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The Ultrastructural Changes in the Liver Cells Induced by High Doses of Benzodiazepine Tranquilizing Drugs: An experimental transmission Electron Microscopic Study on Male Guinea Pigs

التغيرات التركيبية الدقيقة في الخلايا الكبدية الناتجة عن الجرعات المرتفعة من عقاقير البنزوديازبين المهدئة : دراسة تجريبية بالمجهر الالكتروني النافذ على ذكور خنازير غينيا

Ameen Saleh Ahmed Bin Bisher

أمين صالح أحمد بن بشر

King Abdul Aziz University

Jeddah 21454 P. O. Box 15758 Saudi Arabia

E-Mail: Ameenbisher@yahoo.com

ABSTRACT: Benzodiazepines are tranquilizing psychotropic drugs. Unfortunately, despite their therapeutic benefits, they are illegally consumed in high doses by some addicts to reach a sedative, exhilarative and euphoria state similar to that produced by narcotic substances. The present study, using transmission electron microscope on male guinea pigs, aims to investigate the potential ultrastructural changes in the liver cells induced by the high doses of Benzodiazepines. Animals in three treated groups administrated a daily combined dose consisted of (10mg Alprazolam with 10mg Diazepam/day/animal) for three different treatment periods: 7, 15, and 25 days. The ultrastructural examination of the hepatocytes of the animals treated for 15 days showed limited changes in the form of marginal heterochromatine accompanied with marginal nucleoli enlargement. On the other hand, severe ultrastructural damages are observed in the hepatocytes of the animals treated for 25 days, which appeared in the following various patterns: fatty degeneration of the hepatocytes as indicated by the accumulation of large number of lipid droplets in the cytoplasm, marked nuclear atrophy in some necrotic hepatocytes, massive nuclear degeneration in other hepatocytes, mitochondrial damages in the form of cristea destruction accompanied with abnormal oval shape, massive lysis of the cytoplasmic organelles with severe plasma membrane rupture. In conclusion, the observed ultrastructural damages in the present study may refer to the potential hepatotoxic effects of the high dose of Benzodiazepines. It is recommended that much more official restrictions should be applied on the pharmacies sector to prevent any illegal selling of these drugs in order to prevent abusers from obtaining them, as unfortunately in some developing countries the illegal selling of these drugs is known to occur due to the absence of official control.

Keywords: *Benzodiazepines drugs, Ultrastructural changes, Hepatocytes, Necrosis, fatty degeneration.*

المستخلص: تعتبر عقاقير البنزوديازبين من العقاقير النفسية المهدئة. ومن المؤسف أنه بالرغم من الفوائد العلاجية لهذه العقاقير إلا أن بعض المدمنين يتعاطونها بجرعات عالية بهدف الوصول الى حالة من الخدر والسرور الزائف تشبه تلك التي تولدها المُخدرات. تهدف الدراسة الحالية، باستخدام المجهر الالكتروني النافذ على ذكور خنازير غينيا، إلى التعرف على التغيرات التركيبية الدقيقة المحتملة في الخلايا الكبدية الناتجة عن تعاطي الجرعات المرتفعة من هذه العقاقير. تعاطي كل حيوان في المجموعات المعاملة الثلاث جرعة يومية مكونة من (10 ملجم البرازولام مع 10 ملجم ديازبام/يوم/حيوان) على مدى ثلاث فترات معاملة مختلفة: 7 ايام، 15 يوم، 25 يوم. وقد أظهر الفحص التركيبي الدقيق للخلايا الكبدية في الحيوانات التي عوملت بالبنزوديازبين لمدة 15 يوم تغيرات متفاوتة الشدة، حيث ظهرت هذه التغيرات في صورة توزيع غير منتظم للمادة الكروماتينية مصحوب بتوضع للنويات بالقرب من الغلاف النووي. وعلى الجانب المقابل، كانت أكثر الأضرار الخلوية شدة هي تلك التي شوهدت في خلايا الكبد في الحيوانات التي عوملت بالبنزوديازبين لمدة 25 يوم، وظهرت هذه الأضرار الخلوية في صورة عدة أنماط شملت: تحلل فجوي دهني للخلايا الكبدية دل على حدوثه تراكم أعداد كبيرة من القطرات الدهنية في السيتوبلازم، صاحب ذلك تضرر بالغ في أنويه الخلايا حيث أصيب بعضها بالضمور والإنكماش، في حين حدث تحلل كامل للأنوية الاخرى في الخلايا الكبدية المتنكرزة. وفي الوقت ذاته شمل التضرر عضيات الميتوكوندريا والتي ظهرت فيها الحواجز الميتوكوندرية (Cristea) محطمة كليا مع إتخاذ الميتوكوندريا لأشكال كروية غير منتظمة وغير طبيعية، ورافق ذلك تمزق بالغ الشدة للغشاء البلازمي المحيط بالخلايا الكبدية المتنكرزة. إن هذه الأضرار التركيبية الدقيقة التي رصدت خلال الدراسة الحالية في خلايا الكبد تشير إلى وجود تأثيرات سمية كبدية محتملة ناتجة عن تعاطي الجرعات المرتفعة من عقاقير البنزوديازبين. توصي الدراسة بضرورة فرض المزيد من القيود الرسمية على الصيادلة لمنع أي بيع غير قانوني بدون وصفات طبية لهذه العقاقير لمنع وصولها إلى من يسيء استخدامها، إذ أنه من الثابت والمؤسف انه في بعض الدول النامية توجد الكثير من المخالفات حيث تباع هذه العقاقير بشكل غير قانوني بدون وصفات طبية بسبب غياب الرقابة الرسمية.

