

## Pathological study on the effect of vitamin D<sub>3</sub> on sepsis experimentally induced in rats by cecal ligation and punctures

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(Received October 21, 2013; Accepted December 18, 2013)

### Abstract

The aim of this study was to investigate the effects of the Vitamin D<sub>3</sub> on the rats with sepsis that experimentally induced by cecal ligation and puncture. 100 Rats were divided into 5 groups, these include untreated control group, sham-operated group, CLP group and 2 treated groups pretreated daily a Subcutaneous injections of 1,25-dihydroxyvitamin D<sub>3</sub> 100 ng/kg for 3 days, then one of the pretreated groups subjected to sepsis accomplished by abdominal surgery comprising a cecal ligation and puncture. The following parameters were recorded: survival rate, hematological examinations and histopathological changes of the liver and heart were examined. It was found that vitamin D<sub>3</sub> pretreated showed improvement in the survival rats and enhancement in the blood leukocyte count, also protect the rats from thrombocytopenia and Disseminated Intravascular Coagulation (DIC), but vitamin D<sub>3</sub> pretreated show slight improvement in the histopathological lesions in the liver and heart due to cecal ligation and puncture sepsis.

**Keywords:** Pathology; Vitamin D<sub>3</sub>; Sepsis; Rats

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### دراسة مرضية حول تأثير فيتامين D<sub>3</sub> على الإنتان المحدث تجريبياً في الجرذان بواسطة ربط الأعور وثقبه

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### الخلاصة

الهدف من هذه الدراسة هو الكشف عن تأثير فيتامين D<sub>3</sub> على الجرذان المحدث بها الإنتان تجريبياً بواسطة ربط الاعور وثقبه. تستخدم في هذه التجربة 100 جرذاً، قسمت الى 5 مجاميع شملت مجموعتين من السيطرة السالبة والموجبة، مجموعة الانتان المحدث بطريقة ربط الاعور وثقبه ومجموعتين عوملت بالحقن تحت الجلد بفيتامين د<sub>3</sub> 100 نانوغرام/كغم من وزن الجسم ولمدة ثلاثة أيام، بعدها واحدة من هذه المجموعتين احدثت بها الانتان ايضاً عن طريق ربط الاعور وثقبه. تم حساب معدل البقاء على قيد الحياة، التغيرات في صورة الدم والتغيرات المرضية النسجية لكل من الكبد والقلب. حيث وجد أن هنالك تحسن في معدل البقاء على قيد الحياة وزيادة في اعداد كريات الدم البيضاء، فضلاً عن حماية الجرذان من نقص اعداد الصفيحات الدموية والتخثرات داخل الاوعية الدموية المنتشرة في الجرذان المعاملة بفيتامين د<sub>3</sub> قبل احداث الانتان، أظهر الفحص النسجي تحسناً بسيطاً في الصورة النسجية لكل من الكبد والقلب.

### Introduction

Vitamin D is a group of fat-soluble secosteroids, can be obtained from the diet or made in the skin after exposure to ultraviolet B radiation from the sun (1,2). Vitamin D is

subsequently hydroxylated by CYP27A1 enzyme into 25-hydroxyvitamin D<sub>3</sub> in the liver and further hydroxylated by CYP27B1 in the kidney into the active metabolite, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), which is the ligand for vitamin D receptor (VDR) (3,4).

As the vitamin D receptor is expressed on immune cells (B cells, T cells and antigen presenting cells) and these immunologic cells are capable of synthesizing the active vitamin D metabolite, vitamin D has the capability of acting in an autocrine manner in a local immunologic milieu. Vitamin D can modulate the innate and adaptive immune responses (5,6).

The hormonally active form of vitamin D, 1,  $\alpha$ 25-dihydroxyvitamin D<sub>3</sub> is an important regulator of the calcium – phosphate homeostasis and thus bone formation and resorption (7). In addition, This compound possesses a number of non – calcaemic effects. These include an effect on the immune response, differentiation and interaction of macrophages and monocytes and regulation of lymphocyte activity (8).

