



**Full Length Research Article**

**OESTRUS RESPONSE AND PREGNANCY RATE AFTER OESTRUS SYNCHRONIZATION WITH EXPOSURE TO LIGHT AND HEAVY HANDLING IN NELORE CATTLE**

**<sup>1,\*</sup>Mohamed Ali, <sup>2</sup>Wahid, H., <sup>2</sup>Rosnina, Y., <sup>3</sup>Abas Mazni, O., <sup>2</sup>Mohd Azam, K. and <sup>4</sup>Hishamfariz, M. A.**

<sup>1</sup>Department of animal production, Qassim University, College of agriculture and veterinary medicine, Buraidah 6622, Saudi Arabia

<sup>2</sup>Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia

<sup>3</sup>Malaysia Agriculture Research and Development Institute, P.O. Box 12301, 50774 KL, Malaysia

<sup>4</sup>Pusat Ternakan Haiwan Ulu Lebar, Pahang, Malaysia

**ARTICLE INFO**

**Article History:**

Received 06<sup>th</sup> November, 2013

Received in revised form

11<sup>th</sup> December, 2013

Accepted 30<sup>th</sup> January, 2014

Published online 21<sup>st</sup> February, 2014

**Key words:**

Oestrus response;

Pregnancy rate;

Light and Heavy handling;

Cortisol and

P<sub>4</sub> concentration.

**ABSTRACT**

This study was conducted to evaluate the effects of oestrus synchronization protocol on oestrus response, pregnancy rate, progesterone (P<sub>4</sub>) and cortisol concentrations in Nelore cattle under two different types of handlings. In Experiment I (heavy handling), 27 cows were selected and subdivided into four groups, namely the Control, Groups 1, 2 and 3. The Control group was treated with only CIDR inserts for 15 days. Cows in Groups 1, 2 and 3 were treated with CIDR inserts for 15 days and given 500 µg of synthetic prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) at 9, 14 and 19 days post CIDR removal, respectively. Oestrus was observed for all cows in the four groups. Forty-eight hours after treatment, Artificial Insemination (AI) was carried out for cows that displayed oestrus, twice at 12 hours interval. Blood samples were collected twice per week beginning on the day of CIDR insertion until after AI and continued for the next 30 days. Ultrasonography was carried out after CIDR removal for the Control group and after PGF<sub>2α</sub> treatment in other groups to determine the ovulation time. In Experiment II (less handling), 30 cows were subdivided into four groups. The protocol for all the four groups in Experiment II followed that of Experiment I and the only difference was the number of blood samples and that there was no ultrasound examination. The difference between Experiment I (11; 40.7 %) and Experiment II (20; 66.6 %) for the expression of estrus were not significant, similarly for the number of pregnant cows (2; 7.4 % and 9; 30 %). The mean P<sub>4</sub> concentrations after AI were higher at Days 7 and 14 in all groups in Experiment II compared with all groups in Experiment I. The mean cortisol concentration on the day of first AI, Days 7 and 14 post AI was higher in all groups in Experiment I than all groups in Experiment II. The results from this study showed a negative correlation between the P<sub>4</sub> and cortisol concentrations ( $r = -0.267$ ,  $P < 0.01$ ) after AI in Experiment I thus, indicating an inverse relationship between P<sub>4</sub> and cortisol concentrations. The normal P<sub>4</sub> profile was observed in cows from different groups in both experiments, but delayed P<sub>4</sub> production was recorded only in G3 of Experiment I. In conclusion, heavy handling may have altered the expression of oestrus, reduced the pregnancy rate and altered the P<sub>4</sub> profiles. On the other hand, less handling and gentle interaction with animals could improve the reproductive performance.

Copyright © 2014 Mohamed Ali et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**INTRODUCTION**

It is a growing concern in many parts of the world that fertility of cattle is diminishing. Stress is believed to be one of the

important causes or stressors. It can be chronic or acute and can be divided into 2 types of stressors: physiological (physical, chemical or biological agent) and psychological (anxiety, fear, competition, etc). Stress is said to be elicited by one or more stressors whereby physiological function is compromised or the ability to meet energetic requirements is challenged (Balm, 1999). The stressors in cow husbandry include handling factors such as mechanical irritation due to improper rectal palpation or manipulation for artificial

**\*Corresponding author: Mohamed Ali,**

Department of animal production, Qassim University, College of agriculture and veterinary medicine, Buraidah 6622, Saudi Arabia

insemination (AI) (Karg, 1981). Prolonged or repeated exposure to stressors can shift the performance of an animal. It is noteworthy that researchers and people making decisions about animal welfare must understand the importance during non-painful routine handling (Coulon *et al.*, 2011) and transportation is considered as a very strong stressor (Grandin, 1997). On other hand, gentle interactions with the animals to enhance animal welfare can reduce the risk of accidents that can occur (Coulon *et al.*, 2011). It is also demonstrated that, gentler handling methods could improve animal management, the ease of handling of these animals, diminishing losses due to injury of the animals, as well as labor costs.

Interactions between the hypothalamus-pituitary-adrenal axis and the hypothalamus-pituitary ovarian axis have recently been demonstrated (Hollenstein *et al.*, 2006; Fergani *et al.*, 2012) and the most important effect is a disturbance to the hypothalamic function. Normal pulsatile patterns of GnRH release and frequency and amplitude of LH pulses secreted from the pituitary are reduced by exposure to acute stressors such as transportation (Tadich *et al.*, 2005) or high-dose of insulin administration (Saifullizam *et al.*, 2010). This results in abnormal ovarian function and hence either delays or abolishes the LH surge (Hollenstein *et al.*, 2006). Thus, stressors disrupt the correct functioning of each part of the hypothalamus-pituitary-ovarian axis (Hollenstein *et al.*, 2006). The objectives of this study were to determine the effect of the stressor handling on oestrus response, pregnancy rate, progesterone (P<sub>4</sub>) and cortisol concentrations between heavy-stressed group and less-stressed group of Nelore cows.

