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Monitoring of early embryonic death associated growth factors as insulin like growth factors binding proteins (ILGFBP) 1-4 and Cytokines in dairy cattle

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A B S T R A C T

The current study was designed to evaluate the growth factors and cytokines as insulin like growth factors binding proteins (IGFBP 1-4), Interferon Tau (IFNT) and Interleukins 6 and 13 (IL-6, -13) concentrations which associated with early embryonic deaths (EED) and fetal death (FD) in dairy cattle. A total number of nineteen (n=90), non-pregnant crossbreed dairy cows, 40-days postpartum were included in this study. Cows were examined using ultrasonographic examination to ensure absence of any uterine affections. Five cows were excluded due to uterine affections during examination. Estrus synchronization using GnRH, PGF2α and GnRH (GPG) regimen were applied however, another 10 cows were excluded due to showing an estrus signs after the first GnRH injection. The timed artificial insemination (AI) were applied to other cows blindly at the 10th Day of the protocol. Rectal ultrasonographic examination were applied at beginning of the synchronization, then at the 9th day of the protocol (at the second injection of GnRH). For pregnancy diagnosis, ultrasonographic examination were performed at the 20, 25, 30, 40, 50, 60, 70, 80 and 90th day after AI in all cows. Blood sampling were collected at days 0, 7, 9, 11 during synchronization then at each ultrasonographic examination post insemination. Our results revealed that thirty cows (n=30) showed endometrial thickness and some fluctuation on uterine lumen at day 20 post insemination, however only 26 cows were confirmed as pregnant but other 4 cows showed an estrus. The twenty-six (n=26) were showed endometrial thickness and some fluctuation on uterine lumen at day 25 post insemination with a percentage of 43.3% conception rate. Early Embryonic Death (EED) in one cow was monitored at day 40 post-insemination with a percentage of 0.038 % (1/26) and another cow (n=1) showed an Early Fetal Death (EFD) with a percentage 0.04 % (1/25) at the 60th day post-insemination. Samples were analyzed for progesterone (P4), IGFBP1, IGFBP4, IL-6, IL-13 and IFNT concentrations; for P4 during synchronization raise and decrease but usually under 3 ng/ ml while during pregnancy were more than 3 ng/ ml, for IGFBP1 and IGFBP4 ranged from 55-80 ng /ml and 63-99 ng/ml in pregnant cows respectively and increase over that range in cows had EED and FD, for IL-6 and IL-13 ranged from 1-3.5 ng/ml and 44-65 ng/ ml respectively and decrease under that range in cows had EED and FD and for IFNT range from 0.5-18 ng/ml and decrease under 0.5 ng/ml in cows had EED and FD.

Keywords: Growth factors, Cytokines, Embryonic death, Pregnancy.

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1. Introduction

Fetal growth is a complex dynamic process controlled by various maternal and fetal factors. Insulin like growth factors (IGF) are polypeptide hormones produced by the placenta and by maternal and fetal tissues (Gicquel and Le Bouc, 2006). The maternal IGF system can modulate placental growth and functional development, the delivery of substrates to the fetus, the partitioning of nutrients between maternal tissues and the conceptus (Sferruzzi Perri et al., 2007). The IGFBP control the biological activities of IGF by modulating IGF receptor interaction and by increasing the half-life of circulating IGF (Baxter, 1993). The IGF system is regulated by many factors, such as nutritional protein (Perry et al., 2002) and endocrine signals (Fowden, 2003) including bovine placental lactogen (BPL), which has stimulatory effects on the maternal IGF system in cattle (Weber et al., 2007).

Dairy cows should achieve a new pregnancy within 2–3 months of calving in order to maintain high milk production (Han et al., 1996), therefore, the gestational period begins during lactation. Insulin-like growth factors (IGFs) play a crucial role not only for metabolic adaption but also in fetal growth (McCarthy et al., 2012). In this regard, the local IGF system, comprising insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) and their respective IGFBPs are expressed in the endometrium prior to implantation and in the placenta (Robinson et al., 2000). These are important as well as maternal IGF-1 and IGFBPs in the blood circulation. The maternal IGFBP concentrations because of the bioavailability of IGF-1 in the blood is regulated by the binding of IGF-1 to four high-affinity binding proteins (IGFBP1-4) (Clemmons, 1998).

