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Multiple Insulin Injections to Improve Ovarian Activity and Pregnancy of Repeat Breeder Friesian Cows

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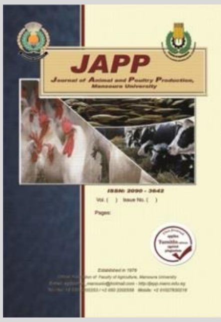

ABSTRACT

This study aimed to evaluate the effect of exogenous multiple insulin injections on ovarian and estrus activity, and pregnancy rate in repeat breeder Friesian cows. Cows (n=12) in heat (Day 0) were divided into three groups. Cows were subcutaneous injected with 0.2 IU insulin/kg LBW on day 9 (G1), 8, 9 and 10 (G2), or 7, 8, 9, 10 and 11 (G3) of the normal estrous cycle. In G1, G2 and G3, cows were i.m. injected with 3 ml PGF2α on day 12. All treated cows in the next heat within 48-72 h post- PGF2α treatment were artificially inseminated with proven fertile semen. Based on overall mean, results showed that follicular number was the highest in G1 and the lowest in G3 ($P<0.05$). Follicular diameter was higher ($P<0.05$) in G3 than in G1. Largest follicles diameter was not affected. Diameter of CL was higher ($P<0.05$) in G2 and G3 than in G1. Serum P4 concentration was higher ($P<0.05$) in G2 and G3 than in G1. Serum P4 concentration was highest in G3. Pregnancy rate was higher ($P<0.05$) in G3 (75%,) than in G1 and G3 (50%). The administration of multiple injections of insulin at days 7, 8, 9, 10 and 11, followed by 3 ml PGF2α on day 12 from initiation of normal estrous cycle improved ovarian and estrous activity, and pregnancy rate of repeat breeders Friesian cows.

Keywords: Insulin, repeat breeder cows, follicular dynamic, progesterone, pregnancy rate.

INTRODUCTION

One of the goals of dairy management programs is to achieve short postpartum period and yielding one calve each year. Delaying the fertilization increases the days open length and postpartum period and decreases longevity of dairy cows. Poor fertility in repeat breeder cows is a multi factorial problem. Life time milk production of cows depends upon re-occurring pregnancy because pregnancy initiates and renews the lactation cycle (Lucy, 2001).

According to Purohit *et al.* (2008), a repeat breeder is typically defined as any cow that has not conceived after three or more services. This syndrome can be one of the more frustrating problems affecting reproductive management of a dairy herd. Garnsworthy *et al*. (2009) reported that insulin has a physiological relationship with the reproductive system, depending on the stage of the reproductive cycle. Insulin is considered as an indicator of energy homeostasis by maintaining peripheral level of glucose via increasing uptake, oxidation, and storage of glucose (Donato *et al*., 2012). Growth factors and metabolic hormones like somatotropins, insulin and IGF-1 had received attention in regulation of ovarian function (Totey *et al*., 1996).

The reduction in follicular responsiveness to gonadotrophic factors by decreasing IGF-I and/or insulin may causr a reduction in estradiol releasing from the dominant follicle leading to negative effect on pulsetile release of LH (Butler, 1999). Spicer *et al*., (1990) observed a positive correlation between serum IGF-I and P4 levels in lactating cows (McGuire *et al*., 1992). In accordance with the present results, Selvarajua *et al.* (2002) reported a marked effect of insulin on pregnancy occurrence in repeat breeding cows.

In different species, Silva and Price (2002) indicated that insulin has direct stimulatory effects on *in vitro* granulosa cell, estradiol production and indirect stimulatory effects via amplification of gonadotropin action. In buffaloes, Bakr and Ramoun (2000) found that insulin treatment may modulate the energy effect on the ovaries to increase response to GnRH treatment. In post-partum cows, feeding a diet that increased insulin level increased the pregnancy rate (Gong *et al*., 2002).

Accordingly, it is possible that insulin injection in the moment of P4 withdrawal in postpartum cows can improve the final follicular development, ovulation, pregnancy rate of repeat breeder cows. In protocols of superovulation in beef cows, insulin increases large follicles diameter and estradiol level (Simpson *et al*., 1994). In cattle, embryo donors treated with insulin to modulate progesterone (P4), insulin, and IGF-I (Sheetal *et al*., 2018).