## MATERIALS AND METHODS

### Animals and Experiments

The study was conducted at the Pusat Ternakan Jabatan Perkhidmatan Haiwan center, Ulu Lepar farm, Pahang state, Malaysia, from May 2006 to August 2006. The season during May was the dry season, while Jun, July, and August were the rainy season. The average daily temperature was 31 °C with a humidity level of about 91 %. Fifty seven Nelore cows were selected for the study. The age of animals were ranged 3-5 years. These cows were healthy, multiparous, cycling normally and non-lactating dry cows that had calved down before (> 2months). Non-pregnancy status in these cows was confirmed through rectal palpation. The ovaries were also palpated for the presence of either follicles or a corpus luteum (CL). This herd was grazing on *Brachiaria decumbens* pasture and supplements of commercial concentrates of palm kernel cake at the rate of 2 kg/cow/day. All 57 cows were inserted with a Controlled Internal Drug Release device (CIDR) for 15 days. This device is coated with 1.38 g of progestagen in a silicon rubber elastomer. After 15 days, the CIDR was removed and the cows were randomly divided for Experiments I and II. In Experiment I, 27 cows were randomly selected and divided into four groups- Control, Groups 1, 2 and 3. In the Control group (n=8 cows), the CIDR was removed on Day 15 and ultrasonography was carried out immediately. For G 1 (n=5 cows), ultrasonography was done on Day 24, i.e. 9 days after the CIDR was removed. As for Groups 2 (n=6 cows) and 3 (n=8 cows), ultrasonography was conducted on Day 29 and Day 34 respectively, i.e. 14 and 19 days after CIDR removal. For groups 1, 2 and 3, after ultrasonography, each cow was

administered intramuscularly with 2 mL (500 µg) of cloprostenol (Estrumate<sup>®</sup>, Schering-Plough Animal Health). Cloprostenol was not given to the Control group. For the Control group, the oestrus detection was performed twenty-four hours after removal of the CIDR and for groups 1, 2 and 3 immediately after PGF<sub>2α</sub> was given. Cows were observed visually for behavioral oestrus twice daily once at 08:00 h and then at 16:00 h until the end of the experiment. The signs observed for oestrus were clear mucus discharge, congested vulva, mounting and standing to be mounted.

All cows that showed oestrus were artificially inseminated with frozen-thawed Nelore semen of the same bull that was obtained from the Institut Bioteknologi Haiwan Kebangsaan, Jabatan Perkhidmatan Haiwan, Jerantut, Pahang state. A 0.25 ml straw containing 20 × 10<sup>6</sup> sperm with 85% individual motility rate was used for insemination of these cows. A.I. was performed by the same professional inseminator immediately twice at 12 h intervals in all the oestrus cows. Blood samples were collected every three to four days beginning on the day of CIDR insertion, until after AI, and continued for the next 30 days. After estrus appearance, ovaries were examined by ultrasound (Aloka SSD-500, Japan) using a 5 MHz trans-rectal probe to determine the time of ovulation. Each ovary was scanned and the image of the largest follicle was recorded on a videotape. Ultrasound examinations of the ovaries were conducted (1) immediately after PGF<sub>2α</sub> injection and (2) at standing oestrus twice daily for the next 5 days. Ovulation was defined as the sudden disappearance of the follicle identified as a dominant follicle during the preceding examination. For confirmation that ovulation had occurred the cow was reexamined 12 hours later. Cows suspected to be pregnant were determined approximately 30 days following the second insemination.

In Experiment II, the remaining 30 cows were subdivided into four groups, Control, Groups 1, 2 and 3. Similarly, the CIDR was inserted in all cows for 15 days. The protocol for all the four groups in Experiment II followed the protocol in Experiment I. The number of cows allotted for Control, G1, G2 and G3 were 7, 9, 6 and 8, respectively. All cows that showed oestrus were artificially inseminated with same frozen-thawed Nelore semen. One cow from G2 that displayed estrus 10 days after treatment was discarded in the calculation of mean onset of oestrus. Blood samples were not collected from all cows during the 15 days of CIDR insertion to minimize animal handling and stress. However, blood samples were collected at AI for the Control Group while in the remaining groups the blood samples were collected after day 9 of CIDR removal. After AI, blood samples were collected in all cows at weekly intervals for the next 30 days. Ultrasonography was not done during this experiment to minimize handling and stress.

### Definition of manual handling

- Heavy handling was carried out in Experiment I when the cows were incurred for blood collection, restraint, and contact with people was twice per week. In addition, ultrasonographic examination was performed at the time of PGF<sub>2α</sub> injection and at the interval from displayed oestrus to ovulation for 5 days.

- Less handling was carried out in Experiment II when the cows were incurred for blood collection, restraint, and contact with people was once per week. However, there was no ultrasound examination in Experiment II.

### Storage of blood samples

Each experiment was grazed alone in large paddock. For blood collection, cows should be collected into a small paddock and then were restrained in a cattle-crush. Blood samples were collected from the coccygeal vein into 5 ml vacutainers containing sodium heparin. The period from restraining the cow to collection of the blood was ten to thirty minutes. Following collection, labeled blood samples were immediately stored in ice (3°C) and centrifuged for 15 min at 1340 x g. The plasma was transferred into a 2 ml polypropylene tube and stored frozen at -20 °C until assay.

### Hormone assay

Plasma P<sub>4</sub> and cortisol concentrations were measured using a commercial radioimmunoassay (RIA) kit (Diagnostics Product Corporation, USA). For the P<sub>4</sub> hormone assay, the sensitivity of the assays was 0.035 ± 0.22 ng/ml. Three Quality Controls (QC), i.e. low, medium and high values of known concentration were used in the assay system. The intra-assay coefficient variation of QC-low was 7.67%, QC-medium was 0.29%, and QC-high was 1.20%. The inter-assay coefficient variation of QC-low was 23.35%, QC-medium was 15.98%, and QC-high was 4.93%. For the cortisol hormone assay, the sensitivity of the assays was 14.2 ± 1.39 ng/mL. Three quality controls (QC), i.e. low, medium and high values of known concentration were used in the assay system. The intra-assay coefficient variation of QC-low was 2.57%, QC-medium was 0.02%, and QC-high was 1.20%. The inter-assay coefficient variation of QC-low was 15.95%, QC-medium was 8.16%, and QC-high was 8.40% (Burtis *et al.*, 1994, Smith, 1985, Reimers, *et al.*, 1983, and Abraham, 1981).

### Statistical Analysis

The numbers of cows in oestrus and of inseminated cows were expressed as a percentage of the total number and of each group. Mean onset of oestrus and P<sub>4</sub> and cortisol concentrations were presented as the arithmetic mean ± SEM. Because the data were not normally distributed, a kruskal-Wallis one-way ANOVA (non parametric statistical test) was used to test for the presence of significant difference among all four groups. The following parameter was analysed mean onset of oestrus. The comparison between two Experiments (I and II) were made using Mann-Whitney U test. The following parameters were analyzed: mean onset of oestrus, mean P<sub>4</sub> concentration and mean cortisol concentration. Since the P<sub>4</sub> and cortisol hormones are two continuous variables, they should be measured using the degree of relationship via linear correlation. Proportional data (pregnancy rate and expression of oestrus) were analyzed by chi-square tests to identify the presence of significant difference among all four groups and comparing responses of Experiment I and II. Data were analyzed using SPSS statistical software release 12.0.

