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ORIGINAL RESEARCH ARTICLE

Protective Effects of Antioxidant Vitamins against Diethyl Hexyl Phthalate-Induced Changes in Insulin Signaling Molecules in Adult Wistar Rats

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ABSTRACT

Diethyl hexyl phthalate (DEHP) is an environmental endocrine disrupting chemical and a widely used plasticizer which influences various organ systems in human beings and animals. DEHP is known for its reproductive toxicity and it also causes variable effects on liver. However, effects of DEHP on insulin signaling and glucose uptake in liver are not known. 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); liver was dissected out and subjected to assessment of various parameters. Our results suggest that DEHP treatment induces lipid peroxidation, defective glucose uptake and oxidation in liver due to impaired insulin signal transduction. DEHP enhanced the GLUT2 expression in liver and favours hepatic glycogenolysis. Oral intubations of vitamins (C and E) possess a protective role against DEHP-induced changes.

Key words: Liver, DEHP, Vitamin C and Vitamin E.

Abbreviations: DEHP, diethyl hexyl phthalate; IR, insulin receptor; IRS, insulin receptor substrate; AS160, Akt substrate 160; GLUT2, glucose transporter protein2; LPO, lipid peroxidation; MDA, Malondialdehyde; H_2O_2, Hydrogen peroxide; OH-, Hydroxyl radical.

1. INTRODUCTION

Liver plays a central role in maintaining glucose homeostasis. Under fasting condition, hepatic glucose production (HGP), which represents 90% of total glucose release, is regulated by insulin by direct and indirect mechanisms. Conversely, glucose utilization by the liver is reduced to minimum (8% of total glucose utilization). [1] reported that the deregulation between glucose uptake and production by the liver contributes to the onset of type-2 diabetes.

Insulin facilitates glucose clearance from the blood by activating glucose uptake into peripheral tissues as well as through the inhibition of hepatic glucose production [2]. Insulin receptor (IR) is expressed in almost all mammalian tissues, highest concentrations are found in insulin target tissues like muscle, adipose tissue and liver [3]. The IR exists as a heterotetrameric (α2β2) structure with the α-subunits containing the insulin binding site and the β-subunits holding the transmembrane domain with tyrosine kinase activity [4]. Insulin binding to the extracellular portion of IR leads to autophosphorylation of specific intracellular tyrosine residues. Once phosphorylated, IR is able to phosphorylate and activate intermediate docking proteins such as insulin receptor substrate (IRS)-1,-2,-3 and -4 which initiate internalization or activation of PI3 kinase which results in phosphorylation of phosphatidylinositol-4, 5 bisphosphate to form phosphatidylinositol-3,4,5-triphosphate; this in turn, activates ser/thr kinase i.e. phosphoinositide dependent kinase-1 (PDK-1) [5,6]. Activated PDK-1 phosphorylates Akt/PKB (ser/thr kinase). Akt

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plays a central role by phosphorylating one of its substrates, AS160 which is essential for effective translocation of glucose transporter protein 2(GLUT2) to the plasma membrane, for the transport of glucose into the cell [7].

Diethyl hexyl phthalate (DEHP) is an aromatic diester widely used as plasticizer in polyvinyl chloride (PVC) resins for fabricating flexible vinyl products [8]. DEHP containing plastics are also commonly used in hospital supplies such as blood storage bag, transfusion and dialysis tubes [9]. It is an environmental endocrine disruptor which has been suspected to confer toxic effects on the reproductive organs in humans and animals [10]. Administration of DEHP to adult male rats interfered with carbohydrate metabolism by reducing the blood glucose utilization, hepatic glycogenesis and glycogenolysis [11]. The DEHP-fed rats had altered glucose tolerance, associated with abnormal glucose intermediate metabolites in liver and skeletal muscle. Reduction in muscle glucose, lactate transport, hexokinase, hepatic glucokinase activities and glycogen synthesis were also recorded in DEHP-fed rats [12]. Oral administration of DEHP to rats significantly increased the serum marker enzymes, the level of total bilirubin and hepatic lipid peroxidation [13]. A previous study from our laboratory showed that DEHP has adverse effects on insulin receptor and glucose oxidation in Chang liver cells in vitro suggesting that DEHP exposure may have a negative influence on glucose homeostasis [14]. [15] have reported that phthalates exposure may contribute to obesity, insulin resistance and related clinical disorders in adult U.S. males.

