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Screening of Some Changes in Hematological, Serum Biochemical, Inflammatory and Oxidative Parameters Associated with Pathogenesis of Retained Placenta in Holstein Dairy Heifers

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ABSTRACT

Retained placenta is still one of the major problems facing dairy farms, and up until now, haematological and biochemical changes related to the condition of retained placenta have been mysterious. Our study aimed to screen the presumptive serum biochemical and haematological alterations in relation to the pathogenesis of retained placenta in dairy Holstein heifers. Twenty heifers were used in this study; ten of them suffered from retained placenta, and the others were kept under control as they expelled their placenta within the reference time range after parturition. Concerning serum biochemical changes, heifers affected by retained placenta suffered from disturbances in the redox state and exhaustion of enzymatic and non-enzymatic antioxidants. In addition, the serum concentration of anti-inflammatory interleukin-13 was increased in association with an elevated level of serum mucin-1, C-reactive protein and alpha-1 anti-trypsin in these heifers. Also, retained placenta induced a decrement in the serum levels of inteleukin-8 (IL-8), prostaglandin F-2 alpha (PGF-2α) and an increment in the serum level of prostaglandin-E2 (PGE-2). Moreover, serum concentrations of non-esterified fatty acids (NEFA) and creatine kinase enzyme activity were elevated in heifers with retained placentas. Haematological results did not show any significant change in RBCs count, haemoglobin concentration, hematocrit%, or platelet count between the two groups. Controversially, the total leukocytic count, granulocytes, lymphocytes and monocytes counts were elevated in cows with retained placentas. In conclusion, the retained placenta condition is accompanied by oxidative stress and inflammatory disturbances that directly affect the metabolic and hormonal states of dairy heifers.

Keywords: Hematology, Holstein dairy heifers, Interleukins, Oxidative Stress, Prostaglandins, Retained placenta.

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INTRODUCTION

The failure of foetal membrane expulsion within 24 hours after parturition is called retained placenta (RP) or retained foetal membranes (RFM). Retained placenta is still one of the most serious and common multi-factorial postpartum diseases, as it may affect the whole profitability of the herd through different ways (increased risk of metritis development, subsequent infertility, and decreased milk yield and quality) **(Benedictus *et al.*, 2013, Mahnani *et al.*, 2015, Moretti *et al.*, 2015, Mahnani *et al.*, 2021).** Additionally, affection with a retained placenta would decrease the future conception rate, increase days open and increase service per conception **(McDougall, 2001).** Also, affection with retained placenta may increase the animal's susceptibility to mastitis and abomasal displacement **(Zhang *et al.*, 2021).** In cattle, the incidence of retained placenta may reach about 5–10% **(Dervishi *et al.*, 2016; Tucho and Ahmed, 2017).**

Several factors would affect the incidence of retained placenta in the cows, including; age, cow physiological state, parity, environmental conditions, nutrition, the presence of twins, and stillbirth **(Han and Kim, 2005; Zobel and Tkalcic, 2013; Qu *et al.*, 2014; Dubuc and Denis-Robichaud, 2017).** Concerning the pathogenesis of retained placenta, a growing body of research has supported the role of changes in serum biological constituents and haematological indices during the course of the disease **(Moretti *et al.*, 2015, Endler *et al.*, 2016, Yazlik *et al.*, 2019).** The oxidative stress **(Yazlik *et al.*, 2019; Li *et al.*, 2021),** the disturbance in energy state **(Esposito *et al.*, 2014)** and the activated inflammatory pathways **(Dervishi *et al.*, 2016)** were proved to be strongly implicated in the occurrence of retained placenta.

Until now, it is difficult to understand the complex pathogenesis of retained placenta, so, our study aimed to investigate the changes in some hematological parameters, oxidative and metabolic state, in addition to changes in different inflammatory cytokines and proteins in relation to the pathogenesis of retained placenta in Holstein dairy heifers.

MATERIALS AND METHODS

Ethical approval

The study protocol was approved by institutional animal care and use committee (IACUC) of Alexandria University.