The main hypothesis of the current study was to determine the optimal times of multiple insulin treatments during estrous cycle for improving fertility of repeat breeder cows synchronized to estrus by PGF2α injection.

MATERIALS AND METHODS

This study was carried out at Sakha Animal Production Research Station; belong to Animal Production Research Institute, Agriculture Research Center, Ministry of Agriculture, Egypt, during the period from January to May 2017.

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Animals:

Total of 16 multi-parous repeat breeder cows had three or more inseminations without conceiving. All experimental cows had 400-550 kg live body weight (LBW), 3-5 parities, 2.5-3.5 body condition score, and normal parturition with placental drop duration from 8 to 12 hours, and.

Cows were submitted to the ordinary system applied at the station, being indoors all over the year and were fed on diet that met both maintenance and production requirements. The type of offered feeds was green feeding system including concentrate feed mixture (CFM) plus fresh Egyptian berseem (*trifolium alixandrinum, 2nd -4th cut*) and rice straw (RS).

Experimental design:

At the beginning of the experiment, all cows (n=12) were noticed for normal estrus incidence, then the experimental cows were divided into three similar groups (4 cows in each), according to their LBW and BCS, (Day 0). Cows in the 1st group (G1) were subcutaneous injected on day 9 of the normal estrous cycle with 0.2 IU/kg LBW of purified insulin (Human Insulin-Mix. VACSERA (30% regular insulin + 70% isophane insulin (10 ml – 40 IU/ml)), followed by intramuscularly injection on day 12 with 3 ml PGF2α analogue (Estromate, Novartis pharma S.A.E Cairo, under licence from Novartis Pharma, AG., Basle, Switzerland). Cows in 2nd group (G2) were subcutaneous injected with insulin (0.2 IU/kg LBW) on days 8, 9 and 10 of the normal estrus, followed by intramuscularly injection with 3 ml PGF2α on day 12.

Cows in 3rd group (G3) were administrated with insulin (0.2/IU/ kg LBW) on days 7, 8, 9, 10 and 11, followed by 3 ml PGF2α on day 12. Estrus was observed in all treated cows within 48-72 h post- PGF2α treatment and animals in heat were artificially inseminated with proven fertile semen.

All treated cows were ultrasonography examined and blood sample were taken on days 0 (at estrus), 8, 12 and AI. The experimental design is summarized as the following:

Diagram showing the experimental design of the study.

Group	Day 0	– – – ➤	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Heat
G1	Estrus	– – ➤			INS	– – – – – – – ➤		$PGF_2\alpha$	AI
G2	Estrus	– – – ➤		INS	INS	INS	– – ➔	$PGF_2\alpha$	AI
G3	Estrus	– – – ➤	INS	INS	INS	INS	INS	$PGF_2\alpha$	AI
USE+BS	•	– – – ➔	•	•	– – – – – – – – – – – ➤			•	•

INS: Insulin (0.2 IU/kg LBW). PG: PGF2α analogue (3 ml *Estromate*/animal).
USE: *Ultrasonography* examination. BS: Blood samples. AI: Artificial insemination • Sampling day.

Experimental procedures:

Estrus was detected by the visual observation for each cow twice daily at 6 a.m. and 6 p.m., Cows get in heat were artificially inseminated after 12 hours from the onset of estrus by good quality frozen semen of the same bull.

Real time ultrasonography equipment (Aquila, pie medical company) with 8.0 MHz liner rectal transducer was used for determination of the ovarian structures including number and diameter of all follicles and CLs on days 0, 8, 12 and on day of AI. Pregnancy was diagnosis on day 35 post-AI by ultrasonographic examination.

Blood sampling:

Blood samples were collected on days 0, 8, 10, 12 of the estrous cycle, and on day of AI to estimate P4 concentration in blood serum. Blood samples were taken into clean test tubes without anticoagulant, and then left for 2-3 h for serum separation, which was stored at -20°C until analysis of P4. The radioimmunoassay of P4 is a competition assay (RIA PROGESTERONE, IM1188, BECKMAN COULTER).

