Research Article

The Effects of Vitamin D on L-arginine-induced Acute Pancreatitis in Rats

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Received: 18-07-2017; **Revised:** 26-08-2017; **Accepted:** 16-09-2017.

ABSTRACT

This study was aimed to investigate the protective effect of vitamin D on L-arginine induced acute pancreatitis in rats. Twenty eight white Albino rats of both sexes were divided into 4 equal groups. Negative control rats (group I) intra-peritoneally injected with normal saline, positive control (group II) rats induced acute pancreatitis with L- arginine, group III rats treated with a single dose of vitamin D, and group IV treated with single dose of vitamin D prior to first dose of L- arginine. Serum amylase, lipase and cytokines such as tumour necrosis factor –alpha (TNF-α), interleukin-1 beta (IL1β); in addition, my eloperoxidase (MPO) enzyme activity in the pancreas were measured. In acute pancreatitis group (group II), there was a significant elevation in all of previously-mentioned parameters compared to negative controls. Administration of vitamin D before first dose of arginine (group IV) produced a significant reduction in serum -amylase and -lipase as well as TNF-α and IL1β and MPO activity in pancreatic tissue compared to acute pancreatitis group (group II). In conclusion, the findings obtained from this study demonstrate the amelioration of pancreatic injury by vitamin D which may suggest the anti-inflammatory property of the intended vitamin and can be utilized in prevention of acute pancreatitis.

Keywords: Vitamin D, acute pancreatitis, L-arginine, enzymes activity, cytokines.

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disorder of the pancreas characterized by severe pain in the upper abdomen and increased serum concentration of pancreatic amylase and lipase [1]. There are many causes of A.P, but gallstones constitute the common cause [2]. Less common cause is abuse of alcohol [3]. Investigators demonstrated that the pathophysiology of A.P considered in three phases. In the *first phase*, there is premature activation of trypsin within pancreatic acinar cells; in the *second phase*, there is intra-pancreatic inflammation [4]. In the *third phase*, there is extra-pancreatic inflammation [5]. Experimental studies suggested that in course of A.P, the overproduction of inflammatory mediator such as tumour necrosis factor-alpha (TNF-α), interleukin 1 beta (IL1β), interleukin-6 (IL6), oxygen free radical and nitric oxide adhesion molecule were observed; additionally, these cytokines are also responsible for the systemic complication of A.P such as vascular leakage, hypovolaemia, system inflammatory response syndrome (SIRS), shock, and organ failure [6, 7].There are no specific therapies for A.P. The medical management aimed to control sign and symptom [8, 9.] Studies on new therapeutic approach directed to its pathogenesis are still in progress.

L-Arginine is classified as a semi-essential amino acid [10]. Its exact mechanism for induction of A.P not clears yet, but the available evidence indicated that oxidative stress and metabolic acidosis contribute to the pancreatic injury in this model [11]

Vitamin D is secosterol, a fat soluble vitamin. It is responsible for the elevation of plasma calcium and phosphate levels, which are required for bone mineralization [12]. It has been reported that low levels of vitamin D are associated with increased -cardiovascular (CV) mortality, -cancer incidence and -mortality, and -autoimmune diseases such as multiple sclerosis [13].

Besides, vitamin D has been reported to act as an antioxidant; where, its antioxidant effect can be mediated through the -induction of expression of several molecules including reduced glutathione (GSH), GSH peroxidase and superoxide dismutase (SOD) enzymes, and -suppression of the NADPH oxidase expression; furthermore, such vitamin can be considered as -a powerful anti-inflammatory and -a promising anti-cancer agent [14].

Vitamin D is a regulator of the immune system by modulating innate and adaptive immunity. It has been reported vitamin D may interact with its receptor (VDR) this may result in -the inhibition of the expression of inflammatory cytokines, including IL-1, IL-6, and TNF-α and interferon- gamma (IFN-γ) can be in accessory cells and in the serum and -the up-regulation of IL-10 release (a cytokine exerting anti-inflammatory activity) from lymphocytes which can be consequently produced [15].

This study is designed to investigate whether vitamin D may possess protective role against pancreatic injury induced by L-arginine.

