RESEARCH ARTICLE

Relationship of the Pregnancy, Stress Biomarkers and Embryonic Losses in Jaffrabadi Buffaloes during Summer Season

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Abstract

The current study sought to explore the relationship between various pregnancy and stress-related biomarkers, and embryonic mortality in Jaffrabadi buffaloes during summer season. A total of 24 multiparous Jaffarabadi buffaloes were subjected to Ovsynch estrus synchronization and fixed time artificial insemination (FTAI) program, and pregnancy was ascertained by trans-rectal ultrasonography on day 35, and reconfirmed by per rectal palpation on day 60 post-AI. The plasma levels of INF-τ and PGFM were recorded on days 14, 16, 18, and 20 post-AI, whereas the pregnancy specific protein-B (PSPB), heat shock protein 70 (HSP 70), and progesterone were evaluated on days 20, 24, 27, 30, and 40 post-AI. The conception rates obtained were 33.34% (08/24) at 35 days and 16.67% (04/24) at 60 days post-AI, indicating that 50.00% of conceived animals experienced embryonic losses. The overall INF-τ level was significantly ($p<0.05$) higher in animals with embryonic mortality than in conceived and non-conceived animals. The overall PSPB levels of the conceived animals were significantly higher than that of the non-conceived animals. Moreover, animals with embryonic mortality had PSPB levels significantly higher than those of non-conceived animals and significantly lower than those of conceived animals. The overall PGFM level was significantly ($p<0.05$) lower in the animals that experienced embryonic death, however, HSP70 concentrations were statistically identical among conceived, non-conceived, and embryonic mortality groups across sampling days. The progesterone levels were significantly higher in animals who conceived and those who had embryonic mortality compared to those not conceived. The plasma levels of all pregnancy and stress biomarkers, with the exception of PSPB, were similar in conceived animals and those that experienced embryonic losses; therefore, poor or retarded embryonic development could be the cause of the embryonic losses that occur in Jaffarabadi buffaloes during the summer.

Key words: Embryonic loss, Heat stress, Interferon-tau, PSPB, PGFM, Summer Season.

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Introduction

The reproductive performance of the buffaloes is diminished significantly during the summer season, which leads to huge economic losses to dairy farmers (Borakhatariya *et al.,* 2024). The high surrounding temperature has an impact on embryonic development which can result in early or late embryonic death or losses, which reduces reproductive efficiency. A temperature humidity index (THI) of more than 68 induces heat stress, the hormonal environment changes, which may lead to the suboptimal secretion of progesterone (P_4) and $PGF_2α$. The higher temperatures may also hinders embryonic development, resulting in inadequate embryonic IFN-τ (interferon-tau) concentrations for maternal detection and perhaps failing to block $PGF_2α$ synthesis (Ribeiro *et al.,* 2016). There are still many unanswered questions exist, one of which is whether or not heat stress has any impact on secretion of the pregnancy and stress biomarkers, and whether or not it would affect the pregnancy rate in buffaloes. Understanding how early embryonic development is compromised by heat stress may allow researchers to develop methods to overcome it. By assessing the summer heat stress effect on the secretion of interferon-τ (IFN-τ), pregnancy specific protein B (PSPB), heat shock protein 70 (HSP70), progesterone, and 13, 14-dihydro-

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15-keto $PGF_2α$ metabolites (PGFM), and the interrelationship of these amongst pregnant and non-pregnant animals, can provide information to improve reproductive performance of buffaloes even under an unfavourable or non-breeding season. Hence, this study was planned to assess relationship of the pregnancy and stress biomarkers, and embryonic losses in Jaffrabadi buffaloes during summer season.

Materials and Methods

The experiment was conducted at Cattle Breeding Farm, Kamdhenu University, Junagadh (Gujarat, India), during the summer season (March to June 2023) following approval of Institutional Animal Ethics Committee (IAEC) of the College (Protocol No.: KU-JVC-IAEC-LA-100-22). Geographically, the city of Junagadh is located in the southwest of the state of Gujarat, near the foothills of Mount Girnar, at latitudes of 21.52°N to 21°23' North and 70.47°E, with the Arabian Sea to the southwest at an elevation of 107 meters above mean sea level. In Junagadh, there are two different seasons: a dry season from October to May and a wet season from June to September. The city has a tropical wet and dry climate that leans on a hot, semi-arid environment. The maximum temperature throughout the summer, which lasts during the months of March, April, May, and June ranges from 28 to 45°C.

