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Original Article

The Novel Anti-Inflammatory Mechanism of Calcitriol in Recurrent Spontaneous Abortion in Experimental Mice

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ABSTRACT

Background: In many instances, Recurrent Spontaneous Abortion (RSA) has unexplained etiology and does not have any prognostic clues. Antioxidant vitamins are closely related to a series of diseases, including RSA. However, the function and underlying mechanism of calcitriol in the treatment of RSA has not been described. **Materials and Methods:** The pregnant mice were injected intraperitoneally with Lipopolysaccharide (LPS) to induce abortion. Calcitriol was used for the treatment and to avoid RSA. Pathological analysis was done to assess the abnormalities during RSA and its treatment using calcitriol. **Results:** After LPS administration, the mice exhibited high embryo absorption and pathological alterations, as well as, presented an increase in inflammation and apoptosis. Moreover, higher levels of NF-κB and inflammatory cytokines (TNF and IL-6) were noticed in serum and in isolated macrophages. Furthermore, we found alterations in the Nrf2 levels, enzymatic antioxidants and non-enzymatic antioxidants in serum. All these abnormalities were attenuated during calcitriol treatment in the appropriate groups of mice. **Conclusion:** Our results indicate that calcitriol may be involved in the modulation of inflammatory signaling and NF-κB pathway-mediated inflammation in RSA. Therefore, calcitriol could serve as a better prognostic regimen in the RSA treatment.

Keywords: Anti-Inflammatory, Macrophages, NF-κB tab, Nrf2, Spontaneous Abortion.

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INTRODUCTION

RSA is the condition of 2 to 3 consecutive miscarriage within 20-28 weeks gestational age in humans.[1,2] RSA is a major issue affecting human population. This has serious effect not only on couple's suffering from RSA but also the whole society. RSA is associated with many other pre-existing maternal pathological conditions.[3] The underlying causes for RSA include chromosomal aberration,[4] hormonal imbalance[1] infections,[5] abnormal maternal immune cell response[6] and immune cell imbalance.[7] Apart from the above factors, unexplained incidence RSA are also reported worldwide.[1] The poly-etiological nature of RSA made the prognosis and treatment a challenging task. This demanded an effective treatment to overcome RSA and bring positive pregnancy outcome.

The mechanism of inflammation mediated RSA with various etiology was explained in various studies.[1,5,6] In order to develop effective therapeutic agent, molecular mechanism behind inflammation mediated process should be studied using animal models. LPS induced RSA animal model was widely used to study the therapeutic effects of various components.[8] Infections caused by gram negative bacteria is the major cause for LPS induced RSA.[5] LPS induced RSA is an inflammation mediated process.[5] Inflammation is regulated by innate immunity and notably vitamin D modulated the innate immune system,[9] specifically in maternal-fetal interface.[10] LPS induced RSA caused oxidative stress accompanied by decreased anti-oxidant levels in various murine models.[8,3]

Vitamin D deficiency was reported in many cases of RSA[9,11] and vitamin supplement had positive effect on pregnancy in many earlier research.[5,6,9] calcitriol, the most active form of vitamin D3, exerted immunomodulatory effect by inhibiting inflammation, thus prevents RSA in women.[12] LPS induced RSA is characterised by embryo absorption[13] Various components were reported to reverse the LPS-induced embryo absorption thus promoting pregnancy progression.[14] Antioxidant imbalance, macrophage polarization and increased inflammatory cytokines were the characteristic features of LPS-induced RSA.[3,5,15,16] Attenuation of these immunological malfunction could be a promising treatment strategy. Elevated inflammatory responses were the characteristic

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feature of LPS induced RSA.[5] LPS caused changes in body weight, tissue damage, embryos loss and elevated inflammatory cytokines IκB Kinase (IKK) α/β, NF-κB, Nrf2, TNF-α, Interleukin-6 were reported earlier.[3,5,9]

Our study investigated the efficacy of calcitriol in the treatment of Recurrent Spontaneous Abortion (RSA) using LPS-induced mice model. Also, our study provided insight on the molecules involved in RSA, induced by LPS and immunomodulatory effect of calcitriol by evaluating the levels of cytokines in the experimental mice model.

MATERIALS AND METHODS

Reagents and kits

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127: B8) 1,25 Vit.D3 (Product No 17936), 1,1,3,3-tetramethoxypropane (product no.108383), hydrochloric acid (product no.1.93001), trichloroacetic acid (Cas. No. 76-03-9), thiobarbituric acid (Cas no. 504-17-6), Tris-HCl buffer (product no 41573), H_2O_2 (Cas No.7722-84-1), ammonium molybdate (Cas no. 12054-85-2) PBS (P. No P2272) and GSH/GSSG Assay Kit (MAK440) were purchased from Sigma Chemical Co, USA. The following kits were procured from Abcam, UK. AST assay kit (ab105135), ALT assay Kit (ab105134), SOD activity assay kit (ab65354), Glutathione Peroxidase Assay Kit (ab102530), Glutathione Peroxidase Assay Kit (ab102530), GST assay kit (ab65326). The following kits were procured from Mybiosource, USA. Nuclear Factor Kappa B (NfκB) ELISA Kit (MBS2023542), Nrf2(Nuclear factor erythroid 2-related factor 2) ELISA Kit (MBS2516218), IKK-alpha/beta ELISA Kit (MBS9501748), Mouse TNF-α ELISA Kit (MBS825075).