MATERIALS AND METHODS

Animals

Twenty eight white Albino rats of both sexes weighing 180-220g were utilized in this study; they were obtained from the Animal House of the College of Pharmacy / Baghdad University and maintained locally under controlled conditions of temperature, humidity and light/dark cycles. They were kept in plastic cages three animals per cage, fed standard rodent pellets and have free access to water

Study design

The study was approved by the Scientific- and the Ethical-Committees of the College of Pharmacy/ University of Baghdad. The animals utilized in this study were divided into four groups as follows:

Group I: Seven rats were intraperitoneally (I.P) administered 0.5ml normal saline. This group served as negative controls.

Group II: Seven rats were I.P injected L-arginine 2.5gm/kg body weight twice at 1 hour interval this group served as positive controls of A.P.

Group III: Seven rats were orally administered with 25µcg/100g vitamin D alone by oral gavage tube.

Group IV: Seven rats were orally administered single dose of vitamin D 25µcg/100g by oral gavage tube 30 minutes before the first L-arginine injection.

The animals in each group were euthanized by anesthetic diethyl ether after 24 hour of the end of the treatment.

Sample Collection

After euthanization of rats by anesthetic ether, blood was collected by heart puncture and put in gel tube then centrifuged at 3500 rpm for 15 minute; the supernatant was separated by using micropipettes and stored at -20 Cº until the day of analysis. The supernatant (serum) was used for the estimation of amylase, and lipase enzyme activities; in addition to the estimation of interleukin IL-1β, and TNF-α level. Moreover, four pancreas from each group utilized in this study was quickly excised, placed in chilled phosphate buffer saline (PBS) solution (PH 7.4) at 4 ^{0}C to remove excess blood then the tissue dried with filter paper and weighed, then minced to small pieces; where, 1g pancreatic tissue was put in a tube, which is contained 10 ml of PBS solution prepared at the previously-mentioned pH value, to obtain 10% tissue homogenate. The tube containing the pancreatic tissue was put in a beaker containing ice then homogenized with the aid of homogenizer (Success Technic Industry, Malaysia) at set 3 for 1 minute at 4 °C. After that, the homogenate was centrifuged by the cooled centrifuge (Eppendorf AG, Germany) for 15 minutes at 1500×g [or 5000 revolution/minute (rpm)] at 4 °C. The supernatant is separated using micropipette and stored at -20Cº until the analysis day; where, it is utilized for the assessment of myeloperoxidase (MPO) enzyme activity.

Analysis

Estimation of serum amylase enzyme activity

Serum amylase activity was estimated with a spectrophotometric technique [16] by kenza auto-analyzer (Biolabo, France) using commercial kits (Biolabo, France) for this purpose. The results were expressed as U/I.

Determination of serum lipase

Determination of serum lipase activity employs the quantitative sandwich enzyme linked immunosorbent (ELISA) assay; where, antibody specific for pancreatic lipase (PL) has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PL present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for PL is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells, incubate and then wash to remove any unbound avidin-enzyme reagent. A substrate solution is added to the wells then incubates and color develops in proportion to the amount of PL bound in the initial step. The product of such reaction forms a blue colored complex. Lastly, an acidic solution (sulphuric acid solution) is added to stop the reaction and will turn the color of the complex solution to yellow [17]. The intensity of color was spectrophotometrically measured at 450nm by means of a microplate reader. The activity of serum lipase was expressed as pg/ml

Determination of interleukin 1 beta IL-1ß

The principle of IL-1β determination in serum is based on ELISA. This assay employs an antibody specific for IL-1β coated on well plate. Standards and samples are pipetted into the wells and IL-1β present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotin conjugated antibody is added. Then incubated and washed to remove any unbound substance. HRP-conjugated streptavidin is pipetted to the wells the wells are again incubated and washed, then tetramethylbenzidine TMB substrate solution is added to the wells after incubation. The color develops in proportion to the amount of IL-1 beta bound. The stop solution (acidic solution) changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. [17] Serum IL1β level was expressed as pg/ml

Determination of tumor necrosis factor alpha TNF-α

The principle of this assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipette into the wells and any TNF-α present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for TNF-α is added to the wells. After washing, avidin conjugated Horseradish

Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound in the initial step. The color development is stopped and the intensity of the color is measured[18]

The intensity of color was spectrophotometrically measured at 450nm by means of a microplate reader. Serum TNF-α level was expressed as pg/ml.