Vitamin C is an essential micronutrient required for normal metabolic functioning of the body as an antioxidant. Vitamin C may help to prevent the oxidative damage to organs such as eyes, brain and kidneys that frequently occur in type-2 diabetes [16]. Vitamin E interacts with the cell membrane, traps free radicals and inhibits reactive oxygen species-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of polyunsaturated fatty acid in membrane phospholipids. It acts as a donor antioxidant (reductant) reacting with peroxyl radicals to inhibit the propagation of lipid peroxidation (LPO) in the cell membrane by scavenging peroxyl (RO*) and alkoxyl (ROO*) radicals [17]. [18] reported that administration of vitamin E prevents DEHP-induced deleterious effects, such as degenerative changes in the brain and thyroid.

The literature survey shows that DEHP has an adverse effect on endocrine and other organs and it affects the normal metabolism in experimental animals and human beings. However, the effect of DEHP on insulin signaling molecules and glucose uptake in liver remains elusive. Therefore, the present study was designed to address the DEHP-induced changes in insulin signaling molecules, glucose uptake and oxidation, GLUT2 and glycogen level in liver. In addition to this, the protective role of antioxidant vitamins (C and E) against DEHP-induced changes was also assessed.

2. 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. Glucose estimation kit was supplied by Linear Chemicals, Barcelona, Spain. ^{14}C-glucose, ^{14}C-2-deoxyglucose and [^{125}I] 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), IRS-1, GLUT2, RPL-19, β-actin primers and the β-actin monoclonal antibody were purchased from Sigma (USA). Polyclonal insulin receptor -subunit, IRS-1, phospho IRS-1(ser 636/639), Phospho IRS-1 (tyrosine 632), Akt1/2/3 and phospho Akt (ser 473), β-Arrestin-2, and GLUT2 antibodies were purchased from Santa Cruz Biotechnology (USA). Akt Substrate160 (AS160) monoclonal antibody was purchased from Cell Signaling technology (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); liver was dissected out and subjected to assessment of various parameters.

Determination of reactive oxygen species

Lipid peroxidation (LPO) was measured by the method of [19]. The malondialdehyde (MDA) content of the sample was expressed as nM of MDA formed/mg protein. Hydrogen peroxide generation (H_2O_2) was assessed by spectrophotometric method of [20]. The H_2O_2 content was expressed as µM/min/mg protein. Hydroxyl radical (OH-) production was quantified by the method of [21] expressed as µM/min/mg protein.

Glucose uptake

^{14}C-2-deoxyglucose uptake in tissues was estimated by the method of [22]. Briefly, after the control and experimental rats were anesthetized, liver 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 [23]. 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.

Insulin receptor assay

Insulin receptors were quantified as per the method published by [24]. Briefly, 100mg of liver was homogenized in 2.5-fold 0.001M $NaHCO_3$ and centrifuged at 600×*g* for 30 min. The supernatant was centrifuged for 30 min at 20,000×*g*. The membrane was washed twice with 0.001 M $NaHCO_3$. The final pellet was resuspended in 0.04 M Tris-HCl buffer (pH 7.4) containing 0.1% BSA. All procedures mentioned above were carried out at 4°C. Protein concentration was estimated [Lowry *et al*., 1951] using BSA as a standard. Membrane preparation (100µg protein) was incubated at 4°C for 16h with increasing concentration of ^{125}I-labeled porcine insulin in a final volume of 0.5 ml of 0.04M Tris buffer (pH 7.4) containing 0.1% BSA. Nonspecific binding was determined in the presence of excess (1000-fold) unlabeled insulin. Bound and free fractions of insulin were separated by centrifugation at 20,000×*g* for 10 min and then the radio activity of the pellets was determined using a gamma counter. Total number of receptors as a binding capacity was determined by the Scatchard analysis [25]. The receptor concentration is expressed as fmol/mg protein.

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 liver 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 (GLUT2, IR, IRS-1, β-actin and RPL-19) 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 or RPL19).

Western blot analysis

Isolation of plasma membrane and cytosolic fractions

Plasma membrane and cytosolic fractions from liver of control and experimental animals were prepared as described previously [26]. 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×*g* for 10 min at 4°C. The resultant supernatant was centrifuged at 20,000×*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×*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×*g* for 1h at 4°C. Pellets were resuspended in buffer A, and protein concentration was estimated [27] using bovine serum albumin (BSA) as a standard. IR levels were estimated in plasma membrane and GLUT2 levels were estimated in both plasma membrane and cytosolic fractions.

