Ultrasonographic, Morphologic and Biochemical Alterations in Experimentally Induced Steroid Hepatopathy in Dogs

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Abstract: Hepatic affections are life threatening conditions in human and pet medicine. Therefore, glucocorticoid induced hepatopathy model was studied in 15 Mongrel dogs. The purpose of the study was to evaluate the clinical, ultrasonographic, cytological, histopathological and clinicopathological changes associated with the uses of dexamethasone (2.2mg/kg) once daily for 28 days. Ultrasonographic examination, percutaneous ultrasound guided liver biopsy and serum samplings were performed before the start of the study day 0 and at 7,14,21,28 of the experiment. Ultrasonographic examination revealed a marked increase in hepatic echogenicity when compared with splenic echogenicity. Cytological and histopathological analysis showed hepatocellular swelling due to accumulation of cytoplasmic vacuoles. There were significant (P<0.05) and progressive increases in serum activities of alkaline phosphatase (ALP), gamma glutamyltransferase (GGT) and alanine transaminase (ALT) by day 28. It was concluded that even with short period, glucocorticoid administration cause vital adverse effects on hepatic parenchyma.

Key words: Glucocorticoid • Liver • Echogenicity • Biopsy • Liver Enzymes

INTRODUCTION

Glucocorticoids are commonly used on a large scale as anti-inflammatory and immuno suppressive drug for treatment of wide range of diseases in human and veterinary medicine [1]. However, excessive use of glucocorticoids has been demonstrated to be the cause of various side effects in different species [2]. The prolonged use of Glucocorticoids, even at low dosages, can lead to development of the symptoms of Cushing’s disease (Hyperadrenocorticism) [3]. Dogs are especially vulnerable to develop glucocorticoid induced hepatopathy [4], which characterized histologically by cytoplasmic vacuolation [5], biochemically by increases in serum activities of alkaline phosphatase (ALP) isoenzymes especially Liver alkaline phosphatase (LALP) and Corticosteroid induced alkaline phosphatase (CIALP), Gamma-glutamyltransferase (GGT) and alanine transaminase (ALT) [6]. Ultrasonography has been considered to be one of the most accurate non-invasive methods for evaluating the parenchymal structure of liver in animals [7]. The liver parenchyma is less echogenic than the splenic parenchyma and ranges from similar to more echogenic than the renal cortex [8]. Diseases that typically cause diffuse increase in hepatic echogenicity include lymphosarcoma, fatty infiltration, cirrhosis, or steroid hepatopathy [9]. Although, the ultrasound is an important method for diagnosis of different liver diseases; the US guided biopsy is required to reach a definitive and final diagnosis [10]. Consequently, the present study was planned to evaluate the effect of dexamethasone administration on dog’s liver by evaluation of ultrasonographic, cytological, histopathological and biochemical findings.

MATERIALS AND METHODS

Animals and Experimental Design: Fifteen apparently healthy mongrel dogs aged from 1-2 years and weighed 11-20 kg were used in the present study.
Steroid hepatopathy was induced by daily administration of dexamethasone sodium phosphate (2.2 mg/kg, body weight. IM) according to Lu et al. [11]. The experiment continued for 28 days, through which clinical examination, ultrasonography, ultrasound guided biopsy and collection of serum samples were carried out at day 0, 7, 14, 21, 28. The animals allowed free access for food and water and consumption rate was recorded daily throughout the experiment period.

Clinical Observation: The following aspects included demeanor, appetite, urination, defecation, general body condition as well as hair growth at sheaved ultrasound and biopsy sites were monitored and reported daily.

Ultrasonography: Hepatic ultrasonography was performed at day 0, 7, 14, 21, 28 of experiment according to Nyland et al. [12]. The hepatic echogenicity was compared with splenic echogenicity according to Nyland and Matton [8].

Biopsy Collection: Prior to biopsy procedure, dogs were sedated by intramuscular injection of Xylazine hydrochloride (1.1 mg/kg) according to Bednarski [13]. The liver biopsies were collected at day 0, 7, 14, 21, 28 of experiment according to Hager et al. [14] and Hoppe et al. [15], by using Semi-automatic biopsy needles.

Cytologic Examinations: The materials obtained from biopsies were smeared into several slides which were air dried for field staining according to method described by Tankeyul et al. [16], then referred for cytological examination according to Rick et al. [17].

