RESEARCH ARTICLE

Impaired Insulin Signaling Molecules in Triceps Muscle of Diethyl Hexyl Phthalate Treated Rat is Amelioreted by Antioxidant Vitamins

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ABSTRACT

Available toxicological testing in animals and *in vitro* test, supported by limited human data, provide evidence linking Diethyl hexyl phthalate (DEHP) and its metabolites to a wide range of adverse effects in the reproductive tracts, liver, skeletal muscle, lungs, kidney and fetus. Recently there has been growing concern for the impact of plastic; polyvinylchloride (PVC) based endocrine disruptors like DEHP affects the function of endocrines and other organs in human beings and animals. Previous studies have shown that exposure to DEHP results in elevated level of blood glucose, decrease in serum insulin and testosterone level. However, specific effects of DEHP on insulin signaling molecules in triceps muscle; an organ involved in the regulation of glucose homeostasis has received only little attention. Healthy adult male albino rats of Wistar strain (*Rattus norvegicus*) were divided into four groups: Group I: Control; Groups II and III: DEHP treated (dissolved in olive oil at a dose of 10 and 100 mg/kg body weight, respectively, once daily through oral intubation for 30 days); and Group IV: DEHP (100 mg/kg body weight) plus vitamins E (50 mg/kg body weight) and C (100 mg/kg body weight) dissolved in olive oil and distilled water, respectively, once daily through oral intubation for 30 days. After the completion of treatments, animals were euthanized and perfused (whole body); triceps was dissected out and subjected to assessment of various parameters. Our results demonstrate that DEHP treatment induces ROS and lipid peroxidation. Furthermore, DEHP treatment significantly decreased the levels of insulin receptor, membrane GLUT4 protein as well as it reduces glucose uptake, oxidation and glycogen in skeletal muscle due to decreased serum insulin level. Antioxidant vitamins (C & E) have significant protective role against the adverse effect of DEHP on these tissues of adult male albino rat. All together, these results suggest that DEHP exposure induces lipid peroxidation which disrupts the membrane integrity and thus the insulin receptor and membrane GLUT4 proteins leading to reduced glucose oxidation in triceps muscle. Supplementation of vitamins (C & E) prevented the DEHP-induced changes.

Key words: Triceps, DEHP, Insulin Signal, Vitamin, Glucose, GLUT4, Type-II diabetes.
Abbreviations: DEHP, diethyl hexyl phthalate; IR, insulin receptor; GLUT4, glucose transporter protein4; LPO, lipid peroxidation; MDA, Malondialdehyde; H_2O_2, Hydrogen peroxide; OH-, Hydroxyl radical.

INTRODUCTION

Diethyl hexyl phthalate (DEHP) in an aromatic diester used primarily to soften and plasticize the rigid polymer poly vinyl chloride (PVC). DEHP imparts to PVC flexibility, strength, optical clarity and resistance to broad range of temperature variations (Shea, 2003; Green et al., 2005). DEHP has been used as a plasticizer in a variety of medical products such as bags containing blood, plasma and intravenous fluids, nasogastric tubes, enteral feeding tubes, umbilical catheters, extracorporeal membrane oxygenation (ECMO) circuit tubes, endotracheal tubes and examination gloves (Marcel, 1973; Schettler, 2006). It is an environmental endocrine disruptor which has been suspected to confer toxic effects on the reproductive organs in humans and animals [Matsumoto et al., 2008; Wittassek et al., 2011].

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Skeletal muscle is an important tissue for the proper maintenance of glucose homeostasis and Glucose transport in skeletal muscle is the rate-limiting step for glucose utilization under physiological condition (Zierath and Kawano, 2003; Muretta and Mastick, 2009). The triceps is an extensor muscle of the elbow joint, and is an antagonist of the biceps and brachialis muscles. It can also fixate the elbow joint when the forearm and hand are used for fine movements, e.g., when writing. The triceps accounts for approximately 60 percent of the upper arm's muscle mass (Madsen et al., 2006).

Insulin is a peptide hormone secreted by pancreatic β-cells in response to an elevation in blood glucose, and it plays a vital role in the control of glucose homeostasis by regulating carbohydrate, lipid, and protein metabolism. These pancreatic β-cells are clustered into the islets of Langerhans, indicating that these islets play a critical role in regulating blood glucose homeostasis (Baudry et al., 2002). GLUT4 is a member of the facilitative monosaccharide transporter gene family, and is expressed exclusively in insulin target tissues of adipocytes, skeletal and cardiac muscles (Mueckler, 1994; Thorens and Mueckler, 2010). Upon acute insulin stimulation, GLUT4 transporters are rapidly translocated from an intracellular vesicular compartment to the plasma membrane, and mediate the bulk of glucose transport activity. The level of GLUT4 expression is a major determinant of insulin sensitivity. On the other hand, decreased expression of GLUT4 is associated with insulin resistance in both skeletal muscles (Garvey et al., 1994; Hansen et al., 1995). GLUT4 displays the unique characteristic of a mostly intracellular disposition in the unstimulated state that is acutely redistributed to the plasma membrane in response to insulin and other stimuli (Bryant et al., 2002).

