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Expression Profile of Angiogenic and *RANTES* Genes and Serum Biochemical Changes in Holstein Dairy Cows with Retained Fetal Membranes in Egypt

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Abstract

The changes in the expression profile of the angiogenic genes and serum biomarkers were investigated in the cows with the retained placenta after normal parturition. Retained fetal membranes (RFM) is considered one of the main reproductive disorders in dairy cattle. A total of 16 cows were allocated into two groups: group 1 (n=8) the cows that have a normal loosed placenta, and group 2 (n=8). The cows that have retained fetal membranes for more than 24 h after parturition. Blood with EDTA was collected from the tail vein for studying the expression of angiogenic proteins including vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor1 (VEGFR1), and *RANTES* genes. Serum was collected at a one-week interval before/after parturition for the study of biochemical changes. The VEGF expression decreased ten folds in cows with retained than normally dropped placentae ($p < 0.0001$). The vascular endothelial growth factor receptor2 (VEGFR2) showed a threefold increase in cows with normal loosed than retained placentae ($p<0.01$). The *RANTES* was increased in cows with retained rather than normal placentae by about 7 folds ($p<0.01$). Phosphorus and total proteins were significantly lower ($p<0.05$) in RFM cows one week after parturition. The serum albumin, TG, HDL, and cholesterol were significantly lower ($p<0.05$) in RFM before, at, and after parturition. CPK was significantly higher in RFM before, at, and after parturition ($p<0.05$). Increased values of MDA and decreased values of CAT, SOD, and R-GSH were detected in the blood of cows suffering from RFM ($p<0.05$). In conclusion, the translation of VEGF and its receptor and *RANTES* mRNA at the time of parturition in dairy cows could be proxy biomarkers for the prediction of retained fetal membranes (RFM). In the same line, the activity of total proteins, serum albumin, TG, HDL, cholesterol, and CPK would be useful in the prediction of the RFM in dairy cows.

KEYWORDS
Retained fetal membranes, Angiogenic proteins, Biochemical changes, Dairy cows

INTRODUCTION

The retained fetal membrane is one of the main reproductive disorders in dairy cattle. The incidence of RFM in cattle varied between 5 and 10 % (Dervishi *et al*., 2016; Tucho and Ahmed, 2017). It causes considerable economic losses in the herd due to decreased milk production, illness, and cost. The risk factors for retained placenta in dairy cows include twine birth, nutritional deficiency, low immunity, and environmental and hormonal causes (Tucho and Ahmed, 2017).

The concentration of serum calcium, glucose, and other biochemical parameters were lower after 12 hours post-partum and 7 days post-partum in cows suffering from fetal membrane retention compared to normal calved cows (Semacan and Sevinç, 2005). In addition, the lower concentration of serum total protein in pregnant cows may be responsible for the retention of the placenta. The concentration of serum albumin in cows with fetal membrane retention was lowered significantly (Hirayama *et al*., 2020; Al-Rikabi *et al*., 2021; Chebel, 2021b).

Oxidative stress in cows is one of the main risk factors that increase the incidence of retained fetal membranes in dairy cows (Sordillo and Aitken, 2009). The metabolic demand is increased during late pregnancy, parturition, and the initiation of lactation (Chebel, 2021a). Also, the retained fetal membranes in dairy cows are associated with the defect in the translation of angiogenic proteins (Peter, 2013).The chemokine ligand 5 is one of several cytokine genes involved in immunoregulatory and inflammatory processes (Choi *et al*., 2014). Therefore, the objectives of this study were to assess biochemical changes and translation of angiogenic proteins during the retained placenta in dairy cows.

MATERIALS AND METHODS

Ethics statement

The collection of samples and care of the animals used in this study followed the guidelines for experimental animals established by Research Ethics Committee, Faculty of Veterinary Medicine; Mansoura University (Code Ph.D. /52), Egypt.

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Animals and data collection

A total of 107 parturient dairy cows aged 3–6 years of age and weighing between 400 and 650 kg were studied. This study used lactating primiparous (n = 35) and multiparous (n = 72) Holstein dairy cows. The cows aged 2–7 years old (4.6±1.8) and experiencing 1–5 parities were employed. Of all experimental cows, 16 were randomly selected for studying gene expression. Animals were classified into two groups. The first one with retained fetal membranes and the second one normal dropping placenta. All selected dairy cows from 15 farms in Dakahlia Governorate, Egypt, were kept in semi-open sheds and fed a diet that fulfilled NRC requirements (NRC, 2001). The current study was conducted during the period of September 2019 to June 2021. The cows were fed twice daily and milked three times a day, at roughly 8h intervals, according to the farm's administration. Vaccinations and deworming were given to all animals to protect them from infectious and parasitic diseases.