Statistical analysis:

Data were statistically analyzed according to Snedecor and Cochran (1982) using computer programme of SAS system (2002). A factorial design (3 groups x 4 sampling times) was used and the statistical model was: $Y_{ijk} = U + A_i + B_j + AB_{ij} + e_{ijk}$. Where: Y_{ijk} = Observed values, U=Overall mean, A_i = group, B_j = sampling time, AB_{ij} = Interaction due to group x sampling time, and e_{ijk} = Random error.

RESULTS AND DISCUSSION

Results

Follicular dynamics:

Data in Table 1 revealed that overall mean of follicular number (FN) was significantly (P<0.05) the highest in G1 and the lowest in G3, but both groups did not differ significantly (P<0.05) from G2. Overall mean of follicular diameter (FD) was significantly (P<0.05) higher in G3 than in G1, but did not differ significantly in G2 from those in other groups. On the other hand, overall mean of FN significantly (P<0.05) increased, while FD significantly (P<0.05) decreased following estrus and insulin treatment. The FN reached to the maximum number on day of AI, while FD showed marked reduction post-insulin treatment, and then significantly (P<0.05) increased post-PGF2α and on day of AI. However, overall mean of largest follicles (LFD) was not affected by insulin treatment. As affected by sampling time, LFD significantly (P<0.05) decreased post-insulin and Post-PGF2α as compared to at estrus and on day of AI.

Table 1. Follicular number, follicle diameter and largest follicle diameter per cow as affected by insulin treatment and treatment time.

Variable	Follicle number /cow	Follicle diameter (cm)	Largest follicle diameter (cm)
Effect of insulin dose:			
G1 (Insulin D9)	$3.44{\pm}0.24^a$	$0.74{\pm}0.06^b$	1.31±0.50
G2 (Insulin D8-10)	$2.91{\pm}0.23^{ab}$	$0.92{\pm}0.06^{ab}$	1.37±0.50
G3 (Insulin D7-11)	$2.34{\pm}0.29^b$	$1.06{\pm}0.09^a$	1.39±0.06
Effect of treatment time:			
At estrus	$2.13{\pm}0.24^b$	$1.19{\pm}0.07^a$	$1.47{\pm}0.05^a$
Post-insulin	$2.97{\pm}0.19^a$	$0.67{\pm}0.05^c$	$1.21{\pm}0.05^b$
Post-PGF2α	$3.25{\pm}0.43^a$	$0.86{\pm}0.07^b$	$1.25{\pm}0.04^b$
On day of AI	$3.44{\pm}0.35^a$	$0.96{\pm}0.08^b$	$1.45{\pm}0.03^a$
Interaction (P value)	0.243^{NS}	0.759^{NS}	0.068^{NS}

a-c: Means with different superscripts within the same column for each variable are significantly different at P<0.05. NS: Not significant.

As affected by the insignificant effect of interaction on all parameters, G1 showed the greatest FN, and the shortest FD and LFD following insulin treatment (on day PGF2α), in an opposite trend for G3. However, G2 showed moderate values between G1 and G3 (Figs. 1-3).

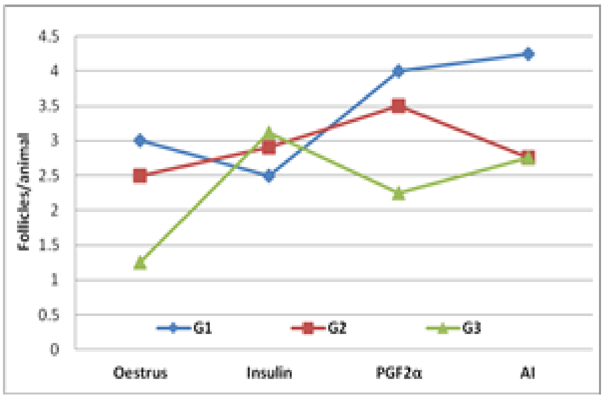

Fig. 1. Average number of follicles/ovary of the experimental groups at treatment days.

