Indian Journal of Animal Sciences **92** (8): 950–956, August 2022/Article
https://doi.org/10.56093/ijans.v92i8.115682

Changes in ovarian structures, blood biochemical and hormonal profile in double Ovsynch treated conceived and non-conceived Jaffarabadi buffaloes

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Received: 20 September 2020; Accepted: 13 July 2022

ABSTRACT

The present study comprised 12 post-pubertal acyclic buffalo heifers (40-50 months of age) and 6 postpartum lactating acyclic buffalo cows. Animals were monitored for ovarian changes by ultrasonography along with blood sampling on different days of hormonal treatment, on day 27 of protocol and on day 12, 21 and 35 post-insemination. Total 10 animals (55.56%; 9/12 heifers; 1/6 buffalo) conceived at fix timed artificial insemination (FTAI) and remaining 8 animals were considered as non-conceived for comparison. Significantly higher number of large follicles was observed on day 7 with reduction in mean diameter of subordinate follicles at day 26, decreased plasma levels of FSH on day 17, 24 and 26; increased plasma level of LH on day 27, with higher insulin level in conceived animals as compared to non-conceived animals. Significant reduction in number of large follicles was recorded on day 21 post-AI in conceived animals. The difference in the levels of progesterone was statistically significant between conceived and non-conceived animals at day 35 post-AI. There were highly significant positive correlations among population of different size follicles, total follicles and largest and subordinate follicles diameter, whereas they had negative correlation with plasma protein and cholesterol both in conceived and non-conceived animals, but their correlations with plasma LH, insulin and estrogen were significant and positive only among non-conceived group.

Keywords: Double Ovsynch, Hormonal profile, Jaffarabadi buffalo, Ovarian changes

Jaffarabadi buffalo is considered as one of the economically important and heaviest breeds of buffalo in the world. It is regarded as an asset to the farmers of Saurashtra region in Gujarat, India. Reproductive efficiency of Jaffarabadi buffaloes is mainly affected by late maturity, poor estrus expression, anestrous, prolonged inter-calving interval and reduced ovarian activity. Age at first heat, age at first calving, calving interval and service period of Jaffarabadi buffaloes were reported to be 1312.8, 1622.7, 502.2 and 197.4 days, respectively (Anonymous 2016). Researchers tried various protocols for estrous induction and ovulation synchronization using combinations of gonadotropin-releasing hormone and prostaglandin $F_2\alpha$, viz. Ovsynch (Pursley *et al*. 1995), Heatsynch (Mohan *et al*. 2009) and Doublesynch (Dhindsa *et al*. 2016) to improve its reproductive efficiency. Double Ovsynch protocol has been developed at the University of Wisconsin and used to improve fertility in cattle (Cirit *et al*. 2007, Stevenson 2017), which combines two standard Ovsynch protocols one week apart with timed artificial insemination (AI) on day 27. Though it takes longer time and is expensive, the conception rates are much better in primiparous cattle (Souza *et al*. 2008, Dirandeh *et al*. 2015). Therefore, the present study was undertaken to evaluate the effect of double Ovsynch protocol on fertility of Jaffarabadi buffaloes through changes in ovarian structures and blood biochemical/hormonal profiles among conceived and non-conceived animals.

MATERIALS AND METHODS

Animals: The study was carried out on 18 Jaffarabadi female buffaloes housed at Cattle Breeding Farm, JAU, Junagadh, India under tropical climate. All the animals were maintained under standard feeding and healthcare management practices.

*Experimental design***:** Twelve post-pubertal acyclic Jaffarabadi buffalo heifers about 40-50 months of age and six lactating acyclic buffaloes (>90 days postpartum) constituted the experimental animals. The experimental animals were in good body condition (BCS = 3.5-4.0 on a 5 point scale, Anitha *et al.* 2011). The animals were maintained under loose housing system with provision of optimum floor space in both covered and open area. The long-axis of the loose house was oriented in East-West direction. The covered area had *pucca* roof with concrete floor and provided with common manger for feeding of animals. The open paddock, with stone slab floor was provided for free movement of animals. The concentrate mixture and the roughages (green and dry) were provided in the common manger present at the covered area of loose