Preparation of tissue lysate

Tissues were homogenized in buffer containing 20 mM Tris-HCl (pH 7.8), 300 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 2% NP-40, 0.2% SDS, 0.2% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM Sodium fluroide (NaF), 25 mM sodium pyrophosphate, 40 mM β-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×*g* for 10 min at 4ºC. The supernatant was centrifuged at 12,000×*g* for 15 min at 4°C. The resultant supernatant was sampled as the total protein for IRS-1, phospho IRS-1, Akt, phospho Akt, AS160 and β-Arrestin-2.

Table 1: Oligonucleotide primers used for RT-PCR.

Name of the gene(rat)		Sequence of the primer	Number of cycles	Product size(bp)	References/ Accession number
GLUT2	Sense primer	5'-TTTCAGACAGCTGGCATCAGCC-3'	32	396	NM012879.2
	Anti-sense primer	5'-GAGGAAGTCCGCAATGTACTGG-3'			
IR	Sense primer	5'-GCCATCCCGAAAGCGAAGATC-3'	35	224	[52]
	Anti-sense primer	5'-TCTGGGTCCTGATTGCAT-3'			
IRS-1	Sense primer	5'-GCCAATCTTCATCCAGTTGCT-3'	37	336	[53]
	Anti-sense primer	5'-CATCGTGAAGAAGGCATAGGG-3'			
β-actin	Sense primer	5'-GCCATGTACGTAGCCATCCA-3'	32	374	[54]
	Anti-sense primer	5'-GAACCGCTCATTGCCGATAG-3'			
RPL-19	Sense primer	5'-CTGAAGGTCAAAGGGAATGTG-3'	35	194	[55]
	Anti-sense primer	5'-GGACAGAGTCTTGATGATCTC-3'			

The protein concentration was determined by [27]. 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, IRS-1, p-IRS-1$^{Tyr\ 632}$, Akt1/2/3, p-Akt^{Ser473}, AS160, β-Arrestin-2 and GLUT2 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 [28]. 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×*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 $p<0.05$.

3. RESULTS

Effects of DEHP on free radical production and LPO in the liver

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 liver. These levels were significantly increased (Figs. 1A, B and C) due to DEHP treatment when compared to control. Oral intubation of antioxidant vitamins (C and E) proved to be beneficial in reducing the free radical production and LPO considerably.

^{14}C-2-deoxy glucose uptake and ^{14}C-glucose oxidation in the liver

The ultimate purpose of insulin signaling is stimulation of glucose uptake from the blood stream. To analyze the influence of DEHP on this process, ^{14}C-2-deoxy glucose uptake was studied. 100 mg DEHP significantly decreased the ^{14}C-2-deoxy glucose uptake (Fig. 2A) but 10 mg had no effect. The rate of glucose oxidation in cells depends on the rate of entry of glucose into the cells. 100 mg DEHP alone brought down the ^{14}C-glucose oxidation level (Fig. 2B) significantly. Vitamins (C and E) oral intubation prevented the DEHP-induced decrease in glucose uptake and oxidation.

Effects of DEHP on the IR concentration and gene expression in the liver

DEHP (10 mg and 100 mg dose) caused a significant decrease in the insulin receptor concentration (Fig. 3A) in liver but vitamins oral intubation maintained it at control level. On DEHP treatment, IR mRNA level (Fig. 3B) was not altered but IR protein (Fig. 3C) was significantly decreased compared to that of control. Due to vitamins oral intubation, normal pattern of IR protein level was observed suggesting their protective effect.

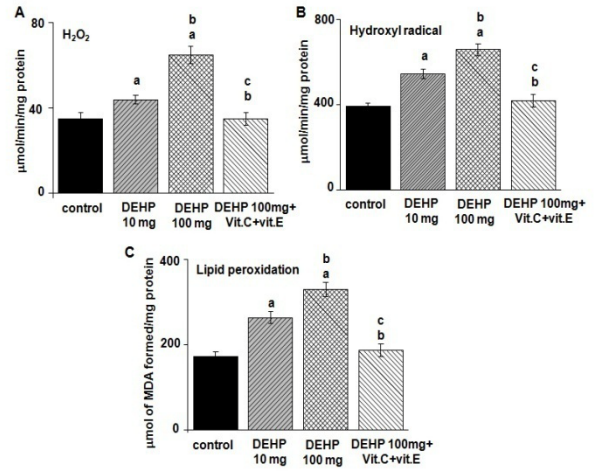

Fig.1. Effect of DEHP and oral intubation of vitamins (C and E) on H_2O_2 generation (A), OH- production (B) and LPO (C) in the liver of adult male rat. Each bar represents Mean ± SEM of six animals (n=6). Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100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

