THE EFFECT OF VITAMIN E AND DI METHYL DIPHENYL BICARBOXYLATE ON ARSENIC HEATOTOXICITY IN ADULT RATS

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ABSTRACT
Summary: Arsenic, a widely studied medicinal and toxicological element, is known to induce oxidative stress and damage cells. The present study aimed at assessing the effect of vitamin E (Alpha-tocopherol) and / or DDB (Dimethyl Diphenyl Bicarboxylate) with or without the chelator Mesot 2,3-dimercaptosuccinic acid (DMSA) on arsenic-induced hepatotoxicity. Fifty four adult male albino rats were divided into nine groups. Histochemical and histopathological parameters were undertaken to assess the structural changes of both intoxicated group and antioxidant-treated groups. The results of this study showed that vitamin E and DDB were almost similar in their antioxidant hepatoprotective effects while the greatest effect was achieved by their combination with a chelating agent (DMSA), with restoration of almost normal hepatic histology.

Key words: Vitamin E, Dimethyl diphenyl bicarboxylate, Arsenic Hepatotoxicity, Experimental Study
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INTRODUCTION
The metalloid arsenic (As) is an industrial element, medicinal agent, and homicidal poison. It also may unfortunately become an unintentional self-poison in contaminated drinking water (1). Arsenic may cause black foot disease and malignancies of skin, lung, liver and urinary bladder (1, 2, 3). Arsenite toxicity may be caused by the generation of the metabolic reactive oxygen species (ROS) or via reactions with intracellular thiols particularly vicinal dithiols (4). In human cells methylated inorganic arsenic is changed to monomethylarsonic acid (MMA) and dimethyl arsenic acid (DMA), while reduced glutathione (GSH) stimulates the methylation of arsenic and augments the excretion of DMA (5). Glutathione, the most abundant cellular non-protein thiol, exerts many physiological roles such as detoxification reactions, storage and transport of amino acids especially cysteine (6). The tripeptide reduced glutathione (GSH) is an essential antioxidant agent against many free radicals which may damage membrane proteins and lipids (7). GSH may form a complex with arsenite, a thiol-reacting element, and is excreted in bile as GSH complex, so the level of cellular GSH will be decreased (8). Glutathione peroxidase (GPx) plays a critical role in protecting the cell from free radicals damage particularly that related to lipid peroxidation (9). Alpha-tocopherol or vitamin E is the most important natural antioxidant working at the membrane level which is the first defense against free radicals-induced lipid peroxidation (10). Dietary vitamin E has a marked effect in delaying atherosclerotic progression in hyperlipidemic persons, hence its protective role against many degenerative diseases (11). Zinc, as a constituent of the antioxidant superoxide dismutase (SOD), is essential for maintenance of vitamin E in the blood by helping its absorption which augments tissue repair and wound healing (12). Glutathione and ascorbic acid can regenerate vitamin E in the liver after its oxidation (13). Dimethyl diphenyl bicarboxylate (DDB), extracted from seeds of (Schisandra, chinensis, endemic) is a beneficial antioxidant used in the treatment of many diseases of cardiovascular and neurological systems as well as diabetes mellitus, and neoplastic diseases. It is beneficial in liver disease especially viral hepatitis (14). It has been suggested to use DDB with other herbs to increase its therapeutic effects and prevent relapse (15). DDB enhances the hepatic mitochondrial glutathione redox status and mitochondrial glutathione reductase (mtGRD) and has a significant ability to suppress the hepatotoxic rise of the enzymes ALT and AST (14). DDB also inhibits hepatotoxic lipid peroxidation and decreases carbon monoxide production and cofactor (NADPH&O2) utilization in the liver microsomes (16). The aim of the present study was to investigate the antioxidant protective capability of...
arsenic-induced hepatotoxicity with regard to histopathological and histochemical parameters.

