Evaluation the Effect of Novel L-arginine Derivative and Cadmium Chloride on Reproductive Efficiency in Male Rats.

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ABSTRACT
The present study aimed to investigate the effect of new compound L-arginine derivative (AVO) alone or in combination with cadmium chloride on the reproductive efficiency in male rats. For these experiment 40 adult male rats and 16 adult female rats, the male were divided in to 4 groups (10 rats each for each group); Group (1): was daily received normal saline by i.p injection and served as a control. Group (2) was daily received AVO only at 72 mg/kg B.W. by ip injection. Group (3) was daily administered CdCl₂ at a dose of 225 mg/kg B.W. by i.p injection. Group (3) was daily administered of CdCl₂ at a dose of 225 mg/kg B.W. by i.p injection and after one hour of CdCl₂ administration the treated rats given the new compound AVO at a dose of 72 mg/kg B.W. by i.p injection. After 28 days 8 of each group of male were sacrificed for biochemical study of reproductive hormones (FSH, LH, and Testosterone), while other 2 male of each groups were mating with 4 females. This experiment is lasted for 21 days, the mating duration was 10 days. One male were mate with two females, the females are separated in individual cages till the parturition once the female’s rats are given birth, and the number of letters are calculated. The fertility percent are documented. The obtained results indicated that cadmium chloride possesses a deleterious effect on reproductive system, induce oxidative damage, histological changes of testes, and cause clear changes on the sexual hormone. The administration of AVO with cadmium chloride is relived the hazard effects of cadmium chloride, it improved, sexual hormones includes FSH, LH and testosterone, with ameliorating the histological changes in the testes, in-addition to positive effect of AVO on the fertility.

KEYWORDS
L-arginine derivatives, cadmium chloride, reproductive efficacy.
1. INTRODUCTION

Cadmium (Cd) is one of the abundant metal, it commonly found in an environmental such as: air, water and earth\(^1\). It is potent toxic agent\(^2\), arising from an industrial, it consider as contaminator agent for environmental, using in pigments, batteries, plastic, and also it found in the smoking of cigarette. When consumption it by human it rapidly absorbed and accumulate in various tissues and produce its harmful effects\(^3\). It binding with the membrane of red blood cell and albumin of the plasma, rapidly after entrance into the body\(^4\). It cause toxic effects of the cells\(^5\), or it acting by changing intracellular glutathione levels\(^6\). Resulting in dysfunction of liver, kidney, testes\(^7\), also it cause pulmonary emphysema and osteoporosis\(^8\). It causes hypothalamus, pituitary gland and testicular damages\(^9\). In our study we used L-arginine and vanillin as antioxidant substances. L-arginine (2-Amino-5-guanidinopentanoic acid), is a semi-essential amino acid with 174.2 molecular weight\(^{10}\), L-arginine acts in mediating of several important function of the body, including; increases immunity, enhances cell division, promotes wound healing, immunity to illness, and the modified secretion of important hormones\(^{11}\). Also it serves as substrate for the Nitric Oxide (NO) synthesis by NOS (nitric oxide synthase), the letter is a radical involved in such divergent effects as relaxation of smooth muscle and host protection\(^{12}\), although, NO has essential effect on the vascular, but also L-arginine exert nitric oxide –independent hemodynamic effects\(^{13}\). The vasodilation effects is promotes by L-arginine NO-independent which occur through: stimulate released of histamine from mast cells\(^{14}\), attenuate of norepinephrine activity, thus it on the other hand enhance the effect of vasodilation substance such as NO\(^{15}\), and increased secretion insulin\(^{16}\), in addition to release NO, all these effects will improved vasodilation effect of L-arginine. Vanillin (4-hydroxy-3- methoxybenzaldehyde), is a substance that has a pleasant odour aromatic, naturally it is occur in vanilla beans\(^{17}\). Generally vanillin is known as flavoring substance in many foods such as desserts like cake, biscuits, ice cream; however, vanillin is not used only as a flavoring substance, but also is more potent antioxidant than antioxidant agent\(^{18,19}\). It has many medical effects, it protects chromosomes from damage induced by radiation, thus it consider as anticlastogenic agent\(^{20}\). Vanillin is a potent antioxidant. It inhibits protein oxidation and lipid peroxidation by quenching singlet oxygen\(^{21,22}\). It scavenges free radicals, protects DNA and mitochondrial membrane against oxidative stress in vitro\(^{23}\), and inhibits oxidation of protein and lipid\(^{21,24}\). It has been reported to show significant brain protective property by reducing the levels of reactive oxygen/ nitrogen species and augmenting the activities of antioxidant enzymes\(^{25}\).

