Predictive biochemical and oxidative markers for dairy cows with and without retained fetal placenta

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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) are considered one of the main reproductive disorders in dairy cattle. Highlight the effects of Excede® (Ceftiofur Crystalline Free Acid: Zoetis, USA) in combination with Oxytocin (Oxytocin®, each ml contains 10 IU oxytocin, ADWIA, Egypt), Estrumate® (250µg cloprostenol: synthetic analogue of PGF2α) and Flunixin Meglumine® (nonsteroidal anti-inflammatory drug) on the reproductive performance in dairy cows. 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).

Reproductive performance parameters for all studied cows were days to first estrous, number of service per conception and pregnancy rate. All reproductive parameters demonstrated significant (P < 0.05) changes between treated animals. 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. Altogether, the obtained results indicated that retained fetal membranes in cows could be treated successfully without any interference by systemic injection of cephalosporin with caring of temperature. The reproductive performance criteria for all cows studied were the time of first estrus, the number of services per conception, and pregnancy rates. All parameters of reproductive performance showed significant changes (P < 0.05) among the treated animals.

The results also indicated that cephalosporin can be used by systemic injection to treat placental retention with caring of the animal's temperature.

Introduction

Anatomy of placenta in dairy cows
The surface of the endometrium in a pregnant cow has four rows of specialized endometrial regions known as caruncles along the long axis of both horns, with a total of 60 to 80 convex and ovoid structures (Duello et al., 1986; Kohan-ghadr, 2011).

Hydrostatic pressure of the allantoic and trophoblastic membrane against the uterine wall is believed to facilitate chorioallantoic attachment to the caruncle and stimulate the fetal chorion to vascularise and hypertrophy. As the cotyledons mature, the remodeling of the endometrium that leads to the development of the caruncles is necessary to accommodate the specialized folding of the chorioallantois. The surface of contact between maternal and fetal tissues is increased by the development of outgrowths on the surface of chorion, known as the villi. These chorionic villi consist of vascular mesenchymal cones surrounded by cuboidal trophoblastic and giant binucleate cells bring the fetal (allantoic) vessels into proximity with the maternal blood vessels. The temporal and spatial changes of the extracellular matrix (ECM) during apposition, adhesion and attachment increase the complexity of placental formation and implantation process (Kohan-ghadr, 2011; Santos et al., 2017).

**Placentomes:**

Placentomes appear as smooth, flattened, and semicircular domed elevations. At first, they are detected only in the area immediately surrounding the embryo. By Day 60, they are present in the uterine horn, both proximal and distal to the embryo, although those near the embryo are larger. Placentomes are not macroscopically observable until approximately Day 37. Initially, from Days 37 to 40, a maximum of 20 placentomes are present on the chorionic surface in the embryo. From Days 40 to 50, the number of placentomes triples. Subsequently, the number increases gradually up to Day 70, averaging approximately 80 to 90 (range, 20–120), based on the number of cotyledons observed on the chorioallantoic surface (Neto et al., 2010, Adeyinka, 2012; Lemley and Camacho, 2021).

The placentomes are linked together by areas of flat apposition between trophoblast and glandular uterine epithelium (interplacentomal areas). Gland openings are covered by domes of phagocytic trophoblast, which facilitate histotrophic uptake of endometrial gland secretions by small endocytotic vesicles or much larger phagocytic vesicles. The trophectoderm is remarkably versatile, with great capacity for invasion, cell fusion, hormone production, specific nutrient absorption, selective transport, active metabolism, and finally for its ability to resist maternal immunological attack. This epithelium, together with internal membranes and blood vessels, form the chorioallantoic placental structures from Day 30 to term (Peter, 2013).

The sizes of the placentomes vary depending on location. For example, placentomes closer to the middle of the uterine horn containing the fetus and nearest to the attachment of the middle uterine artery are the largest (Roberts, 2004, Blankenvoorde, 2011; Redifer et al., 2021).

Placentomes are larger and more numerous closer to the fetus and in the horn containing the fetus relative to those located in the uterine extremities and those found in the contralateral horn. Total placentome weight and length increase until Day 190, attaining 13 approximately 4.5 kg and 10 to 12 cm (Kohan-ghadr, 2011; Schlafer et al., 2000) with most acquiring a mushroom-like shape with an occasional flat configuration (Miles et al., 2004).
The total number of placentomes does not correlate with the increased fetal nutritional demands of late pregnancy suggesting that there is an alteration in the pattern of vasculature to increase feto/maternal exchange without an increase in placentome number (Laven and Peters, 2001; Leiser et al., 1997).

**Fetal membrane**

The placenta is literally a "sac" covering and allowing the fetus to be nourished and developed until the process of parturition. The placenta is considered an essential organ for the prenatal transfer of nutrients and oxygen from the dam to the fetus. The other functions of the placenta are to provide a reservoir of blood for the fetus (Elmetwally and Bollwein, 2017; Gohar et al., 2018). Nutritional delivery to the fetus is determined by several essential parameters, including placental growth and development, utero-placental blood flow, nutrient availability, and placental metabolism and transport capacity (Dunlap et al., 2015). Previous research from Dr. Fuller W. Bazer's lab and others has highlighted the importance of amino acids and their metabolites in normal fetal growth and development, as well as the importance of amino acid transporters in nutrient delivery to the fetus (Elmetwally et al., 2022).

During the first stages of pregnancy, the placenta secretes amino acids. It is well known that amino acids are essential for the conceptus's (the embryo/fetus and related placental membranes) growth (Elmetwally et al., 2018; Lenis et al., 2018). Amino acids are essential for the production of tissue proteins and also act as cell signalling molecules, hormone secretion regulators, antioxidants, and hormone secretion regulators (Wu et al., 2013). Biologically significant non-protein molecules such as nitric oxide, polyamines, neurotransmitters, amino sugars, purine and pyrimidine nucleotides, creatine, carnitine, porphyrins, melatonin, melanin, and sphingolipids are all synthesised using amino acids as key building blocks (Wu, 2009).

The main hormones produced by the placenta are human chorionic gonadotropin, equine chorionic gonadotropin, lactogenic hormone, progesterone, and estrogen hormone. When the fetus is born, the placenta normally attaches within a short time and is expelled. That is why it is referred to as "afterbirth". (Ball and Peters, 2004; Napso et al., 2018).

The drop of fetal membranes postpartum is a physiological process that involves loss of feto-maternal adherence, combined with contraction of the uterine musculature, and is usually accomplished within 6 hours of calving. (El-Malky et al., 2010 and Gaafar et al., 2010).

During pregnancy, nitric oxide (NO), a byproduct of arginine catabolism and secreted by the trophectoderm cells, is essential for controlling placental angiogenesis and fetal-placental blood flow (Chen and Zheng, 2014; L P Reynolds et al., 2005). Polyamines (polycationic compounds made from ornithine) was proved to control cell division, differentiation, and function as well as controlling DNA and protein synthesis, ion channel activity, signal transmission, and gene expression, (Wu et al., 2005). It should be highlighted that a thorough collection of studies looking at the function of specific nutrients and their transporters during the early peri-implantation period in the ruminant conceptus' development have been reported (Berkane et al., 2017; Raguema et al., 2020; Reynolds et al., 2005).

**Retained placenta in Holstein dairy cows**
Retained placenta is a pathological condition defined as failure to expel fetal membranes within 24 hr after parturition (Dervishi et al., 2016). Retained placenta is one of the most common puerperal disorders affecting the reproductive performance of dairy cows (LeBlanc, 2008; Mahnani et al., 2021b). The incidence varies from 4.0-16.1% but can be much higher in problem herds (Mahnani et al., 2021a). Abortion, stillbirth, and twin calving resulted in increased incidence rates to 25.6, 16.4, and 43.8%, respectively (Tucho and Ahmed, 2017).

Fetal membrane retention and metritis are linked to substantial immunological and metabolic alterations that predispose cows to periparturient illnesses. Kimura et al. (1999) revealed that parturition-related colostrogenesis/lactogenesis impacted CD62L expression prepartum, leukocyte count postpartum, and neutrophil killing activity postpartum (Kimura et al., 1999). Cows with a negative energy balance during early lactation exhibit lower blood polymorphonuclear leukocyte (PMNL) phagocytosis, chemotaxis, and diapedesis than cows with a positive energy balance by mid-lactation (Stevens et al., 2011). As a result, it is not unexpected that roughly 60% of viral illnesses affecting dairy cows during lactation are observed in the first 30 days of lactation (Pinedo et al., 2020).