Determination of pancreatic myeloperoxidase

Assay of myeloperoxidase activity in the pancreatic tissue employs the quantitative sandwich enzyme linked immunosorbent (ELISA) technique. . Antibody specific for MPO has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any MPO present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for MPO is added to the wells. After incubation and washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following incubation and washing to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of MPO bound in the initial step. The color development is stopped by means of acidic solution and the intensity of the color is spectrophotometrically measured at 450nm.[18] The activity of MPO enzyme was expressed as ng/ml

Statistical Analysis

The significance of differences between the mean values was calculated using unpaired Student's t-test and comparisons among groups were made using analysis of variance (one way ANOVA) by SPSS statistics. Numeric data were expressed as Mean ± Standard error of the mean (SEM). *P*-values less than 0.05 were considered significant for all data presented in this study.

RESULTS

Effects of vitamin D on serum amylase and lipase enzymes activity

Table 1 showed that, there was significant elevation ($P<0.05$) in serum amylase activity in L-arginine- induced acute pancreatitis (group II) rats compared to normal controls (group I). Besides in group of rats administered vitamin D at a dose of 25µcg/100gm alone (group III), table 1 showed that there was no significant difference ($P>0.05$) in serum amylase activity compared to normal control animals. By comparing serum amylase activity among groups II, III, and IV, table 1 showed that, serum amylase activity was significantly reduced in group of rats treated with vitamin D 25µcg/100gm thirty minutes prior to L-arginine (group IV) compared to L-arginine-induced acute pancreatitis (group II) rats; while a significant elevation of the intended activity was observed in group IV rats compared to rats of group III that treated with vitamin D 25µcg/100gm alone ($P<0.05$).

Concerning serum lipase activity, table 1 showed that there were significant elevation in serum lipase activity in rats IP injected with L-arginine-induced acute pancreatitis (group II) compared to normal control group I animals ($P<0.05$). Furthermore, in group of rats administered vitamin D at a dose of 25µcg/100gm alone (group III), table 1 showed that there was no significant difference ($P>0.05$) in serum lipase activity compared to normal control animals. By comparing serum lipase activity among groups II, III, and IV, table 1 showed that, the intended activity was significantly reduced in group of rats treated with vitamin D 25µcg/100gm thirty minutes prior to L-arginine (group IV) compared to L-arginine-induced acute pancreatitis (group II) rats; while anon significant elevation of serum lipase activity was observed in group IV rats compared to rats of group III that treated with vitamin D 25µcg/100gm alone ($P<0.05$).

Effects of vitamin D on serum TNF-α and IL 1β levels

Table 2 showed that, there was a significant elevation in serum TNF-α level in group II rats IP injected with L-arginine model of acute pancreatitis compared to negative control group I rats ($P<0.05$). Furthermore, in group of rats administered vitamin D at a dose of 25µcg/100gm alone (group III), table 2 showed that there was a significant reduction ($P<0.05$) in TNF-α level compared to normal control animals. By comparing serum TNF-α level among groups II, III, and IV, table 2 showed that, the intended level was significantly reduced in group of rats treated with vitamin D 25µcg/100gm thirty minutes prior to L-arginine (group IV) compared to L-arginine-induced acute pancreatitis (group II) rats; while anon significant elevation of serum TNF-α level was observed in group IV rats compared to rats of group III that treated with vitamin D 25µcg/100gm alone ($P<0.05$).

Concerning serum IL-1β level, table 2 showed that there were significant elevation of the intended level in rats IP injected with L-arginine-induced acute pancreatitis (group II) compared to normal control group I animals ($P<0.05$). Moreover, in group of rats administered vitamin D at a dose of 25µcg/100gm alone (group III), table 2 showed that there was no significant difference ($P>0.05$) in serum IL-1β level compared to normal control animals. By comparing serum IL-1β level among groups II, III, and IV, table 2 showed that, the intended level was significantly reduced in group of rats treated with vitamin D 25µcg/100gm thirty minutes prior to L-arginine (group IV) compared to L-arginine-induced acute pancreatitis (group II) rats; while a significant elevation of serum IL-1β level was observed in group IV rats compared to rats of group III that treated with vitamin D 25µcg/100gm alone ($P<0.05$).

