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Research Paper

Effect of 1,25-dihydroxy-vitamin D_3 in experimental sepsis

Søren Møller[1], Finn Laigaard[1], Klaus Olgaard[2], Claus Hemmingsen [1]

1. Department of Anaesthesiology, Frederiksberg Hospital, University of Copenhagen, Denmark
2. Department of Nephrology, Rigshospitalet, University of Copenhagen, Denmark

Correspondence to: Claus Hemmingsen, MD DMSc, Department of Anaesthesiology, Frederiksberg Hospital Nordre Fasanvej 57, DK-2000 Copenhagen Frederiksberg, Denmark. Phone: +45 3816 3310, E-mail: clhe@dadlnet.dk

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Background: In addition to the regulation of calcium homeostasis, vitamin D affects the cellular immune system, targets the TNF-α pathway and increases vasoconstrictor response to angiotensin II. We therefore examined the effect of 1,25-dihydroxy-vitamin D_3 on coagulation and organ failure in experimental sepsis in the rat.

Methods: Three series of placebo-controlled studies were conducted. All rats were pre-treated with daily SC injections of 1,25-dihydroxy-vitamin D_3 100 ng/kg or placebo vehicle for 3 days. In study 1, sepsis was accomplished by abdominal surgery comprising a coecal ligation and puncture with a 1,2 mm needle, or sham surgery. In study 2, the rats had a single IP injection of lipopolysaccharide from E. Coli 0111:B4 (LPS) 8 mg/kg, or placebo. In study 3, an hour-long IV infusion of LPS 7 mg/kg, or placebo was given.

Results: All three models of sepsis showed significant effects on coagulation and liver function with reduced thrombocyte count and prothrombin time together with elevated ALT and bilirubin ($p<0.05$) as compared to controls. In study 1, the vitamin D treated rats maintained normal platelet count, whereas the vehicle treated rats showed a significant reduction ($p<0.05$). This effect of vitamin D on platelets was not found in the LPS-treated groups. We found no significant differences between vitamin D and placebo-treated rats with regards to liver function.

Conclusion: The present data suggest a positive modulating effect of 1,25-dihydroxy-vitamin D_3 supplementation on sepsis-induced coagulation disturbances in the coecal ligation and puncture model. No such effect was found in LPS-induced sepsis.

Key words: 1,25 Vitamin D, calcitriol, sepsis, rats, coagulation, thrombocytes

1. Introduction

The hormonally active form of vitamin D, 1,α25-dihydroxyvitamin D3 (1,25-vit D) is an important regulator of the calcium-phosphate homeostasis. In addition, this compound possesses a number of non-calcaemic effects. These include an effect on the immune system cell differentiation and the interaction of macrophages and monocytes and regulation of lymphocyte activity [1].

Sepsis may be complicated by a variety of conditions such as disseminated intravascular coagulation (DIC), circulatory collapse and multiple organ failure. DIC is characterized by simultaneous micro thrombosis and expenditure of clotting factors causing increased bleeding tendency and organ failure. Monocytes play an important role in the induction of tissue-factor expression [2], which is seen as a key event in the development of DIC in septic patients. Therefore, there is reason to expect that 1,25-vit D could be useful in the treatment of DIC caused by sepsis.

1,25-vit D also targets the TNF-a pathway to suppress experimental bowel disease[3] and increases the vasoconstrictor response to noradrenalin and angiotensin II [4] . Recently, evidence has emerged that vitamin D also enhances the function of the innate immune system by stimulating the formation of the cathelicidin antimicrobial peptide [5]. Theoretically, treatment with 1,25-vit D may therefore also reduce the septic response to intestinal perforation and reduce the circulatory effect of the septic condition.

Only two studies have previously addressed this potential association, and both showed a beneficial effect of 1,25-vit D against lipopolysaccharide (LPS)-induced DIC in rat models [6,7]. In the present study, we aimed to demonstrate a possible effect of 1,25-vit D in three different models of experimental sepsis and DIC in rats, using a controlled and clinically relevant administration of vitamin D.

2. Material and methods

Animals

Male Wistar rats weighing 300 g were obtained from Charles River, Sulzfeld, Germany and were kept in plastic cages in a controlled environment with a 12-hour light/dark cycle and a constant temperature (22° C) and humidity (70%), with free access to food and water. The diet was Altromin 1324 (Altromin GmbH, Lage, Germany). The experimental studies on rats were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the local ethics committee for re-

search on animals. The rats were anaethetized by either fentanyl-midazolam for abdominal surgery or by pentobarbital for LPS infusion. Postoperative analgesia was provided with buprenorphin. All animals were euthanized immediately after blood sampling.