Retained placentae typically lead the cow to miss the next pregnancy by 2–6 months, have a later calving date the following year, and may result in an open cow the following year. The prevalence of retained placenta in dairy cows is related to breed, age, parity, and body condition score (Newby et al., 2014).

**Risk factors affecting the retained placentae in dairy cows:**

**Metritis**

Metritis is considered a symptom of a poorly functioning immune system during the transition period (Goff, 2008), and it predisposes to an increase in the incidence of RFM. Shortly after parturition, foetal membranes become a “foreign body,” and their ejection is dependent on the maternal immune system detecting and destroying them (Gunnink, 1984). Cotyledons from RFM cows were less leukocyte chemoattractant than cotyledons from cows that ejected the placenta regularly after calving (Gunnink, 1984). Furthermore, in vitro tests revealed that leukocytes from cows with RFM were less able to detect cotyledon tissue in a chemotaxis experiment before, during, and after calving than leukocytes from cows without RFM (Gunnink, 1984).

From the prepartum period until 1–2 weeks after calving, polymorphonuclear leukocytes PMNL from cows with RFM have diminished reactivity to chemoattractants and lower phagocytic capability (Cai et al., 1994; Kimura et al., 2002). Delayed foetal membrane evacuation exposes the uterus to environmental infections and pollution as well as increasing the risk of metritis. Furthermore, the majority of cows have postpartum uterine contamination, (LeBlanc, 2010), and macrophages and PMNL serve as the first line of defense against this contamination, moving from the bloodstream to the uterus. The immune system is unable to quickly clear bacterial contamination in immunocompromised cows, and bacterial proliferation in the uterus causes metritis (LeBlanc, 2010).

Metritis is distinguished by a foul-smelling, red-brown watery discharge from the uterus within the first 21 days following calving, which may or may not be followed by systemic symptoms (i.e. pyrexia, anorexia, depression). Hammon et al., (2006) found that PMNL function was decreased during the periparturient phase in cows with puerperal metritis and subclinical endometritis compared to healthy cows.
During the last few decades, many hypotheses have been proposed and a great number of studies have been conducted to understand the etiological factors and pathobiology of RP. In addition, several blood biomarkers have been proposed as indicators of the risk of RP in dairy cows (Dervishi et al., 2016). Retention of the placenta (ROP) denotes failure of the fetal villi to separate from the maternal crypts i.e. the lack of placental dehiscence. (Tucho and Ahmed, 2017).

The effects of infectious diseases, hormonal imbalance, and inflammatory status on the incidence of retained placenta in cows:

Infectious disease plays an important role as a causative agent for retained placenta in dairy cows. In order to preserve the placenta, four primary ideas have been offered throughout the years: uterine atony, chorionic villi edema, inflammatory conditions, and neutrophil inactivation (Elmetwally, 2018; Lai et al., 2022). It was proposed that the decrease in neutrophil functions prior to parturition supported the latter idea (Kimura et al., 2002; Mordak and Stewart, 2015). Later, LeBlanc (2008) and Wagner et al. (2017) proposed that RP, metritis, and endometritis are illnesses of immune function that begin at least 2 weeks before birth (LeBlanc, 2008; Wagener et al., 2017). Additionally, Ametaj et al., (2010) postulated that endotoxin may be involved in all four states by decreasing uterine atony, generating chorionic villi edema, commencing the inflammatory state, and promoting neutrophilia due to the neutrophil's inability to migrate to inflammatory tissues (Ametaj et al., 2010).

Pathogenic bacteria that induce localized tissue damage and inflammation are present in the uterus after calving, which is a key characteristic of uterine disorders. The bacteria Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum, and Prevotella melaninogenica are frequently cultured from cows with uterine illness (Sheldon et al., 2020).

Infectious causes are associated with Brucellosis, Salmonellosis, Leptospirosis, and Listeriosis. Such retention creates a number of problems by allowing microorganisms to grow inside the uterus, causing inflammation, fever, weight loss, decreased milk yield, longer calving intervals, and possibly an open cow during the next year; if the infection is so bad, the animal may actually die (Han and Kim, 2005).

Steroids are important in pregnancy maintenance and parturition because they regulate prostaglandin production. Progesterone promotes the production of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), and estrogens raise the levels of prostaglandin F\textsubscript{2} (PGF\textsubscript{2}) in the uterus (Wango et al., 1992; Wooding et al., 1996). Although progesterone levels are higher in animals with placental retention (Rasmussen et al., 1996), estrogen levels are lower (Beagley et al., 2010). Of note, cows with retained fetal membranes had higher cortisol and lower estrogen levels in late pregnancy, which affect immune function by exerting local and systemic immunosuppressive effects. Uncomplicated fetal membrane retention is unattractive and difficult for animal workers and milkers, but it is often not damaging to the cow (Hartmann et al., 2013; Kindahl et al., 2002).

The effect of management, nutrition, and genetic traits on the incidence of retained placenta in cows:

Manegamental causes of retained placentas include stress, genetic traits, inbreeding, and obesity (Joosten et al., 1991). Lack of exercise and hypocalcemia are the most frequent causes of decreased myometrial contractility. Stress (Transportation, rough handling, poor feed conditions, isolation from group, lameness,)
results in elevated corticosteroids and increased risk of placental retention. Dairy producers have suggested that poor health management in herds can predispose animals to the retention of the placenta (Fricke, 2001).

In addition to this deficiency of antioxidants, vitamin E and selenium may decrease chemotaxis and leukocyte numbers at the fetomaternal junction, thus contributing to the retention of fetal membranes (Amin and Hussein, 2022; Bourne et al., 2007). Over-condition and under the condition as well as managemental defects and environmental factors can result in the retention of the placenta (Hayirli et al., 2002).

Retained placenta incidence would be reduced through dietary management of older cows for optimum body condition and minimal incidences of milk fever. The most crucial management factors for preventing retained placenta in heifers are proper growth rates that cause heifers to calve at 600 kg and the selection of calving ease sires (Barański et al., 2021; Mahnani et al., 2021a).

Nutritional causes of RP are primarily due to the deficiency of feed during the last 6 to 8 weeks before calving, especially when there is a deficiency in the content of minerals and vitamins in the diet. (Alšić et al., 2008; Spears and Weiss, 2008)

Heavy grain feeding may be associated with both higher milk production and an increased risk of reproductive disorders such as dystocia, retained placenta, cystic ovaries, and metritis. (Jorritsma et al., 2003). Vitamin and mineral deficiency conditions such as selenium, vitamin E and vitamin A, β-carotene, and a disturbed Ca/P ratio can impair general immunity, alter the competence of the cellular self-defense mechanism, and increase the risk for placental retention and metritis (Ahmed et al., 2009). High milking cows with a greater degree of negative energy balance prepartum and higher NEFA concentrations were more likely to suffer from RP (LeBlanc, 2010).

On the other hand, over-conditioned cows were shown to be more sensitive to a retained placenta and subsequent infertility than cows with normal body condition scores. (Madushanka and Ranasingha, 2016; Markusfeld et al., 1997).

No significant differences were found in the incidence of the retained placenta during green (winter) and dry (summer) feeding (26.20% vs. 22.90%), respectively. Also, Deyab, (2000.) and Gabr, et al., (2005) found non-significant differences between feeding systems in the percentage of retained placenta.

In regards to the genetic traits, it was proven in the past, a concentration on production traits resulted in declining trends in health, reproduction, and longevity traits, which can lead to a decrease in herd profitability (Fleming et al., 2019, Erasmus and van Marle-Köster, 2021; Luo et al., 2021). To minimize the harmful impact of years of choosing primarily productivity and conformation traits, several countries began to integrate health traits into their breeding objectives (Egger-Danner et al., 2015).In Egypt, the dairy herd health monitoring system has tracked fertility-related diseases and reproductive abnormalities, and RP was first incorporated with other health features (Hamed and Kamel, 2021).

