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SUBCHRONIC TOXICOLOGY STUDY OF ORALLY ADMINISTRATED DIETHANOLAMINE IN MICE KIDNEY

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ABSTRACT
Diethanolamie (DEA) considers one of the toxic ingredients in cosmetic products, Its concentrations were measured in 50 lipstick samples (25 new and 25 used); and it recorded highly significant differences (P< 0.01); 68% from new lipsticks had DEA concentrations ranging between (ND- 977.97 ppm) while 12% from used lipsticks samples had DEA concentrations ranging between (ND- 383.3 ppm). For evaluation the subchronic effects of this chemical on kidney tissues, 60 mice the average age of (8- 10 weeks) and weight (27- 32 gm); were divided into 6 groups (control, 603, 1250, 2500, 5000, 10000 ppm) treated for 12 weeks. After observing the external toxicity signs, the results showed appearance of lethargy, weight gain except the higher concentrations, ruffled fur, pale foot pads and abortions were recorded. The is reversible relationship between the mice water consumption and the DEA value concentrations in drinking water, resulting in severe dehydration which causes death. The histopathological sections showed the increasing in weight of kidneys. Kidneys showed sever degeneration and necrosis of hepatocytes, degenerative vacuoles within epithelium of renal tubules, membranous glomerulonephritis with thickening of Bowman capsule, sever degeneration and necrosis of renal tubules, hemorrhage with congestion of intertubular blood vessels, glomerular depletion and renal cells degeneration.

KEY WORDS: Diethanolamine, lipsticks, subchronic toxicity

INTRODUCTION

Cosmetic products include different chemical substances which may be possible carcinogen and endocrine disruptors (Marie *et al*., 2016). One of these chemicals ie Diethanolamine (DEA) from family of ethanolamines which consist of A family of three chemicals including monoethanolamine (MEA), diethanolamine (DEA), triethanolamine (TEA) are using in spread industries. This group of chemicals showed to have double functional groups (amino and hydroxyl) which make them play as intermediates of surfactants in soaps and pharmaceuticals applications and other (Knaak *et al*., 1997). Diethanolamine is an organic compound resulting from ethylene oxide and ammonia reacting, it is not occurring in nature. It is used for the preparations of amides and amides salts of DEA which are forming cosmetics, detergents, shampoos and hair conditioners. The chemical structure of DEA is: (OH -CH_2 – CH_2- NH- CH_2 – CH_2- OH) Chemical formula ($C4H_{11}NO_2$) with molecular weight is 105.14 (IARC, 2012). DEA is using to make the cosmetic product more creamy or sudsy. Also it has been used as pH adjuster to naturalize the other ingredients with high acidity (David Suzuki Foundation, 2010). It is using lipsticks industry about 3- 10% (CIR, 2011). DEA promoted hepato carcinogenicity via the continuous decreasing of SAM (a donor in DNA methyltransferase). These changes lead to DNA hypomethylation that alter gene expression alteration (Knaak *et al*., 1997), when mice exposed to higher DEA tolerated doses, this was resulting in choline levels reduction in the liver (Lehman-Mckeeman *et al*., 2002). According to (Knaak *et al*., 1997) DEA was a carcinogenic of lab animals (rats, mice, and Drosophila) because of liver choline uptake decreasing. However there was weak evidence to be possibly carcinogenic to human (group 2B). DEA half-life long in tissues is (7 days); in blood (54 days) but according to the FDA (2013) DEA absorption increased by increasing dose. NTP (1999) showed the clear evidence of DEA carcinogenicity in male and female mice by inducing liver neoplasms in both sexes. In addition to renal tubule increasing cytoplasmic alternation, syncytial alternation, renal tubule hyperplasia and thyroid gland follicular cell hyperplasia, skin hyper keratosis in males under 2- year's dermal injection studies.