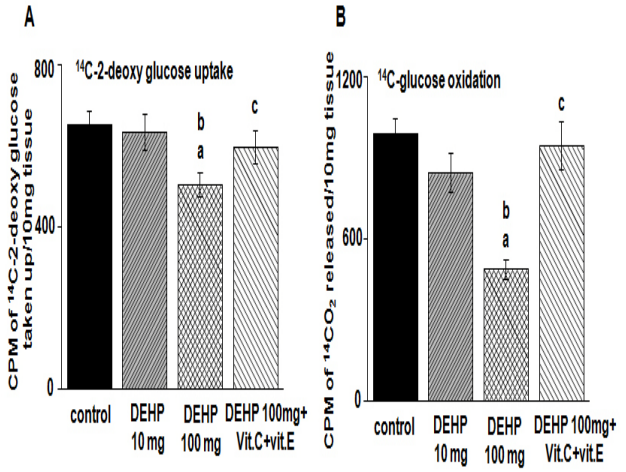

Fig.2. Effect of antioxidant vitamins (C and E) against DEHP-induced changes in ^{14}C-2-deoxy glucose uptake (A) and ^{14}C-glucose oxidation (B) in the liver of adult male rat. Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

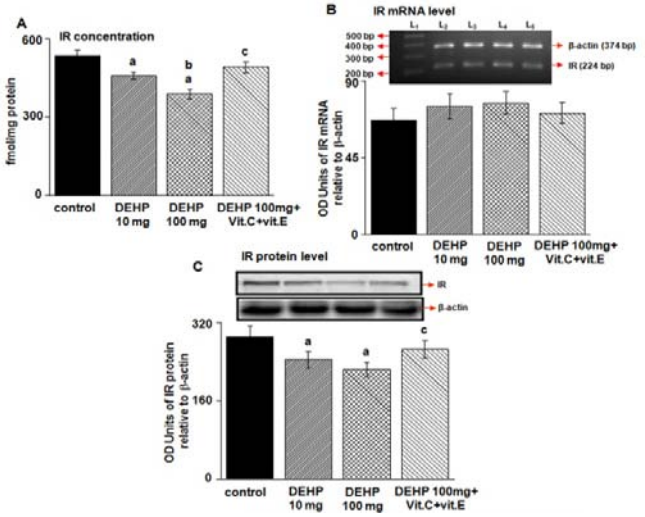

Fig.3.Effect of antioxidant vitamins (C and E) against DEHP-induced changes in the IR concentration (A), mRNA (B) and protein (C) levels in the liver of adult male rat. Lane 1: 100 bp marker; Lane 2: Control; Lane 3: 10 mg DEHP; Lane 4: 100 mg DEHP; Lane 5: 100 mg DEHP+Vit C and E. significance at p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

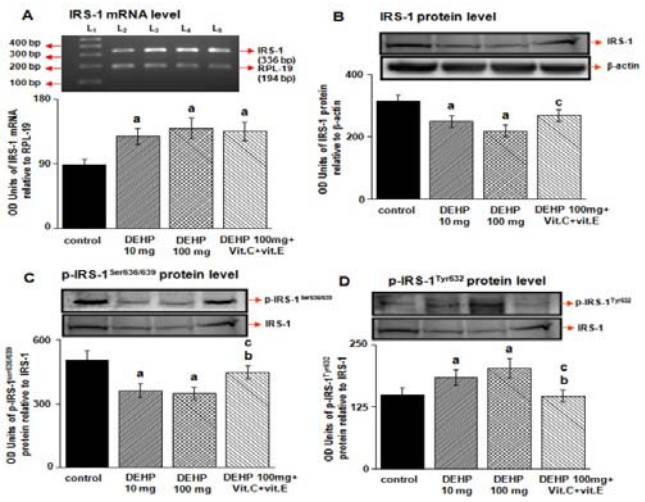

Fig.4. Effect of antioxidant vitamins (C and E) against DEHP-induced changes in IRS-1 mRNA (A) and protein (B), p-IRS-1$^{Ser636/639}$ (C) and p-IRS-1^{Tyr632} (D) protein levels in the liver of adult male rat. Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