Blood sampling

Two types of peripheral blood samples (10 ml each) were taken from the selected dairy cows via tail vein puncture 7 days before and after parturition as well as time of parturition. The first sample was collected in a plain tube devoid of anticoagulant for immediate centrifugation at 3000 rpm for 15 minutes to separate serum, which was stored at 20°C for further biochemical analysis. In the meantime, the second blood sample was collected into a tube containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and stored at 80°C for analysis of angiogenic protein translation according to (Elmetwally *et al*., 2018; Elmetwally *et al*., 2020; Elmetwally *et al*., 2022).

Quantitative real-time PCR

The quantitative real-time PCR method was used to analyze gene expression quantitatively (qRT-PCR). Primer-BLAST was used to design all primers for genes encoding angiogenic proteins (VEGF, VEGFR2, and *RANTES*). Software RNA was extracted from peripheral blood cells using trizol reagent (Puregene, Genetix brands). The RNA pellet was eluted with 50 µL of RNase-free water and incubated for 10 min at 55°C to be dissolved completely. The extracted RNA was reverse transcribed to cDNA in a 20 µL reaction using the SensiFAST cDNA synthesis method. kit (Bioline, London, U.K.), where 5 µL of the RNA sample was added to 4 µL of 5x Trans-Amp Buffer, 1 µL of reverse transcriptase enzyme and 10 µL of Ultra-Pure DNase/RNase-free water. The reaction mixture was incubated at 25°C for 10 min, then 42°C for 15 min, and heated to 85°C for 5 min in a thermal cycler. Finally, the cDNA samples were diluted at 1:10 in sterile DNase-free water and stored at -20°C.

The reaction consisted of 2 µl of cDNA template, 10 l SYBR Green PCR Master mix (SensiFAST SYBR NO- ROX kit, Bioline, London, UK), 0.8 µl of 10 µM of each forward and reverse primers (Vivantis Technologies Sdn Bhd., Malaysia) and adding 6.4 µL of sterilized Ultra-Pure DNase- free water to bring the total volume to 20 µL. The reaction mixtures were subjected to the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and 72°C for 15 s. The specificity of each primer was assessed by gel electrophoresis and melting curve analysis. In addition, the efficiency of each primer was calculated via the equation 'Efficiency = −1+10 (-1/slope)'. Relative quantification of mRNA transcripts was determined using the 2−ΔΔCt method described by Livak and Schmittgen (2001) where the β-actin gene was used as the housekeeping gene.

Biochemical analysis

After collecting all the required samples, biochemical analysis of all the selected serum parameters was performed. Spectrophotometric measurements of serum calcium and phosphorus were made with commercial kits (Human Gesellschaft Für Biochemica und Diagnostic ambH, Max-Planck-Ring 2165205, Wiesbaden, Germany). Kits of total protein (TP), total cholesterol (TC), triglyceride (TG), and high density lipoprotein (HDL), albumin, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and oxidative stress kits were purchased from Biodiagnostic, Co. (Egypt).

Exclusion criteria were used in this step, as instances with any biochemical anomalies were eliminated to guarantee that the data attribution was valid.

Statistical analysis

Data analyses were performed using a statistical software program (SPSS for Windows V.22, SPSS, Chicago, USA). The data was tested for normal distribution using the Shapiro-Wilk test. The data was normally distributed; therefore, the mean and SD for each variable were calculated. Data were subjected to repeated measures analysis of variance (ANOVA), one- way ANOVA was used to identify which time (one week prior to calving, at the day of parturition, and one week postpartum) was statistically different. The correlation between the on the profile of VEGF, VEGFR2, and *RANTES* and different biochemical paCorrelation parameters was analyzed using Spearman correlation. The differences between the means at a p-value of $p<0.05$ were considered significant.

Table 1. Oligonucleotide primers sequence, accession number, annealing temperature and PCR product size of genes used in real time PCR.

Gene	Primer	Annealing temperature (°C)	Accession number
RANTES	F5'-CACCCACGTCCAGGAGTATT-3 R5'-CTCGCACCCACTTCTTCTCT-3'	60	NM_175827
VEGF	F5'-GAAACCCCACGAAGTGGTGA R5'-GCGCTCCAGGATTTATACCG	60	NM_001316956.1
VEGFR2	F5'AGTATGAGAGGCTGGGAGCA R5'-AGCAATTACACCTCAAGCCAGA	60	NM_001110000.3
ß-actin	F5'-GGCATCCTGACCCTCAAGTA-3' R5'-CACACGGAGCTCGTTGTAGA-3'	60	NM_173979.3

RANTES= Regulated on Activation, Normal T Expressed and Secreted; VEGF= vascular endothelial growth factor; VEGFR2= vascular endothelial growth factor receptor 2; ß-actin= housekeeping gene (human gene and protein abbreviation ACTB/ACTB)

RESULTS

The incidence of RFM according to cow parity was recorded as 50% 2,3parous 50% 5,6 parous in the NRFM group versus 37.5% 2,3 parous 62.5% 5,6 parous in the RFM group with a non-significant statistical difference between calved cows used for the current work (Table 2).

