters, molecular mechanisms and gene expression in rat model islet cells

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Abstract

Styrene is an aromatic hydrocarbon con
through plastic industry. The current stu
parameters in rat plasma fasting blood g
oxidative stress, and inflammatory cytok
corn oil and administered at different do
each rat, for 42 days. In treated groups,
insulin ($p < 0.001$) and glucose tolerance
were found to be the main consequence
significant enhancement of oxidative str
and concentration-dependent manner in
9 of the islet cells were significantly up-r
styrene administrated groups ($p < 0.001$
0.05) and upregulated at 1500 and 2000
GLUT2 were down regulated at 250 and
doses of styrene ($p < 0.01$). The expres
mg/kg and downregulation of relative fol
change at 2000 mg/kg of styrene for GC
pancreatic islet cells in styrene exposed
and insulin secretory functions. The pres
pathway, oxidative balance, inflammator
metabolism are susceptible to styrene, which consequently lead to other abnormalities in various organs.

Keywords

Author Keywords: Styrene; Insulin; Glucose; Oxidative stress; Inflammatory cytokines; Gene expression

KeyWords Plus: VOLATILE ORGANIC-COMPOUNDS; PANCREATIC BETA-CELLS; LUNG EPITHELIAL-CELLS; NECROSIS-FACTOR-ALPHA; OXIDATIVE STRESS; INSULIN-RESISTANCE; HUMAN-LYMPHOCYTES; IN-VITRO; GLUTAMATE-DEHYDROGENASE; GLUCOSE-TRANSPORTER

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