The effects of DEHP on fetal testosterone production and expression of protein and genes involved in steroid hormone synthesis in fetal testis was investigated. Phthalate esters are classified as peroxisome proliferators and they interact via peroxisome proliferators activated receptors γ to reduce mRNA expression of steroidogenesis related factors like Steroidogenic Acute regulatory protein (StAR), and the nuclear receptor Steroidogenic Factor 1, which regulates certain steps in steroidogenesis (Borch et al., 2006). Akingbemi et al., (2004) had conducted experiments to investigate the ability of DEHP to affect leydig cell androgen biosynthesis. DEHP interferes with carbohydrate metabolism by reducing the blood glucose utilization and hepatic glycogenesis and glycogenolysis in rat (Mushtaq et al., 1980). Martinelli et al. (2006) they investigated effect of DEHP on the glycolytic metabolites in the liver. They found there will be a decreased glucose-6-phosphate, fructose-6-phosphate, pyruvate, and lactate. At low doses, DEHP reduced the serum insulin and increased the blood glucose T_3 (Triiodothyronine) and T_4 (Thyroxine) in rats (Gayathri et al., 2004). DEHP exposure leads to decreased insulin receptor concentration and glucose oxidation in Chang liver cells and liver of adult male rat. These reports suggested that DEHP exposure may have a negative effect on glucose homeostasis (Rengarajan et al., 2006; Srinivasan and Balasubramanian, 2013). Stahlhut et al. (2007) studied the concentrations of urinary phthalate metabolites are associated with increased waist circumference, insulin resistance and related clinical disorders in adult U.S. males.

The importance of vitamin C is a well known antioxidant and it is an essential micronutrient required for normal metabolic functioning of the body (Kojo, 2004). Vitamin C is known for its beneficial effects on oxidative damage to organs such as the eyes and kidneys that frequently occur in type-II diabetes (Afkhami-Ardekani and Shojaoddiny-Ardekani, 2007; Lee et al., 2007). It may also reduce the risk of cardiovascular complications by several mechanisms including lowering blood pressure and preventing hemodynamic changes induced by hyperglycemia (Mullen et al., 2002). Vitamin C significantly decreases the adverse effect of oxidative damage to macromolecules namely, lipids, DNA and proteins, which are implicated in chronic diseases including neurodegenerative diseases (Halliwell and Gutteridge, 1999; Ishihara et al., 2000). Supplementation of antioxidant vitamins significantly accelerated regeneration of the injured seminiferous epithelium in DEHP treated animals, suggesting that the vitamins have a therapeutic effect on DEHP-induced aspermatogenesis (Ablake et al., 2004).

Vitamin E is a major non-enzymatic antioxidant, exerting its effect by scavenging free radicals directly and thereby stabilizing membranes containing polyunsaturated fatty acids and or by down-regulating mitochondrial superoxide generation (Burton, 1983). Vitamin E supplementation might be effective in preventing early endothelial damage in type-diabetes mellitus by reducing plasminogen activator inhibitor type-1 activity, intracellular adhesion molecule and

vascular cell adhesion molecule-1 concentrations and increasing the nitric oxide production (Vignini et al., 2008). Dhanya et al. (2004) reported that administration of vitamin E prevented DEHP-induced deleterious effects like degenerative changes in the brain and thyroid and decrease in the concentration of serum insulin. Moreover, DEHP promotes lipid peroxidation and incorporation of vitamine E along with DEHP into the culture medium containing hepatocytes counteracted these effects (Santhosh et al., 1998). Previous studies have shown that exposure to DEHP results in elevated level of blood glucose and decrease in serum insulin (Srinivasan et al., 2011; Rajesh et al., 2013). However, very few studies has been conducted on the effects of DEHP on insulin signaling molecules in triceps muscle; an organ involved in the regulation of glucose homeostasis. Therefore, the aim of this study was to explore the dose-dependent effect of DEHP on insulin signaling molecules, ROS generation and glucose transport in triceps muscle of adult male albino rat. Further, we have evaluated the scavenging effect of antioxidant vitamins (C & E) against DEHP-induced toxicity.

MATERIALS AND METHODS

Chemicals: All chemicals and reagents used in the study were of molecular and analytical grade; and they were purchased from Sigma Chemical Company, St. Louis, MO, USA; Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom; and Sisco Research Laboratories, Mumbai, India. ^{14}C-glucose and ^{14}C-2-deoxyglucose were purchased from the Board of Radiation and Isotope Technology, Mumbai, India. Total RNA isolation reagent (TRIR) and one-step reverse transcriptase-polymerase chain reaction (RT-PCR) were purchased from ABgene (UK) and Siegen (Germany). The insulin receptor (IR), GLUT4, β-actin primers and the β-actin monoclonal antibody were purchased from Sigma (USA). Polyclonal insulin receptor β-subunit, Akt1/2/3 and GLUT4 antibodies were purchased from Santa Cruz Biotechnology (USA).

Animals and experimental treatment: Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethical Committee (IAEC No. 03/030/07). Healthy adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180 to 200 g (100 days old) were used. Animals were housed in polypropylene cages under specific humidity

(65% ± 5%) and temperature (21°C ± 2°C) with constant 12 h light and 12 h dark schedule. They were fed with standard rat pelleted diet (Lipton, Mumbai, India), and clean drinking water was made available *ad libitum*. Rats were divided into four groups, each consisting of six animals: Group I: Control (vehicle treated at a dose of 2 ml/kg body weight); Group II: DEHP treated (dissolved in olive oil at a dose of 10 mg/kg body weight, daily at 10 am through oral intubation for 30 days); and Group III: DEHP treated (100 mg/kg body weight, daily at 10 am through oral intubation for 30 days). Group IV: DEHP (100 mg/kg body weight), Vitamin E (dissolved in olive oil at a dose of 50 mg/kg body weight) and Vitamin C treated (100 mg/kg body weight dissolved in distilled water daily at 10 am through oral intubation for 30 days). After the treatment period, animals were anesthetized with thiopentone (40mg/kg body weight) and perfused with normal saline (whole body); triceps was dissected out and subjected to assessment of various parameters.

Determination of reactive oxygen species: Lipid peroxidation (LPO) was measured by the method of Devasagayam and Tarachand [1987]. The malondialdehyde (MDA) content of the sample was expressed as nmol of MDA formed/mg protein. Hydrogen peroxide generation (H_2O_2) was assessed by spectrophotometric method of Pick and Keisari [1981]. The H_2O_2 content was expressed as µmol/min/mg protein. Hydroxyl radical (OH-) production was quantified by the method of Puntarulo and Cederbaum [1988] expressed as µmol/min/mg protein.