Sepsis is a syndrome involving the systemic host response to an inflammatory or infectious agents. It is a common and frequently fatal condition that occurs as a result of severe infection often leading to overwhelming systemic inflammation. (9) Sepsis has been shown to develop when the initial, appropriate host response to an infection becomes amplified and subsequently dysregulated, leading to an imbalance between pro-inflammatory and anti-inflammatory responses (10). Sepsis can be experimentally induced in lab animals by different methods, but cecal ligation and puncture (CLP) has been considered to be the gold-standard model in poly-microbial sepsis (11)

In the present study, we aimed to demonstrate the effect of 1,25-vit D<sub>3</sub> on experimental sepsis induced in rats using cecal ligation and puncture procedure.

## **Material and methods**

### **Experimental Groups**

This study was carried out on (100) albino rats weighing 200-250 g. Animals were supplied by the animal house of the college of Veterinary Medicine in Mosul university – Iraq. The rats were housed under the same environmental conditions. Fed normal laboratory diet and they had free access to tap water. The animals were randomly divided into five groups of 20 animals each; The group I: control animals (negative control), with free access to food and water, without any treated. Group II: Sham-operated animals (positive control), this group underwent a ventral laparotomy under general xylazine and ketamine anesthesia, with the handling of the cecum only without ligation or puncture. Group III: Cecal ligation and puncture (CLP), this group was subjected to cecum ligation and puncture, under xylazine and ketamine anesthesia (by a single i.p. Injection (0.5 ml) of ketamine (90 mg/kg b.w) and xylazine (10 mg/kg b.w). A laparotomy was performed through a midline abdominal incision, then ligated the cecum below

the ileocecal valve with 3-0 silk ligature, So that intestinal continuity was maintained. Using a 20-gauge needle, the cecum was punctured in two locations, then the cecum was gently compressed until feces were extruded. The bowel was then returned to the abdomen, and the incision was closed with a layer of catgut for the muscle and silk for the skin. All rats (including the sham-operated) were resuscitated with saline solution (5 ml per 100 g B.W.) injected subcutaneously at the time of the operation. The rats had free access to food and water after the recovery period. Group IV: received daily Subcutaneous (s/c) injection and for 3 days of 1,25-dihydroxy-vitamin D<sub>3</sub> (100 ng per kg body weight) (12) and this dose consider the highest dose not causing hypercalcemia (11) dissolve in vegetable oil. Group V: The rats in this group were pre-treated with daily SC injections of 1,25-dihydroxyvitamin D<sub>3</sub> 100 ng/kg for 3 days. Then laparotomy was performed for CLP as group III. Then the rats had free access to food and water after the recovery period.

### **Hematologic Examination**

The blood samples for the hematological study were collected from the ophthalmic venous plexus located in the orbital sinus of the rats after 24 days after the experiment, by using a micro-capillary pipette in tubes containing Ethylene Diamine Tetraacetic Acid (EDTA), The blood picture was measured by blood culture.

### **Histopathology**

Tissue specimens, were collected from the liver and heart after 24h, 3days, 7days and 14 days. then fixed in 10 % Neutral Buffer formalin solution for 72 hours, trimmed to suitable sizes, washed, dehydrated, cleared in xylene, Embedded in paraffin wax, Sectioned at 5-6  $\mu$ M, stained with hematoxylin and Eosin and examined under a light microscope (13).

## **Results**

No mortality was observed in the group I and IV. In group II, 10% mortality rate after 24h from the operation, then survival rate was 100 %. In group III, survival was 75 % after 24h and decreasing until no survival rate after 7days.

While in group V survival was 80% after 24h. And also decreasing until all animals die within 14 days after CLP (Fig. 1).

There were severe reduction in thrombocyte count of rats with sepsis by CLP comparing to the control rats, and these thrombocytopenia was significantly counteracted in rat treated with Vit. D<sub>3</sub> before induction of sepsis with CLP (Fig. 2).