Identification of non-pregnant animals at the earliest day post-breeding is of prime importance for maintaining an optimum calving interval. Pregnancy diagnosis as early as possible after insemination helps in better management of pregnant animals and early submission of non-pregnant animals for subsequent breeding (Fricke, 2002). Although several methods like non-return to estrus, estimation of progesterone in blood or milk, and ultrasonography are used to diagnose pregnancy in dairy animals. Rectal examination during 45-60 days post-breeding continue to be the most common method used for pregnancy diagnosis, especially under field conditions. By rectal examination, it is difficult to identify the pregnancy before 45 days. Ultrasonography offer a great scope for early pregnancy diagnosis and additionally the cases of early embryonic mortality, which can be misdiagnosed as pregnant by serum progesterone estimation, can be detected by ultrasonography more accurately (Silva et al, 2007 and Walsh et al, 2007).

The conceptus inherits half of its genetic materials from the mother, and the maternal immune system can detect the presence of the conceptus (embryo/fetus and associated membranes) as alloantigen's or receptor ligands. During early pregnancy in the bovine, however, the conceptus usually thrives within the maternal uterine environment without suffering any deleterious immune attack from the mother. It is necessary for the mother that a variety of immunological adjustments are essential to limit maternal immune response against the conceptus (Hansen, 2011).

The chemokines and cytokines (Interleukins) produced by the conceptus can modify maternal immune system, and these immunological events promote conceptus development. Interferon-tau (IFNT) is the primary

pregnancy recognition signal which is produced by conceptus in the bovine. IFNT also is inhibitory to lymphocyte proliferation, protecting the allogeneic conceptus from immune responses to fetal antigens (Skopets et al., 1992)

Embryonic mortality refers to the losses which occur in the period between fertilization and the completion of the stage of differentiation at approximately day 45. Embryonic death or mortality denotes the death of fertilized ova and embryo up to the end of implantation. Early embryonic mortality is a major source of embryonic and economic loss through repeat breeding and increased cost of artificial insemination. EED also leads to extended calving intervals and prolonged dry period resulting in reduced life time milk production and reduced net calf production (Maurer and Chenault, 1983).

The aim of the present study was to examine whether the maternal IGFBP concentration in blood during early pregnancy in cows; to compare the IGFBP-4 concentration between pregnant and non-pregnant cows to examine cows undergoing embryonic/fetal mortality and to determine whether proteolytic activity for IGFBPs can be detected in pregnant cows. Also, Synchronization program in dairy cattle which generally beneficial to reproductive performance, detection of P4 levels, and to determine IGFBP-2, IGFBP-3, and IGFBP-4, IFNT, IL-6, and IL-13 concentrations that sign pregnancy in cows and their curves with detection early embryonic death. Therefore, we aim to improve the reproductive performance of dairy cattle in our country.

2. Materials and Methods

2.1. Animals and management

This study was carried out in a private animal farm (Aba-Hussein farm) Nubaria city in Elbehira governorate, Egypt. Nineteen (n=90) non-pregnant crossbreed dairy cows 40-days postpartum were used in this study, Cows were examined using ultrasonographic examination to ensure absence of any uterine affections. The animals were kept indoor at night and had access to natural grazing area for the most day. During indoor the animals take a diet of concentrated feed with wheat straw and barseem. Water and mineral licks are available in the farm. The experiment was conducted during the winter season from November 2020 until February 2021 when the ambient temperature was 26 ℃.

2.2. Experimental design

Cross breed dairy cows (n=90) were examined at first rectally by ultrasound using (Sonoscape A6, Guangdong, China) to exclude cows had uterine affection as pyometra and endometritis. The cows had clean uterus were included in the experiment.

2.2.1. Estrus synchronization and Timed AI; GnRH-PGF2α-GnRH (GPG) regimen

All cows were subjected to intramuscular treatment with gonadotropin releasing hormone GnRH (5 ml receptal) (Zootis, New Zealand) at day 0 then cows were injected intramuscularly with Cloprostenol sodium (PGF2α) 2 ml Estrumate (MSD) at day 7 then at day 9 repeat the GnRH (5 ml receptal) then after timed AI at the 10th day (16-20 hours after the 2nd GnRH injection.

2.2.2. Estrus detection and timed AI

All animals were subjected to timed AI at the 10th day (16-20 hours after the 2nd GnRH injection. Of note, most of injected cows were exhibiting estrus (bellowing, estral mucous and mounting other cows) which detected by workers during 10th day of synchronization. Cows were inseminated by frozen semen from the examined farm with ensuring estrus at insemination.

2.2.3. Ultrasonographic examination

Ultrasound examinations were performed using (Sonoscape A6, Guangdong, China) rectal probe at 5.5 frequency. all examinations were done in special stanchion specific for gynecological examination in the farm with assistants for securing the cows by one operator.