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house. Experimental animals were fed as per Indian Council of Agricultural Research (ICAR) feeding standards to meet their nutrient requirements (ICAR 2013). These animals were subjected to Double Ovsynch protocol (Stevenson 2017, Dirandeh *et al*. 2015), consisting of intramuscular injections of 20 µg Buserelin acetate, a GnRH analogue (Receptal, Intervet India Pvt. Ltd.) on days 0, 10, 17 and 26, and injections of 500 µg $PGF_2\alpha$ (Cloprostenol, Zydus Animal Healthcare Ltd., India) on days 7 and 24, with a timed insemination at 16 and 24 h after the last GnRH Inj. (i.e. on day 27). The animals inseminated at induced estrus/ fix timed artificial insemination (FTAI) were followed for return to estrus, if any, and pregnancy was confirmed on day 35 by ultrasonography and on day 70 by per rectal examination.

Animal Ethics Committee Approval: The experiment protocol was approved by the Institutional Animal Ethics Committee (IAEC), Animal Welfare Division, New Delhi vide approval No. JAU/JVC/IAEC/LA/28/2018. All experimental animals were maintained as per CPCSEA Standard Guidelines, 2003.

Ultrasonography: Transrectal ultrasonography was performed using real-time B-mode scanner equipped with a 5.0-7.5 MHz rectal probe (DB355M, IMAGO.S, ECM, France). The transducer was placed over the ovaries through rectal wall and scanning was accomplished in several planes to identify all the follicles with diameter <4 mm (small follicles), 4-8 mm (medium follicles) and >8 mm (large follicles) and corpus luteum (CL) (Malik 2005). Scanning was performed on each day of hormone injection and on days 0, 12, 21 and 35 post-artificial insemination (AI).

Blood sampling and hormonal-biochemical assay: Blood (7 ml) was collected aseptically from the jugular vein in sterile glass vials containing potassium EDTA as anticoagulant on each day of hormone injection and on days 0, 12, 21 and 35 post-artificial insemination (AI) from all animals. Plasma was separated by centrifugation of blood samples at 3,000 rpm for 10 min and stored at –20°C with a drop of 0.01% merthiolate as preservative until analysed. Plasma concentrations of follicle stimulating hormone (FSH, Cloud-Clone Corp., USA); and luteinizing hormone (LH), estrogen and insulin (MyBio Source Inc., USA) were determined using concerned Enzyme Linked Immuno Sorbent Assay (ELISA) kits as per manufacturers' instructions. Plasma progesterone level was determined through standard Radio Immuno Assay (RIA) technique using Labelled antigen (I^{125}), antibody-coated tubes and standards procured from Immunotech-SAS, Marseille Cedex, France. Blood glucose levels were determined in freshly collected whole blood samples using Morepen Glucometer (Morepen Lab. Ltd, Delhi). Plasma total cholesterol and total protein levels were determined by CHOD/PAP and Biuret methods, respectively using kits procured from Diatek Healthcare Pvt. Ltd., Kolkata, India.

*Statistical analysis***:** Animals confirmed as pregnant at FTAI were considered as conceived (n=10) and others as non-conceived animals (n=8). Data on ovarian changes were analyzed group-wise using General Linear Model using SPSS and mean differences within group were compared by Duncan's multiple range test (DMRT) and between groups by 't' test. Pearson's correlations were studied between ovarian structures and plasma hormonal/ biochemical parameters using methods described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

*Ovarian changes and conception rate***:** The findings on the effect of double Ovsynch protocol on follicular population and diameter at different time intervals in conceived and non-conceived animals, irrespective of age group, are presented in Figs 1 and 2. In the present study, the first service (FTAI) conception rate in double Ovsynch treated Jaffarabadi buffaloes was 55.56% (n=10). The results were better in acyclic buffalo heifers (9/12) than the acyclic lactating buffalo cows (1/6). There was significantly ($p<0.05$) greater number of large follicles on day 7 of treatment and reduced number on day 21 post-FTAI in conceived animals as compared to non-conceived animals, but no such differences were observed for small and medium size follicle population (Fig. 1). Further, significant ($p<0.05$) reduction in diameter of only sub-ordinate follicles was recorded on day 26 of treatment protocol in conceived animals as compared to non-conceived animals. However, the mean total number of follicles and diameter of large follicles were statistically similar among them, though apparently larger in non-conceived group on day 12 and 21 post-AI. Both these traits however showed gradual and significant ($p<0.05$) increase over treatment days from day 0 to day 26 (Fig. 2).

