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Impact of Vitamin D Supplement on Reproductive Performance in Pre-mating Ewes: Presumptive Regulatory Role of Some Inflammatory Cytokines and Serum Biomarkers

Gamal El-Amrawi[1] and Dina Gad El-Karim[2*]

[1] *Department of Theriogenology, Faculty of Veterinary Medicine, Alexandria University, Egypt.*

[2]*Department of Pathology and Clinical Pathology, Faculty of Veterinary Medicine, Alexandria University, Egypt.*

SHEEP is substantial source of meat in many countries all over the world. But early embryonic death is a great problem negatively affects sheep production. This study was designed to investigate the effect of vitamin D_3 administration before mating on reproductive performance of Rahmani ewes, referring to its impact on some biomarkers related to uterine receptivity during implantation period. Injection of single dose of vitamin D (300,000 IU/ ewe), resulted in less significant increment in serum pro-inflammatory cytokines (TNF-α and IL-6) and higher significant elevation in serum level of anti-inflammatory cytokines (IL-4 and IL-10), insulin like growth factor-1 (IGF-1), sterodogenic regulatory protein (StAR), macrophage inflammatory protein-1β (MIP-1β) and progesterone as compared to the ewes which did not receive vitamin D on 20th day after injection. Also, the treated group recorded an increased number of pregnant ewes upon pregnancy diagnosis on 40th day post-service and increased number of twins feti at delivery. On conclusion, vitamin D administration to ewes before mating has enhanced the reproductive performance of them through induction of some biochemical changes which would improve fertility and uterine embryos receptivity.

Keywords: Ewes, Vitamin D, Reproduction, Cytokines, Implantation.

Introduction

Meat is an essential source of protein in the diet for many peoples, especially in developed countries [1]. By the year 2050, the world demand for animal protein will be increased by about 60%, and this challenge will be overcome through increasing meats production from poultry, pigs, bovine and sheep (Food and Agriculture Organization of the United Nations) [2], so sheep meat market will be recovered [3]. One of the critical problems facing sheep productivity is embryos mortality during elongation period prior to attachment to endometrium [4]. During this critical period, up-regulation of maternal immune system and rejection of embryos can be considered as one of the main causes for early embryonic death and embryonic loss[5], where implantation in ewes starts between days 14th and 16th and ends approximately on day 20th after fertilization [6]. Vitamin D may act as the main regulator of calcium and phosphorus homeostasis by enhancing calcium and phosphate absorption in the gut and regulating their deposition in bones [7]. However, the most active metabolite of vitamin D (1,25-dihydroxyvitamin D (1,25$(OH)_2D_3$) exerts

*Corresponding author: Dina Gad El-Karim E-mail: dina.shabaan@alexu.edu.eg,Tel.:000201117476977
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a wide range of functions other than calcium and phosphorus homeostasis [8]. Correlation between low level of vitamin D and occurrence of many diseases as diabetes, autoimmune and thyroid diseases was proved [9]. The importance of vitamin D as an endocrine regulator for reproduction process has been cleared since 1990s [10,11]. Vitamin D was proved to affect folliculogenesis, estrogen synthesis [12,13], endometrial health [14] and fetal development [15].Moreover, vitamin D receptors (VDR) are present on ovary, uterus and placenta of human and animals [16,17], in addition to its presence on different cells of male reproductive tract as germ cells and spermatozoa [18]. Also, the action of $1,25(OH)_2D_3$ as an immune system regulator is important for successful implantation and pregnancy development as it may facilitate shifting of uterine immunity from cell-mediated to humoral immunity [19,20], while proper early implantation needs an adjusted level of pro-inflammatory cytokines and chemokines [21,22]. Many reports have demonstrated the positive correlation between hydroxyl vitamin D level and successful in-vitro fertilization (IVF) [23,24]. On contrary, low serum level of vitamin D is thought to be linked with preeclampsia which may illuminate its role in maintenance of successful pregnancy [25], as it could mitigate oxidative stress, which is implicated progressively during preeclampsia course [26]. In this context, the impact of vitamin D on reproduction enhancement in ewe has never been reported before, so our study aimed to explore the effect of vitamin D_3supplementation on reproductive performance efficiency and pregnancy in Rahmani ewes and highlighting its role during implantation period.