Ultrasound examination were carried as follow: before synchronization to ensure non pregnant clear uterus of cows; at day 9 of synchronization to detect mature Graffian follicles; at day 20 post-insemination to detect endometrial thickness; at days 25-27 and 28-32 post-insemination to detect pregnant cows (embryonic sacs); and days 33-45, 50-60, 61-67, 68-75, 76-82, and 83-90 post-insemination.

2.3. Maternal Blood Sampling and Hormone Assays

Blood were collected by vein-puncture of the Jugular vein or tail vein directly into 10-mL heparin vacutainer tubes (Lithium Heparin Tube) using 1.2 mm × 38 mm needles. Vacutainer tubes were gently rotated by hand for 5 to 10 s, labeled, and stored on ice box for 1 to 2 h before centrifugation at 3,000 rounds / min. for 10 min using centrifuge (Centrifuge- CL008, CYAN), and plasma were harvested. Samples were stored frozen at −20°C until analyzed.

The blood samples were collected at the following times: days 0, 7 and 9 of synchronization (GPG) to detect P4 concentration; and every 10 days intervals blood sampling from 1st week post-insemination till third month of pregnancy to detect P4, IGFBP1, IGFBP4, IFNT, IL-6 and IL-13 concentrations.

Samples that analyzed for P4 concentrations were 90 samples during synchronization; 30 samples at day 0, 30 samples at day 7 and 30 samples at day 9. Another 90 samples were analyzed for P4 concentrations during pregnancy; 10 samples at day 7 post insemination, 10 samples at day 21 post insemination, 10 samples at day 30 post insemination, 10 samples at day 40 post insemination, 10 samples at day 50 post insemination, 10 samples at day 60 post insemination, 10 samples at day 70 post insemination, 10 samples at day 80 post insemination, 10 samples at day 90 post insemination. Totally 180 samples were examined using 2 P4 kits (96T).

Samples that analyzed for ILGFBP1, ILGFBP4, IL-6, IL-13 and IFNT concentrations were 90 samples for each one during pregnancy; 10 samples at day 7 post insemination, 10 samples at day 21 post insemination, 10 samples at day 30 post insemination, 10 samples at day 40 post insemination, 10 samples at day 50 post insemination, 10 samples at day 60 post insemination, 10 samples at day 70 post insemination, 10 samples at day 80 post insemination, 10 samples at day 90 post insemination. Totally one kit (96T) for each item.

2.4. Hormonal assay

Plasma P4, IGFBP 1-4, IFNT, IL-6, and IL-13 concentrations were measured by Enzyme linked-immune sorbent assay (ELISA; Erba Lisa Scan II) and adjusted at 450 nm wavelength, by using bovine P4, IGFBP 1-4, IL-6, IL-13 and IFNT ELISA (96T) kits (BT LAB Bioassay Technology Laboratory), seeing the difference between plasma concentration in different reproductive states.

2.5. Statistical analysis

All the data were presented as the means ± S.E.M. The statistical significance of differences in plasma P4, IGFBP 1-4, IL-6, IL-13 and IFNT concentrations throughout the different days of study were determined by student t-test. All data were analyzed by using the Graph-Pad Prism (Graph Pad Software, San Diego, CA, USA).

3. Results

Ninety (n=90) cross breed dairy cows examined with ultrasound rectally to ensure clear uterine lumen and free from any uterine affections eighty-five (n=85) cows were had clear uterus and uterine lumen free from any exudate with no thickness in uterine wall. These animals were subjected for synchronization. While five (n=5) cows were had different uterine affection like; endometritis (thick uterine wall and uterine lumen had some exudate with turgidity), pyometra (thick wall and lumen had pus with doughy texture). These animals were had a history of retained placenta and metritis post-partum.

During synchronization with GPG Programme, some cows (n=10) were noticed in estrus and explained estrus signs after 1st GnRH injection with-in 36-48 hrs, but these animal were excluded (not inseminated) and the experiment was completed.

3.1. Rectal examination of the uterus

After 2nd GnRH injection in GPG Program (between day 10, 11), sixty (n=60) cows were subjected to rectal palpation before insemination. The result showed a clear estrus mucus at vulvar lips once touching the cervix, turgid uterine wall with rings and mature graffian follicle (MGF) on the ovary. Of note, some estrus signs were detected by worker like; bellowing, oestral mucus, erection of teats, drop in milk, excessive movement, mounting other animals and later standing heat. The results showed a percentage of 70.5% (60/85) heat detection rate.