![Scheme 1](image_url): Schiff base compound derived from L-arginine and Vanillin\(^{26}\)
2. MATERIALS AND METHODS

2.1. Reproductive study

Animal and experimental design:
Rats are divided into 4 groups (8 rats in each group) as following:

Control group: In this group, 8 male rats and were injected I.P with 0.9% normal saline (N.S) 0.5 ml daily for 28 days.

Treated group 1: This group consisted of 8 male rats which were injected intraperitoneally (I.P) with AVO only 0.5 ml daily for 28 days.

Treated group 2: This group consisted of 8 male rats were injected I.P with 225 mg/kg CdCl₂ daily for 28 days.

Treated group 3: In this group, 8 male rats and 10 female rats were injected I.P with 1/10 of LD₅₀ (72) mg/kg AVO complex daily after one hour of 225mg/kg CdCl₂ administration for 28 days.

Blood samples were collected from the heart by heart puncture by the use of the disposable syringes of 5cc capacity. After anesthesia of the rats, blood collected and analyzed according to Sood.

♦ 4 ml of blood was poured into a tube containing the ethylene diamin tetra acetic acid (EDTA) as an anticoagulant for RBC, Hb, PCV and WBC, differential WBC analysis.

♦ 6 ml of blood was poured into test tubes free from anticoagulant to isolate blood serum to estimate serum reproductive hormones FSH, LH and testosterone.

Semen fluid analysis

Sperm count: The sperms were counted according to method of Evan and Maxwell. The tail of epididymis was put in Petri dish containing 5ml normal saline (0.89% NaCl) and minced with sharp curved scissor. The resulting suspension was filtered by clean piece of gauze to get rid of clumped sperms. The hemocytometer loaded with semen diluted by normal saline (1/200) and sperm calculated over the central square. The obtained number is multiplied by 10000 to obtain the number of sperms per ml of diluted sample and then multiplied by the dilution factor (200). The result will be the sperm number/ml of seminal fluid.

Histology of testes

Livers and testes were collected from all groups and prepared for histological study as following.

Fixation: The specimens were fixed in 10% buffered formalin. Washing and dehydration: The specimens washed with water to remove the fixative and prevent its interaction with the staining materials used later. Specimens dehydration achieved by bathing them successively in an increasing concentration of ethanol (70 %, 80 %, 90 %, and 100 % ethanol).

Clearing: Bathing the dehydrated fragments in xylene for 30-60 minutes, this step was repeated 3 times. Cleared tissues looks transparent.

Infiltration and embedding: After clearing, the tissue fragments were placed in melted paraffin in an oven (52-60 °C). The heat causes the solvent to evaporate, and the space within the tissues becomes filled with paraffin.
Sectioning: The specimens removed from the oven and allowed at room temperature to be solid. Five microns thickness sections obtained by microtome from paraffin-embedded tissue were floated on water bath (50-55 °C), then transferred into glass slides and left to dry.

Staining: The histological sections of the studying organs were stained with Hematoxylin-Eosin stain.

