GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

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ADVERSE EFFECT OF DIAZEPAM ON CYTOGENETIC AND BIOCHEMICAL EFFECTS IN WHITE MICE FED DIET SUPPLEMENT WITH CHITOSAN

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ABSTRACT
The objective of the study was to know the impact of diazepam on the cytogenetic (mitotic, blast index) and biochemical parameters. For this purpose 75 mice, both sexes, aged (7-8) weeks were randomly divided into 5 groups and treated as follows. The first group (n=15) was administrated orally with diazepam (0.6mg/kg B.W.) for a period of 8 weeks by stomach tubes. The second group (n=15) was administrated with diazepam as in the first group and fed a diet containing Chitosan 1gm/kg b.w. The third group (n=15) was immunized by the killed vaccine of *Pasteurella multocida* injected (0.1ml) I/P at a dose of 3×10^8 cfu/ml two doses at two weeks interval and administrated daily with diazepam for a period of eight weeks. The fourth group (n=15) was administrated as in the first group and vaccinated as in the third group and fed a diet containing Chitosan (1gm/kg). The fifth group (n=15) was administrated with given 0.25ml distill water and was considered as negative control. Biochemical tests (ALT and Cholesterol) were measured at 60 days. Cholesterol was measured 1st group (206.4±11.97), 2nd group (168.2±3.54), 3rd group (192.2±10.19), 4th group (176.60±10.19), and 5th group (132.4±3.92). ALT was measured 1st group (87.0±3.05), 2nd group (53.40±11.98), 3rd group (72.80±1.43), 4th group (53.6±3.75), and 5th group (24.20±2.56). The results showed that there was an adverse change in the groups treated with diazepam, while improvements in the groups treated with Chitosan. Cytogenetic study (mitotic index) and (blast index) to cell bone marrow were measured at 60 days post treatment 1st group (12.1±0.4 and 27.33±0.1), 2nd group (15.6±0.4 and 38.33±0.8), 3rd group (17.2±0.1 and 32.67±0.3), 4th group (19.0±0.4 and 40.12±0.3) and 5th group (14.0±0.1 and 29.33±0.2) negative effect of diazepam on the ratio of mitotic index and blast index.

KEYWORDS: diazepam, Chitosan, *Pasteurella multocida*, Cytogenetic study, blast index.

INTRODUCTION

Diazepam is a member of a group of medications that belong to a group known as benzodiazepines it is commonly used to treat anxiety, panic attacks, insomnia, seizures (including status epileptic), muscle spasms (such as in tetanus cases), restless legs syndrome, alcohol withdrawal,benzodiazepine withdrawal, opiate withdrawal syndrome and Meniere's disease[1]. It may also be used before certain medical procedures (such as endoscopies) to reduce tension and anxiety, and in some surgical procedures to induce amnesia[2]. It possesses anxiolytic, anticonvulsant, hypnotic, sedative, skeletal muscle relaxant, and amnestic properties[3]. Incorrect uses of the diazepam with overdose may be led to impairment in immune system principal against opportunistic bacterial infections or decrease infinity program vaccination[4]. Chitosan has widely investigated for many biomedical and pharmaceutical applications it is insoluble in water, but becomes soluble and cationic in aqueous acidic solution (PH<6.5)[5]. Due to the few researches on the effects of diazepam on the immune response and influence strength of the immune response against toxic effects of diazepam and effect of diazepam on biochemical, cytogenetic parameters. The aims of the present study are to determine the toxic effects of high dose of diazepam on cytogenetic and biochemical parameters in white mice.

MATERIALS & METHODS

Experimental Design

Seventy five (75) healthy mice both sexes (7-8) weeks old were randomly divided into five groups and treated as follows:

1. First group: (n=15) was administrated with diazepam 0.25ml orally containing 0.6mg/kg b.w daily for 8 weeks.
2. Second group: (n=15) was treated as the 1st group but at the same time received (1gm/kg of diet) Chitosan for 8 weeks.
3. Third group: (n=15) was administrated with diazepam 0.6mg/kg b.w orally daily but at the same time immunized with *P. multocida* vaccines, I/P with 3×10^8 cfu/ml two doses at two weeks intervals.
4. Fourth group: (n=15) was administrated with diazepam as the 1st group and received (1gm/kg of diet) Chitosan and immunized with *P. multocida* vaccines (I/P with 3×10^8 cfu/ml two doses at two weeks intervals).
5. Fifth group: (n=15) was administrated with 0.25ml distal water as a negative control group.