3.2. Ultrasound examination

3.2.1. First ultrasonographic examination

Cows were examined before synchronization to ensure clear uterus and structures on ovaries some cows were had abnormal uterine content and the other cows were had normal uterus as shown in (Image 1A, B). The eighty-five (n=85) cows were had different ovarian structures. Fifty

(n=50) cows were had functional corpus luteum (CL) on the ovaries, fifteen (n=15) cows were had smooth in active ovaries, seventeen (n=17) cows were had growing follicles on their ovaries, one (n=1) cow was had cyst on ovary (follicular cyst) and two (n=2) cows were had luteal cyst on their ovaries.

3.2.2. Second ultrasonographic examination

Cows were examined at day 9 of synchronization for detection of MGF on ovaries and ensuring estrus, sixty (n=60) cows were had MGF on their ovaries and some animals were had more than one MGF with a history of twining. The size of MGF ranged from 1.8 mm – 2.5 mm (Image 1C).

3.2.3. Third ultrasonographic examination

Cows were examined at day 20 post insemination, thirty (n=30) were showed endometrial thickness and some fluctuation on uterine lumen as shown in (Image 2A). Only 26 cows were confirmed as a pregnant, however the other 4 cows showed a signs of estrus.

3.2.4. Fourth:Eleventh ultrasonographic examination

Cows were examined at day 25 post insemination, twenty-six (n=26) were positive pregnancy; they had embryonic sac demarcated with fluctuation in uterine lumen with a percentage 43.3 % conception rate (26/60). Then after at 30 days pregnant cows were ensured pregnant with well-developed embryonic sacs and embryos were appeared clear and measure the Crown Rump Length (CRL) of each embryo from crown of embryo till rump or coccygeal.

Cows were measured at day 37-45 post insemination, embryos were developed and pulsations were noticed with movement of embryos and can detect embryonic vesicles by hand. But one cow (n=1) by examination was had disappearance to fluid and embryonic sac and embryo were replaced by turbid fluid showing EED (Image 2B) at day 40 with a percentage of 0.038 % (1/26).

Another ultrasound examination was applied at 55-60th days. Fetal development was measured by Bi-Parietal Diameter (BPD) and Trunk Diameter (TD) and with rectal palpation, Fetal Membrane Slip (FMS) were applied (+ve). Another animal (n=1) was detected pregnant before had an EFD showing fragmentation to fetus and surrounded with scanty turbid fluid without FMS and pulsation, the EFD was with a percentage 0.04 % (1/25).

The ultrasound examination (Image 2C, D) were continued to the pregnant cows (n=24) at day 60, 70, 80 and 90 post insemination showing enlarging to fetuses and well growth with measuring to BPD and TD and appearance of placentomes.

3.3. Hormonal Assay

Blood samples were collected, centrifuged, stored at -20o c and then analyzed by Enzyme Linked Immuno-Sorbant Assay (ELISA) to measure the concentration of Progestrone (P4), Insulin Like Growth Factor Binding Protein 1 (ILGFBP1), insulin like growth factor binding protein 4 (ILGFBP4), Inter-Lukeins 6 (IL6), Inter-Lukeins 13 (IL13) and Interferon Tau (IFNT).

The results revealed that the concentrations of P4 during synchronization raise and decrease in the amount but usually under 3 ng/ml while during pregnancy were more than 3 ng/ ml as shown in (Figure 1), (Figure 2), and (Figure 3).

The levels of both IGFBP1 and IGFBP4 ranged from 55-80 ng /ml and 63-99 ng/ml in pregnant cows respectively, and increase over that range in cows had EED and FD as shown in (Figure 4), and (Figure 5) respectively.

For the concentrations of IL-6 and IL-13 the result revealed that its level ranged from 1-3.5 ng/ml and 44-65 ng/ ml respectively and decrease under that range in cows had EED and FD as shown in (Figure 6), (Figure 7) respectively.

The data of IFNT concentrations showed its level ranged from 0.5-18 ng/ml and decrease under 0.5 ng/ml in cows had EED and FD (Figure 8).

4. Discussion

Improvement of the reproductive ability and increasing fertility rate among cows are the most important challenges in dairy cattle industry (Walsh et al., 2011). Old methods of management and breeding systems of dairy cattle are still used in dairy cattle farms in developing countries worldwide including Egypt and this could be reflected on the productivity and health of animals resulting in high economic losses (Khatib, 2014).