2.2. Fertility study

Animals and experimental design
In this part of the experiment, 24 mature rats are used 16 female and 8 male. Females and males have been separated for the 16 days before the beginning of the experiment to insure that the females are not conceived. The rats divided to three groups as follow:
1. Control group: In which male rats were treated with normal saline intraperitoneally injected (0.5 ml / day) for 4 weeks.
2. AVO group: In which male rats were treated with 72 mg / kg intraperitoneally injected of New derivative of L-arginine (0.5 ml/day) for 4 weeks.
3. CdCl₂ group: In which male rats were treated with 225 / kg of CdCl₂, intraperitoneally injected (0.5 ml / day) for 4 weeks.
4. AVO and CdCl₂ group: In which male rats were treated with 225/kg of CdCl₂ and after one hour, the rats treated with 72 mg/kg of new derivative of L-arginine intraperitonealy injected (0.5 ml/day for each material) for 4 weeks.

2.3. Measured parameters
The fertility rate (number of pregnant females/ 4 = number of females in each group ×100), mean birth number (number of births/ 4 = number of females in each group) and mean birth weights were recorded.

2.4. Statistical analysis:
Computerized SPSS (Statistical Package for Social Sciences) (V.13) program were used for analysis of results of the present study. The data were expressed as mean ± standard deviation (mean ± SE). Least significant difference test (LSD) was used to test the difference between means (groups); P≤ 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. The ameliorative effects of AVO on CdCl₂ treated rats on serum FSH, LH, Testosterone
The results in Table (1) revealed that that administration of AVO alone caused clear increased in FSH, LH, Testosterone levels when compared with the normal group. While Cadmium chloride administration resulted in a decrease in FSH, LH, Testosterone levels compared with control group and AVO group. Concomitant administration of AVO with Cadmium chloride ameliorated the effects of CdCl₂ on the above hormones levels and their values nearly approach to the values in the control group.
Table 1. The ameliorative effects of AVO on CdCl$_2$ treated rats on serum FSH, LH, Testosterone.

<table>
<thead>
<tr>
<th>Group</th>
<th>FSH (ng/ml)</th>
<th>LH (ng/ml)</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.42 ± 0.17 a</td>
<td>4.46 ± 0.13 a</td>
<td>4.52 ± 0.16 a</td>
</tr>
<tr>
<td>AVO (72mg/kg)</td>
<td>10.57 ± 0.15 a</td>
<td>4.60 ± 0.11 a</td>
<td>4.53 ± 0.14 a</td>
</tr>
<tr>
<td>CdCl$_2$ (225mg/kg)</td>
<td>4.65 ± 0.25 b</td>
<td>0.94 ± 0.05 b</td>
<td>1.14 ± 0.066 b</td>
</tr>
<tr>
<td>AVO (72mg/kg) + CdCl$_2$ (225mg/kg)</td>
<td>9.29 ± 0.238 c</td>
<td>3.66 ± 0.15 c</td>
<td>3.88 ± 0.063 c</td>
</tr>
<tr>
<td>LSD</td>
<td>1.12</td>
<td>0.94</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Different letters indicate significant difference at (P≤0.05). M ± SE

3.2. The ameliorative effects of AVO on CdCl$_2$ treated rats on sperm count, motility, viability and reproductive efficiency in male rats.

A significant reduction (P<0.05) in sperm count and motility in male rats treated with Cadmium chloride were recorded compared with control one. The dead sperms elevated significantly due to cadmium chloride injection. The deleterious effects of Cadmium chloride are ameliorated in male rats treated by AVO, after an hour of cadmium chloride administration, and values of sperm count and motility increased significantly, but still less significant compared with the control group (Table 2). Also the data from the table show the fertility and offspring number were not affected by administration of AVO alone to the male rats. Data also revealed that Cadmium chloride administration to male rats resulted in significant reduction to zero % (P<0.05) in the fertility of the normal female rats and therefore, no given birth. The reduction in normal female fertility and birth number were ameliorated by concomitant administration of AVO after an hour of Cadmium chloride injection to male rats, but they still less significantly from those of control values.