Glucose uptake: ^{14}C-2-deoxyglucose uptake in tissues was estimated by the method of Valverde et al. [1999]. Briefly, after the control and experimental rats were anesthetized, triceps was dissected out and rapidly cut into pieces of 10 mg. The tissues were put into a 12 well plate, containing 2ml Krebs-Ringer bicarbonate (KRB) buffer (119mM NaCl, 4.8mM KCl, 1mM KH_2PO_4, 1.2mM $MgSO_4$, 1mM $CaCl_2$, 24mM $NaHCO_3$, 12mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1% BSA, and 2mM sodium pyruvate) supplemented with 8mM glucose and incubated at 37°C for 60 min. Then, the tissues were incubated for 20 min using KRB buffer supplemented with (for measurement of insulin-stimulated glucose uptake) or without (for measurement of basal glucose uptake) insulin (2□U/ml). Following, tissues were rinsed using

KRB buffer and further incubated for 20 min at 37°C in 2 ml KRB buffer, which contained 8mM ^{14}C-2-deoxyglucose (0.05 µCi). Plates were continuously supplied with 95% O_2 and 5%CO_2 throughout the experiment. Then the tissues were removed and rapidly rinsed in isotope-free KRB buffer, solubilized with 1N NaOH. Radioactivity was counted using liquid scintillation counter. Results are expressed as CPM of ^{14}C-2-deoxyglucose taken up/10mg tissue.

^{14}C-Glucose oxidation: ^{14}C-Glucose oxidation was estimated as per the standard method [Kraft and Johnson, 1972]. Briefly, 10 mg tissue was weighed and placed in a 2 ml ampoule containing 170 µl DMEM (Dulbecco's modified Eagle's medium, pH 7.4), 10 IU penicillin and 0.5 µCi ^{14}C-glucose. The ampoules were aerated with a gas mixture (5% CO_2, 95% air) for 30s and tightly covered with rubber cork containing CO_2 trap (a piece of filter paper was inserted into the rubber cork and 0.1 ml of diethanolamine was applied to the filter paper before closing the ampoule). This closed system with CO_2 trap was placed in an incubator at 37°C. CO_2 trap was replaced every 2h. After removing the second trap, 0.01 ml of 1N H_2SO_4 was added to halt further metabolism and release of any residual CO_2 from the sample. The system was again closed for 1h before the third and final trap is removed. All the CO_2 traps were placed in the scintillation vials containing 10 ml of scintillation fluid and the radioactivity was assayed in a Beta counter. Results are expressed as cpm of $^{14}CO_2$ released/10mg tissue.

RT-PCR: Total RNA was isolated from control and experimental samples using TRIR (Total RNA Isolation Reagent) kit from ABgene, UK. The concentration and purity of RNA were determined spectrophotometrically at $A_{260/280}$ nm. The purity of RNA obtained was 1.8-1.9. The yield of RNA was expressed in □g. Total RNA (2 µg) extracted from triceps muscle of control and experimental animals were reverse-transcribed by Qiagen One step RT-PCR kit, according to the manufacturer's instructions and further amplified by PCR. The details of the primers used, number of cycles and size of the PCR-amplified products are listed in Table 1. 10µl of each RT-PCR product was subjected for the analysis of the DNA by electrophoresis on 2% agarose gel. The molecular size of the amplified products (GLUT4, IR and β-actin) were determined by comparison with molecular weight marker (100bp DNA ladder), run parallel with RT-PCR products. Then the bands were detected by using gel documentation image scanner. The bands were quantified with quantity one software (Bio Rad) and normalized against the internal control (β-actin).

Table 1: Oligonucleotide Primers Used For RT-PCR

Name of the gene(rat)	Sequence of the primer	Number of cycles	Product Size(bp)	References
GLUT4	**Sense primer** 5'- GGG CTG TGA GTG AGT GCT TTC-3'	35	150	Liu et al., 2006
	Anti-sense primer 5'- CAG CGA GGC AAG GCT AGA-3'			
IR	**Sense primer** 5'- GCC ATC CCG AAA GCG AAG ATC-3'	35	224	Gonzalez et al., 2002
	Anti-sense primer 5'- TCT GGG TCC TGA TTG CAT-3'			
β-actin	**Sense primer** 5'- GCC ATG TAC GTA GCC ATC CA-3'	32	374	Oaks and Raff, 1995
	Anti-sense primer 5'- GAA CCG CTC ATT GCC GAT AG-3'			

Western blot analysis:

Isolation of plasma membrane and cytosolic fractions: Plasma membrane and cytosolic fractions from triceps muscle of control and experimental animals were prepared as described previously [Dombrowski et al., 1996]. Briefly, tissues were homogenized in buffer A containing 10 mM/l $NaHCO_3$ (pH 7.0), 250 mM/l sucrose, 5 mM/l NaN_3, protease inhibitor cocktail (Sigma Chemical Company, USA), and 100 µM/l phenylmethylsulfonyl fluoride (PMSF) using a Polytron-equipped homogenizer (Model PT 3000, Kinematica, Littau, Switzerland) at a precise low setting on ice. The resulting homogenate was clarified at $1,300 \times g$ for 10 min at 4°C. The resultant supernatant was centrifuged at $20,000 \times g$ for 30 min at 4°C. The pellet was resuspended in buffer A, applied on discontinuous sucrose gradients (25%, 32%, and 35%, wt/wt), and centrifuged at $1, 50,000 \times g$ for 16h at 4°C. Membranes at 25-32% (plasma membrane) and 32-35% (cytosolic fraction) interfaces were recovered, diluted with sucrose-free buffer A, and centrifuged at $1,90,000 \times g$ for 1h at 4°C. Pellets were resuspended in buffer A, and protein concentration was estimated [Lowry et al., 1951] using bovine serum albumin (BSA) as a standard. IR levels were estimated in plasma membrane and

GLUT4 levels were estimated in both plasma membrane and cytosolic fractions.

