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EVALUATION OF ACUTE TOXICITY OF GENABILIC ACID (MENBUTONE 10%) IN RABBITS

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Abstract: A complete investigation of the acute toxicity of a choleretic compound, menbutone, was performed in rabbits, including lethal dose for 50% of rabbits determination, clinical signs observation and *in vivo* and *post-mortem* examinations. Haematological, biochemical and histopathological changes resulting from intramuscular injection of menbutone were also investigated at dose 400 mg/kg body weight. Acute toxicity of menbutone at dose of 400 mg/kg BW induced interstitial myocarditis and multifocal necrosis, whereas serum creatine phosphokinase, creatinine phosphokinase-MB isoenzyme and aspartate aminotransferase activities were significantly increased. Elevation of serum alanine aminotransferase and alkaline phosphatase activities and total bilirubin level associated with lowered albumin content was consistent with histopathological changes of hepatic tissues; hepatic necrosis and fatty infiltration were pronounced indicators of injuries. Renal tubular necrosis and interstitial nephritis were also observed in intoxicated rabbits. Menbutone also induced variations in some haematological parameters. We concluded that acute toxicity of menbutone in rabbits occurred at accidental high doses, as the lethal dose was about 50 fold over the recommended therapeutic dose for other animals. Cardiac muscle, liver and kidneys are the main target organs for menbutone toxicity. Menbutone is not recommended for use in rabbits suffering from any cardiac and hepatic disorders, especially in overdosing situations.

Key Words: menbutone, lethal dose, necrosis, rabbits.

INTRODUCTION

Genabilic acid is a white, odourless and tasteless powder which is soluble in water and alkaline solution. Chemically, it is diethanolamine salts of 4, 4-methoxynaphthalene-1-(4) oxybutyric acid and provided as menbutone 10%. In veterinary practices, menbutone was used as a choleretic and appetiser drug for the treatment of digestive disorders in bovine, ovine, porcine and equine species (Symonds, 1982; Bishop, 2005). Menbutone was also used as an additional treatment in some cases of toxicity, such as calves intoxicated with sodium monensin due to feeding concentrate containing sodium monensin. The calves exhibited severe oedema, hydrothorax, hydropericardium, pulmonary oedema, enlargement of heart and dilated ventricles with fatty degeneration, followed by sudden death. The calves treated with mixture of oral administration of sodium carbonate (1 g/L), intravenous (i.v.) saline solution (3 L/animal d), intramuscular (i.m.) injection of genabilic acid (menbutone 5 mg/kg) and sorbitol (25 mg/kg) showed a recovery from intoxication (De La Cruz-Hernández *et al.*, 2012). In rats, gastrointestinal absorption of ^{14}C-menbutone is complete as, 50-60% of the administered dose is eliminated via the urine after 4-8 h, while 79% is excreted via the urine and 4.4% via the faeces after 24 h (Lund and Lassen, 1969).

To our knowledge, there are no available data regarding the use of menbutone in rabbits and this may be related to the absence of toxicological evaluation of this drug in this species. Therefore, the present study aimed to evaluate the acute toxicity of menbutone in rabbits after single intramuscular injection and also to assess the haematological, biochemical and histopathological changes after drug injection.

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MATERIALS AND METHODS

Animals and experimental design

Thirty six healthy adult male rabbits, weighing 1500-1800 g and aged 3 mo, were provided from the Faculty of Veterinary Medicine, Alexandria University, Egypt. All animals were housed in stainless steel cages under controlled environmental conditions with a 12-h light-dark cycle. They were fed on standard commercial diet and water was provided *ad libitum.* The experiment was carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the local authorities. After 1 wk of acclimatisation, all animals were randomly divided into 6 groups of 6 rabbits (1 control and 5 treated groups).