Material and Methods

This study was carried out during June-November, 2022 in a private farm located in Cairo-Alexandria desert road. The study procedures have been approved ethically by Institutional Animal Care and Use Committee (IACUC), Alexandria University (178/2022).

Experimental animals

Thirty Rahmani ewes, aged between 1-2 years, weight about 45-50 kg were selected randomly to accomplish this study. All the animals were fed on balanced diet composed of 60% concentrates (in form of 30% wheat bran, 35% corn, 15% cotton seed meal, 15% sunflower meal, 3% molasses, 1.5% limestone powder and 0.5% common salt), in addition to 40% roughage in form of clover hay or rice straw. Water and mineral blocks were provided *ad-libtum.* According to farm records, vaccination and de-worming programs were applied routinely. The animals were kept under normal conditions of temperature and photoperiod.

Experimental design

To induce and synchronize estrus in non-breeding season (June), controlled release vaginal sponges were inserted in vagina of the ewes, each sponge impregnated with 20 mg flugestone acetate (Chronogest® CR, Intervet, Holland), and sponges were maintained in vagina for 14 days. 600 IU of equine chorionic gonadotropin (eCG) (Folligon®, Intervet, Holland) was injected intramuscularly to each ewe after sponge removal and the animals were divided equally into two groups. Group-I: kept without any treatment after sponge removal, Group-II: injected with single dose of vitamin D_3 (300,000 IU/ewe) (Decapreno®, Minapharm, Egypt) intramuscularly in the day of vaginal sponge removal [27].

Natural mating

Three apparently healthy rams of the same breed (average 50 kg body weight, three years old) (1 ram/10 ewes in separate yard) were selected for natural mating with the ewes. To make sure of their semen quality, semen samples were collected from rams using electric ejaculator one week before gathering with females and checked for sperm concentration using hemocytometer [28], mass motility using hot stage microscope, and percent of abnormalities [29], in addition to life/dead (viability) percent using nigrosin–eosin stain [30]. The rams were left with the ewes for 5 days starting from day of sponge removal. Mating harness was fixed to each ram to make sure of mating achievement with all of the females in each group.

Blood sampling and analysis

Blood samples were drawn from jugular vein of all ewes on the first day after sponge removal (before vitamin D administration) and again on 20^{th} day after vitamin D injection. Blood samples were left to coagulate, and serum aliquots were separated through centrifugation (3000 rpm for 15 minutes) and kept at -20°C for further analysis. Serum level of the evaluated parameters were detected using highly specific ELISA kits including tumor necrosis factor-alpha (TNF-α) (Cusabio®, China, CSB-E13853Sh), interleukin-6 (IL-6) (Mybiosource®, USA, MBS738671), interleukin-4 (IL-4) (Abcam®, USA, ab273254) and interleukin-10 (IL-10) (Mybiosource®, USA, MBS733885). In addition, serum levels of steroidogenic acute regulatory protein (StAR) (Mybiosource®, USA, MBS1607792), macrophage inflammatory protein-1β (MIP-1β) (Mybiosource®, USA, MBS006000), insulin like growth factor-1 (IGF-1) (Cusabio®, China, CSB-E13753Sh) and progesterone (Biorbyt®, UK, orb568116) were detected.

Evaluation of reproductive indices

Number of animals which came into estrus from each group was detected. In addition, pregnancy diagnosis was done by the aid of trans-abdominal ultrasonography (Sonoscape®, China, model A6V, B-mode, 7.5 MHz) on 40th day post-service to detect number of pregnant animals [31]. Also, number of aborted or still birth lambs and number of ewes which delivered with difficulties (dystocia) were determined for each group.