Experimental animals and Ethical approval

The experimental mice were purchased and ethical approval was obtained from Medical ethics committee of Shijiazhuang Obstetrics and Gynecology Hospital (Approval number: 20090007-2019). All the animals were maintained and samples collection was done by following the instructions approved by the institutional animal ethical guidelines.

Study design

The mice strain (BALB/c) of 8-9 weeks old (male *n*=20; only for mating); female *n*=40; weight 20-24 g) were maintained 12 hr dark/light cycle, with free access of food and water for a week. After acclimatization, female and male mice were housed together overnight in laboratory condition at a ratio of 2:1. On next day morning, the female with vaginal plug was considered as pregnant and the day was considered as Gestational Day 0 (GD0). Pregnant mice were randomly divided into 4 groups (*n*=10 in each group). Group 1 was normal control mice, fed with olive oil daily and saline. In group 2, the mice were treated with olive oil daily and LPS (150 µg/kg in saline) given intra-peritoneal on GD6. In group 3, the mice were treated with calcitriol (50 g/kg in olive oil; p.o.) orally daily. In group 4, the mice were with both calcitriol and LPS i.p. injection.[5] Animal distress were monitored by the locomotor activities and grooming behaviour changes in the mice group that received LPS.

Serum collection

On GD8 mice blood samples were obtained from abdominal aorta under anaesthesia.[6] The samples were centrifuged at 1500 g at 4°C for 30 min and stored at -80°C. The samples were used for further analysis.

Body weight changes

All the experimental groups were measured for its weight from GD0 to GD8. On GD8 body weight was measured just before sample collection and sacrifice.

Sample collection for examination of pathological alterations

On GD8, after collection of blood the mice were sacrificed by cervical dislocation and the uteri were collected. They were examined for number of implant sites and viable embryos, after careful dissection and visualizing uteri inner-wall under compound microscope. The number of embryo loss and integrate embryo were recorded in all mice groups. Using the following formula, the embryo loss percentage (%) was calculated. Embryo loss % = (number of lost fetuses/number of fetuses)x100, as described earlier.[6] The pathological finds namely necrosis, hemorrage and size change were recorded.[7,13] The rate of embryo resorption was calculated using formula embryo Resorption rate (R)=Reabsorbed embryos/(surviving embryos+resorbed embryos)×100.[7,16]

Tissue damage markers in serum (AST and ALT)

The assessment of tissue damage was done by quantifying tissue damage markers AST and ALT. The quantification of AST was done AST assay kit (ab105135, Abcam, UK). The quantification of ALT was done by ALT assay Kit (ab105134, Abcam, UK). Serum samples were processed as per instruction given in the kit manual. The AST assay protocol was based on conversion of colourless probe to a coloured product by glutamate produced as a result of AST activity. The product was read at OD 450 nm. In the ALT assay protocol, pyruvate produced by ALT converted the colourless probe to produce colour which was read at 570 nm. Their concentrations were calculated by comparing with standard curve obtained for each enzyme substrate and using formula provided in manufacturer's instruction. The concentration of enzymes was represented as U/L.

Quantification of oxidative stress marker

The Thiobarbituric Acid (TBA) reaction method was used to quantify MDA level. This was based on reaction between MDA

and TBA in acetic acid at high temperature. The MDA-TBA adduct formed was measured at 530-540 nm. The standard MDA was prepared by measuring MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. To 250 µL serum, 500 µL working solution (15% trichloroacetic acid, 0.375% Thiobarbituric acid and 0.25 N hydrochloric acid) were mixed and kept in boiling water for 10 min. These samples were cooled and centrifuged at 3000 rpm for 10 min. The supernatant was separated and OD measured at 535nm. The concentration of MDA was expressed as µmol/L.[17]

The Glutathione GSH/GSSG Assay Kit (MAK440, Sigma-Aldrich, USA) was used quantify GSSG and GSH. The samples were prepared as per kit instruction. In this method Ellman's Reagent (DTNB) and Glutathione Reductase (GR) were used. DTNB reacted with reduced glutathione to form a yellow product. The rate of change in the optical density at 0 min and 10 min, were measured at 412 nm and this is directly proportional to the glutathione concentration in the sample. The oxidized Glutathione (GSSG) was quantified using a specific protocol which first scavenges all existing GSH using 1-methyl-2-vinylpyridinium triflate as a scavenging reagent. The amount of GSH and GSSG were calculated as per formula given in the kit manual.

