Cloud Publications
International Journal of Advanced Veterinary Science and Technology
2016, Volume 5, Issue 2, pp. 285-292, Article ID Sci-487
ISSN 2320-3595

Research Article | Open Access

Antisperm Antibodies in Blood Serum and Cervical Mucus of Cross-Bred Cows With Respect to Age, Parity and Number of Inseminations

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Publication Date: 15 October 2016

Article Link: http://scientific.cloud-journals.com/index.php/IJAVST/article/view/Sci-487

Abstract Role of antisperm antibodies in infertility of animals is still controversial. A study was planned with an objective to detect antisperm antibodies (ASA) by Immunoperoxidase assay (IPA), Sperm Mar test and ELISA in blood serum and cervical mucus of 53 cross-bred cows, which were interrelated with age, parity and number of inseminations. Cows were grouped according to age (years), parity- and insemination- number as G-1(< 3, 0 and 0), G-II (3.0 - 4.5, 1.0 and 1- 3), G-III (4.6 - 6.0, 2.0 and 4.0 – 6.0) and G-IV (> 6.0, ≥ 3.0 and > 6.0). In Sperm Mar test, 40% reaction between motile spermatozoa and coated latex particles of IgG and IgA is considered as lower limit of significant activity. Therefore, percentage of cows with higher level of ASA (> 40% IPA / IgG / IgA, 3200 - 6400 / 200 - 800 titre) was calculated in each group. Proportion of cows with higher serum-IgG and ELISA-titre in cervical mucus and that with higher ELISA titre in serum / cervical mucus increased with increased age and parity, respectively. According to number of inseminations, percentage of cows with higher level of ASA (IPA), IgG and ELISA titre in blood serum was maximum in G-IV, whereas that with IgA in G-III in comparison to other groups. Percentage of cows with higher level of ASA (IPA), IgG, IgA and ELISA in cervical mucus was higher in G-III as compared to other groups. This study exposed a significant increase in ASA in serum /cervical mucus of cross-bred cows only with increase in number of inseminations.
Keywords *ASA; Cross Bred Cattle; Age; Parity; Artificial Insemination*

1. Introduction

Role of anti-sperm antibodies (ASA) in infertility of animals is controversial. Studies done on prevalence of ASA by some authors revealed that ASA may inhibit fertility [1]. But other authors are of the opinion that ASA are irrelevant to infertility [2]. In females, the epithelium of the cervix is capable of producing a local immune response. Antibodies in cervical mucus or oviductal fluid could obstruct sperm transport or interfere with the capacitation / acrosome reaction [3, 4]. Immune responses vary from one animal to another and certain animals may have a genetic tendency for a more response. It has been suggested that if spermatozoa are present during an infection of the reproductive tract, the

infectious agents have an adjuvant effect and somehow potentiate the immune response against spermatozoa [3]. Development of cross-reacting antibodies (antibodies that are formed against one antigen but can also react with a second antigen) is another possibility, which may impair fertility in animals [5]. Occurrence of ASA in blood serum or CM of cows has been related to age [6], parity [6, 7], repeat breeding / number of inseminations and infertility [1, 7-11]. Therefore, ASA detected by Immunoperoxidase assay, Sperm Mar test and ELISA in blood serum (BS) and cervical mucus (CM) of cross-bred cows were interrelated with age, parity and number of inseminations.

2. Materials and Methods

2.1. Collection of Samples

Blood and cervical mucus were collected from 53 cross-bred cows from dairy farm, Guru Angad Dev Veterinary and Animal Sciences University and private dairy farms around Ludhiana city. Blood was collected in sterilized vials from jugular vein without anticoagulant, centrifuged at 3000 rpm for 5 min to separate serum. At the time of estrus, CM was collected with the help of AI pipette, sonicated at 20 watts, 3 X 20 secs. BS and CM were inactivated at 56^{o} C for 30 min and stored in aliquots at -20^{o} till further use. Data regarding age, parity and number of inseminations of 40, 40 and 53 cows could be obtained from the dairy farms at the time of sample collection.

2.2. Procurement of Semen

Frozen semen straws were procured from GADVASU, dairy farm.