Table 2. The ameliorative effects of AVO on CdCl$_2$ treated rats on sperm count, viability, and fertility %. and birth number.
Different letters indicate significant difference at (P≤0.05). NS= non-significant. \( M \pm SE \)

### 3.3. Histopathological changes in the testis (Fig: 1,2,3,4)

In control group, the testes showed normal structure of seminiferous tubules and normal spermatogenesis and supporting cells (Fig: 1). While there is no apparent changes in testes were associated with separate administration of New derivative of L-arginine (Fig: 2). Treatment with Cadmium chloride resulted in disorganization of germinal epithelium, impaired spermatogenesis, widening and congestion of interstitial space (Fig: 3). Cadmium chloride induced histological changes are obliterated by treatment with new derivative of L-arginine (Fig: 4).

![Image](image_url)  
**Fig. 1.** Normal structure of seminiferous tubules and normal spermatogenesis and supporting cells
Fig. 2. Changes in testes associated with separate administration of new derivative of L-arginine.

Fig 3. Disorganization of germinal epithelium, impaired spermatogenesis, widening and congestion of interstitial space.
4. CONCLUSION

4.1. Effect of CdCl₂ and AVO on reproductive efficiency in male rats.

The results showed sharp reduction in the serum FSH, LH and Testosterone levels, which agreed with results of Younessi and Sadeghi\textsuperscript{32}, Abdel Hady et al.\textsuperscript{33} and Saeed\textsuperscript{34}, in decreasing LH and Testosterone concentration. Previous studies have shown that cadmium affects plasma gonadotropin levels), and its prominent inhibitory action on testosterone production by interference with hypothalamic-hypophyseal-testis axis\textsuperscript{(35-40)}, demonstrated that the cadmium cause decreased in testosterone production through reduction in testicular leutilizing hormone receptor, in addition, cadmium chloride decreased sperm viability and reproductive efficiency when injected to rats compared with control. The current study result is agreed with Boscolo\textsuperscript{41} and Kataranovski\textsuperscript{42}. Cadmium is one of the important agents that cause hypothalamus, pituitary gland and testicular damages\textsuperscript{9}. According on present results, there are different possible mechanisms exist by which Cadmium induces oxidative stress affects the gonadotropin and steroid hormones from their glands. The first mechanism involves the depletion of glutathione content. Glutathione, an important non-enzymatic antioxidant, can accept two electrons from free radicals, thus oxidizing to form oxide glutathione under the action of glutathione peroxidase. Through this action, glutathione could inactivate the destructive effects of the free radical. After production, oxide glutathione obtains two protons from NADPH and converts back to glutathione under the action of glutathione reductase, thus forming an antioxidant cycle. It was reported that Cadmium inhibits the activity of glutathione peroxidase and glutathione reductase\textsuperscript{43}, which indicates this heavy metal, can stimulate depletion of glutathione and inhibit production of it. The other mechanism by which Cadmium can induce oxidative stress is through inhibition of antioxidant enzyme activity. Superoxide dismutase, an important enzymatic antioxidant, lies in cytoplasm and mitochondrion. It was reported that the superoxide dismutase...
activity was significantly decreased by Cadmium treatment, which indicates Cadmium can inhibit superoxide dismutase activity\(^43\). The interaction of Cadmium and Zinc may also cause the testicular zinc content to decrease, which also decreases superoxide dismutase activity\(^44\). The injection of AVO retained the fertility percentage and correct the damage that caused by CdCl\(_2\), to its normal compared to the control fertility percentage. L-arginine supplementation significantly improved sperm motility without any side effects\(^45,46\). Many factors caused abnormality effects on the sperm. As lipid peroxidation as well as accumulation of free radicals cause morphological damage of sperm. Supplementation with L-arginine will provides protection power against lipid peroxidation. When NO predominates or in high amount, it cause inactivation of superoxide; while when superoxide predominates or in high amount, it cause inactivation of NO thus in general, higher NO concentration is expected to reduce lipid peroxidation by inactivating superoxides. As it mention above that the L-arginine has is the main source of NO. Based on this, it can be postulated that L-arginine protects spermatozoa against lipid peroxidation through increased NO production\(^45\). Nitric oxide has been shown to abolish free radical anions\(^47\). These anions cause peroxidative damage to membrane phospholipids. Sperms are known to be sensitive to such lipid peroxidation, resulted in impaired sperms function\(^48\). Study by Kisa showed a correlation between increased NO level and sperm motility in rat testicular tissue\(^49\). L-arginine, which acts as protection against lipid peroxidation, is acted as an antioxidant. On the other hand, when NO predominates, it inactivates superoxide; when superoxide predominates, it inactivates NO. Thus in general, higher NO concentration is expected to reduce lipid peroxidation by inactivating superoxides. It has been shown that L-arginine increased generation of NO. Based on this, it can be postulated that L-arginine protects spermatozoa against lipid peroxidation through increased NO production. These results strongly support the present study that L-arginine exhibits its effects on spermatozoa through increased biosynthesis of nitric oxide. These results provide a good understanding of the factors affecting fertility. Also it reported that the vanillin in the dose of 200 mg/kg demonstrated aphrodisiac properties in male Wistar rats\(^50\).