Chemicals and reagents

Menbutone 10% was obtained from Adwea pharmaceutical company, Egypt. Diagnostic kits used for determination of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine phosphokinase (CPK), creatinine phosphokinase-MB (CPK-MB), free albumin, total bilirubin and creatinine were purchased from Vitro Scient Company (Germany). All other chemicals and solvents used in this experiment were of analytical grade.

Acute toxicity of menbutone 10%

Acute toxicity of a single dose of menbutone 10% was investigated by giving the drug intramuscularly to rabbits in various doses ranging from 400, 450, 500, 550 and 600 mg genabilic acid (menbutone)/kg body weight (BW). Animals in the control group were injected with a single dose of normal saline. After menbutone administration, animals were closely observed for the appearance of toxic symptoms, especially those related to hepatic and cardiac lesions as the target of menbutone toxicity and mortality, hourly during the first 6 h, then every 12 h for 7 d. After the end of the observation period, the lethal dose for 50% of rabbits (LD_{50}) was calculated according to Kerber formula (Pershin, 1971) using the following equation: $LD_{50}=LD_{100}-\Sigma(z\times d)/n$, where z is half the sum of the number of animals that died from 2 consecutive doses; d is the interval between doses; n is the number of animals.

Haematological and biochemical analysis

Animals injected with 400 mg/kg BW were used to investigate the haematological, biochemical and histopathological effects of menbutone. Blood samples were collected after 12 h fasting from the ear vein pre and 1, 3 and 7 d post injection and equally separated into 2 test tubes. In the dry tube, blood was centrifuged at 3000 *g* for 10 min (after 30 min at room temperature). Then serum was separated, transferred to Eppendorf tubes, and stored at –20°C for further biochemical analysis. The second test tube containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant was used for haematological studies. Haematological parameters were measured in whole EDTA blood using cell counter apparatus (Exgo Vet apparatus). Serum activity of ALT, AST, ALP, CPK, CPK-MB enzymes, and albumin, bilirubin and creatinine concentrations were spectrophotometrically assessed following the manufacturer's instructions, using Biochemical analyser AE-600N, ERMA-INC-Japan.

Histopathology

After necropsy of animals sacrificed at the end of the 7-d observation period, tissue specimens of liver, kidneys, heart and brain were fixed in 10% neutral buffered formalin solution for at least 24 h. The fixed specimens were processed using the conventional paraffin-embedding technique. From the prepared paraffin blocks, 5-mm-thick sections were obtained and stained with haematoxylin and eosin (H&E) for light microscopic examination, according to the method described by Culling (1983).

Statistical analysis

Data were expressed as mean±standard error. The significance of the difference between pre and post injection parameters was analysed using one way ANOVA test by computerised Costat programme.

Table 1: Respiratory rate at 30 min after drug injection.

	Control	400 mg/kg	450 mg/kg	500 mg/kg	550 mg/kg	600 mg/kg
Respiratory rate (breaths/m)	55.8±2.3	112.5±4.4*	121.2±2.7*	125.0±1.8*	125.0±1.8*	127.6±1.8*

All values represented as mean±standard error.
*Significantly different (P<0.001) to the control value.

RESULTS

Acute toxicity of menbutone 10%

Thirty minutes after drug administration, rabbits in all experimental groups presented tachypnea (Table 1). For 1 to 2 h, the treated rabbits suffered from generalised weakness, salivation, off food (anorexia), inability to move (apathy) and frequent defecation with small amounts of dark faecal matter. We also observed lameness and signs of inflammation at the site of drug injection. The severities of signs were dose dependent. The surviving rabbits gradually returned to normal clinical condition after 24-48 h.

No death was detected among rabbits receiving 400 mg/kg genabilic acid (menbutone), while in those injected with 450 mg/kg BW, 2 animals died after 5-6 h. In the 500 and 550 mg/kg BW groups, 5 animals died within 2-6 h. Menbutone at a dose of 600 mg/kg BW, killed all treated rabbits within 2-3 h. After the end of experiment the calculated LD_{50} of menbutone was 475 mg/kg BW.