**Cow’s Body Weight, Calves’ Birth Weight:**

The percentage of retained placenta increases significantly with increasing live body weight in cows due to the increment in fat adipose tissues (Madushanka and Ranasingha, 2016; Markusfeld et al., 1997), which
may result in trapping the steroid sex hormones. With increasing fetal birth weight, a major rise in retained placental issues occurs. (Gaafar et al., 2010). The cause may be due to fetal pressure on the placenta and fetal membrane. This increases the incidence of placental retention to reinforce the bond between the cotyledons and the fetal membrane.

Estrone sulphate content did not significantly correlate with gestational length, calf birth weight, placenta weight, or newborn calves’ survivability. These findings suggest that variations in plasma estrone sulphate content between Japanese beef cattle and Holstein dairy cattle are very similar. Estrone sulfate's plasma concentration is related to the breed of the pregnant cow, and depending on the breed of bull, it is also influenced by calf birth weight. In Japanese beef cattle, it appears to be possible to predict the incidence of retained placenta but not the calf birth weight or survivability of newborn calves (Isobe et al., 2003).

**Twining and Sex of calves:**

It suggested that with twinning birth, the retained placenta incidence (37.9% vs. 24.20 in Friesian cows was higher than with singleton %) (Gaafar et al., 2010). Increased management of twin-producing dams and their calves during the calving season is required in order to achieve increased productivity with twinning in cattle due to the shorter gestation period and the increased incidence of retained placenta and/or dystocia. The periparturient identification of twin pregnancies can enable obstetrical help to allow delivery of twin calves and to increase newborn survival (Echternkamp and Gregory, 1999).

In Friesian cows, the percentage of retained placenta was insignificantly higher with born male rather than female calves (26.50 vs. 23.20) respectively; the slight increase in the percentage of retained placenta observed with born male calves may mean that the fetal androgenic hormone from the fetal testes may have partially affected the placenta retention process. (Gaafar et al., 2010).

**Parity, body condition scores and seasons:**

Parity had a major impact on the occurrence of RFM milk for cow, 5th parity were reported as the highest occurrences of retained placenta (Islam et al., 2012). Furthermore, it was proven that calf birth weight has a positive correlation with placental weight, (Echternkamp and Gregory, 1999).

On the other hand, dystocia and stillbirth in primiparous cows raised the odds ratios (OR) of RP by 4.30 and 3.33 times, respectively. Dystocia, twinning, and stillbirth in multiparous cows raised the OR of RP by 4.36, 3.94, and 1.29 times, respectively (Mahnani et al., 2021a).

The state of the body had a major influence on the incidence of RFM following the birth of milk cows. In fair, good, and bad conditions, the incidence of retained placenta in cows is 7.1%, 3.2%, and 3.1%, respectively. However Sarder et al., (2010) reported that in good health, the prevalence of retained placenta was higher and lower in fair and other cows in bad condition, respectively. These variations may be due to several risk factors, such as uterine contractility, last type of calving, pregnancy body condition, postpartum estrus period of onset, stillbirth, twin birth, calving month and season, hereditary, gestation duration, dietary deficiency, difficult delivery, age and parity of the cow, and other uterine infections that predispose the cows to retention after birth, carotene, vitamin A, progesterone, and estrogen imbalance at parturition. (Sarder et al., 2010)
Researchers have found that in fall parturitions, the incidence of retained incidence is less than 40% relative to spring (Binabaj et al., 2014; Echternkamp and Gregory, 1999). The occurrence of retained placenta was lower in the cold months of the year than in the warm months. (DuBois and Williams, 1980; Mahnani et al., 2021a). In contrast, Ghavi Hossein-Zadeh and Ardalan, (2011) and Wetherill, (1965) reported that the incidence of retained placenta in parturitions was lower in the summer in comparison to the winter and spring.

Birth comparisons were made between the weights of calves born during cold and warm times. Calves born in cold periods have a higher birth weight relative to calves born in warm periods (1.12 kg) (Binabaj et al., 2014). Moreover, heavier birth weights of calves born during the cold period suggest that they have a larger, more physiologically distinguished, and mature placenta, resulting in decreased placenta retention. (Echternkamp and Gregory, 1999).

The effects of gestational length on the incidence of retained placenta in dairy cows:

The increased risk of placenta physiological immaturity at a delivery time is due to the shortening of the gestation period because the relations of cotyledon and caruncle do not fully separate and part of the placenta remains in the genital period.

It was reported that the gestation period in cows with retained placenta was 3.3 to 5.25 days shorter than in cows without retained placenta (Muller and Owens, 1974., DuBois and Williams, 1980). A gestation period shorter than 274 days caused a doubling of the incidence of retention rates (Mahnani et al., 2021a; Vieira-Neto et al., 2021). However it is often noticed that the length of the gestation period does not affect the incidence of retained placenta (Kok et al., 2021)

Induced parturition had a higher incidence of placenta retention than spontaneous parturition (21.0 vs 0.0%), but there was no difference between cows treated with prostaglandin F\textsubscript{2α} or fenprostalene (19.2 vs 22.6%). By administering prostaglandin F\textsubscript{2α} or fenprostalene together with dexamethasone, an acceptable level of parturition synchrony was achieved (Königsson and Gustafsson, 2002; Wischral et al., 2001).

Different Concepts Of Placental Separation In Dairy Cows:

Fetal membranes are normally expelled within two to eight hours of parturition. Pathological retention of fetal membranes beyond 12 hours can be considered (Hooshmandabbasi et al., 2018; Kindahl et al., 2004). Usually, the uterus contracts approximately 14 times/hour immediately after parturition, but at 42 hours, the frequency steadily decreases to one per hour. Delayed involution of the uterus is normally connected with membrane retention (Hartmann et al., 2013; Wiebe et al., 2021).

Maternal immunological recognition by trophoblast cells of fetal MHC class I proteins triggers an immune/inflammatory response that leads to placental separation during parturition. According to Tucho and Ahmed, (2017), normal separation and placenta delivery processes are multifactorial and begin before parturition. (Fig. 2).
The maturation of the placenta plays an important role in the detachment loosing and/or detachment process of the placenta in dairy cows. The decreased number of binucleate trophoblast giant cells in the fetal chorion is an important feature that facilitates the separation of the placenta (Fig. 2, Seo et al., 2019).

Significant differences between RFM cows and cows that shed their fetal membranes on time have been identified in terms of serum/plasma cholesterol, urea nitrogen, glucose, total protein, non-esterified fatty acids, and -hydroxybutyric acid (Zhang et al., 2002).

Following a normal parturition, foetal membranes should be evacuated in less than 8 hours; hence, their retention for more than 8 to 12 hours is inappropriate (Amin and Hussein, 2022; Gohar et al., 2018). RFM frequently develops from abortions that happen during the second part of pregnancy, whether they are random or contagious. When compared to normal parturitions, hydrops, uterine torsion, twinning, and dystocia generally increase the incidence of RFM (Beagley et al., 2010; Szelényi et al., 2022). It is also made worse by heat stress and peripartum hypocalcemia. RFM should be expected in cows that have been induced to calve pharmacologically, such as through the administration of exogenous corticosteroids. Nutritional factors like carotene and selenium deficiency as well as over-conditioning of dry cows have been implicated (Garcia-Ispierto et al., 2022; Klisch and Schraner, 2021).

RFM, metritis, and miscarriage are all linked to low levels of vitamin A, which occur in hyperkeratosis and polybrominated biphenyl poisoning. Low selenium cattle may be more susceptible to RFM, metritis, and cystic ovaries in selenium-deficient locations. Vitamin E, which has been demonstrated to improve neutrophil function, may potentially be at play. Selenium levels in cattle fed stored diets from selenium-deficient areas should be regularly checked, and supplements should be given as needed. Vitamin E and selenium may be linked to RFM either through altered neutrophil activity or a simple lack (Chebel, 2021a; Moghimi-Kandelousi et al., 2020). After giving birth, cows with RFM may be more susceptible to developing the condition the following year. What's more, epidemiological research indicates that cows with RFM are more likely to have metabolic disorders, mastitis, metritis, and subsequent abortions (Amin and Hussein, 2022; Chebel, 2021b; Melendez et al., 2021). Therefore, related disorders pose a risk even if many cows with RFM remain asymptomatic with regard to their immediate uterine health. RFM-related neutrophil dysfunction in periparturient cows has been linked to the condition, which helps to explain why animals with the condition have decreased resistance to uterine and other infections (Mordak and Stewart, 2015; Moretti et al., 2016).