2.3. Detection of Antisperm-Antibodies in Blood Serum and Cervical Mucus

2.3.1. *Immunoperoxidase assay* [IPA, 12]

Sperm smears on clean slides were incubated with 1% bovine sperm albumin for 2 hours at 4°C. Slides were washed thrice with PBS, pH 7.4, incubated with 1:200 diluted BS / 1:50 diluted CM for 1 hour at 37°C, again washed thrice with PBS. Smears were incubated with rabbit anti-bovine IgG (Sigma) for 45 minutes at 37°C and washed thrice with PBS. Colour was developed with 3, 3'-Diaminobenzindine tetrahydrochloride in Tris buffer (0.05M, pH 7.6 at 25°C) and 27µl of 3% hydrogen peroxide for 5 minutes at room temperature. Washed with distilled water, slides were mounted in 10% glycerol in PBS, covered with coverslip and examined under the microscope at 10 x 100 X for dark brownish colouration of the sperm. About 200 sperms with browning on acrosome, post acrosomal cap or whole head were counted in different fields and percentage of IPA positive sperms was calculated.

2.3.2. *Indirect Sperm Mar Test* [Sperm Mar kit, 13]

Diluted inactivated BS/CM 1/4 with TALP medium, pH, 7.4 and incubated at 37°C for 30 min. Collected the motile sperms by centrifugation through Histopaque, suspended the sperm pellet in TALP and adjusted the sperm conc. to 20 X 10^{6}. Incubated 100 µl of the sperm suspension of motile spermatozoa with 100 µl of inactivated ¼ diluted BS or CM, incubated for 1 hour at 37°C. Added 2 ml of TALP, mixed well and centrifuged for 10 minutes at 400 g. Re-suspended the pellet with 50 µl of TALP. On a slide, mixed 10 µl of sperm suspension and 5 µl of sperm Mart latex particles IgG / IgA, mixed, covered with cover slip, kept in humid chamber for 5 min and observed under microscope at 400 X. Attachment of latex particles to the head/ tail or whole sperm was observed. About 200 sperms in different fields were counted and percentage was calculated.

2.3.3. *Preparation of Sperm Antigen for ELISA*

Sperm antigen was prepared by suspending washed spermatozoa in 62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 1mM PMSF, 25 mM benzidine, 10mM aprotinin, 10mM pepstatin and 5mM soya bean trypsin inhibitor, sonicated (3 bursts of 20 sec each) and centrifuged at 15,000 rpm for 30 minutes.

2.3.4. *ELISA* [14]

ELISA plates were coated with 5 µg protein (sperm antigen) per well by incubating at 37ºC for three hrs. After washing thrice with PBS, antigen coating was blocked by incubating with 300 µl of 2% BSA per well for overnight at 4ºC. Again washed thrice with PBS pH 7.4 and added serial dilutions of BS/ CM into the wells and incubated at 37ºC for three hours. Washed again with PBS and incubated with 100 µl/well of HRP conjugated anti bovine IgG for three hours at 37ºC. Washed the plate twice with PBS and incubated with 100 µl of o-phenyldiamine + 0.06% H_2O_2 as a substrate for 20 min at room temperature. Stopped the reaction with 5 N H_2SO_4 and measured the absorbance at 492 nm using ELISA reader.

2.4. Statistical Analysis of Data

The data obtained was analyzed statistically according to Independent Sample T-Test and One-Way ANOVA using difference between means of two groups and means of different group application at 5 percent level of significance (SPSS, Version 16.0).

3. Results and Discussion

ASA were detected in all the tested cows irrespective of age, parity and number of inseminations. Cows were grouped according to age (years), parity and insemination (number) as G-1(< 3, 0, 0), G-II (3.0 - 4.5, 1, 1-3), G-III (4.6 - 6.0, 2, 4-6) and G-IV (>6, ≥3, >6). In Sperm Mar test [13], 40% reaction between motile spermatozoa and coated latex particles of IgG and IgA is considered as lower limit of significant activity. Therefore, percentage of cows with higher level of ASA (> 40% IPA / IgG / IgA, 3200 - 6400 / 200 - 800 titre) was calculated in each group. Table 1, 2 and 3 depicts the values of ASA in relation to age, parity and number of inseminations, respectively.

3.1. ASA in blood serum and cervical mucus of cows according to age (n=40)

ASA detected by IPA / Sperm Mar-IgG and -IgA were non-significantly ($p<0.05$) / significantly ($p>0.05$) and non-significantly ($p<0.05$) higher in BS of G-II and G-III, respectively (Table 1). ELISA titre in BS showed a significant ($p>0.05$) increase with increase in age. There was a non-significant ($p < 0.05$) higher percentage of IPA, IgG and IgA in CM of G-I, G-IV and G-I, respectively. ELISA- titre was also non-significantly (<0.05) higher in CM of G-IV as compared to G-I and G-III. The presence of circulating sperm antibody was significantly associated with age in cows [$P<0.001$, 6].