كلمات مدخلية: *عقاقير البنزوديازبين، التغيرات التركيبية الدقيقة، خلايا الكبدية، تنكرز، تحلل دهني.*

INTRODUCTION

Benzodiazepines are a class of commonly prescribed tranquilizing drugs which are widely used by physicians in treating many neurological and psychiatric disorders, such as sleeping disorders, acute anxiety, depression, muscular convulsions and gastric stress ulcers (Harvey and Champe, 2002; Martire, *et al.* 2002). All different types of benzodiazepine drugs are characterized by the high similarity in their chemical structure (McKim, 1986). According to Schlatter, *et al.* (2001) despite these therapeutic benefits of benzodiazepines, there is a negative phenomenon related to them which is the abuse problem, as they are unfortunately consumed in high doses by benzodiazepines addicts to reach a sedative, exhilaration and euphoria state (a pleasure feeling state similar to that produced by narcotic substances) (Kapczinski, *et al.* 2001; Wang, 2002; Hertz and Knight, 2006). This illegal misbehavior induces serious health hazards such as withdrawal symptoms, overdose intoxication and death in some cases (Haw and Stubbs, 2006; Jonson, *et al.* 2007). Isbister, *et al.* (2004) study provided a clear image about the health hazards of this illegal phenomenon as it recoded 2063 intoxication cases due to benzodiazepines abuse in Australia. A number of previous investigators confirmed the existence of this negative phenomenon in many countries including Canada, USA, Brazil, UK, Germany, Sweden, Turkey, Egypt, Kuwait, India, Pakistan and Australia (Bilal and Khattar, 1992; Roger, *et al.* 1997; Kapczinski, *et al.* 2001; Hertz and Knight, 2006). In fact, part of the complexity of this problem results from the confusing conflict between the necessary therapeutic benefits of these drugs on one side, and the health risks induced by abusing them on the other (Roger, *et al.* 1997; Sjogren and Rindom, 2006). In addition, there is a disagreement between the investigators regarding the hepatic adverse effects of benzodiazepines, as some investigators still believe that benzodiazepines are safe drugs even in the overdose cases (Berthold, 2007). In regard to the hepatic adverse effects in particular induced by benzodiazepines abuse,

Serna, *et al.* (1997), Jadallah, *et al.* (2003) and Calarasu, *et al.* (2004) reported some cases of acute hepatic disorders caused by high doses of certain types of benzodiazepines. However, none of the previous studies have provided a clear ultrastructrual evidences proving these potential toxic adverse effects. Such ultrastructural study will be very helpful in determining the exact internal organelles in liver cells which are very sensitive and susceptible to the potential toxicity of these drugs. Therefore, the present electron microscopic study has been designed to investigate the potential ultrastructural changes in the liver cells induced by repeated administration of two benzodiazepine drugs (Alprazolam and Diazepam) in male Guinea pigs during three different treatment periods (7, 15, and 25 days).