As demonstrated by the degenerative left shift in the leukogram seen in some septic metritis patients, cattle with acute metritis associated with RFM may also have a depletion of neutrophils in the peripheral blood as a result of acute recruitment of neutrophils to the infected uterus (McNaughton and Murray, 2009). Although most calves with RFM do not develop septic metritis or chronic endometritis, the need to treat RFM stems mostly from the inability to foresee which cows may experience clinically severe consequences (Moretti et al., 2015; Pathak et al., 2015).

The idea that RFM is mediated by reduced neutrophil function starting in the late dry period is supported by recent findings (Moretti et al., 2016). In cows that later develop RFM, decreased neutrophil migration into tissue extracts of placentomes can be seen as early as two weeks before calving. These cows are also less able to perform other neutrophil tasks, such as oxidative burst, which is a part of the neutrophils' bacterial
killing action (McNaughton and Murray, 2009; Mordak and Stewart, 2015). In hypocalcemic cows, impaired neutrophil function has also been seen. In fact, a large number of the etiological factors linked to RFM, such as vitamin and mineral deficiencies, heat stress, or the use of exogenous corticosteroids, have also been linked to impaired neutrophil activity (Daros et al., 2020; Ehsanollah et al., 2021).

**Molecular Changes Associated With Placental Dysfunction In Dairy Cows**

It was hypothesized that the separation of the placenta depends mainly on the uterine and umbilical blood perfusion during the peri-parturient period (Elmetwally and Bollwein, 2017; Hartmann et al., 2013).

Intrauterine fetal growth depends mainly on placental function and blood perfusion. Vascular remodeling of the placenta plays an important role either during the gestational period and/or directly after parturition. Even while placental weight in ruminants reaches its peak by about midgestation, placental vascular beds continue to grow throughout pregnancy (Reynolds et al., 2005a; Reynolds et al., 1985). Rates of uterine and umbilical blood flows, as well as fetal oxygen and nutrition uptakes, rise in tandem with the formation of placental vascular beds throughout gestation (Reynolds et al., 2005b). Despite the fact that angiogenesis is crucial to placental health, little is understood about how the placental blood supply develops. However, there is some evidence to suggest that the placenta's own production of vasoactive substances, such as prostaglandins and glycosaminoglycans, may be the source of the stimulation for the development of placental vascular beds (Reynolds et al., 2005b).

Angiogenesis in particular plays a crucial part in the correct progression of healing since the newly formed blood vessels provide nutrients and oxygen to the growing tissue (Raguema et al., 2020; Song et al., 2020). The secretion of angiogenic proteins from the placenta depends mainly on the NO, INFT (interferon tau), and polyamines secreted by the placenta (trophectoderm cells) (Wu, 2013). The angiogenic response has been attributed in part to several angiogenic regulators, such as growth factors and chemokines, which can also be released by mesenchymal stromal cells (MSCs), normal T cell expressed and presumably secreted (RANTES), and VEGF in fibroblasts and macrophages (Porwal et al., 2021). 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).

The 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, RANTES likely participates in the normal physiology of the endometrial immunological system. (Saed et al., 2022).

One of the most important angiogenic factors in the placenta is the VEGF, which is essential for embryonic vasculogenesis (Porwal et al., 2021) and its expression increases as gestation proceeds. The hypoxia-inducible factor 1 (HIF1A) controls the expression and transcription of VEGF, therefore, the clock network regulates angiogenesis and embryonic development (Johnson and Mowa, 2021). Under low oxygen conditions, HIF1A activates multiple transcription factors, including VEGF which promotes angiogenesis and vascularization in the placenta (Fong, 2008, Agaoglu et al., 2015).
VEGF was characterized by its ability to induce vascularity, and permeability, and promote vascular endothelial cell proliferation. Three families of VEGF proteins and their corresponding receptors have been characterized, and the main receptors involved in the first steps of signal transduction cascades comprise different tyrosine kinase receptors, such as VEGFR-1, VEGFR-2, and VEGFR-3. It was proven that miRNA-185 plays an important role in the regulation of the VEGF signaling pathway in cows suffering from a retained placenta (Zheng et al., 2018).

Across species, some VEGF family members and receptors are found in placentomes, uterus tissues, and oviducts, and in different species including humans, mice, rats, cattle, sheep, pigs, and rabbits (Cheung et al., 2017; Gabler et al., 1999; Kaczynski et al., 2020; Kalkunte et al., 2009; Kazi and Koos, 2007; Llobat et al., 2012; Pfarrer et al., 2006; Złotkowska et al., 2019).

In the fifteen days before parturition, a substantial decrease in IL-1β gene expression was observed and continued to the fifteenth day after delivery, which indicates a disrupted immune response with subsequent RP. Also, Shimizu et al., (2018) found that at 4 weeks postpartum, IL-1β gene expression in peripheral blood mononuclear cells was significantly lower in RP dairy cows than in control cows.

In the normal physiological processes of parturition, IL-6 plays a role as it is expressed in the female reproductive tract and gestational tissues and also regulates placenta growth, and helps in embryo implantation. (Gomez-Lopez et al., 2016).

It was reported that IL-6 affects low P4 levels and activates the uterus genes responsible for normal delivery (Robertson et al., 2010). Moreover, IL-6 stimulates the adaptive immune response, which may take place throughout parturition (Jaworska and Janowski, 2019).

A process called placental maturation involves a decrease in CE and a decrease in TGC numbers and is necessary for the release of bovine fetal membranes. Impaired regulation of the process can lead to the retention of fetal membranes, one of the major reproductive disorders in cattle (RFM). The condition in which the fetal membranes are not expelled from the uterus within 12–48 h postpartum is described as this disturbance. (Dilly et al., 2011).

The loosening of the adhesion of the fetal membranes in the maternal compartment could involve local factors. Both the maternal and fetal compartments are exposed during gestation to rapid growth, angiogenesis, and tissue remodeling. Proteolytic enzymes and the subsequent degradation of extracellular matrix (ECM) components are required for these processes, as well as for the proper release of fetal membranes. Matrix metalloproteinases (MMPs) play a pivotal role in tissue remodeling and ECM breakdown processes during placentation and implantation in several species. (Beceriklisoy et al., 2007).

**Biochemical changes during retained placenta**

For dairy cows to behave very productively and reproductively in the later postpartum phase, lactation must transition smoothly from pregnancy to lactation. However, due to hampered reproduction and productivity, a bad transition frequently causes dairy farmers to suffer significant financial losses (Roche et al., 2017). Understanding the reasons behind and effects of metabolic alterations during this time is crucial for
Variations in serum metabolic marker concentrations for systemic inflammation, liver function, mineral, and energy status, and blood neutrophil counts are associated with healthy postpartum dairy cow neutrophil function. (Bogado et al., 2021).

The most important biomarkers are those related to liver and kidney function, lipid profile, minerals, and oxidative stress.

**Kidney and liver functions**

As a result of tissue destruction and inflammation during the RFM period in attempts to expel the placenta, the production of protein in the liver is partially altered towards the increased synthesis of positive acute phase proteins and reduction of albumin, which is a negative acute phase protein, and globulin elevation, thereby reducing A/G (Hassan et al., 2019). With altered energy metabolism and systemic inflammation, there is a state of reduced liver function in postpartum dairy cows (Osorio et al., 2014). Concentrations of hepatic proteins such as albumin and globulin can alter physiological and pathological conditions. Albumin is a negative acute phase protein, and albumin synthesis in the liver is expected to decrease in the case of infection to facilitate the production of globulin (Trevisi and Bertoni, 2008; Trevisi et al., 2011).

To evaluate the source of the tissue insult, AST activity should be viewed in combination with that of a liver-specific enzyme, such as GGT, or a muscle-specific enzyme, such as CPK (creatine phospho kinase). For clinical and subclinical diagnosis, serum activity of AST is a very sensitive predictor of liver disorders. There were higher levels of GGT and AST in cows with fatty livers. The increase in AST and GGT activity in cows with retained placenta relative to control cows may be due to lipid accumulation in hepatocytes. (Hashem and Amer, 2008; Semacan and Sevinç, 2005b).