In Figure 2, the gene expression profiles of VEGF, VEGFR2, and *RANTES* were depicted. A significant decrease of VEGF and VEGFR2mRNA expression in retained placenta affected dairy cows compared to healthy ones (Fig.1: $p < 0.001$). The mRNA expression for *RANTES* showed a significant decrease in the case of cows suffering from retained placentae compared to other normal dropped placentae (Fig. 1: $p < 0.001$).

According to serum biochemical changes in the week before parturition, at parturition, and one week after parturition, the mean of studied Ca_2^+, phosphorous, urea, and total protein values showed a significant reduction pattern ($p < 0.05$). Phosphorus

Table 2. Incidence of RFM according to cows' parity

Cow parity	Group I n= (8)	Group II n= (8)	p-value
2,3 parous	4 (50.0%)	3 (37.5%)	0.614
5,6 parous	4 (50.0%)	5 (62.5%)	

Data were presented as number and %. *p <0.05 is a significant value

Table 3. Serum biochemical changes of group I and II on during timetable.

	7 days pre-parturition	At parturition	7 days post-parturition	p-value			
				P1	P2	P3	P4
Calcium (mmol/L)							
Group I	2.56±0.02	2.08±0.24	2.19±0.25	0.001*	0.109	0.872	0.42
Group II	2.54±0.03	2.06±0.24	2.07±0.30	0.001*			
Phosphorous (mmol/L)							
Group I	2.02±0.13	1.64±0.18	1.65±0.19	0.004*	0.233	0.288	0.038*
Group II	1.92±0.17	1.56±0.13	1.44±0.14	0.001*			
Urea (mg/dL)							
Group I	8.98±1.29	8.99±2.20	8.29±1.66	0.127	0.096	0.079	0.062
Group II	10.71±2.40	11.36±2.77	10.31±2.26	0.008*			
Total protein (g/L)							
Group I	63.30±0.99	60.00±3.57	59.00±2.95	0.007†	0.023*	0.077	0.094
Group II	64.47±0.83	62.95±2.52	61.57±2.76	0.025§			
Albumin (g/L)							
Group I	38.57±1.27	38.13±1.24	36.12±1.74	0.001†	0.003*	0.001*	<0.001*
Group II	34.65±2.63	31.70±3.55	28.28±2.48	0.001§			
Aspartate aminotransferase (AST) (U/L)							
Group I	56.50±8.97	81.12±11.15	120.25±14.82	0.001†	0.057	0.001*	0.027*
Group II	67.87±12.60	108.75±15.69	142.00±19.97	0.001§			
Alanine transaminase (ALT) (U/L)							
Group I	30.62±3.20	43.37±8.91	45.12±8.25	0.001†	0.795	0.001*	<0.001*
Group II	30.12±4.25	70.87±5.89	76.62±7.91	0.001§			
Bilirubin (mg/dL)							
Group I	0.97±0.21	1.03±0.36	1.13±0.46	0.418	0.394	0.205	0.128
Group II	0.80±0.51	1.33±0.52	1.71±0.91	0.057			
CPK (U/L)							
Group I	99.87±39.74	115.97±43.20	121.75±44.06	0.075	0.023*	0.016*	0.001*
Group II	154.87±45.98	186.75±59.15	231.87±43.01	0.001§			
Triglycerides (TG) (mmol/L)							
Group I	0.46±0.06	0.45±0.07	0.43±0.06	0.063	0.013*	0.012*	0.022*
Group II	0.39±0.01	0.37±0.03	0.35±0.06	0.076			
High Density Lipoprotein (HDL) (mmol/L)							
Group I	1.44±0.27	1.49±0.22	1.42±0.0.25	0.063	0.017*	0.001*	0.002*
Group II	1.14±0.16	1.05±0.06	1.01±0.05	0.16			
Cholesterol (mmol/L)							
Group I	3.68±0.54	3.69±0.83	3.47±0.77	0.08	0.002*	0.024*	0.030*
Group II	2.79±0.35	2.81±0.51	2.70±0.47	0.325			

Data were presented as mean±SD., Group I: Non-RFM, Group II: RFM., *p <0.05 is a significant value., P1: comparison of different interval time in each group, †: for Group I and §: Group II., P2: comparison between Group I and II7 days pre-parturition., P3: comparison between Group I and II at parturition. P4: comparison between Group I and II 7 days post-parturition.

showed a significantly lower value in group II compared to group I at one-week post-parturition (p = 0.038). the total protein showed a significantly high value in group II compared to group I at one-week pre-parturition (Table 3, p=0.023).