Quantification of enzymatic antioxidants

The SOD activity was quantified using Superoxide Dismutase activity assay kit (ab65354, Abcam, UK). The assay was based on decrease in formation of reduced tetrazolium salt (a water-soluble formazan dye) caused by SOD. The dismutation of superoxide anion catalysed by SOD results in decrease of tetrazolium salt reduction. This inhibition activity of SOD was measured by spectrophotometrically at OD 450 nm. The SOD activity was calculated as per kit manual and expressed as U/mg protein.[18]

The Catalase (CAT) catalyses the decomposition of H_2O_2. The catalase activity was quantified based on the principle of formation of ammonium molybdate and residual H_2O_2. To 10 µL of serum sample 100 μmol/mL of H_2O_2 in 0.05 mmol/L Tris-HCl buffer pH=7 was added and incubated for 10 min. The reaction was terminated by rapidly adding 50 µL of 4% ammonium molybdate. The absorbance of this yellow was read at 410 nm. The standard H_2O_2 were prepared along with sample and control. The activity of CAT was calculated and expressed in µmol H_2O_2 decomposed/min/mg protein.[18]

The glutathione peroxidase assay was performed using GPx assay Kit (ab102530, Abcam, UK). This is based on the quantification of level of decreased NADPH. Oxidation of GSH to GSSG is catalysed by GPx, cumene hyperperoxide is the reducing agent in this reaction. NADPH is consumed by Glutathione reductase to reduce GSSG to GSH. The decrease of NADPH (measured at OD=340 nm) is proportional to GPx activity. The calculations were done as per formula given in kit manual and GPx concentration expressed as µmol GSH oxidized/min/mg protein.

The quantification of GST was done using GST assay kit (ab65326, Abcam, UK). In this reaction, GST catalyses formation of GS-DNB conjugate, dinitrothioester. The absorbance was read at 340 nm. One unit of GST activity is defined as the amount of enzyme producing 1 µmol of GS-DNB conjugate/min under the conditions of the assay. 50 µL of reaction mix (CDNB (1-chloro-2,4-dinitrobenzene) and buffer), added to sample (5-50 µL). The absorbance at OD 340 nm was measured at an interval of 2-3 min, 5 readings were recorded. The activity of GST was calculated using formula given in the instruction manual and expressed as nmol CDNB conjugated/min/mg protein.

Quantification of NF-κB, Nrf2, TNF-α, IL-6 in serum

The levels of NfκB in serum were determined by using mouse Nuclear Factor Kappa B (NF-κB) ELISA Kit (MBS2023542, Mybiosource, USA). The assay was based on formation of coloured complex due to NfκB, biotin-conjugated antibody and enzyme-conjugated Avidin reaction. The samples were prepared using instructions given in the kit. The samples/standards were added to ELISA reader plate pre-coated with an antibody specific to NF-κB. Conjugated horseradish peroxidase was added to the wells and incubated. TMB substrate solution was added, coloured complex was read spectrophotometrically at a wavelength of 450 nm. The volume of sample, reaction condition and assay protocol were followed as per instructions given in the manual. The concentration of NF-κB in the samples were determined by comparing the O.D. of the samples to the standard curve. The concentration of NF-κB was expressed as ng/mL.

The levels of Nrf2 in serum were determined using mouse Nrf2 (Nuclear factor erythroid 2-related factor 2) ELISA Kit (MBS2516218, Mybiosource, USA). The assay was based on Nrf2 antibody-Nrf2 antigen interactions and an HRP colorimetric detection system to detect Nrf2 antigen targets in samples. The assay was performed as per instruction given instruction manual. The concentration of Nrf2 in the sample was calculated by comparing the absorbance of the samples to the standard curve and expressed as ng/mL.

The quantification of TNF-α was done using mouse TNF-α ELISA Kit (MBS825075, Mybiosource, USA). The standards and samples were added to plate coated with antibody specific for Mouse TNF-α and incubated. After washing, biotinylated anti-Mouse TNF-α antibody was added. The plates were incubated and washed. HRP-conjugated streptavidin was added to the plates, incubated and washed. TMB substrate was added to the plates, colour developed was proportional to concentration of bound TNF-α. Stop solution was added and intensity of the colour was measured at 450 nm. A similar procedure was followed for the quantification of IL6 in the samples using kit (MBS2023471, Mybiosource, USA). The concentration of TNF-α and IL6 were determined by comparing the standard curve and

the concentration was calculated as per instructions given in the manual.

Isolation of macrophages

The macrophages were isolated from mouse peritoneal cavity. The mice were euthanized and incision was made inner lining skin was exposed. 5 mL of ice-cold PBS (with 3% FCS) was injected and massaged gently to dislodge attached cells into PBS. 25 g needle was used to collect the cells and the same was repeated. Then, incision was made in the inner skin of peritoneum to collect remaining fluid. Samples were discarded if contaminated with blood cells. The cell suspensions were centrifuged at 1500 rpm for 8 min. The supernatant was discarded and cell pellet was resuspended in PBS and stored 4°C until usage.[19]

Quantification of NF-κB, TNF-α and IL-6 in macrophage

The macrophage samples were prepared as per kit manual. The quantification of NF-κB, TNF-α and IL-6 in macrophage was done by using mouse Nuclear factor kappa B (NF-κB) ELISA Kit (MBS2023542, Mybiosource, USA), mouse TNF-α ELISA Kit (MBS825075, Mybiosource, USA) and IL6 ELISA Kit (MBS2023471, Mybiosource, USA). The assay procedure was same as that of serum sample.

Statistical analysis

The experimental data were expressed as mean±SD. The significant difference of parameters among the study groups were analyzed by one-way ANOVA using the SPSS 19.0 software package. A value of atleast $p \leq 0.05$ was considered statistically significant.