Recently, new technologies were applied in Egyptian dairy farms to enhance the management and increase the reproductive ability among cattle. These new technologies simply including estrous detection methods, estrous synchronization and ultrasound application in detection of reproductive problems as well as pregnancy diagnosis. In the discussion section of this study we will focus on the application of these new technologies in an Egyptian dairy farm in El-behaira governorate for the first time. Our discussion will be classified into three main categories; firstly, improvement of heat detection rate (estrous detection rate) and conception rate with estrous synchronization and AI respectively. Secondly, drastic changes in the hormonal level and immunological factors of the reproductive tract in pregnant cows during first stage of pregnancy. Finally, the application of ultrasound examination in detection of reproductive problems and pregnancy diagnosis including early embryonic death as well as fetal death and its association with the changes in hormonal level and immunological factors of the reproductive system.

Totally, ninety (n=90) dairy cows were examined using ultrasound rectally for detection of the health of the reproductive system, the results showed eighty-five (n=85) cows had a clear uterus and uterine lumen free from any exudate with normal thickness in the uterine wall. However, five (n=5) cows had a different uterine affection. This could be indicative for the importance of the ultrasound application in Egyptian dairy farms because imaging is considered the most accurate diagnostic method to identify ovarian and uterine structures. Furthermore, the trans-rectal palpation is a complement to the ultrasound examination and remains a practical means of evaluating the contours and texture of these organs. During the ultra-sonographic examination of the ovaries, it is important to be able to distinguish a follicle which appears black due to hypo-echogenic follicular fluid and the mature CL which is hypo-echogenic compared to the ovarian stroma due to extensive vascularization.

A detection of the health of the uterus by ultrasound is a more accurate than palpation. Naturally, animals with reproductive tract pathology will have poor conception regardless of the synchronization method used. An ultrasound examination prior to beginning a synchronization protocol will identify these animals before time and money is spent on drugs and insemination (Silva et al, 2007; Walsh et al, 2007).

The normal uterine structure, eighty-five (n=85) cows were subjected to estrus synchronization with GPG Program, ten of them (n=10) were detected in estrus by traditional methods after 1st GnRH injection within 36-48 hrs. Only, 75 cows were continued and passed to the step of second shot of GnRH. Out of them, sixty (n=60) cows were showed signs of estrus with a percentage 70.5% heat detection rate. This percentage is considered a high rate of estrus rate and very beneficial because of the synchronized estrus and ovulation reduce the time and labor associated with estrus detection (Carvalho et al., 2018).

The first GnRH injection alters follicular growth by inducing ovulation of the largest follicle (dominant follicle) in the ovaries after the GnRH injection to form a new or additional CL (Pursley et al., 1995). Thus, estrus usually does not occur until a PGF2a injection regresses the natural CL and the secondary CL (formed from the follicle induced to ovulate by the second GnRH injection). Therefore, a new group of follicles appear on the ovaries (based on transrectal ultrasonographic within 1 to 2 days after the first injection of GnRH (Vasconcelos et al., 1999). From that new group of follicles, a newly developed dominant follicle emerges, matures, and can ovulate after estrus is induced by PGF2a or it can be induced to ovulate after a second injection of GnRH. The GnRH injections release pituitary luteinizing hormone (LH), the natural ovulation-inducing hormone of the estrous cycle (Islam, 2011).

Sixty (n=60) cows were examined at day 9 of synchronization for detection of MGF on ovaries, all of them had one or more MGF on their ovaries with the size of ranged from 1.8 mm – 2.5 mm. Those 60 cows were subjected to AI and examined for pregnancy using ultrasound at days 18 after insemination for uterine thickening and confirmed pregnancy at day 25 post-inseminations as the uterus had an embryonic sac demarcated with fluctuation in uterine lumen. The result showed out of 60 cows 26 cows were diagnosed as pregnant (26/60) with a conception rate 43.3 %. This conception rate is considered higher than previous studies used the same protocol (Taponen, 2009).

Originally the Ovsynch protocol for synchronization of ovulation was developed for reproductive management in dairy herds (Pursley et al., 1995). Many studies have evaluated the fertility of lactating dairy cows following the Ovsynch protocol, and pregnancy rates per AI have varied from 27% to 39% (Taponen, 2009). These pregnancy rates have been

similar to (Pursley et al., 1997) or only slightly lower (Peters and Pursley, 2003) than the pregnancy rates with AI after estrus detection or after estrus detection following synchronization of estrus with PGF2α in the control cows.