MATERIALS & METHODS

Twenty- five samples of the most popular brands of lipsticks were purchased from the various shops from local markets of Baghdad city while the same number of samples for same types of cosmetics was collected from Iraqi women who had been used for a period of time (the storage period ranged between from few months to several years). Each lipstick sample had been given a brief name with sequence from (LP1-50); from each sequence number 1 to no.25 were given to the were given to the used samples (LP_1-LP_{50}).To Extract Diethanolamine from lipstick samples: fifty samples from different brands were diluted to 50% level (5 mg/ml) in an appropriate solvent (typically, aceton or dichloromethane was used). The samples were placed in an ultrasonic bath for 15 minutes to completely dissolve in target solutes of solvents. After extraction and dissolution, the sample could be centrifuged and the supernatant transferred to an autosampler vial to analysis (David, 2007).

Diethanolamine (DEA) (31590) was obtained from SIGMA ALDIRCH Company with purity 99.5% resulting

from gas chromatography peak. The chemical with prepared solutions were stored at room temperature for 20 days than refrigerated and protected from light (NTP, 1992). Oral DEA doses were given in drinking water, these doses were prepared with deionized water; the pH was adjusted to 7.4 ± 0.2 with (1N) hydrochloric acid (NTP, 1992). To study the DEA subchronic toxicity for twelve weeks, sixty healthy females of albino mice (in mature age of 5-6 weeks) and (20 ± 5 gm as average weight) were separated in six groups, each group of ten mice in separated polypropylene cage and treated with DEA doses, food was given ad libitum.Ten female of each cage received drinking water solutions containing diethanolamine at concentrations of (control, 630, 1250, 2500, 5000, and 10000 ppm) *ad libitum* daily. The observations for mortality/moribundity 2x/ day for; the body weight was taken every week regularly, all mice had been monitored for other toxicity signs, and water consumption was measured twice in every week. After mice body autopsied, the kidney samples were isolated and washed with normal saline twice and kept immediately in 10 % formalin for the microscopic study of the expected histological changes (NTP, 1992).

DISCUSSION

Twenty from the total number of samples had DEA concentrations while thirty brands did not have any concentration, as it showed in table (1). This table shows eight brands from new samples had not any DEA concentrations (32%). While Twenty two (88%) from used brands had not DEA concentrations. There were highly significant differences (P< 0.01) between new and used lipstick brands as it shows in table (2). The new brands recorded much higher differences in DEA levels than used brands. The levels of DEA in all fifty brands of lipstick were ranging between ND- 977.97 ppm as it shows in (table 3). The highest concentration was found in sample 16 from new brands with extremely high concentration (977.97 ppm). While in new brands were ranging between (ND- 977.97 ppm); but in most of used brands clear from DEA concentration except three brands samples 29, 34, and 47 with extremely high concentrations (383.3, 380.2 and 271.8 ppm). according to French acceptable limit in cosmetics which is 3 ppm or 15 mg/ m3 (Cayman chemical company, 2015), All lipstick brands were analyzed in this study had DEA levels above this limit enormously, while the other thirty brands had not any DEA concentrations.

TABLE 1: Comparison between new and used sequence in DEA percentages in lipsticks

Sequence	No.	Yes	No.	%
New	25	17	8	32.00
Used	25	3	22	88.00
Total	---	20	30	---
Chi-square	---	---	---	9.416 **
** (P<0.01).				

TABLE 3- 12: Comparison between new and used sequence (Lipsticks)

Sequence	No.	Mean ± SE of DEA
New	25	266.48 ± 57.63
Used	25	43.08 ± 24.07
T-Test value	---	127.60 **
P-value	---	0.0010
** (P<0.01).		