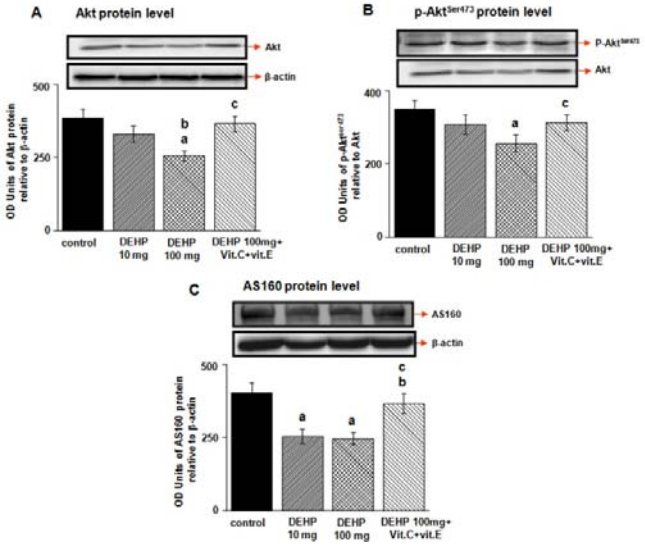

Fig.5. Effect of antioxidant vitamins (C and E) against DEHP-induced changes in Akt (A), p-Akt^{Ser473} (B) and AS160 (C) protein levels in the liver of adult male rat. Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

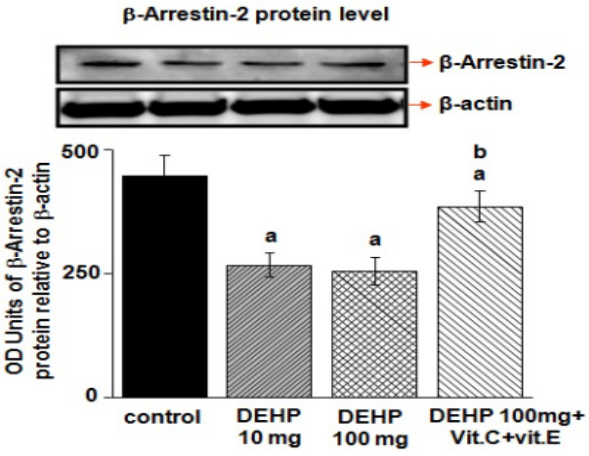

Fig.6. Effect of antioxidant vitamins (C and E) against DEHP-induced changes in β-Arrestin-2 level in the liver of adult male rat. Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

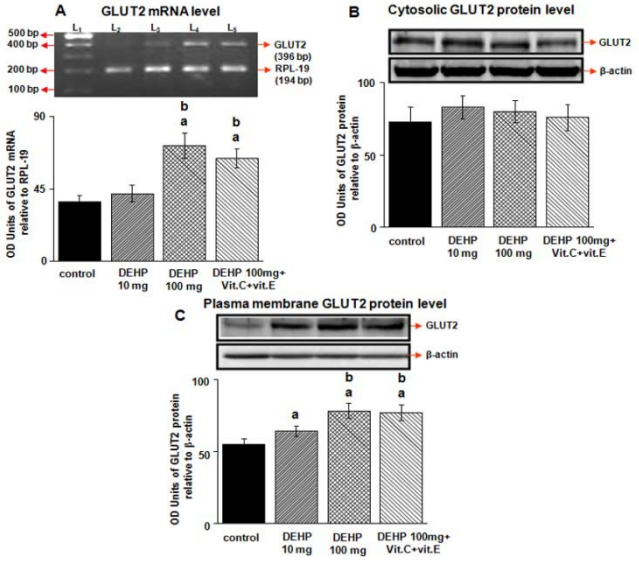

Fig.7. Effect of antioxidant vitamins (C and E) against DEHP-induced changes in the GLUT2 mRNA (A), cytosolic GLUT2 protein (B) and plasma membrane GLUT2 protein levels (C) in the liver of adult male rat. Lane 1: 100bp marker; Lane 2: Control; Lane 3: 10 mg DEHP; Lane 4: 100 mg DEHP; Lane 5: 100 mg DEHP + Vit.C and E. Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP.