Experimental Animals and their Management

Twenty four Jaffarabadi buffaloes, aged from 4 to 8 years and weighing 400-500 kg on average, were incorporated in the present experiment, imposing a postpartum voluntary waiting period (VWP) of 45-60 days. Prior to being included in the study, animals were clinically examined, and only the cyclic animals that had no apparent reproductive abnormalities were included in the study. Experimental animals were kept in loose housing, and exposed to regular climate conditions with continuous access to *ad libitum* water. During the course of the experiment, the buffaloes were given seasonal fodder, hay, and compounded concentrate. Apart from this, the wallowing was discontinued for those buffaloes that were part of the study.

The selected animals were subjected to a protocol of estrus induction cum synchronization, *i.e.,* the Ovsynch protocol, followed by FTAI twice, at 24 and 48 h later. Animals were then regularly observed for the return of estrus after insemination. For those animals who didn't show the estrus post-AI, on day 35 after AI, pregnancy status was determined using a real time B-mode trans-rectal ultrasound scanner equipped with a 5-8.5 MHz linear array rectal transducer (MINDRAY, Model: Z5 Vet USG), and reconfirmation of pregnancy was done by palpation per rectum on day 60 post-AI. Conception rate and embryonic losses were recorded during the study period. The temperature and humidity data were obtained from the Department of Agronomy, Agrometeorological Cell, College of Agriculture, Junagadh. The THI index was calculated using the formula: $THI = (1.8 \times AT + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times AT - 26.8)]$, where, AT = air temperature (°C) and RH = relative humidity (%).

Blood Sampling and Assays Procedures

The experimental animals were examined for the blood plasma levels of INF-τ and PGFM on days 14, 16, 18, and 20 post-AI, whereas the plasma levels of pregnancy marker (PSPB; pregnancy specific protein-B), and heat shock protein 70 (HSP70) were evaluated on days 24, 27, 30, and 40 post-AI. Further, plasma progesterone was evaluated on days 20, 24, 27, 30, and 40 post-AI. To determine levels of these plasma profiles, blood samples (6-8 mL) were withdrawn from the jugular vein in 10 mL vacutainers (EDTA vials) by veni-puncturing on pre-determined days post-AI, *i.e.,* on days 14^{th}, 16^{th}, 18^{th}, 20^{th}, 24^{th}, 27^{th}, 30^{th}, and 40^{th}. Collected blood samples were placed in a portable mini cooler (4-6 °C), and brought to the laboratory immediately. The blood samples were centrifuged at 1372 g for 15 min, and the separated plasma was stored deep frozen at -20 °C with a drop of merthiolate (0.1%) until assayed. Plasma levels of all the parameters were analyzed using ELISA assay kits procured from MyBiosource, San Diego, USA, and procedures were performed in accordance with the standard techniques laid down by the manufacturer.

Statistical Analysis

The plasma profiles of INF-τ, PSPB, PGFM, HSP70, and progesterone were analyzed using a two-way ANOVA, and the groups mean differences were compared using Duncan's *post hoc* multiple range test, using Sigmaplot version 11.0 (Systat Software Inc, USA). The relationship between the parameters was examined using correlation matrix analysis and the 'p' value <0.05, was considered as significant.

Results and Discussion

During the study period, thermal humidity index (THI) values ranged from 75.0 to 83.0 with extreme during May-June months. This range denotes moderate to severe heat stress to the animals. The results of the current study showed that 50.00% of experimental animals that were conceived had embryonic losses, with conception rates of 33.34% (08/24) at 35 days post-AI and 16.67% (04/24) at 60 days (Table 1).

Table. 1: Conception rates in estrus synchronized Jaffarabadi buffaloes during summer season

No. of animals	Conception Rate (%)		Embryonic losses (%)
	at 35 days post-AI	at 60-days post-AI	
24	33.34 % (08/24)	16.67 % (04/24)	50.00 % (04/24)

Plasma Profile of Interferon-τ and Prostaglandin $F_2α$ Metabolite (PGFM)

The overall mean INF-τ level (pg/mL) in the animals with embryonic mortality was significantly (p<0.01) higher (11.73±0.47) than in the conceived animals (9.80±0.28) and both were higher (p<0.01) than in non-conceived animals (6.48±0.06) (Table 2). The present finding of a statistically significant difference in INF-τ levels between conceived and non-conceived animals is in close agreement with the findings of Amri *et al.* (2021) and Panjaitan *et al.* (2021), who also reported significantly higher INF-τ levels in pregnant animals compared to non-pregnant animals. Melia *et al.* (2020) observed numerically higher INF-τ concentrations in the cows that had embryonic mortality than in the pregnant animals on the 14^{th}, 15^{th}, 16^{th}, 17^{th}, and 18^{th} days post-AI. Sheikh *et al.* (2018) on the contrary perceived a significantly (p<0.05) lower concentration of plasma INF-τ in the Karan Fries (KF) cows in which late embryonic mortality (LEM) occurred as compared to pregnant animals.