TABLE 3: Concentration of DEA in new lipstick brands

Sample no.	Concentration	Sample no.	Concentration
LP1	259.104	LP26	ND
LP2	ND	LP27	DN
LP3	941.301	LP28	DN
LP4	129.07	LP29	383.318
LP5	139.807	LP30	ND
LP6	94.958	LP31	ND
LP7	563.025	LP32	ND
LP8	343.915	LP33	ND
LP9	ND	LP34	380.205
LP10	409.586	LP35	ND
LP11	519.764	LP36	ND
LP12	ND	LP37	ND
LP13	ND	LP38	ND
LP14	555.898	LP39	ND
LP15	ND	LP40	ND
LP16	977.965	LP41	ND
LP17	214.504	LP42	ND
LP18	ND	LP43	ND
LP19	356.801	LP44	ND
LP20	ND	LP45	ND
LP21	ND	LP46	ND
LP22	509.244	LP47	271.802
LP23	148.646	LP48	ND

LP24	423.094	LP49	ND
LP25	84.501	LP50	ND

When lipstick has been eaten or swallowed through by drinking, there is a possibility of DEA reacting with sodium nitrite forming NDEL (N- nitrosodiethanolamine) in stomach in the presence of supplemental source of nitrite in stomach's acidize environment (NTP, 2002). Diethanolamine will react with nitrites or nitrogen oxides in cosmetics resulting in N- nitrosamines (potential carcinogenic). These nitrites are used sometimes as anti-corrosive agents in products or occured as contaminants (Bibra Toxicity Advice and Consulting, 1998; Zhang *et al*., 2008). The mechanism comprises the replacement of ethanolamine by DEA in phospholipids. Phosphotidyl DEA did not supply a major phosphotidyl choline synthesis that considered an endogenous origin for choline molecules in mammals (NTP, 2002). Oral human administration of DEA can cause diarrhea and changes in fat levels in blood. However, dogs and cats when treated with DEA for two days or more orally, this resulted in acute neurological effects such as paralysis (Bibra Toxicity Advice and Consulting, 1993).

TABLE 4: Effect of DEA concentrations in mice weight (12 weeks)

CONC.	Weeks											
	first	second	third	fourth	fifth	sixth	seventh	eighth	ninth	tenth	eleventh	twelfth
Cont.	28.9 ±1.6	26.2 ±1.8	26.45 ±1.5	25.22 ±1.4	25.77 ±1.6	25.3 ±1.4	23.23 ±1.2	28.89 ±1.9	26.96 ±1.5	32.5 ±2.5	31.48 ±1.9	31.05 ±2.3
630	31.3 ± 2.4	27.0 ±1.3	26.45 ±1.6	27.33 ±1.8	26.08 ±1.4	26.1 ±1.7	24.62 ±1.5	28.18 ±2.0	31.53 ±2.4	34.36 ±2.7	33.02 ±2.7	34.59 ±2.6
1250	28.2 ±1.6	27.0 ±1.6	26.45 ±1.4	26.34 ±1.4	22.19 ±1.5	26.4 ±1.7	24.87 ±1.3	23.07 ±1.4	30.23 ±1.9	32.87 ±2.1	28.23 ±1.9	-
2500	28.0 ±1.7	25.3 ±1.2	24.34 ±1.2	24.37 ±1.2	22.45 ±1.1	25.0 ±1.3	-	-	-	-	-	-
1250	20.1 ±1.2	22.17 ±1.4	20.01 ±1.1	-	-	-	-	-	-	-	-	-
10000	22.96 ±1.4	24.83 ±1.2	23.85 ±1.5	-	-	-	-	-	-	-	-	-
LSD	3.28 *	2.69 *	2.75 *	2.04 *	2.55 *	2.14 NS	2.09 NS	3.26 *	2.31 *	2.93 NS	2.42 *	2.54 *
P-value	0.034	0.042	0.049	0.027	0.044	0.215	0.094	0.041	0.039	0.169	0.047	0.050

* (P<0.05), NS: Non-significant.