Animals and experimental design

This study was performed from December 2022 to February 2023 on an intensive dairy farm on the Cairo-Alexandria desert road, Alexandria, Egypt. Twenty Holstein dairy heifers, weighing about 500–550 kg, were selected to complete this study. The animals were apparently free from any affections or diseases, and they were fed a total mixed ration (18% crude protein) **(NRC, 2009)** with free access to water and salt cubes. All the heifers were around parturition, and their delivery was accomplished without any difficulties or birth help. These animals were grouped into two equal groups, as follows: **Group-I:** including the heifers that successfully expelled the placenta within 6–8 hours after parturition and represented as control healthy group. **Group-II:** the heifers that suffered from retained foetal membranes, as the placenta dropping did not occur up to 12 hours after the parturition **(Hashem and Amer, 2009).**

Blood sampling

Blood samples were obtained from the tail vein of the animals in groups I and II, 24 hours after parturition. Part of the blood was drained into EDTA containing tubes to perform the haematological studies (evaluation of the erythrogram and leukogram). Another part of the blood was drained into a plain vacutainer and left to coagulate at room temperature to obtain serum through centrifugation at 3000 r.p.m. for 10 minutes. The serum aliquots were kept at -20 °C for further evaluation of the biochemical parameters.

Detection of inflammatory cytokines and proteins

The serum concentration of interleukin-8 (IL-8), interleukin-13 (IL-13) and mucin-1 (MUC-1) were evaluated using species specific ELISA kits (Cusabio, China). Also, alpha-1anti-trypsin (AAT) concentration was determined using species specific ELISA kit (Mybiosource, USA).While serum level of C-reactive protein (CRP) was detected using rapid latex slide test method (Spectrum, Egypt).

Evaluation of serum oxidant/antioxidant state

The serum levels of total anti-oxidant capacity (TAC) **(Koracevic *et al.*, 2001),** malondialdehyde (MDA) **(Ohkawa *et al.*, 1979),** reduced glutathione (GSH) **(Beutler *et al.* 1963)** and the serum activity of catalase enzyme (CAT) **(Aebi, 1984)** were detected using commercially available kits (Biodiagnostic, Egypt) according to the manufacturer's instructions.

Detection of prostaglandins level

ELISA based serum level of prostaglandin-E2 (PGE-2) (Abcam, USA) and prostaglandin F-2 alpha (PGF-2α) (Arch Biotech Pvt, Ltd, India) were determined according to the manufacturer's instructions.

Evaluation of some metabolic parameters

The level of non-esterified fatty acids (NEFA) **(Duncombe, 1964)** (Zen-bio, Inc., USA) and creatine kinase enzyme activity (CK) according to **Foreback and Chu (1981)** (Biosystem, Spain) were detected in serum samples according to the manufacturer's instructions. .

Hematological studies

Red blood cells (RBCs) count, hematocrit percent (HCT %), hemoglobin concentration (Hb) and platelets count (PLT) were detected. In addition, total leukocytic count (TLC), granulocytes, lymphocytes and monocytes counts were evaluated using special veterinary automated cell counter (Exigo®, H400, Swedan).

Statistical Analysis

Independent samples *t-test* was used to detect the difference between means of the evaluated parameters by the aid of SPSS 16.0 software package for widows. All the values are expressed as mean ± standard deviation (SD).

RESULTS

Serum inflammatory cytokines and proteins

The heifers affected with retained placenta (Group-II) recorded a significant decrease in the serum level of IL-8 with a significant elevation in the serum level of IL-13, MUC-1, CRP and alpha-1anti-trypsin (AAT) when compared to control group as present in Table (1).

Table 1: Changes in the serum concentration of some inflammatory cytokines and proteins among the healthy Holstein dairy heifers and those suffering from retained placenta.

Parameter	Group-I (Healthy)	Group-II (Retained placenta)
IL-8 (pg/ml)	209.40±13.71	101.90±10.00***
IL-13 (pg/ml)	54.70±6.75	129.60±10.23***
MUC-1 (ng/ml)	27.90±2.95	65.10±5.70**
CRP (mg/L)	9.67±0.81	21.63±1.95**
AAT (µM/L)	18.90±2.46	54.30±5.41***

All values are expressed as mean ± SD.
Significant ($P < 0.01$), * Significant ($P < 0.001$)

Serum oxidant/ antioxidant state

Table (2) showed that, serum level of TAC recorded a significant decrement in the heifers affected with retained placenta; this decrement was associated with a significant decrease in the serum activity of CAT enzyme and GSH level and a significant elevation in malondialdehyde (MDA) when compared to the control group.

Table 2: Changes in the serum concentration of some oxidant/ antioxidant parameters biomarkers among the healthy Holstein dairy heifers and those suffering from retained placenta.