The mean PGFM level in the animals, which experienced embryonic death during the experiment was much lower (1780.13±165.44 pg/mL) than in the animals that were conceived and not conceived (Table 2). The PGFM levels of conceived and non-conceived animals, on the other hand, did not differ significantly from one another (2711.21±172.35 vs. 3387.32±183.61 pg/mL). Thatcher *et al.* (1984) found significantly lower PGFM levels (pg/mL) in pregnant (362.8±84.3) and non-pregnant (724.3±136.8) Holstein cows on day 19 of the cycle, which contradicts the PGFM levels found in the conceived (2711.21±172.35) and non-conceived (3387.32±183.61) animals under current investigation. Pinaffi *et al.* (2018) discovered higher PGFM concentrations in non-pregnant animals than in pregnant animals, which is consistent with the present results. The correlations between levels of PGFM and INF-τ in the present study were found to be statistically non-significant and negligible in conceived, non-conceived and embryonic mortality groups (r = 0.184, p=0.495; 0.116, p=0.363; & 0.187, p=0.488, respectively).

Plasma Profile of Pregnancy Marker (PSPB) and Heat Shock Protein 70 (HSP70)

The overall mean PSPB level (ng/mL) of conceived animals was significantly higher (13.58±2.19) than that of non-conceived animals (0.88±0.05), and the animals with embryonic mortality (7.77±0.97), the levels of latter two groups also differed significantly. Moreover, among conceived animals, PSPB levels varied throughout the sampling days and increased from day 24 to day 40 (Table 3). On the other hand, in the animals with embryonic mortality, there was no significant increase in PSPB levels from day 27 to day 40. Gabor *et al.* (2016), Pohler *et al.* (2016) and Filho *et al.* (2019) also found that animals that were conceived had higher levels of these pregnancy markers than those that had embryonic mortality, and similar were the findings of other researchers (Barbato *et al.,* 2017; Ayad *et al.,* 2020; Peixoto *et al.,* 2021; Casano *et al.,* 2022). On the other hand, no significant difference in levels of pregnancy markers was observed between the conceived and embryonic mortality animals in the studies conducted by Karen *et al.* (2014) and Reese *et al.* (2018).

HSP70 values were found to be statistically the same across sampling days and among the groups of conceived, non-conceived, and embryonic mortality (Table 4). Hence, HSP70 was not found to affect embryonic survival in this study. Apart from this, no additional information about the study looking into the seasonal differences in HSP70 levels between conceived and non-conceived buffaloes could be found. Further, the correlations between levels of PSPB and HSP70 were moderate yet statistically non-significant and negative in conceived and embryonic mortality groups (r = -0.347, p=0.187 & -0.465, p=0.069, resp.), and negligible in non-conceived group (r = 0.088, p=0.486), may be due to small sample size of observations.

Table. 2: Levels of plasma INF-τ and plasma PGFM concentrations in Jaffarabadi buffaloes on various days after AI during the summer season in conceived, non-conceived, and animals that experienced embryonic mortality (Mean ± SEm)

Parameter	Status of Animals	No.	Days post-AI				Overall
			Day-14	Day-16	Day-18	Day-20	
Plasma INF-τ (pg/mL)	Conceived	04	9.61±1.08^{b}	9.27±0.66^{b}	10.59±1.56^{b}	9.73±1.24^{b}	9.80±0.28^{b}
	Non-conceived	16	6.62±0.39^{a}	6.51±0.40^{a}	6.46±0.42^{a}	6.34±0.40^{a}	6.48±0.06^{a}
	Embryonic mortality	04	12.23±1.28^{b}	12.76±1.31^{c}	11.19±1.25^{b}	10.73±1.45^{b}	11.73±0.47^{c}
Plasma PGFM (pg/mL)	Conceived	04	2273.76 ±506.91	3105.04 ±920.18	2800.26 ±685.08	2665.79 ±746.59	2711.21 ±172.35^{ab}
	Non-conceived	16	2853.92 ±529.47	3648.38 ±615.06	3609.51 ±572.28	3437.49 ±601.20	3387.32 ±183.61^{b}
	Embryonic mortality	04	1341.05 ±277.80	1858.16 ±526.36	1781.22 ±374.83	2140.09 ±467.30	1780.13 ±165.44^{a}

Figures bearing superscripts (a,b,c) for a parameter within a column differ significantly at p<0.05

Table. 3: The average peripheral levels of plasma PSPB and HSP70 assessed in Jaffarabadi buffaloes on various days after AI during the summer season in conceived, non-conceived, and animals that experienced embryonic mortality (Mean ± SEm)

