PROTECTIVE ROLE OF VITAMIN C AND E AGAINST SODIUM ARSENATE INDUCED CHANGES IN DEVELOPING KIDNEY OF ALBINO MICE

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Background: Arsenic is a teratogenic agent present in the environment as oxides and arsenate and humans are exposed to it through contaminated drinking water, food, soil and air. This investigation was undertaken to evaluate protective role of Vitamin C and E against teratogenic injury produced by sodium arsenate in developing kidney of the mouse. **Methods:** Twenty-four pregnant albino mice of BALB/c strain, were randomly divided into 4 groups of 6 each: A_1, A_2, A_3 and A_4. Group A_1 served as the control and received weight related distilled water by intra-peritoneal (I/P) injection, group A_2 was given a single doses of 35 mg/kg on 8th GD whereas groups A_3 and A_4 were treated with Vitamin C and E by IP injection, 9 mg/kg/day and 15 mg/kg/day respectively, starting from 8th day and continued for the rest of the pregnancy period. The foetal kidneys were weighed and histological studies carried out including micrometry on different components of nephron. **Results**: Sodium arsenate toxicity manifested as an increase in weight of the kidneys, wider nephrogenic zone and significant reduction in the mean of number of mature renal corpuscles as compared to the control group (p<0.000). There were moderate to severe necrotic and degenerative changes in proximal and distal convoluted tubules; glomeruli were hypercellular, the Bowman's spaces were obliterated. There was a statistically significant difference in mean diameter of renal corpuscles of group A_2 when compared with groups A_1, A_3 and A_4, (p<0.000). **Conclusions:** The findings implied that groups receiving Vitamin C and E along with sodium arsenate showed an overall improvement in all parameters, indicating the protective role of Vitamin C and E against arsenic induced teratogenicity in developing kidney and are safe to use during pregnancy without deleterious effect on human conspectuses in arsenic exposed areas.

Keywords: Arsenic, teratogenic, ascorbic acid, nephrogenesis.

INTRODUCTION

Heavy metals and their chemical salts are reputed to produce serious toxic effects, in addition to congenital malformations in animals and human beings.[1] The metal form of arsenic is non toxic where as its inorganic salts as oxides and arsenate are highly toxic and are water soluble.[2] Sodium arsenate ($Na_2HAsO_4.7H_2O$) is a pentavalent form of arsenic which is used as a pesticide and has mutagenic potential.[3] The presence of high levels of arsenic in industrial waste processed for use as fertilisers is of growing concern.[4]

Arsenic has been used extensively in medicine, agriculture and preparation of pigments and electrical devices and is commonly used as pesticide and wood preservative.[5,6]

Accidental arsenic poisoning occurs from ingestion of insecticides and pesticides; its acute poisoning results in nausea, vomiting, diarrhoea, renal and respiratory failure; its chronic poisoning leads to accumulation of arsenic compounds in liver, kidneys, heart, lungs, muscles, nervous system; it produces hyper pigmentation and keratosis of skin.[2–7] Long term exposure to it leads to hypertension, diabetes mellitus and respiratory conditions like Asthma.[8] Malignancies of skin, liver, kidney, bladder and prostate have been reported after long term arsenic exposure.[9] Arsenic toxicity depends upon its geographical distribution and individual nutritional habits.[3] Malnourished individuals have higher risk of arsenic toxicity.[8,10] Children and immuno-compromised individuals are more susceptible to arsenic toxicity. Chronic arsenic poisoning is difficult to treat.[5]

Arsenic contamination of drinking water, food, soil and air has become a global problem.[11] In Pakistan 5.6 million tons of pesticides are used annually, containing arsenic as main constituent, this mixes with water and leaches to underground aquifers.[12]

Food is also the major source of arsenic exposure; its organic form in high level are found in fish and sea food.[6] Arsenic accumulates in rice plant through contaminated irrigation water.[13] Cooking rice in such water increases its arsenic content by 10–30% and by 200–300% in case of beans and grains.[11]

Epidemiological and animal studies have revealed the association between arsenic exposure through drinking water and occurrence of spontaneous abortion, still and premature birth, low birth weight babies as compared to those who were not exposed to water with arsenic contamination.[8,10,14]