RESULTS

Effect of calcitriol on body weight

In our study there was gradual increase in body weight of all groups of mice. Table 1 shows the weight of mice in all the 4 groups. There was consistent weight increase in both control and group 3 mice. In group 2 mice there was decrease in body weight on GD8 compared to all other groups. In group 4 animals the weight gain was more than group 2 mice. The average weight on GD8 in group 2 was less compared to group 1,3 and group 4. The percentage body weight changes were notable the groups and were 8.48%, -4.04%, 10.22% and 3.13% respectively. The body weight change in group 2 vs group 4 was statistically significant. ($p \leq 0.05$)

Effect of calcitriol on embryo resorption

Table 1 shows the rate of embryo resorption in different groups of mice. The embryoresorption rate was more in group 2 compared to all other groups. The group 4 showed more embryo survival compared to group 2. The group 1 and 3 mice showed normal embryonic development. In LPS model, group 2 the implantation sites were small and even distribution was seen. There were necrotic lesions, prominent bruising of uterine cavity. The group 4 mice showed beaded structure and colour of uterus was similar to group 1 and 3. The bruising was less in group 4 compared to group 2. The embryo resorption rate was more in group 2 compared to all other groups.

Effect of calcitriol tissue damage markers AST and ALT

The levels of tissue damage markers AST and ALT (Table 2) were increased, almost doubled in group 2 experimental mice. The group 1 and group 3 showed low level of AST and ALT. The AST and ALT levels were decreased in group 4 compared to group 2. The difference in levels of these markers group 4 and group 1,3 was statistically insignificant. There was drastic difference in enzyme level in group 2 and group 4 and it was statistically significant. ($p \leq 0.05$).

Effect of calcitriolon oxidative stress markers

Oxidative stress markers were quantified in all experimental groups (Table 3). Group1,3 showed basal level of MDA and GSH; and group 4 showed a slightly higher level. There was striking increase of MDA and decrease of GSH in group 2 mice. The level of GSSG was at basal level in group 1 and 3. The level of GSSG was very low in group 4 compared to other groups and difference was statistically significant. ($p \leq 0.05$).

Effect of calcitriol on enzymatic antioxidants

Enzymatic antioxidants SOD, CAT, GPx, GST (Table 4) were at basal levels in group 1 and group 3. In group 2 mice these levels were very low. In group 4 the levels of these enzymatic antioxidants

Table 1: Effect of calcitriol on body weight and embryo resorption.

Parameters	Normal Control	LPS	Calcitriol	Calcitriol+LPS
Initial body weight (g)	22.4±1.26	22.3±1.25	22.5±1.27	22.4±1.17
Final body weight (g)	24.3±1.34	21.4±1.43$^{\#}$	24.8±1.32	23.1±1.66*
Change in body weight %	8.48%	-4.04%	10.22%	3.13%
Embryo resorption rate %	10.05±0.83	27.17±5.07$^{\#}$	10.56±0.79	15.32±6.53$^{\#}$

Data were expressed as mean±SD. One-way ANOVA by SPSS software was done to compare LPS with NC group and Cal+LPS with LPS groups. Level of statistical significance: * $p \leq 0.05$, $^{@}p \leq 0.01$ and $^{\#}p \leq 0.001$.

were restored. The changes in the levels of investigated enzymatic antioxidants were statistically significant ($p \leq 0.05$).

Effect of calcitriol on serum NF-κB, Nrf2, TNF-α and IL-6

The levels of NF-κB, Nrf2, TNF-α and IL-6 were shown in Figure 1. The levels of NF-κB, Nrf2, TNF-α and IL-6 were at basal level in group 1. The group 2 exhibited high levels of NF-κB, TNF-α and IL-6. The NF-κB, TNFα and IL-6 in group 4 mice were found to be decreased. The levels of Nrf2 were at basal level in group1. Nrf2 was almost nil in group 2 mice. The group 3 mice showed increased Nrf2 levels but less than group 4. The Nrf2 level in group 4 mice was very high compared to other group of mice. The difference in levels of NF-κB, Nrf2, TNF-α and IL-6 were statistically significant. ($p \leq 0.05$).

Effect of calcitriol on macrophage NF-κB, TNF-α and IL-6

In Figure 2, the levels of macrophage NF-κB was at basal level in group 1 and 3. The levels of macrophage NF-κB was very high in group 2. The group 4 macrophage NF-κB was lower compared to group 2. TNF-α and IL6 levels were same in group 1, 3 and increased in group 2. There was a notable decrease in TNF-α and IL-6 in group 4 macrophage, compared to group 2. The difference in the levels of NF-κB, TNF-α and IL-6 were statistically significant. ($p \leq 0.05$).

DISCUSSION

Our study demonstrated the efficacy of calcitriol on protection of LPS induced recurrent spontaneous abortion. The mechanism of LPS mediated inflammation by the cytokines and antioxidant system was elaborated.[5] LPS model for RSA successfully induced oxidative stress which created redox imbalance. This caused cascade of inflammatory events programmed by macrophage produced cytokines.[4,5,16] This resulted in body weight changes, embryo loss by resorption and uterine morphology change. calcitriol (dosage) administration had restored the redox balance by decreased levels of pro-inflammatory cytokine by altering macrophage polarisation. The alteration of immune cell balance restored uterine morphology, number of implantation sites, thus

Table 2: Effect of calcitriol tissue damage markers.