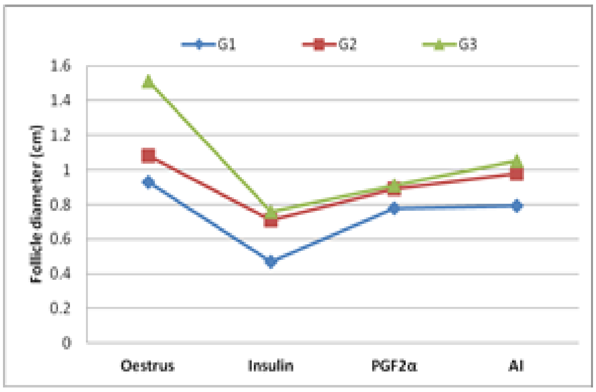

Fig. 2. Average diameter (cm) of follicles on ovaries of the experimental groups at treatment days.

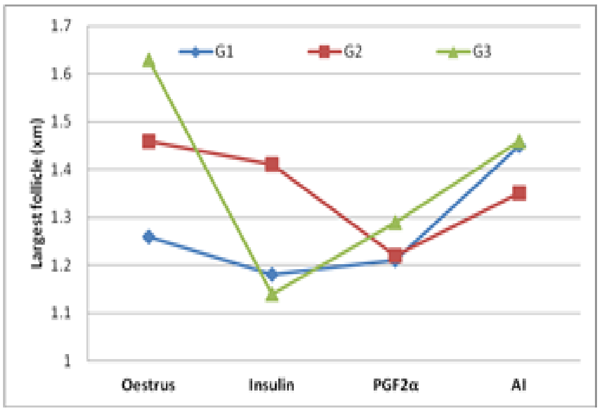

Fig. 3. Average diameter (cm) of largest follicle on ovaries of the experimental groups at treatment days.

Corpus luteum:

Ultrasonoghraphic examination of cows revealed presence of one complete functional CL/animal (on the right or left ovarian side) of all cows in each group only following insulin and PGF2α treatments and with significant differences in CL diameter in each group.

However, no CLs were observed at estrus or on day of AI. After insulin and PGF2α treatments, diameter of CL was significantly (P<0.05) higher in G2 and G3 than in G1 and G4 (Table 2).

Table 2. Effect of insulin doses on number and diameter of corpus luteum per cow at different treatment times.

Item	G1	G2	G3
CL number/animal:			
Estrus	0.00	0.00	0.00
Insulin	1.00	1.00	1.00
PGF2α	1.00	1.00	1.00
AI	0.00	0.00	0.00
CL diameter (cm):			
Estrus	0.00	0.00	0.00
Insulin	0.78 ± 0.04^b	1.33 ± 0.12^a	1.57 ± 0.12^a
PGF2α	0.80 ± 0.05^c	1.86 ± 0.10^a	1.99 ± 0.04^a
AI	0.00	0.00	0.00

a-c: Means with different superscripts within the same column for each variable are significantly different at P<0.05.

Progesterone profile:

Effect of treatment:

Effect of treatment on overall mean of plasma P4 concentration of cows was significant (P<0.05), being higher in G2 and G3 than in G1. The highest overall mean of P4 concentration was obtained for cows in G3 (Fig. 4).

Plasma P4 level was higher in pregnant than in non-pregnant cows, being higher in G2 and G3 than in G1. However, the differences were not significant within each experimental group (Fig. 5).

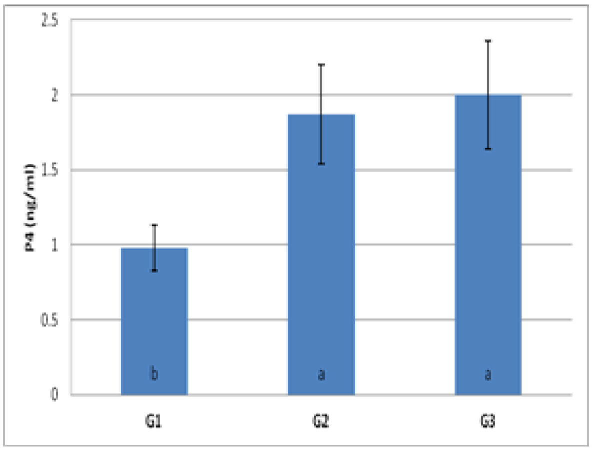

Fig. 4. Overall mean of plasma P4 concentration of cows in experimental groups.

