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Leptin, Insulin like growth factor -1 and progesterone hormones during superovulation and early pregnancy of Arabian Dromedary camels

[1]Amal M. Abo El-Maaty, [1]Heba F. Hozyen, [1]Mahmoud A.I. Elgabry, [1]Hany A. Hussein

[1]Animal Reproduction and AI Dept., Veterinary Division, NRC, Dokki, Giza, Egypt

Abstract

Background

Assisted reproductive techniques are not commonly used in Arabian dromedaries due to the lower responses to multiple ovulation techniques and conception rate compared to bovine. This study aimed to relate responses to superovulation and circulating leptin and insulin like growth factor-I (IGF-1).

Results

Results revealed that 14, 21 and 28 days pregnant conceived recipient camels had significantly (P=0.0001) high leptin but significantly (P=0.0001) low IGF-1 concentrations compared to non pregnant cyclic. Superovulation treatment significantly (P=0.0001) affected leptin, IGF-1 and P4 concentrations. Leptin concentrations were significantly (P=0.0001) low at breeding of superovulated camels. IGF-1 concentrations were significantly (P=0.0001) low at the day of first GnRH treatment, Last day of FSH treatment and days of breeding. Finally, responded donors had significantly (P=0.0001) high IGF-1, leptin but low P4 concentrations

compared to non- responded ones and pregnant recipient camels had significantly higher leptin but significantly low IGF-1 and P4 compared to non-responsive recipients.

Conclusions

In conclusion, leptin, IGF-1 and P4 play significant role during superovulation program and conception in dromedary camels. Leptin and IGF-1 are necessary during follicle growth, ovulation, response to superovulation and early embryonic development in dromedaries.

Keywords: superovulation, IGF-1, Leptin, progesterone, camel

Introduction

Old Arabian dromedary camel's reproductive physiology has received little interest compared to bovine and equine species. Female dromedary camels' reproduction is characterized by a seasonal activity and ovulation is induced [1]. Using ultrasonography, it was better to induce ovulation with GnRH, when the dominant follicle diameter measures between 13 and 18 mm, with depositing semen into the uterus 24 hours later, and pregnancy could be diagnosed from day 18 after breeding [2].

Leptin is a 16kDa hormone that is mainly secreted by white adipose tissue of camels [3]. Leptin and its receptor were expressed by adipose tissue, mammary alveolar epithelial cells, liver hepatocytes, and the lining epithelium of the bile duct of the one-humped camel (Camelus dromedaries) [4]. Circulating leptin is related to fat mass content in ruminants [5], horses [6], and camels [3]. In cows [7], and mares [8,9], leptin levels increased as ovulation approached. Leptin also played a role during follicle growth, ovulation, and corpus luteum development and modulating uterine blood flow before and after ovulation in mares [9]. Moreover, leptin has immunoregulatory and angiogenic effects [10]. Yet relation of leptin to reproduction in Arabian camels received no interest.

Insulin-like growth factors (IGFs) are small peptides, of 7 kDa size and similar to insulin in structure [11]. They act systemically and locally as autocrine/ paracrine factors [12,13]. They are essential for normal growth and development [14]. Insulin-like growth factor I (IGF-1) promotes cell proliferation and differentiation of several tissues including ovarian tissue and function [15], and its biological availability is regulated by IGF binding protein-1 (IGFBP-1)

[16]. IGFBP-1 levels reflect changes related to nutrition, insulin secretion and fetal development [17]. Components of the IGF system were found within equine [18] and camel [19] ovarian follicle. IGF-1 concentrations were related to growth and body weight of Shami Dromedary prepubertal Heifers [20], and nutritional supplementation of postpartum camels [21]. IGF-I is synthesized by granulose cells of ovarian follicles [22], and had a stimulatory effect on leptin receptor expression of luteal cells [23]. Because of the rareness of data on relation of both leptin and IGF-1 concentrations to reproduction of dromedary camels, this study aimed to investigate the circulating concentrations of leptin and IGF-1 in superovulated camels and early pregnant Arabian camels and find out their relation to superovulation responses.