Effects of vitamin D on pancreatic myeloperoxidase (MPO) enzyme activity

Table 3 showed that, there were significant elevation in pancreatic MPO activity in the acute pancreatitis (group II) IP injected with L-arginine compared to negative

control rats (group I) (P<0.05). Besides, in group of rats administered vitamin D at a dose of 25µcg/100gm alone (group III), table 3 showed that there was non-significant difference (P>0.05) in pancreatic MPO activity compared to negative control animals. By comparing pancreatic MPO activity among groups II, III, and IV, table 3 showed that, the intended activity was significantly reduced in group of rats treated with vitamin D 25µcg/100gm thirty minutes prior to L-arginine (group IV) compared to L-arginine-induced acute pancreatitis (group II) rats; similarly, a significant increase in pancreatic MPO activity was observed in group IV rats compared to rats of group III that treated with vitamin D 25µcg/100gm alone (P<0.05).

Table 1: Effects of various treatments on serum amylase and lipase enzymes activity in rats (N=7). Data presented as mean ± SEM

Groups	Treatment	Serum amylase activity (IU/L)	Serum Lipase activity (Pg/ml)
Group I	Negative Control (normal saline)	2170.7±130.6	0.44±0.099
Group II	L-arginine 250mg/100gm	6243.3±298.6^{*,a}	1.75±0.255^{*,a}
Group III	25µcg/100gm vitamin D	2114.2±135.55^{b}	0.82±0.12^{b}
Group IV	vitamin D 25µcg/100gm thirty minutes prior to L-arginine	3212±421.8^{*,c}	0.98±0.118^{*,c}

- $^{*}P$<0.05: significant difference compared to the negative control group.

- Values with non-identical small letter superscripts (a, b, and c) are considered significantly different $^{*}P$<0.05.

Table 2: Effects of various treatments on serum levels of TNF-α and IL1β in rats (N=7). Data presented as mean ± SEM

Groups	Treatment	Serum TNF-α (Pg/ml)	Serum IL1β (Pg/ml)
Group I	Negative Control (normal saline)	54±8.37	233.67±11.97
Group II	L-arginine 250mg/100gm	78.5±12.2^{*,a}	502.83±19.14^{*,a}
Group III	25µcg/100gm vitamin D	31.38±1.78^{*,b}	241.83±15.66^{b}
Group IV	vitamin D 25µcg/100gm thirty minutes prior to L-arginine	43.53±2.53^{,c}	412.17±29.15^{*,c}

- $^{*}P$<0.05: significant difference compared to the negative control group.

- Values with non-identical small letter superscripts (a, b, and c) are considered significantly different $^{*}P$<0.05.

Table 3: Effects of various treatments on pancreatic myeloperoxidase (MPO) enzyme activity in rats (N=4). Data presented as mean ± SEM

Groups	Treatment	Pancreatic MPO (Pg/ml)
Group I	Negative Control (normal saline)	**0.87±0.1**
Group II	L-arginine 250mg/100gm	**1.93±0.09**^{*,a}
Group III	25µcg/100gm vitamin D	**1.08±0.1**^{b}
Group IV	vitamin D 25µcg/100gm thirty minutes prior to L-arginine	**1.54±0.09**^{*,c}

- $^{*}P$<0.05: significant difference compared to the negative control group.

- Values with non-identical small letter superscripts (a, b, and c) are considered significantly different $^{*}P$<0.05.

DISCUSSION

The treatment strategies of AP are limited to supportive care; and, more effective therapeutic options should be developed for better management of this disorder [19]. In this study the effects of vitamin D on AP rats' model induced by L-arginine were investigated. L-arginine was chosen for induction of AP because it is highly reproducible, non-invasive and may produce biochemical parameters changes in addition to possible histological changes in pancreas resemble that of human situation [20]

In the present study administration of L- arginine significantly developed A.P that characterized by raised serum activities of amylase and lipase enzymes. The results of this study are consistent with those of other study [21]. Serum amylase and lipase enzymes are important diagnostic marker for A.P; where their levels may at least be tripled compared to upper limit of

normal; however, their serum level may not depend on the disorder severity [22]. Furthermore, it has been reported that the elevation of serum amylase can be detected for up to 3-5 days while, serum lipase activity level may remain high for 8-14 days; thus, lipase enzyme can be considered as a more sensitive parameter for diagnosis in delayed presentation [23].