## RESULTS

**Oestrus response and pregnancy rate after oestrus synchronization treatment:** Table 1 shows the effect of CIDR treatment on oestrus response in the Nelore cows. In

Experiment I, 11 (40.7%) out of the 27 treated cows exhibited oestrus. Overall, the percentage of cows observed in oestrus was highest in the Control group (62.5 %) followed by G1 (60.6 %), G2 (33.3 %), and G3 (12.5 %). We observed that 62.5 % (Control) and 60.6 % (G1) of cows showed oestrus compared to 33.3 % G2 and 12.5 % G3 (P<0.05). The mean onset of oestrus after CIDR and PGF<sub>2α</sub> treatment among the groups ranged between 57 and 96 h with G2 (96.0 ± 0.0 h) having significantly longer (P<0.05) onset compared to the other groups (Table 1). As for pregnancy rate only 1 cow from the Control, G2 and G3 became pregnant. None of the cows in G1 was pregnant.

In Experiment II, 20 out of the 30 (66.6%) cows exhibited oestrus after treatment. All cows from the control group (P<0.05) showed oestrus in comparison to other treatments groups (Table 1). The onset of oestrus after CIDR and PGF<sub>2α</sub> treatment among the groups ranged between 51 and 72 hours with G2 (72.0 ± 0.0 h) and G3 (72.0 ± 13.0 h) displaying oestrus about 18 hours later than the other two groups (Control and G1). Nevertheless, the difference was not significant. The number of cows that became pregnant after AI ranged between 1 and 3 cows only from all the groups and when comparison was made between Experiment I and Experiment II, Experiment II produced a significant number of cows showing oestrus after treatment.

**Table 1. Ostrus response and pregnancy rate of Nelore cows that were heavy and less handled following synchronization treatment**

Group	No. of treated cows	No. of cows in oestrus after treatment (%)	Mean onset of oestrus ± SEM (h)	Percentage of pregnant cows (%)
<b>Exp. I (Heavy Handling)</b>				
Control	8	5 (62.5) <sup>a</sup>	57.6 ± 2.3 <sup>b</sup>	12.5 <sup>a</sup>
G1	5	3 (60.6) <sup>a</sup>	64.0 ± 4.0 <sup>b</sup>	00.0 <sup>a</sup>
G2	6	2 (33.3) <sup>b</sup>	96.0 ± 0.0 <sup>a</sup>	16.6 <sup>a</sup>
G3	8	1 (12.5) <sup>b</sup>	72.0 ± 0.0 <sup>b</sup>	12.5 <sup>a</sup>
Total	27	11 (40.7) <sup>b</sup>		11.1 <sup>a</sup>
<b>Exp. II (Less Handling)</b>				
Control	7	7 (100.0) <sup>a</sup>	51.4 ± 2.2 <sup>a</sup>	28.5 <sup>a</sup>
G1	9	6 (66.6) <sup>b</sup>	54.0 ± 4.0 <sup>a</sup>	33.3 <sup>a</sup>
G2	6	4 (66.6) <sup>b</sup>	72.0 ± 0.0 <sup>a</sup>	50.0 <sup>a</sup>
G3	8	3 (37.5) <sup>b</sup>	72.0 ± 13.0 <sup>a</sup>	12.5 <sup>a</sup>
Total	30	20 (66.6) <sup>a</sup>		30.0 <sup>a</sup>

<sup>a,b,c</sup>: Values with different superscripts in the same column and experiment differ significantly at P<0.05, Chi-square and kruskal-Wallis one-way ANOVA.

<sup>A,B</sup>: Values with different superscripts in the same column between experiments differ significantly at P<0.05, Chi-square.

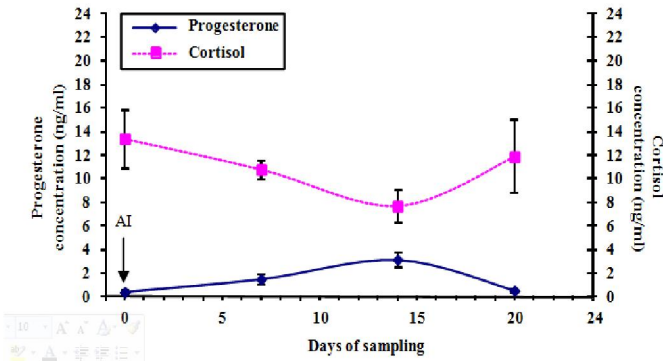
### Effect of different handling types on progesterone and cortisol measurements of non-pregnant cows after oestrus synchronization

#### Control group

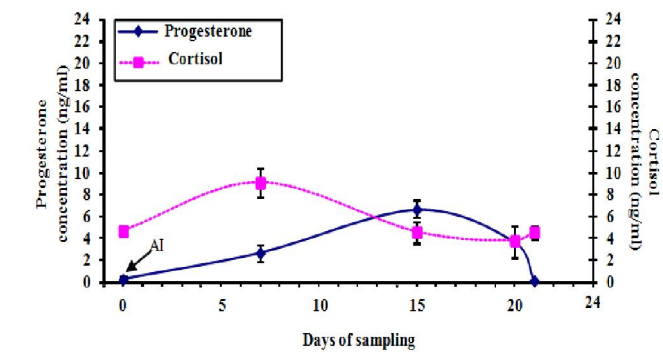
The mean concentrations of P<sub>4</sub> and cortisol in the peripheral circulation of non-pregnant cows in the Control group of Experiments I and II are shown in Figure 1. In Experiment I, the P<sub>4</sub> levels were 0.4 ± 0.1, 1.5 ± 0.4 and 3.1 ± 0.6 ng/ml on the day of AI, Day 7, and Day 14 post-AI, respectively. On the day of AI the cortisol level was 13.4 ± 2.5 ng/ml, Day 7 was 10.8 ± 0.8 ng/ml, and Day 14 was 7.7 ± 1.4 ng/ml post-AI. Whereas, in Experiment II, the P<sub>4</sub> levels were 0.2 ± 0.1, 2.6 ± 0.6 and 6.6 ± 0.6 ng/ml on the Day of AI, Day 7, and Day 14 post-AI, respectively. On the day of AI, the level of cortisol was 4.7 ± 0.5 ng/ml, Day 7 was 9.1 ± 1.3 ng/ml and Day 14 was 4.6 ± 1.0 ng/ml post-AI. The average P<sub>4</sub> concentration from the day of AI to 14 days post-AI was significantly higher (P<0.05) in Experiment II than in Experiment I (4.6 ± 0.8

ng/ml vs  $2.7 \pm 0.2$  ng/ml). On the other hand, the average cortisol concentration from the day of AI to 14 days post-AI was significantly higher ( $P < 0.001$ ) in Experiment I than in Experiment II ( $9.96 \pm 0.76$  ng/ml vs  $5.39 \pm 0.43$  ng/ml). The current study showed a significant ( $P < 0.05$ ) negative correlation ( $r = -0.14$ ) between the cortisol and  $P_4$  concentrations in the Control group of Experiment I. On the other hand, there was no correlation between the cortisol and  $P_4$  concentration in the Control group of Experiment II.