Parameters	Normal Control	LPS	Calcitriol	Calcitriol+LPS
AST (U/L)	166.2±9.26	227.8±23.79$^{\#}$	153.6±17.98	203.2±19.42*
ALT (U/L)	92.1±8.03	171.6±12.89$^{\#}$	85.6±9.19	141.4±10.73$^{\#}$

Data were expressed as mean±SD. One-way ANOVA by SPSS software was done to compare LPS with NC group and Cal+LPS with LPS groups. Level of statistical significance: $^{*}p \leq 0.05$, $^{@}p \leq 0.01$ and $^{\#}p \leq 0.001$.

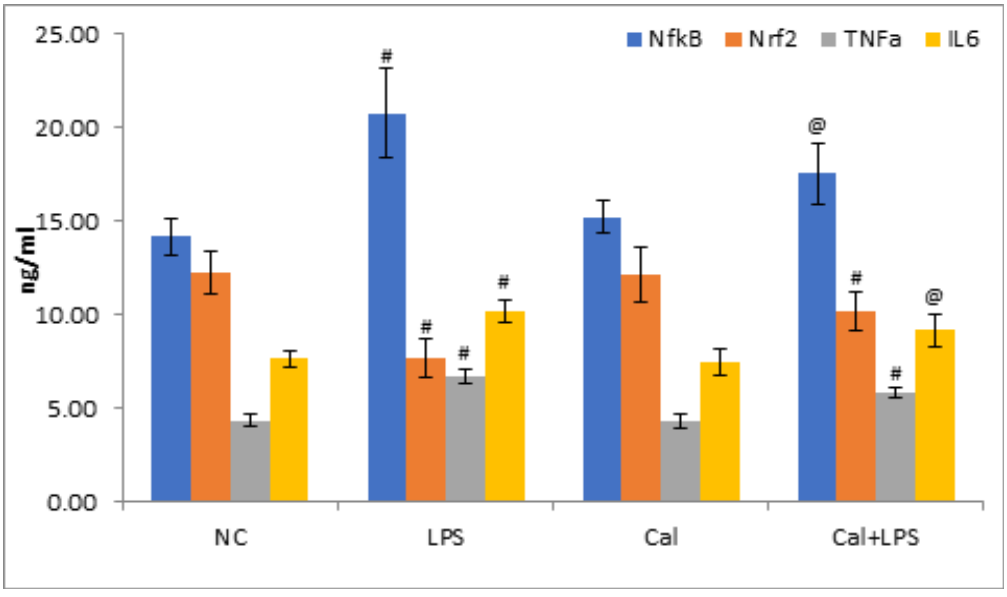

Figure 1: Effect of calcitriol on serum NF-κB, Nrf2, TNF-α and IL-6.

Data were expressed as mean±SD. One-way ANOVA by SPSS software was done to compare LPS with NC group and Cal+LPS with LPS groups. Level of statistical significance: $^{*}p \leq 0.05$, $^{@}p \leq 0.01$ and $^{\#}p \leq 0.001$.

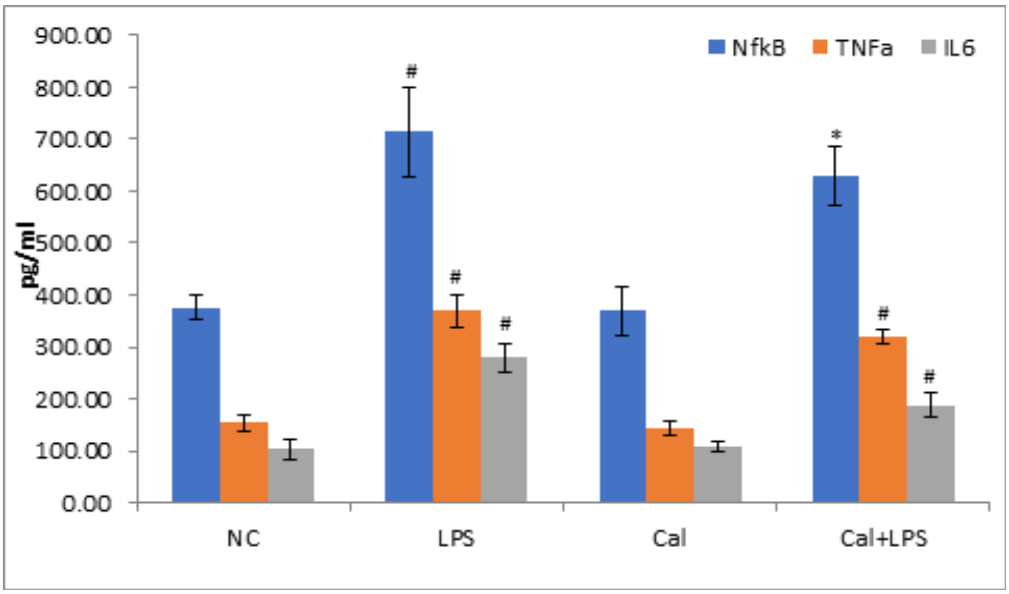

Figure 2: Effect of calcitriol on macrophage NF-κB, TNF-α and IL-6.