The animals ovulated out of 12 buffalo heifers and 6 buffalo cows after 1st, 2nd, 3rd and 4th GnRH injection were 6 vs 2; 3 vs 0; 7 vs 5 and 10 vs 4, respectively, being better after 3rd and 4th GnRH injection of the protocol. Further, the animals that maintained CL on days 12, 21 and 35 post-AI in two groups were 10 vs 4; 10 vs 2; and 9 vs 1, respectively, giving conception rates of 75% and 16.6% at FTAI in buffalo heifers and buffalo cows, respectively. The result showed better response in acyclic postpubertal buffalo heifers than the acyclic multiparous lactating buffalo cows.

Souza *et al*. (2008) and Dirandeh *et al*. (2015) reported increased pregnancy per AI (P/AI) with double Ovsynch in primiparous as compared to multiparous cows which is supported by the present study. Further, more number of cows in the double Ovsynch protocol had CL at first GnRH injection, greater ovulatory response to second GnRH injection, larger mean diameter of ovulatory follicle at the time of AI (TAI) and greater percentage of pregnancy at 32 days after AI compared with other protocols (Dirandeh *et al*. 2015). It is generally accepted that primiparous cows are more likely to be anovular as compared to multiparous cows (Silva *et al*. 2007). Improved fertility during double Ovsynch may be partially related to the hormonal milieu

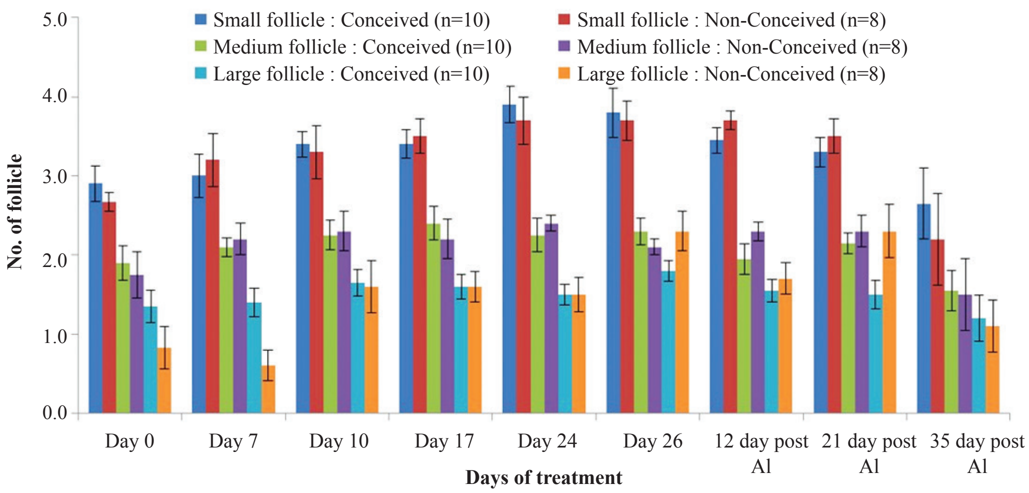

Fig. 1. Average number of follicles per ovary in conceived (n=10) and non-conceived (n=8) Jaffarabadi animals at various time period of double Ovsynch treatment protocol.

during follicular development. Greater circulating progesterone during follicular development could decrease LH pulsatility possibly improving the competency of the dominant follicle and/or the quality of the ovulated oocyte (Silva *et al*. 2007). The success of ovulation synchronization protocol has been related to ovulatory response to first GnRH injection of Ovsynch (Vasconcelos *et al*. 1999) and progesterone concentration prior to $PGF_2\alpha$ injection (Martins *et al*. 2011). A lack of follicle turnover due to failure to respond to the initial GnRH administration might compromise the quality of embryos and consequently reduce P/AI.