Preparation of tissue lysate: Tissues were homogenized in buffer containing 20mM Tris-HCl (pH 7.8), 300mM NaCl, 2mM ethylenediaminetetraacetic acid (EDTA), 2mM dithiothreitol (DTT), 2% NP-40, 0.2% SDS, 0.2% sodium deoxycholate, 0.5mM phenylmethylsulfonyl fluoride (PMSF), 50mM Sodium fluoride (NaF), 25mM sodium pyrophosphate, 40mM □-glycerophosphate, 2mM Sodium Orthovandate (Na_3Vo_4) and protease inhibitor cocktail (Sigma) using a polytron equipped homogenizer at a precise low setting on ice. The homogenate was centrifuged at $1,300 \times g$ for 10 min at 4ºC. The supernatant was centrifuged at $12,000 \times g$ for 15 min at 4°C. The resultant supernatant was sampled as the total protein. The protein concentration was determined by Lowry et al. [1951]. Briefly, each sample (25µg) was subjected to heat denaturation at 96°C for 5 min with Laemmli buffer. The proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and then transferred to PVDF membrane (Amersham Biosciences, UK). The membrane was blocked with 5% blocking buffer (Amersham Biosciences, UK) in TBS-T (Tris-Buffered Saline and Tween 20), for 1h at room temperature followed by incubation with primary antibody to insulin receptor, Akt1/2/3 and GLUT4 at a dilution of 1:1000. The membrane was subjected for repeated wash for 3 times with TBS-T and then incubated for 1h in horseradish peroxidase (HRP)-conjugated mouse/rabbit secondary antibody by 1:7500 dilutions in TBS-T. The membrane was again subjected for repeated wash for 3 times with TBS and TBS-T. The protein bands were visualized in Chemidoc using Enhanced Chemiluminescence Reagents (ECL; Amersham Biosciences, UK). The detected bands were quantified by using Quantity one software (Bio Rad). Later, the membranes were incubated in stripping buffer (50 ml, containing 62.5mM Tris HCl 62.5mM (pH 6.7), 1g SDS and 0.34 ml β-mercaptoethanol) at 55˚C for 40 min. Following this, the membrane was re-probed using a β-actin antibody (1:2000). In the present study, rat β-actin was used as the invariant control.

Estimation of glycogen: Glycogen was estimated by the method of Hassid and Abraham (1957). 5 mg of tissue was digested with 1 ml of 30% KOH for 20 min in a boiling water bath. The contents were cooled in an ice bath and 1.25 ml of 95%

ethanol was added, thoroughly mixed and gently brought to boil in a hot water bath. This was cooled and centrifuged at $1287.9 \times g$ for 15 min. The supernatant was decanted and the tubes were allowed to drain on a filter paper for few min. The precipitate was redissolved in 1 ml of distilled water, reprecipitated with 1ml of 95% ethanol, centrifuged and drained as stated before. The precipitate was dissolved in 5 ml distilled water and 10 ml of 0.2% anthrone reagent was added under ice-cold conditions. 5 ml of distilled water and series of standards with a final volume of 5 ml were treated with anthrone reagent and subjected to the same procedure. The tubes were covered with glass marbles and heated for 10 min, in boiling water bath. The contents were cooled immediately and the color developed was read at 680 nm. The amount of glycogen is expressed as mg/g wet tissue.

Statistical Analysis: The data were expressed as mean ± SEM. The data were subjected to statistical analysis using one-way analysis of variance (ANOVA), Tukey and HSD multiple range test to assess the significance of individual variations between the control and treatment groups using a computer based software (SPSS 7.5 for windows student version). In Tukey and HSD multiple comparison test, the significance was considered at the level of $p < 0.05$.

RESULTS
Dose-dependent effect of DEHP on free radical production and LPO in the triceps muscle: To gain insights into the free radical production due to exposure to DEHP, we examined the H_2O_2, OH- and LPO levels in the triceps muscle. DEHP treatment (10 and 100 mg) significantly increased these levels (Fig. 1A, B and C) when compared to control. Simultaneous administration of vitamins (C & E) proved to be beneficial in reducing the free radical production and LPO considerably.

Fig.1. Effect of DEHP and oral intubation of vitamins (C & E) on H_2O_2 generation (A), OH- production (B) and LPO (C) in the triceps muscle of adult male rat. Each bar represents Mean ± SEM of six animals (n=6). Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