MATERIAL and METHODS: Fifty-four adult male Sprague-Dawley albino rats weighing 170-200 grams were bred at the animal house of the Histology Department, Faculty of Medicine, El Minya University, El Minya, Egypt, then experimented upon at Faculties of Medicine, El Minya University, Egypt and Faculty of medicine, Benghazi, Libya. The rats were fed with standard laboratory chow and water ad libitum for two weeks of acclimatization, then randomly divided into nine groups of 6 rats each. Group I rats; were the control group and received only an intra-peritoneal injection of 2 milliliters of normal saline twice a week for two weeks. Group II rats, were injected with sodium arsenite in normal saline at a dose of 80 mmol/kg body weight twice a week for 2 weeks. Group III rats, were injected with the same aforementioned dose of sodium arsenite and received DMSA orally through gavages at a daily dose of 50mg/kg body weight (17). Group IV rats; were injected the same aforementioned dose of sodium arsenite and received vitamin E (alpha-tocopherol) orally at a daily dose of 100 mg/kg body weight for 2 weeks. Group V rats; were injected the same aforementioned dose of sodium arsenite and received DDB orally at a daily dose of 150 mg/kg body weight for 2 weeks (18). Group VI rats; received the same aforementioned doses of sodium arsenate, vitamin E and DDB for 2 weeks. Group VII rats; received the same aforementioned doses of sodium arsenite, DMSA and vitamin E for 2 weeks. Group VIII rats, received the same aforementioned doses of sodium arsenate, DMSA and DDB for 2 weeks. Group IX rats, received the same aforementioned doses of sodium arsenate, DMSA, vitamin E and DDB for 2 weeks. On the fifteenth day of the experiment, the rats were anesthetized by ether inhalation and sacrificed. Liver biopsies were taken and fixed in 10% buffered normal saline, dehydrated in ascending grades of ethanol, cleared in xylene, infiltrated and embedded in paraplast paraffin wax. Blocks were cut into 5 millimetre thick sections, stained with Harris's, hematoxylin and eosin, histochemical periodic acid Schiff (PAS) stains, and finally photographed with an Olympus microscope digital camera (19).

RESULTS

The control group I showed a normal hepatic stroma and parenchyma where the radially-arranged hepatocytes appeared normal with rounded nuclei and normally dense cytoplasm. The central vein, hepatic blood sinusoids and portal tract showed normal distribution and regular continuous endothelium (Fig. 1A, B). The glycogen or polysaccharides content of the control group I appeared normally high with the histochemical PAS-reaction (Fig. 1C). The arsenic group II showed central lobular necrosis around the central veins with many vacuolated-necrotic hepatocytes with fatty infiltration, cell lysis, indistinct boundaries, pyknotic nuclei, and other nuclei which appeared fragmented (karyorrhexis) or even karyolyzed (Fig. 2A, B, C). Periportal (periportal) hepatocytes tended to appear slightly flattened with decreased granularity and density of the cytoplasm while some hepatocytes showed cytomegaly and karyomegaly. The damage around the portal tract (periportal or peribular) was less prominent than that in the centrilobular area. The blood sinusoids appeared dilated and some of the lining endothelial cells showed irregularity and necrosis while other areas showed focal infiltration with inflammatory cells. Hepatocytes manifested a very weak PAS-reaction (pale pink) especially at the centrilobular necrotic areas (Fig. 2D). Administration of DMSA to the arsenic-treated animals (group III) yielded less centrilobular necrosis with less incidence of vacuolations and less frequent nuclear pyknosis and karyolysis. Hepatocytes of the periportal areas showed mild improvement while the blood sinusoids and portal blood vessels showed mild congestion (Fig. 3A). The centrilobu-
lar areas showed a weak PAS-reaction while the periportal areas showed mild to moderate PAS-reaction (Fig. 3B). The treatment of the arsenic-intoxicated animals with alpha-tocopherol (vitamin E) i.e. group IV showed hepatocytes of the centrilobular areas with a more or less similar amount of vacuolations when compared to the arsenic group II, while the perilobular areas showed mild improvement of the affected hepatocytes (Fig. 4A). PAS-reaction was very weak at the centrilobular areas but it was moderate at the perilobular or periportal areas (Fig. 4B). The arsenic-treated animals when given DDB (group V) showed hepatocytes of the centrilobular areas with a low amount of vacuolations, and lower incidence of nuclear pyknosis and karyolysis while the perilobular areas showed a mild improvement of the affected hepatocytes (Fig. 5A). The centrilobular areas showed a weak PAS-reaction while this reaction was mild to moderate at the perilobular areas (Fig. 5B). The administration of both vitamin E and DDB to the arsenic-intoxicated animals (group VI) showed hepatocytes of the centrilobular areas with less vacuolations, nuclear pyknosis, karyorrhexis and karyolysis while the perilobular areas manifested moderate improvement of the affected hepatocytes (Fig. 6A). PAS-reaction was weak among the centrilobular areas while the perilobular areas showed mild to moderate reaction (Fig. 6B). The concomitant treatment of arsenic-intoxicated animals with DMSA and vitamin E (group VII) resulted in less centrilobular necrosis with a low amount of vacuolations, nuclear pyknosis, karyorrhexis and karyolysis. The perilobular areas showed moderate improvement of the affected hepatocytes with mild congestion of the blood vessels and sinusoids (Fig. 7).