Statistical analysis

Data was checked for normality using Shapiro-Wilk test. Independent *t-test* was applied to detect the significant changes in level of the estimated serum parameters between the experimental groups with the aid of SPSS 16.0 for windows. All the results were represented as means ±SEM

Results and Discussion

Vitamin D has gained high attention during the last few years due to its identified multiple physiological actions other than calcium homeostasis [8], including its role in successful reproduction process [12-15]. As present in tables (1 and 2), serum level of TNF-α and IL-6 recorded a significant increase in both groups. This increase may be attributed to the effect of early implantation which induces an inflammatory state to improve uterine receptivity and pregnancy outcome [21, 22]. During trophoblast invasion into endometrium, it will injury or break through endometrial epithelial and stromal cells, these events will be followed by healing and repair upon placental growth. Endometrial injury is mediated by an alteration in the level of pro-inflammatory cytokines as TNF-α and IL-6 [32, 33]. In details, pro-inflammatory cytokine TNF-α plays a key role in induction of uterine receptivity through initiation of inflammatory condition which facilitates implantation [34-36]. But, over production of TNF-α is related to implantation failure and early embryonic death [31]. In the same manner, excessive level of IL-6 was proved to be associated with miscarriage and fertility problems [38]. On the other hand, serum level of IL-4 and IL-10 in the ewes of the experimental groups (I and II) increased significantly after 20 days from sponge removal (Tables 1 and 2).

This increment can be explained on the basis of the ability of macrophages to secrete number of anti-inflammatory cytokines as IL-4 and IL-10 to inhibit excessive inflammation and for angiogenesis and re-modelling of the tissues after implantation process [39, 40].

TABLE 1. Serum concentration of different detected parameters in non-vitamin D treated group (group I) before and 20 days after service

Parameters	1st Day	20th Day	t-value	P-value
TNF-α (pg/ml)	56.40±3.86^{B}	78.73±4.04^{A}	3.99	0.0014**
IL-6 (pg/ml)	49.00±3.75^{B}	71.00±3.40^{A}	4.34	0.001**
IL-4 (pg/ml)	156.40±7.90^{B}	181.80±7.41^{A}	2.34	0.026*
IL-10 (pg/ml)	32.27±2.82^{B}	47.87±3.27^{A}	3.61	0.0014**
IGF-1(ng/ml)	10.98±0.78^{B}	14.87±0.71^{A}	3.69	0.002**
StAR (ng/ml)	1.46±0.17^{B}	2.33±0.18^{A}	3.48	0.002**
Progesterone (ng/ml)	1.21±0.13^{B}	4.00±0.21^{A}	11.27	0.001*
MIP-1β (pg/ml)	165.07±12.58^{B}	197.80±12.67^{A}	1.87	0.025*

TNF-α: tumor necrosis factor alpha, IL-6: interleukin-6, IL-4: interleukin-4, IL-10: interleukin-10, IGF-1: insulin like growth factor-1, steroidogenic acute regulatory protein and MIP-1: macrophage inflammatory protein-1.

Means within the same raw of different litters are significantly different.

* = Significant at ($P < 0.05$) ** = Significant at ($P < 0.01$)

TABLE 2. Serum concentration of different detected parameters in vitamin D treated group (group II) just before and 20 days after service

Parameters	1st Day	20th Day	t-value	P-value
TNF-α (pg/ml)	54.40±4.52^{B}	71.80±4.45^{A}	2.75	0.01**
IL-6 (pg/ml)	48.87±3.69^{B}	65.47±3.37^{A}	3.32	0.002**
IL-4 (pg/ml)	170.73±9.71^{B}	202.67±10.10^{A}	2.27	0.03*
IL-10 (pg/ml)	38.00±3.42^{B}	60.80±3.25^{A}	4.83	0.000**
IGF-1(ng/ml)	12.26±0.87^{B}	17.63±0.74^{A}	4.68	0.001**
StAR (ng/ml)	1.56±0.14^{B}	3.07±0.17^{A}	6.96	0.0001**
Progesterone (ng/ml)	1.26±0.13^{B}	4.93±0.28^{A}	11.86	0.001**
MIP-1β (pg/ml)	169.27±14.14^{B}	207.07±14.91^{A}	2.01	0.049*