The effect of Vit. D<sub>3</sub> on sepsis induced by CLP is shown in table (1). The blood picture showed significant decrease

in white blood cell count in the CLP sepsis group compared to the control and sham groups, while in Vit D<sub>3</sub> and CLP sepsis group showed improvement in white blood cells count.

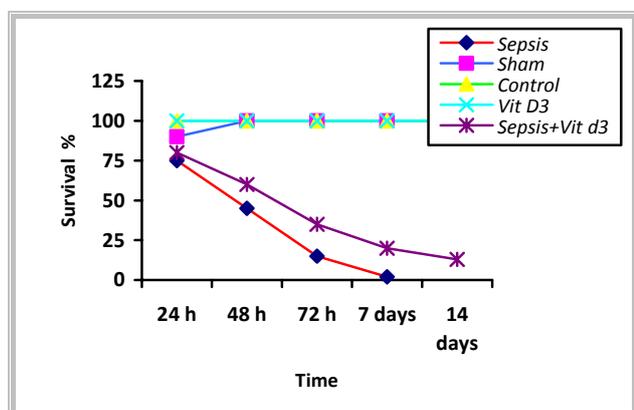


Figure 1: Effect of Vit. D<sub>3</sub> on the Survival rate in the different groups of the experiments.

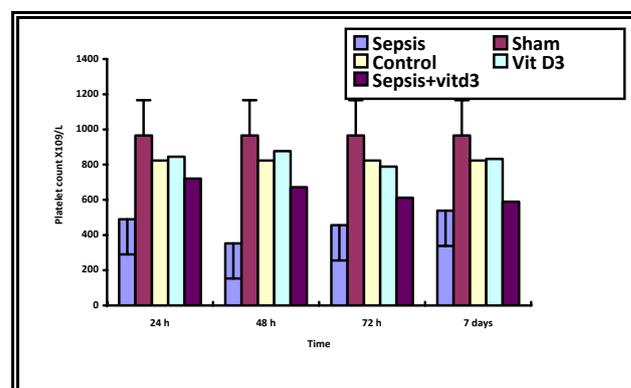


Figure 2: Effect of Vit. D<sub>3</sub> on platelet count in rats 24 h. after sepsis by CLP.

Table 1: Effect of vit. D<sub>3</sub> on some Hematological parameters 24 h. after induction of sepsis in rats with CLP. (mean ± SE)

Parameters	control	Sham	Sepsis	Vit D <sub>3</sub>	Sepsis+Vit D <sub>3</sub>
WBC, 10 <sup>9</sup> /L	11.2±0.51	11.6±0.57	7.5 ±0.38*	11.11±0.56	9.08±0.51*
RBC, 10 <sup>9</sup> /L	7.43±0.24	7.81±0.18	8.15 ±0.26*	8.28±0.17*	7.89±0.1
Hemoglobin, g/dl	14.1±0.41	16.1±0.57	15.8 ±0.38*	18±1.7*	16.7±0.28*
Hematocrit %	42.9±0.63	43.3±1.04	45.6 ±2.24*	43.9±0.06	47.3±0.39*
MCV, fL	55.7±0.93	56.5±1.34	55.38 ±0.96	54.6±1.76	56.7±1.2
MCHC, g/dl	35.3±0.42	35.2±0.71	35.28 ±0.46	34.30.61	35.8±0.74

\* P< 0.05.

The Microscopic evaluation of liver sections 24h. After CLP sepsis revealed congestion of central veins, swelling of the hepatocytes with infiltration of polymorphonuclear inflammatory cells (Fig. 3). After 3 days of CLP procedure, the liver sections revealed severe necrosis of the hepatocytes with infiltration of inflammatory cells in the portal area and around central veins with kupffer cell hyperplasia (Fig. 4), and the microscopic lesions became more severe after 7 days from CLP sepsis, which was characterized by massive necrosis of the hepatic lobule with the presence of thrombi in some blood vessels (Fig. 5). The microscopic changes of sham – operated group after 24h showed vacuolar degeneration and coagulative necrosis of other hepatocytes, with dilatation of the sinusoids (Fig. 6), and after 3 and 7days no significant histopathological changes occurs.