Materials

1,25-dihydroxyvitamin D_3 was a gift from Leo Pharmaceuticals (Ballerup, Denmark) and was dissolved in a vehicle consisting of water/propylene glycol/ethanol 50/40/10. The dose of 1,25-dihydroxyvitamin D_3 of 100 ng/kg/day was shown in previous studies from our laboratory to be the highest dose not causing hypercalcemia [8] . Lipopolysaccharide from E. Coli 0111:B4 was obtained from Sigma Chemicals (St. Louis, MO, USA).

Analytical methods

Thrombocytes were counted on a Sysmex Kx-21 (Sysmex Corp, Mundelei, IL, USA), prothrombin, ALT, bilirubin, creatinine and urea were measured on an ACL 9000 (Instrumentation Laboratory, Lexington, MA, USA) kindly provided by ILS Scandinavia, and ionized calcium was measured on an ABL 77 (Radiometer, Bronshoj, Denmark). The blood to be used for measurement of coagulation parameters were sampled directly in a citrate dilution (9:1) to avoid early coagulation. Data were analyzed using non-parametric analysis of variance. All data are shown as the mean ± confidence interval. *P* values < 0.05 were considered statistically significant.

Study design

Three series of studies were conducted. In studies 1 and 2, which were conducted simultaneously, groups 1 and 2 were identical but are in the following sections and in the tables described as separate groups to ease comparison between controls (not septic) and septic animals.

Study 1 (Abdominal sepsis).

Six groups of rats were pre-treated with daily SC injections of 1,25-vit D 100 ng/kg or vehicle for 3 days. The rats were then allocated to 6 groups:

1. Controls + vehicle.
2. Controls + 1,25-vit D.
3. Sham surgery comprising laparotomy and exposure of coecum but without ligation and puncture + vehicle.
4. Sham surgery + 1,25-vit D.
5. Surgery comprising a laparotomy with a coecal ligation and puncture with a 1,2 mm needle (CLP) + vehicle
6. CLP + 1,25-vit D.

At the end of surgery 10 ml isotonic saline was administered SC as early fluid resuscitation. Blood samples were obtained 24 h post treatment.

Study 2 (24 hours chronic sepsis).

Four groups of rats were pre-treated with daily SC injections of 1,25-vit D 100 ng/kg or vehicle for 3 days. The rats were then allocated to 4 groups:

1. Controls + vehicle.
2. Controls + 1,25-vit D.
3. Single IP injection of lipopolysaccharide from E. Coli 0111:B4 (LPS) 8 mg/kg + vehicle.
4. Single IP injection of lipopolysaccharide from E. Coli 0111:B4 (LPS) 8 mg/kg + 1,25-vit D.

10 ml isotonic saline was administered SC as early fluid resuscitation. Blood samples were obtained 24 h post treatment.

Study 3 (4 hours acute sepsis).

Four groups of rats were pre-treated with daily SC injections of 1,25-vit D 100 ng/kg or vehicle for 3 days. A catheter was placed in the left femoral vein with the use of microscopic surgery. The anaesthesia was maintained for the whole duration of the experiment (4 hours) by supplemental administration of pentobarbital. The rats were allocated to 4 groups:

1. Controls had hour-long placebo infusion of 20 ml isotonic saline through the femoral catheter + vehicle.
2. Controls + 1,25-vit D.
3. Hour-long IV infusion of LPS 7 mg/kg in 20 ml isotonic saline via a catheter in a femoral vein + vehicle.
4. Hour-long IV infusion of LPS 7 mg/kg + 1,25-vit D.

Blood samples were obtained 4 h post treatment.

3. Results

The effect of 1,25 vit-D or vehicle on sepsis induced by coecal ligation and puncture (CLP) or sham operation is shown in table 1.

The CLP model induced a septic condition in the rats as shown by reduced thrombocyte count and increased ALT and bilirubin ($p<0.05$). The thrombocytopenia was significantly counteracted in the the vitamin D treated rats with thrombocytes 515 ± 53 10^9 /L as compared to 418 ± 49 10^9 /L in the vehicle treated rats ($p<0.05$). Plasma bilirubin showed a minor but significant increase from 0.68 ± 0.29 µmol/L to 1.23 ± 0.11 µmol/L among the sham operated rats, this was however counteracted in the 1,25 vit-D treated sham operated rats to 0.73 ± 0.39 µmol/L ($p<0.05$). This effect of 1,25 vit-D was also found among the CLP operated animals where the bilirubin value increased to 3.51 ± 1.87 µmol/L, but among the 1,25 vit-D treated and CLP operated bilirubin only increased to 2.36 ± 1.14 µmol/L. This difference did not reach statistical significance due to high variance of data. Plasma ALT levels increased among both sham operated and CLP operated rats. The ALT elevation was only significant among the 1,25-vit D treated CLP operated rats. No significant differences were found in the measuremets of PT, creatinine, or urea. Ionized calcium values were significantly reduced in LPS treated rats ($p<0.05$), and no difference in ionized calcium values was found between 1,25-vit D or vehicle treated rats.