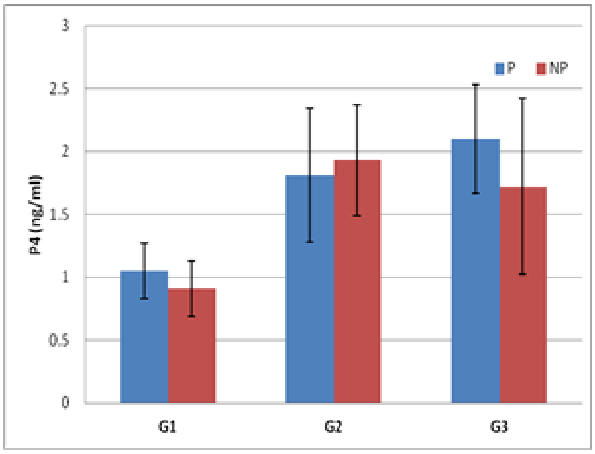

Fig. 5. Average of plasma P4 concentration of pregnant and non-pregnant cows in the experimental groups.

Effect of sampling time:

Effect of treatment time also had significant effect on overall mean of plasma P4 concentration of cows. After estrus, overall men of plasma P4 level showed significantly (P<0.05) gradual increase from <1 ng/ml to > 1 ng/ml post insulin and PGF2α treatments, then significantly (P<0.05) decreased to <1 ng/ml on day of AI (Fig. 6).

Through different treatment times, plasma P4 level showed similar trend of change, but was higher in pregnant than in non-pregnant cows, being higher after insulin and PGF2α than at estrus and on day of AI. However, the differences were not significant at each treatment time (Fig. 7)

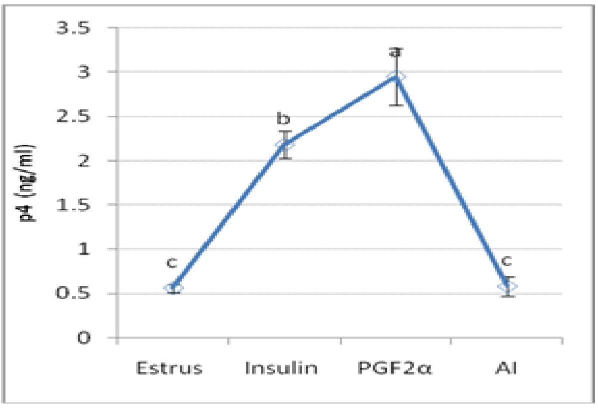

Fig. 6. Overall mean of plasma P4 concentration at different treatment times.

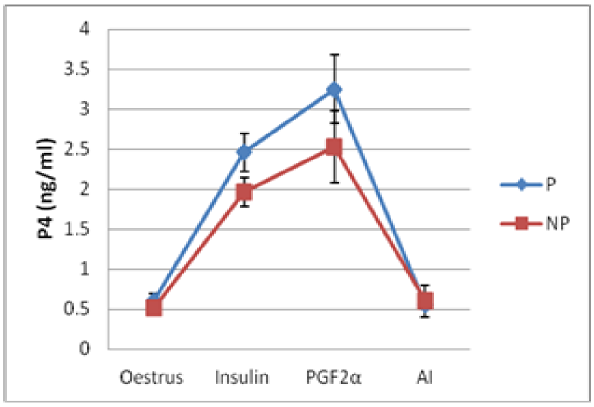

Fig. 7. Average of plasma P4 concentration of pregnant and non-pregnant cows at different treatment times.

Effect of interaction:

Analysis of variance revealed that the effect of interaction between insulin treatment and treatment time on P4 concentration was non-significant. This effect was reflected in similar trend of increase in P4 level following incidence of estrus up to day of AI in all groups, being the highest in G3, followed by G2 and G4, respectively, and the lowest in G1. Generally, cows in all groups showed P4 level of <1 ng/ml at estrus and on day of AI, and P4 concentration was >1 ng/ml in G1, and >3 ng/ml in G2 and G3, following insulin and PGF2α treatments (Fig. 8).