Percentage of cows with higher level of IPA / IgG and IgA / ELISA titre in BS was more in G-III and G-II as compared to other groups (Figure 1a). But percentage of cows with higher level of IPA / IgG and IgA / ELISA titre in CM was more in G-I, G-II, G-III and G-IV, respectively (Figure 1b). It indicated an increase in proportion of cows with higher serum-IgG and cervical mucus-ELISA-titre with age. Fayemi [12] were also of the opinion that the proportion of Zebu cattle with sperm antibodies increased significantly with age ($P < 0.001$).

3.2. ASA in blood serum and cervical mucus of cows according to parity (n=40)

ASA, detected by IPA / ELISA titre in BS increased non-insignificantly ($p < 0.05$) / significantly ($p < 0.05$) from G-I to G-III and IgG/IgA were non-significantly higher ($p < 0.05$) in G-II (Table 2). Zraly et al., [7] detected higher concentrations of ASA in 537 pluriparus cows by ELISA. Waziri and Fayemi [6] postulated that the presence of circulating sperm antibody was significantly associated with parity ($P < 0.001$). There was no difference in IPA-ASA and IgG type antibodies in cervical mucus of cows with respect to parity number. A decrease in IgA in CM from G-I to G-III and again an increase in G-IV was observed. ELISA titre in CM was also non-significantly maximum ($p < 0.05$) in cows of G-III.

Table 1: *Presence of ASA (evaluated by IPA, Sperm Mar test and ELISA, Mean ± SE) in blood serum and cervical mucus of cross-bred cattle according to age*

G. N0./ Age (Years)	IPA (%)		SpermMar Test				ELISA titre (%)	
			IgG (%)		IgA (%)			
	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus
I/ <3 (n=7)	39.3±6.1[a] (19.7-66.2)	47.9±2.6[a] (43.4-52.6)	36.5±2.4[a] (30.1-46.8)	38.5±3.1[a] (32.5-42.5)	32.9±2.2[a] (26.3-42.6)	50.5±5.3[a] (40.8-59.1)	1228±529[a] (100-3200)	226±108[a] (50-800)
II/ 3-4.5 (n=16)	53.1±3.4[a] (29.3-65.5)	40.6±4.4[a] (29.6-65.5)	43.0±2.6[b] (29.6-65)	44.6±2.5[a] (31.1-57.8)	36.3±3.7[a] (18.7-63.7)	41.7±3.6[a] (18.6-65.7)	2675±1181[b] (400-6400)	290±183[a] (25-800)
III/ 4.6-6.0 (n=7)	45.7±5.4[a] (28.9-64.7)	34.8±6.3[a] (14.3-58.1)	26.7±6.6[c] (17.6-57.7)	40.5±4.5[a] (27.4-56.8)	38.5±5.3[a] (19.8-52.1)	46.1±6.8[a] (20.6-64.1)	2971±657[c] (1600-6400)	253±105[a] (25-800)
IV/>6.0 (n=9)	43.7±5.3[a] (13.5-63.7)	33.3±6.3[a] (16.9-64.2)	35.5±5.8[c] (23.3-63.5)	45.1±7.9[a] (24.1-88.1)	31.6±6.3[a] (13.2-65.8)	39.1±8.4[a] (17-78.7)	3555±1273[c] (1600-12800)	300±78[a] (100-800)

- Figures in parentheses represent range of values
- Values with different superscripts are significant ($p<0.05$)

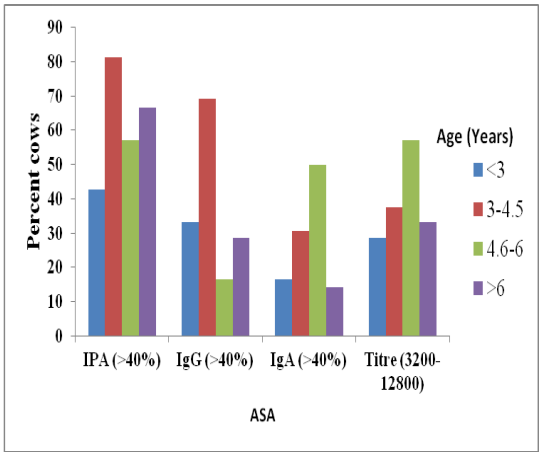

Figure 1a: *Occurrence of significant level of ASA in blood serum of cows according to age*