The effect of 1,25 vit-D or vehicle on chronic sepsis induced by single IP injection of LPS is shown in

table 2.

The LPS injection induced a septic condition in the rats with effects on coagulation and liver function as shown by reduced thrombocyte count and increased ALT and bilirubin ($p<0.05$). We found no differences in thrombocyte count, ALT and bilirubin between controls receiving 1,25 vit-D or vehicle, and we found no differences in PT and creatinine between controls and LPS treated rats. Plasma urea increased from 8.2 ± 0.5 mmol/L in the control rats in group 1 to 11.9 ± 2.5 mmol/L among the 1,25 vit-D and LPS treated rats ($p<0.05$), but no difference was demonstrated between the rats that received LPS and either vehicle or vitamin D. Ionized calcium values were significantly reduced in LPS treated rats ($p<0.05$), and no difference in ionized calcium values was found between 1,25-vit D or vehicle treated rats.

The effect of 1,25 vit-D or vehicle on acute sepsis induced by an hour-long infusion of LPS is shown in table 3.

Table 1. Sepsis accomplished by abdominal surgery comprising coecal ligation and puncture with a 1,2 mm needle (CLP), or sham surgery + pre-treatment with 1,25 vit-D or vehicle.

	Control +vehicle	Control + 1,25 vit-D	Sham-operation +vehicle	Sham-operation + 1,25 vit-D	CLP +vehicle	CLP + 1,25 vit-D
n=	8	8	8	8	8	8
Thrombocytes (10^9/L)	647 ± 95	606 ± 139	631 ± 48	551 ± 53	418 ± 49 [b]	515 ± 53 [c]
PT (seconds)	12,0 ± 0,2	12,2 ± 0,7	11,4 ± 0,6	11,0 ± 0,8	12,5 ± 1,2	12,7 ± 1,0
ALT (units/L)	53 ± 7	53 ± 8	59 ± 13	64 ± 5	71 ± 17	77 ± 13 [b]
Bilirubin (µmol/L)	0,68 ± 0,29	0,62 ± 0,27	1,23 ± 0,11 [a]	0,73 ± 0,39 [c]	3,51 ± 1,87 [b]	2,36 ± 1,14 [b]
Creatinine (µmol/L)	41 ± 7	46 ± 19	46 ± 14	36 ± 3	40 ± 2	42 ± 5
Urea (µmol/L)	8,2 ± 0,5	8,4 ± 0,7	7,0 ± 0,5 [a]	6,5 ± 0,6 [a]	6,9 ± 0,8	8,9 ± 2,6
Ca^{++}(7,4) (mmol/L)	1,34 ± 0,02	1,36 ± 0,02	1,35 ± 0,02	1,35 ± 0,02	1,26 ± 0,06 [b]	1,28 ± 0,03 [b]

[a] Significant effect of sham operation vs control. (P<0,05)

[b] Significant effect of CLP vs. sham operation. (P<0,05)

[c] Significant effect of 1,25 vit-D vs. vehicle. (P<0,05).

Table 2. Sepsis accomplished by a single IP injection of lipopolysaccharide from E. Coli 0111:B4 8 mg/kg (LPS), or control + pre-treatment with 1,25 vit-D or vehicle.

	Controls +vehicle	Controls +1,25 vit-D	LPS + vehicle	LPS +1,25 vit-D
n=	8	8	6	6
Thrombocytes (10^9/L)	647 ± 95	606 ± 139	259 ± 198 [a]	208 ± 214 [a]
PT (seconds)	12,0 ± 0,2	12,2 ± 0,7	11,4 ± 0,4 [a]	12,0 ± 0,4
ALT (units/L)	53 ± 7	53 ± 8	124 ± 167	94 ± 24 [a]
Bilirubin (µmol/L)	0,68 ± 0,29	0,62 ± 0,27	1,77 ± 1,10	2,12 ± 0,53 [a]
Creatinine (µmol/L)	41 ± 7	46 ± 19	44 ± 7	57 ± 11
urea (µmol/L)	8,2 ± 0,5	8,4 ± 0,7	10,66 ± 3,09	11,90 ± 2,47 [a]
Ca^{++}(7,4) (mmol/L)	1,34 ± 0,02	1,36 ± 0,02	1,36 ± 0,02	1,37 ± 0,02

[a] Significant effect of LPS vs control. (P<0,05).