Histopathological Examination: For histopathological examination another biopsy sample was obtained from each animal at day 0, 7, 14, 21, 28 of experiment. Liver biopsy samples were fixed in 10% neutral-buffered formalin, routinely processed and embedded in paraffin. Sections (4-6 µm) were prepared and stained with hematoxylin and eosin as described by Bancroft et al. [18].

Serum Samples: Blood Samples (without anticoagulant) were collected on day 0, 7, 14, 21, 28 of experiment for serum separation and clear non hemolysed supernatant was harvested for biochemical studies.

Measurement of Enzymatic Activity: Kinetic determination of ALP, GGT, ALT and AST were performed within 24 hours from serum separation according to Bowers and Mccomb [19], Szasz [20] and Bergmeyer et al. [21].

Statistical Analysis: Values were expressed as mean and ± SD. Statistical comparisons among the means of different experimental groups were made with completely randomized two ways ANOVA "Student-Newman-Keuls test" by COSTAT program version one. A probability "P" value of <0.05 was assumed for statistical significance.

RESULTS

Clinical Signs: The clinical signs of cushing’s syndrome developed on day 7 included lethargy, semisolid feces, polyphagia, polydipsia and polyuria. The lethargy became more pronounced by day 14, while other signs persist. The rough hair with the presence of skin rash and impaired hair growth were detected by day 21 (Figure 1.a,b). Skin abscess, impaired wound healing, generalized muscle wasting; and pendulous abdomen were observed by day 28 of experiment (Figure 1.d,c).

Ultrasoundographic Findings: At day 0, sonographic examination of liver revealed a uniform intermediate echopattern, which interrupted by round to oval anechoic linear structures represent the portal veins while anechoic linear structures represent the hepatic veins (Figure 2. a). At day 7, a slight increase in hepatic echogenicity was noticed as the liver and spleen appeared relatively isoechoic (Figure 2. b). At day 14, the increased hepatic echogenicity became more pronounced (Figure 2 c). At day 21, the hepatic parenchyma became markedly increased and hepati c parenchyma became more echoic than splenic parenchyma. (Figure 2. d). At D28, the hepatic echogenicity was progressively increased and hepatic parenchyma became significantly more echoic than splenic parenchyma (Figure 2. e) and the clearance of hyperechoic portal vein walls were decreased (Figure 2. f).

Cytologic Findings: At day 0, the microscopic findings showed normal hepatocytes, which appeared as large, round to polyhedral cell with basophilic cytoplasm.

(1) Pie Medical Scanner (Maastricht, Netherlands) ultrasound and sector transducer with multifrequent probe (5.0-7.5 MHz)
(2) Xylaject manufactured by Epico pharmaceuticals Egypt.
(3) Stero-cut needle (16-gauge 150 mm needles)
(4) Reagent kits supplied by Stanbio Laboratories incorporation, USA.
Fig. 1: Clinical signs of steroid induced hepatopathy
Treated dog at day 21 of experiment with (a) Rough hair, impaired hair growth (arrows). (b) Skin rashes and impaired hair growth at sheaved areas (arrows).
Treated dog at day 28 of experiment with (c) Impaired wound healing and skin abscess (arrow). (d) Generalized muscle wasting and pendulous abdomen (arrow).

Fig. 2: (A) Normal sagittal scan of the liver parenchyma that appeared homogenous moderately echoic interrupted by anechoic gall bladder (GB) and short, highly echogenic paired parallel lines surrounding an anechoic lumen that represent the portal veins (PV) at day 0. (B) Double B scan at day 7 displaying that hepatic and splenic echogenicity appeared nearly equivalent. (C) Double B scan at day 14 revealed moderate increase in hepatic echogenicity in relation to splenic echogenicity. (D) Double B scan at day 21 showed marked increase in hepatic echogenicity in comparison with splenic parenchymal echogenicity. (E) Double B Scan of liver and spleen at day 28, hepatic parenchyma showed marked increase in the echodensity than the spleen. (F) Hepatic sagittal scan at day 28, displayed marked diffuse hyper-echogenicity and less clearance of echogenic portal vein walls.
Fig. 3: Cytologic findings of liver biopsy specimens (Field stain x 1000)

(a) Normal hepatocyte (polyhedral cell with round nucleus) at day 0. (b) Mild to Moderate cytoplasmic vacuolation (arrow) in hepatocytes at day 7. (c) Moderate cytoplasmic vacuolation (arrow) in hepatocytes at day 14. (d) Marked cytoplasmic vacuoles with different sizes scattered within the hepatocytes (arrow) at day 21. (e) Single large vacuole displacing the nucleus to the periphery of hepatocyte (arrow) at day 28.