MATERIALS AND METHODS

Dose preparation

According to Juergens (1991), Alprazolam and Diazepam have been found to be the most benzodiazepine types abused by abusers in USA and in many other countries. Therefore, in this study a combination of Alprazolam and Diazepam have been used in preparing the daily dose given to each treated animal, to create an experimental model simulating the cases of benzodiazepines abuse in humans (Haw and Stubbs, 2006). The LD50 of Diazepam and Alprazolam in mice were found to be 620 mg/Kg and 960 mg/Kg, respectively (McKim, 1986). Alprazolam tablets (manufactured by Amoun Pharmaceutical Co, Egypt) and Diazepam tablets (manufactured by Nile Co. for pharmaceuticals, Egypt) have been obtained for research purpose. The daily dose given to each treated animal was prepared by dissolving 10mg of Alprazolam together with 10mg of Diazepam in 4ml of physiological saline according to the method of Fukazawa, *et al.* (1975). This daily dose was given orally to each treated animal by using a gastric feeding tube.

Animals used and treatment

Twenty-four adult male Guinea pigs (Dunkin Hartely strain) weighing 800-830g have been obtained from the animal house unit of King Fahed Medical Research Center at King Abdull Aziz University in Jeddah. The animals were kept under standard normal conditions of temperature, dark/light cycle, water and diet, wide clean cages. After the acclimatization period, the animals were divided into 4 groups (6 animals each), the first group (G I) was the control group, while the second group (G II), third group (G III) and fourth group (G IV) were the three treated groups, in which animals were treated daily with the tested dose for three different treatment periods of 7, 15 and 25 days, respectively.

Preparation of the transmission electron microscopic sections

The protocol used in the preparation was similar to that described by Hummdi (2002) study. All the preparation steps were preformed in the Electron Nicroscope Unit in King Fahed Medical Research Center, Jeddah. At the end of the treatment period of each treated groups (G II, G III, G IV), each animal was killed by sudden neck decapitation (Al-Tayib, 2004). Similar method was used with control group (G I). Liver samples were collected within few minutes and cut into small pieces (1mm thickness), and fixed immediately in glutaraldehyde at 4°C for 24 hours, then liver pieces were washed in phosphate buffered solution (0.1 M). The samples were then post-fixed in buffered solution of 1% osmium tetroxide for 3 hours, and blocked in liquid epoxy resin and incubated at 60° C for 24 hours. The semi-thin sections were prepared using Reichert ultramicrotome (at 1 μm thickness) and stained with 1% toluidine blue for preliminary investigation. Approved fields were further cut into ultrathin sections (60-90nm). The sections were stained with uranyl acetate and lead citrate and visualized using Philips-cm100 transmission electron microscope.

RESULTS

The ultrastructural investigation

Figures 1-14 show the election micrographs for the 4 animal groups. The results of each are given below.

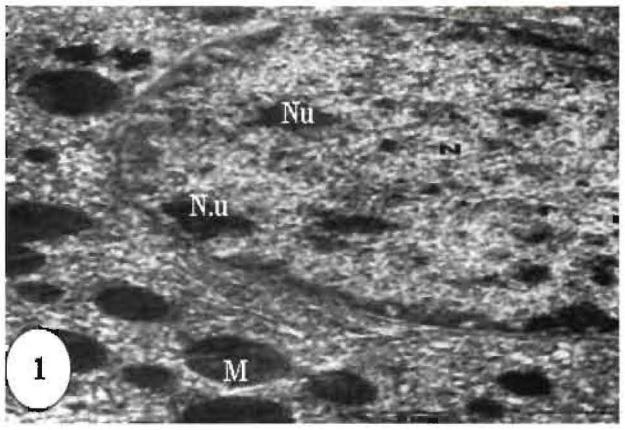
Control group

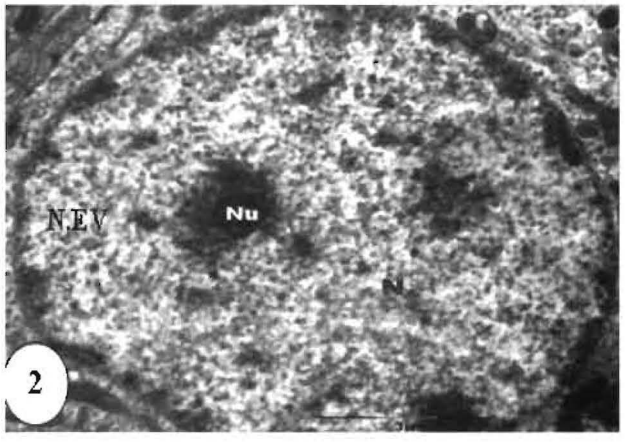
The ultrastructural examination of liver cells (hepatocytes) of the control animals showed normal cellular structure. The hepatocytes appeared