Albumin or globulin concentrations are correlated with neutrophil activity, but total protein concentrations at 5 d postpartum were positively linked to the release of reactive oxygen species. (Bogado et al., 2021). In healthy and RP cows, albumin and urea concentrations were similar, but total protein concentrations were higher at 30 ± 4 days after calving in healthy cows. (Yazlık et al., 2019).

**Serum minerals**

Decreased levels of Ca can cause uterine atony, which leads to RP. Furthermore, subclinical hypocalcemia in milk cows is an issue for the first few days after parturition due to excessive demand for calcium for colostrum synthesis and milk production and an insufficient bone response to restore Ca concentration. (Reinhardt et al., 2011).

Serum total calcium concentrations at 5, 10, 14, or 21 d postpartum were > 2.2 mmol/L. So, he did not observe associations of total calcium with neutrophil function, but samples were collected after postpartum hypocalcemia would have passed in almost all cows. RFM and reduced circulating levels of calcium are believed to be associated with one another (Vallejo-Timaran et al., 2021).

Normal ranges for blood calcium (1.97–2.5 mmol/L), blood phosphorus (4.6 to 9 mg/dL), blood magnesium (1.4 to 2.3 mg/dL), milk urea nitrogen (9 to 18 mg/dL), milk BHB (< 0.1 mM), and milk fat/protein ratio (1 to
1.2) were defined a priori (Lu et al., 2020; Vallejo-Timaran et al., 2021).

**Serum Lipid Profile**

Severe RP hyperlipidemia leads to lipid accumulation inside the hepatocytes and subsequently to an increase in liver enzyme activity and liver injury. (Joksimovic-Todorovic and Davidovic, 2013). Liver disorders are often associated with abnormal lipid and lipoprotein concentrations. Several authors reported that the concentrations of triglycerides, cholesterol, and HDL-cholesterol decreased in fatty liver cows. For cows with retained placenta, serum levels of cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol are lower than those of control cows. Several mechanisms, including a decrease in the conversion of VLDL to LDL, could result in a decrease in LDL-cholesterol serum concentration in cows with retained placenta. An increase in LDL catabolism could be another explanation for the decrease in LDL. The decrease in serum HDL-cholesterol levels in cows with the retained placenta may be related to lower serum cholesterol levels because HDL-cholesterol consists of about 33% cholesterol. (Civelek et al., 2011; Semacan and Sevinç, 2005a).

**Different Protocols Used For The Treatment Of Retained Placenta In Dairy Cows**

Retained placenta remains a therapeutic challenge in cattle. The negative effects of retained placenta include reduced milk yield, increased incidence of metritis, and impaired subsequent fertility. Therefore, effective treatments for retained placenta are crucial for improving puerperal health care in cows (Cui et al., 2014).

High levels of prostaglandin F$_{2\alpha}$ or PGE$_2$ are released from the uterus during the early post-calving time in cows and may play an important role in both placental separation and uterine involution (Slama et al., 1994).

For the prevention or treatment of retention of the placenta, injections of ecbolic drugs such as oxytocin, (PGF$_2\alpha$), or methylergometrine have been administered within 24 hours of parturition (Nosier et al., 2012; Solanki et al., 2019). Time elapsed from parturition to complete fetal membrane drop was shorter in cows receiving oxytocin or methylergometrine maleate/ intramuscular injection than in untreated cows (Azad, 2010; Madhwal et al., 2007).

Also, the usage of methylergometrine or PGF2α immediately postpartum reduced the incidence of retained fetal membranes and improved reproductive performance in cows (Solanki et al., 2019). Additionally, the administration of 500 µg PGF2α or 50 I.U. oxytocin via an intramuscular injection immediately after the expulsion of the fetus induces early expulsion of the placenta and improves the reproductive and productive efficiency of cows experiencing retention of fetal membranes (Abou-Aiana et al., 2019; El-Hawary et al., 2020).

**Manual Removal And Antibiotic Therapy:**

Many broad-spectrum antibiotics and hormonal therapies have been used to treat retained placenta in dairy cattle. However, the efficacy of these approaches is controversial, and some treatments might negatively
affect subsequent reproductive performance (Drillich et al., 2006). Furthermore, the administration of antibiotics in livestock should be minimized to reduce the prevalence of resistant bacteria (Peter et al., 2018).

The manual removal of the RFM, although commonly practiced by owner of the animals (Drillich et al., 2006) has been critically discussed for many years. Among the downsides of manual removal as a routine procedure for RFM treatment includes traumatic injury to uterine mucosa, intrauterine bacterial contamination, disturbance of intrauterine cellular defenses, and impairment of subsequent fertility (Peter et al., 2018).

Although intrauterine therapy such as tetracycline/sulfonamide boluses is used in most RFM cases (Hehenberger et al., 2015), its application does not reduce the incidence of metritis or improve fertility, and it inhibits the metalloproteinase matrix and possibly perpetuates bacterial resistance (Peter, 2013).

Systemic antibiotics are believed to be beneficial in RFM cases where fever is also present, although it is not clear whether the resolution of fever is due to antibiotics or the cow’s own immune defense mechanisms (Amin et al., 2013; Drillich et al., 2006). Only antibiotic treatment is beneficial in cases of acute postpartum metritis (Chenault et al., 2004). Prostaglandins and oxytocin are the most commonly used hormones in treating RFM, which play a role in uterine contraction and are thus effective in treating RFM following uterine atony (Han and Kim, 2005; Patel and Parmar, 2016). However, it has been reported that uterine atony accounts for a very small percentage of retained placenta cases (Peters and Laven, 1996). Therefore, these hormones are not supported for their use in the treatment of RFM (Drillich et al., 2006).

**Effects Of Retained Fetal Membranes On The Fertility Traits In Dairy Cows**

The retained fetal membranes are associated with a great variation in the postpartum reproductive performances in dairy cows (Gohar et al., 2018). RFM abrogates endometritis, puerperal metritis, and mastitis, and these diseases ultimately lead to a decrease in cattle fertility and milk production (Vallejo-Timaran et al., 2021).

Post-calving time (puerperium) is of paramount value in the reproductive and productive performance of cows (El-Hawary et al., 2020; Waheeb et al., 2014). Aberrations of the postpartum period, including retained placenta, cystic ovaries, metritis/ endometritis, uterine prolapse, and pyometra, are major causes of infertility in the postpartum period (Alharoon, 2018; El-Hawary et al., 2020; Waheeb et al., 2014).

**First estrus**

Placenta retention increased the time between parturition and the first postpartum estrus of Friesian cows by approximately 5 days. In a previous study, it was proven that approximately 72% of the normally calved cows showed first estrus after parturition by 25 days. Meanwhile, of the delivered cows suffering from retained placenta, the corresponding percentage was 63%. There were long periods between calving and first estrus for cows with retained placenta. (Shiferaw et al., 2005).
Days open

Following RFM, the uterus becomes infected with bacteria, which has a detrimental effect on cattle's ability to reproduce, including delaying uterine involution, lengthening the time until the first service, increasing the number of services required for each conception, and lengthening the time the uterus is open (Mahnani et al., 2021b). RFM has also been linked to a higher incidence of mastitis, metritis, endometritis, and ketosis (Mahnani et al., 2022). These conditions may also result in reduced fertility and possible milk production losses. In the retained placenta, open days were longer by about 17 days relative to normal cows. This is because about 52% of normally calved animals had days open within the first three months postpartum, while only 45% of cows exhibiting retained placenta conceived during the same period. This is in addition to 28.20% of the retained cows that had been open for more than 120 days. (Shiferaw et al., 2005).

The number of services per conception

The time from parturition to the first service was longer than 42 days, while during the same period, 37.1% of the cows exhibiting retained placenta were served. (Shiferaw et al., 2005). In Holstein cows with retained placenta, the interval from calving to the first service was extended, meaning that cows with retained placenta had longer intervals from calving to the first service (Han and Kim, 2005). The length of service was longer (P < 61 days). The corresponding percentage of retained placenta in cows was around 57%. (Gaafar et al., 2010). For cows exhibiting retained placenta, the number of services per conception was higher. These may be attributed to the longer time of service as well as the lower rate of cow conception with retained placenta, which required more services. (Gaafar et al., 2010; Komba and Kashoma, 2020).