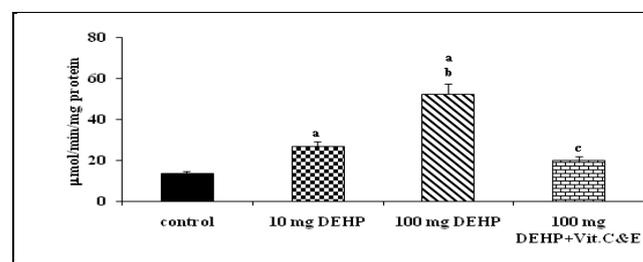**Fig.1A H_2O_2**

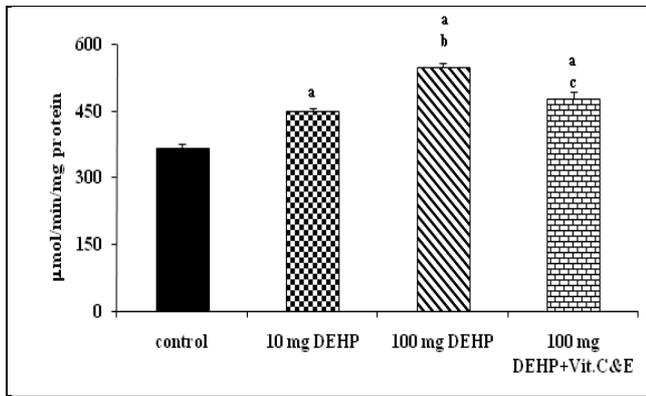**Fig.1B Hydroxylradical production**

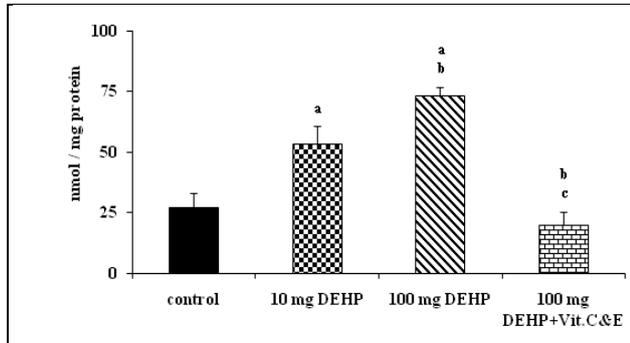**Fig. 1C Lipid peroxidation**

Estimation of ^{14}C-2-deoxy glucose uptake and ^{14}C-glucose oxidation in the triceps muscle:

The ultimate purpose of insulin signaling is stimulation of glucose uptake from the blood stream. We investigate the influence of DEHP on this process; ^{14}C-2-deoxy glucose uptake was studied. Both doses of DEHP significantly decreased the ^{14}C-2-deoxy glucose uptake (Fig. 2A) when compared to control. The rate of glucose oxidation in cells depends on the rate of entry of glucose into the cells. ^{14}C-glucose oxidation level (Fig. 2B) significantly brought down in DEHP (10 and 100 mg dose) treated rat. Vitamins (C & E) oral intubation prevented the DEHP-induced decrease in glucose uptake and oxidation.

Fig.2. Effect of antioxidant vitamin (C & E) against DEHP-induced changes in ^{14}C-2-deoxy glucose uptake (A) and ^{14}C-glucose oxidation (B) in the triceps muscle of adult male rat. Significance at $p < 0.05$, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.
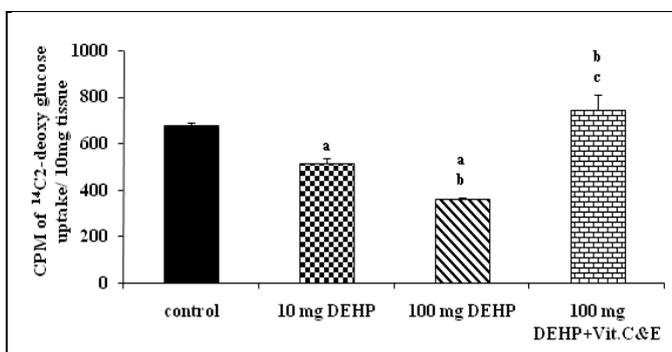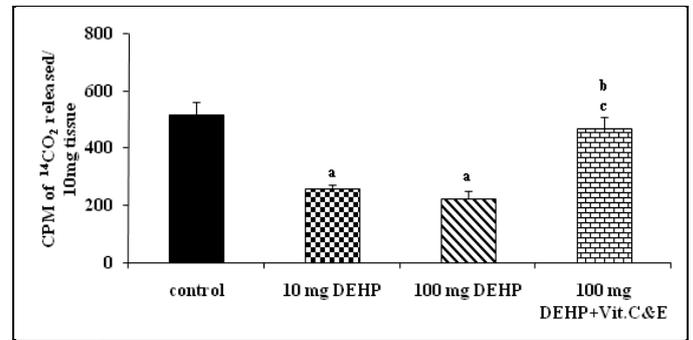

Fig. 2A ^{14}C-2-deoxy glucose uptake

Fig.2B ^{14}C glucose oxidation

Effects of DEHP on the IR gene expression in the triceps muscle:

Insulin is an essential hormone for maintaining whole-body glucose homeostasis. Insulin binds to its receptor, leading to receptor autophosphorylation and activation of downstream signaling molecules. We evaluate DEHP treatment, IR mRNA level (Fig. 3A) was not altered but IR protein (Fig. 3B) was significantly decreased compared to that of control. Due to vitamins (C & E) oral intubation, normal pattern of IR protein level was observed suggesting their protective effect.

Fig.3. Effect of antioxidant vitamins (C & E) against DEHP-induced changes in the IR mRNA (A) and protein (B) levels in the triceps muscle of adult male rat. Lane 1: 100bp marker; Lane 2: Control; Lane 3: 10 mg DEHP; Lane 4: 100 mg DEHP; Lane 5: 100 mg DEHP + Vit.C & E. Significance at $p < 0.05$, a-compared with control, c-compared with 100 mg DEHP.
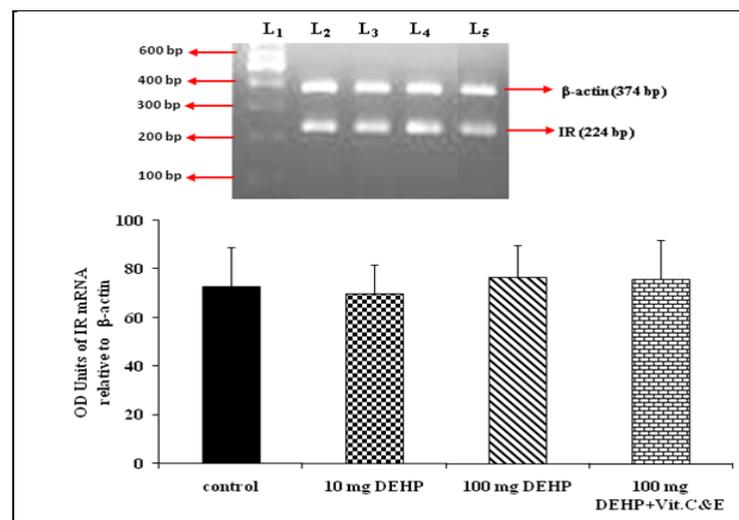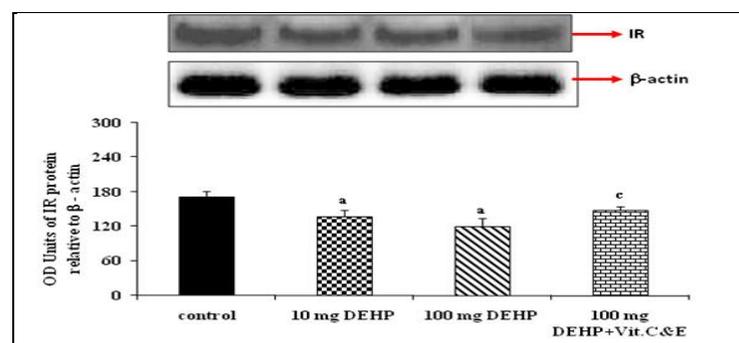