Haematological analysis

The haematological values as shown in (Table 2) revealed a non significant decrease in the mean of total leukocyte count 24 h post injection, while after 3 and 7 d it tends to increase. Differential leukocyte count revealed significant granulocytosis and lymphocytopenia 7 d following drug administration. We also detected a non significant decrease in haematocrit (HCT) value and total haemoglobin (HGB) concentration between days 1 and 3 post injection.

Total erythrocyte count, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) varied little along the course of the experiment, while mean corpuscular haemoglobin concentration (MCHC) was significantly decreased at the end of experiment.

Biochemical analysis

Table 3 shows that administration of genabilic acid (menbutone) at 400 mg/kg BW. induces a perturbation in hepatic and biliary functions, as indicated by a significant increase in serum ALT, AST, ALP activities and a total bilirubin level increase together with a significant decrease in albumin concentration after 24 h. Serum activity of ALP was significantly decreased at 3rd and 7th d post injection as compared to its value before injection. Also, menbutone administration led to significant increase in the activities of total CPK, CPK-MB isoenzyme and creatinine level as soon as 24 h after administration, indicating cardiomyopathy.

Table 2: Effect of menbutone toxicity (400 mg/kg body weight) on haematological parameters in rabbits.

	WBCs (10^3/µL)	Lym (%)	Gran (%)	RBCs (10^6/µL)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (%)
0 d	5.8±0.7	68.6±1.4	23.9±1.6	4.8±0.1	10.1±0.2	21.4±0.6	44.0±0.8	20.9±0.4	47.6±0.2
1st d	4.6±0.3	65.6±3.1	29.0±2.8	4.5±0.3	9.2±0.6	19.4±1.3	43.4±0.6	20.6±0.4	47.5±0.6
3rd d	6.3±0.5	63.7±4.0	28.3±3.1	4.4±0.1	8.7±0.3	18.5±0.6	45.4±0.6	21.3±0.1	46.9±0.4
7th d	7.4±0.8	56.1±4.6*	37.6±4.6*	4.8±0.3	9.7±0.4	21.2±1.0	44.4±1.1	20.3±0.4	45.8±0.3*

All values represented as mean±standard error.
WBCs: White blood cells; Lym: lymphocytes; Gran: granulocytes; RBCs: red blood cells; Hb: haemoglobin; Hct: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration.
*Significantly different (P<0.05) to the value at 0 d.

Table 3: Effect of menbutone toxicity (400 mg/kg body weight) on biochemical parameters in rabbits

	Albumin (g/L)	ALT (U/L)	AST (U/L)	CPK (U/L)	CK-MB (U/L)	ALP (U/L)	Bilirubin (mg/dL)	Creatinine (mg/dL)
0 d	4.2±0.13	20.0±0.73	21.5±3.09	409.9±14.86	179.2±2.80	381.5±2.44	0.33±0.02	0.86±0.02
1st d	3.6±0.0[a]	35.5±1.66[b]	55.4±2.31[b]	28872.3±1167.77[b]	362.4±10.41[b]	425.7±5.60[b]	0.52±0.04[a]	1.18±0.03[b]
3rd d	3.8±0.12	28.6±0.72[b]	29.6±3.40	3915.4±163.41[b]	241.0±9.66[b]	256.8±10.64[b]	0.33±0.01	1.03±0.04[a]
7th d	3.9±0.10	21.2±0.96	25.1±1.61	1856.6±44.44[b]	187.8±3.17	225.5±8.51[b]	0.31±0.03	0.95±0.02

All values represented as mean±standard error.

ALT: alanine aminotransferase; AST: aspartate aminotransferase; CPK: creatine phosphokinase: CK-MB: creatinine kinase; ALP: alkaline phosphatase. U/L: units per L.

[a]$P<0.005$, compared to the corresponding value before injection.