Methods

Animals and treatment

Female dromedary camels (N=30) were equally divided into either donors or recipients, aged 7-10 years, were selected for performing this experiment which was conducted during the physiological breeding season (from October to April, Al-Eknah et al., 1997). All camels were in good physical condition and were fed a diet of mixed concentrates and hay twice a day, and water was available ad libitum. During the breeding season, the camels were selected after ultrasonographic examination and proved to be free from reproductive abnormality. On March 1st2013, all donor and recipient camels received intramuscular injection of 8 µg Buserelin (Synthetic Gonadotrophin Releasing Hormone, GnRH, Receptal, Schering-Plough Animal Health Ltd) for synchronizing estrus. On the 7th day, donor camels were treated with 80 mg Urofollitropin FSH (Fostimon, IBSA Farmaceutici Italia S.r.l) every 12 hour with 2000 i.u. equine chroionic gondaotropins, (eCG, Folligon, Intervet, The Netherlands). On 8th day (60mg), 9th day (40mg), and 10th day (20mg) decreasing doses of Urofollitropin were administered twice a day, respectively. On the 10th day, all camels were treated with 7.5 mg intramuscular injections of Lutalyse (Upjohn Veterinary Products, Inc., Kalamazoo, MI, USA). Donor camels were naturally bred twice on 16th day and 17th day and treated with 8 µg Buserelin. Donor camels produced embryos were classified as responded but those did not produce embryos were classified as non-responded. Recipient camels were treated as donors except for FSH and breeding and became pregnant are classified as pregnant recipient but those received embryos but detected non-pregnant were classified as non-responded recipients.

Embryo-flushing and transfer processing

Embryos were recovered on days 23th and 24th and transferred directly to recipients after evaluation of recovered embryos [24].

Ultrasonographic examination

Ultrasonography was conducted using an Aloka 210 Ultrasound (Corometrics Medical Systems, Inc., Wallingford, CT, USA) supplied with a 5-mHz probe for selecting suitable animals before conducting this experiment. Following transfer of embryos, recipients were examined with ultrasound at weekly interval for detecting early pregnancy. Detection of Pregnancy started from day 18-21 and confirmed on day 28 to 40 of gestation.

Blood samples

Blood samples (10ml) were collected from non pregnant donors and recipients by jugular venepuncture from Days 1 to 17 and from conceived recipients on days 14, 21 and 28 of pregnancy. Samples were placed on ice, transported to the laboratory and centrifuged at 3000g for 15min. Sera was harvested and stored at -20 ℃ until hormone analysis.

Hormone analysis

Progesterone hormone (P4) was assayed using commercial ELISA (DRG, International, Inc., USA). P4 sensitivity was 0.045ng/mL. Intra- and inter-assay coefficients of variation of progesterone were 6.86 and 5.59%.

Camel leptin Cat.No: MBS091173 and camel IGF-1 Cat.No: MBS077229 were assayed using Quantitative Sandwich ELISA kit (MyBioSource.com). The detection range is 0.5ng/ml-16ng/ml for leptin and is 15.6ng/ml-500ng/ml For IGF-1. Sensitivity of the assay was 0.1 ng/ml for leptin and 2.0 ng/ml for IGF-1. Both Intra-assay CV (%) and Inter-assay CV (%) of both leptin and IGF-1 was less than 15%. [CV(%) = SD/mean ×100].

Statistical analysis

Data are presented as Mean ± SEM (Standard error) using SPSS software [25]. Analysis of variance simple one way ANOVA was used to compare between levels of progesterone, leptin and IGF-1 during different days of superovulation of donor and recipient camels and weeks of early pregnancy and Duncan's Multiple Range test was used to differentiate between significant means. Independent sample t-test was used to compare between cyclic superovulated and early pregnant camels, in addition to donors and recipients.

Results

Results revealed that the concentrations of leptin (Table 1) of cyclic superovulated camels was decreasing from the start of the superovulation program linearly and significantly (P=0.0001) till day of mating but 14 and 28 days pregnant recipient superovulated camels had significantly higher leptin. Concentrations of IGF-1 of pregnant recipient camels were significantly low (P=0.0001) compared cyclic superovulated camels (Table 1). Generally, early pregnant camels had higher leptin but lower IGF-1 concentrations than non pregnant superovulated ones (Table 1).

It is clear from table (2) that superovulation treatment significantly (P=0.0001) affected concentrations of leptin, progesterone and IGF-1. Leptin concentrations were significantly low on the day of breeding compared to the first day of treatment with Buserelin, 2nd and 4th day of treatment with Urofollitropin and insignificantly lower than the 1st and 3rd day of treatment with Urofollitropin. IGF-1 concentrations were significantly low on the day of breeding, the first day of starting superovulation program and 4th day of treatment with Urofollitropin compared to 1st 2nd, and 3rd days of treatment with Urofollitropin. It is obvious that mean progesterone concentrations were higher than 2ng/ml from the 1st day of the program till the day of breeding. However, P4 concentrations were significantly low (P=0.0001) on the ay of treatment with Buserelin,2nd and 3r days of treatment with Urofollitropin compared to 1st , 4th days of treatment with Urofollitropin and day of breeding (Table 2).