Fig. 3A IR mRNA level

Fig. 3B IR protein level

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Effects of DEHP on insulin signaling downstream molecule of Akt protein level in the triceps muscle:

Insulin, downstream of Akt/PKB activation to promotes glucose uptake into skeletal muscle to lower level of postprandial blood glucose, an enforced change in cellular metabolism to maintain glucose homeostasis. We next examined the expression of Akt protein (Fig. 4). DEHP treatment as well as administration of vitamins (C & E) had no effect on the Akt protein expression.

Fig.4. Effect of antioxidant vitamins (C & E) against DEHP-induced changes in Akt protein levels in the triceps muscle of adult male rat. Significance at p<0.05.
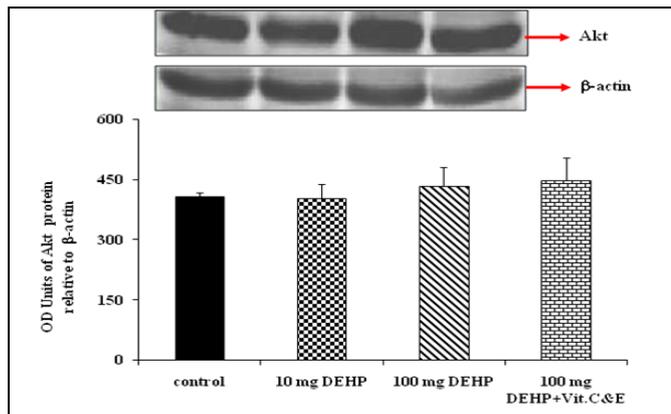
**Fig.4 Akt protein level**

## Effects of Antioxidant vitamins and DEHP on GLUT4 expression in the triceps muscle:

GLUT4 is a 12-transmembrane facilitative glucose transporter, primarily expressed in muscle and adipose tissues. It is responsible for insulin-stimulated glucose disposal and for the entry of glucose to muscles during contraction and exercise. The dysfunction of insulin-stimulated GLUT4 translocation is highly related to peripheral insulin resistance and non-insulin-dependent diabetes mellitus in human beings. We next performed the GLUT4 gene expression. GLUT4 mRNA (Fig. 5A) level and cytosolic GLUT4 protein (Fig. 5B) expression was not altered due to all treatment groups but in 100 mg DEHP treatment caused a significantly decreased the plasma membrane GLUT4 protein (Fig. 5C) when compared to control. Vitamins (C & E) supplementation maintained the plasma membrane GLUT4 protein level at par with control rats.

Fig.5. Effect of antioxidant vitamins (C & E) against DEHP-induced changes in the GLUT4 mRNA (A), cytosolic GLUT4 protein (B) and plasma membrane GLUT4 protein levels (C) in the triceps muscle of adult male rat. Lane 1: 100bp marker; Lane 2: Control; Lane 3: 10 mg DEHP; Lane 4: 100 mg DEHP; Lane 5: 100 mg DEHP + Vit.C & E. Significance at p<0.05,
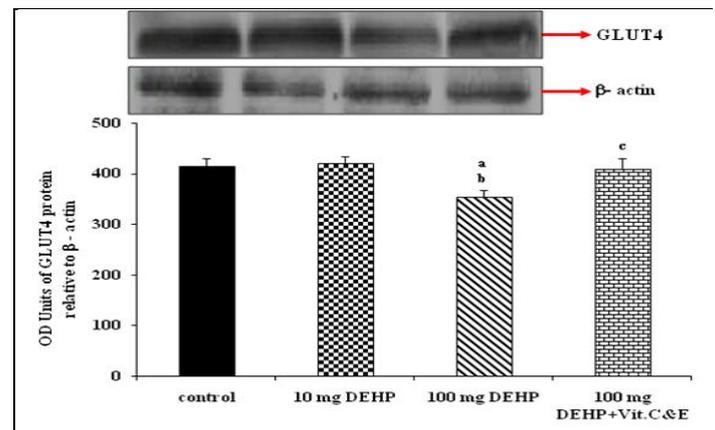
a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.
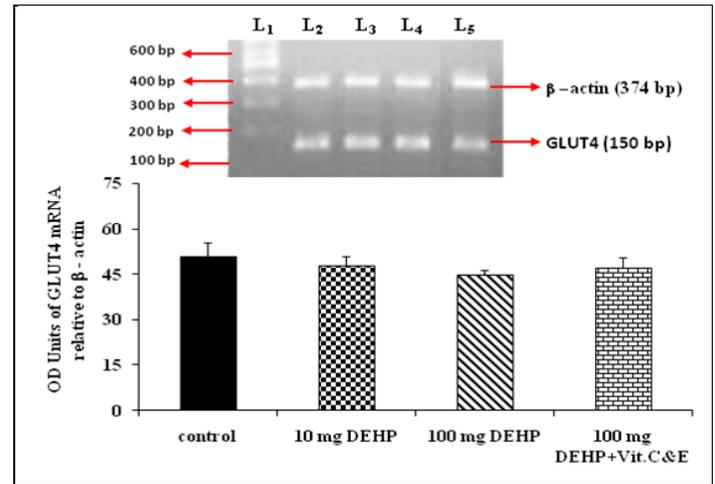
Fig. 5A GLUT4 mRNA level
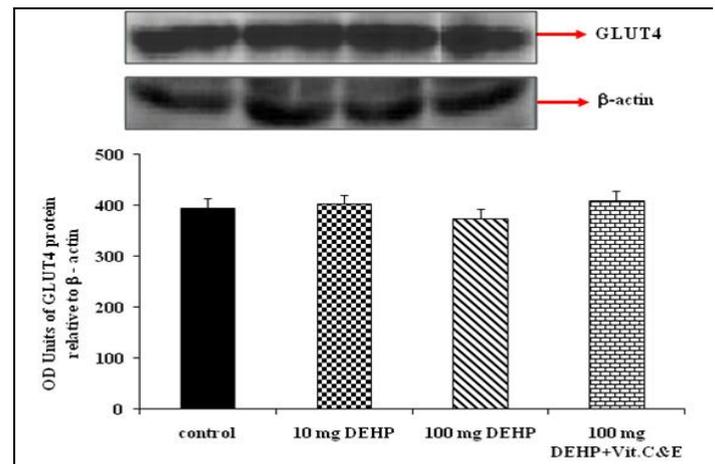
Fig. 5B Cytosolic GLUT4 protein level