[b]$P<0.001$, compared to the corresponding value before injection.

Histopathology

Histopathological findings of liver, kidney, heart and brain were evaluated under light microscopy. The incidence and severities of detected lesions in selected organs are summarised in (Table 4). Menbutone administration induces moderate to severe hepatic necrosis infiltrated with inflammatory cells (Figure 1a), along with moderate sharp outline cytoplasmic vacuolation of the hepatocytes (Figure 1b). Extravasation of erythrocytes in hepatic parenchyma was also noticed (Figure 1c). Moreover, menbutone toxicity led to formation of hyaline cast in the lumen of renal tubules (Figure 2a).

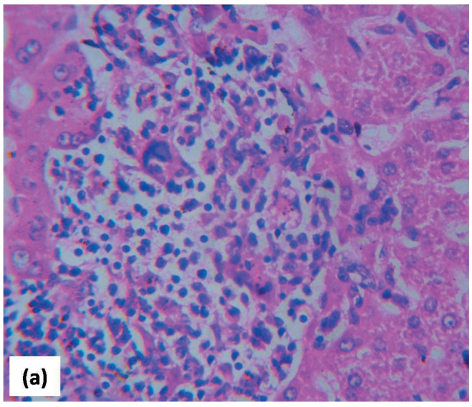
In addition, moderate tubular necrosis (Figure 2b) and tubulointerstitial nephritis were observed, characterised by infiltration of interstitial mononuclear cells (Figure 2c). Furthermore, the lesions encountered in cardiac muscle were moderate to severe interstitial myocarditis, associated with interstitial infiltrates of mononuclear cells (Figure 3a), and multifocal necrotic myocytes (Figure 3b). Moreover, mild inflammatory cells infiltrate necrotic myofibres (Figure 3c) and haemorrhages (Figure 3d) were noticed. Finally, the detectable brain lesion was haemorrhages (Figure 3e).

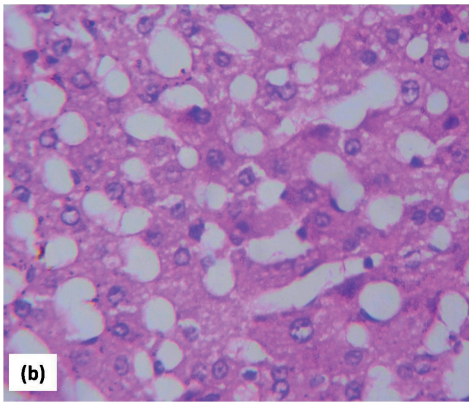
Table 4: Incidence and severity of histopathological lesions in liver, kidney, heart and brain after acute menbutone toxicity.

	Incidence[1] and Severity[2] of histopathological Lesions			
Organ / Lesions	Absent (-)	Mild (+)	Moderate (++)	Severe (+++)
Liver				
Necrotic hepatocytes	1	1	2	2
fatty infiltration	0	3	3	0
haemorrhage	0	2	4	0
Kidney				
tubular necrosis	1	1	3	1
interstitial nephritis	1	1	3	1
hyaline caste	2	2	2	0
Heart				
interstitial myocarditis	1	1	2	2
lytic necrosis	0	1	3	2
necrotic myofibres with inflammatory cell infiltration	2	3	1	0
haemorrhage	0	2	4	0
Brain				
haemorrhage	3	2	1	0

[1]Number of rabbits with lesions per total examined (6 rabbits).

[2]Severity of lesions was graded by estimating the percentage area affected in the entire section. Lesion scoring: (0) absence of the lesion=0%, (+) mild=5-25%, (++) moderate=26-50% and (+++) severe ≥50% of the examined tissue sections.