No significant effects (P<0,05) of 1,25 vit-D were seen in this study.

Table 3. Sepsis accomplished by an hour-long IV infusion of lipopolysaccharide from E. Coli 0111:B4 7 mg/kg (LPS), or control + pre-treatment with 1,25 vit-D or vehicle.

	Control +vehicle	Control + 1,25 vit-D	LPS + vehicle	LPS + 1,25 vit-D
n=	6	8	10	10
Thrombocytes (10^9/L)	1056 ± 196	980 ± 260	480 ± 80 [a]	415 ± 63 [a]
PT (seconds)	21,4 ± 3,5	17,9 ± 2,0	29,8 ± 6,1	26,6 ± 3,6 [a]
ALT (units/L)	53 ± 18	78 ± 30	66 ± 8	93 ± 33

Bilirubin (µmol/L)	1,27 ± 0,35	1,27 ± 0,13	2,66 ± 0,65 [a]	3,26 ± 0,60 [a]
Creatinine (µmol/L)	55 ± 4	62 ± 5 [b]	76 ± 6 [a]	103 ± 19 [a,b]
Urea (µmol/L)	6,3 ± 1,1	7,8 ± 0,7	11,8 ± 0,8 [a]	13,3 ± 1,0 [a,b]
Ca^{++}(7,4) (mmol/L)	1,34 ± 0,02	1,42 ± 0,05 [b]	1,24 ± 0,04 [a]	1,28 ± 0,04 [a]

[a] Significant effect of LPS vs. controls. (P<0,05)

[b] Significant effect of 1,25 vit-D vs. vehicle. (P<0,05).

The LPS infusion induced a septic condition in the rats with effect on coagulation and liver function as shown by reduced thrombocyte count and increased bilirubin ($p<0.05$). ALT values did not increase in the short 4 hours observation time, but in this model renal failure occurred as expressed by significantly elevated creatinine and urea values in the LPS treated rats ($p<0.05$). The 1,25-vit D treated rats developed higher values of creatinine and urea than the vehicle treated rats ($p<0.05$) indicating a higher level of renal susceptibility to sepsis after 1,25 vit-D administration. Ionized calcium values were significantly reduced in LPS treated rats ($p<0.05$), and no difference in ionized calcium values was found between 1,25-vit D or vehicle treated rats.

The mortality rate in the present study was low. In study 1, all rats survived. In study 2, Two out of 8 rats died in both LPS + 1,25 vit-D and in LPS + vehicle groups. In study 3, due to the expected mortality, 8 rats were included in both control groups and 10 in both LPS-infused groups. However, no animals died in the observation period in the LPS infused groups, whereas 2 animals died in the control + vehicle group.

4. Discussion

1,25 $(OH)_2$ vitamin D_3 regulates the differentiation from stem cells towards monocytes and macrophages by interacting with specific vitamin D receptors in the myeloid tissue cells [9,10]. The macrophages themselves produce and excrete this active form of vitamin D and, therefore, by both autocrine and paracrin stimulation influence their own macrophage activity in addition to the T- and B-lymphocyte differentiation and activity [11,12,13].

The inflammatory cytokines, TNF-alpha and certain interleukins play a key-role in initiating systemic inflammatory response syndrome (SIRS) and it is known that vitamin D may modulate the cytokine expression from the monocytes and macrophages, even though the action is complex and unclarified [14,15,16]. vitamin D also targets theTNF-alpha pathway to suppress experimental inflammatory bowel disease [3].

Arteriolar and myocardial walls contain specific vitamin D receptors, and vitamin D exerts a direct effect on the vasculature causing an enhanced effect of inotropic drugs [4,17]. The hemodynamic shock response to induction of sepsis may therefore be reduced by administration of 1,25-vit D.

In addition to these effects, Vitamin D may also inhibit the cytokines effect on target cells. vitamin D has an antagonistic effect on TNF-alpha stimulation of monocytes in cultures by down-regulating the surface protein tissue factor (TF) and up-regulating thrombomodulin (TM) expression in monocytic cells [18]. These proteins are well-known activators and controllers of coagulation.