After five years of consuming new cosmetic products, N-nitrosamines concentrations are higher than the new cosmetic products of the same products (Matyska *et al*., 2000). These compounds end the cosmetic product's shelf life and must be assessed as a routine safety evaluation (Jones and Glover, 2016). European Union (EU) (2009) in Annexes II and III established limited levels for N-nitrosamines in cosmetic products. No more than 0.05 ppm for both FDA and Cosmetic Act in (1938) and Fair Pack aging and Labelling Act in (1966) deemed any cosmetic product comprising N- nitrorsamines would be a cheated product and the markets must be cleaned from it. Except the experiments on laboratory animals and in vitro studies on human skin, there was no data on DEA absorption, metabolism or excretion by human (NTP, 2002).

Dethanoamine had been found as foaming agents in soaps, detergents, hand wash, conditioners and shampoos and it gives the lathery smooth texture and other cosmetic products (USEPA, 2006). Loretz *et al*. (2006) found that the person exposed to 12- 80 grams DEA resulting from daily shampoos application. Herrman *et al*. (2015) found the highest concentrations of N- nitrosamines after cooking meats and cooked sausages resulting from preservative material of nitrite. However, Rubashvili and Tsitsishvili (2015) found highest levels of volatile N-nitrosamines in tobacco smoke (cigarette).

After 12weeks of oral administration, only control and treated mice receivied 630 ppm groups lived almost till to the experiment termination. All these mice after 12 weeks had been autopsied. In the same time all mice in 5000 and 10000 ppm were died after the third week from the beginning of experiment except one mouse from both doses sacrificed in abnormal conditions by 3 weeks (table 4).

The other toxic signs in treated groups except the control are weight gain except the two high doses, thin appearance, icterus (or yellow skin), abnormal condition, hypoactivity and ruffled fur, in severe cases animals suffering from being incapable of standing, eating or drinking, clear dehydration with recessed eyes and pale foot pads as one of toxic signs of anemia with some cases of abortions. According to table (4) weight gain had been found in control and treated female mice groups except those which received high doses 5000 and 10000 ppm. Control and treated groups with received 630 and 1250 ppm only increased after 11 and 12 weeks treatment with DEA. Body weight reduced in the other treated groups involving the dose of (2500 ppm). According to table (4) mean value recorded in control was 28.9± 1.6 in the first week, while in the experiment termination was 31.05± 2.3 and the lowest level was recorded 22.96± 1.4 in mice receiving 10000 ppm in the first week; but 23.85± 1.5 in third week. According to the table there were significant differences (P< 0.05) between the control and all treated groups after DEA treatment with different number of weeks. According to table (5) and due to LSD value 177.01 the highest water consumption was found in

control group 389.33± 45.91 while lowest value in mice group received 10000 ppm with 59.75± 35.05 whenever the concentration of DEA in drinking water increased, the less consumed by mice, water consumption was reduced in high doses remarkably because of the minimized palatability of 5000 and 10000 ppm in the drinking water. This leads the animal to abstain from drinking water and gets severe dehydration (NTP, 1992).

In subchronic toxicity experiment, the kidney weight showed an increasing as it given in (table 5). The lowest mean value recorded was 0.220± 0.023 in control group; however, its highest value in the treated group with 10000 ppm was 0.483± 0.063. The results of this table show highly significant differences (P< 0.01) in kidney weight after a long period of time of oral DEA treatment due to LSD value of 0.154. As a result of oral administration, 27% from DEA deposit in liver and 5% in kidney than (< 1.0%) in blood, brain, spleen and heart (Artom *et al*., 1949). DEA has specific affinity for liver and kidney (Mathews *et al*., 1995). While the increasing in kidney weight because of minimal renal tubular necrosis and severity of nephropathy especially in the survived mice till to the end of subchronic toxicity experiment (NTP, 1992).

Figure (1) shows the normal kidneys structure comprises from several corpuscles, each of these corpuscles has an outer cover from simple epithelial cells which named (Bowman's capsule); in an overhang glomerulus (a net of blood capillaries). The renal tubules have two kinds: proximal convoluted tubules which covered with simple cuboidal epithelium from inside; there are microvilli as a brush in their borders. The other kind of renal tubules is the distal convoluted tubules which covered also by simple cuboidal tissues but without brushes microvilli.