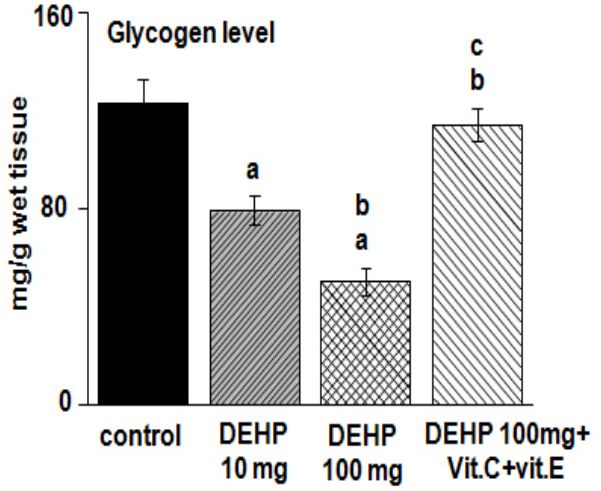

Fig.8. Effect of antioxidant vitamins (C and E) against DEHP-induced changes in glycogen level in the liver of adult male rat. Significance at p<0.05, a-compared with control, b-compared with 10 mg DEHP, c-compared with 100 mg DEHP.

Effects of DEHP on insulin signaling molecules in the liver

IR phosphorylation is followed by recruitment of IRS and its phosphorylation. DEHP (10 and 100 mg) treatment significantly increased the IRS-1 mRNA (Fig. 4A). Simultaneous administration of vitamins had no effect on IRS-I mRNA level. We next examined the expression of its protein. IRS-1 protein (Fig. 4B) level was significantly decreased in both 10 and 100 mg DEHP treated rat. However, administration of vitamins was able to prevent the diminution. Further study, DEHP (10 and 100 mg) treatment significantly diminished the p-IRS-1$^{Ser636/639}$ (Fig. 4C) but oral intubation of vitamins prevents the DEHP-induced decrease in p-IRS-1$^{Ser636/639}$. Further study, DEHP (10 and 100 mg) treatment significantly increased p-IRS-1^{Tyr632} protein (Fig. 4D) level. But, administration of vitamins along with DEHP partially maintains the protein level when compared with control. Akt protein (Fig. 5A) level and its phosphorylation of Akt (serine 473) (Fig. 5B) were significantly reduced in 100 mg DEHP treated rat. DEHP plus antioxidant vitamins maintained Akt protein level and phosphorylation of Akt (serine 473) when compared to control. DEHP treatment (10 and 100 mg) significantly decreased the AS160 (Fig. 5C) and □-arrestin-2 proteins (Fig. 6). The protective role of vitamins was evident with both AS160 and □-arrestin-2 proteins.

Effects of Antioxidant vitamins and DEHP on GLUT2 expression in the liver

GLUT2 is a family of facilitative glucose transporter which is highly expressed in liver. 100 mg DEHP treatment significantly increased the GLUT2 mRNA (Fig. 7A) level when compared to control. No alterations were seen in all treatment groups in cytosolic GLUT2 protein (Fig. 7B) level but DEHP (10 and 100 mg) treatment caused a significant increase in plasma membrane GLUT2 protein (Fig. 7C). Vitamins (C and E) oral intubation did not show any protective role in GLUT2 mRNA and protein levels.

Glycogen concentration in the liver

DEHP treatment (10 and 100 mg) lowered the glycogen concentration in the liver but oral intubation of vitamins (C and E) prevented the same (Fig. 8).

4. DISCUSSION

A number of recent studies support the idea that reactive oxygen species and the redox state of certain proteins play an important role in gene regulation. First we examined the beneficial effects of antioxidant vitamins, against DEHP-induced changes in lipid peroxidation, glucose uptake and oxidation, insulin signal transduction and glycogen level in liver of adult male albino rat. Our results demonstrate that DEHP treatment significantly rises the generation of H_2O_2, OH- and subsequent LPO. Evidence suggested that phthalate induces free radical production *in vivo* via NADPH oxidase complex activation that generates superoxide anion which is rapidly transformed to H_2O_2 and then to OH- [29]. Oral intubation of vitamins (C and E) significantly reduced the LPO level, attesting their antioxidant property which quenches the free radicals produced by DEHP. Decline in LPO levels were observed in DEHP induced hepatotoxicity after

the treatment with methanolic extract of *Apium graveolens* in rats [13].