Parameter	Status of animals	No.	Days post-AI				Overall
			Day-24	Day-27	Day-30	Day-40	
Plasma PSPB (ng/mL)	Conceived	04	^{p}8.58±2.02^{c}	^{q}12.12±1.56^{c}	^{r}14.63±1.25^{c}	^{s}18.98±1.29^{c}	13.58±2.19^{c}
	Non-conceived	16	0.85±0.07^{a}	0.84±0.07^{a}	0.85±0.08^{a}	0.97±0.05^{a}	0.88±0.05^{a}
	Embryonic death	04	^{p}5.17±0.67^{b}	^{q}7.41±0.95^{b}	^{q}9.41±0.61^{b}	^{q}9.11±1.24^{b}	7.77±0.97^{b}
Plasma HSP70 (ng/mL)	Conceived	04	117.33±10.48	92.34±16.08	96.68±19.10	88.71±14.33	98.77±6.40
	Non-conceived	16	105.71±9.64	105.07±10.94	102.23±11.38	99.68±8.51	106.41±9.07
	Embryonic death	04	110.24±24.26	111.64±15.70	80.60±14.38	123.16±19.71	103.17±1.39

Significant differences exist at p<0.05 across values in the same row (p,q,r,s) and column (a,b,c) for a parameter.

Plasma Profile of Progesterone Hormone

The progesterone levels (ng/mL) in animals that were conceived and those that had embryonic mortality were 7.25±0.27 and 6.01±0.39 (p>0.05), respectively, being significantly higher from those that did not conceive (1.99±0.23). Throughout the duration of the sampling days, the progesterone profiles of the conceived and embryonic mortality animals were significantly higher than those of the non-conceived animals (Table 4). These findings closely aligned with the reports of Ghanem and Nishibori (2015), Saini *et al.* (2021), and Thanh *et al.* (2023), where it was observed that pregnant animals had higher plasma progesterone levels than non-pregnant ones. Lopez-Gatius *et al.* (2007) and Karen *et al.* (2014) reported no change in progesterone levels between the pregnant and embryonic mortality animals. Our results, where we came across statistically equal plasma progesterone between conceived and embryonic mortality animals, however contradicted the earlier reports (Abu El-Hamd *et al.,* 2019; Husnurrizal *et al.,* 2020; Peixoto *et al.,* 2021) that animals with embryonic losses had lower levels of progesterone than pregnant animals.

The correlations of plasma progesterone with HSP70 were negative in all groups, being non-significant in conceived (r = -0.190, p=0.480) and embryonic mortality groups (r = -0.312, p=0.239), and but significant in non-conceived group (r = -0.335, p<0.006). Further, the association between progesterone and PSPB was strong positive (p<0.05) in conceived group (r =0.613; p=0.114) compared to that in embryonic mortality (r =0.198; p=0.460) and non-conceived groups (r =-0.166; p=0.189), though all were statistically non-significant.

Conclusion

The current investigation perceived that the conceived and embryonic mortality animals showed higher levels of INF-τ, suggesting that the embryonic INF-τ secretion remained unchanged and was not the reason behind the summer embryonic mortality in Jaffarabadi buffaloes. Retarded or poor embryonic growth, however, may be one of the contributing factors to the summertime embryonic losses, considering the significantly lower concentration of PSPB among animals with embryonic mortality. HSP70 levels did not influence the pregnancy outcome. Since there was no difference in PGFM levels between the conceived and embryonic mortality groups, it can be said that summer stress does not result in an increase in premature prostaglandin production during the course of the study, which could lead to embryonic death. Diminished progesterone was also not found to be the cause of the embryonic losses during the experiment, as levels were the same in animals that were conceived and those that had embryonic mortality.

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Table. 4: The Mean ± SEm of plasma progesterone levels (ng/mL) estimated in Jaffarabadi buffaloes on days 20, 24, 27, 30, and 40 post AI during the summer season in conceived, non-conceived, and animals that suffered embryonic mortality

Status of animals	No.	Days post-AI					Overall
		Day-20	Day-24	Day-27	Day-30	Day-40	
Conceived	04	6.63±1.38^{b}	7.04±1.51^{b}	7.51±1.91^{b}	7.84±1.84^{b}	8.31±1.73^{b}	7.25±0.27^{b}
Non-conceived	16	2.2±0.29^{a}	1.63±0.25^{a}	2.01±0.18^{a}	2.72±0.18^{a}	1.38±0.06^{a}	1.99±0.23^{a}
Embryonic mortality	04	5.33±0.83^{b}	6.08±0.36^{b}	6.79±0.57^{b}	6.94±0.56^{b}	4.93±0.52^{b}	6.01±0.39^{b}

Figures bearing asterisk within a column (a, b) differ significantly at p<0.05

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