Arsenic salts can cross placental barrier and produce malformations, primarily neural tube defects, growth retardation and increased neonatal mortality when exposed during embryogenesis; also reduced gain in body weight of dams during pregnancy. Sodium arsenate or arsenite, when administered by a single dose I/P injection or oral route, caused maternal toxicity and

foetal malformations, depending upon dose and gestation period when the salt was administered.[15]

In mice, foetal kidneys are fully differentiated and able to function by 17th and 18th gestational days, however nephrogenesis continues after birth.[20] The developing kidney is vulnerable to the environmental toxins, proportional to the degree of exposure of the renal tissue to them and the stage of development.[20] Arsenic induced toxicity is due to the production of free radicals like super oxide and hydrogen peroxide which cause DNA damage.[5] Antioxidants facilitate in the methylation and excretion of heavy metals.[4] Vitamin E is fat soluble vitamin and act as free radical scavenger in lipophilic environment and has a role in protecting polyunsaturated fats.[17] Vitamin E, due to its antioxidant properties, is believed to prevent diseases associated with oxidative stress like cancer, atherosclerosis, premature aging, cataract formation and arthritis.[18,19] Vitamin C (Ascorbic acid) is a water soluble antioxidant which scavenges the reactive oxygen species including super oxide and plays an important role in regulation of intracellular redox state.[20]

Although teratogenic effects of arsenate and arsenite had been extensively studied by number of workers using different doses, routes and variable exposures, prevention of its teratogenicity has met with limited attention in the past. The chelating agents have been used to treat arsenic toxicity but they are foetotoxic and teratogenic, consequently they cannot be used during pregnancy. The present study is therefore, designed to have a morphometric analysis of various histological parameters and evaluate the protective role of Vitamin C and E in combating teratogenicity of arsenic in mice developing kidney.

MATERIALS AND METHODS

It was an experimental study, using mice as an animal model and was carried out at the Experimental Research Laboratory of University of Health Sciences Lahore.

Thirty-two Albino mice of BALB/c strain (twenty four females and eight males), 10 weeks old and weighing 30–35 gm were procured from National Institute of Health Islamabad. They were kept under controlled environments (temperature 22±1 ºC and humidity 40%–60%). The animals were allowed to acclimatize for 7 days and were fed on standard rodent diet, distilled water ad libitum and a 12 hour light/dark cycle. Three female mice were kept overnight with a single male for purpose of mating; the day, when vaginal plug appeared, was regarded as gestational day (GD) zero. The pregnant females were given a permanent picric acid mark of identification on the body with cotton buds; these were randomly divided into four groups of six each, named A_1, A_2, A_3 and A_4 respectively and, placed in respective cages (n=6); A_1 served as a control and the other three as experimental groups. Group A_1 received weight related distilled water by I/P injection, for 18 days. Group A_2 animals were treated with sodium arsenate 35 mg/kg ($Na_2HAsO_4.7H_2O$) by a single I/P injection on 8th day of gestation; sodium arsenate was dissolved in distilled water before injecting. Groups A_3 and A_4 animals received sodium arsenate 35 mg/kg on 8th GD and Vitamin C and E by I/P injection, 9 mg/kg/day and 15 mg/kg/day respectively, from 8th day for the rest of the pregnancy period.

The animals were sacrificed on 18th day of gestation by anaesthetising with ether. The foetuses were extracted from the uterus; foetal kidneys were examined macroscopically, noting their colour, size and any other discernable malformations, using Wolfe stereo dissecting microscope, ER-59-1828. Combined weight of right and left foetal kidneys was calculated and recorded using analytical balance (AY220). The foetal kidneys were washed with distilled water and fixed in 10% formalin solution and processed for histological study in a usual way. Sections were cut at 5 µm thickness and were mounted on the albumenised slides; these were then stained with hematoxylin and eosin by standard procedures. Stained sections were studied under a light microscope (Leica DM 1000) at varying magnifications.

A morphometric analysis of histological parameters included width of nephrogenic zone, and calculating the diameter and number of renal corpuscles. The tubules were randomly selected from ten fields in corticomedullary region. They were assessed for cellular distension, cellular vacuolation and condition of their lumen as histological variables and scored as:

1–3= Mild, 4–6= Moderate, 7–9= Severe

Mean scores of histological changes were calculated and the frequency of histological changes in renal tubules was expressed in percentage.