At day 37-45 post insemination, embryos were developed and pulsations were noticed with movement of embryos and the embryonic vesicles were detected in gravid uteruses. Furthermore, at day 55-60 days, fetuses were developed and Bi-Parietal Diameter (BPD) and Trunk Diameter (TD) were measured and with rectal palpation of Fetal Membrane Slip (FMS) were positive. However, one cow showed Early Embryonic Death (EED) at day 40 with a percentage of 0.038 % (1/26) and another one was detected as Early Fetal Death (EFD) with a percentage 0.04 % (1/25). EED and EFD could be occur during fertilization and during the process of implantation due to infectious and non-infectious causes. Major attention has often been given to the infectious causes, but non-infectious causes probably accounts for more than 70% of the cases of early embryonic death (Vanroose et.al, 2000). Endometrial receptivity and asynchrony, progesterone insufficiency, maternal age, lactation, oocyte age, immunologic and endocrine factors could be the major causes of embryonic death as well as environmental stresses, illness, nutrition, season/climate, transrectal palpation / ultrasonography and expression of lethal genes, chromosomal anomalies of the embryo (Dey et al, 2004).

Regarding to progesterone concentration during synchronization, we have detected the P4 level using ELISA; 30 samples at day 0, 30 samples at day 7 and 30 samples at day 9. Furthermore, P4 levels were detected among other pregnant animals as 10 samples were measured every time for 10 days intervals starting from day 7 until day 90 post-insemination. The results showed that Mean P4 level during synchronization is nearly duplicated in day 7 and drastically decrease in day 9. However, the mean P4 concentration were gradually increased from day 7 until day 90 post-insemination in all animals except in 2 cows that showed early embryonic deaths in days 30 and 60 respectively, the level of P4 was very low. These measurements are an indicator for the importance of P4 concentration in estrus cycle as well as during pregnancy. Progesterone stimulates and maintains endometrial functions necessary for embryonic survival, conceptus growth, implantation, placentation, and development to term. A strong positive association exists between the postovulatory rise in concentrations of P4 and embryonic development in cattle. The effect of P4 on conceptus growth and elongation in cattle is indirectly mediated by the endometrium (Lonergan and Forde, 2014).

During the estrous cycle and pregnancy, P4 induces a set of genes in the endometrium that establish uterine receptivity, which is the physiological state of the uterus when conceptus growth and implantation is possible. The absence of a sufficiently developed conceptus to signal pregnancy recognition results in those genes being turned off as luteolysis ensues and the animal returns to estrus for another opportunity to mate (Spencer et al., 2015).

ILGFBP-1, and ILGFBP-4 levels were detected among those pregnant animals as 10 samples were detected every time for 10 days intervals starting from day 7 until day 90 post-insemination. The concentrations did not show a significantly change along the experiment but a slight increase were observed. However, for the 2 cows showed an early embryonic and fetal deaths were recorded a very high increase in the level of both ILGFBP-1, and ILGFBP-4 from day 40 until the end of experiment. This have indicated that IGF may be useful as a predictive marker for uterine diseases (Piechotta et al., 2012). Therefore, the insulin like growth factors plays an important function in tissue repair processes (Bitar, 2000). This increase of such factors could be associated with many pathogenic bacteria for the uterus causing infection and inflammation as well as endometritis (Sheldon et al, 2006).

An immunoregulatory cytokines IL-6, and IL-13 that produced primarily by activated Th2 cells, and is involved in regulating inflammatory and immune responses were determined among the pregnant cows. Ten samples were detected every time for 10 days intervals starting from day 7 until day 90 post-insemination. The levels of both IL-6, and IL-13 were non-significantly changed along the experiment but a slight increase was recorded. However, in both cows that showed an early embryonic and fetal deaths a clear increase in the levels of both IL-6, and IL-13 was observed. This results could explain that the plasma concentration of interleukins in the early embryonic death cows have shown a positive correlation with the severity of the inflammatory response (Trevisi et al., 2012).

IFNT levels were demonstrated for 10 times starting from day 7 until day 90 post-insemination using ELISA. The results revealed that the level of IFNT is gradually decreased along the experiment this is due to the function of IFNT which play an important role in maternal recognition of pregnancy during the first days. However, its level in 2 animals that showed early embryonic deaths was very low. Maternal recognition of pregnancy is the physiological process whereby the conceptus signals its presence to the maternal system and prolongs the lifespan of the ovarian CL (Bazer et al., 1991). In ruminants, IFNT is the pregnancy recognition signal secreted by the elongating conceptus that acts on the endometrium to inhibit development of the luteolytic mechanism. The trophectoderm cells of the elongating conceptus secrete IFNT predominantly before implantation (Spencer et al., 2007; Bazer et al., 2010).