Data were expressed as mean±SD. One-way ANOVA by SPSS software was done to compare LPS with NC group and Cal+LPS with LPS groups. Level of statistical significance: *$p \leq 0.05$, @$p \leq 0.01$ and #$p \leq 0.001$.

prevented embryo loss. This resulted in amelioration of RSA induced by LPS.[3,5]

The success of pregnancy dependents on intake of balanced nutrient, hormonal balance, environmental condition, age, physiological conditions and other pathological condition.[2] Nutrient supplementation, treatment for hormonal imbalance and other pathological condition are possible strategies for successful pregnancy outcome.[3] Miscarriage reduces the success of pregnancy rates worldwide. The RSA is challenging risk factor in pregnancy because of its unexplained and polyetiological nature.[2,13] The immune factor imbalance especially at maternal-fetal interface is one of the major mechanisms involved in most of the pathological conditions related to RSA.[6,7] Treatment with effective, readily available, low-cost compound with immunomodulatory property is appropriate for successful pregnancy.[1,6,10] Deficiency of vitamin D_3 was reported in many cases of unexplained RSA and supplement of same improved embryo survival.[1,5,9] Vitamin D_3 mediated immunomodulatory in unexplained RSA. (10) Moreover, ratio of serumcalcitriol to 25-vitamin D_3 is indicatory of pregnancy status.[12] At concentration of 10^{-4} mmol/L calcitriol reduced risk of RSA by ameliorating the inflammatory signalling pathway in placental cell culture.[20] Based on these statements we conducted studies for effective treatment of RSA with calcitriol in LPS mice model.

Calcitriol improved the body weight

The body weight changes are indicators of health status of an organism.[16] The gradual weight increase in control group (group 1) showed the mice were in healthy pregnancy and physiological status. The LPS treatment induced body weight changes had been reported in many mice model studies and plant flavonoids has been reported for its ability to improve body weight and thus promoting embryo survival.[16] The body weight in group 4 was less compared group 1,3 but more than the weight in group 2, this showed the efficacy of calcitriol (dosage) in prevention of RSA.

Calcitriol decreased embryo resorption and restored uterine morphology

The increase in embryo absorption/resorption is characteristic feature of LPS induced RSA.[2,5,8] This is accompanied by abnormal uterine change and decrease in implantation site size. The embryo loss rate was also a cause of RSA.[20] Embryo atrophy and necrosis were seen in implantation sites in case of LPS induced RSA in mice.[14] The unsuccessful pregnancy was caused due to series of inflammatory events involving pro-inflammatory cytokines.[21] The group 2 mice exhibited the characteristic features of uterine pathology of LPS induced RSA. The restoration of uterine morphology, reduction of embryo absorption, improving implantation size and thus mitigation of LPS or other factors induced damage by vitamin D_3, aspirin,[5,6] sinomenine,[7] quercetin,[16] SR9009[8] and various other gene expression upregulation and downregulation.[4,14,15,20] These alterations by LPS treatment were also seen in pregnant rat.[22] Calcitriol supplementation in our study had maintained pregnancy and also restored the embryo damage caused by LPS in our study.

Calcitriol restored tissue damage

AST and ATL are first line tissue damage markers in almost all organisms. The increased levels of AST and ALT were reported in LPS model mice.[23] Reversal of tissue damage was evident from restoration of serum AST and ATL. The restoration of

tissue integrity by inhibiting inflammatory pathways is the major mechanism of action by various components and this treated the pathological conditions.[24] The obvious increase in serum AST and ATL in group 2 is evident for tissue damage induced by LPS. Dosage of calcitriol used in this study was nontoxic. The restoration of tissue damage caused by LPS was ameliorated by calcitriol (dosage) was supported by decrease in AST and ALT in group 4 mice. This is also supported by uterine pathology findings in our study.

Calcitriol restored redox balance

LPS-induced redox imbalance is the cause for the cascade of events leading to RSA. Oxidative stress activated lipid peroxidation is measured by MDA quantification. Increased lipid peroxidation and redox imbalance was reported earlier.[24] MDA was increased in LPS model (group 2) suggesting activated lipid peroxidation. The oxidative stress caused decreased GSH in various inflammatory conditions. The decreased GSH and increased GSSG in group 2 mice indicated the oxidative stress induction. The safeness of dosage used in our study was confirmed by the level of MDA in group 3. Reduced levels of MDA protected the uterus from inflammation resulted in successful pregnancy outcome in group 4 mice. The mitigation of oxidative stress by decreasing the extent of lipid peroxidation was reported earlier.[22,25] Decreased GSH indicates the risk of miscarriage in human subjects.[26] Pretreatment with calcitriol (dosage) had successfully combated the oxidative stress induced by LPS-mice model in our study. The level of GSSG was significantly reduced in our study. The oxidized glutathione plays important role in maintenance of redox balance in cellular system. The alterations in GSH and GSSG indicated the interruption of glutathione redox system by LPS. The increased GSSG levels, induced by LPS, was successfully mitigated by calcitriol in our study.