Through different treatment times, plasma P4 level showed similar trend of change, but was higher in pregnant than in non-pregnant cows at each treatment time and each group. However, the differences were not significant at each treatment time (Fig. 9)

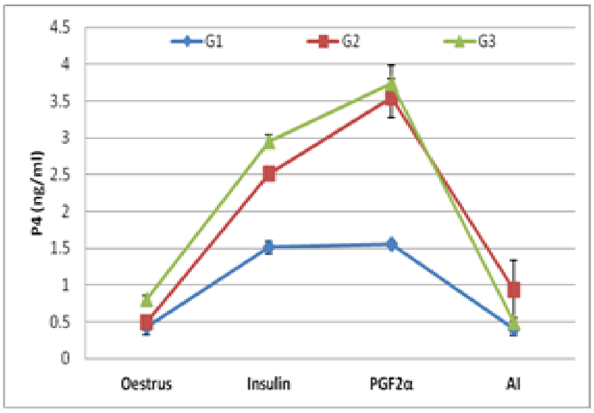

Fig. 8. Average of plasma P4 concentration in experimental group at different treatment times.

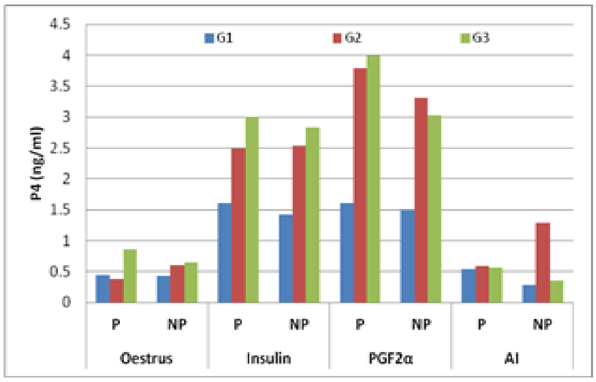

Fig. 9. Average of plasma P4 concentration of pregnant and non-pregnant cows in each group at different treatment times.

Pregnancy rate:

Pregnancy rate was significantly (P<0.05) higher in G3 (75%, 3 out of 4 cows) than in G1 and G3 (50%, 2 out of 4 cows I each, Fig. 10).

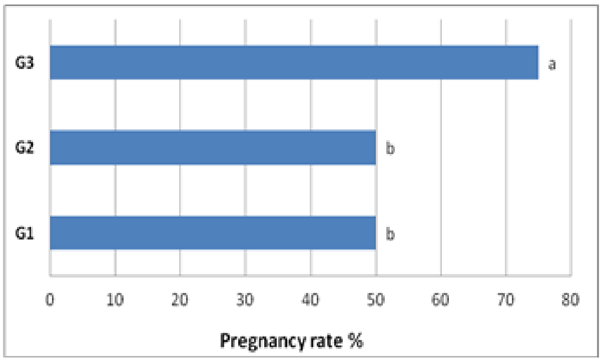

Fig. 10. Pregnancy rate of cows in different experimental groups (statistically analyzed by Chi-square test).

Discussion

The main aim of the current study was to determine the optimal times of multiple insulin treatments during estrous cycle for improving fertility of repeat breeder cows.

Treatment of insulin on the 9th day (G1) or from the 8th to 10th day (G2), following by PGF2α significantly increased pregnancy rate (PR) of repeat breeder cows to 50% in G1 and G2 and to 75% in G3. The present results indicated similar PR of insulin treatment on the 9th (G1) or on the 8^{th,} ^{9th} and 10th day (G2). However, the highest improvement in PR achieved with insulin treatment on days from 7 to 11 of the estrous cycle, followed by PGF2α.This was indeed the best strategy of insulin treatment under the conditions of this experiment.

Pregnancy responses of cows treated with insulin can, therefore, be attributed to direct and indirect effect of insulin on the reproductive system.