After mice exposed to different doses of DEA foe thirteen weeks; the sections of mice kidney treated with 630 ppm showed that the epithelial cells of proximal convoluted tubules were exhibiting mild vacuolated epithelial cells while their nuclei have displayed nuclear pyknosis (figure 2). In mice treated with 1250 ppm, the kidneys sections showed similar alteration in those of 360ppm as it shows in (Figure 3).

TABLE 5: Effect of difference DEA concentration in Water consumption and Liver weight

Concentration	Mean ± SE (ml)	
	13 weeks	Kidney weight
Control	389.33 ±45.91	0.220 ±0.023
630	438.42 ±36.09	0.234 ±0.012
1250	292.78 ±63.76	0.271 ±0.024
2500	281.33 ±64.27	0.462 ±0.126
5000	89.00 ±48.29	0.456 ±0.071
10000	59.75 ±35.05	0.483 ±0.063
LSD value	177.01 **	0.154 **
P-value	0.0003	0.0014

** (P<0.01).

Changes of kidneys in mice treated with 2500 ppm showed congestion of interlobular blood vessels (figure 4). The cortical region was displaying moderate tubulonphrosis which showed degenerative vacuoles and necrosis (figure 5).

In mice treated with 50000 ppm of DEA; more histological alterations in the section of kidney were observed which shows membranous glomerulonephrosis that characterized by proliferation of the epithelial cells of parietal layer of Bowman capsule. There were sever degeneration and necrosis of renal tubules and multiple hemorrhagic foci with congestion of intertubular blood vessels (figure 6).

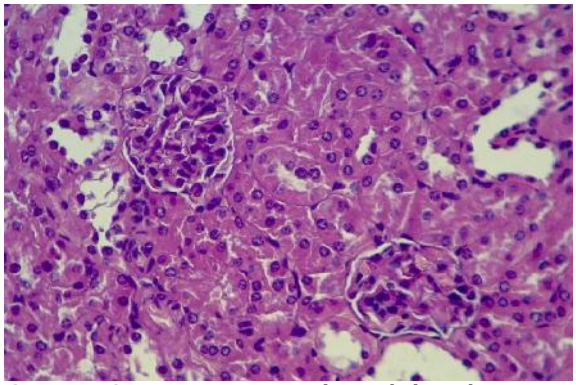

FIGURE 1: Cross section in normal mice kidney shows normal structure in control mice treated with distal water for 12 weeks (H&E) (400X).

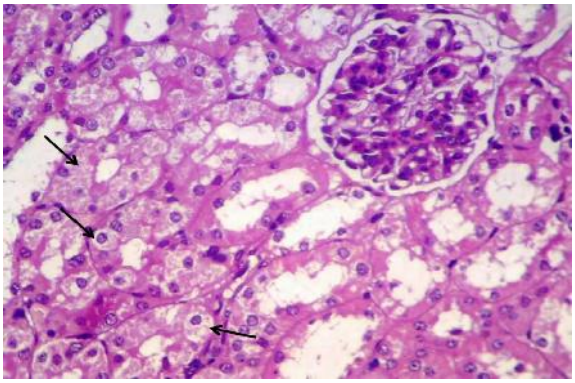

FIGURE 2: Renal cortex for 12 weeks in mice kidney treatment with (630ppm of DEA) Shows: vacuolated epithelial cells of proximal tubule (black arrows). (H&E) (400X).