The data was entered and analysed using SPSS version 13.0. Mean and standard deviations were calculated for normally distributed quantitative variables. One way ANOVA was applied to assess the significance of difference among the groups. The post-hoc test Tukey was applied to assess the difference of means between the groups. The p value of <0.05 was considered as statistically significant.

RESULTS

The kidneys treated with sodium arsenate group (A_2) appeared to be enlarged as compared to groups A_1, A_3 and A_4 (Figure-1). Comparison of the mean of the combined left and right foetal kidneys weight among different groups showed significant statistical difference ($p<0.000$) (Figure-2).

In group A_2 arsenic exposure resulted in glomerular enlargement which fills the capsular space; there were cluster of cells in centre indicating hyper

cellularity due to the proliferation of mesangial cells whereas the endothelial cells showed evidence of swelling. The capillary loops showed hemorrhages, there was also deposition of eosinophilic material in Bowman's spaces. The parietal layer of capsules were intact, adhesions between capillary tuft and capsule were common (Figure-4). In group A_1 the glomeruli and Bowman's capsular spaces remained normal (Figure-3).

In groups A_3 and A_4 the renal corpuscles appeared to be spherical the cells of visceral layer grouped together in centre surrounded by narrow Bowman's spaces, the haemorrhage in capillary tuft and adhesions between capillary tuft and capsule were not observed as observed in group A_2 (Figure-5 & 6).

The difference of mean width of nephrogenic zone among various groups showed statistically significant difference (Table-1). Multiple comparisons according to Tukey test showed significant difference between groups (Table-1a). In group A_1 the sub capsular nephrogenic zone (NZ) showed nephrons in different developmental stages (Figure-7). In group A_2, the sub capsular nephrogenic zone (NZ) depicted the arrest of development (Figure-8). In groups A_3 & A_4 the nephrogenic zone showed nephrons in different developmental stages (Figure-9, 10).

The diameter of renal corpuscles increased considerably in group A_2 as compared to other groups , the data are given in (Table-2, Figure-11). The multiple comparisons of mean diameter of renal corpuscles according to post-hoc test Tukey showed statistical significant difference between groups (Table-2a).

There was a marked reduction in mean number of renal corpuscles in group A_2 as compared to other groups (Table-3). The Tukey test showed that group A_1 differs significantly with groups A_2 & A_4, while group A_2 showed statistically significant difference with groups A_3 & A_4 (Table-3a).

Group A_2 showed more number of hyper cellular renal corpuscles than normal as compared to other groups. The data are given in (Figure-12). The multiple comparisons among the groups by post-hoc test Tukey showed statistically significant difference among different groups (p<0.000).

In group A_2 degenerative changes were observed in proximal and distal tubules in the cortex. The tubules showed vacuolation and presence of eosinophilic material in lumen, in some areas there was loss of nuclei and disruption of basement membranes (Figure-4). In groups A_3 and A_4 the treatment with Vitamin C and E respectively had reduced the arsenic induced tubular necrotic changes. Dilatation of tubules was reduced and degenerative changes were reversed, with intact tubular epithelium the nuclei of epithelial cells were present. The mean score in group A_2 was 6.9±0.5 the frequency of histological changes in tubules from 10 fields showed that in group A_2 21% tubules showed moderate changes while 19 % showed severe changes as compared to group A_1 . In group A_3 100% tubules showed mild changes while in group A_4 17% showed mild changes and 82 % moderate changes (Table-4).

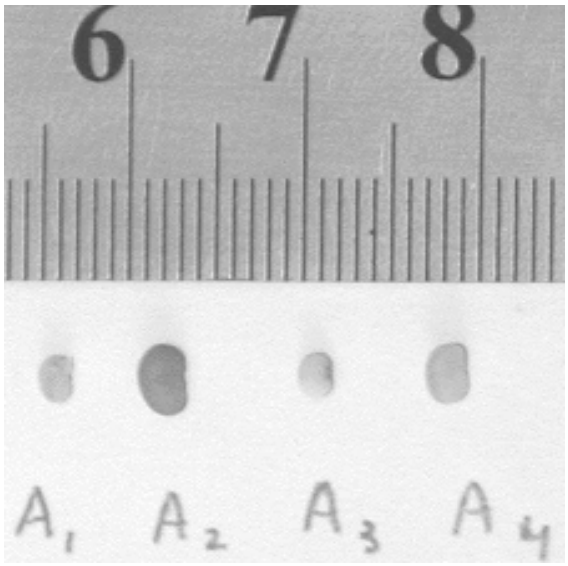

Figure-1: Photograph depicting the comparison of mice foetal kidneys taken from various groups.