Experiment I (Control)



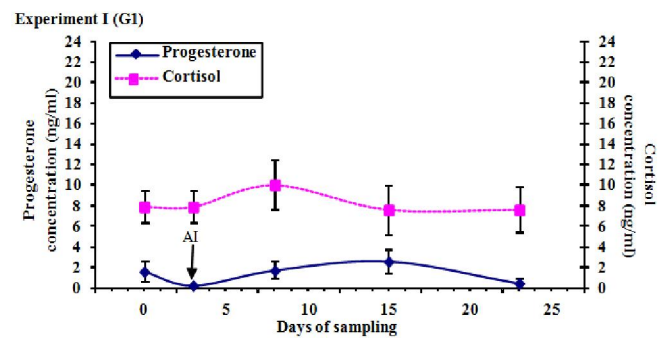
Experiment II (Control)



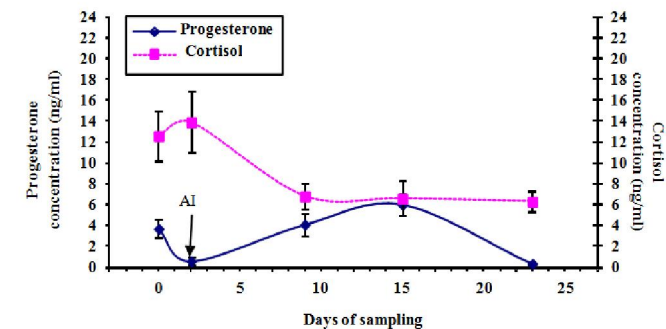
**Figure 1. Mean progesterone and cortisol concentrations during the oestrous cycle of non-pregnant cows in Control group of Experiments I and II**

## G1

The mean concentrations of  $P_4$  and cortisol in the peripheral circulation of non-pregnant cows in G1 of Experiments I and II are shown in Figure 2. In Experiment I, the  $P_4$  level from the day of AI ( $0.2 \pm 0.04$  ng/ml) to 13 ( $2.5 \pm 1.2$  ng/ml) days post-AI increases. The cortisol levels were  $7.9 \pm 1.6$ ,  $10.0 \pm 2.4$  and  $7.6 \pm 2.4$  ng/ml on the day of AI, Day 7 and Day 14 post-AI, respectively. In Experiment II, the  $P_4$  levels were  $0.6 \pm 0.3$ ,  $4.1 \pm 1.1$  and  $6.0 \pm 1.1$  ng/ml on the Day of AI, Day 7, and Day 14 post-AI, respectively. The levels of cortisol were  $13.9 \pm 2.9$ ,  $6.8 \pm 1.2$  and  $6.7 \pm 1.5$  ng/ml on the day of AI, Day 7 and Day 14 post-AI, respectively. The average  $P_4$  concentration from the day of AI to 14 days post-AI was significantly higher ( $P < 0.05$ ) in Experiment II than in Experiment I ( $5.8 \pm 0.6$  ng/ml vs  $3.0 \pm 0.3$  ng/ml). On the other hand, the average cortisol concentration from the day of AI to 14 days post-AI between Experiment I and Experiment II was not significantly different. The results show no correlation between the  $P_4$  and cortisol concentrations in G1 of Experiment I nor of Experiment II.



Experiment II (G1)

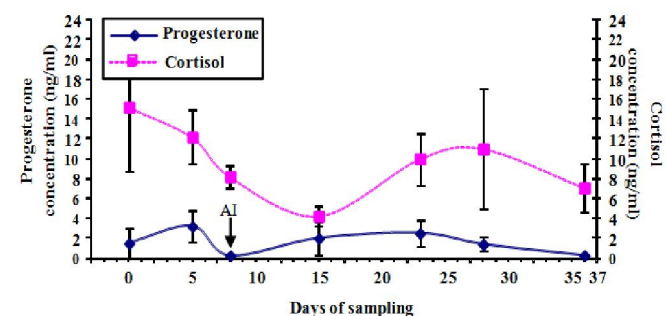


**Figure 2. Mean progesterone and cortisol concentrations during the estrous cycle of non-pregnant cows in G1 of Experiments I and II**

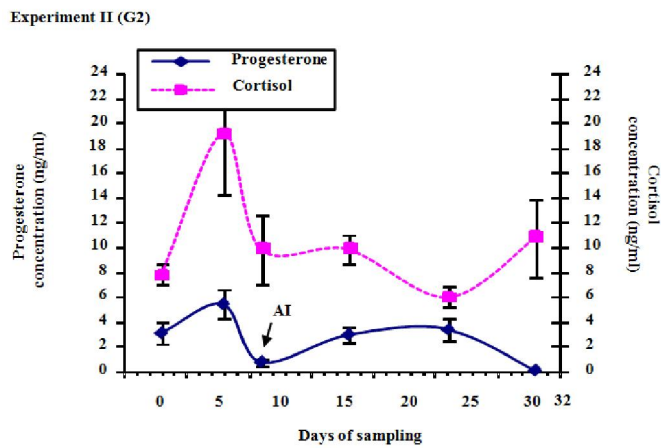
## G2

The mean concentration of  $P_4$  and cortisol in the peripheral circulation of non-pregnant cows in G2 of Experiments I and II are shown in Figure 3. In Experiment I, the  $P_4$  levels were  $0.2 \pm 0.1$ ,  $2.0 \pm 1.7$  and  $2.5 \pm 1.3$  ng/ml on the Day of AI, Day 7, and Day 14 of the post-AI, respectively. The cortisol levels were  $8.2 \pm 2.7$ ,  $4.2 \pm 1.1$ , and  $10.0 \pm 1.0$  ng/ml on the Day of AI, Day 7, and Day 14 of the post-AI, respectively. In Experiment II, the  $P_4$  levels were  $0.2 \pm 0.1$ ,  $2.9 \pm 0.6$  and  $3.4 \pm 0.9$  ng/ml on the Day of AI, Day 7, and Day 14 post-AI, respectively. On the day of AI, the cortisol level was  $9.8 \pm 2.8$  ng/ml, Day 7 was  $9.8 \pm 1.2$  ng/ml and Day 14 was  $6.0 \pm 0.8$  ng/ml post-AI. The average  $P_4$  concentration from the day of AI to 14 days post-AI between Experiment I ( $2.0 \pm 0.4$  ng/ml) and in Experiment II ( $2.5 \pm 0.4$  ng/ml) was not significantly different. Similarly, the average cortisol concentration from the day of AI to 14 days post-AI in Experiment I was ( $7.2 \pm 1.3$  ng/ml) and in Experiment II was ( $6.9 \pm 1.1$  ng/ml), thus not significantly different. The results show no correlation between the  $P_4$  and cortisol concentrations in G2 of Experiment I nor of Experiment II.