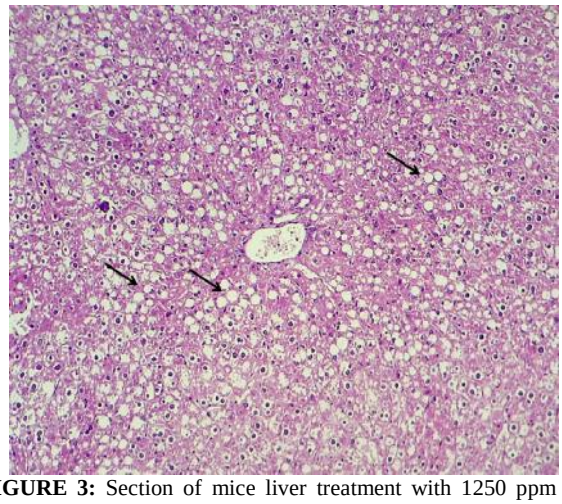

FIGURE 3: Section of mice liver treatment with 1250 ppm of DEA for 12 weeks shows diffused moderate fatty changes (arrows) (H&E) (400X).

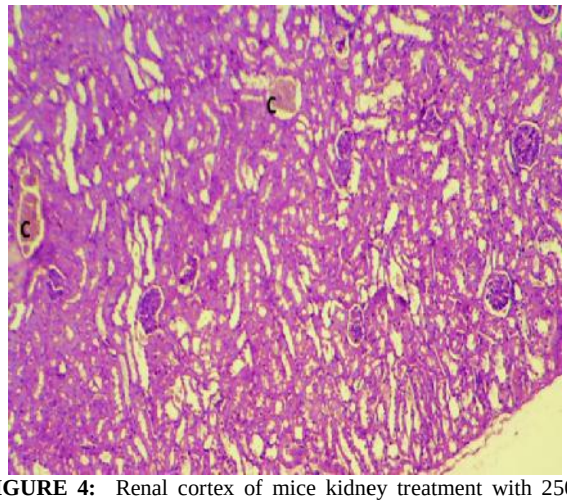

FIGURE 4: Renal cortex of mice kidney treatment with 2500 ppm for 12 weeks shows: congestion of interlobular blood vessels (N) (H&E) (100X).

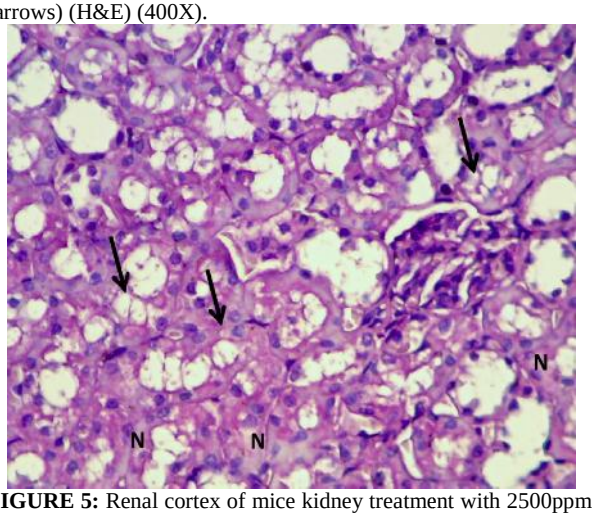

FIGURE 5: Renal cortex of mice kidney treatment with 2500ppm of DEA for 12 weeks shows: degenerative vacuoles within epithelium of renal tubules (arrows) and necrosis (N) (H&E) (400X).

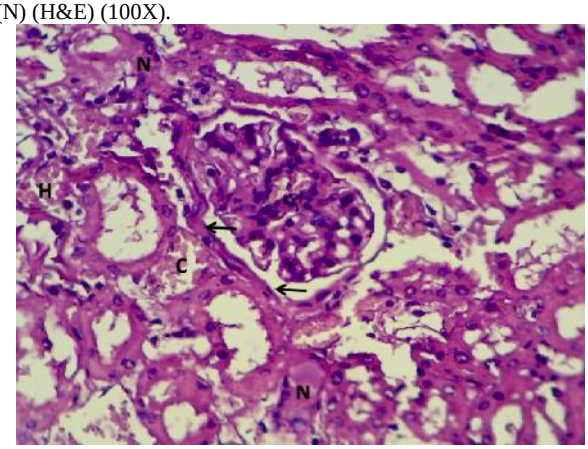

FIGURE 6: Renal cortex of kidney in mice treatment with 5000 ppm of DEA for 12 weeks shows membranous glomerulonephrosis with thickening of Bowman capsule (arrows), sever degeneration and necrosis of renal tubules (N) and there were hemorrhage (H) with congestion of intertubular blood vessels (C) (H&E) (400X).