EXPERIMENTAL DESIGN

Total number 75 mice divided into 5 groups

Group 1	Group 2	Group 3	Group 4	Group 5
N=15	N=15	N=15	N=15	N=15
Diazepam 0.006%	Diazepam 0.006%	Diazepam	Diazepam	Control
Orally	Chitosan	*Pasteurella*vaccine	Vaccine	
60 days	0.1% in diet	60 days	Chitosan	
	60 days		60 days	60 days

Cytogenetic Diazepam in Bone Marrow

Direct Method: Mitotic and Blast index (MI, BI)

The protocol of [6] was done to study as follows:

1. After the bone was washed, both ends of the bones were cut and the bone held vertically above test tube, 5ml at 37C° sterile PBS injected into the bone until the bone being clear, collected the bone marrow in a test tube.
2. Cell suspension mixed with 1ml of colchicines added for about 10 minutes and then the tube was put in an incubator at 37C°.
3. The tubes centrifuged which contain bone marrow at 2000 RPM for 10 minutes. The suspension was withdrawn and the sediments mixed very well and then 10 ml of 37C° KCL was added gently at the beginning and incubated for about 40 minutes.
4. After ending the incubation period, the tubes were centrifuged at 2000 RPM for 10 minutes and withdraw the suspension, the sediments mixed well and fixed by fresh glacial acetic acid methanol (1:3 v/v) by dropping gently until reach (5ml).
5. The tubes left in 4C° for 30 minutes and then centrifuged at 2000 RPM for 10 minutes, the washing by saline process return for 6 hours and then the sediment suspend in 1-2 ml.
6. The suspension mixed by pipette and then dropped the suspension on oil free slide at a distance (30-50cm) and the slides were left to dry. The slide stained with Giemsa stain for about 15 minutes and then after dry, the slides were examined under light microscope. By this technique the MI (mitotic index) and BI (blast index) can be calculated.

$$MI = \frac{\text{Number of Chromosomal Metaphase}}{1000} \times 100$$

$$BI = \frac{\text{Number of Blastocytes}}{1000} \times 100$$

Biochemical study

Blood samples were collected from mice directly by using syringe 1ml in at 60th day post treated, blood samples were transferred into a epindrof tubes, after that kept in refrigerator overnight in a stand position then centrifuged at 1500 RPM for 3 minutes, the serum were stored in the frozen at -20 C° until biochemical analysis. Biochemical analysis of ALT enzyme and cholesterol were measured using special kits[7].

RESULTS & DISCUSSION

Mitotic and Blast index

The data of table (1) explained the effects of diazepam on mitotic and blast index (MI) and (BI) in groups treated with diazepam (12.1±0.4) and (27.33±0.1) showed a significant decrease ($p<0.05$) in MI and BI when compared with a control group which MI and BI were (14.0±0.1) and (29.33±0.2), while group treated with diazepam and Chitosan were (15.6±0.4) and (38.33±0.8) showed significant ($p<0.05$) increase as a comparison with the control group. The results showed a significant increase ($p<0.05$) in MI and BI values in the group treated with diazepam and vaccine (17.2±0.1) and (32.67±0.3) as a comparison with the control group, in addition to the results showed a significant increase in group treated with diazepam, the 4th group treated with diazepam, Chitosan and vaccine (19.0±0.4) and (40.12±0.3) was high significant ($p<0.05$) as compared with the control group.

TABLE 1: the effects of diazepam (mean ± SE) on mitotic (%) and blast index (%)

No	Group	MI	BI
1	Diazepam	12.1±0.4	27.33±0.1
2	Diazepam + Chitosan	15.6±0.4	38.33±0.8
3	Diazepam + vaccine	17.2±0.1	32.67±0.3
4	Diazepam + Chitosan Vaccine	19.0±0.4	40.12±0.3
5	Control	14.0±0.1	29.33±0.2

The results of the group diazepam showed low significantly ($p<0.05$) in mitotic and blast index may be due to the adverse effects of active metabolites called desmethyldiazepam of the diazepam most of the drug is metabolized, very little diazepam is excreted, these results agreements with[8] who reported accumulation of the drug during repeated administration. The present study results showed that the second group leads to increase the ratio of MI and BI in this study may be due to the action of Chitosan immune stimulation effects, this results agreement with[9] who found mechanism involves immunostimulating effects of chitin and its carboxymethyl derivatives via stimulation of cytolytic T-lymphocytes. Also the results of the group treated with diazepam and Chitosan showed increased gradually in mitotic index and blast index this results may be that Chitosan play role as antioxidant this agreement with[10] who showed that antioxidant provides protection to living organism from damage caused by uncontrolled production of free radicals, reactive oxygen species (ROS) and concomitant

lipid peroxidation, protein denaturation and DNA-strand breaking. The present results showed that the group administrated with (DZ) and immunized group lead to increase in (MI) and (BI) this results may indicate that immunization may be neutralized the cytogenetic effects of diazepam on bone marrow by activation of immune system so that leads to remove the dead cells and prevent formation of free radical and this observation was in agreement with[11] who found vaccination that stimulates immune cells to recognize and attack foreign bodies, especially through antibody production.

BIOCHEMICAL TESTES

Serum cholesterol level

The serum cholesterol levels in the 1st group (206.4± 11.97) and 3rd group (192.2 ± 10.19) groups recorded significantly ($p<0.05$) higher values than all other groups table (2), while the 2nd group (168.2±3.54) and 4th group recorded significantly ($p<0.05$) higher values compared with the 5th group (control group) (132.4±3.92).