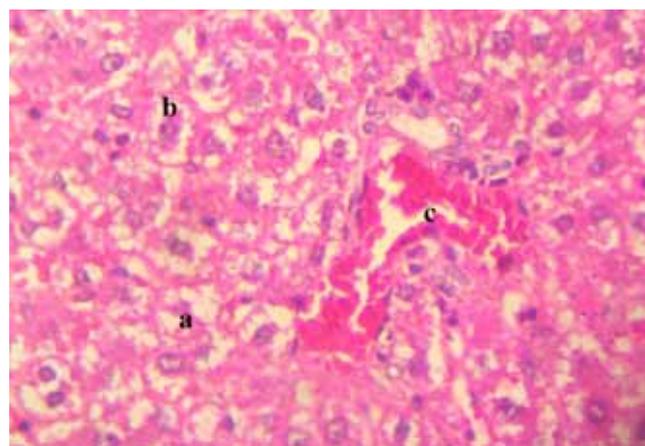


Figure 3: Rat liver section, 24 hours after CLP, show acute cell swelling (a), centrilobular necrosis of hepatocytes (b) congestion of central vein (c). H&E X105.

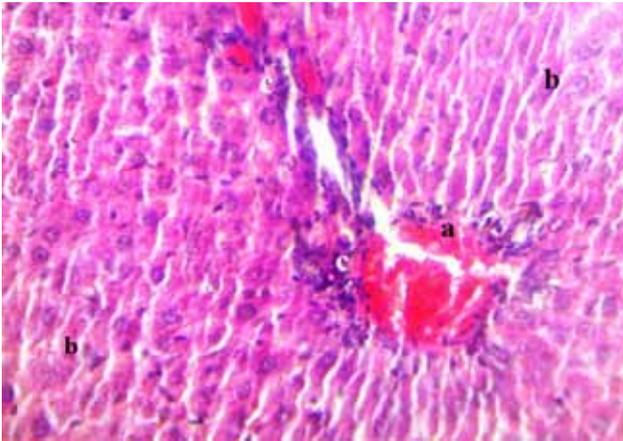


Figure 4: Rat liver section 3 days after CLP, show congestion of central vein (a), severe necrosis of hepatocytes (b) infiltration of inflammatory cells (C). H & E. X105.

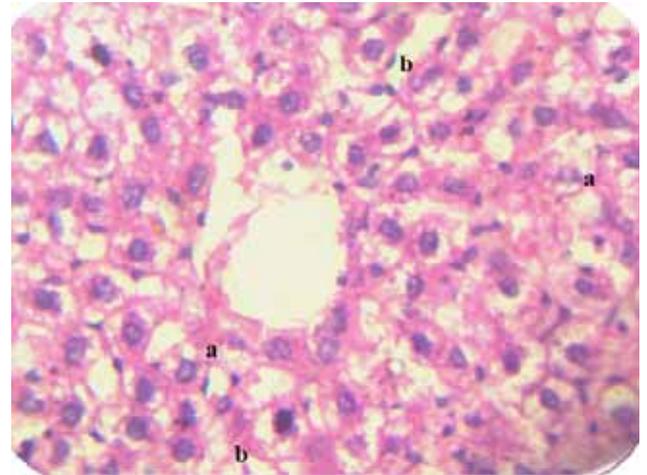


Figure 6: Rat liver section of sham – operated group, showed vacuolar degeneration of hepatocytes, and coagulative necrosis (a) with dilatation of sinusoids (b). H & E X 350.