Generally, recipient camels had significantly high concentrations of leptin (P=.05), slightly high IGF-1 and low P4 (P=0.024) as compared to donors (table 3). Moreover, treatment significantly affected concentrations of leptin (P=0.002), IGF-1(P=0.007) and P4 (P=0.02) of superovulated donor and also leptin (P=0.019), IGF-1(P=0.001) and P4 (P=0.001) of synchronized recipient (Table 3). Superovulated donor camels had higher P4 concentrations (>2ng/mL) at all the treatment days but synchronized recipients even not mated but comparably had higher progesterone (>2ng/mL) during 9th, 10th and days of breeding. Leptin concentrations of donor camels were >2ng/mL during 8th, 10th days (Table 3). However, leptin concentrations of synchronized recipient camels were significantly higher than >2ng/mL on the start of study 1st and 8th day of the study but significantly lowest concentration was observed at the days of breeding. The lowest leptin concentrations of superovulated donor camels and synchronized recipient camels were observed on days 16th

and 17th. The highest leptin concentrations of both donors and recipients were recorded on the 8th day of the experiment following the first FSH treatment of only donors (table 3).

The lowest IGF-1 concentrations of superovulated donor camels were observed on days of breeding but those of synchronized recipient camels were observed on the 10th day of study. The highest IGF-1 concentrations of both donors and recipients were recorded on the 7th day of the experiment (table 3).

Leptin and IGF-1 concentrations of responded donor were significantly (P=0.0001) higher but P4 concentrations were lower than those of non-responded donor or recipient (table 4). Both non-responded donors and pregnant camels had significantly (P=0.001) the lowest IGF-1 concentrations (table 4).

Progesterone had a negative significant correlation with IGF-1 (r=-0.25; P= 0.001), but had a low positive significant correlation with leptin (r=0.18; P= 0.01). Leptin had a low negative correlation with IGF-1 (r=-0.12; P= 0.026)

Discussion

It was important during camels' embryo transfer program to find good quality recipients [26]. Camels responded differently to the superovlation [27], but season did not affect response of camel to superovulation but combining both pFSH and eCG was better than pFSH alone [26]. Camels superovulated with decreasing doses of pFSH for 4 days combined with LH on day 0 and day 12 an eCG on the first day of pFSH responded better than compared to those received no LH and inserted with CIDR for 7 days before the same pFSH regime in producing more corpora lutae and less corpora follicles [27]. A possible cause for increasing progesterone of dromedary camels treated with a lower dose of GnRH analogue (8 µg Buserelin) during this study is the induction of ovulation with such lower dose similar to the induced ovulation of Bactrian camels [28], or dromedary camel [29-31] using higher doses (20 µg) of the GnRH analogue (Buserelin), that ovulated within 1-2 days. Another possible explanation of the increased progesterone concentration at the start of the experiment is the occurrence of spontaneous ovulation [32], and mean serum progesterone levels were higher after induced ovulation but compared to induced ovulation by a GnRH, functional development of the corpus luteum (CL) after spontaneous ovulation might be altered but the morphological development is not affected [32]. Confirming both suggestions, Ismail et al., [33]