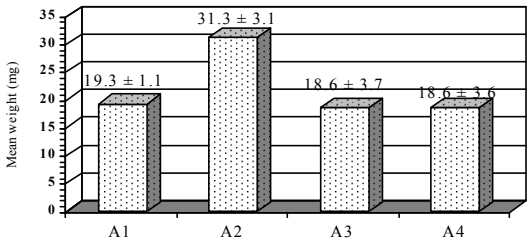

Figure-2: A bar chart showing comparisons of mean weight of both foetal kidneys among various groups

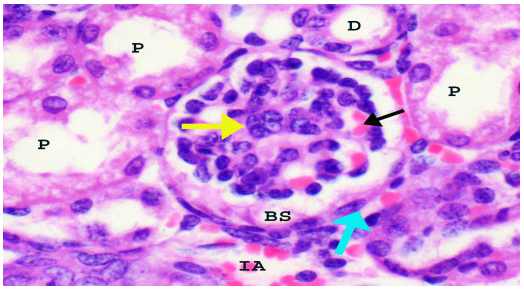

Figure-3: Photomicrograph of foetal kidney.

Group A_1: depicting renal corpuscle, the glomerulus showing mesangial cell nuclei grouped in centre (yellow arrow) capillary tuft showing RBC (black arrow). Bowman's capsule lined by squamous epithelium showing flattened nuclei (blue arrow), surrounded by proximal (P) and distal (D) convoluted tubules and interlobular arteries (IA) filled with red blood cells. H & E, ×400

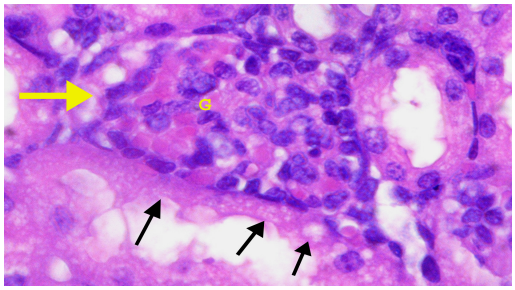

Figure-4: Photomicrograph of foetal kidney.

Group A_2: showing glomerulonephritis (G) obliterating the Bowman's space (yellow arrow), surrounded by tubules showing vacuolation, (Black arrows) indicating the progressive loss of nuclei. H & E, ×400

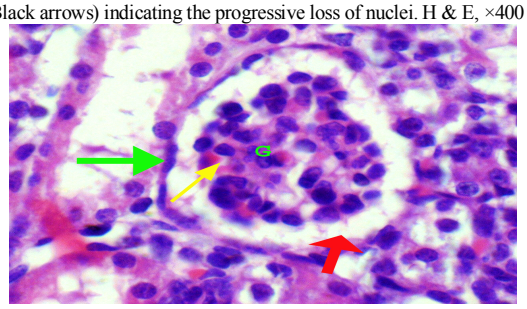

Figure-5: Photomicrograph of foetal kidney.

Group A_3: showing renal corpuscle with intact parietal layer (green arrow), the visceral layer shows cluster of cells grouped in centre. (G), the capillary tuft showing RBC (yellow arrow) surrounded by clear Bowman's space (red arrow). H & E, ×400

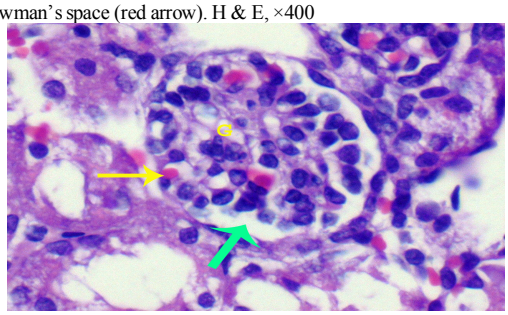

Figure-6: Photomicrograph of foetal kidney.