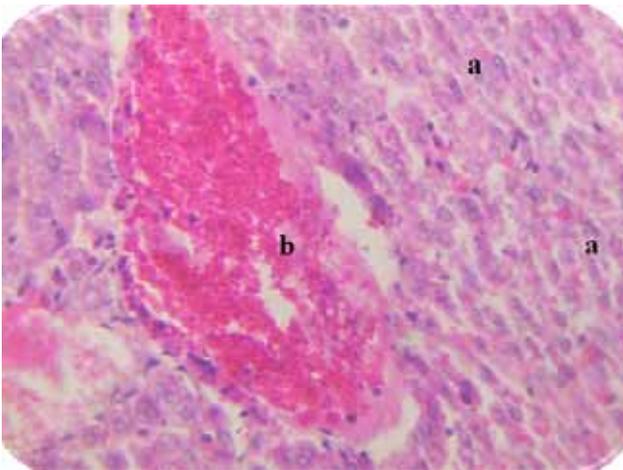


Figure 5: Rat liver section 7 days after CLP, show massive necrosis of the liver parenchyma (a) with recent thrombus in central vein (b). H & E. X105.

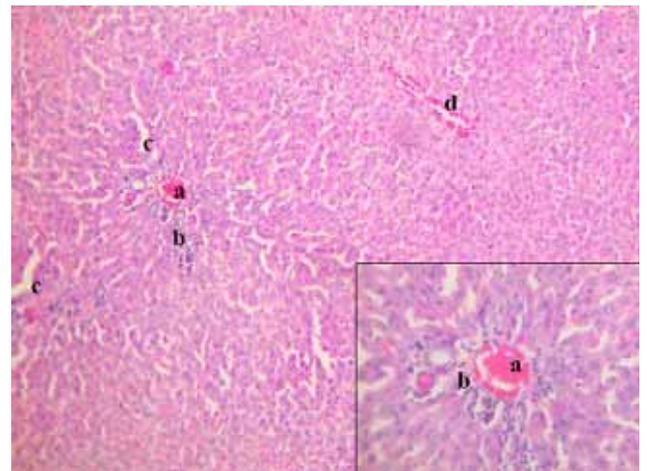


Figure 7: Rat liver section, Vit D3 treated, 24 h. after CLP, showed congestion of central vein (a), infiltration of inflammatory cells (b), dilatation of sinusoids (c), and hemorrhage in parenchyma (d). H & E. X 42.

In the Vit. D<sub>3</sub> treated by CLP sepsis group, after 24h. The days, the liver microscopic sections revealed dilatation of sinusoids and hemorrhage in liver parenchyma with perivascular cuffing of polymorphonuclear inflammatory cells (Fig. 7), while after 3 days from CLP procedure and treated with Vit. D<sub>3</sub> the characteristic lesions revealed coagulative necrosis of hepatocytes with severe infiltration of polymorphonuclear inflammatory cells (Fig. 8). After 7 days, there was an increase in necrosis of hepatocytes with infiltration of inflammatory cells in portal area and around central veins, and after 14 days the lesions became more severe with massive necrosis in hepatocytes (Fig. 9).

Histopathological study of heart of CLP sepsis rats, 24h., exhibited varying stages of mild degenerative and necrosis of the heart muscles, besides these changes there was evidence of infiltration of multinuclear inflammatory cells in the myocardium (Fig. 10). While after 3 days, there was increased the zenkers necrosis of the myocardium with presence of edema between the muscle fibers (Fig. 11), and after 7 days, there was massive necrosis of the myocytes, with thickening of blood vessels wall and thrombi in others (Fig. 12).

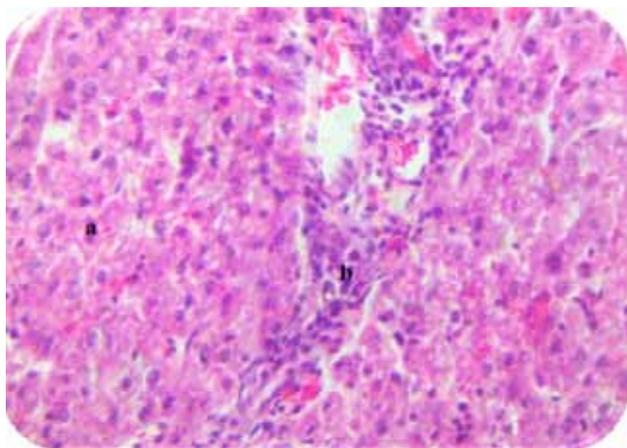


Figure 8: Rat liver section, Vit D3 treated, 3 days after CLP, showed coagulative necrosis of the hepatocytes (a), with infiltration of PMNs in the portal areas (b). H & E. X 105.