The combination of DMSA and DDB treatment to the arsenic-intoxicated animals (group VIII) showed a very little amount of vacuolations at the centrilobular areas, less nuclear pyknosis, karyorrhexis and karyolysis with some degree of regeneration. The perilobular areas showed moderate improvement and regeneration of the affected hepatocytes with mild congestion of the portal blood vessels and sinusoids (Fig. 8A). PAS-reaction was moderate at the centrilobular areas while the reaction was good at the perilobular areas (Fig. 8B). The concomitant

Fig. 2A: A section in the liver of an arsenic-treated animal. Centrilobular necrosis with vacuolated (O) hepatocytes around central veins (C) while the perilobular (P) areas are less affected (x200, H&E).

Fig. 2B: A section in the liver of an arsenic-treated animal. Centrilobular necrosis with vacuolated (O) hepatocytes with fatty infiltration (F). Note the inflammatory cell infiltration (arrow) around the central (C) vein (x400, H&E).

Fig. 2C: A section in the liver of an arsenic-treated animal. Centrilobular necrosis with vacuolated hepatocytes (O) and abnormal acidophilic (D) due to increased binding capacity of the denaturated proteins to Eosin in contrast to the acidophilic of normal hepatocytes (N). Some endothelial cells lining the central vein (C) appeared injured, irregular and discontinuous (x400, H&E).

Fig. 2D: A section in the liver of an arsenic-treated animal. Faint hepatocytes granulation and density with very weak PAS reaction especially at the centrilobular areas (C) in contrast to the mild PAS reaction at the periportal areas (P) (x200, PAS)
treatment of the arsenic-intoxicated animals with DMSA, vitamin E and DDB (group 1X) resulted in a good regeneration of the centrilobular areas with less vacuoles and nuclear pyknosis, while the cytoplasm showed a normal density and acidophilia. The periportal (peripheral) areas showed marked regeneration with almost normal hepatic architecture and no cellular infiltration. Cytomegaly and karyomegaly almost disappeared. The hepatic sinusoids appeared normal with no endothelial injury while the portal blood vessels were mildly congested (Fig. 9A, 9B). The periportal areas manifested a highly positive PAS-reaction while the centrilobular areas showed good positive PAS-reaction (Fig. 9C, 9D), reaction (Fig. 9C, 9D).