The use of double Ovsynch protocol has been tested only in exotic cattle under controlled farm conditions with observation that more cows ovulate in response to GnRH-3, and the future egg to be fertilized after timed AI develops within its follicle in higher progesterone environment under this protocol. Both of these factors have been found to increase fertility when breeding cows with timed AI. Another advantage of this protocol is its ability to initiate first ovulation in anovulatory cows in response to either GnRH-1 or GnRH-2 before the breeding (Stevenson 2017). Low conception rate in postpartum lactating acyclic buffalo cows observed may be due to lack of ovulatory response to GnRH injections due to multi-parity or induced estrus being anovulatory or with luteal insufficiency for lactation stress. In all probability, this was primarily a consequence of embryonic mortality after maternal recognition of pregnancy since all animals had well developed CL on day 12 post-AI. Moreover, during early lactation, reproductive performance of dairy animals, particularly conception rate, may be negatively associated with the magnitude of the negative energy balance (Neble and Mcgilliard 1993). Wiltbank *et al*. (2011) suggested that lower fertility in cows with a small ovulatory follicle was associated with reduced serum estradiol concentrations before AI and ovulation of a less mature oocyte. Ovulation of smaller follicles might result in smaller CL that produce less progesterone (Vasconcelos *et al*. 1999) and perhaps exhibit a delayed responsiveness to $PGF_2\alpha$ due to expression of fewer $PGF_2\alpha$ receptors.

In present study, the diameters of large follicles were apparently higher in non-conceived than conceived animals at most intervals of treatment with concurrent higher FSH and lower LH leading to delayed ovulation or anovulation causing conception failure at fixed time AI.

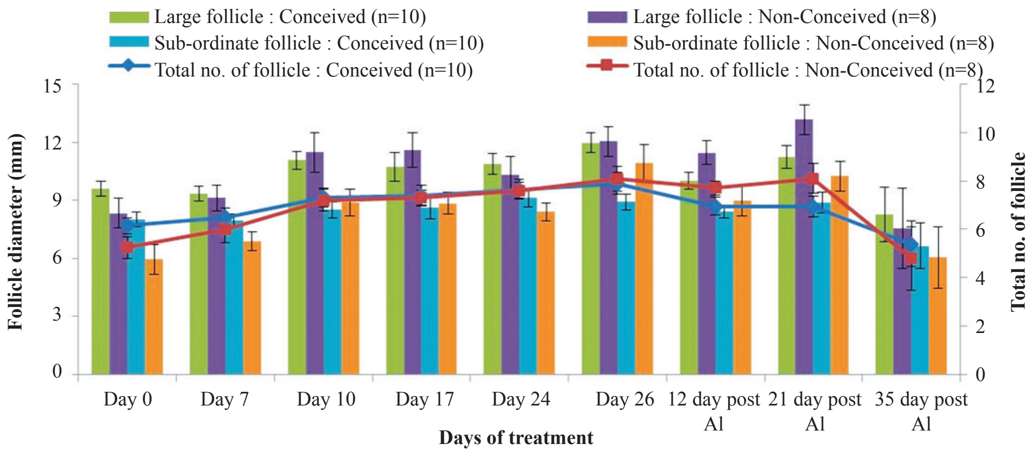

Fig. 2. Average number of follicles, diameter of large and subordinate follicles per ovary of conceived (n=10) and non-conceived (n=8) Jaffarabadi animals during different period of double Ovsynch protocol.

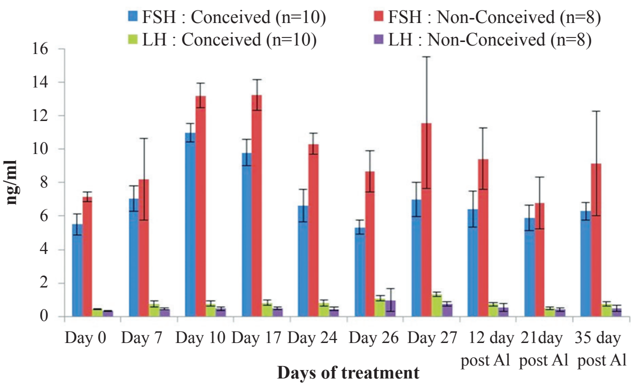

Fig. 3. Plasma FSH and LH profile of conceived (n=10) and non-conceived (n=8) Jaffarabadi animals during different period of double Ovsynch protocol.