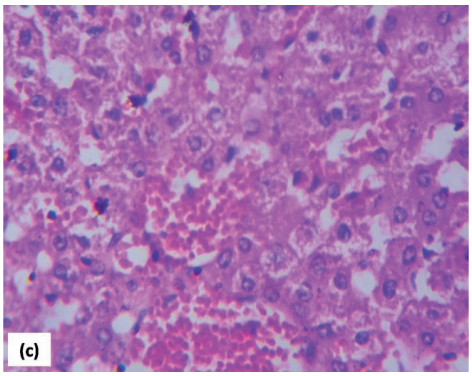
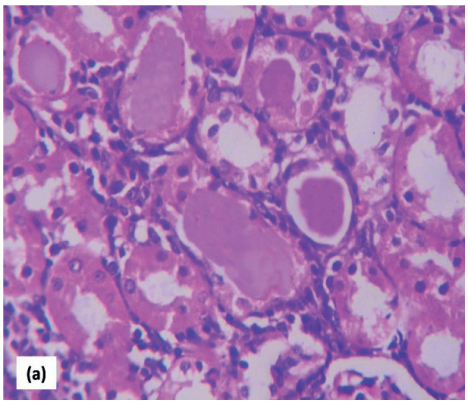
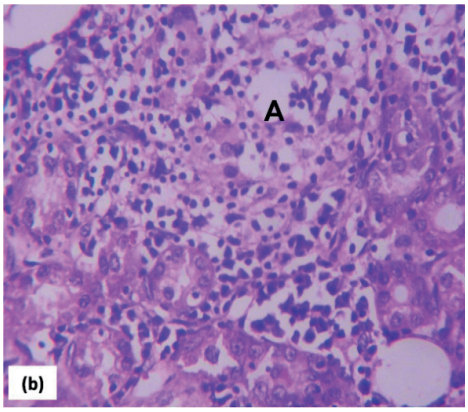


Figure 1: Photomicrograph of rabbit liver that received 400 mg/kg body weight menbutone (10%) (a) Necrotic hepatocytes infiltrated with inflammatory cells. Haematoxylin and eosin (H&E). (×250). (b) Sharp outline cytoplasmic vacuolation of the hepatocytes. H&E. (×250). (c) Haemorrhage. H&E. (×250).





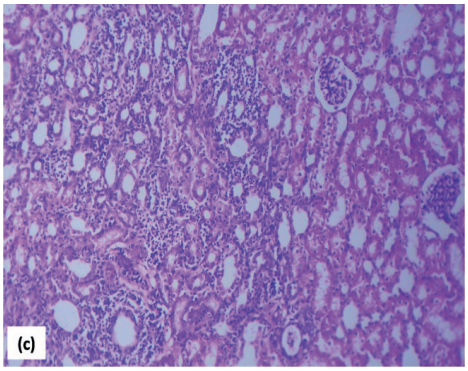


Figure 2: Photomicrograph of rabbit kidney that received 400 mg/kg body weight menbutone (10%) (a) Hyaline caste in the lumen of renal tubules. Haematoxylin and eosin (H&E). (×250). (b) Tubular necrosis with inflammatory cells infiltration (A). H&E. (×250). (c) Tubulointerstitial nephritis. H&E. (×160).