The obtained results at estrus, showed a variation in FN, FD and DLF of the 1st follicular wave, with nearly similarity in P4 level in all groups prior to treatment application, indicated that all animals had different follicular dynamics with similarity in CL cases (no CLs were found on the right or left ovary). The positive impact of insulin through the presence its receptors on the ovary, oocyte, embryo, oviduct and uterus could improve the development of follicles, quality of oocyte and early development of embryos (Robinson *et al*., 2000). In accordance with reducing the follicular diameter in G1 than in other groups, Maffi *et al*. (2019) indicated that a single dose of insulin does not promote an increase in follicular size and conception rate in dairy cows. In response to GnRH, insulin treatment significantly increased the largest follicle diameter to ovulate (Beam and Butler 1997, 1999; Rhodes *et al*. 2003). The present results cleared insignificant effect of insulin treatment on diameter of the largest follicle in all groups. This means that insulin treatment significantly enhanced the largest follicle size (Simpson *et al*., 1994). Insulin has a direct effect on its own receptors on the ovary and subsequence follicular growth (Poretsky and Kalin 1987) or an indirect effect on increase of the follicular sensitivity to FSH (Simpson *et al*. 1994). In cows, insulin was found to increase the follicular growth (Gupta *et al*., 2010; Schneider *et al*., 2010; Chaves *et al*., 2012). In heifers, increasing insulin level through different feeding systems (hyperinsulinemia) may increase the follicular growth and follicular recruitment (Gamarra *et al*., 2014). The increase in insulin and IGF-1 levels had a stimulatory effect on follicular growth (small follicle).

It is of interest to note that PR with different values had occurred following insulin and PGF2α treatments in G1, G2 and G3. Schneider *et al*. (2010) found that administering both insulin and eCG during the timing AI protocol lead to an increase in pregnancy rate in Angus cattle in comparison to animals receiving only eCG. The recorded diameter in all groups following insulin treatment was the larger in G2 and G3 than in G1. This was associated with the times of insulin treatment and follicular size. The population of ovarian follicles has been reported to be more during 9-13 days of cycle or during CL dominance or at a higher level of P4 (Sheetal *et al*., 2018). Also, Vasconcelos *et al*. (2001) indicated that smaller ovulatory follicles generate smaller CL in the subsequent estrous phase and secrete less P4 than larger follicles. Inducing ovulation of small follicles may result in low pregnancy rate and increased embryonic mortality rate (Perry *et al*., 2005). The presence of insulin receptors has been reported on bovine CL (Sauerwein *et al*., 1992; Lucy *et al*., 1993), therefore, insulin regulates CL function, through glucose availability and hormone production in cattle (Sousa *et al*., 2016).

The obtained results revealed that the mean concentration of plasma P4 was nearly similar at estrus, then level of P4 showed marked increase up to day 12th (PG treatment), as functional CL was present on the ovary of each cow. The increase observed in P4 level following insulin treatment in each group was in depending on CL diameter manner. Concentration of P4 was the highest in G3, moderate in G2, and the lowest in G1, following insulin and PG treatments (Fig. 8). These results are in association with average CL diameter (Table 2), in agreement with Pandey *et al.* (2016) and Sheetal *et al.* (2018), who reported values of P4 <2 ng/ml on the 5th and 9th day of estrous cycle. Increasing P4 concentration in G3 to the highest level in comparing with other groups was mainly attributed to longer time of insulin (7-11 d) than in G2 (8-10 d), while the lowest P4 concentration in G1 was due to the short time treatment with insulin (the 9th day).

Similarly, some authors (Shukla *et al.,* 2005; Gupta *et al*., 2011) reported increase of serum P4 level by exogenous insulin, which is considered as a luteotrophic factor. More follicular recruitment was reported by increasing P4 concentration at the 2nd follicular wave leading to more yield and recovery rate of embryos (Rivera *et al*., 2011). Additionally, increasing P4 level prior to estrus incidence is very important for fertilization in repeat breeder animals as mentioned by Abo-Farw *et al*. (2009) in repeat breeder Egyptian buffalo heifers. The P4 declined significantly following PGF2α injection in G1, G2 and G3 reached its basal level on the day of AI (<1 ng/ml) was reported by many authors (Siddiqui *et al.,* 2011; Sheetal *et al*., 2018).