ALT enzyme

Enzyme activities in the table (2) of the 1st group (87.0±3.05) showed significant ($p<0.05$) higher values than all other groups except the 3rd group (72.8±1.43) which revealed significant ($p<0.05$) higher values than the 5th group (24.20±2.56) which showed no significant difference among them. The 4th group (53.6±3.75) and the 2nd group result showed (53.40±11.98) significant ($p<0.05$) higher than the control group.

TABLE 2: (Mean±SE) of serum of cholesterol and ALT post 60 days from the experimenter.

No	Group	Cholesterol(mg/dl)	ALT (I.U)
1	Diazepam	206.4±11.97	87.0±3.05
2	Diazepam + Chitosan	168.2±3.54	53.40±11.98
3	Diazepam + vaccine	192.2±10.19	72.80±1.43
4	Diazepam + Chitosan + vaccine	176.60±10.19	53.6±3.75
5	Control	132.4±3.92	24.20±2.56

The present study gave indication that animals in the 1st group diazepam administrated for 8 weeks induced increase level cholesterol in serum of animals, these results may be the diazepam cause disturbances in liver metabolism, these results were agreements with[12] who found a significant increase of cholesterol synthesis was observed with much higher level of diazepam-treated. In addition[12] suggested a positive correlation between the increased cholesterol synthesis and the formation of areas smooth endoplasmic reticulum. These findings suggest an early stimulation of the liver cells microsomal system by diazepam in man, they also point to side effects of some drugs. These investigations explain the reason of high level of cholesterol in serum of mice received administrated with diazepam. The present study gave indication that animals fed diet supplement with Chitosan expressed decreased in level cholesterol these result may be indicate Chitosan prevent intestinal absorption of cholesterol supplement and these investigation was agreed with[13] who found that the Chitosan combined with lipid absorption from intestine and decrease the amount of fat absorption. The result of this study showed that mice fed on a diet with Chitosan 1 gm/kg with diazepam administrated orally for 8 weeks, showed reduction in concentration of the serum total cholesterol and ALT, and as compared to mice received a diet supplement only. These results may indicate that Chitosan is a good hypocholesterolemic agent. The result was consistent with [14] who showed that Chitosan had a potent lipid lowering effect in the mice. And also agreed with[15] who found that Chitosan has a hypocholesterolemic effect. The present study gave indication that diazepam administrated for 8 weeks in the 1st group induced increase level ALT in serum of animals as compared with the negative control group these result may indicate that the treated with diazepam that attributed to the hepatocellular injury and this results agreement with[16] who showed that diazepam caused increase in liver enzymes activity (ALT), also agree with[17] who showed that diazepam caused increase in liver enzyme activity (ALT, AST) and revealed that the administration of this toxic drug induces oxidative tissue damage indicated by the marked elevated activity of a xanthine oxide (XO) accompanied by increased nitric oxide (NO) levels in the liver[18] who showed repeated oral administration of diazepam significantly elevated serum concentrations of ALT, AST, ALP, LDH and bilirubin in cats. The results agree with[19] who found acute hepatic injury may be caused by direct toxic effects of the drug or its metabolites on the hepatocyte, producing predictable dose dependent effects, or by idiosyncratic drug reactions, which occur unpredictably in a small number of cats exposed to a particular drug such as diazepam, in addition[20] suggested that the drug was hepatotoxic in cats when a cat serum AST and ALT rise following therapy due to formation free radicals and cells damage.In the current study, serum concentrations of ALT decreased significantly ($p<0.05$) in 2nd , 3rd and 4th groups as compared with the 1st group these results may indicate that Chitosan cause decrease in liver toxicity these resulting agreements with [21] who found that Chitosan stabilized the cellular membrane structure and regulated the activity of AST, ALT and ALP. The results of this study showed serum concentrations of ALT decreased significantly ($p<0.05$) in immunized animals fed diets supplement groups as compared with 1st group these results may indicate that synergism effects between Chitosan and the immune response that may be improving the efficacy of liver cells and remove damage cells these results a

greeting with [22] who found a mixture of lower molecular weight Chitosan induced cellular proliferation and IgM secretion, Chitosan play important role in innate defense mechanisms of the host through activated phagocytic cells and by up-regulated IL-1, TNF-α. The result in this study showed that mice fed on a diet with Chitosan 1gm/kg with diazepam administrated orally for 8 weeks, showed reduction in concentration of the serum total cholesterol and ALT, as compared to mice received a diet supplement with diazepam only, the results of the group treated with Chitosan showed a decrease in liver enzyme activity and that may be repair of injured hepatic parenchyma and that agreed with[23] who found that Chitosan decreases liver enzyme activity ALT, glutamyltranspeptidase (GGT) and total bilirubin (TB), and reduces liver necrosis and inflammation.

CONCLUSION: The diazepam has side effects on biochemical, cytogenetic parameters in white mice.

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