DISCUSSION:
Arsenic, a double-edged weapon, is a medicinal element and an industrial agent but unfortunately a homicidal poison and unintentional self-poison in contaminated drinking water (20, 21). Arsenic toxicity may also come from E-waste (electronic disposal) resulting from the disposal of computers, televisions, mobile phones and other electronic devices which can reach the soil and groundwater or may become an airborne toxin after being incinerated (22). Arsenic is classified as a definite -Group 1- human carcinogen with strong evidence of causation of skin, liver, lung and urinary bladder cancers (23, 3, 21, 25, 24). Arsenic, particularly the inorganic trivalent form (As III) causes toxic effects to the parenchymal cells of many organs especially the liver, lung, skin, urinary bladder and peripheral blood vessels that may lead to dangerous black foot disease (BFD) and gangrene (1, 2). It was observed that arsenic and mercury deliver a large host of external and internal eye signs of toxicity, hence the consideration of utilizing the liver and the eye as beneficial clinical biomarkers to measure heavy metals toxicity (26). Arsenic exerts its toxicity by binding to sulphydryl groups on enzymes and other cellular proteins and also through reactions with intracellular thiols (27). Reduced glutathione (GSH) stimulates the methylation of inorganic arsenic to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) and augments excretion of the latter, hence the decreased GSH and cytochrome P-450 levels in the
liver and the increased lipid peroxidation with arsenic toxicity (28). Arsenic toxicity is also exerted via increasing the production of ROS i.e. reactive oxygen species (29). These previous studies encouraged us to formulate a study design and model to specifically define the role of two promising antioxidants with or without a chelating agent against arsenic-induced hepatotoxicity with the emphasis on histophysiological, histopathological and histochemical parameters. Arsenic toxicity whether acute, subacute or chronic, produces accumulation of arsenic in the liver and kidney which leads to elevation of hepatic enzymes and histopathological changes in the form of inflammatory infiltration, steatosis and necrosis (30). Glutathione, an antioxidant against lipid peroxidation is present in lower concentration at the centrilobular area in comparison with the perilobular area (31). Arsenic toxicity leads to Glutathione depletion which rendered the rat hepatocytes highly sensitive to cell death as being manifested in arsenic group II liver tissue. This makes the centrilobular area less protected against toxins than the perilobular area which may explain the incidence of centrilobular necrosis with vacuolations, cell lysis and pyknotic, fragmented or lysed nuclei of hepatocytes. These results are in agreement with previous studies (32) which concluded that arsenic increased lipid peroxidation and protein oxidation which both represent strong indices of oxidative stress. Some endothelial cells appeared slightly vacuolated, and irregular with indistinct cell boundaries which may be an explanation for the dilation noted in the blood sinusoids. This is in agreement with previous studies. (31). This endothelial injury explains the complication that may come out of arsenic toxicity – especially the chronic form – causing the peripheral vascular black foot disease (BDF) which usually leads to gangrene (2). Centrilobular hepatocytes and to a lesser extent perilobular ones showed vacuolations and fatty infiltration which appeared similar to steatosis. This has been confirmed by others in a previous study (30). Steatosis is a common cellular response to toxic insult and is normally reversible while its prevalence in the liver is particularly common as this organ has a major role in lipid metabolism (33). The present study showed the arsenic-intoxicated hepatocytes had a lightly...

Fig. (5A): A section in the liver of an arsenic-treated animal given DDB. Centrilobular (C) necrosis with less vacuolated hepatocytes around central vein (C) while the cells are less affected near the perportal (P) areas (x250), Hx & E

Fig. (5B): A section in the liver of an arsenic-treated animal given DDB. Centrilobular areas (C) showed weak PAS-reaction while the reaction is mild to moderate near the perilobular areas (P) (x200), PAS

Fig. (6A): A section in the liver of an arsenic-treated animal given Vit.E + DDB. Centrilobular (C) necrosis with some necrotic and vacuolated (O) hepatocytes having pyknotic nuclei (arrowhead). Focal inflammatory cell infiltration (arrow) is near the centrilobular area while the cells are less affected near the perilobular areas (P) (x600, Hx & E

Fig. (6B): A section in the liver of arsenic-treated animal given Vit. E + DDB. Centrilobular areas (C) showed weak PAS-reaction while the reaction is moderate near the perilobular areas (P) (x200, PAS