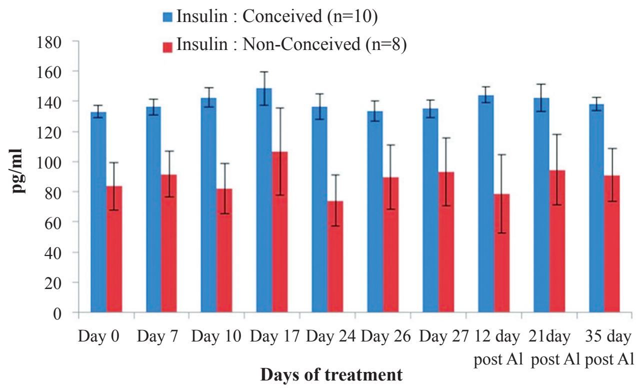

Fig. 5. Plasma insulin concentration in conceived (n=10) and non-conceived (n=8) Jaffarabadi animals during different period of double Ovsynch protocol.

Moreover, CL size recorded by ultrasonography, was also non-significantly larger on all days of monitoring. This may be associated with relatively larger size of dominant follicles in these animals. Further, the greater size of follicles and CLs noted in non-conceived sub-group over conceived one could also be due to pooling of data of heifers and multiparous buffaloes, and the fact that most of the multiparous buffaloes with larger dominant follicles (5/6) failed to conceive at FTAI in relation to heifers. We could see higher conception rate in animals with smaller/normal follicles compared to larger ones (10.5 vs 12.0 mm) concomitant to even larger subordinate follicles. This is in contradiction to the findings of De Rensis *et al*. (2005), who observed a relatively high conception rate in buffaloes with follicle size ≥ 10 mm compared to those with follicle size of ≤ 10 mm. Pregnant buffaloes were reported to have a larger pre-ovulatory follicle (POF) and subsequent CL than non-pregnant buffaloes and they also exhibited higher blood progesterone concentration on day 7 post-AI as compared to non-pregnant animals (Barlie *et al*. 2007). Heifers that were induced to ovulate follicles <10.7 mm in diameter had decreased pregnancy rates compared to heifers that ovulated follicles ≥12.8 mm and less than 15.7 mm (Perry *et al*. 2007). It is attributed to competence of the oocyte ovulated, post-ovulation progesterone production by the CL, and the uterine environment. Larger preovulatory follicle results in larger CL development, which secretes more progesterone and has a net positive effect on pregnancy recognition, pregnancy rates and less chance of early embryonic mortality (Barile *et al*. 2007, Lopes *et al*. 2007).

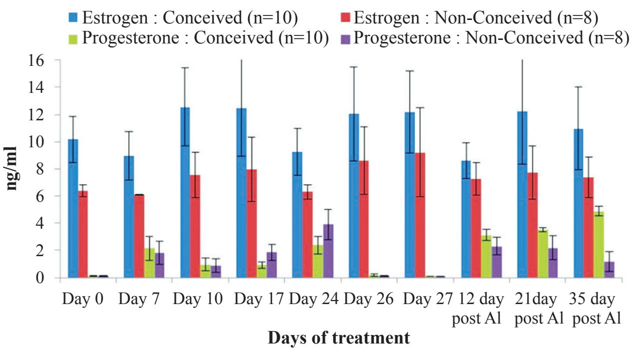

Fig. 4. Plasma estrogen and progesterone profile of conceived (n=10) and non-conceived (n=8) Jaffarabadi animals during different period of double Ovsynch protocol.