Calving interval and conception rate

The calving period was longer than 375 days, whereas only 44% of cows with exhibition retained placentas had open days of the same duration reported (Elmetwally et al., 2016). Retained placenta causes the dairy industry to suffer significant financial losses by lengthening days open, calving to first heat interval, services per conception, and days from calving to first service (Kimura et al., 2002). With an estimated cost of $285 per case and an average incidence rate of 7.8%, RP is regarded as an economically significant problem for the dairy sector since it increases the likelihood of developing metritis, infertility, mastitis, and poorer milk supply (Dervishi et al., 2016). Retention of the placenta resulted in a reduction in conception rate of about 7% compared to a healthy one (74.10 vs. 66.70%, respectively). The conception rates of normally calved animals and those exhibiting retained placentas were 15.20 and 12.90% before 60 days, 23.60 and 16.80% for 61–90 days, 20.40 and 18.10% for 91–120 days, and 14.90 and 18.80% for more than 120 days postpartum, respectively. Moreover, the highest percentage (31.80%) of normal cows was conceived 61 to 90 days after parturition, while the highest percentage (28.20%) of retained placenta cows was conceived more than 120 days after parturition. (Gaafar et al., 2010).

Material And Methods

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

2. Animals and data collection:

A total of 107 parturient dairy cows aged 3–6 years old and weighing between 400 and 650 kg were studied. This study used lactating primiparous (n = 35) and multiparous (n = 72) Holstein dairy cows. 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.

Out of 35 primiparous cows, (13 cows were presented retained placenta and 26 cows were retained placenta from 72 multiparous cows. So this study had a total number of 39 cows were retained placenta and 68 cows were controlled with normal dropping fetal membranes. after classification of animals 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 and biochemical changes. Animals were classified into two groups. The first one with retained fetal membranes (n = 8) and the second one normal dropping placenta (n = 8). For treatment part of the study and the effect of retention of the placenta on reproductive performances (number of services per conception, interval from calving to 1st estrus, calving interval and pregnancy rate), 39 cows with retained placenta subclassified into 3 subgroups: group І (n = 13) received 5–10 ml oxytocin intramuscular injection (Oxytocin®, each ml contains 10 IU oxytocin, ADWIA, Egypt) ca + 2, group І І (n = 13) received oxytocin at 24 hours after calving, estrumate 2 ml I/M injection (Estrumate® (250µg cloprostenol: synthetic analog of PGF2α) at 48 hours and repeated at 72 hours without fever and normal lochia, group І І І (n = 13) received oxytocin at 24 hours, exceede (cephalosporin 3ml/100kg subcutaneous injection at the base of the ear) Excede® (Ceftiofur Crystalline Free Acid: Zov ), estrumate (2 ml I/m injection MERCK), Flunixin Meglumine® (nonsteroidal anti-inflammatory drug) (2 ml/45kg I/m injection MSD) at 48hrs and repeated dose of estrumate at 72hrs suffered from fever and offensive odor uterine discharge.

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 without 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, 2020, and, 2022).

Quantitative Real-time Pcr
The quantitative real-time PCR (qRT-PCR) method was used to analyze gene expression quantitatively. Primer-BLAST was used to design all primers for genes encoding angiogenic proteins (VEGF, VEGFR2, and RANTES) which described (Table 1). 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 SensiFASTcDNA 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-PureDNase/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, 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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Annealing temperature (°C)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES</td>
<td>F5'-CACCCACGTCCAGGAGTATT-3'</td>
<td>60</td>
<td>NM_175827</td>
</tr>
<tr>
<td></td>
<td>R5'-CTCGCACCCACTTTCTTCT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>F5'-GAAACCCACGAAGTGTGA</td>
<td>60</td>
<td>NM_001316956.1</td>
</tr>
<tr>
<td></td>
<td>R5'-GGGCTCCAGGATTATACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFR2</td>
<td>F5'AGGTATGAGGGCTGGGAGCA</td>
<td>60</td>
<td>NM_001110000.3</td>
</tr>
<tr>
<td></td>
<td>R5'-AGCAATTACCTCAAGGCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F5'-GCGATCTGACCTACAGGTA-3'</td>
<td>60</td>
<td>NM_173979.3</td>
</tr>
<tr>
<td></td>
<td>R5'-CACACGGAGCTTGTTAGA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 GesellschaftFürBiochemica und DiagnosticambH, Max-Planck-Ring 2165205,
Wiesbaden, Germany). Kits of total protein (TP), total cholesterol (TC), triglyceride (TG), and high density lipoprotein (HDL), albumin, urea, creatinine, CPK, 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, and 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 .Correlation parameters was analyzed using Spearman correlation. The differences between the means at a p-value of p < 0.05 were considered significant.

**Results**

1. **Effect of parity on retained fetal membranes**

   The incidence of RFM according to cow parity (Table 2) was recorded as 50% 2, 3 parous 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 two groups (NRFM and RFM).

2. **Vegf, Vegfr2 And Ccl5 Expression In The Whole Blood**

   In Fig. 5, the gene expression profiles of VEGF, VEGFR2, and RANTES were depicted. A significant (p < 0.001) decrease of VEGF and VEGFR2 mRNA expression in retained placenta affected dairy cows compared to healthy ones. The mRNA expression for RANTES showed a significant (p < 0.001) decrease in the case of cows suffering from retained placentae compared to other normal dropped placentae.

3. **Biochemical Changes With And Without Retained Fetal Membranes**

   Table (3) showed that according to serum biochemical changes in the week before parturition, at parturition, and one week after parturition, the mean of studied Ca2+, phosphorous, urea, and total protein values showed a significant (p < 0.05) reduction pattern. Phosphorus showed a significantly (p = 0.038) lower value in group II compared to group I at one-week post-parturition. The total protein showed a significantly high (p = 0.023) value in group compared to group at one-week pre-parturition.
Serum albumin showed a significant ($p > 0.001$) decrease in value at one-week post-parturition compared to one-week pre-parturition 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 ($p > 0.003, 0.001, \text{and} 0.001$, 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 compared to group 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 reduction (R-GSH) (mmol/L) and catalase (CAT) (U/ml) were significantly lower in group compared to group (Table 4: $p < 0.05$).

4. The effect of treatment on reproductive performances in retained cows:

The time needed for the descent of the placenta was significantly shorter in group 1 compared to both group 2 and 3 with $p$ value $< 0.001$. Moreover it was significantly $p$ value $< 0.001$ shorter in group 2 than group 3 (Table 7 and Fig. 6).

Days to first estrus from calving: was significantly $p$ value $< 0.001$ lengthened in group 3 compared to both group 2 with and 3 (Table 7 and Fig. 6).

Calving interval was significantly $p$ value $< 0.001$ shorter in group 1 compared to both group 2 and 3. Also it was significantly $p$ value $< 0.001$ shorter in group 2 than group 3 (Table 7 and Fig. 6).

Number of services per conception: was shorter in group 1 but showed no significant statistical difference between the three groups (Table 7 and Fig. 7).

Pregnancy rate: was higher in group 1 and showed significant statistical difference between the three groups (Table 7).

<table>
<thead>
<tr>
<th>Cow parity</th>
<th>Group II</th>
<th>Group III</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3 parous</td>
<td>4 (50.0%)</td>
<td>3 (37.5%)</td>
<td>0.614</td>
</tr>
<tr>
<td>5,6 parous</td>
<td>4 (50.0%)</td>
<td>5 (62.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Data were presented as number and %.