superovulated dromedary camels using a closely program similar to that used during the present study and recorded also a progesterone concentrations >2ng/mL just before GnRH (20µg Buserelin) treatment and >4ng/mL at the superovulation treatment 7 to 10 days after priming with GnRH. Also, the dromedary camels of this study were having high progesterone concentrations 7 to 10 days after the first GnRH treatment confirmed the assumption of either spontaneous or induced ovulation. After the inducing stimulus, the CL of camels needed 3-4 days for developing with low progesterone concentrations, become mature 8-9 days with maximum P4 values [34] and started regressing 9-10 days [35,36], or 11-12 days after ovulation [34,35], then P4 decreased to basal concentrations. The follicular wave length of induced ovulators lasts from 17.2 to 23.4 days in India [37], 24.2 days in Egypt [38], and 28 days in Sudan [39], and also increased toward the start and the end of the breeding season [40]. A last explanation of the increased progesterone at and after treatment with GnRH analogue is the production of progesterone by overlarge follicles that sometimes become luteinized and produce levels of progesterone similar to that observed in presence of a CL [35,36]. Meanwhile, the CL developing after ovulation, the linear growth rate, duration of growth and mature phases of the dominant follicle and the development of the dominant follicle to its maximum size during its mature phase and inter-wave interval were not affected by the P4 secreted by the induced CL [41]. Although progesterone concentrations of large follicles were higher but serum concentration was many folds lower than those of follicular fluid [42]. Moreover, follicular fluid of follicles ranging from 0.5 to 3cm [35], and atretic follicles of 2 to 3cm in diameter contained lower P4 concentration [43]. In addition, recent studies of female dromedary camels recorded higher concentrations of progesterone in the follicular fluid as the diameter of the ovulatory-sized follicle increases with no differences between serum concentrations of progesterone of camels with different ovulatory-sized follicles from 10 to >30 mm diameter and the final stages of oocyte maturation *in vivo* was also associated with increasing progesterone concentrations [19]. In agreement with our results where higher progesterone concentrations observed in superovulated camels, the diameter of the dominant follicle could continue increasing when it starts to lose its dominance, it allows the emergence of the next follicular wave [44]. As well as, 50% camels having dominant follicles continue to grow, even after losing their dominance, reaching a mean maximum diameter of 42 mm (range 40-64 mm), sometimes become luteinized and produce levels of progesterone similar to that observed in presence of the corpus luteum [35,36]. However, when both exogenous progesterone and eCG were used to maintain

pregnancy of recipient dromedary camels, those could not maintain their pregnancy was due to irresponsive to induction of ovulation using eCG or shortly after stoppage of exogenous progesterone [45]. Even when ovulation was induced using hCG in dromedary camels [44], the peak progesterone recorded 7 days after ovulation was still higher than that recorded during the current study. Although ovarian follicular status and ovulation rate was similar among dromedary camels either mated at random, treated with GnRH analog (Buserelin, 20 μg/animal) then were mated 14 days later, or treated twice with GnRH 14 days interval and mated 28 days later but favorable pregnancy rate was achieved following ovarian follicular wave synchronization with repeated GnRH analog and fixed-time natural mating at 14 days [29]. In agreement with the increase of progesterone during the superovulation of donor and recipient camels of this study, Egyptian camels superovulated with single or multiple decreasing doses of eCG after 13 days of CIDR insertion had high progesterone on the day of CIDR removal even though all superovulated and control camels treated with prostaglandine F2α analogue 24 hours before CIDR removal [46]. In contrast to the higher P4 concentrations recorded during follicular phase of superovulated camels of this study; both Homeida et al. [47] and Skidmore [48] reported higher P4 only after mating and ovulation of dromedary camels, reaching 3ng/mL by day 8-9 [35]. However, high incidence of overstimulation, irresponsive females and lutinized follicles, in addition to wide variability in response to ovulation and embryos collected are the main factors adversely encourage using superovulation in camels [49]. While the linear increase of P4 significantly increased the number of CL [50]. The increased progesterone concentrations detected in non responsive camels of this study may be due to the induction of ovulation at the start of the treatment similar to the induced ovulation using hCG [35]. An 85% ovulation rates was achieved when the dominant follicle diameter was ≤19 mm, and declined to 12.5% when the dominant follicle diameter ranged from >19 and >29 mm, but follicles of diameter >30 mm and follicles during the regression phase cannot ovulate [35].

Camels are different from other mono-ovular ruminants where progesterone could be synthesized from large atretic follicles, small follicles other than corpora lutae in levels. Similar to induced ovulation either hormonally or via natural mating, the increased P4 concentrations recorded during superovulation confirmed such suggestion and indicated that growing large follicle or superovulation needed deep investigations using Doppler ultrasound, OPU parallel to the superovulation program. The progesterone concentrations recorded in

pregnant recipient dromedary camels of the current study is similar to progesterone concentrations increased in pregnant Iraqi dromedary camels from day 20 to day 180 [51].