Hormonal/biochemical profile: The plasma FSH concentration was consistently lower and LH level was higher with significant (p<0.05) reduction in the levels of FSH on day 17, 24 and 26 of protocol and significant increase in level of LH on day 27 of protocol (the day of estrus/FTAI) in conceived animals as compared to non-conceived animals (Fig. 3). The higher FSH was also observed to be associated with greater size of follicles in non-conceived buffaloes. The levels of estrogen remained statistically at par in both conceived and non-conceived animals during treatment period, though the values were apparently lower for conceived animals at all intervals. The difference in the levels of progesterone was statistically significant (p<0.01) between conceived and non-conceived groups at day 35 post-AI. The plasma P_4 pattern showed ovarian follicle/CL response to GnRH/PG injections at defined periods over the treatment cycle (Fig. 4). The plasma levels of insulin throughout treatment protocol were significantly (p<0.001) higher in conceived than non-conceived animals, except on day 17 of treatment (Fig. 5). The overall mean values of blood glucose, plasma cholesterol and protein in double Ovsynch treated conceived and non-conceived animals were however statistically similar and did not vary much between periods (Fig. 6).

The insignificant influence of Double Ovsynch protocol noted on blood biochemical profile concurred well with the report of Patel *et al*. (2013) in cattle following Ovsych and CIDR treatment protocols.

The concentrations of plasma FSH have been reported to be high prior to the emergence of each follicular wave and remains low during the growing phase of a dominant follicle (Kaneko *et al*. 1995). Plasma estradiol concentrations are generally high on the day of estrus in conceiving animals as compared to non-conceiving cows/buffaloes (Perry *et al*. 2007, Lopes *et al*. 2007, Kumar 2015), but no such trend was observed in the present study, though apparently the levels

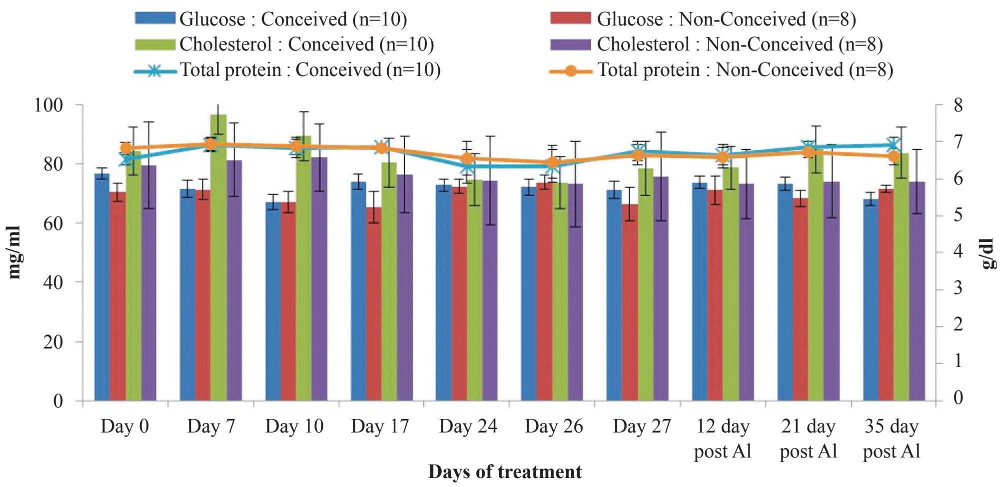

Fig. 6. Blood glucose, plasma total cholesterol and total protein profile of conceived (n=10) and non-conceived (n=8) Jaffarabadi animals during different period of double Ovsynch protocol.

were high in conceived than non-conceived animals during treatment period. In the present study, plasma progesterone levels, which were lowest during estrus, increased after 1st GnRH and 3rd GnRH injections on day 7 and day 24, respectively, and also increased levels were noticed on days 12, 21 and 35 post-AI in both conceived and non-conceived animals suggesting ovulatory estrus in both the subgroups, but perhaps with higher luteal insufficiency and/or early embryonic mortality in non-conceived group. These results are in agreement with earlier observations in buffaloes (Campanile *et al*. 2007, Prajapati *et al*. 2018).