Group A_4 showing renal corpuscle with enlargement of glomerular tuft (G) surrounded by narrow Bowman's space (green arrow), the capillary tuft showing RBC (yellow arrow) the tubules showing vacuolation. H & E, ×400

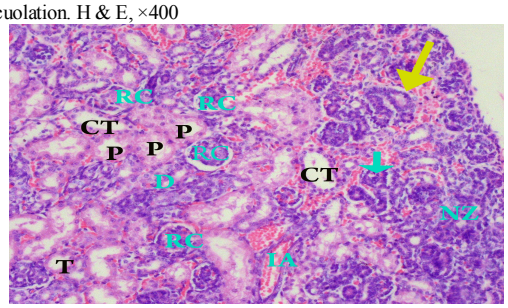

Figure-7: Photomicrograph of foetal kidney.

Group A_1 on 18th GD: The sub capsular nephrogenic zone (NZ) showing Vesicular (yellow arrow) and Comma shaped (green arrow) stages respectively of developing nephrons surrounded by interstitial tissue. Mature renal corpuscles (RC) are located in deeper cortex. The distal (T) and proximal (P) tubules with interstitium and interlobular arteries (IA) filled with blood and (CT) collecting tubules are also shown. H & E, ×100

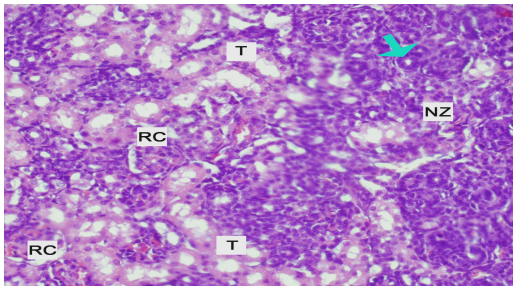

Figure-8: Photomicrograph of foetal kidney.

Group A_2 on 18th GD: showing darkly stained sub capsular nephrogenic zone (NZ) , which appears to be wider than in the control group A_1 and shows majority of immature nephrons (green arrow) . The cortex show darkly stained areas surrounded by tubules (T); some mature renal corpuscles (RC) are also sparsely present, H & E, ×100.

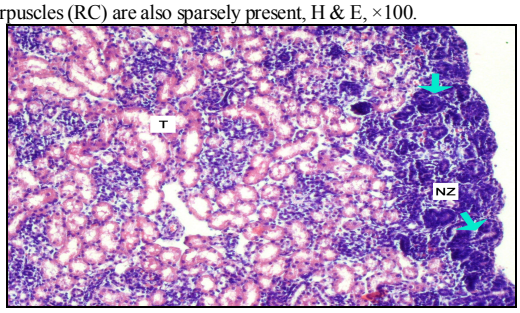

Figure-9: Photomicrograph of foetal kidney.

Group A_3 on18th GD: showing darkly stained sub capsular nephrogenic zone (NZ), is narrower than in group $A_2$with patches of darkly stained areas, indicative of developing renal corpuscles, the cortex shows well developed tubules (T) .The dark subcapsular area contains many developing nephrons with renal corpuscles in vesicular and coma stages of development (green arrows), indicative of more advanced stage of developing kidney than in group A_2 H & E, ×100.

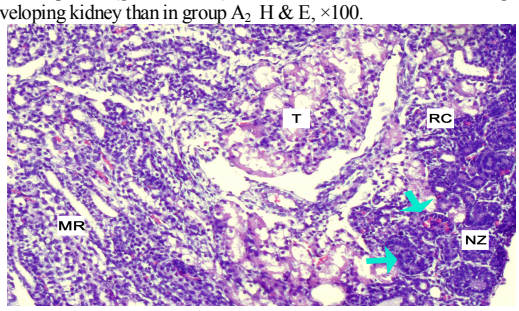

Figure-10: Photomicrograph of foetal kidney.

Group A_4 on18th GD: showing darkly stained sub capsular nephrogenic zone (NZ), renal corpuscles (RC) in different developmental stages (green arrows). The cortex shows well developed tubules (T) and medullary rays (MR) showing medullary tubules, indicative of more advanced stage of developing kidney than in group A_2 . H & E, ×100

Table-1: Comparison of mean width of nephrogenic zone in µm of foetal kidneys at the level of pelvis among various groups.

Parameter	Width of nephrogenic zone (µm, Mean±SD)
A_1 Cnotrol (n=40)	164.2±39.9
A_2 Sodium arsenate (n=40)	236.6±42.3
A_3 Sodium arsenate + Vit C (n=40)	168.6±45.7
A_4 Sodium arsenate + Vit E (n=40)	166.3±27.4
***p*-value**	0.000*

*The mean difference is statistically highly significant.