Means within the same raw of different litters are significantly different.
* = Significant at ($P < 0.05$) ** = Significant at ($P < 0.01$)

Table (3) illustrated that vitamin D administration was associated with less decrease in pro-inflammatory cytokines and more increase in anti-inflammatory cytokines as compared to non-treated group. Supplementation of vitamin D was associated with enhancement of anti-inflammatory environment and balancing anti-inflammatory/pro-inflammatory ratio [41,42] and many studies have illuminated the role of Vitamin D as a key regulator of immune cells cytokine secretion [43,44] with an enhanced anti-inflammatory cytokines secretion in trophoblasts [45,46] and this would explain the increase in anti-inflammatory cytokines (IL-4 and IL-10) and decrease in pro-inflammatory cytokines (TNF-α and IL-6) in ewes treated with vitamin D. The recorded increase in serum concentration of IGF-1in both of groups could be due to importance of IGF system for early development of mammalian embryo [47], as IGF-1 is important for proliferation of trophoblast during early pregnancy [48]. Moreover, the increase in IGF-1 was higher in vitamin D_3-adminstratedgroup (Table 3), as vitamin D_3 was proved to enhance production of IGF-1 [49].

During early pregnancy, progesterone hormone is produced by ovary to maintain conceptus growth and survival [50], and this would elucidate the increase in its level in both experimental groups (Tables 1, 2). Steroidogenic acute regulatory protein (StAR) is important for steroid hormones biosynthesis as it is involved in transport of cholesterol (substrate of steroid hormones) to inner mitochondrial membrane [51,52], so, its level was elevated in the ewes after service (Tables 1, 2). Vitamin D_3 is a powerful regulator of sex steroid hormones production as progesterone [53] through regulation of transcription of progesterone biosynthesis linked enzymes and proteins including StAR [54] and/or via increasing 3β-hydroxysteroid dehydrogenase (3β-HSD) in granulose cells which would stimulate and enhance secretion of progesterone [55]. As a result, vitamin D was proved to increase progesterone output through up regulation of StAR mRNA in goat follicles [56]. In this consistency, the higher serum level of progesterone upon administration of vitamin $D_3$is thought be due to the increased level of steroidogenic acute regulatory protein which was recorded in group-II (Table3).Macrophage inflammatory protein-1β (MIP-1β) is one of progesterone regulated endometrial chemokines which is produced by epithelial and stromal cells of endometrium [57, 58].MIP-1βwas proved to facilitate migration of trophoblast through endometrium [59]. The higher elevation in MIP-1β upon treatment with vitamin D (Table 3) may be linked with the increased progesterone level in these ewes.

As shown in Table (4), the net result of the previously mentioned biochemical changes in relation to vitamin D administration may explain the enhancement of uterine receptivity which was reflected in form of increased number of ewes which proved to be pregnant at 40th day post-service in vitamin D treated group as compared to control group. The increase in number of twins feti may be attributed to the ability of vitamin D to enhance redox balance of follicular granulose cells which may prevent follicular atresia [56],allowing more follicular growth and ovulation. One of the ewes which suffered from dystocia has lost the lamb (stillbirth), as dystocia is strongly related to

TABLE 3. The mean increase in serum concentration of the detected parameters in Group-I and Group-II at 20th day post-service:

Parameters	Group-I	Group-II	t-value	P-value
TNF-α (pg/ml)	22.07±1.78^{A}	17.40±2.04^{B}	2.04	0.041*
IL-6 (pg/ml)	21.33±1.32^{A}	16.93±1.27^{B}	2.40	0.023*
IL-4 (pg/ml)	25.40±1.75^{A}	29.60±1.78^{A}	2.23	0.049*
IL-10 (pg/ml)	16.00±1.76^{B}	22.27±1.52^{A}	2.69	0.012*
IGF-1(ng/ml)	3.89±0.34^{B}	5.47±0.38^{A}	3.08	0.005**
StAR (ng/ml)	0.86±0.06^{B}	1.51±0.09^{A}	6.11	0.0001**
Progesterone (ng/ml)	2.85±0.18^{B}	3.66±0.32^{A}	2.22	0.034*
MIP-1β (pg/ml)	32.73±2.65^{B}	37.60±2.89^{A}	2.24	0.024*