Significantly higher insulin levels at different period of treatment observed in conceived animals compared to non-conceived animals, throughout treatment protocol could be attributed to age of animals and its association with fertility, since 90% of conceived animals were heifers, whereas among non-conceived group, 83.3% were multiparous lactating buffaloes. Insulin profile in general is reported to be higher in heifers than buffaloes. Moreover, low insulin perhaps reduced follicular responsiveness to gonadotrophic stimuli, causing low production of estradiol by the dominant follicle and affecting LH pulsatility in non-conceived animals (Butler 1999).

Blood plasma glucose level was recorded to be non-significantly lower in fertile than infertile estrus in Surti buffaloes (Kavani *et al*. 2005). Cholesterol serves as a precursor for the synthesis of progesterone by ovarian luteal cells. In a recent study, significantly higher mean plasma cholesterol concentration was documented in non-conceived than conceived Gir heifers treated with Doublesynch and Estradoublesynch protocols (Chaudhary *et al*. 2018). Moreover, the mean plasma protein levels on the day of FTAI and day 12 post-AI were significantly higher compared to day 0 and 9 of the Estradoublesynch protocol. In present study, however, the mean values of glucose, protein and cholesterol did not differ significantly between conceived and non-conceived status and even between periods of treatment with double Ovsynch protocol.

Correlations between ovarian structures and plasma profile: Pearson's correlations observed between the parameters of ovarian structures and plasma endocrine/biochemical profiles in conceived (n=10) and non-conceived (n=8) Jaffarabadi buffaloes under double Ovsynch protocol and their follow up till 35 days post-FTAI are shown in Table 1.

The small follicle population showed significant positive correlations with medium, large and total number of follicles, diameter of large follicle, diameter of subordinate follicle and significant negative correlation with plasma cholesterol and protein in conceived animals. However, in non-conceived animals, it showed significant positive correlation with number of large follicles, total number of follicles, diameter of large follicle and diameter of subordinate follicle. The medium sized follicle population was significantly correlated with large follicular population, total number of follicles, and plasma estrogen level in conceived animals, while in non-conceived animals, it was significantly positively correlated with total number of follicles and plasma progesterone level. Large size follicle population revealed highly significant positive correlation with total follicle population, diameter of large follicle and diameter of subordinate follicle in conceived animals, while in non-conceived animals, it showed significant positive correlation with total follicle population, diameter of large follicle and diameter of subordinate follicle, plasma LH, estrogen levels and had significant negative correlations with plasma total protein level. Further, the total number of follicles in conceived animals had highly significant positive correlations with diameter of large and subordinate follicle, plasma insulin level and significant negative correlations with plasma total cholesterol and plasma total protein levels. In non-conceived animals, total number of follicle showed significant positive correlations with diameter of large and subordinate follicle and plasma insulin level. Diameter of large follicle showed significant positive correlation only with diameter of subordinate follicle in conceived animals, while in non-conceived animals, it showed positive correlation with diameter

Table 1. Correlation matrix (r) of ovarian structures and plasma biochemical/hormonal profiles of conceived and non-conceived Jaffarabadi buffaloes following double Ovsynch treatment protocol