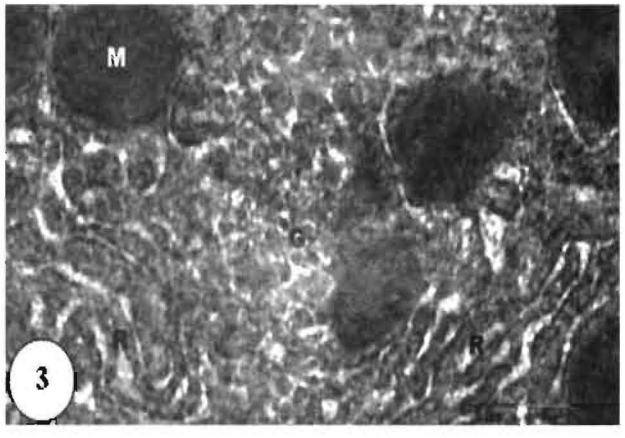
in normal round shape with well defined plasma membrane, abundant endoplasmic reticula, normal mitochondria with their regular spherical shape as shown in Figs.1 and 3. The nuclei appeared in normal regular spherical shape surrounded by intact regular nuclear envelope, the chromatinic material showed normal distribution, and nucleoli appeared in their round shaped as shown in Fig. 2.

Treated groups

The ultrastructural examination of the hepatocytes of the animals treated for 7 days (G II) showed normal ultrastructure similar to that of the control group. On the other hand, limited ultrastructural changes have been observed in the hepatocytes of the animals treated for 15 days in the form of marginal nuclear heterochromatine and abnormal enlargement of marginal nucleoli as shown in Fig. 4. The worst damages were observed in the hepatocytes of the animals treated for 25 days. In this group, both hepatocytes necrosis and fatty degeneration were observed. The necrotic hepatocyte appeared shrunken (Fig. 12). The fatty degenerated cells appeared containing large number of lipid droplets in the cytoplasm (Figs. 5, 6 and 7). These lipid droplets occupied most of the cytoplasmic space causing cellular swelling as shown in Figs. 5, 6, 7 and 8. This fatty degeneration was associated with massive lysis of most of the cytoplasmic orgranelles (Figs. 6 and 7). Meanwhile, nuclei deformities have been observed either in the form of nuclear atrophy or in the from of nuclear degeneration. The atrophied nuclei appeared shrunken with irregularity of the outer surface of the nuclear envelope (Figs. 6, 10, 13 and 14), while the degenerative nuclei appeared with multiple lysis as shown in Figs. 7 and 9. Some of these degenerative nuclei appeared in "crescent" shape (Figs. 8 and 9). The damage extended to include the nucleoli, as they appeared fragmentized (Figs. 13 and 14). Severe plasma membrane rupture was observed as well (Fig. 11). Mitochondrial injuries have been also observed in the form of cristea destruction (Figs. 9 and 10). Meanwhile, destruction of the of the endoplasmic reticula has been also observed (Fig. 10). These observed ultrastructural damages in the liver cells may refer to potential hepatotoxic effects induced by the high doses of benzodiazepines.