In experimental pancreatitis performed by others, serum levels of TNF-α, IL-1ß, and IL-6 were reported to be elevated and their blockade can attenuate the disorder[24-26].

In the present study, serum levels of TNF-α, IL-1ß were elevated after induction of pancreatitis by L-arginine (group II); in addition, decreased levels of pro-inflammatory TNF-α and IL-1β cytokines in serum of rats' group treated with vitamin D prior to L-arginine (group IV) were observed.

Tumour necrosis factor-alpha (TNF-α) and IL-1β are the major and the primary pro-inflammatory mediators and are accountable for all other subsequent systemic complications [27]. Activation of granulocytes and other pro-inflammatory mediators can be regulated by TNF-α, which may activate intracellular protease (trypsinogen) and thus cellular necrosis [28]. Besides, IL-1 can play an important detrimental role in AP, since it is noted as the main mediator in sterile necrosis and local and systemic tissue destruction, with 82% accuracy in prediction of severity [27].

Studies indicated that vitamin D is a key modulator of immune function and inflammation [29, 30]. It may exert broad regulatory effects on cells of the adaptive and innate immune system [31]. The conversion of 25(OH) D to its active form 1, 25$(OH)_2$ D3 can be locally produced in immune system cells which has been reported to possess an anti-inflammatory effect on the inflammatory profile of monocytes [32,33] and it may down-regulate the expression and production of several pro-inflammatory cytokines including TNF- α, IL-1β, IL-6, and IL-8 [32,33]. Although, some of the immediate effects of vitamin D may occur in cells that possess the membrane vitamin D receptor (mVDR) [34] but, the majority of vitamin D's biological functions are mediated through the regulation of gene expression. The active metabolite of vitamin D [1, 25(OH) 2D3] was reported to bind to its nuclear vitamin D receptor (nVDR) with high affinity and specificity. The vitamin D-nVDR can form a heterodimer with the retinoid X receptor and this complex may -amplify or -repress the transcription of the target genes through its binding to vitamin D responsive elements (VDRE) on DNA [35]. The nVDR is found in multiple cells of the immune system such as human Treg cells [36] neutrophils [37]dendritic cells, B cells [38] and macrophages [39].

Myeloperoxidase (MPO) enzyme is one of the enzymes contained in azurophilic granules. Increased MPO blood levels reflect neutrophil activation [40]. The results of the current study showed that significant elevation (P<0.05) in pancreatic tissue MPO enzyme activities in L-arginine – injected group (group II) compared to negative control group (group I). These results are in agreement with the results of other study; where, an increased MPO activity in A.P [41]. Additionally, the MPO enzyme activity were significantly reduced (P<0.05) in group IV rats treated with vitamin D prior to (group II and III). The finding obtained from the present study are disagree with the results of others; where, they demonstrated that the conversion of 25(OH)D3 to the active form 1,25(OH)2D3 was produced by the activated macrophages in tissue undergoing inflammation and that the elevation of the blood level of the intended active form can cause hypercalcaemia and A.P; and a down regulation of blood level of vitamin D and its active form in patient with pancreatitis can be considered as a protective mechanism to prevent the development hypercalcaemia which may consequently exacerbate the pancreatitis [42].

CONCLUSION

According to the results obtained from the present study, it could be concluded that vitamin D can efficiently reduce the severity of L-arginine-induced acute pancreatitis that is evidenced by significant reduction in the measured serum biomarkers enzymes amylase and lipase, serum TNF-α and IL1β levels; with significant reduction in MPO pancreatic tissue enzyme activity; this may indicate a protective effect of such vitamin at early stage of the disease progression.

Acknowledgment: Authors express sincere thanks and appreciation to the College of Pharmacy, Baghdad University for help and support. Special thanks to Dr. Saleema for her statistical assistance.

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Source of Support: Nil, **Conflict of Interest:** None.