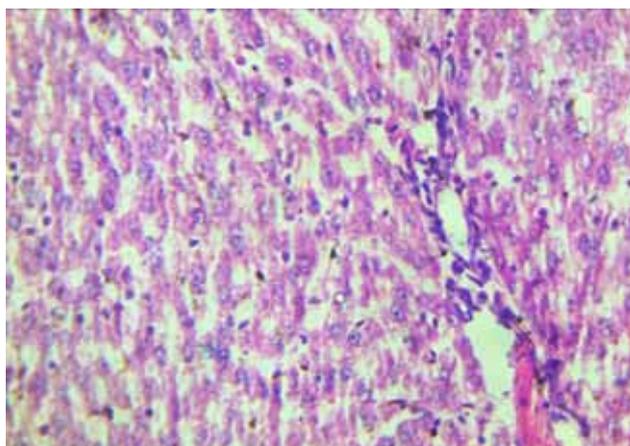


Figure 9: Rat liver section, Vit D3 treated, 14 days after CLP, showed massive necrosis of the hepatocytes. H & E. X 350.

In the Vit. D<sub>3</sub> treated by CLP sepsis group, The microscopic lesions characterized by marked focal infiltration of inflammatory cells with hemorrhage between the cardiac muscle fibers, and after 3days there was necrosis in muscle fibers with edema and congestion of blood vessels with perivascular cuffing of inflammatory cells (Fig. 13), and the lesions became more severe after 7 and 14 days which was characterized by severe necrosis of cardiac muscle and thrombi in the blood vessels (Fig. 14).

The microscopic changes of sham – operated group after 24h, showed only mild degenerative changes in muscle cells with mild infiltration of inflammatory cells in comparing with the control group.

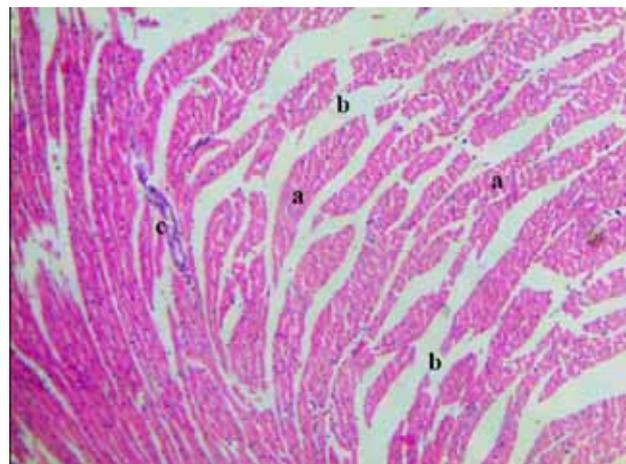


Figure 10: Rat heart section 24 days after CLP, showed degeneration and necrosis of heart muscles (a), with edema between the muscle fiber (b) and focal aggregation of inflammatory cells (c). H & E. X350.