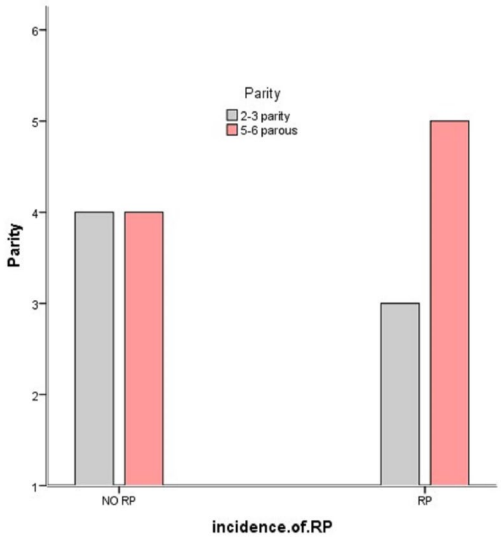


Figure 1. Incidence of RFM according to cows' parity. No RP= non retained placenta; RP= retained placenta

Serum albumin showed a significant decrease in value at one-week post-parturition compared to one-week pre-parturition (p = 0.001) in groups I and II. Moreover, serum albumin was significantly lower in cows with the retained placenta when compared to the cows with the normal loss of placenta at one-week pre-parturition, parturition, and one week post-parturition (Table 3: p = 0.003, 0.001, and 0.001, respectively).

ALT and AST activities, bilirubin, and CPK concentration were significantly elevated at one-week post-parturition compared to one-week pre-parturition (p = 0.001) in groups I and II. Additionally, serum ALT and AST activities and CPK concentration were significantly higher in RTP compared to NP cows at one-week pre-parturition, parturition, and one week post-parturition (Table 3). The Serum TG and HDL and cholesterol were significantly lower in group II compared to group I at one-week pre-parturition, at parturition and one week post-parturition (Table 3: p< 0.051). Serum Malondialdehyde (MDA) was significantly higher in RTP compared to NP cows (p< 0.05), but the Superoxide dismutase (SOD) (U/ml), Glutathione (GSH) reduction (R-GSH) (mmol/L) and catalase (CAT) (U/ml) were significantly lower in group II compared to group I (Table 4: p< 0.05).

DISCUSSION

According to Sethi *et al*. (2021), retained fetal membrane commonly occurs in animals. It occurs when the calf's side of the placenta (the fetal membranes) fails to separate from the mother's side. Separation of the membranes normally occurs after the calf is born. Retained placenta is usually defined as the failure to

Table 4. Stress oxidant markers of group I and II after parturition

	Group I n= (8)	Group II n= (8)	p-value
Malondialdehyde (MDA) (mmol/ml)	1.25±0.36	2.39±0.46	0.001*
Superoxide dismutase (SOD) (U/ml)	330.63±19.89	266.91±13.01	0.001*
Glutathione reduced (R-GSH) (mmol/L)	6.16±0.26	1.93±0.35	0.001*
Catalase (CAT) (U/ml)	2.21±0.11	0.94±0.13	0.001*

Data were presented as mean+SD, Group I: Non-RFM, Group II: RFM. *p <0.05 is a significant value.

Table 5. Correlation between chemical and angiogenic proteins in NRFM.