Experiment I (G2)



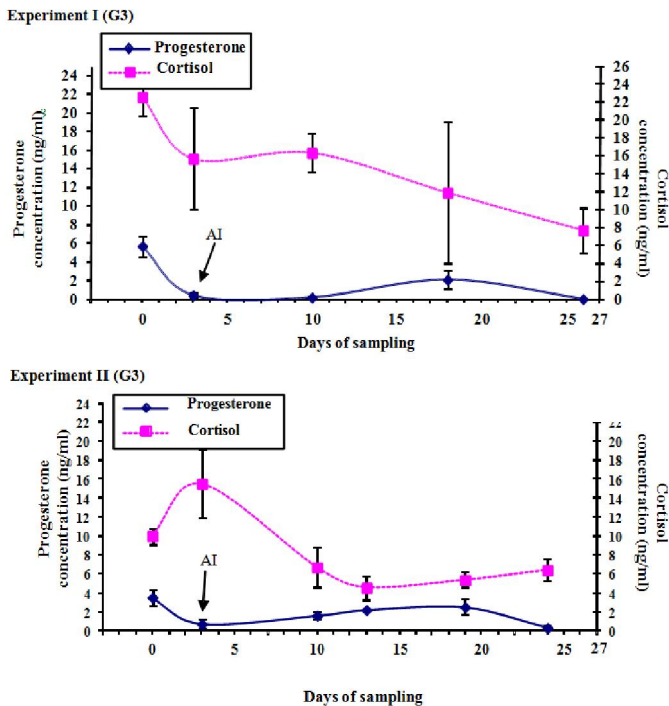




**Figure 3. Mean progesterone and cortisol concentrations during the oestrous cycle of non-pregnant cows in G2 of Experiments I and II.**

### G3

The mean concentration of P4 and cortisol in the peripheral circulation of non-pregnant cows in G3 of Experiments I and II are shown in Figure 4. In Experiment I, the P<sub>4</sub> levels were  $0.4 \pm 0.3$ ,  $0.1 \pm 0.07$  and  $2.1 \pm 1.0$  ng/ml on the Day of AI, Day 7, and Day 14 post-AI, respectively. The cortisol levels were  $15.7 \pm 5.7$ ,  $16.3 \pm 2.1$ , and  $11.9 \pm 7.9$  ng/ml on the Day of AI, Day 7, and Day 14 post-AI, respectively. In Experiment II, the P<sub>4</sub> levels were  $0.7 \pm 0.5$ ,  $1.6 \pm 0.4$  and  $2.5 \pm 0.8$  ng/ml on the Day of AI, Day 7, and Day 14 post-AI, respectively. On the day of AI, the cortisol level was  $15.5 \pm 3.6$  ng/ml. Thereafter, the cortisol level at Days 7 and 14 post-AI were  $6.6 \pm 2.1$  and  $5.3 \pm 0.8$  ng/ml, respectively. The average P<sub>4</sub> concentration from the day of AI to 14 days post-AI between Experiment I ( $1.8 \pm 0.3$  ng/ml) and Experiment II ( $2.0 \pm 0.4$  ng/ml) was not significantly different.



**Figure 4. Mean progesterone and cortisol concentrations during the oestrous cycle of non-pregnant cows in G3 of Experiments I and II**

The average cortisol concentration from the day of AI to 14 days post-AI was significantly higher in Experiment I ( $14.1 \pm 1.4$  ng/ml) than in Experiment II ( $9.0 \pm 0.9$  ng/ml) ( $P < 0.05$ ). The results show a significant ( $P < 0.05$ ) negative correlation ( $r = -0.17$ ) between the P<sub>4</sub> and cortisol concentrations in G3 of Experiment I. In contrast, there was no correlation between the P<sub>4</sub> and cortisol concentrations in G3 of Experiment II.

### Total mean P<sub>4</sub> and cortisol concentration in Experiment I and II

Table 2 shows the total mean P<sub>4</sub> and cortisol concentrations in non-pregnant cows of all groups of Experiment I and II. The level of P<sub>4</sub> concentration on Day 0 (AI) was  $0.2 \pm 0.05$  ng/ml and  $0.1 \pm 0.04$  ng/ml in Experiment I and Experiment II, respectively. The P<sub>4</sub> concentration was significantly higher ( $P < 0.05$ ) on Day 7 after AI in Experiment II ( $3.6 \pm 0.4$  ng/ml) than in Experiment I ( $1.2 \pm 0.2$  ng/ml). Similarly, the P<sub>4</sub> concentration was significantly higher on Day 14 in Experiment II than in Experiment I in the estrous cycle:  $5.3 \pm 0.4$  ng/ml and  $3.7 \pm 0.3$  ng/ml, respectively ( $P < 0.05$ ). The cortisol concentration on the Day of AI was not significant between Experiment I and Experiment II,  $14.6 \pm 2.4$  ng/ml and  $9.3 \pm 1.4$  ng/ml, respectively. Similarly, the cortisol concentrations on Day 7 ( $8.8 \pm 1.1$  ng/ml and  $7.2 \pm 0.8$  ng/ml) and Day 14 ( $8.2 \pm 1.0$  ng/ml and  $7.2 \pm 1.0$  ng/ml) post AI were not significant between Experiment I and Experiment II. Significant ( $P < 0.05$ ) negative correlations ( $r = -0.26$ ) were found between the total mean P<sub>4</sub> and cortisol for all groups after AI in Experiment I. In contrast, no correlation was found in Experiment II.

**Table 2. The total means of progesterone and cortisol concentrations in non-pregnant cows of Experiments I and II (mean  $\pm$  SEM)**

Days	P <sub>4</sub> concentration (ng/ml)		Cortisol concentration (ng/ml)	
	Experiment I	Experiment II	Experiment I	Experiment II
Day 0 (AI)	$0.2 \pm 0.05^a$	$0.1 \pm 0.04^a$	$14.6 \pm 2.4^a$	$9.3 \pm 1.4^a$
Day 7	$1.2 \pm 0.2^b$	$3.6 \pm 0.4^a$	$8.8 \pm 1.1^a$	$7.2 \pm 0.8^a$
Day 14	$3.7 \pm 0.3^b$	$5.3 \pm 0.4^a$	$8.2 \pm 1.0^a$	$7.2 \pm 1.0^a$

<sup>a, b</sup>. Values with different superscripts in the same row differ significantly at  $P < 0.05$ ; Mann-Whitney U test.