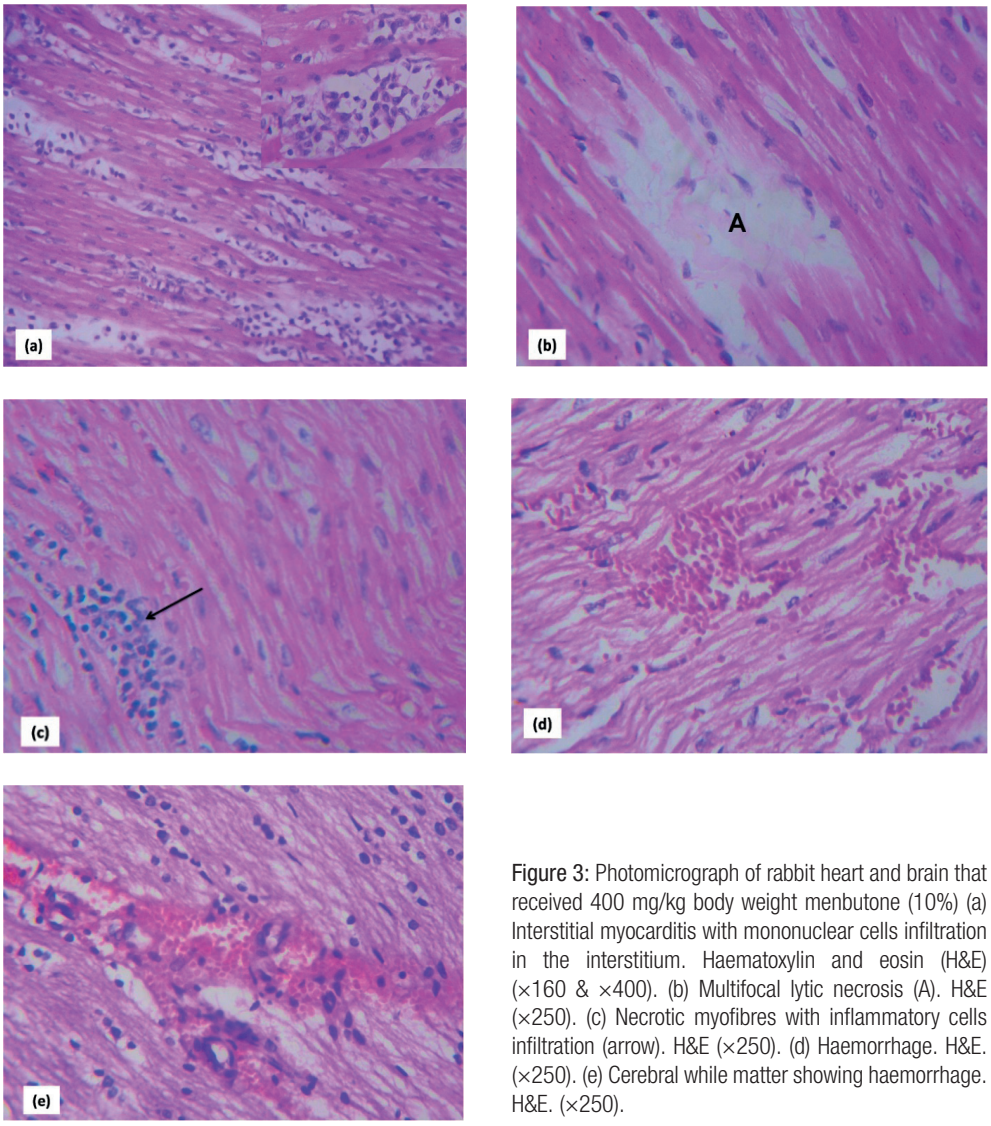


Figure 3: Photomicrograph of rabbit heart and brain that received 400 mg/kg body weight menbutone (10%) (a) Interstitial myocarditis with mononuclear cells infiltration in the interstitium. Haematoxylin and eosin (H&E) (×160 & ×400). (b) Multifocal lytic necrosis (A). H&E (×250). (c) Necrotic myofibres with inflammatory cells infiltration (arrow). H&E (×250). (d) Haemorrhage. H&E. (×250). (e) Cerebral while matter showing haemorrhage. H&E. (×250).

DISCUSSION

Our results revealed that the calculated LD_{50} was 475 mg/kg BW, indicating that menbutone has a relatively wide safety margin in rabbits, as lethal dose was roughly 100 fold over the recommended therapeutic dose for other animals, such as calves, at 5 mg/kg BW. (De La Cruz-Hernández *et al.*, 2012). These were in accordance with AEPPAE (1954) that the intramuscular lethal dose in rats was 500 mg/kg BW and details of toxic effects were not reported. Irritant effect of menbutone clinically demonstrated by the appearance of severe inflammatory response at the site of injection may be the cause of the high respiratory rate reported shortly after injection, which may be attributed to the histamine release after exposure to the drug. It was established that exposure to histamine led to rapid and shallow breathing in cats and rabbits due to pulmonary vagal stimulation (Miserocchi *et al.*, 1978, 1979).