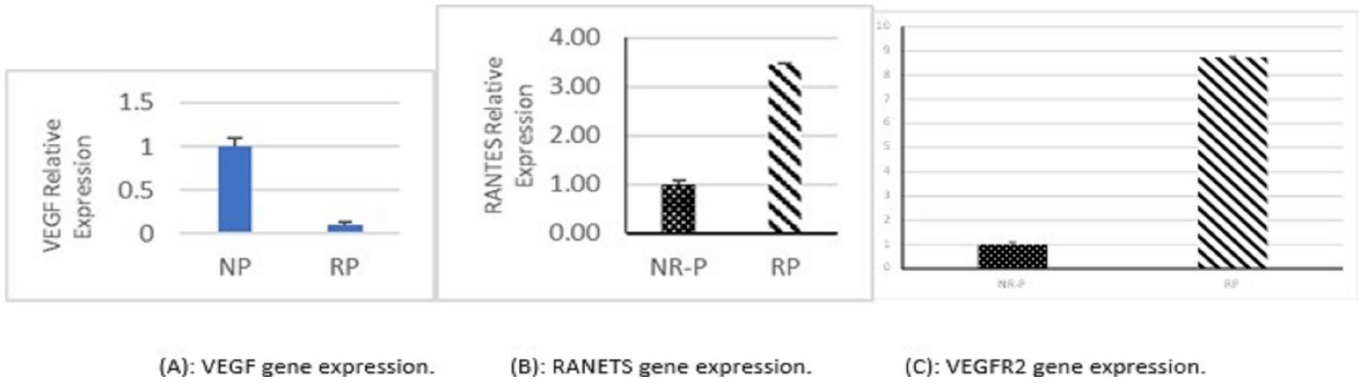
	VEGF		*RANTES*		VEGFR2	
	R	p-value	R	p-value	R	p-value
PO4	0.326	0.431	0.285	0.494	-0.504	0.202
Urea	0.659	0.075	0.307	0.459	0.069	0.871
Ca	0.753	0.031	0.614	0.105	-0.204	0.627
Cholesterol	-0.055	0.897	0.3	0.471	-0.356	0.386
TG	-0.041	0.922	-0.044	0.918	0.022	0.958
HDL	-0.059	0.889	0.123	0.771	-0.169	0.689
Bilirubin	-0.312	0.452	-0.186	0.659	0.011	0.979
GOT	-0.527	0.18	-0.557	0.151	0.28	0.501
GPT	-0.21	0.618	-0.165	0.696	0.05	0.906
Albumin	0.046	0.915	0.261	0.532	-0.254	0.545
CK	0.128	0.763	-0.542	0.166	0.661	0.074
Total protein	0.064	0.881	0.339	0.412	-0.326	0.431
SOD	0.422	0.298	-0.306	0.461	0.586	0.127
MAD	-0.45	0.263	-0.335	0.417	0.087	0.837
GR	-0.197	0.641	-0.412	0.311	0.324	0.434
CAT	-0.612	0.107	0.024	0.956	-0.397	0.33

VEGF= vascular endothelial growth factor; *RANTES*= Regulated on Activation, Normal T Expressed and Secreted.
VEGFR2= vascular endothelial growth factor receptor 2; PO4= phosphate; Ca= calcium; TG= triglycerides

Table 6. Correlation between chemical and angiogenic proteins in RFM.

	VEGF		*RANTES*		VEGFR2	
	R	p-value	R	p-value	R	p-value
PO4	-0.054	0.899	-0.168	0.961	-0.145	0.731
Urea	0.497	0.21	0.606	0.111	0.586	0.127
Ca	-0.266	0.525	-0.28	0.502	-0.278	0.505
Cholesterol	-0.097	0.819	-0.021	0.961	-0.036	0.932
TG	-0.375	0.361	-0.349	0.396	-0.356	0.387
HDL	0.381	0.351	0.333	0.421	0.343	0.405
Bilirubin	-0.07	0.87	-0.017	0.968	-0.028	0.948
GOT	-0.135	0.75	-0.221	0.599	-0.204	0.628
GPT	-0.414	0.309	-0.482	0.226	-0.47	0.24
Albumin	0.124	0.77	0.064	0.881	0.076	0.858
CK	0.422	0.297	0.331	0.423	0.351	0.395
Total protein	0.273	0.514	0.199	0.637	0.214	0.611
SOD	-0.457	0.255	-0.559	0.15	-0.54	0.167
MAD	0.262	0.531	0.241	0.565	0.246	0.557
GR	0.13	0.759	0.021	0.96	0.043	0.919
CAT	0.301	0.469	0.386	0.344	0.37	0.367

VEGF= vascular endothelial growth factor; *RANTES*= Regulated on Activation, Normal T Expressed and Secreted.
VEGFR2= vascular endothelial growth factor receptor 2; PO4= phosphate; Ca= calcium; TG= triglycerides



(A): VEGF gene expression. (B): RANETS gene expression. (C): VEGFR2 gene expression.

Figure 2. mRNA levels of VEGF, VEGFR2 and RNATES in retained placenta and non-retained placenta affected dairy cows.

expel fetal membranes within 24 h after parturition. As far as its causes are concerned it is most associated with dystocia, milk fever and twin births. However, it can be prevented by good dry cow management. This includes the supply of correct nutrients, particularly magnesium, and fat-soluble vitamins, maximizing dry matter intake, maintaining the correct body condition score, and supplying a clean dry environment.

In this context, the expression profile of VEGF was significantly downregulated in retained placenta dairy cows compared to healthy ones. However, mRNA levels of VEGFR2 and *RANTES* were significantly upregulated. To our knowledge, there is little information on the gene expression profiles of VEGF, VEGFR2, and *RANTES* genes in retained placenta affected dairy cows. However, Zheng *et al.* (2018) found that VEGFA mRNA levels in caruncle tissue were significantly down-regulated in RFM cows, as compared to healthy cows. The observed down-regulation of VEGF may be attributed to the fact that oxygen (O2) concentration in antral ovarian follicles is below that found in most tissues, which is important for adequate granulosa cell function. The VEGF system is linked to angiogenesis and responds to changing O2 by stimulating neovascularization when levels are low.