Table-1a: Multiple comparisons of mean width of nephrogenic zone in µm among various groups according to Tukey test.

Comparison among groups		Mean Difference	Level of Significance
Groups (I)	Group compared (J)	(I-J)	*p*-value
(A_1)	(A_2)	-72.4	0.000*
	(A_3)	-4.4	0.960
	(A_4)	-2.2	0.995
(A_2)	(A_3)	68.0	0.000*
	(A_4)	70.3	0.000*
(A_3)	(A_4)	2.2	0.994

*statistically highly significant

Table-2: Comparisons of mean diameter of the renal corpuscles (m) among various groups (Mean±SD, n=40)

	Groups				
Parameter	A_1	A_2	A_3	A_4	*p*
Diameter of Renal corpuscles	55.3±4.4	73.2±5.5	47.2±4.0	52.7±4.5	0.000*

*statistically highly significant

Table-2a: Multiple comparisons of mean diameter of renal corpuscles in µm among various groups according to Tukey test.

Comparison among groups		Mean Difference	Level of Significance
Groups (I)	Group compared (J)	(I-J)	*p*-value
(A_1)	(A_2)	-17.8	0.000*
	(A_3)	8.12	0.000*
	(A_4)	2.59	0.063
(A_2)	(A_3)	26.0	0.000*
	(A_4)	20.4	0.000*
(A_3)	(A_4)	-5.51	0.000*

*highly significant

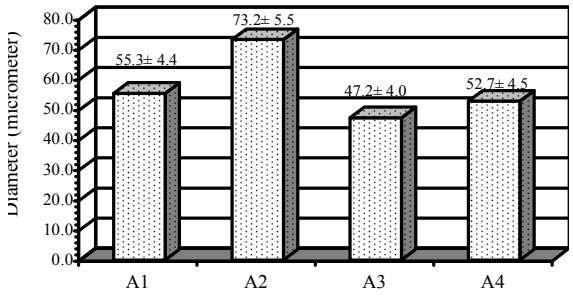

Figure-11: A bar graph showing comparisons of mean diameter of renal corpuscles among various groups.

Table-3: Comparisons of mean of the number of renal corpuscles/mm^2 from 4 fields among various groups (n=40)

	A_1	A_2	A_3	A_4	
Parameter	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*-value
Number of Renal corpuscles.	5.1±1.3	2.0±0.6	4.6±1.2	4.0±1.2	0.000*

*The mean difference is statistically highly significant.

Table-3a: Multiple comparisons of mean number of renal corpuscles/mm^2 from 4 fields among various groups.

Comparison among groups		Mean Difference	Level of Significance
Groups (I)	Group compared (J)	(I-J)	p-value
(A_1)	(A_2)	3.12	0.000*
	(A_3)	0.45	0.277
	(A_4)	1.05	0.000*
(A_2)	(A_3)	-2.67	0.000*
	(A_4)	-2.07	0.000*
(A_3)	(A_4)	0.60	0.081

*The mean difference is statistically highly significant between groups A_1 & A_2, A_2 & A_3, A_2 & A_4. The mean difference is statistically insignificant between groups A_1 & A_3, A_3 & A_4.

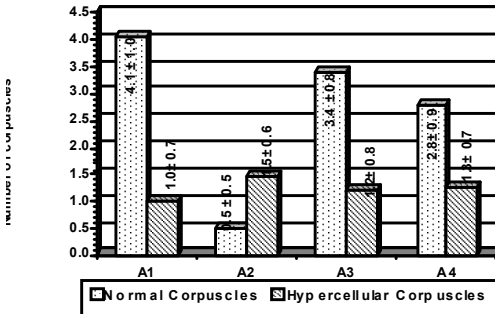

Figure-12: A bar graph showing comparisons of mean of the number of normal and hyper cellular corpuscles/mm^2 from 4 fields among various groups.

Table-4: Multiple Comparisons of mean Scoring for Histological changes in foetal Kidney tubules from 10 fields among various groups according to Tukey test.