Calcitriol restored enzymatic antioxidants

The enzymatic antioxidants SOD plays a pivotal role in scavenging oxygen free radical. Catalase plays inevitable role in scavenging hydrogen peroxide. GPx catalyses the reduction of peroxide radicals. The potential of SOD in treatment of various pathological condition was exploited in various studies.[25] Decreased SOD, CAT and GPx levels were reported in case of pregnancy complications.[22,25] Restoration of uterine status suitable for embryo survival, accompanied by increased SOD and CAT on zinc nanoparticle was reported.[22] Vitamin D3 increased antioxidant capacity by increasing expression of GPx, CAT and SOD.[27] GPx plays important role in keeping ROS at very low level in uterus tissue. Expression of GPX gene is not only required in early stages but also in progression. GPx decreased embryo resorption and GPx gene knockout mice had pregnancy complications.[28] GST catalyses the conjugation of toxic molecules to GSH and detoxification is proceeded further by antioxidant system.[29] LPS treatment caused remarkable decline in GST[30,31] and recombinant GST inhibited inflammatory response in LPS treated human cell line RAW246.7.[30] Exposure to various metals induced increased GST, there by created pregnancy issues.[31] GST genetic polymorphism had various effects on pregnancy.[32] Increased GST in stages of early pregnancy plays vital role in implantation in association with progesterone.[33] In our study the defects of implantation site might be due to decreased GST in LPS treated mice. Restoration of LPS induced implantation site in calcitriol treated mice is the evidence of mitigation of ROS induced inflammation.

Table 3: Effect of calcitriol on oxidative stress markers.

Parameters	Normal Control	LPS	Calcitriol	Calcitriol+LPS
MDA (nmol/L)	173.4±11.69	299.7±20.31$^{\#}$	157.9±13.67	210.5±22.11$^{\#}$
GSH (mg/dl)	93.6±10.76	72.5±5.46$^{\#}$	90.3±9.24	83.3±10.84*
GSSG (mg/dl)	28.8±2.9	39.5±6.36$^{\#}$	23.9±5.22	31.1±4.43$^{@}$

Data were expressed as mean±SD. One-way ANOVA by SPSS software was done to compare LPS with NC group and Cal+LPS with LPS groups. Level of statistical significance: $^{\star}p\leq0.05$, $^{@}p\leq0.01$ and $^{\#}p\leq0.001$.

Table 4: Effect of calcitriol on enzymatic antioxidants.

Parameters	Normal Control	LPS	Calcitriol	Calcitriol+LPS
SOD (U/mg protein)	52.5±7.53	40.5±5.06$^{\#}$	52.4±5.48	46.1±4.91*
CAT (µmol H_2O_2 decomposed/min/mg protein)	223.5±19.57	140.8±15.96$^{\#}$	214.4±23.6	188.1±12.03$^{\#}$
GST (nmol CDNB conjugated/min/mg protein)	104.4±14.93	77.3±10.76$^{\#}$	103.9±9.06	94.5±10.32$^{@}$
GPx (µmol GSH oxidized/min/mg protein)	1.29±0.27	0.79±0.14$^{\#}$	1.19±0.21	1.12±0.18$^{\#}$

Data were expressed as mean±SD. One-way ANOVA by SPSS software was done to compare LPS with NC group and Cal+LPS with LPS groups. Level of statistical significance: $^{\star}p\leq0.05$, $^{@}p\leq0.01$ and $^{\#}p\leq0.001$.

Calcitriol exhibited cytokine modulatory role

Inflammation is the major factor involved in RSA.[2,4] The inflammatory cytokines conduct series of events leading to embryo loss in RSA induced by LPS.[5,16,25] Vitamin D_3 mitigated LPS induced RSA by restoring redox balance and cytokine levels, thereby creating micro environment suitable for embryo survival.[5,6,9,10,27] NF-κB plays a key role in controlling fetal membrane alterations, myometric contractions and progression of maternal inflammation in pregnancy disorders. Phosphorylation of NF-κB is needs Ikk α/β kinases, phosphorylated NF-κB p50 gets translocated into the nucleus and induces activation of pro-inflammatory cytokines.[34] $1,25(OH)_2D3$ inhibited NfκB p65 translocation from cytoplasm to nucleus in human JEG-3 cell lines and this is dependent placental Vitamin D Receptor (VDR) signalling. VitD3 (25μg/kg) attenuated LPS induced inflammation by inhibiting NF-κB translocation.[35] Increased NF-κB by LPS and decrease of the same by calcitriol (dosage) was observed in our study. This suggests the translocation inhibition of NF-κB may be possible mechanism behind action of calcitriol.