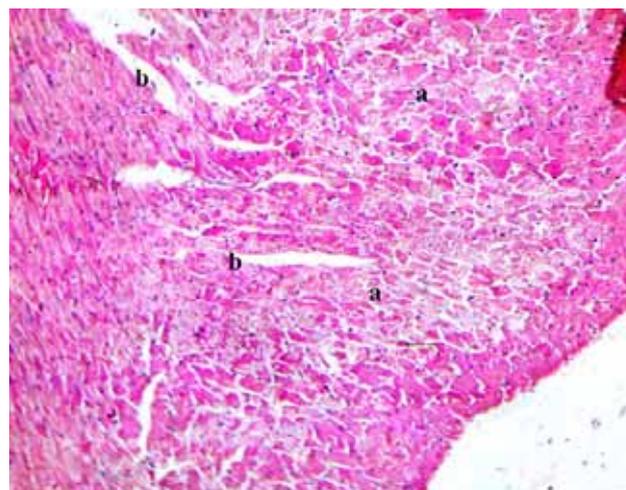


Figure 11: Rat heart section 3 days after CLP, showed severe zencers necrosis of the myocardium (a) with edema between the cardiac muscle fibers (b). H & E. X 42.

## Discussion

One of the most promising extra-skeletal role of the vitamin D with sepsis is in the functioning of the immune system (14). This was initially indicated by the discovery of the Vitamin D receptors in nearly all types of the immune cells, including activated CD4<sup>+</sup> and CD8<sup>+</sup>T cells, B cells, neutrophils, macrophages and dendritic cells (15). These cells span the body's innate and adaptive immune responses to pathogens.

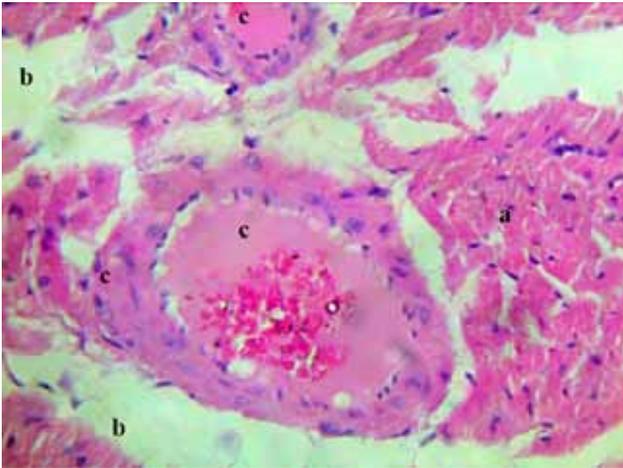


Figure 12: Rat heart section 7 days after CLP, showed massive necrosis of the myocytes (a), with severe edema (b) and thickening of blood vessels with recent thrombus (c). H & E. X 420.

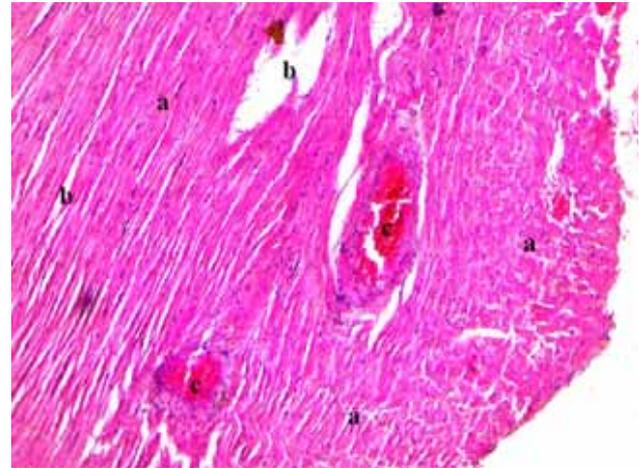


Figure 14: Rat heart section treated with Vit. D3, 3 days after CLP, showed severe necrosis of the cardiac muscles (a), edema between muscle fibers (b) and congestion and thrombus in blood vessels (c). H & E. X 105.