It seems that under low oxygen concentrations, the hypoxia-induced factor-1a (HIF-1a) binds to the hypoxia response element (HRE) located in the promoter region of the VEGF gene, hence inducing its expression Kim and Lee, (2017). While follicle mRNA expressions for VEGFR1 is higher in dominant follicles collected at Day 4 of the follicular wave and subsequently decreases as the dominant follicle consolidates (Days 6 and 9 of the follicular wave), an inverse relationship is found for the expressions of the VEGFR2 gene. We hypothesize that under low oxygen culture At VEGF ligand concentrations resembling those found in the antral follicle, granulosa cells will upregulate VEGF ligand and downregulate VEGF receptor mRNA expression (Hernández-Morales *et al*., 2021). Estradiol increases the expression of the VEGF gene in normal human endometrium, also prostaglandin is another factor known to up-regulate VEGF expression (Johnson and Mowa, 2021).

MMP-2 is an important collagenase in both the maternal and fetal compartments of the bovine placentome that is involved in the breakdown of extracellular matrix components, such as collagen. It has been reported that ARA metabolites act as signal mediators of MMP-2 activation. Abnormal concentrations of ARA are key to the elucidation of the underlying mechanisms of RFM. Many studies have identified PLA as a rate-limiting enzyme in ARA production. PLA has activated in the VEGFA signaling pathway through a series of cascades (Kamada *et al*., 2012).

In the VEGFA signaling pathway, VEGFA binds to the epithelial cell membrane kinase insert domain protein receptor to activate

PLC, which then activates PKC and promotes the release of Ca_2^+ within the cytoplasm. Once released, Ca_2^+ then binds to the C2 target region of PKC to activate PKC. Activated PKC then activates the MAPK cascade through RAF. Phospho-MAPK activates extracellular MAPK signaling and phospho-MAPK promotes the synthesis of PLA. PLA was down-regulated in the caruncles of cows with RFM (Zheng *et al*., 2018). Tasaki *et al*., (2010) showed that VEGF mRNA expression at estrus was significantly higher than at the early 1; early 2 and late luteal stages. VEGFR2 mRNA expression was higher at mid and late luteal stages than at the early 1 and 2 luteal stages also, VEGFR2 protein was higher at the mid and late luteal stages than at estrus. Punyadeera *et al*. (2006) showed that the expression of VEGF was greatest at estrus and the expression of the protein was highest at the early 1 luteal stage. Estradiol-17b (E2) regulates VEGF mRNA expression in human endometrial cells and the rat uterus (Forsythe *et al*., 1996).

It is worth mentioning that, Dervishi *et al*. (2016) showed that cows that retained their fetal membranes had activation of innate immunity starting at −8 weeks before parturition and up to +8 weeks after parturition. The latter study may decipher the significant up-regulation of *RANTES* in case of retained placenta-affected dairy cows. *RANTES*, a chemokine for monocytes and activated T cells, is actively synthesized by stromal cells derived from normal endometrium and endometriosis implants (Khorram *et al*., 1993). It was suggested that convergent chemokine pathways contribute to a feedforward cascade that perpetuates the infiltration of inflammatory cells associated with endometriosis (Hornung *et al*., 1997). The subtle increase in *RANTES* gene expression observed in normal endometrium during the ovulatory cycle was less than that noted for endometrial vascular endothelial growth factor mRNA. Nevertheless, it is likely that *RANTES* participates in the normal physiology of the endometrial immunological system (Shifren *et al*., 1996). Increased cellularity (Hofbauer cells and TCD8+ lymphocytes), expression of local pro-inflammatory cytokines such as IFN-γ and TNF-α, and other markers, such as *RANTES*/CCL5 and VEGFR2, supported placental inflammation and dysfunction (Rabelo *et al*., 2018). Hirayama *et al*. (2020) reported that the expression levels of inflammatory cytokine and receptors genes in the caruncles at parturition exhibited two-fold higher mRNA expression in the spontaneous group than in the group induced parturition with DEXA and showed RFM such as *RANTES* mRNA expression in the caruncles was lower in the SP group than DEX group.