Glucose uptake and oxidation are important processes which provide energy to the cells to perform various functions. The rate of glucose oxidation in a cell depends on the rate of entry of glucose into the cells. Glucose uptake and oxidation also depends upon glycogen synthesis and storage. The release of glycogen phosphorylase from glycogen partially accounts for the observed reversal in glycogenolysis. As glycogen breakdown diminishes during continued contraction, the unfavorable conditions for extracellular glucose uptake and utilization are dissipated. Glucose transport into the cell increase, correlated acutely with enhanced GLUT2 translocation to the plasma membrane, and upregulation of GLUT2 mRNA and protein levels [30]. In the current study, 100 mg of DEHP treatment significantly decreased the glucose uptake and oxidation whereas 10 mg dose had no such effect suggesting that DEHP treatment alters the rate of glucose uptake and oxidation levels in a dose-dependent manner. Simultaneous administration of vitamins (C and E) maintained them at control level. Insulin plays a central role in maintaining cellular glucose uptake and oxidation. Our recent study also showed an impaired glucose uptake and oxidation in gastrocnemius muscle as a result of decreased serum insulin level due to DEHP treatment [31]. In support of this, [32] have shown that DEHP treatment decreases serum insulin level in rat.

Insulin, the dominant glucose-lowering hormone stimulates glucose utilization by acting on insulin-sensitive tissues, thereby lowering the plasma glucose concentration. Effects of insulin on glucose uptake are mediated by efficient signal transduction process, which is initiated by its binding to the extracellular domain of the insulin receptor [33]. IR is the master switch of the signaling pathway of insulin and therefore, the effect of DEHP on IR concentration and IR gene expression were assessed. The concentration of IR was significantly reduced due to DEHP treatment whereas simultaneous oral intubation of vitamins (C and E) prevented the decrease in IR concentration. Reduction in the concentration of IR might be due to impaired expression of IR gene. Although the IR mRNA level was unaltered, there was a significant decrease in the IR protein level in plasma membrane which was maintained at control level by vitamins oral intubation. [14] have also shown that DEHP impairs the IR concentration in cultured Chang liver cells.

IRS proteins play a central role in the propagation of insulin signal. DEHP treatment significantly increased the IRS-1 mRNA, while vitamins oral intubation had no effect on IRS-I mRNA level. DEHP-mediated diminution in IR protein is associated with decreased IRS-1 protein. Since degradation of IRS-1 by ROS has been reported by [34], DEHP-induced ROS production probably degraded the IRS-1 in liver. Ser/Thr phosphorylation of IRS protein is a sensitive mechanism for downregulation of insulin signaling after insulin stimulation. DEHP treatment significantly diminished the phosphorylation of IRS-1$^{Ser636/639}$ but increased p-IRS-1^{Tyr632} protein level whereas, administration of vitamins along with DEHP maintained the protein at control level. IR mediated tyrosine phosphorylation of IRS-1 activates the metabolic signaling pathway by binding to the regulatory subunit (p85) of the PI3K. Ser636/639/307 is a well-recognized phosphorylation site in IRS-1 and the preponderance of evidence suggests that Ser636/639/307 phosphorylation can negatively influence insulin signaling [35]. In this regard, it is worth to recall the work of [36] who reported that Ser/Thr phosphorylation can have both a positive and a negative regulatory role on tyrosine phosphorylation of IRS-1 and IRS-2 by insulin and IGF-1 receptors. Moreover, Ser/Thr hypo- and hyperphosphorylated IRS proteins are poor substrates for the insulin receptor, whereas IRS-1 or IRS-2 phosporylated to an intermediate or basal level represents the optimal substrate; and some Ser/Thr phosphorylation of IRS-1 and IRS-2 is required for optimal tyrosine phosphorylation [36]. Therefore, the observed hyperphosphorylated tyrosine might have masked the necessary Ser phosphorylation sites thereby resulting in subsequent diminished downstream signaling.

A major downstream target of phosphoinositide 3-kinase is the serine/threonine protein kinase Akt (protein kinase B), which regulates diverse processes, including glucose homeostasis, transcription, apoptosis, cell motility, angiogenesis, proliferation and growth [37]. Akt is completely active on phosphorylation at the Thr$^{308/309}$ and Ser$^{473/474}$ residues [38]. In the present finding showed that DEHP diminished the Akt and p-Akt ^{Ser473} in liver whereas, on vitamins (C and E) oral intubation, the DEHP-induced changes in Akt and p-Akt ^{Ser473} levels were maintained at