Comparison among groups		Mean Difference	Level of Significance
Groups (I)	Group compared (J)	(I-J)	p-value
(A_1)	(A_2)	-6.0	0.000*
	(A_3)	-1.1	0.000*
	(A_4)	-3.8	0.000*
(A_2)	(A_3)	5.0	0.000*
	(A_4)	2.3	0.000*
(A_3)	(A_4)	-2.7	0.000*

* Mean difference is statistically highly significant among all groups

DISCUSSION

The sodium arsenate exposure resulted in hypertrophy of foetal kidneys as compared to control and other experimental groups. Hypertrophy of kidney and higher kidney/body weight ratio after arsenic exposure had been reported.[21] In groups A_3 and A_4 the kidneys appeared to be comparable to control suggesting that Vitamin C and E prevented the hypertrophy of kidney. It had been documented that co-administration of Vitamin C and succimer reduces arsenic burden of liver and kidney as Vitamin C acts as a detoxifying agent by forming poorly ionised but soluble complexes.[22]

In mice nephrogenesis continues from 11th GD to postnatal 5–7 days; whereas in humans it is completed before birth.[24] On 18th GD the fetal kidney showed wider nephrogenic zone in group A_2 as compared to control group A_1, indicating arrest of development or impaired nephrogenesis. The nephrogenic zone is not present when nephrogenesis is completed.[24] The treatment with Vitamin C and E in groups A_3 and A_4 respectively, considerably reduced the width of nephrogenic zone ,which was comparable to that in group-A_1 suggesting thereby that Vitamin C and E had prevented the arsenic induced arrest of development of nephrons. These effects are attributed to the production of free radicals which possibly interfered with the inductive influences of ureteric bud on metanephric mesenchyme, resulting in arrest of development, Vitamin C and E, on other hand, due to their antioxidant properties; scavenge free radicals, reviving the nephrogenesis. It had been reported that arsenic exposure in mice on 9th GD resulted in impaired growth of ureteric bud with subsequent failure of differentiation of metanephric blastema [23].

The mean number of renal corpuscles in group A_2 was reduced as compared to groups A_1, A_3 and A_4 respectively. There were lesser number of normal than hyper cellular renal corpuscles in group A_2 when compared to control and groups A_3 & A_4. Treatment with Vitamin C and E of groups A_3 and A_4 considerably increased the nephron number. Sodium arsenate exposure resulted in impaired nephrogenesis and reduction in number of mature renal corpuscles and compensatory hypertrophy of remaining corpuscles and their hyper cellularity. It had been reported that the reduction in nephron number during nephrogenesis is more critical than after the completion of nephrogenesis, the latter situation would produce compensatory hypertrophy of remaining nephrons leading to hypertension in adult life.[25] The developed cortical renal corpuscles in sodium arsenate treated group (A_2) showed increase in diameter as compared to control group A_1.This indicated glomerular hypertrophy possibly due to direct injury of endothelial cells leading to constriction of efferent arterioles resulting in compensatory hypertrophy of glomeruli. In groups A_3 and A_4 the mean diameter of renal corpuscles were comparable to control group suggesting that Vitamin C and E had prevented the endothelial damage and congestion of efferent arterioles, thus reducing the glomerular hypertrophy.

In sodium arsenate exposed group, epithelial cells were present in discernibly distended tubules of the cortex; epithelial cell vacuolation and nuclear changes, karyolysis and pyknosis were observed in proximal convoluted tubules. In group A_2 21% of tubules showed epithelial cells distension and vacuolation, while 19%, in addition, showed disruption of epithelial cell basement membranes. The treatment with Vitamin C and E in groups A_3 and A_4 respectively, considerably reduced the tubular degenerative changes. Epithelial cell vacuolation and tubular atrophy after chronic arsenic exposure had been reported by (Liu J, 2000).[21] The tubular degenerative changes were maximum in cortical PCT due to direct effects of arsenic which is absorbed and concentrated here to the extent as to injure the epithelium.[26] The proximal tubular cells had been investigated in human after arsenic exposure and showed apoptosis and necrotic changes in cytoplasm and depolarization of mitochondria.[27] The current study suggested that Vitamin C and E were discernibly effective in preventing arsenic induced teratogenicity in mice, the protective role of these antioxidants is presumably based on the mechanism as reported by Patrick.[4]

CONCLUSIONS

The present study demonstrates Vitamin C and E to be protective against arsenic induced teratogenecity in mice developing kidney, seemingly is equally applicable to human beings. It can therefore be presumed that Vitamin C and E can be used in pregnant women in arsenic contaminated areas.

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