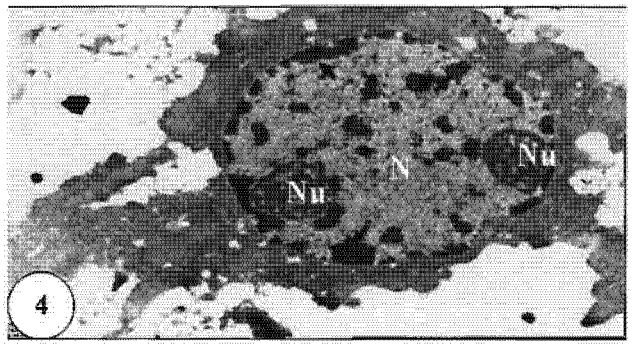


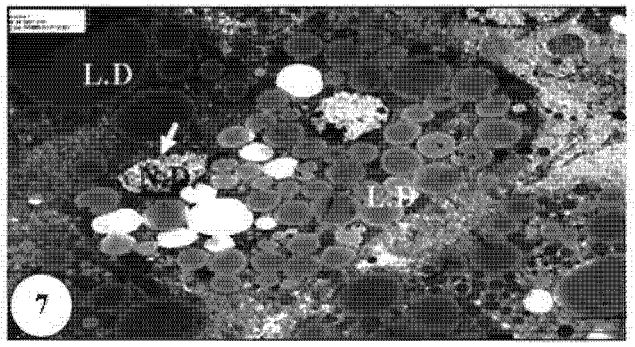


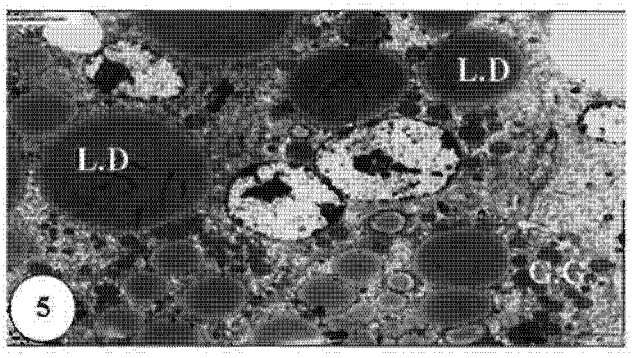


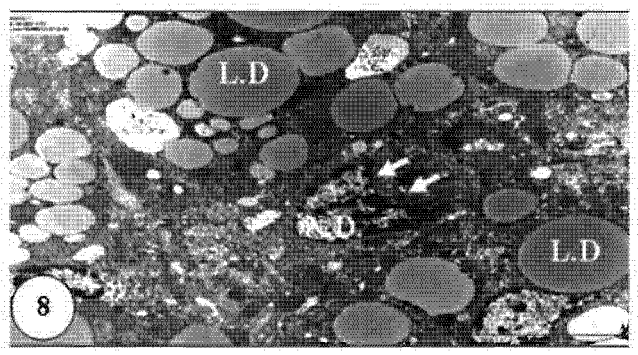
Figs. 1 and 2. Two transmission electron micrographs of liver cells of control animal, showing the normal cellular structure of the hepatocytes, note the regular round shape of the nucleus (N) with normal chromatine distribution, normal nucleoli (Nu), regular intact nuclear envelope (N.EV.), and spherical shape of the mitochondria (M). Scale bar = 5μm and 3μm respectively.

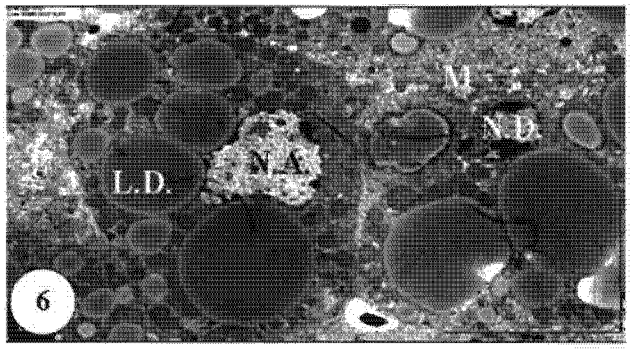
Fig. 3. Electron micrograph of liver cell of control animal, shows some of the cytoplasmic organelles, observe the normal shape of smooth endoplasmic reticulum (R), Golgi apparatus (G) and spherical shape of mitochondria (M). Scale bar = 5μm.