Up on the previous results, the early detection of the pregnancy in the dairy farms and the application of ultrasonography for pregnant cows to differentiate between early embryonic death and healthy pregnancy are very important. This study was concluded a higher estrous detection rate and conception rate with GPG synchronization program than the previous studies used the same methods. The level of pregnancy hormones such as P4 and cytokines such as ILGFBP1-4, interleukins IL-6, and IL-13, and IFNT were strongly associated with pregnant cows, in contrast, its levels were completely different in early embryonic and fetal death. These conclusion was recommended that application of new technologies in Egyptian dairy farms such as synchronization system and ultrasound have become an urgent issue.

5. **Authors contributions**: KS, shared in all steps of experiment and manuscript preparations; HH, NB, NY, AH supervisors, planning and revision of manuscript; SM, MA help in experiment proceeding; SM, preparation of manuscript figures.

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Figure 1. The mean concentrations of progesterone during synchronization period including days 0, 7, and 9.

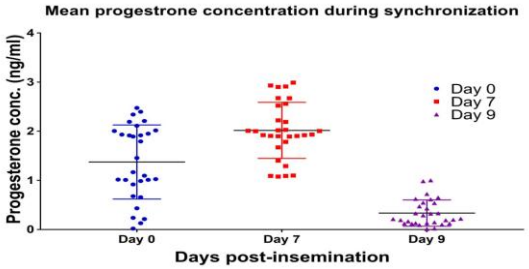


Figure 2. The mean concentrations of progesterone post-insemination including days 7, 14, 21, 30, 40, 50, 60, 70, 80 and 90.

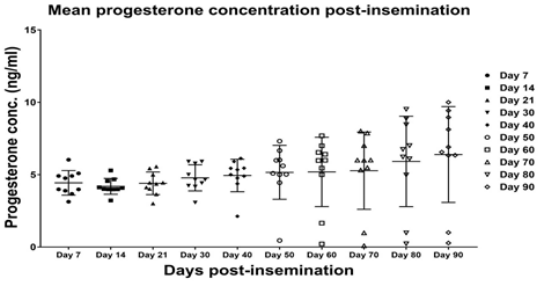


Figure 3. The mean concentrations of progesterone along the experiment; during the synchronization program before insemination in days 0, 7, and 9; and post-insemination including days 7, 14, 21, 30, 40, 50, 60, 70, 80 and 90.

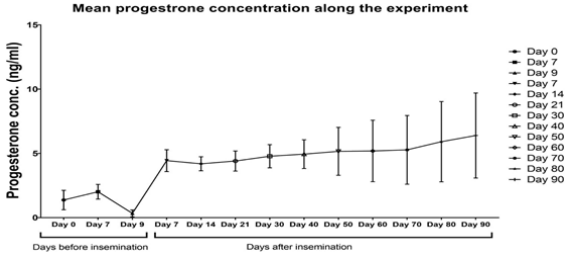


Figure 4. The mean concentrations of ILGFBP-1 post-insemination including days 7, 14, 21, 30, 40, 50, 60, 70, 80 and 90.

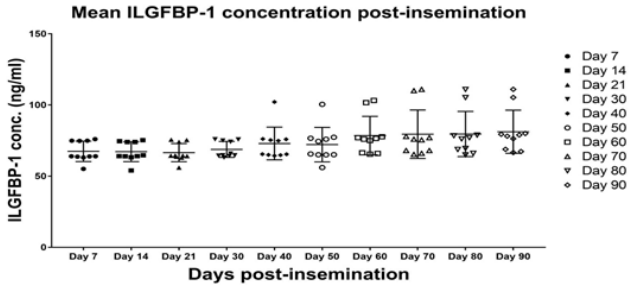


Figure 5. The mean concentrations of ILGFBP-4 post-insemination including days 7, 14, 21, 30, 40, 50, 60, 70, 80 and 90.

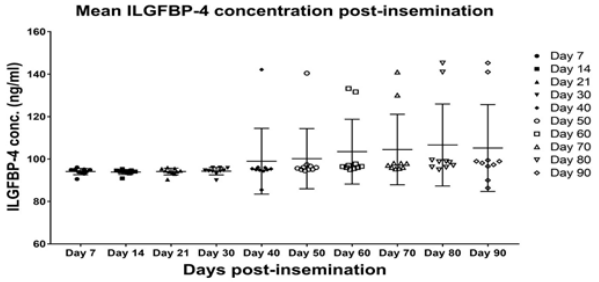


Figure 6. The mean concentrations of IL-6 post-insemination including days 7, 14, 21, 30, 40, 50, 60, 70, 80 and 90.