Xiao *et al*. (1998) found that in cows, E2 Secretion is highest at estrus thus, the greatest expression of VEGF mRNA expression at estrus may be stimulated by E2. Saed *et al*. (2020) elicited that prior to parturition by 3 weeks the expression level of *RANTES* gene increased while at time of parturition the expression of the gene was down regulated. Porwal *et al*. (2021) found that inflammation, as well as the expression of *RANTES* and VEGF, were significantly reduced in the treated human hemorrhoid and fistula tissues as compared to untreated ones. Vacinova *et al*. (2021) found that *RANTES* levels were up to 100 times higher in AD (Alzheimer's disease) patients compared to control subjects also, this finding agrees with others (Iarlori *et al*., 2005; Stuart and Baune, 2014). Kimura *et al*. (2003) showed that RT–PCR analysis on *RANTES* mRNA in the skin of cats with eosinophilic plaque revealed that its expression was higher in the eosinophilic plaque skin lesions than in the normal skin. The result suggested that *RANTES* might play a role to induce eosinophil infiltration in feline eosinophilic plaque lesions.

In the current study, the incidence of RFM according to cow parity was higher in 5,6 parous cows (62.5%) versus 37.5% 2,3 parous of calved cows used for the current work. In the same way, Mahnani *et al*. (2021) found that the incidence of RFM is higher in multiparous cows than in primiparous ones which may be attributed to the reduction of calcium in these animals collected from a dairy farm. Moreover, a survey was conducted by Sharma *et al*. (2017). At Uttarakhand India from the year 2003 to 2013 on total cases of retention of placenta 339 to observe the effect of parity of animals on the rate of retention of placenta in dairy cattle and found that 25.95% cases occurred in primiparous cows and 74.04% in pluriparous cows which is in accordance with our results. The highest percentages of incidence of retained placenta were detected in the spring and summer seasons. Sarder *et al*. (2010) found that the incidence of retained placenta was 8.5, 13.3, 6.1, 9.4, 20, and 28.7 % at 1st, 2nd, 3rd, 4th, 5th, and >6th parity, respectively, which may be due to the higher incidence of metritis and dystocia with the increased parity which is similar to the obtained findings.

According to Azad (2010), placenta retention rates in the first, second, third, and fifth parties were 15, 15, 33.3, and 37.5 %, respectively. According to Gaafar *et al*. (2010), the incidence of retained fetal membranes in Friesian cows increased considerably from 14.20 percent for the first parity to 54.60 percent for the second parity. It could be explained based on the uterine muscles. Cows with uterine diseases can develop severe acute inflammation and reduced dry matter intake, contributing to a greater reduction of albumin in the bloodstream. In addition, Ruprechter *et al*. (2018) highlighted that the monitoring of albumin contributed to predictions for uterine health in pre-metritis cows. Low serum albumin levels were observed in dairy cows with retained fetal membranes. Albumin is considered an acute negative inflammatory protein, and it assists in the diagnosis of animals that undergo intense inflammation (Bertoni *et al*., 2015). The current work showed that serum albumin showed a significant decrease in value at one-week post-parturition compared to one-week pre-parturition ($p = 0.001$) in groups II and I and was significantly lower in group II compared to group I during the timetable. Similarly, Nogalski *et al*. (2012) found the same results as regards albumin but with no significant statistical differences.

ALT and AST activities, bilirubin, and CPK concentration were significantly elevated at one-week post-parturition compared to one-week pre-parturition ($p = 0.001$) in groups I and II. Additionally, serum ALT and AST activities and CPK concentration were significantly higher in group II compared to the control group during the timetable. In addition to the greater concentration of GGT enzyme activity, a higher serum activity of the AST enzyme was observed, indicating that cows with metritis had alterations in liver function. Elevated serum AST activity is indicative of liver tissue damage (Paiano *et al*., 2020). In the study of Yazlık *et al*. (2019), cows with RFM had higher AST levels during the prepartum period and around calving than healthy cows. Concurrent with AST, CK levels were higher in cows with RFM. CK may have increased because of muscle tissue degradation due to increased demand for energy and insufficient lipomobilization, which also increases AST activity. Although GGT concentrations were higher in cows with RFM, they were at physiological levels in the groups. The mechanism underlying this difference might be related to some degree of hepatic lesions. Also, Nogalski *et al*., (2012) found the same results as regards AST but with only significant statistical differences between the 2nd and 3rd-week post parturition when compared to other times.

Furthermore, in the current study, serum TG and cholesterol were significantly lower in group II compared to group I during the timetable. Serum cholesterol levels have the potential to be indicators of disease risk in dairy cows (Kaneene *et al*., 1997). In study of Rayan *et al*. (2019) study, cholesterol levels increased very slightly directly in the prepartum, then they were below average reference values in cows with RFM. Cows with RFMs had significantly lower blood total cholesterol concentrations on the day of parturition and day 1 of calving than did cows that expelled fetal membranes normally. In another study, the authors proved higher triglyceride levels in RFM cows (in prepartum, partum, and postpartum periods) compared with the control group (Rayan *et al*., 2019). The higher concentrations of triglycerides in RFM cows might have resulted from more energy needs (Seifi *et al*., 2007).