## DISCUSSION

The current study shows that a high percentage of cows in less handling expressed oestrus compared with cows in heavy handling. A factor that may contribute to this outcome is the heavy handling that was performed on the cows in Experiment I as a result of a more frequent blood sampling (twice per week) and ultrasound examinations were carried out. In this case, animal handling led to the increase in cortisol secretion. Thus, contributing to decrease reproductive efficiency (Burns *et al.*, 2010). Report by Roelofs and colleagues (2004), demonstrated that frequent ultrasound examinations every 3 hours from the onset of oestrus until time of ovulation does not affect behavioral oestrus characteristic or peri-ovulatory hormone profiles. Furthermore, cows were subjected to repeated restraint for gynecological exam, blood collection procedures and all animals were kept in the same pen for the entire experiments (Roelofs *et al.*, 2004). In contrast, repeated restraint for gynecological exam and blood collection

procedures were not performed in cows used in our study. Therefore, it is possible that there may be differences in LH secretion as the cortisol relationship observed in our study among the Nelore cows during heavy-handling (Experiment I) and less-handling (Experiment II). As demonstrated by other studies, cortisol released by stress may influence the follicular secretion of estradiol-17 $\beta$ , and P<sub>4</sub> secretion (Kawate *et al.*, 1993) and the timing and amplitude of the preovulatory LH surge (Dobson *et al.*, 2001; Fergani *et al.*, 2012). Therefore, the increase of cortisol results in negative feedback on other hormones, and thus, leads to lower oestrus expression and low pregnancy rates in Nelore cattle. We also demonstrated that the percentage of cows that failed to display oestrus was higher in Experiment I than in Experiment II. All cows in Experiment I showed a decrease in P<sub>4</sub> after CIDR and PGF<sub>2 $\alpha$</sub>  treatment. These cows were heavily handled with blood sampling done twice a week and ultrasound examination performed daily for 5 days depending on the time of ovulation. Stress can shorten or inhibit estrus (Orihuela, 2000; Sood and Nanda, 2006; Fergani *et al.*, 2012).

In previous studies it is reported that stressors can disrupt the correct functioning of each part of the hypothalamus-pituitary-ovarian axis and this includes the disruption of the pulsatile pattern of LH, a lower than normal oestradiol secretion, delay of LH surge, and thus, late ovulation (Dobson *et al.*, 2001; Hollenstein *et al.*, 2006; Saifullizam *et al.*, 2010; Fergani *et al.*, 2012). In addition, a study by Stoebel and Moberg (1982) reported that the infusion of high cortisol during the preovulatory period prevented the LH surge and also oestrus behavior in three of four heifers. However, ovarian function may have been altered by the infusion of cortisol and the stimulus responsible for estrus behavior (estrogen) may have been absent. In addition, one injection of 4 mg of dexamethasone (a synthetic glucocorticoid) to estradiol-treated ovariectomised heifers caused a decrease in the percentage of heifers in estrus, but the behavior of those heifers displaying oestrus was not altered (Cook *et al.*, 1987; Allrich *et al.*, 1989). Therefore, we can assume that the natural glucocorticoids like cortisol that were produced from the stressed Nelore cows in our study could also inhibit oestrus behavior therefore causing a low percentage of oestrus response.

Artificial insemination does not affect our oestrus behavior and hormonal profile when carried out with minimal stress. However, restraining cows frequently for blood collection and ultrasound examinations around estrus/AI will induce stressed animals and will result in low oestrus response and low a pregnancy rate of these animals, since acute stress around this period might influence the timing and amplitude of the preovulatory LH surge (Kawate *et al.*, 1993; Dobson *et al.*, 2001; Saifullizam *et al.*, 2010; Fergani *et al.*, 2012), oestradiol-17 $\beta$  (Saifullizam *et al.*, 2010; Fergani *et al.*, 2012) and progesterone secretions (Kawate *et al.*, 1993). Although cows in this study were raised freely on pasture, it was evident that fertility was affected by handling. In Experiment I, there was a low percentage of pregnant cows and a low percentage return to oestrus compared with cows in Experiment II. This could be due to one or more of the following reasons: high incidence of post-insemination luteal sub-function (Hommeida *et al.*, 2004); high incidence of embryo deaths correlating with the sub-function of corpus luteum (Taponen *et al.*, 2003);

ovulation without oestrus (Leyva-Ocariz *et al.*, 1996); and anovulatory follicle (Stevenson *et al.*, 1997; Wiltbank *et al.*, 2002). In heavily-handled animals, 26 out of 27 animals showed P<sub>4</sub> concentration of less than 1 ng/ml at oestrus after CIDR removal followed or not by PGF<sub>2 $\alpha$</sub>  injection. The significantly low P<sub>4</sub> levels in these animals imply that the treatment was effective and that there was luteolysis of the corpus luteum. The P<sub>4</sub> profiles were observed for different treatment groups. In the Control and G1 groups, the P<sub>4</sub> concentration peak was observed on Day 4 after oestrus. In these animals, oestrus was observed at 56 and 64 hours after treatment and the development of corpus luteum occurred at approximately 4 days after oestrus. However, the P<sub>4</sub> peak for G2 and G3 groups was observed on days 8 and 10 respectively after estrus. In these animals, oestrus was observed at 96 hours for G2 and 72 hours for G3 after treatment. Three out of 8 cows in G3 had basal P<sub>4</sub> level and 5 cows in this group showed abnormal P<sub>4</sub> profiles resulting in delay of the development of corpus luteum that caused a delay in P<sub>4</sub> peak. Similarly, in G2, oestrus was observed at 96 hours after treatment and therefore the P<sub>4</sub> concentration peak was observed on Day 8 after estrus. Delayed and insufficient P<sub>4</sub> productions may result from ovulation of immature follicles (Hunter, 1991) or from partial withdrawal of luteotrophic support during the luteal phase (Southee *et al.*, 1988). Delayed and insufficient P<sub>4</sub> profiles can lead to a stronger luteolytic signal and thus may predispose to a higher incidence of embryo loss (Lonergan, 2011). These abnormal P<sub>4</sub> profiles were also associated with decline in pregnancy rate due to asynchrony between uterus and embryo (Hommeida *et al.*, 2004; Lonergan, 2011).

In less-handled animals, 27 out 30 animals showed a P<sub>4</sub> concentration of less than 1 ng/ml at oestrus after CIDR removal followed or not by PGF<sub>2 $\alpha$</sub>  injection. The significantly low P<sub>4</sub> levels in these animals imply that the treatment was effective and that there was luteolysis of the corpus luteum. Different P<sub>4</sub> profiles were observed in the different treatment groups. In the Control and G1 groups, the P<sub>4</sub> concentration peak was observed 7 days after oestrus and showed high P<sub>4</sub> levels (6 ng/ml) at 14 days after treatment. In these animals, oestrus was observed at 51 and 54 hours after treatment and the development of corpus luteum occurred at approximately 7 days after oestrus. Therefore, the P<sub>4</sub> peak occurred on the same day. Only one cow in the Control group showed follicular cyst and basal P<sub>4</sub> profiles after CIDR removal. Similarly, the P<sub>4</sub> peak for G2 and G3 groups was observed on Day 7 after oestrus and showed a slight increase of P<sub>4</sub> levels (3 ng/ml) at 14 days after treatment. In these animals, oestrus was observed at the same time of approximately 72 hours after treatment. The P<sub>4</sub> level of all cows in G2 ranged from 2 to 5 ng/ml throughout the estrous cycle.