*p $< 0.05$ is a significant value.
Table 4: Stress oxidant markers of group ♂ and ♂ after parturition:

<table>
<thead>
<tr>
<th>Stress oxidant markers</th>
<th>Group ♂ n= (8)</th>
<th>Group ♂ n= (8)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde (MDA) (mmol/ml)</td>
<td>1.25±0.36</td>
<td>2.39±0.46</td>
<td>0.001*</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD) (U/ml)</td>
<td>330.63±19.89</td>
<td>266.91±13.01</td>
<td>0.001*</td>
</tr>
<tr>
<td>Glutathione reduced (R-GSH) (mmol/L)</td>
<td>6.16±0.26</td>
<td>1.93±0.35</td>
<td>0.001*</td>
</tr>
<tr>
<td>Catalase (CAT) (U/ml)</td>
<td>2.21±0.11</td>
<td>0.94±0.13</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

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

Table 5: Correlation between chemical and angiogenic proteins in NRFM
<table>
<thead>
<tr>
<th></th>
<th>VEGF</th>
<th></th>
<th>RANTES</th>
<th></th>
<th>VEGFR2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p-value</td>
<td>R</td>
<td>p-value</td>
<td>R</td>
<td>p-value</td>
</tr>
<tr>
<td>PO4</td>
<td>0.326</td>
<td>0.431</td>
<td>0.285</td>
<td>0.494</td>
<td>-0.504</td>
<td>0.202</td>
</tr>
<tr>
<td>Urea</td>
<td>0.659</td>
<td>0.075</td>
<td>0.307</td>
<td>0.459</td>
<td>0.069</td>
<td>0.871</td>
</tr>
<tr>
<td>Ca</td>
<td><strong>0.753</strong></td>
<td><strong>0.031</strong></td>
<td>0.614</td>
<td>0.105</td>
<td>-0.204</td>
<td>0.627</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-0.055</td>
<td>0.897</td>
<td>0.300</td>
<td>0.471</td>
<td>-0.356</td>
<td>0.386</td>
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<tr>
<td>TG</td>
<td>-0.041</td>
<td>0.922</td>
<td>-0.044</td>
<td>0.918</td>
<td>0.022</td>
<td>0.958</td>
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<tr>
<td>HDL</td>
<td>-0.059</td>
<td>0.889</td>
<td>0.123</td>
<td>0.771</td>
<td>-0.169</td>
<td>0.689</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>-0.312</td>
<td>0.452</td>
<td>-0.186</td>
<td>0.659</td>
<td>0.011</td>
<td>0.979</td>
</tr>
<tr>
<td>GOT</td>
<td>-0.527</td>
<td>0.180</td>
<td>-0.557</td>
<td>0.151</td>
<td>0.280</td>
<td>0.501</td>
</tr>
<tr>
<td>GPT</td>
<td>-0.210</td>
<td>0.618</td>
<td>-0.165</td>
<td>0.696</td>
<td>0.050</td>
<td>0.906</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.046</td>
<td>0.915</td>
<td>0.261</td>
<td>0.532</td>
<td>-0.254</td>
<td>0.545</td>
</tr>
<tr>
<td>CK</td>
<td>0.128</td>
<td>0.763</td>
<td>-0.542</td>
<td>0.166</td>
<td>0.661</td>
<td>0.074</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.064</td>
<td>0.881</td>
<td>0.339</td>
<td>0.412</td>
<td>-0.326</td>
<td>0.431</td>
</tr>
<tr>
<td>SOD</td>
<td>0.422</td>
<td>0.298</td>
<td>-0.306</td>
<td>0.461</td>
<td>0.586</td>
<td>0.127</td>
</tr>
<tr>
<td>MAD</td>
<td>-0.450</td>
<td>0.263</td>
<td>-0.335</td>
<td>0.417</td>
<td>0.087</td>
<td>0.837</td>
</tr>
<tr>
<td>GR</td>
<td>-0.197</td>
<td>0.641</td>
<td>-0.412</td>
<td>0.311</td>
<td>0.324</td>
<td>0.434</td>
</tr>
<tr>
<td>CAT</td>
<td>-0.612</td>
<td>0.107</td>
<td>0.024</td>
<td>0.956</td>
<td>-0.397</td>
<td>0.330</td>
</tr>
</tbody>
</table>

**Table 6: Correlation between chemical and angiogenic proteins in RFM**
Correlation between gene expression pattern and serum profile of biochemical markers

In the NRFM group, the serum levels of urea, PO4, albumin, CK, total proteins and SOD were positively correlated with mRNA levels of VEGF with strong positive correlation with serum Ca ($r = 0.753, p=0.031$) (Table 5), while, the serum levels of cholesterol, TG, bilirubin, GOT, GPT, MAD, GR and CAT were negatively correlated with mRNA levels of VEGF.

The serum levels of urea, PO4, Ca, cholesterol, HDL, albumin, total proteins and CAT were positively correlated with mRNA levels of RANTES, while, the serum levels of TG, bilirubin, GOT, GPT, CK, SOD, MAD and GR were negatively correlated with mRNA levels of RANTES.

The serum levels of urea, TG, bilirubin, GOT, GPT, CK, SOD, MAD and GR were positively correlated with mRNA levels of VEGFR2, while the serum levels of PO4, Ca, cholesterol, HDL, albumin, total proteins and CAT were negatively correlated with mRNA levels of VEGF.

<table>
<thead>
<tr>
<th></th>
<th>VEGF</th>
<th></th>
<th>RANTES</th>
<th></th>
<th>VEGFR2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p-value</td>
<td>R</td>
<td>p-value</td>
<td>R</td>
<td>p-value</td>
</tr>
<tr>
<td>PO4</td>
<td>-0.054</td>
<td>0.899</td>
<td>-0.168</td>
<td>0.961</td>
<td>-0.145</td>
<td>0.731</td>
</tr>
<tr>
<td>Urea</td>
<td>0.497</td>
<td>0.210</td>
<td>0.606</td>
<td>0.111</td>
<td>0.586</td>
<td>0.127</td>
</tr>
<tr>
<td>Ca</td>
<td>-0.266</td>
<td>0.525</td>
<td>-0.280</td>
<td>0.502</td>
<td>-0.278</td>
<td>0.505</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-0.097</td>
<td>0.819</td>
<td>-0.021</td>
<td>0.961</td>
<td>-0.036</td>
<td>0.932</td>
</tr>
<tr>
<td>TG</td>
<td>-0.375</td>
<td>0.361</td>
<td>-0.349</td>
<td>0.396</td>
<td>-0.356</td>
<td>0.387</td>
</tr>
<tr>
<td>HDL</td>
<td>0.381</td>
<td>0.351</td>
<td>0.333</td>
<td>0.421</td>
<td>0.343</td>
<td>0.405</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>-0.070</td>
<td>0.870</td>
<td>-0.017</td>
<td>0.968</td>
<td>-0.028</td>
<td>0.948</td>
</tr>
<tr>
<td>GOT</td>
<td>-0.135</td>
<td>0.750</td>
<td>-0.221</td>
<td>0.599</td>
<td>-0.204</td>
<td>0.628</td>
</tr>
<tr>
<td>GPT</td>
<td>-0.414</td>
<td>0.309</td>
<td>-0.482</td>
<td>0.226</td>
<td>-0.470</td>
<td>0.240</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.124</td>
<td>0.770</td>
<td>0.064</td>
<td>0.881</td>
<td>0.076</td>
<td>0.858</td>
</tr>
<tr>
<td>CK</td>
<td>0.422</td>
<td>0.297</td>
<td>0.331</td>
<td>0.423</td>
<td>0.351</td>
<td>0.395</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.273</td>
<td>0.514</td>
<td>0.199</td>
<td>0.637</td>
<td>0.214</td>
<td>0.611</td>
</tr>
<tr>
<td>SOD</td>
<td>-0.457</td>
<td>0.255</td>
<td>-0.559</td>
<td>0.150</td>
<td>-0.540</td>
<td>0.167</td>
</tr>
<tr>
<td>MAD</td>
<td>0.262</td>
<td>0.531</td>
<td>0.241</td>
<td>0.565</td>
<td>0.246</td>
<td>0.557</td>
</tr>
<tr>
<td>GR</td>
<td>0.130</td>
<td>0.759</td>
<td>0.021</td>
<td>0.960</td>
<td>0.043</td>
<td>0.919</td>
</tr>
<tr>
<td>CAT</td>
<td>0.301</td>
<td>0.469</td>
<td>0.386</td>
<td>0.344</td>
<td>0.370</td>
<td>0.367</td>
</tr>
</tbody>
</table>
In the RFM group, the serum levels of urea, HDL, albumin, total proteins, MAD, GR and CAT were positively correlated with mRNA levels of VEGF, RANTES and VEGFR2, while, the serum levels of PO4, Ca, TG, bilirubin, GOT, GPT, CK and SOD were negatively correlated with mRNA levels of VEGF, RANTES and VEGFR2.