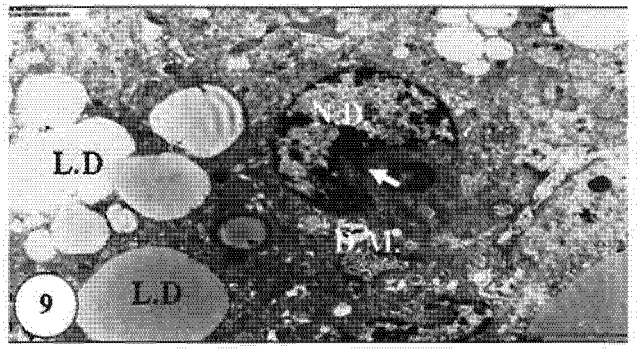
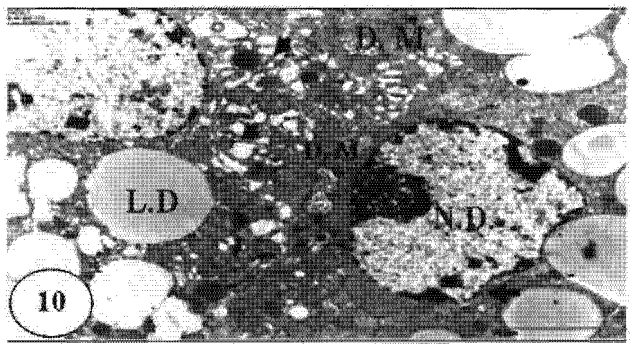
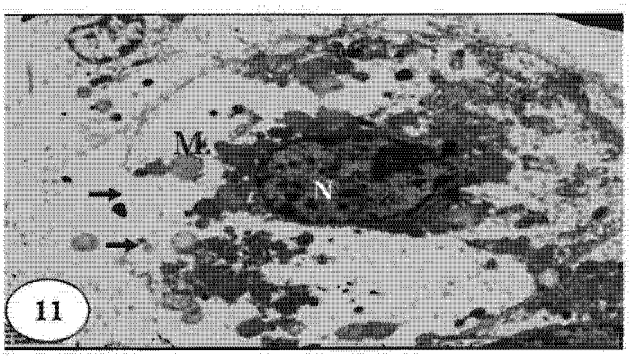


Fig. 4. Electron micrograph of liver cells in animal treated for 15 days with benzodiazepines, showing abnormal marginal heterochromatine (arrows) accompanied with marginal nucleoli (Nu) close to inner surface of the nuclear envelope. Scale bar = 5μm.

Figs. 5 and 6. Two electron micrographs of damaged liver cells in animal treated for 25 days, showing accumulation of large number of Lipid Droplets (D.L.) due to fatty vacuolar degeneration accompanied with both of nuclear atrophy (N.A.) and nuclear degeneration (N. D.) (arrows), also observe mitochondrial destruction (M) and presence of few glycogen granules (G.G.). Scale bar = 10μm.

Figs. 7, 8 and 9. Three electron micrographs of necrotic hepatocytes in animal treated for 25 days, showing severe nuclear degeneration (N. D.) (arrows), the degenerated nuclei appeared in « crescent « like shape, also note accumulation of many Lipid Droplets (L.D.) in the cytoplasm and mitochondrial destruction (D.M.) with cristea disorganization,. Scale bar = 20μm and 10μm, respectively.





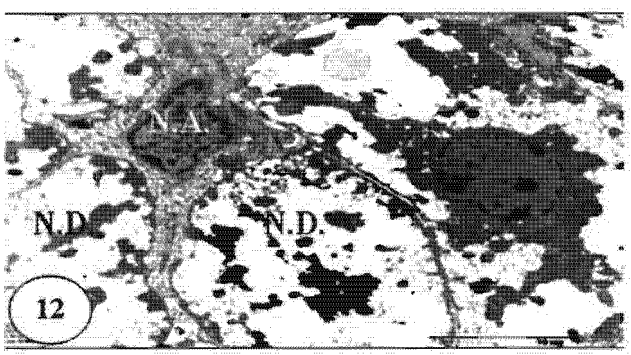
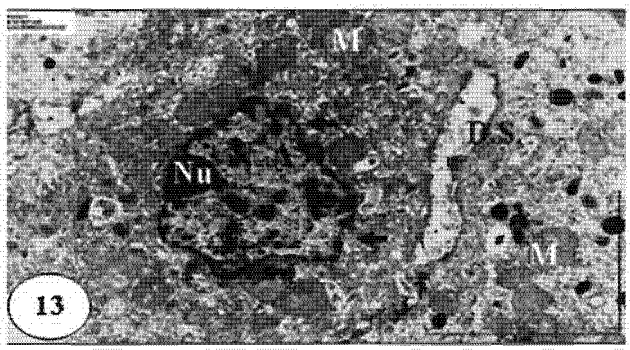


Fig. 10. Electron micrograph of damaged liver cell of animal treated for 25 days, showing two hepatocytes suffering from fatty degeneration as indicated by the accumulation of lipid droplets (L.D.) with nuclear degeneration (N.D.) (arrow), and mitochondrial destruction (D.M.) with endoplasmic reticulum destruction (heads). Scale bar = 10μm.

Fig. 11. Electron micrograph of damaged liver cell of animal treated for 25, showing cell membrane rupture (arrows) accompanied with nuclear deformity (heads) and few damaged oval shape mitochondria (M). Scale bar = 10μm.