However, mice were given 10000 ppm, the sections of kidney showed sever and generalized glomerulonephrosis with multiple foci of infiltrated mononuclear leukocytes (figure 7). The renal tubules showed sever vacuolar degenerative changes and necrosis (figure 8). While the glomeruli was showed deterioration with collapsed of bowman space (figure 9).

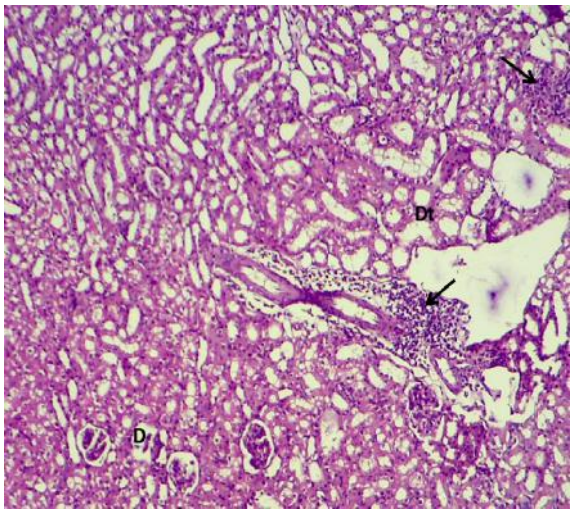

Figure (7): Section of kidney in mice treatment with 10000 ppm of DEA for 12 weeks shows degenerated renal tubules (Dt),

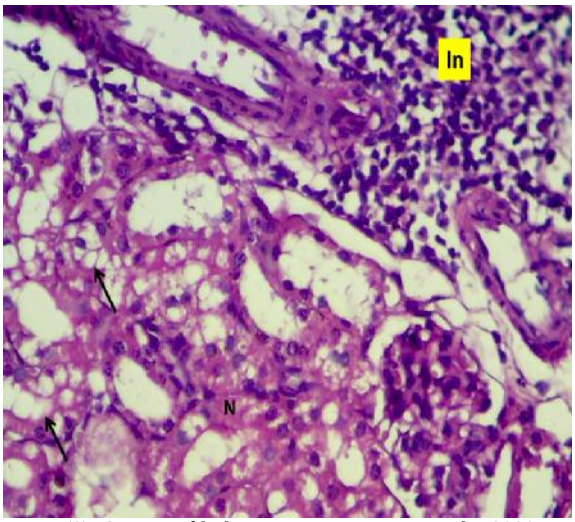

Figure (8): Section of kidney in mice treatment with 10000 ppm of DEA for 12 weeks shows degenerated renal tubules (arrows) and

glomerular depletion (D) with multiple foci of infiltration of mononuclear leukocytes (arrows) (H&E) (400X).

necrosis (N) with glomerular deterioration. Focus of infiltration of mononuclear leukocytes (In) (H&E) (400X).