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stained cytoplasm, less density, decreased granulations and histochemically low glycogen content manifested by hematoxylin and eosin stain and PAS-reaction. This may be explained by loss of ribosomes from rough endoplasmic reticulum (RER) and swelling of mitochondria with loss of their cristae as the high metabolic activity and rapid membrane transport of mitochondria may expose them as a target to heavy metal toxicity (34). It is concluded that the upset in the structure and inhibition of enzymes in mitochondria might reduce their capacity to provide energy for active transport in the liver and kidney exposed to arsenic toxicity. The observed focal inflammatory cell infiltration especially in the centrilobular areas was seen in other studies (30, 35). Cytomegaly and karyomegaly seen among some of the arsenic-intoxicated hepatocytes may be explained by necrotic vacuolations that expand the cytoplasm. However, karyomegaly is more likely explained by perturbations in DNA synthesis accompanying chemical or toxic-induced cellular proliferation. The administration of DMSA to arsenic-intoxicated animals expressed less necrotic parameters among the centrilobular areas with weak PAS-reaction while the perilobular areas showed mild improvement of the affected hepatocytes with mild to moderate PAS-reaction. This confirms the role of chelators in lowering the toxic effects of heavy metals to the extent of being used as antidotes in such cases in spite of their lacking an antioxidant role (17). Alpha-tocopherol (vitamin E) is the most important natural antioxidant working at membrane level (10). Vitamin E administration to the arsenic group of animals revealed almost no effect among the centrilobular necrotic areas which showed a very weak PAS-reaction, while the perilobular areas showed mild improvement of the affected hepatocytes with moderate PAS-reaction. This indicates the protective effect of the antioxidant vitamin E (on the less affected perilobular areas) rather than a regenerative effect as manifested by non improvement of the heavily affected necrotic centrilobular areas. This result is contradictory to some previous studies (36) which postulated that vitamin E did not have any effect in reducing hepatic lipid peroxidation and has no role to ameliorate the structural damage. Combined administration of alpha-tocopherol (vitamin E) and DMSA to the arsenic group animals showed mild improvement of the centrilobular hepatocytes with weak PAS-reaction while the perilobular areas showed moderate improvement of hepatocytes with moderate PAS-reaction. It has been suggested that the use of dimethyl diphenylcarboxylate (DDB) extracted from the seeds of Schisandra, chines, endemite, especially when used in combination with other herbs may increase the therapeutic antioxidant effects during the treatment of many diseases of the liver, cardiovascular and neurological systems, as well as diabetes mellitus (16, 14, 15). In the present study, DDB administration to the arsenic animals showed mild improvement in hepatocytes of both the centrilobular and perilobular areas while PAS-reaction was weak at the centrilobular areas and moderate among the perilobular areas. This indicates the better regenera-
tive effects of DDB in comparison with vitamin E as manifested by their different effects on the centrilobular affected hepatocytes. These results are in accordance with many previous studies (16, 37). Ip et al. (1996) postulated that the role of DDB may involve facilitation of both antioxidant and detoxification processes in the liver against heavy metals toxicity (37). On the other hand, Ip et al. (2000) observed the role of DDB was in decreasing the elevated levels of ALT (alanine amino-transferase) and AST (aspartate amino-transferase) but not SDH (sorbitol dehydrogenase) induced by CCl4 intoxication, concluding that DDB had no role in the treatment of hepatic disorders (14). The results of the present study (14 days treatment) are contradictory to Ip et al (2000) analysing the effect of a short therapy of DDB (3 days treatment) which was not enough to produce a hepatoprotective or detoxifying effect. The concomitant administration of vitamin E, DDB and DMSA to arsenic-intoxicated animals resulted in a good regeneration of hepatocytes at the centrilobular areas with good positive PAS-reaction while the perilobular areas showed marked regeneration of hepatocytes with almost normal architecture and a highly positive PAS-reaction with normal granulation and density of the cytoplasm. This indicates that the hepatoprotective and regenerative effects of DDB and vitamin E in combination with the chelator DMSA may almost reverse and neutralize the hepatotoxic effects of arsenic. The histopathological results were parallel with the histochemical ones as polysaccharides or glycogen metabolism is affected accordingly with the structural changes that affect the mitochondrial, microsomal and GERL (Golgi-Endoplasmic Reticulum-Lysosomes) systems of the cell. CONCLUSION: The results of the study support the role of oxidative stress induced by arsenic toxicity which can be measured by the histopathological and histochemical degree of changes. This study explored the hepatoprotective and / or regenerative effect of two promising antioxidants (vitamin E or alpha-tocopherol and DDB) and their mutual cumulative influence against arsenic-induced hepatotoxicity especially when these are combined with a chelating agent such as DMSA. Finally, the liver with regard to its histopathology and histochemistry is a very useful provider of biomarkers to measure the degree of heavy metals toxicity.

Fig. (9A): A section in the liver of an arsenic-treated animal given DMSA + Vit.E + DDB. Centrilobular areas are moderately regenerated with minimal amount of vacuolated hepatocytes (O) and nearly normal acidophilic. The endothelium (arrow) appeared regular and continuous. (x500, Hx & E)

Fig. (9B): A section in the liver of an arsenic-treated animal given DMSA + Vit.E + DDB. The portal vein (PV) endothelium is regular and continuous and surrounded with well regenerated hepatocytes while minimal amount of vacuolated (O) hepatocytes are seen near the centrilobular areas (x250, Hx & E)

Fig. (9 C): A section in the liver of an arsenic-treated animal given DMSA + Vit.E + DDB. Perilobular area (P) with highly positive PAS-reaction (x250), PAS

Fig. (9 D): A section in the liver of an arsenic treated animal given DMSA + Vit.E + DDB. Centrilobular area (C) appeared with good positive reaction (x250), PAS
REFERENCES