Fig. 12. Electron micrograph of damaged liver cells of animal treated for 25 days, shows two lower necrotic hepatocytes suffered from massive nuclear degeneration (N.D.), while the upper cell appeared shrunken with irregular atrophied nucleus (N.A.). Scale bar = 10μm.



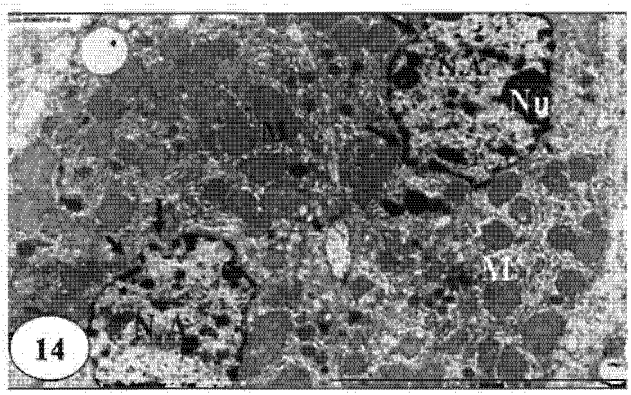


Fig. 13. Electron micrograph of Kupffer cell in animal treated for 25 days, showing deformed nuclear atrophy (N.A) (arrows), irregular chromatine distribution and marginal fragmented nucleoli (Nu), also note deformed mitochondria (M) with oval shape and dilated sinus (D.S.). Scale bar = 5μm.

Fig. 14. Electron micrograph of damaged liver cells in animal treated for 25 days, showing shape irregularity of nuclear atrophy (N.A) (arrows) with marginal fragmented nucleoli (Nu), also note deformed mitochondria (M) which appeared in various shapes. Scale bar = 10μm.

DISCUSSION

At the outset, it is important to mention the fact that all different types of Benzodiazepine drugs have a very highly similar pharmacological and chemical structure, i.e., composed of benzene ring and diazepine ring (McKim, 1986; Harvey and Champe, 2002). Therefore any conclusions about the adverse effects of one type of these drugs can be generalized to include the other types due to this highly chemical and pharmacological similarity. The current study is similar to Chatterjees, *et al.* (1997)

study as it attempted to investigate the hepatic ultrastructural changes induced by high doses of benzodiazepines based on electron microscopic examination. In comparison with previous studies, the present results seem to correspond to the findings of Ozcanli, *et al.* (2006) and Atasoy, *et al.* (2007) who reported some cases of hepatic toxic adverse effects due to benzodiazepines abuse. Similarly, the results also match the histological observations of Calarasu, *et al.* (2004) and Andrade, *et al.* (2006) who reported additional cases of hepatocellular injuries caused by long term of benzodiazepines treatment (i.e., several years) in humans.

Furthermore, the results also support the observations of Roybyrne, *et al.* (1983), Farrell (1994) and Andrade, *et al.* (2000) who documented a number of induced acute cholestatic hepatitis cases by high doses benzodiazepines in humans. On the other hand, in regard to the cellular pathological mechanisms which can explain how these hepatic ultrastructural damages have been induced, it seems that the exact mechanism is still unknown yet. However, previous study of Mollendroff (1973) suggested that the accumulation of lipid droplets in the damaged hepatocytes usually represent an indicator of early stage of cellular autolysis due to long term of exposure to toxic compounds. According to this hypothesis, the injured hepatocytes usually uses these lipid vacuoles to collect the invading toxic compounds in these vacuoles prior of removing them outside the cells. In addition, Cheville (1983) suggested that there is a correlation between the cellular injuries of the different organelles, for instance, mitochondrial damages could cause lipid accumulation in the cytoplasm due to the marked decrease in the process of fatty acids oxidation that occur in the mitochondria. Similarly, Deutsch, *et al.* (2001) provided another hypothesis which stated that high doses of benzodiazepines induced their severe cellular damages in the hepatocytes through the action of certain metabolites such as oxazepam which is able to cause severe DNA damages leading to nuclear damages and cellular necrosis. In conclusion, the current observed ultrastructural damages may prove the potential hepatotoxic effects of the high doses of benzodiazepines. Thus, it can be predicted that abusing high doses of benzodiazepine drugs for long duration (i.e., several years) may cause some hepatic disorders. Therefore, it is recommended that much more restrictions should be applied to prevent the abusers from obtaining these drugs, specially that in some of the developing countries these drugs are illegaly sold without prescriptions due to the absence or weak official control.

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