Parameter	SF	MF	LF	TF	DLF	DSF	FSH	LH	E_2	P_4	Insulin	Glucose	TC	Protein
Correlations: Conceived Animals														
No. of small follicle (SF)	1	0.24*	0.31**	0.79**	0.36**	0.37**	0.05	0.15	-0.04	-0.07	0.20	0.13	-0.33**	-0.21*
No. of medium follicle (MF)	0.01	1	0.23*	0.66**	-0.02	-0.01	-0.01	0.02	0.23*	-0.17	0.17	0.11	-0.19	-0.15
No. of large follicle (LF)	0.35*	0.07	1	0.68**	0.47**	0.60**	0.12	-0.01	-0.03	-0.15	0.07	0.16	0.02	-0.08
Total no. of follicles (TF)	0.71**	0.40**	0.82**	1	0.39**	0.45**	0.07	0.09	0.06	-0.17	0.21*	0.18	-0.25*	-0.21*
Diameter of large follicle (DLF)	0.31*	0.19	0.64**	0.62**	1	0.80**	0.05	0.11	-0.05	-0.17	0.02	0.16	0.01	-0.11
Diameter of subordinate follicle (DSF)	0.39**	0.04	0.81**	0.71**	0.74**	1	0.03	0.19	-0.07	-0.12	-0.10	0.18	0.08	-0.12
Plasma FSH	0.06	0.03	-0.05	0.01	-0.06	-0.06	1	-0.11	0.05	-0.04	-0.19	-0.04	0.34**	0.30**
Plasma LH	-0.03	-0.08	0.32*	0.16	0.30*	0.47**	0.01	1	-0.15	-0.16	0.21*	-0.27**	-0.15	-0.24*
Plasma estrogen (E_2)	0.02	0.01	0.44**	0.29	0.52**	0.65**	-0.01	0.76**	1	-0.03	0.03	-0.16	-0.23*	0.04
Plasma progesterone (P_4)	0.03	0.30*	0.03	0.14	0.12	0.01	0.04	0.06	-0.04	1	-0.10	-0.23*	0.06	0.02
Plasma insulin	0.18	0.23	0.24	0.32*	0.49**	0.47**	-0.16	0.48**	0.64**	0.07	1	-0.15	-0.63**	-0.34**
Blood glucose	0.08	-0.15	0.25	0.14	0.16	0.19	-0.23	0.23	0.29	-0.04	0.10	1	0.03	-0.02
Plasma total cholesterol (TC)	-0.03	-0.24	0.01	-0.10	-0.18	-0.19	-0.24	-0.20	-0.15	-0.37*	-0.37*	0.15	1	0.55**
Plasma total protein	0.01	-0.01	-0.41**	-0.26	-0.55**	-0.42**	0.10	-0.69**	-0.53**	-0.14	-0.39**	-0.40**	0.16	1
Correlations: Non-Conceived Animals														

*Significant at p<0.05 level; **Significant at p<0.01 level (2-tailed); n = Conceived (90 observations), Non-conceived (72 observations).

of subordinate follicle, plasma LH, estrogen and insulin levels and negative correlation with plasma total protein level. Diameter of subordinate follicle showed significant positive correlation with plasma LH, estrogen and insulin levels and significant negative correlation with plasma total protein level in non-conceived animals.

In conceived animals, plasma FSH concentration had significant positive correlation with total cholesterol and protein levels, while plasma LH concentration had significant positive correlation with plasma insulin and negative correlation with blood glucose level. In non-conceived animals, LH showed significant positive correlation with plasma estrogen and insulin and negative correlation with plasma total protein. Plasma estrogen level revealed significant negative correlation with plasma total cholesterol in conceived animals, while in non-conceived animals, it showed significant positive correlation with plasma insulin and negative correlation with plasma protein. Plasma progesterone concentration had significant positive correlation with medium follicles and negative correlation with blood glucose level in conceived animals. However, in non-conceived animals, it had significant negative correlation with plasma total cholesterol, and total cholesterol had positive correlation with total protein level in conceived animals. The correlation findings observed in Jaffarabadi females were physiological and as per expectations.

Kumar (2015) observed positive correlation on the day of estrus between POF diameter and plasma estradiol in buffaloes, however, Lynch *et al*. (2010) failed to observe such correlation and even subsequent conception in cattle. The literature reviewed however did not reveal comparable correlation studies of conceived and non-conceived animals following synchronization protocols in dairy animals.

In conclusion, the double Ovsynch protocol, though lengthy and expensive, was significantly better for improving conception rates in nulliparous than the multiparous Jaffarabadi buffaloes. The buffaloes conceived following double Ovsynch protocol had higher numbers of large follicles on day 7, with reduction in mean diameters of subordinate follicles on day 26, decreased plasma levels of FSH on days 17, 24 and 26, increased plasma levels of LH on day 27 (day of estrus/FTAI), with higher plasma levels of insulin, and increased plasma levels of progesterone on days 12-35 post-AI. There was reduced number of large follicles on day 21 post-AI in conceived than non-conceived animals. The interrelationships observed among different parameters in conceived and non-conceived animals were physiologically expected.

ACKNOWLEDGEMENTS

We thank the Principal and Dean, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh and Research Scientist (AGB), Cattle Breeding Farm, Junagadh Agricultural University, Junagadh for providing necessary facilities for carrying out this work.

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