### Table 7: Effect of type of Treatment on descending placenta

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Time of descending</th>
<th>Number of service/conception</th>
<th>Interval from calving to 1st estrus</th>
<th>Calving interval</th>
<th>Pregnancy rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(n=13)</td>
<td>7.16±1.16</td>
<td>1.83±0.75</td>
<td>32.00±8.62</td>
<td>363.166±5.19</td>
<td>9(69.23%)</td>
</tr>
<tr>
<td>2(n=13)</td>
<td>52.80±38.51#</td>
<td>2.40±1.14</td>
<td>50.00±19.86</td>
<td>386.80±7.69#</td>
<td>5(38.46%)</td>
</tr>
<tr>
<td>3(n=13)</td>
<td>126.00±12.00†§</td>
<td>2.50±1.29</td>
<td>104.50±13.20†§</td>
<td>405.25±3.86†§</td>
<td>7(53.85%)</td>
</tr>
</tbody>
</table>

P-value <0.001* 0.550 <0.001* <0.001* <0.001*

Data are presented as mean +SD.

**Group 1**: (n=13) received 5-10ml oxytocin (Oxytocin®, each ml contains 10 IU oxytocin, ADWIA, Egypt), ca+2

**Group 2**: (n=13) received oxytocin (Oxytocin®, each ml contains 10 IU oxytocin, ADWIA, Egypt), Estrumate® (250μg cloprostenol: synthetic analogue of PGF2α) at 24 hours after calving I/m injection, at 48 hours and repeated at72 hours without fever and normal lochia

**Group 3**: (n=13) received oxytocin at 24 hours, Excede® (Ceftiofur Crystalline Free Acid: Zoetis, USA (3ml/100kg subcutaneous injection at base of ear), estrumate (2 ml I/m injection), funixin meglumin (2 ml/45kg I/m injection MSD) at 48hrs and repeated dose of estrumate at 72hrs suffered from fever and offensive odor uterine discharge

- P-value is significant when <0.05 (*between the three groups, # between group 1 and 2, †between group 1 and 3, §between group 2 and 3).

**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 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 concentration (O$_2$) 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 O$_2$ by stimulating neovascularization when levels are low.

It seems that under low (O$_2$), 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 expression of the sVEGFR2 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 placentae 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 found 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. Vaccinova 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., (2021a) 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) and 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 are 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% for the first parity to 54.60% 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 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 a study by 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). Rayan and El-abedeen, (2019) conducted that, cholesterol levels increased very slightly directly in the prepartum, then they were below average reference values in cows with RFM. Cows with RFM 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 and El-abedeen, 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 et al., (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 Ca2+ 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 afterparturition due to excessive Ca demandcolostrum 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 timepoints 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 (Islam and Kumar, 2015; Jovanović et al., 2013).

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, (2019) found that catalase reduction was significantly lower in the RFM group compared to NRFM.

Retained fetal membranes (RFM) is one of the most common periparturient problems that cows face in this period, it can be defined as failure to deliver the fetal membrane within 12 hours after delivery (Khudhair et al., 2021). The normal release of placenta in cows usually occurs within six hours after birth and the incidence rate ranged from 3–12% of dairy cows (Dervishi et al., 2016; Sheldon et al., 2009).

RFM is multifactorial problem, although there is no detection factor that provides scientific explanation to the incidence of this syndrome but there are some evidences for various risk factors represented by genetics, environment, age, nutritional status and hormones that may be interfere with the causes of RFM (Tucho and Ahmed, 2017). The other pathological condition associated with RFM incidence in dairy cattle are abortion, twinning, dystocia, obstetrical complication, infectious disease and nutritional disorder (Chandrashekhar and Singh, 2014; Seifi et al., 2007).

In the current work, the time needed for the descent of the placenta was significantly shorter in group 1 compared to both group 2 and 3 with p value < 0.001. Moreover it was significantly p value < 0.001 shorter in group 2 than group. Reproductive performance is economically important in dairy cattle because it affects milk yield and culling rate. Poor reproductive performance due to postpartum reproductive disorders can reduce the number of born calves and milk production and increase the cost of nutrition, therapy and artificial insemination.

According to the postpartum reproductive performances including days to first estrus from calving: was significantly p value < 0.001 lengthened in group 3 compared to both group 2 with and 3.

Kim and Jeong, (2019; Shiferaw et al., (2005) showed that Cows with reproductive disorders had longer intervals from calving to first service and to conception and required more services per conception and lower pregnancy rate and conception to first service.

Similarly, Amin et al., (2013; Beagley et al., (2010) showed that the time from calving to first service was significantly lower in treated cows than those in untreated cows.

Similarly, Sattar et al., (2007) who found that the buffaloes and cows being treated with vitamin E and selenium during late pregnancy the interval from calving to first insemination was lower than those in control group.

Perera, (2011); Warriach et al., (2015) recorded that the duration of the estrous cycle in cows is ranging from 17 to 26 days with an average of 21 days However, there is a greater variability of the estrous cycle length in buffalo compared to cattle, with a greater incidence of both abnormally short and long estrous cycles. This may be attributed to various factors including adverse environmental conditions, nutrition and irregularities in secretion of ovarian steroid hormones.
Number of services per conception: was shorter in group 1 but showed no significant statistical difference between the three groups. Similarly, Gohar et al., (2018) found that the buffalo cows which suffered from retained fetal membranes showed a greater number of services per conception when compared to the buffalo cows which gave normal birth without retained fetal membranes but with significant tendency. They added that the conception rate of cows presenting retained placenta were significantly lower compared to normally calved cows; the highest proportion of normal cows was conceived during the period from 61 to 90 days after parturition, while cows with retained placenta were conceived at more than 120 days after parturition.

In the present study Calving interval was significantly p value < 0.001 shorter in group 1 compared to both group 2 and 3. Also it was significantly p value < 0.001 shorter in group 2 than group 3.

Han and Kim, (2005; Stevenson and Call, (1988) reported that retention of placenta and metritis may cause prolonged calving interval and permanent infertility. Calving interval remained longer in cows revealing retained placenta as compared to normal cows in general, the financial losses due to retained placenta in dairy cattle existed due to increased calving interval, increased culling rate, reduced conception rate, infertility, loss of milk production, the costs of veterinary service and drugs.

Pregnancy rate: was higher in group 1 and showed significant statistical difference between the three groups, Abdelhameed et al., (2009) showed that Cows with reproductive disorders had longer intervals from calving to the first service and conception, thus they required more service per conception which led to a lower pregnancy rate. Ribeiro et al., (2016) reported low pregnancy rate in cows with uterine diseases than in cows in control group as cows suffered from retained fetal membrane easy to be exposed to uterine infection and considered good media for growth and multiplication of bacteria.

**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.

Retained fetal membranes in cows could be treated successfully without any interference by systemic injection of cephalosporin with caring of temperature.

The use of antibiotic, anti-inflammatory, and hormonal supplies at puberium only for improving and enhancing the reproductive performance of dairy cows not to prevent the occurrence of the retention of the placenta.

**Declarations**

Ethics statement The collection of samples and care of the animals used in this study was approved by the Research Ethics Committee, Faculty of Veterinary Medicine; Mansoura University (Code Ph.D. /52).

Competing interests: The authors declare no competing interests
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Table 3
Table 3 is available in the Supplementary Files section.

Figures
Figure 1

Light micrographs and graphic illustrations of buffalo and cattle placentomes
Figure 2

Modified diagram of physiologic processes leading to the detachment of the placenta in dairy cows (Tucho and Ahmed, 2017).
Figure 3

(Seo et al., 2019) A working hypothesis for the syncytialization of the sheep placenta. Mononuclear trophoblast cells fuse with one-another to become multinucleated TGCs. Large numbers of TGCs migrate to insert themselves between the uterine LE cells that are simultaneously undergoing apoptosis. TGCs then engulf LE cells and carry them to the stroma for elimination by immune cells. The remaining TGCs then fuse with each other to form an extensive trophoblast syncytial layer that fills spaces left by removal of uterine LE
and form the interface between caruncles and cotyledons in placentomes of the functional synepitheliochorial placenta of sheep.

**Figure 4**

Incidence of RFM according to cow’s parity.

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

**Figure 5**
mRNA levels of VEGF, VEGFR2 and RNATES in retained placenta and non-retained placenta affected dairy cows

**Figure 6**

Effect of treatment groups on placental descending.

**Figure 7**

Effect of treatment groups on service/conception.
Figure 8

Effect of treatment groups on interval from calving to 1st estrus.

Figure 9

Effect of treatment groups on calving interval.

Supplementary Files

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- Table3.docx