Means within the same raw of different litters are significantly different.
* = Significant at ($P < 0.05$)   ** = Significant at ($P < 0.01$)

TABLE 4. Reproductive indices of tested ewes of group I and II:

Parameter/ Group	**Group-I**		**Group-II**	
	Number	**%**	**Number**	**%**
Animals came into estrus	15	100	15	100
Pregnant animals at 40th day post-service	9	60	11	73.3
Twin feti	0	0	1	9.09
Still birth	1	11.1	0	0
Dystocia at birth	2	25	3	27.3

stillbirth in sheep [60]. The increase in number of ewes which suffered from dystocia in vitamin D treated group may be owed to presence of twin feti [61].

Conclusions

In conclusion, based on our study findings, administration of vitamin D_3 before mating may enhance reproductive performance of the ewes through regulation of levels of pro-inflammatory/ anti-inflammatory cytokines and some other biological molecules during implantation period.

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Conflicts of interest

The authors have no conflict of interest to be mentioned.

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تأثير حقن فيتامين د علي الكفاءة التناسلية في النعاج: الدور المنظم لبعض سيتوكينات الالتهاب وبعض الجزيئات البيوكيميائية

جمال العمراوي[1] و دينا جاد الكريم[2]

١- قسم الولادة - كلية الطب البيطري- جامعة الاسكندرية - مصر.

٢- قسم الباثولوجيا والباثولوجيا الاكلينيكية - كلية الطب البيطري - جامعة الاسكندرية - مصر.

تعتبر مشكلة الموت المبكر للاجنة هي واحدة من اكبر الصعاب التي تواجه انتاج الخراف. صممت هذه الدراسة بهدف تقييم تأثير حقن فيتامين د ٣ علي الكفاءة التناسلية للنعاج الرحماني بالاشارة الى تأثيره علي بعض الجزيئات البيوكيميائية المنظمة لعملية الغرس الجنيني في جدار الرحم. بعد الاستحداث المتزامن لدورة الشبق في النعاج, تم حقن مجموعة مكونة من ١٥ نعجة ب ٣٠٠,٠٠٠ وحدة دولية من فيتامين د ٣ لكل نعجة ، بينما تركت المجموعة الضابطة بدون اي معالجة. تم تلقيح المجموعتين بواسطة الكباش وتم تجميع عينات الدم عند اليوم الاول واليوم العشرين بعد ازالة الاسفنجة. اوضحت النتائج ان المجموعة المعالجة بفيتامين د سجلت زيادة اقل في مستويات الستوكينات المحفزة للالتهاب و زيادة اعلي في الستوكينات المضادة للالهاب مع زيادة اعلي في مستوي هرمون البروجيستيرون وعامل النمو المشابه للانسولين وكذلك مستوي البروتين المنظم للاستيرويدات وايضا مستوي البروتين الالتهابي المرتبط بالخلايا الضامة عند مقارنتها بمستويات المجموعة الضابطة. كما نتج عن حقن فيتامين د زياددة في عدد النعاج التي اثبت حملها عند اليوم الاربعين من خلال الفحص بالموجات الصوتية وكذلك زيادة في عدد تؤام الحملان. مما سبق يمكننا استخلاص ان فيتامين د ٣ له تأثير محفز ومحسن للكفاءة التناسلية وبطانة الرحم في النعاج مما ينعكس علي تحسين القدرة الاستقبالية للاجنة في الرحم.

الكلمات الدالة: نعاج ، السيتوكينات ، فيتامين د ، الكفاءة التناسلية ، الغرس الجنيني.