LPS treatment in pregnant mice increased serum TNF-α levels within 2 hr.[5] Decrease in TNFα expression inhibited inflammation caused by LPS in RAW246.7 cell line.[30] Increased TNF-α in LPS mice model was reported, it was supported by altered uterine morphology. The increased TNF-α in LPS model supported by uterine morphology in our study indicated inflammatory mediated abortion. Supplement of Vitamin D_3 lowered TNFα and IL-6 in patients with RSA complication.[10] Pretreatment with calcitriol decreased TNF-α, thus improved embryo survival. Down-regulation of the level of proinflammatory factors such as IL-6 and TNF-α by calcitriol *in vivo.*[20] Previous studies had reported Vitamin D_3 inhibited nuclear translocation TNFα-mediated nuclear translocation of NF-κB in HEK293 cells.[36] Our study results propose inhibition of translocation of NF-κB by decreasing TNF-α by calcitriol. IκB Kinase (IKK α/β) activates NfκB by phosphorylating IκBα protein, which facilities nuclear translocation of NF-κB p65/50.[34] LPS increased IκB kinase activity in mice model, suggesting the increase in NF-κB translocation.[37]

IL-6 is a proinflammatory and anti-inflammatory cytokine secreted by Th2 cells and M1 macrophage. In case of unexplained RSA IL-6 was reported to be higher and the effect of vitamin D_3 was unclear.[10] Elevated level of IL-6 in LPS treated human monocyte cell line was reported.[8] Calcitriol reduced the levels of IL-6 in gestational diabetes, thus combated the inflammation.[20] Increased IL-6 in LPS model in our study is supported by earlier studies. Calcitriol had a positive effect on embryo survival by inhibiting pro-inflammatory effect of IL-6. This created a favourable immune-modulation for embryo survival and progression.

Nrf2 regulated redox system by increasing GSH synthesis by regulating glutathione reductase enzyme. Nrf2 caused increased GSH production which impaired inflammatory conditions.[38] The Nrf2 regulation by NF-κB was the mechanism behind glutathione homeostasis restoration in our study. Alterations in GSH, GSSG and glutathione reductase supported these findings. Nrf2 plays vital role in anti-inflammatory signalling by regulating antigen-response element. The M1 induced genes are down-regulated by Nrf2, IL-6 is one of those genes. 1,25-Dihydroxyvitamin D upregulated Nrf2-antioxidant signalling in mice.[39] Gene knockout experiments revealed the presence increased IL-6 and TNF-α, supporting the anti-inflammatory role of Nrf2 in mice models.[40] LPS induced RSA was evident by decreased Nrf2 levels. Calcitriol pretreatment in LPS model increased Nrf2, suggesting activation of anti-inflammatory response. The basal level of Nrf2 in control and calcitriol only mice suggested healthy physiological status throughout the study.

Calcitriol restored macrophage polarization

Macrophages play a vital role in pregnancy maintenance, as its polarization to M1 or M2 cascades series of inflammatory or anti-inflammatory events.[41] LPS mediates production of ROS and alteration in host macrophage polarisation as result of host immune response.[21] Oxidative stress and macrophage polarisation are associated strongly. Macrophage polarization toward M1 was found to be associated with RSA.[15] Increased TNF-α is associated with M1 population, proinflammatory phenotype macrophage.[8] IL-6 plays a key role in macrophage polarisation.[15] Exposure to LPS caused macrophage polarisation to M1, TNFα and IL6 released by these macrophages initiated inflammatory response resulted in embryo loss.[42] Increased macrophage NfκB and IκB kinase in LPS model suggested the M1 polarised inflammatory signalling. Nrf2 suppressed M1 polarisation, thus protected against inflammatory cytokine. Nrf2 also suppressed NF-κB signalling.[43] Our study result suggested the macrophage polarization alteration by calcitriol. This was evident from increased Nrf2, decreased TNF-α and IL-6 in calcitriol pretreated animals. On treatment with calcitriol, LPS induced RSA was reversed, which was evident from the improvement in decreased in tissue damage marker levels, decreased embryo resorption, balanced antioxidant enzyme levels and cytokine homeostasis.

CONCLUSION

Our study is the first report to prove the efficacy of calcitriol (dosage) in mitigating LPS induced RSA mice model. Calcitriol ameliorated the LPS induced oxidative stress and this was evident from the restoration of non-enzymatic and enzymatic antioxidants under investigation. The tissue integrity was restored by calcitriol, this was supported by restoration of tissue damage markers, uterine morphology and decreased embryo absorption. The immunomodulatory effect of calcitriol was proved by altered

levels of Nrf2, NfκB, IL-6 and TNFα in serum and macrophage. The inhibition of M1 polarization by calcitriol demonstrated the protective role of the same in RSA. Study with differential dosage of calcitriol is suggested. Investigation of surface markers of M1/M2 macrophage may give clear picture on macrophage regulation by calcitriol.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

RSA: Recurrent spontaneous abortion; **LPS:** Lipopolysaccharide; **NF-κB:** Nuclear factor kappa B**; Nrf2:** Nuclear factor erythroid 2-related factor 2; **GD:** Gestation day; **i.p.:** Intra-peritoneal; **p.o.:** per orally; **ALT:** Alanine transaminase; **AST:** Aspartate transaminase; **TBA:** Thiobarbituric acid; **MDA:** Malondialdehyde; **GSH:** Reduced glutathione; **GSSG:** Oxidized glutathione; **GR:** Glutathione reductase; **SOD:** Superoxide dismutase**; CAT:** Catalase; **GPx:** Glutathione peroxide; **NADPH:** Reduced nicotinamide adenine dinucleotide phosphate; **ELISA:** Enzyme-linked immunosorbant assay; **TNF-α:** Tumor necrosis factor-α; **IL-6:** Interleukin-6; **IFN:** Interferon.

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