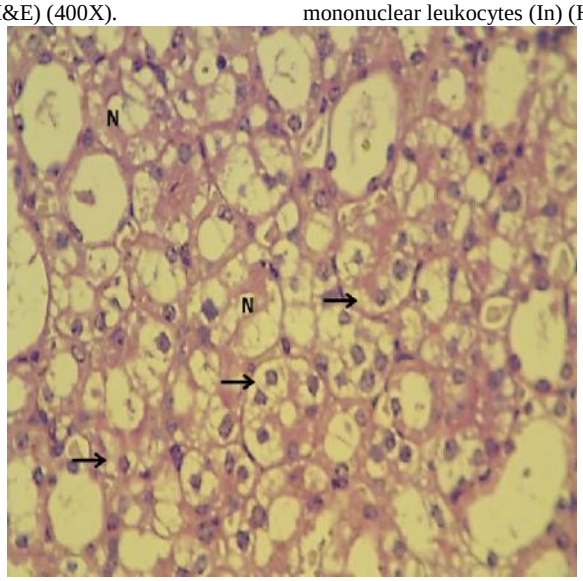

FIGURE 9: Section of kidney in mice treatment with 10000 ppm of DEA for 12 weeks shows: degenerated renal tubules (arrows) and necrosis (N) (H&E) (400X).

National Toxicity Program (2005) assured that kidneys one of the most significant organs because of its serious functions infiltration, metabolism and compound excretion

National Toxicity Program (1992) mentioned that kidney changes in mice were restricted fundamentally on increasing DEA doses; nephropathy can be found in mice survived to the end of the study. While IARC (2012) found there is no mechanistic data are available on tumors formation in kidney by DEA, the chronic progressive nephropathy can present in renal tissues as a result of chemical administration in rodents and consider a factor for renal toxicological and carcinogenic returns (NTP, 2005). It is known that kidney toxicity and reduction of renal function can be caused by choline deficiency in animal's laboratory (Zeisel and Blusztajn, 1994). Young adult mice (10- 12 weeks) are hardly to develop characteristics of totally expressed choline deficiency. The great risk can be resulted from oral DEA dose than dermal administration because the liver receives the toxicant directly through the portal system (Leung *et al*., 2005). Moreover, because the human stomach has low level pH (3-4) which is sufficient to make the synthesis of nitrates very easy with secondary to produce nitrosamine (Bryan *et al*., 2012). It does not metabolized or eliminated easily from liver or kidneys; Diethanolamine replaces for Mono ethanolamine (MEA) in phospholipids and ethylates to produce phospholipids constituted of N- methyl and N, N-dimethyl DEA (Knaak *et al*., 1997). The DEA disposition different across species, is dependent on the dosage and is attributed by relative long elimination for incorporation of DEA into phospholipids (Mathews *et al*. 1995; Mendrala *et al*., 2001). Since the choline is basic nutrient in all mammals, the lowest activity of choline oxidase (which determines choline requirements) can be occurred in humans; choline deficiency in them can be observed only after prolonged fasting (Zeisel and Blusztajn, 1994). There are biomarkers which are indicating on choline deficiency such as (low levels of glycerophosphocholine) have an association with chronic DEA administration (NTP, 2002). Leung *et al*. (2005) assumed a paradox between DEA acute high doses against chronic low via several factors

1. Diethanolamine incorporated into phospholipids, fatty liver may not developed if DEA involving lipids can function in triglyceride secretion
2. Lack of fatty liver can be illustrated by choline deficiency progressive development in animals treated with DEA; human can expose to DEA from including vegetables (Bryan *et al*., 2012); nitrites which added as a preservative to prevent Clostiridium botulinum (Park *et al*., 2015)

It had been assured that effects of chronic exposure may be clear much later than the cusative exposure or resulting from some interaction factors or physiological changes which cause in body deposition alteration (Vahter *et al*., 2002).

According to the data base of FDA, DEA from inactive ingredients which used in approval brand-name and general drug products such as DEA aqueous solutions are used as solvent for intravenously given drugs, topical creams (0.3%) and in ophthalmic solutions (FDA, 2013). HSDB (2002) said that the fate of DEA in atmosphere may be stay in the vapor while expose to biodegradation in water, soil and terrestrial with a half- life of days to weeks. Diethanolmine has ability to affect on structure and functions of biological membranes and this effect depend on DEA dose and time, with swollen hepatic mitochondria in animals treated with DEA alternation of phospholipid metabolism (Barbee and Hartung, 1979).

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