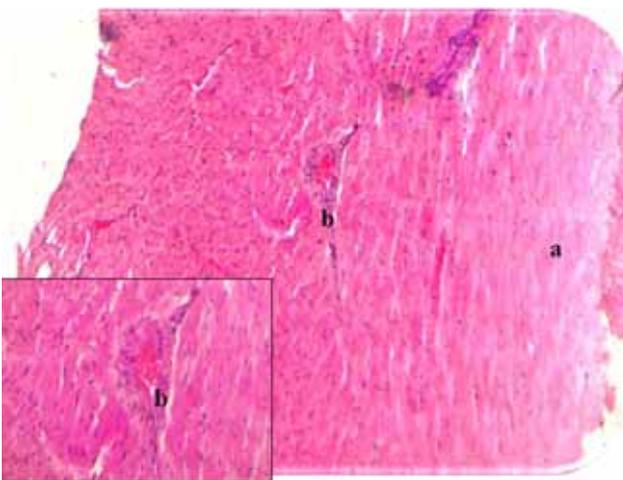


Figure 13: Rat heart section treated with Vit. D3, 3 days after CLP, showed Zenker's necrosis of the cardiac muscle fibers (a), with perivascular cuffing (b). H & E. X42.

Vitamin D acts in the local tissue response to infections and are integral to the production of antimicrobial peptides (CAMPs) Cathelicidin (16). The active fragment of the cathelicidin is IL-37, which has been shown to be produced by phagocytic leukocytes, mucosal epithelium, keratinocytes and to be present in mucosal secretions and plasma, its immune functions includes direct bactericidal activity, promotion of phagocytosis and reactive oxygen species and chemotaxis of other immune cells to the site of infection (17).

The inflammatory cytokines  $TNF-\alpha$  and certain interleukins, play a key- role in the initiating systemic inflammatory response in sepsis (10). The host response towards invading pathogens is characterized by an overwhelming systemic pro-inflammatory response that is primarily mediated by cytokines, which can lead to fatal multiorgan failure and septic shock. Vitamin D modulates the cytokine expressions from the monocytes and macrophages and vitamin D may improve outcomes by reducing both local and systemic inflammatory responses as a result of cytokine responses and reducing Toll-like receptor (TLR) activation (17). Circulating vitamin D levels have a direct influence on macrophages, increase their "oxidative burst", potential (maturation and production of cytokines, acid phosphatase and hydrogen peroxide), and prevent excessive expression of inflammatory cytokines, also facilitates neutrophil motility and phagocytic function (18,19).

In our study, the survival rate showed improvement in rats with sepsis pretreated with Vit. D3, compared with rats with sepsis only and sham-operated rats group, and that may be due to the enhancement in the immune state of rats pretreated with vitamin D3. Also the pretreated of rats with Vit. D3, protect from the CLP sepsis induced thrombocytopenia (12), which is the cause of the Disseminated Intravascular Coagulation (DIC), that consider one of the most causes of death in sepsis animals (10). Also, there was elevated in the white blood cells count in rats pretreated with Vit. D3 compared with rats with sepsis only, and this revealed that it. D3 causing increase in the immune response of the animals (13).

Sepsis causes severe tissue injury and multiple organ dysfunction (20). Cells of the innate immune system (neutrophils, macrophages and dendritic cells) are activated by pathogens and initiated the inflammatory response via interaction with Toll-like receptors (10). Normally, neutrophil – mediated inflammation and phagocytosis occur at the site of infection prior to the apoptosis, during sepsis apoptosis of the neutrophils is delayed, resulting in a prolonged inflammatory phase (20).

The histopathological examination of the liver and heart in rats pretreated with the vitamin D3 before CLP sepsis, showed slight improvement compared to those with sepsis only. Organ lesions in rats with sepsis are due to severe bacteremia and septicemia that result from rapid transfer of septic pathogens and its toxins from the peritoneal cavity into the systemic circulation (9). Vit. D3 through its antimicrobial role and immune functions may decrease the septic pathogens (21).

In conclusion, the results of this study have demonstrated that pretreated rats with Vit. D3 before CLP sepsis, improve the survival of rats and enhancement in blood leukocyte count, also protect the rats from thrombocytopenia and Disseminated Intravascular Coagulation (DIC) but Vit. D3 pretreated show slight improvement in the histopathological lesions in the liver and heart due to CLP sepsis.

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