Parameter	Group-I (Healthy)	Group-II (Retained placenta)
TAC (µM/L)	707.70±25.67	465.80±19.10***
CAT (U/ml)	5.41±0.40	3.09±0.25**
GSH (µM/L)	6.27±0.38	3.43±0.31***
MDA (µM/L)	3.14±0.36	6.22±0.33***

All values are expressed as mean ± SD.
** Significant at ($P<0.01$) *** Significant ($P<0.001$)

Serum concentration of prostaglandins

The serum concentration of prostaglandin-E2 was elevated in the heifers affected with retained placenta, while their serum level of prostaglandin F-2 alpha recorded a significant decrement as compared to the heifers of group-I (Table: 3).

Serum concentration of metabolic parameters

As present in Table (3), in comparison with control group heifers, serum concentration of NEFA and serum activity of CK enzyme recorded a significant increase in heifers affected with retained placenta.

Table 3: Changes in the serum concentration of PGE2, PGF-2α, NEFA and CK among the healthy Holstein dairy heifers and those suffering from retained placenta.

Parameter	Group-I (Healthy)	Group-II (Retained placenta)
PGE(ng/ml)	16.38±1.85	33.76±3.69**
PGF-2α (ng/ml)	3.19±0.22	2.20±0.17**
NEFA (µM/L)	267.40±15.43	333.30±16.66**
CK (U/L)	467.80±37.01	678.20±28.87**

All values are expressed as mean ± SD.
** Significant ($P<0.01$)

Hematological findings

As shown in Table (4), RBCs count, Hb concentration, HCT % and platelets count did not record any significant change in heifers with retained placenta as compared to the healthy cows of group-I. However, and in comparison with control group, the TLC, granulocytes, lymphocytes and monocytes count showed significant increment in the heifers affected with retained placenta.

Table 4: Changes in the hematological parameters among the healthy Holstein dairy heifers and those suffering from retained placenta.

Parameter	Group-I (Healthy)	Group-II (Retained placenta)
RBCs ($\times 10^6$/µL)	5.64±0.17	5.75±0.15 NS
HCT (%)	35.32±0.27	35.66±0.20 NS
Hb (g/dl)	11.09±0.26	11.14±0.28 NS
PLT ($\times 10^3$/µL)	460.00±24.84	463.50±29.99 NS
TLC ($\times 10^3$/µL)	6.01±0.23	7.72±0.21***
Granulocytes ($\times 10^3$/µL)	2.26±0.06	2.66±0.08**
Lymphocytes ($\times 10^3$/µL)	3.80±0.16	5.06±0.23***
Monocytes ($\times 10^3$/µL)	0.30±0.02	0.37±0.03*

All values are expressed as mean ± SD.
NS, ($P<0.05$). *** Significant ($P<0.001$).
** Significant ($P<0.01$)

DISCUSSION

Retained placenta is one of the common problems that affect highly yielding dairy cows in the post-partum period **(Rabbani *et al.*, 2010).** For the researchers, the investigation of the complex alterations that accompany the pathogenesis of retained placenta is a massive challenge **(Li *et al.*, 2021).** Inflammation acts as a cornerstone in the parturition initiation and expulsion of the foetal membranes, as the immune system begins to recognize the foetal antigens as foreign material and begins to attack the uterine placental junction in a similar way as the events of the graft rejection **(Jaworska and Janowski, 2019).** Several inflammatory cytokines share fundamental roles in this process, including IL-8, as it acts as a chemo-attractant to the neutrophils towards the cotyledons at parturition to facilitate the separation process, so a decrease in IL-8 would disturb neutrophil function and chemotaxis and delay the separation of the placenta **(Kimura *et al.*, 2002).** On the other hand, IL-13 is considered one of the most important anti-inflammatory cytokines due to its ability to inhibit monocytes inflammatory actions **(Zurawski and Varies, 1994).** Side by side with IL-13, AAT is one of the serpin protein family members that is produced by the liver in response to inflammation. It has anti-inflammatory and proteinase inhibitory effects **(Janciauskiene *et al.*, 2011),** as well as the ability to inhibit neutrophil migration and chemotaxis **(Al-Omari *et al.*, 2011).**