control level. Therefore, it is suggested that DEHP-mediated diminution of IR and IRS-1 may be responsible for the impaired Akt ^{ser473} phosphorylation. In addition, rats exposed to DEHP in diet showed a significant increase in the peroxisomal fatty acyl-CoA oxidation in liver [39]. The peroxisomal □ -oxidation appears to be independent of an electron transport chain resulting in production of H_2O_2 by direct transfer of electrons to O_2. Any increased production of H_2O_2 would lead to formation of highly reactive oxygen species (ROS) and hydroxy (OH) free radicals [40]. MEHP, a metabolite of DEHP negatively regulates Akt protein levels through cross talk in Akt/NFkB signaling pathway a nd induces oxidative damage in germ cells [41]. H_2O_2 decreases Akt level which leads to impaired insulin signaling and induces oxidative stress in vascular smooth muscles which may be one of the reasons for atherosclerosis and hypertension [42].

Akt is responsible for insulin-stimulated phosphorylation of AS160. Insulin led to the rapid increase in phosphorylation of Akt, with a subsequent elevation in phosphorylation of potential Akt substrate protein, AS160, and these effects were induced by physiological insulin levels [43]. Insulin also activates Akt in skeletal muscle, where AS160 is expressed. Phosphorylation of AS160 is essential to trigger insulin-stimulated translocation of GLUT4 and the associated increase in glucose uptake in skeletal muscles [44]. Results of the present investigation suggest that DEHP impairs insulin signal transduction.

β-arrestin mediates biological functions of G-protein coupled receptor by linking activated receptors with distinct sets of accessory and effector proteins, thereby determining the specificity, efficiency and capacity of signals [45]. In the current investigation, DEHP treated rats showed significant low levels of β-arrestin-2 protein but the protective role of vitamins oral intubation was felt with □-arrestin-2 protein level. Knockdown of β-arrestin-2 exacerbates insulin resistance whereas; administration of β-arrestin-2 restores insulin sensitivity in mice [46]. Loss or dysfunction of β-arrestin-2 results in deficiency of this signal complex and impairs insulin signaling *in vivo* and thereby contributing to the development of insulin resistance and progression of type-2 diabetes [46]. DEHP-induced decrease in β-arrestin-2 in the liver suggests that it may lead to insulin resistance. In this regard, it is pertinent to recall the report of [47] who have shown that a decrease in β-arrestin-2 inhibits insulin-induced stimulation of the Akt pathway in the aorta of diabetic mice with hyperinsulinemia.

Hepatic glucose transport levels are determined by GLUT2, which is a facilitative glucose transporter [48]. [49] suggested that GLUT2 promoter contains glucose responsive elements. This was demonstrated in mhAT3F cells transfected with plasmids containing -338 to +49bp proximal region of GLUT2 promoter fused to the CAT gene. When -338 to +49bp proximal region of GLUT2 promoter were transfected, the reporter activity was 4 fold increased in the presence of glucose, indicating the regulatory role of glucose [49]. In this study, DEHP treated rats showed high plasma glucose level associated with upregulation of GLUT2 mRNA and protein levels but vitamins (C and E) oral intubation had no effect on the DEHP-induced changes in liver. It is therefore suggested that the DEHP-induced hyperglycemia may be one of the reasons for upregulated GLUT2 expression. GLUT2 upregulation is more critical in glucose export from the liver to maintain glucose homeostasis than in import [50]. In support of this study, [51] reported that in diabetic state, GLUT2 in the liver is upregulated, indicating that hyperglycemia may play a positive role in GLUT2 gene expression.

Glycogen content of the liver is the result of glycogenesis and glycogenolysis. In the current study, a significant decrease in glycogen content in DEHP-treated rats was observed. In this respect, it is worth to recall the report of [12] who have shown that DEHP-fed rats had an altered *in vivo* glucose tolerance associated with abnormal glucose intermediate metabolites in liver and skeletal muscle. In these rats, the hepatic content of glucose-6-phosphate (G-6-P), fructose-6-phosphate, pyruvate, lactate, glucose-1-phosphate and glycogen were decreased [12].

5. CONCLUSION

In essence, our results demonstrate that DEHP treatment induces ROS and lipid peroxidation, lowers glucose uptake and oxidation in liver due to impaired insulin signal transduction. Antioxidant vitamins (C and E) have significant protective role against the adverse effect of DEHP-induced changes in liver. It appears that the impaired insulin signaling due to DHEP exposure may be one of the reasons for the onset of insulin resistance/type-2 diabetes in humans.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors as far as this work is concerned.

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