Therefore, in this group there was an increase of P<sub>4</sub> concentration during the oestrous cycle. Two out of 8 cows in G3 had basal P<sub>4</sub> levels after treatment. In addition, 5 cows in this group showed a slight increase of P<sub>4</sub> concentration ranging from 2 to 3 ng/ml at days 7 and 14 post AI. Therefore, in these animals there was probably a slight increase of the P<sub>4</sub> concentration during the estrous cycle. The result from our study demonstrated that the incidence of different types of abnormal P<sub>4</sub> profiles in Nelore cattle under different handling procedures can lead to stress. On the other hand, in less handled cows, the mean P<sub>4</sub> concentration and normal P<sub>4</sub> profile

within the oestrous cycle were higher than with heavily handled cows. Stress factors induce high cortisol secretion (Berghold *et al.*, 2007). It was observed that the post insemination P<sub>4</sub> profile has an effect on pregnancy rates. The importance of P<sub>4</sub> in the survival of the early embryo and maintenance of pregnancy in cattle has been reported (Lonergan, 2011; Kenyon *et al.*, 2012). Therefore, P<sub>4</sub> assays may find useful application in monitoring occurrence of cyclic activities, early pregnancy and repeat breeders in cattle (Rekwot *et al.*, 2000). Low reproductive rates, failures in oestrus detection and high frequencies of abnormal post breeding luteal phases are among the major problems leading to reductions in herd fertility and difficulties in reproductive management (Garcia, 1990). Different types of abnormal post breeding luteal phases could be investigated by hormonal radioimmunoassay which can provide practical values in monitoring ovarian activity in Nelore cows.

In the present study, the normal P<sub>4</sub> profile was recorded in Experiments I and II. The increase in the P<sub>4</sub> concentrations during a few days (4-7 days) after oestrus. Indicates ovulation and subsequently formation of corpus luteum (Karg, 1981). Progesterone concentration is elevated for approximately 2 weeks and is the hallmark of a normal oestrous cycle (Hommeida *et al.*, 2004; Lonergan, 2011). In addition, the embryos of these cows produced larger amounts of interferon tau (INF- $\tau$ ) that would alter the dynamics of PGF<sub>2 $\alpha$</sub>  secretion, and therefore the pregnancy was more likely to be maintained (Mann and Lamming, 2001; Lonergan, 2011). Any deviation from this pattern is likely to be associated with reduced fertility (Rekwot *et al.*, 2000). Therefore, a high percentage pregnancy rate indicates that a herd of cows has normal P<sub>4</sub> profile. In the present study, serum cortisol concentration was elevated in cows in Experiment I compared with cows in Experiment II. Cortisol is commonly used as a stress marker because its production by the adrenal cortex tends to increase as a result of energetic, immunological, and psychological challenges (Nepomnaschy *et al.*, 2006). This is expected with cows that were handled more than those that were handled less. Cows that were heavily handled when gathered in the corral for frequent blood sampling and ultrasound examination of the ovaries.

It is possible that these stressors influenced steroid concentrations. However, the level of cortisol decreased gradually over time. This is because cows can be adapted to the handling procedures and became familiar with the workers. Thus they experienced less stress. In addition, animal handlers became more experienced in restraining and handling the cows. Therefore, causing less stress to animals. It was reported that cows that have been trained and habituated to a handling procedure may be completely calm and have baseline cortisol with normal heart rates during handling and restraint (Grandin, 1997) and is similarly reported to horse mares (Berghold *et al.*, 2007). It is assumed that the animals in the present study adapted and became more familiar with the human contact and handling of the workers with time. This explains the improved oestrus response and pregnancy rate and the gradual decline in cortisol concentration in all cows throughout the experiment. It was observed that there was a negative correlation between cortisol and P<sub>4</sub> hormone level after AI in the estrous cycle in Experiment I. This finding indicated that the high cortisol level can affect the function of the corpus luteum and hence P<sub>4</sub>

production. The relationship between cortisol and luteal activities has been reported in other species. High production of endogenous cortisol decreased P<sub>4</sub> secretion during the early luteal phase in women (Nepomnaschy *et al.*, 2006), bitches (Leyva-Ocariz, *et al.*, 1996) and sows (Liptrap *et al.*, 1989). The discovery of corticotrophin-releasing factor receptors on the ovary is also consistent with the possible existence of a down-regulatory effect of stress on steroidogenesis exerted at the gonadal level (Nepomnaschy *et al.*, 2006). Environmental factors such as the dry season in tropical areas can also cause stress and elevate the concentration of cortisol and decrease P<sub>4</sub> secretion by corpus luteum. This condition has a negative effect on fertility (Leyva-Ocariz *et al.*, 1996). Therefore, it is advisable that the cattle can be trained and habituated to a handling procedure so that they become calm and less stressed. This will lead to less cortisol secretion during the handling and restraint. In conclusion, it appears that in Nelore cows, heavy handling may have altered the expression of oestrus, reduced the pregnancy rate and altered the corpus luteum function. The increased cortisol secretion would be able to start several mechanisms to impair the hypothalamus-pituitary ovarian axis affecting the corpus luteum function to produce P<sub>4</sub>, which would cause different types of abnormal P<sub>4</sub> profiles or decreased P<sub>4</sub> secretion, which were found in Heavily-handled groups. On the other hand, less handling and gentle interaction with animals could improve the reproductive performance, which in return could increase the income of the farm.

#### Acknowledgment

I would like thank Dr. Abdul Wahid Haron and the staff at theriogenology and cytogenetic unit of Veterinary Medicine, UPM for help with samples analyses. We also thank to Dr. Hishamfariz and his staff at Pusat Ternakan in Ulu Lebar, Pahang farm, for their assistance in my research.

#### REFERENCES

- Abraham GE, 1981: The application of natural steroid radioimmunoassay to gynecologic endocrinology. In: Abraham GE, editor. *Radioassay systems in clinical endocrinology*. Basel: Marcel Dekker, 475-529.
- Allrich DR, Cook DL, Horstman LA, Knutson RJ, Winters TA, 1989: Influence of dexamethasone, P<sub>4</sub>, gonadotropin-releasing hormone, and testosterone on estrus behaviour of estradiol-treated ovariectomized heifers. *Journal of Dairy Science* 72: 2707 - 2711.
- Balm PHM, 1999: Stress physiology in animals. Sheffield Academic Press, CRC.
- Berghold P, Mostl E, Aurich C, 2007: Effects of reproductive status and management on cortisol secretion and fertility of estrous horse mares. *Animal Reproduction Science* 102: 276-285.
- Burns BM, Fordyce G, Holroyd RG, 2010: A review of factors that impact on the capacity of beef cattle females to conceive, maintain a pregnancy and wean a calf— Implications for reproductive efficiency in northern Australia. *Animal Reproduction Science* 122: 1-22.
- Burtis CA, Ashwood ER, 1994: Tietz textbook of clinical chemistry. 2<sup>nd</sup> edition. Philadelphia: W.B. Saunders,
- Cook DL, Winters TA, Horstman LA, Allrich RD, 1987: Influence of cortisol and dexamethasone on estrous behaviour of estradiol-treated ovariectomized cows and heifers. *Journal of Dairy Science* 70: 181 - 185.