4.2. Effect of CdCl\(_2\) and AVO on the testes histology

It's known that the Cadmium causes stimulation of free radical releasing, which results in oxidative state of lipids, proteins and DNA, and starting of many pathological conditions in living organisms\(^51\). Exposure to cadmium, acute as well as chronic, is contact with trigger of lipid peroxidation in different tissues as liver, kidney, spleen, testes, lung, brain and erythrocytes\(^52,53\). Treatment with Cadmium chloride resulted in disorganization of germinal epithelium, impaired spermatogenesis, widening and congestion of interstitial space, this result is similar to result obtained by Mohamed \textit{et al.}\(^54\). The mechanism of Cd by which it induced toxicity is oxidative status. It enhances oxidative state by increased released of \(O_2\) of free radicals, which may occur due to interaction of cadmium with the structures of mitochondria\(^55\). Moreover, after absorption of the Cd, it rapidly delivered to the liver by MT. Therefore, MT is responsible for absorption and carrying of Cd\(^56\). Histopathological changes that result after administration of exogenous L-arginine plus vanillin (AVO) following injection of CdCl\(_2\) result prevented the toxic effect that induced by cadmium chloride, this result...
is agreed with the result that obtained by Bauer\textsuperscript{57}, that found partially but significantly repairing of obstructive jaundiced in rats after L-arginine supplementation. It played a protective role by inducing NOS expression and causes NO synthesis. The exogenous arginine was effective in elevating hepatic membrane transport activity and increasing production of nitric oxide\textsuperscript{58}. Arginine could promote hepatic NO synthesis, improve hepatic microcirculation, reduce generation of superoxide catalyzed by NOS, and decrease hepatic damage.

In addition the beneficial effect of vanillin in repair histopathological change in liver\textsuperscript{59}, and histopathological change in kidneys caused by CCl\textsubscript{4}\textsuperscript{60}. Present histological results support the earlier findings where in vanillin was found to improve the macroscopic and microscopic damage in ulcerative colitis\textsuperscript{60}. Oral vanillin not only prevented chemically induced colitis, but was also effective in the amelioration of established colitis through its anti-inflammatory activity\textsuperscript{61}. And supports with recent study by Al-Asmaria\textsuperscript{62}, which were demonstrated the gastro protective effect of vanillin against ulcer induced by ethanol. It protect stomach from damage of the wall and prevent hemorrhagic lesions that induced by ethanol. The ameliorating effect of vanillin against gastric ulcers may be assigned to the observed antisecretory, mucosal strengthening, anti-inflammatory and anti-oxidative properties.

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