Table 1: Activities of serum liver enzymes in 15 Mongrel dogs treated with Dexamethasone (2.2mg/kg IM) once daily for 28 days.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>D0</th>
<th>D7</th>
<th>D14</th>
<th>D21</th>
<th>D28</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (U/L)</td>
<td>82.6±35.7</td>
<td>373±50.2*</td>
<td>1,082±117*</td>
<td>3,074±277*</td>
<td>3,795±324*</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>8.48±1.79</td>
<td>15.4±2.58*</td>
<td>26.2±4.91*</td>
<td>41.6±4.24*</td>
<td>54.0±4.07*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>33.6±3.31</td>
<td>46.6±6.18*</td>
<td>71.0±5.03*</td>
<td>87.2±6.77*</td>
<td>112±9.056*</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>17.3±1.31</td>
<td>15.9±3.55</td>
<td>17.6±2.46</td>
<td>16.5±1.78</td>
<td>36.7±2.48*</td>
</tr>
</tbody>
</table>

*Significantly different from base line value (P<0.05) D= day of experiment, ALP= alkalinephosphatase, GGT= gamma glutamyltransferase, ALT= alanine transaminase, AST= aspartate transaminase, U/L= Units/ Liter

The nucleus is round, centrally located with a distinctive large, single nucleolus (Figure 3.a). At day 7, mild to moderate cytoplasmic vacuolation in hepatocytes that were recognized as discrete, round intracellular vacuoles scattered within the hepatocytes (Figure 3.b). At day 14, moderate cytoplasmic vacuolation in hepatocytes were present (Figure 3.c). At day 21, diffuse cytoplasmic vacuolation were recognized as round intracellular vacuoles of different sizes scattered within multiple hepatocytes (Figure 3.d). At day 28, nearly all the hepatocytes are distended by multiple vacuoles of different size with little visible cytoplasm and often consist of single large vacuole displacing the nucleus to the periphery of cell (Figure 3.e).

**Histopathological Findings:** The microscopic finding of histopathological biopsy samples showed mild to moderate cytoplasmic vacuolation of the hepatocytesat day 7 (Figure 4.a). Moderate cytoplasmic vacuolation were observed at day 14 (Figure 4.b). At day 21, cytoplasmic vacuolation of the hepatocytes became more pronounced (Figure 4.c). At day 28, hepatocytes appeared as large vacuolated cells with compressed nucleus at the periphery as shown in Figure (4.d).

**Clinicopathological Findings:** The mean serum enzymatic activities of ALP, GGT, AST and ALT were within the reference interval at day0 as shown in Table (1) and Figure (5). The mean value of serum ALP activity of
Fig. 4: Histopathologic findings of liver biopsy specimens (H&E X 400)
(a) Hepatic biopsy from treated dogs showing cytoplasmic vacuolation (arrow) at day 7. (b) Hepatic biopsy from treated dogs showing moderate cytoplasmic vacuolation (arrow) at day 14. (c) Hepatic biopsy from treated dogs showing marked cytoplasmic vacuolation (arrow) at day 21. (d) Hepatic biopsy from treated dogs showing severe cytoplasmic vacuolation and the hepatocytes appear as large vacuolated cells with compressed nucleus at the periphery (arrow) at day 28.

Fig. 5: Activities of liver enzymes in dexamethasone treated dogs

- ALP: The activity increased rapidly and showed about 13-fold increase above baseline at day 14, then progressively increased and showed about 46-fold increase above baseline at day 28.
- GGT: The mean value of serum GGT activity of treated dogs increased rapidly and showed about 3-fold increase above baseline at day 14. Then progressively increased and showed 6-fold increase above baseline at day 28.
- ALT: The mean value of serum ALT activity of treated dogs remained within normal limits until day 28 of experiment, when there was a significant (P ≤ 0.05) increase.
DISCUSSION