The significant granulocytosis and lymphocytopenia reported seven days after drug administration may be understood as an inflammatory response to drug injection and not related to the direct effect of the drug. We may also hypothesise that the decrease in the total red blood cells (RBCs) count, Hb content, HCT and MCHC values was due to the loss of appetite which lasts for up to 2 d post injection, or may be related to haemorrhage in the parenchyma of some internal organs, as evidenced by histopathological findings.

Biochemical analysis revealed for the first record that a high dose of menbutone can induce variable toxic effects in different organs of rabbits. Generally, indicators used for monitoring hepatotoxicity include ALT, AST, ALP and gamma glutamil transpeptidasa parameters (Hsu *et al*., 2003). ALT is known clinically to be a marker more specific to liver function than AST. Compared to ALT and AST, ALP tends to be higher in diseases associated with injury to the bile secreting part of the liver's activity. In our study, rabbits exposed to a toxic dose of genabilic acid (menbutone) 400 mg/kg BW had higher activities of ALT, AST, ALP, and total bilirubin level with lowered albumin content than before drug injection, which is indicative of acute liver malfunctions. These results corroborate the histopathological changes observed in the liver because serum aminotransferases are located in the cytoplasm and are released into circulation after sinusoidal dilatation and hepatomegaly (Brent and Rumack, 1993). Our histopathological finding showed fatty changes, hepatocytic necrosis and inflammatory cell infiltration following menbutone injection, which explains the release of hepatic enzymes into blood. All the measured parameters tend to be decreased at 3 and 7 d after drug injection, except ALP, which continued to decrease and even to reach a significantly lower activity at the 7th d than that observed before drug administration; this may be attributed to the choleretic action of menbutone. This hypothesis was supported by Piyachaturawat *et al.* (2002), who demonstrated that serum alkaline phosphatase activity decreased after high doses of some choleretic compounds, such as phloracetophenone.

Regarding cardiotoxicity, we should bear in mind that the cardiac troponins; cardiac troponin T, cardiac troponin I and myocardial isoenzyme of creatine kinase are cardiospecific markers that indicate structural injuries in the cardiomyocytes (Cardinale *et al.*, 2004)

The results of our study reveal that a single high dose of menbutone at 400 mg/kg BW. alters the structural integrity of the cardiac cells in intoxicated rabbits: the activities of cardiac markers; CPK, CPK-MB and AST enzymes were increased after 24 h following drug administration. This may be attributed to the hypothesis that cardiac cell may be damaged by free radicals released from genabilic acid metabolism, and correlated with our histopathological findings which showed an interstitial myocarditis with mononuclear cell infiltration accompanied by necrosis of myocytes and hemorrhages. At day 1, the level of CPK was increased as high as 60 fold the initial one. This may be due to the severe muscular inflammation induced by the drug at the injection site, as observed clinically.

The kidney exhibited histopathological changes such as hyaline casts in the lumen of renal tubules and this may be attributed to the cytotoxic effect of menbutone, which rendered the glomeruli more permeable even to blood albumin in addition to tubular necrosis and interstitial nephritis, which explains the high level of serum creatinine and lowered serum albumin content.

CONCLUSION

We can conclude that acute toxicity of menbutone in rabbits produces variable biochemical and pathological changes in liver, heart, kidneys and brain tissues. Menbutone even at 10% dilution is an irritant drug inducing severe inflammation at the site of injection, high respiratory rate and lameness shortly after injection of large dose. This paper gives an idea of the toxic dose and effects of menbutone in rabbits.

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