In this respect, the present results indicated higher P4 level in pregnant than in non-pregnant cows in each group following insulin and PGF2α treatment (Fig. 7) in all groups, but the differences were not significant.

Insulin induced upregulation of type I IGF receptors would facilitate increased responsiveness to combined stimulation of ovarian steroidogenesis by IGF-I, IGF-II and insulin. Insulin infusion improved energy balance (Butler *et al*. 2003; 2004). The reduction of insulin and IGF-1 levels in blood plasma may be in relation with failure in LH pulsatile release (Brown *et al*., 2012) and delaying the preovulatory follicle development and ovulation (Shimizu *et al*., 2008; Kawashima *et al*., 2012).

It was reported that insulin increase LH the release via the effect of high estradiol levels (positive feedback) resulting from the largest follicles. Undergoing insulin, serum estrogen level increases within 3 days after insulin injection and simulation of LH release in both frequency and amplitude treatments (Ramoun *et al*., 2007).

This study concluded that the administration of multiple injections of insulin at days 7, 8, 9, 10 and 11, followed by 3 ml PGF2α on day 12 from initiation of normal estrous cycle (3rd group) improved ovarian and estrous activity, and pregnancy rate of repeat breeders friesian cows.

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حقنات الأنسولين المتعددة لتحسين النشاط المبيضى والحمل فى الأبقار الفريزيان المتكررة التلقيح

وائل فكرى فؤاد ، وائل عبد المنعم الحمادى ، أحمد محمد شهاب الدين و محمود عبد الغنى الحناوى
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تهدف هذه الدراسة الى تقييم تأثير حقنات متعددة من هرمون الأنسولين على النشاط المبيضى و الشبقى ومعدل الحمل فى أبقار الفريزيان متكررة التلقيح. الشبق بعد حدوث الشياع قسمت الأبقار (عدد = 12) إلى ثلاث مجموعات. حقنت الأبقار تحت الجلد بـ 0.2 وحدة دولية أنسولين / كجم من وزن الجسم في اليوم 9 من دورة الشبق الطبيعية في المجموعة الأولى و الأيام 8، 9 و 10 فى المجموعة الثانية و 7، 8، 9 ، 10 و 11 فى المجموعة الثالثة. تم حقن الابقار فى كل المجموعات عضليا بـ 3 مل من هرمون البروستاجلاندين في اليوم 12 من الشياع وتم تلقيح الأبقار الشائعه صناعيا باستخدام سائل منوى مجمد ذو خصوبة عالية خلال 48-72 ساعة بعد الحقن بالبروستاجلاندين. أظهرت النتائج مايلى: زاد عدد الحويصلات فى المجموعة الاولى وانخفض في المجموعة الثالثة (P<0.05) عن المجموعه الثانيه. أرتفع قطر الحويصلات المبيضيه (P<0.05) في المجموعة الثالثة عن المجموعة الاولى، بينما لم يتأثر قطر اكبر حويصلة مبيضيه بالمعامله بالأنسولين. كان قطر الجسم الأصفر أعلى (P<0.05) في المجموعة الثانية والمجموعة الثالثة عن المجموعة الاولى. زاد تركيز البروجستيرون في سيرم دم الابقار (P<0.05) في المجموعة الثانية والمجموعة الثالثة عن المجموعة الاولى، وكان أعلى تركيز لهرمون البروجستيرون فى سيرم الأبقار في المجموعة الثالثة. أرتفع معدل الحمل (P<0.05) فى المجموعة الثالثة (75%) عن المجموعة الاولى و الثانيه (50% لكل مجموعه). وتوصى الدراسه أن إعطاء حقن متعدد من الأنسولين في الأيام 7، 8، 9، 10 و11 من دورة الشياع، متبوعة بـ 3 مل من البروستاجلاندين في اليوم 12 ادى الى تحسين نشاط المبيضى و الشبقى ومعدل الحمل للأبقار الفريزيان متكررة التلقيح