Both IGF1 and leptin are closely associated with body condition score of cows [52], nutritional levels of mares [53.54]. Within bovine large follicles, IGF-1 concentrations were significantly higher during the luteal phase compared with the follicular fluid [7]. In camels, IGF-1 increased with increasing nutritional supplementation from day 21 to day 90 postpartum [21]. Early pregnant superovulated recipient camels had significantly lower concentrations of IGF-1 compared to superovulated donor camels. In superovulated cows, the linear increase of IGF concentrations significantly increased the number of CL [50]. Higher IGF-I actions in the uterus after superovulation may be responsible for the increase of early embryonic loss. The detrimental factor for embryo development seems a small molecule and is likely a local product of the uterus in which IGF-I actions are enhanced [55]. The significant increase of IGF-1 levels is linked to early pregnancy loss [56]. The lower IGF-1 concentration recorded in pregnant camels of the current study compared to non pregnant superovulated either responded or non- responded camels is similar to that recorded in cows where excess IGF-1 did not improve blastocyst formation and induced higher levels of apoptosis in the bovine embryos [57], and adversely affected endometrial function leading to deteriorating effects on implantation [58,59]. The increased levels of apoptosis in the embryos is triggered upon exposure to high concentration of IGF-I, so like other cell systems, preimplantation blastocyst respond to high concentration of IGF-I by down-regulating the IGF-I receptor and when the blastocyst becomes insulin resistant, it reduced intra-embryonic glucose levels leading to apoptosis [60,61].

In dromedary camels, IGF-I was significantly higher in ovulatory follicles of 18 to 30 mm diameter compared to other groups of follicles and serum concentrations of IGF-I was similar to its follicular fluids concentrations and was linearly increasing with the increase of the ovulatory follicle diameter from 10 to >30mm and the final stages of oocyte maturation *in vivo* is also associated with increasing IGF-I concentrations [62]. Moreover, the increase of IGF-1 levels is associated with significantly increase of CL numbers after superovulation [50]. The significant increase of IGF-1 concentrations in superovulated Arabian dromedary camels of this study compared to the early pregnant recipients indicated the importance of IGF1 for the success of superovulatory treatments of ovine [63], and bovine [64]. Moreover, the significant increase of IGF-1 levels of the responded donors compared to non-responded

donors and recipients confirm the association of oocyte quality and embryo viability with IGF1 concentrations of ovine [63], and bovine [64] subjected to superovulation, and the increase of IGF1 concentrations in ovarian follicular fluid of superstimulated donors treated with recombinant somatotropin and estradiol was associated with increased bovine numbers of viable embryos in vivo [65,66]. As well as, genetically selected double ovulatory cows had greater IGF1 concentrations in their blood and follicular fluid [67]. Although this study found a negative correlation between serum concentrations of IGF-1 and P4, but IGF-1 stimulates the production of P4 on the level of granulose cells [68].

Leptin concentrations is decreased with underfeeding and increased with overfeeding with more significant effects in camels that were previously overfed or underfed, respectively and leptinemia was positively related to hump adipocyte volume [69]. During the luteal phase of cows, serum levels leptin positively was correlated with follicular fluid leptin and progesterone level in the preovulatory follicles, and the concentration of leptin in follicular fluid was associated with atresia in small follicles [7]. Leptin concentrations of follicular fluid and both serum and follicular fluid leptin were nearly similar along the estrous cycle of cows [7], and women [70]. In obese women, leptin caused a concentration-related inhibition of the insulin-like growth factor I (IGF-I). The effect of leptin was specific, because there was no effect on progesterone production can directly inhibit IGF-I action in ovarian theca and granulose cells [70]. In Holstein-Friesian cows, the leptin level was influenced by feeding status, as indicated by the BCS [50]. In contrast to the significantly low leptin concentrations reported in non-responded camels compared to those responded to the superovulation program tried during this study, the superovulated cows prepared for embryo transfer program had optimum level of leptin resulted in conjunction with highest number of CL [50].

Conclusion

In conclusion, leptin and insulin like growth factor-1 play significant roles in response to superovulation and early embryo development in dromedary camels.

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Tables

Table (1): Mean ±SEM of leptin ng/mL and IGF-1 ng/mL during treatment of superovulated camels and weeks of early pregnancy

Condition	Cyclic superovulated			Days of pregnancy			P-value
Treatment	Day 1 before GnRH	Days 7,8.9.10 FSH/12hour	At mating+ GnRH	14	21	28	
Leptin ng/mL	1.94 ±0.88^{b}	1.73 ±0.78^{ab}	1.34 ±0.4^{a}	2.68 ±0.71^{c}	1.93 ±0.81^{b}	2.50 ±0.42^{c}	0.0001
	1.66 ±0.06			2.37 ±0.11			0.0001
IGF-1 ng/mL	157.7 ±36.5^{bc}	178.2 ±36.4^{c}	156.3 ±40.7^{bc}	145.9 ±38.9^{b}	143. ± 47.9^{b}	122.7 ±33.9^{a}	0.0001
	168.2 ±2.5			137.5 ±5.9			0.0001