It is therefore theoretically feasible that vitamin D administration may attenuate the course of sepsis induced coagulation disturbances, but only two in vivo studies have, to our knowledge, previously tested this hypothesis (Horiuchi et al (1991) [6] and Asakura et al 2001 [7]). These studies showed beneficial effects of vitamin D in LPS induced sepsis.

Horiuchi [6] administered a single dose of LPS 20 mg/kg and 1,25-vit D 20 ng/kg simultaneously to mice. He found an improvement in survival rate from 0% to 39% after 48 hours and concluded that this effect might be a result of an inhibition of endotoxemia through regulation of thromboxan and hepatic malondialdehyd. Asakura in his work [7] administered 1,25-vit D 2 mg/kg/day or vehicle orally for 3 days prior to a 4 hour infusion of E. Coli LPS in rats and found beneficial effects of the vitamin D metabolite on thrombocyte count, ALT, creatinine, and glomerular fibrin deposition. Asakura concluded that 1,25-vit D was effective in protecting against DIC in experimental LPS-induced shock.

It has been argued that the experimental model of sepsis induced by infusion of LPS in high doses is unphysiological and differs from a clinical scenario [19], whereas other standardized setups such as the cecal ligation and puncture technique [19], to a greater degree mimic the clinical realities in sepsis. We, therefore, decided to duplicate the experimental protocols used by Horiuchi [6] and Asakura [7] but also to add an examination of a model of abdominal sepsis. To probe the possibility that 1,25-vit D could have therapeutic potential within pharmacologically safe doses, a previously validated dose of 1,25-vit D was chosen [8] . In Asakura´s study, 1,25-vit D was given orally but as intestinal vitamin D uptake is unpredictable, we decided to administer the 1,25-vit D by subcutaneous injections. This study is the first of its kind, and therefore our main priority was to establish a potential relationship between vitamin D (pre)treatment and the effects of sepsis. The effect of 1,25-vit D on the immune system and coagulation has never been time-scaled, and may not follow the pattern of calcium regulation. As the steroid hormone Vitamin D works by gene transcription, the effects are delayed, and could not be expected to be effective on an hour-to-hour basis. For these reasons, we chose to administer vitamin D as a pre-treatment to sepsis rather than an intervention..

Apart from the addition of the surgical, abdominal sepsis (which has not previously been studied in relation to vitamin D) the dosage, timing and administration form of 1,25 vit-D constitute the biggest differences between this study and earlier works, and we attribute much of the difference in results to these factors.

All three models of experimental sepsis in our study worked successfully by inducing a significant affection of coagulation and liver paramenters in the rats. The 1,25-vit D treatment caused a minor increase in ionized calcium levels, however this only reached a significant level in study 3 showing an increase from 1.34 to 1.42 mmol/l ($p<0.05$). Among the septic rats, ionized calcium levels fell significantly in all three models and was unaffected by the1,25-vit D treatment. This is a well described pattern both in clinical and experimental sepsis and may be caused both by insufficient secretion and effect of parathyroid hormone in an acidotic environment, and by insufficient calcium pumps in the cellular membranes changing the balance between the intracellular low calcium and the extracellular high calcium values. The renal parameters showed no sign of uremia after CLP, whereas LPS infusion caused an increase of both creatinine and urea surprisingly further increased among the rats that were pretreated with 1,25-vit D. This state of hyperacute sepsis may cause a higher susceptibility to nephrocalcinosis, which is a well described, harmful effect of vitamin D.

In our study, pretreatment with 1,25-vit D reduced the CLP induced thrombocytopenia significantly ($p<0.05$) indicating a protective role for 1,25-vit D against the development of sepsis induced disseminated intravascular coagulation. In contrast to the studies by Horiuchi [6] and Asakura [7] we were unable to demonstrate a protective effect of 1,25-vit D treatment in the LPS induced sepsis.

The pretreatment with 1,25-vit D had no protective effect on the elevation of plasma bilirubin and ALT. It therefore appears that the septic shock developed equally severely in both 1,25-vit D and vehicle treated rats, but that the secondary effect on coagulation in the CLP treated rats was less severe after 1,25-vit D treatment.

5. Conclusion

The beneficial effects of vitamin D on the response to sepsis in the present study only comprises a lesser development of thrombocytopenia in CLP treated rats. A limiting factor in exploring this subject further is the development of hypercalcemia by the use of higher doses of 1,25-vit D.

In conclusion, the present data suggest a slightly positive modulating effect of 1,25-dihydroxy-vitamin D_3 supplementation on sepsis-induced coagulation disturbances in the coecal ligation and puncture model. No such effect was found in LPS-induced sepsis.

Conflict of interest

The authors have declared that no conflict of interest exists.

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