Glucocorticoids are repeatedly used in companion animals for their anti-inflammatory and immunosuppressive properties. However, these effects are not selective and corticosteroids will affect body systems other than “target tissue,” probably causing serious adverse effects [22]. This study illustrates the rapid occurrence of clinical, ultrasonographic, morphologic and clinicopathologic alterations in dogs after dexamethasone administration. In the present study, observed clinical signs were similar to those reported by Badylak and Van Vleet [23] and Huang et al. [24]. Polyuria and polydipsia may be attributed to cortisol antagonism effect with the antidiuretic hormone (ADH) on the distal renal tubules or increased ADH destruction in the liver. Diuresis reported here may be ascribed to lack of ADH to stimulate water resorption in the renal tubules [25]. The semisolid feces may be attributed to alterations in local immune function in the small intestine which result in bacterial diarrhea [26]. Generalized muscle wasting and pendulous abdomen may be ascribed to excessive stimulation of gluconeogenesis by the liver as a result of increased cortisol values that led to lack of amino acids usually required in protein synthesis and exhaustion in gluconeogenesis for formation of glycogen in the liver [27]. The rough hair, poor hair growth at sheaved areas may be attributed to prominent hyperkeratosis, marked reduction in numbers of hair follicles, apocrine and sebaceous glands and dilation of hair follicles associated with iatrogenic hyperadrenocorticism [28]. Skin rashes and skin abscesses may occur as a consequence of an increase in bacterial infections, reduced fibroblastic activity that led to poor wound healing and the weakness of the blood vessels walls promoting bruising [26]. These clinical signs resemble those of naturally occurring canine Cushing’s disease. So, it could be considered an experimentally induced Cushing’s disease. Ultrasonography proved to be an efficient tool for assessment of the progression of canine hepatopathy due to its ability to evaluate the hepatic parenchymal structure [29] and subjective visual comparison of liver echogenicity with normal spleen is an accepted method for detecting diffuse changes in hepatic echogenicity [8]. In comparing the liver-spleen echogenicity at day 7, a slight increase in hepatic echogenicity was noticed which reflected the hepatic damage due to vacuolation of hepatocytes [30]. These findings were supported by cytologic and histopathologic findings which revealed the presence of mild to moderate hepatic vacuolation. At 14, 21 and 28, a pronounced and progressive increase in hepatic echogenicity was noticed that reflected the advanced hepatic damage due to vacuolation of hepatocytes [30]. The ultrasonographic findings were reinforced by cytologic and histopathologic findings which revealed that nearly all the hepatocytes are distended by multiple vacuoles of different sizes with little visible cytoplasm, where the hepatocytes appeared as large vacuolated cells with peripherally positioned nucleus. These hepatic morphologic alterations have been attributed variably to intercellular edema [31], accumulation of cytoplasmic lipids [32] or glycogen [33]. The findings were similar to those previously mentioned by Pereira et al. [10]; and Syakalima et al. [34]. Ultrasonographic, cytologic and histopthologic findings corresponded with clinicopathologic findings which showed about 46-fold increase above the base line in serum activities of ALP, 6-fold increase in GGT activity, 3-fold increase in ALT level and a significant increase in AST activities by day 28. Increase in ALP serum activity may be ascribed to production of corticosteroid-induced alkaline phosphatase (CIALP) which is an isoenzyme appears in canine serum after the prolonged influence of either endogenous or exogenous corticosteroids [35], this agreed with Tietz [36] and Hoffman [37]. Increase in ALT and AST level may be occur as a consequence of hepatocellular necrosis or altered membrane permeability, moreover the elevation of ALT and AST suggested to be positively correlated with the extent of hepatocytes damage [38], as previously mentioned by Dillon et al. [31], Badylak and Van Vleet [23], Sanicki et al. [39] and DeNovo and Prasse [40]. Increase in GGT level may be attributed to glucocorticoid stimulation of the microsomal oxidase system of the hepatocytes. [41-43] as previously reported by Dillon et al. [31] and Badylak and Van Vleet [23]. The present findings will be useful in diagnosis and prognosis of naturally occurring glucocorticoid induced hepatopathy.

CONCLUSION

The present study confirmed that excessive use of glucocorticoid causes serious adverse effects on hepatic parenchyma. Ultrasonography, liver biopsy and biochemical analysis were complementary to each other in diagnosis of glucocorticoid induced hepatopathy and should be used in conjugation to interpret the obtained results.
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REFERENCES