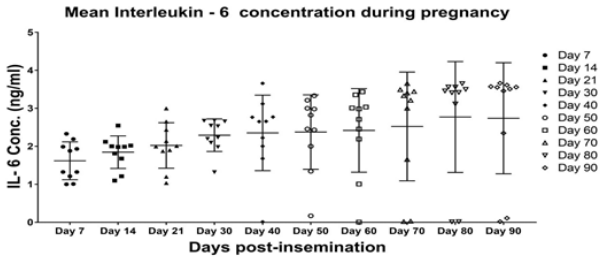


Figure 7. The mean concentrations of IL-6 post-insemination including days 7, 14, 21, 30, 40, 50, 60, 70, 80 and 90.

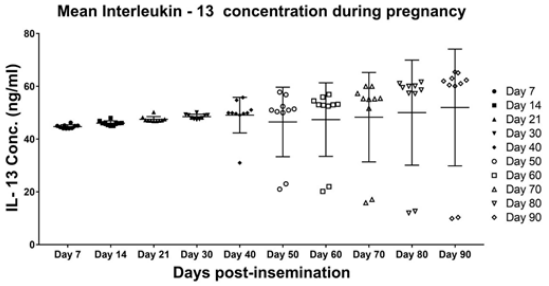
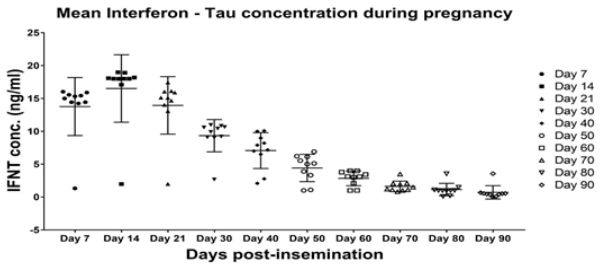


Figure 8. The mean concentrations of IFNT post-insemination including days 7, 14, 21, 30, 40, 50, 60, 70, 80 and 90.



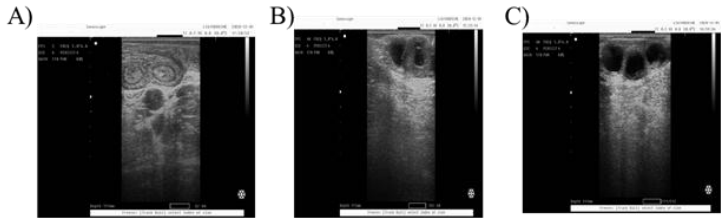


Image 1. Trans-rectal ultrasonography examination of uterine horns and ovarian structures. A) a clear uterine horns. B) Ovarian cyst. C) Ovary showing some mature follicles.

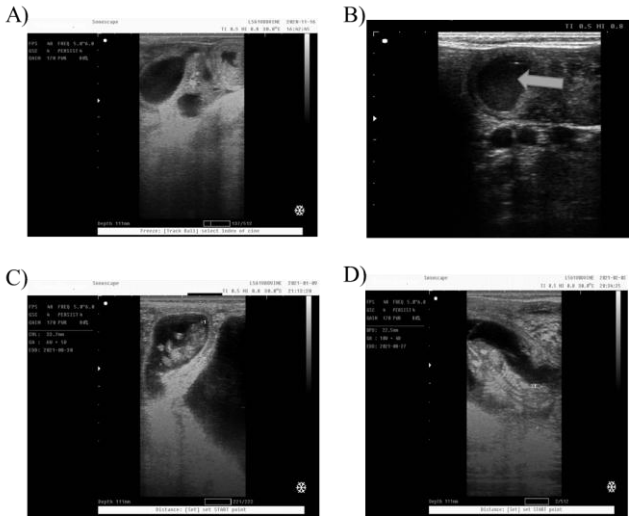


Image 2. Trans-rectal ultrasonography examination of uterus during pregnancy. A) endometrial thickness and some fluctuation on uterine lumen. B) Disappearance to fluid and embryonic sac and embryo were replaced by turbid fluid showing Early Embryonic Death. C) Bi-Parietal Diameter of fetus. D) Trunk Diameter of fetus.