As a result, the recorded decrease in production of IL-8 with the increase in synthesis of IL-13 and AAT in heifers suffering from retained placentas would disturb the inflammatory signalling of parturition and the separation of foetal membranes. MUC-1 is a transmembrane protein that is secreted by the epithelial cells of the endometrium as a defensive mechanism against infections **(Brayman *et al.*, 2004).** In the same manner, CRP is an acute phase protein (produced by hepatocytes) that increases in many cases, including the presence of pathogens and their related inflammation **(Du Clos and Mold, 2001)** and its level would increase in response to metritis **(Li *et al.*, 2010; Kaya *et al.*, 2016).** The increment in MUC-1 and CRP in heifers suffering from retained placenta would support the theory of induction of uterine inflammation (metritis) as a consequence of the presence of retained foetal membranes. Moreover, there is a well proven relationship between oxidative stress and the failure of foetal membrane expulsion **(Endler *et al.*, 2016; Yazlik *et al*., 2019)** as oxidative stress may increase the risk of foetal membrane retention **(McNaughton and Murray, 2009).** Concurrently, the diminished level of antioxidants in the placental tissues prior to parturition was suggested to predispose to the occurrence of retained placenta **(Wischral *et al.*, 2001).** Oxidative stress and redox state imbalance were previously detected in cases of retained placenta in cattle **(Kankofer, 2001; Perumal *et al.*, 2020, Li *et al.*, 2021).** All the previous may serve as clues for the relationship between the retained placenta state and oxidative stress and may offer sufficient explanation for the increase in malondialdehyde (MDA) and the exhaustion of TAC with enzymatic and non-enzymatic anti-oxidants (GSH and CAT).

The decrement in PGF2α level in heifers with retained placenta could be explained on the basis that some researchers have proposed a physio-mechanism for the occurrence of retained placenta, which begins with a decrement in the antioxidant capacity at the level of placenta, which may decrease estradiol production and in turn decrease PGF-2α production **(Wischral *et al.*, 2001; Yasuhara *et al.*, 2019).** The decrease in these hormones may reduce uterine contractility and favour the retention of foetal membranes **(Attupuram *et al.*, 2016).** Additionally, the increase in concentration of PGE-2 in heifers of group II may be due to the fact that PGE-2 is an anti-inflammatory molecule **(Herath *et al.*, 2006; Sugimoto and Narumiya, 2007),** so it would increase in response to the presence of inflammation (metritis). Also, the increase in concentration of PGE2 may be a consequence of the increment in IL-13 production **(Yu *et al.*, 1998)** to potentiate its anti-inflammatory effect. Unfortunately, a PGE-2 increase would suppress the production of PGF2α as the uterine glands switched their production from PGF2α to PGE2, and this may decrease uterine contractions and foetal membrane expulsion **(Manns *et al.*, 1985).** During the early post-partum period, liver gluconeogenesis and fat mobilization would increase to offer sufficient glucose for milk lactose synthesis **(Bauman and Currie, 1980).**

Excessive fat mobilization could increase the serum concentration of NEFA **(Seifi *et al.*, 2007).** The elevated level of NEFA is strongly associated with the retained placenta **(Ospina *et al.*, 2010)** as this increase, as in the case of heifers in group II, would enhance the immune suppression that shares in the retained placenta occurrence **(Hammon *et al.*, 2006).**The increase in serum activity of CK enzymes, which are present in skeletal muscles in heifers suffering from retained placenta, may be owed to muscle protein degradation due to the massive demand for energy with insufficient fat mobilization **(Yazlik *et al.*, 2019).** Concerning the haematological findings of this study, leucocytosis, granulocytosis, and lymphocytosis in the animals suffering from RP may be attributed to the initiation of uterine inflammation, as the delay in the uterine involution could make the uterine lumen more susceptible to the infection, which in turn may enhance and predispose to the occurrence of puerperal metritis and pyometra **(Farzaneh *et al.*, 2006; Beagley *et al.*, 2010).** In the presence of inflammation, the removal of uterine debris requires the presence of scavenger phagocytic cells (monocytes), which may explain the monocytosis detected in RP-affected group **(Perumal *et al.*, 2020).**

CONCLUSION

In summary, we can conclude that, retained placenta is one of the most critical cases that affect the productivity of dairy cows. Based on our findings, heifers with retained placenta were suffering from inflammatory and anti-inflammatory cytokine alterations as well as redox balance disturbances besides metabolic disturbances. Also, prostaglandin concentration changes and some haematological alterations were manifested in these cows. Collectively, the previous changes are fundamentally implicated in the pathogenesis of retained placenta in dairy heifers.

Competing interest

There is no conflict of interests of any sort between authors or elsewhere.

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