Fig. 5C Plasma membrane GLUT4 protein level

Determination of glycogen level in the triceps muscle: Insulin is a one of the vital role on intracellular metabolism and its ability to stimulate the synthesis of glycogen in muscle. We next determined the glycogen level in DEHP-fed rats. DEHP exposure significantly decreased the glycogen level (Fig. 6). However, the co administration of vitamins (C & E) prevents deleterious effects of DEHP-induced changes in muscle glycogen level.

Fig.6. Effect of antioxidant vitamins (C & E) against DEHP-induced changes in glycogen level in the triceps muscle of adult male rat. Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.
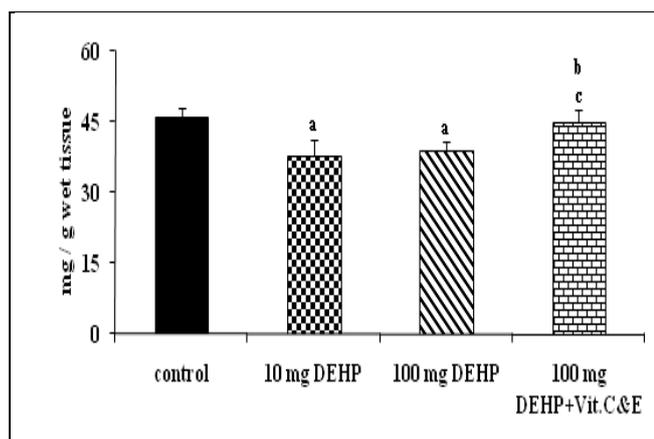

Fig.6 Glycogen level

DISCUSSION

Insulin is an essential hormone and plays a vital role in maintaining of whole-body glucose homeostasis. In normal individuals, increased plasma glucose level stimulates the secretion of insulin from the β-cells of the pancreatic islets (Kahn, 1994), which in turn stimulates glucose transport into peripheral tissues. The important aim of this study, DEHP induced changes in insulin signaling downstream molecules and reduced glucose uptake as well as oxidation leads to increased blood glucose level with the consequence of type-II diabetes. Insulin receptor is composed of 2 extracellular □-subunits and two transmembrane □-subunits linked together by disulphide bonds. Binding of the insulin to the subunits induces conformational changes resulting in the autophosphorylation of a number of tyrosine residues present in the □-subunits (Van Obberghem et al., 2001).These residues are recognized by phosphotyrosine biding (PTB) domains of adapter proteins such as members of the insulin receptor substrate family (IRS). Receptor activation leads to the phosphorylation of key tyrosine residues on IRS proteins, some of which are recognized by the SH2 domain of p85 regulatory subunit of PI3- kinase. The catalytic subunit of PI3- kinase p110, then phosphorylate phosphatidyl inositol (4, 5) biphosphate leading to the formation of phosphatidyl inositol (3, 4, 5) triphosphate. A key downstream effector of phosphatidyl inositol (3, 4, 5) triphosphate is Akt otherwise known as PKB (Lizcano and Alessi, 2002). Upon acute insulin stimulation, GLUT4 transporters are rapidly translocated from an intracellular vesicular compartment to the plasma membrane, and mediate the bulk of glucose transport activity (Bryant et al., 2002).

In the present study, 10 and 100mg DEHP treatment significantly increased the hydrogen peroxide (H_2O_2) generation. Vitamins (C & E) supplementation significantly reduced the hydrogen peroxides attesting their antioxidant property which quench the free radical produced by DEHP. Phthalate induces free radical production *in vivo* by activating NADPH oxidase complex that generate superoxide anion (Rusyn et al., 2001). Super oxide anion was rapidly transformed to H_2O_2 and then to hydroxyl radical. DEHP treatment significantly increased the hydroxyl radical generation but vitamins supplementation significantly scavenged the hydroxyl radicals. An increased production of H_2O_2 would lead to formation of highly Reactive Oxygen Species (ROS). ROS generation in living organism can react with macromolecules like lipids, proteins, nucleic acid and carbohydrate and can damage the structure and function of the same (Barros et al., 2006; She et al., 2017). DEHP treatment significantly increased the lipid peroxidation but supplementations of vitamins markedly decreased the lipid peroxidation suggesting their beneficial effect against DEHP-induced oxidative damages. It is supported by the previous studies that direct exposure of DEHP treated animals induces more oxidative stress and decline in antioxidants defense system (Santhosh et al., 1998; Popovic et al., 2006; Jain et al., 2009).