Means with superscripts (a, b, c) are significantly different at P<0.05

Table (2): Mean ±SEM of progesterone (P4 ng/mL) leptin (ng/mL) and IGF-1 (ng/mL) during treatment of superovulated camels

Day	Treatment	Leptin ng/mL	IGF-1 ng/mL	P4 ng/mL
1	8 µg Buserelin	1.94 ±0.71^{bc}	157.7 ±5.8^{a}	2.21 ±1.04^{a}
7	80 mg Urofollitropin +2000 eCG	1.77 ±0.01^{abc}	193.4 ± 6.2^{b}	9.4 ±1.53^{b}
8	60 mg Urofollitropin	2.23 ±0.09^{c}	183.0 ±5.3^{b}	4.32 ±1.46^{a}
9	40 mg Urofollitropin	1.50 ±0.12^{ab}	179.7 ±4.3^{b}	5.45 ±1.00^{a}
10	20 mg Urofollitropin +7.5mg PGF2α	1.89 ±0.15^{bc}	152.3 ±10.7^{a}	10.29 ±2.38^{b}
16,17	Breeding + 8 µg Buserelin	1.34 ±0.09^{a}	165.3 ±4.8^{a}	11.01 ±0.89^{b}
	P-Value	0.0001	0.0001	0.0001

Means with superscripts (a, b, c) are significantly different at P<0.05

Table (3): Mean ±SEM of progesterone (P4 ng/mL) leptin (ng/mL) and IGF-1 (ng/mL) during days of treatment of superovulated donor and recipient camels

Day	Leptin ng/mL		IGF-1 ng/mL		P4 ng/mL	
	Donor	Recipient	Donor	Recipient	Donor	Recipient
1	1.79 ±0.17^{abc}	2.04 ±0.20 ^{b}	166.4 ±7.8^{ab}	151.8 ±8.0^{ab}	5.15 ±2.19^{a}	0.0 ±0.0
7	1.75 ±0.01^{abc}	1.79 ±0.5^{ab}	187.9 ± 7.1^{b}	215.5 ±05.3^{c}	12.19 ±1.53^{b}	1.08 ±0.0
8	2.11 ±0.09^{c}	2.70 ±0.0^{b}	185.7 ±6.2^{b}	169.6 ±8.5^{b}	5.76 ±1.76^{a}	0.0 ±0.0
9	1.39 ±0.19^{ab}	1.61 ±0.14^{ab}	178.5 ±7.2^{ab}	180.9 ±4.9^{bc}	8.15 ±1.74^{ab}	3.19 ±0.94
10	2.07 ±0.20^{bc}	1.62 ±0.18^{ab}	169.8 ±11.6^{ab}	125.9 ±17.1^{a}	12.42 ±3.37^{b}	6.04 ±0.0
16,17	1.35 ±0.10^{a}	1.01 ±0.0^{a}	156.9 ±5.1^{a}	144.6 ±3.4^{ab}	10.70 ±0.96^{ab}	14.29 ±0.0
P-value	0.002	0.019	0.007	0.001	0.02	0.001
Total	1.59 ±0.07	1.79 ±0.09	165.9 ±3.1	172.6 ±4.5	9.36 ±0.69	2.90 ±0.63
P-value	0.05		0.16		0.024	

Means with different superscripts (a, b, c) within column are significantly different at P<0.05

Table (4): Mean ± SEM of leptin (ng/mL) and IGF-1 (ng/mL) of responded and non-responded donor and recipient camels to superovulation and pregnant recipients

Hormone	**Donors**		**Recipient**		**P-value**
	Non –responded	**Responded**	**Non –responded**	**Pregnant**	
IGF-1	134.1 ±3.9^{a}	195.3 ±2.0^{c}	157.5 ±4.3^{b}	137.5 ±5.9^{a}	0.0001
Leptin	1.53 ±0.13^{a}	1.87 ±0.09^{b}	1.57 ±0.12^{ab}	2.40 ±0.12^{c}	0.0001
P4	13.29 ±1.12^{c}	0.61 ±0.14^{a}	12.58 ±0.55^{c}	7.15 ±0.54^{b}	0.0001

Means with superscripts (a, b, c) are significantly different at P<0.05

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