Cholesterol is considered an acute-phase reactant of inflammation, which can contribute to the diagnosis of intense pro-inflammatory status (Paiano *et al*., 2019). Kim and Suh (2003) showed that dairy cows with lower concentrations of cholesterol

were more likely to develop uterine diseases. Also, Nogalski *et al.* (2012) found that cholesterol was statistically decreased post parturition when compared to other times in both the RFM and control groups, with higher values in control groups when compared with the RFM group.

Results by Yazlık *et al.* (2019) showed that the mean blood Ca concentrations in cows suffering from RFM were at subclinical hypocalcemia levels. The abovementioned mechanism might affect immune capacity negatively during the prepartum period and around parturition in cows suffering from RFM. In our present study, the means of studying Ca_2^+ showed a significant reduction trend in groups I and II at one-week post-parturition. Compared to one-week pre-parturition. Phosphorus showed a significantly lower value in group II compared to group I at one-week post-parturition. Calcium is an important ion with a role in the etiology of RFM. Decreased Ca concentrations can cause uterine atony, which leads to RFM. In addition, subclinical hypocalcemia in dairy cows is an issue for the first few days after parturition due to excessive Ca demand colostrum synthesis and milk production and inadequate response from bones for restoring Ca concentration (Yazlık *et al.*, 2019). In relation to serum calcium concentration, the findings in the present study are in agreement with previous reports which showed lower concentrations of calcium in cows with metritis than in healthy cows (Cui *et al.*, 2019). The calcium concentration in metritis cows was 1.17 mg/dl lower than in healthy cows. Cows with low calcium concentration can present impaired immune function (Kimura *et al.*, 2002).

In the current work, phosphorus, urea, and total protein values showed a significant reduction trend in groups I and II at one-week post-parturition compared to one-week pre-parturition. Phosphorus showed a significantly lower value in group II compared to group I at one-week post-parturition. The results found by Lu *et al.* (2020) indicate that serum concentrations of P and BUN in the cows of the RFM group were markedly greater than in cows of the NRFM group at -7 d, and there was no significant difference at other time points between the cows of the two groups. Serum concentrations of TP were greater in cows of the NRFM than in the RFM group at -7 d.

In the current study, serum malondialdehyde was significantly higher in group II compared to group I. At the same time, superoxide dismutase, glutathione reductase, and catalase were significantly lower in group II compared to group I. The imbalance in ROS production is one of the predisposing factors that cause the improper release of the placenta. Hence, the study of antioxidant defense systems such as TAC, SOD, and GSH was a very crucial matter during the current work. Similarly, Hassan *et al.*, (2020) work showed a reduction of SOD activity, TAC, and GSH level in RFM compared to NRFM which is attributed to multi factors including the reduced production of E2 and PG-F2α and accumulation of arachidonic and linoleic acids in the placental tissue (Tagesu, 2018).

Kankofer (2001) indicated that SOD activity in fetal membrane tissues increased in cows with RFM at preterm and term delivery. Similar to our results, the Yazlık *et al.*, (2019) study found a relationship between elevated prepartum SOD activity and subsequent development of RFM. SOD enzyme activity is a part of the antioxidant defense system and plays a role in animal health status (Kankofer, 2001). Previous studies have revealed different levels of SOD activity in cows with periparturient disorders. Heat stress elevated the SOD activity, while the cows with mastitis had lower SOD activity. Wischral *et al.*, (2001) reported that SOD concentrations were similar in cows with or without RFM. Hassan *et al.*, (2020) work exhibited that RFM is associated with an elevation of MDA concentration, which comes in line with preceding studies (Jovanović *et al.*, 2013; Islam and Kumar, 2015). MDA is an indicator of lipids peroxidation which is associated with the presence of poisonous metabolites and the destruction of free fatty acids and phospholipids (Erisir *et al.*, 2006). We assumed that a high level of MDA was predicted due to metabolic and endocrine changes related to RFM. Similarly, Khudhair *et al.* (2021) found that glutathione reduction was significantly lower in the RFM group compared to NRFM. In the same way, Ahmed *et al.* (2009) found that catalase reduction was significantly lower in the RFM group compared to NRFM.

CONCLUSION

The translation of angiogenic and *RANTES* proteins at the time of parturition in dairy cows could be proxy biomarkers for the prediction of retained fetal membranes. In the same line, the activity of total proteins, serum albumin, TG, HDL, cholesterol, and CPK would be useful in the prediction of the retained fetal membranes in dairy cow.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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