- Coulon M, Hild S, Schroeder A, Janczak AM, Zanella AJ, 2011: Gentle vs. aversive handling of pregnant ewes: II. Physiology and behavior of the lambs. *Physiology and Behavior* 103: 575–584.
- Dobson H, Tebble JE, Smith RF, Ward WR, 2001: Is stress really all that important? *Theriogenology* 55: 65 - 73.
- Fergani C, Saifullizam AK, Routly JE, Smith RF, Dobson H, 2012: Estrous behavior, luteinizing hormone and estradiol profiles of intact ewes treated with insulin or endotoxin. *Physiology & Behavior* 105: 757–765.
- Garcia M, 1990: Milk P4 profiles in crossbred brown swiss x nelore cattle following natural service. *Theriogenology* 33: 1105 - 1111.
- Grandin T, 1997: Assessment of stress during handling and transport. *Journal of Animal Science* 75: 249 - 257.
- Hollenstein K, Janett F, Bleul U, Hassi M, Kahn W, Thun R, 2006: Influence of oestradiol on adrenal activity in ovariectomized cows during acute stress. *Animal Reproduction Science* 93: 292 - 302.
- Hommeida A, Nakao T, Kubota H, 2004: Luteal function and conception in lactating cows and some factors influencing luteal function after first insemination. *Theriogenology* 62: 217 - 225.
- Hunter MG, 1991: Characteristics and causes of the inadequate corpus luteum. *Journal of reproduction and fertility* 43: 91 - 99.
- Karg H, 1981: Physiological impact on fertility in cattle with special emphasis on assessment of the reproductive function by P4 assay. *Livestock Production Science*, 8: 233-246.
- Kawate N, Inaba T, Mori J, 1993: Effects of cortisol on the amounts of estradiol-17 $\beta$  and P4 secreted and the number of luteinizing hormone receptors in cultured bovine granulosa cells. *Animal reproductive science*, 32 : 15-25.
- Kenyon AG, Mendonc LGD, Lopes JrG, Lima JR, Santos JEP, Chebel RC, 2012: Minimal progesterone concentration required for embryo survival after embryo transfer in lactating Holstein cows. *Animal Reproduction Science*, 4645: 1-8.
- Leyva-Ocariz H, Querales G, Saavedr J, HernBndez A, 1996: Corpus luteum activity, fertility, and adrenal cortex response in lactating Carora cows during rainy and dry seasons in the tropics of Venezuela. *Domestic Animal Endocrinology* 13: 297 - 306.
- Liptrap RM, Cummings E, Huether P, 1989: Effect of mid-luteal glucocorticoid injection with pulsatile administration of gonadotropin releasing hormone on follicular growth and ovulation in the sow. *Animal Reproduction Science* 19: 131 - 141.
- Lonergan P, 2011: Advances in Bovine Reproduction and Embryo Technology: Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology* 76: 1594–1601.
- Mann G E, Lamming G E, 2001: Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* 121: 175 - 180.
- Nepomnaschy PA, Welch KB, McConnell DS, Low BS, Strassmann BI, England BG, 2006: Cortisol levels and very early pregnancy loss in humans. *Proceeding of the National Academy of Science (PNAS)* 103: 3938 - 3942.
- Orihuela A, 2000: Some factors affecting the behavioural manifestation of estrus in cattle: a review. *Applied Animal Behaviour Science* 70: 1 - 16.
- Rekwot PI, Ogwu D, Sekoni VO, Oyedipe EO, 2000: Serum P4 profiles of zebu cattle (*Bos indicus*) in relationship to conception and repeat breeding after artificial insemination. *Animal Reproduction Science* 63: 41 - 51.
- Reimers TJ, McCann JP, Cowan RG, 1983: effect of storage times and temperature on T3, T4, LH, prolactin, insulin, cortisol, and progesterone concentrations in blood samples from cows. *Journal of Animal Science* 57: 683-691.
- Roelofs JB, Bouwman EG, Dieleman SJ, Van Eerdenburg FJCM, Kaal-Lansbergen LMTE, Soede NM, Kemp B, 2004. Influence of repeated rectal ultrasound examinations on hormone profiles and behaviour around estrus and ovulation in dairy cattle. *Theriogenology* 62: 1337 - 1352.
- Saifullizam AK, Routly JE, Smith RF, Dobson H, 2010: Effect of insulin on the relationship of estrous behaviors to estradiol and LH surges in intact ewes. *Physiology & Behavior* 99; 555–561.
- Smith RL, 1985: Effect of serum-separating gels on progesterone assays. *Clin chem.* 31:1239.
- Sood P, Nanda AS, 2006: Effect of lameness on estrous behavior in crossbred cows. *Theriogenology* 66: 1375–1380.
- Southee JA, Hunter MG, Law AS, Haresign W, 1988: Effect of hysterectomy on the short life-cycle corpus luteum produced after GnRH-induced ovulation in the anoestrous ewe. *Journal of Reproduction and Fertility* 84: 149 - 155.
- Stevenson JS, Lamb GC, Hoffmann DP, Minton, JE, 1997: Review Interrelationships of lactation and postpartum anovulation in suckled and milked cows. *Livestock Production Science* 50: 57 - 74.
- Stoebel DP, Moberg GP, 1982: Effect of adrenocorticotropin and cortisol on luteinizing hormone surge and estrus behaviour of cattle. *Journal of Dairy Science* 65: 1016 - 1024.
- Tadich N, Gallo C, Bustamante H, Schwerter M, van Schaik G, 2005: Effects of transport and lairage time on some blood constituents of Friesian-cross steers in Chile. *Livestock Production Science* 93: 223 - 233.
- Taponen J, Hjerpe P, Kopra E, Rodriguez-Martinez H, Katila T, Kindahl H, 2003: Premature prostaglandin F<sub>2 $\alpha$</sub>  secretion causes luteal regression in GnRH-induced short estrous cycles in cyclic dairy heifers. *Theriogenology* 60: 379 - 393.
- Wiltbank MC, Gtimen A, Sartori R, 2002: Physiological classification of anovulatory conditions in cattle. *Theriogenology* 57: 21 - 52.

\*\*\*\*\*