Glucose uptake is the crucial step in muscle which involved in disposal of blood glucose and maintains glucose homeostasis. Glucose oxidation is an important process which provides energy to the cells to perform various functions. The rate of glucose oxidation depends on the entry of glucose in to the cells (Joost and Thornes, 2001). Earlier study from our laboratory has shown decreased glucose uptake and oxidation in the gastrocnemius muscle, liver and adipose tissue of DEHP treated adult male rat (Srinivasan et al., 2011; Rajesh et al., 2013; Srinivasan and Balasubramanian, 2013). However, there are no reports on the effect of DEHP in triceps muscle. In the present study, glucose uptake and oxidation was significantly decreased in the DEHP treated rats. Vitamins (C & E) supplementation maintained the glucose uptake and oxidation at control level. In view of these findings, it is suggested that decreased glucose oxidation may be due to impaired glucose uptake as a result of decreased membrane GLUT4.

The rate of glucose uptake is determined by insulin, which is initiated by its binding to the extracellular domain of the insulin receptor (Zaid et al., 2008). A follow-up study showed that no profound effect was observed in the expression of IR mRNA as a result of DEHP treatment. Nevertheless, IR protein expression was significantly decreased in DEHP treated rats. Concomitant supplementation of vitamins (C & E) maintains the IR protein when compared to control level. DEHP-induced lipid peroxidation has been recognized to disrupt the plasma membrane. Since insulin receptor is a membrane bound protein, DEHP-induced lipid peroxidation might have disturbed the plasma membrane that results in decreased insulin receptor protein. In accordance with this, previous study from our laboratory also showed a reduction in IR of Chang liver cells exposed to DEHP *in vitro* (Rengarajan et al., 2007).

The Akt/PKB is one of the insulin signaling molecules and the control of cellular metabolism (Jiang et al., 2003). As such, the current study investigated the expression of Akt protein expression level. DEHP treatment had no effect on the Akt protein level. We previously reported that DEHP treatment decreases the Akt protein expression in gastrocnemius muscle of male rat (Srinivasan et al., 2011). A possible explanation for the unaltered level of Akt protein recorded in this study may be the result of tissue specific influence of DEHP.

GLUT4 is the primary transporter for glucose uptake in muscle and adipose tissue (James et al., 1988). Zhou et al. (2007) demonstrated that defects in GLUT4 expression and translocation associated with acquired insulin resistance of glucose uptake, observed in diabetes. We next performed a GLUT4 mRNA level and it was not significantly altered in the DEHP treated rats. This study provides evidence that PPARγ represses transcriptional activity of the GLUT4 promoter via direct and specific binding of the PPARγ/RXR, heterodimer to a -66/+163 bp of GLUT4 promoter region (Armoni et al., 2003). In particularly, DEHP and its metabolites MEHP induces PPARγ in a time and dose-dependent manner in HRP-1 cells (Xu et al., 2005). However, to our knowledge, further studies on PPARγ and RXR levels and the stability of GLUT4 mRNA would be informative to resolve the issue.

DEHP administration interfered with the expression of plasma membrane GLUT4 protein level. Vitamins (C & E) supplementation maintained the same at control level. The reduced membrane GLUT4 may partly be due to impaired GLUT4 translocation from cytosol to plasma membrane. GLUT4 is a membrane bound protein and are likely to get affected once the membrane integrity is lost (Joost and Thorne, 2001). DEHP is shown to induce lipid peroxidation (Santhosh et al., 1998) and this might have altered the membrane integrity of myocytes resulting in reduced membrane bound GLUT4. Siddiqui et al. (2006) reported the increased level of lipid peroxidation in diabetic rats and decreased membrane fluidity. Emerging evidence indicates that DEHP exposure induces can impair pancreatic beta cells (INS-1 cells) function, which is associated with insulin resistance and type-II diabetes (She et al., 2017). Moreover, previous studies have shown that decreases serum insulin level in rat, this may be due to reduced membrane bound GLUT4 level (Gayathri et al., 2004; Srinivasan et al., 2011).

Lastly, DEHP administration decreased the glycogen level but concomitant supplementastion of vitamins (C & E) restore the control level. These results associated with decreased serum insulin, glucose uptake and oxidation, the elevation of blood glucose level (Srinivasan et al., 2011). However, Martenelli et al. (2006) recorded a significant reduction in glycogen concentration in DEHP-treated rat. Previous work has examined the DEHP and its metabolites MEHP defective GSK3β^{Ser9} phosphorylation and the decreased glycogen synthesis in cultured L6 myotubes (Viswanathan et al., 2017).

In conclusion, our findings demonstrates that DEHP fed male rat induces ROS and lipid peroxidation, lowers glucose uptake and oxidation in triceps muscle due to impaired insulin signal transduction acquire with defective translocation of GLUT4 from cytosol to plasma membrane. Concomitant supplementation of antioxidant vitamins (C & E) have significant protective role against the adverse effect of DEHP-induced changes in triceps muscle. Emerging evidence indicates that impaired insulin signaling due to DHEP exposure may be one of the reasons for the onset of insulin resistance/type-II diabetes in humans and antioxidant vitamins (C & E) act as a scavenging effect of DEHP-induced changes.

CONFLICT OF INTEREST: The authors declare no potential conflicts of interests.

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