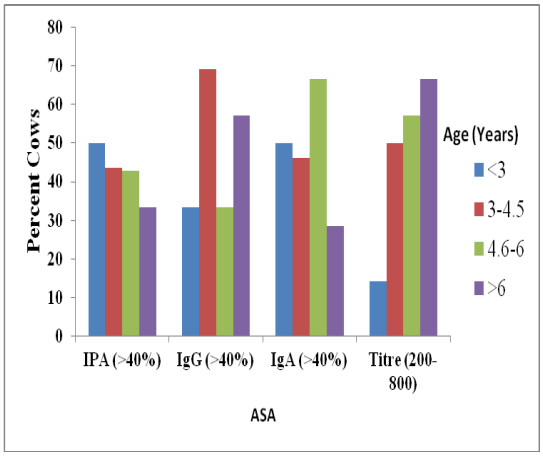

Figure 1b: *Occurrence of significant level of ASA in cervical mucus of cows according to age*

Percentage of cows with higher level of ASA detected by IPA and ELISA-titre in BS was higher in G-III and that of IgG and IgA in G-II and G-III as compared to other groups (Figure 2a). Percentage of cows with higher level of IPA / IgA and IgG / ELISA titre in CM was maximum in G-I and G-III, respectively (Figure 2b). It revealed that proportion of cows with higher ELISA titre in BS and CM increased with increased parity. The proportion of Zebu cows positive for sperm antibodies was significantly associated with increased parity [$P < 0.00$, 7]. Parturition can induce injury to the reproductive tract especially in cases of dystocia and injury to the reproductive tract may play a role in

induction of immunity against sperm [15]. This can be the reason for higher percentage of cows with significant level of ASA in G-I and G-III, as compared to G-II and G-IV.

Table 2: *Presence of ASA (evaluated by IPA, SpermMar test and ELISA, Mean ± SE) in blood serum and cervical mucus of cross-bred cattle according to parity*

Parity No (G. No.)	IPA (%)		SpermMar Test				ELISA titre (%)	
			IgG (%)		IgA (%)			
	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus
0 (G-I) N=7	39±5.6[a] (19.7-62.8)	40.1±10[a] (31.6-59.8)	38±2.8[a] (28.2-49.7)	41.5±9.6[a] (17.8-63.2)	35.4±4.2[a] (27.2-58.2)	49.9±9.2[a] (25-69)	1834±881[a] (100-6400)	200±70[a] (20-200)
1 (G-II) N=9	49.5±5.8[a] (29.3-79.5)	37.7±6.8[a] (17.6-63.5)	44.4±5.9[a] (31.6-65)	41.2±5.0[a] (24.1-57.8)	39.1±5.4[a] (18.7-58.5)	43.9±3.6[a] (30.9-57.5)	2750±798[b] (400-6400)	195±91[a] (40-800)
2 (G-III) N=16	52.1±3.2[a] (28.9-67.4)	37.9±5[a] (11.4-63.5)	37.8±3.9[a] (15.7-57.7)	43.9±5.0[a] (16.4-88.1)	30.7±2.1[a] (19.8-49.2)	37.5±3.6[a] (18.6-57.6)	3437±820[b] (400-12800)	335±65.9[a] (20-800)
≥3 (G-IV) N=9	44.4±5.5[a] (13.5-67)	34.3±6.3[a] (16.9-72.8)	31.1±3.8[a] (17.6-48.6)	41.8±7.1[a] (25.6-88.1)	37.3±6.1[a] (13.2-65.8)	42.7±7.6[a] (17-78.7)	2933±1250[b] (800-12800)	228±79[a] (50-800)

- Figures in parentheses represent range of values
- Values with different superscripts are significant (p<0.05)

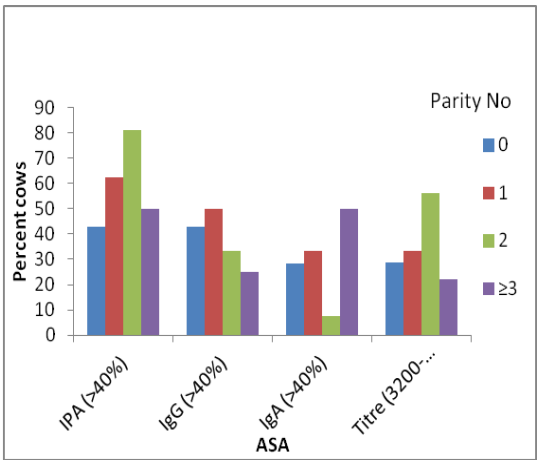

Figure 2a: *Occurrence of significant level of ASA in blood serum of cows according to parity number*

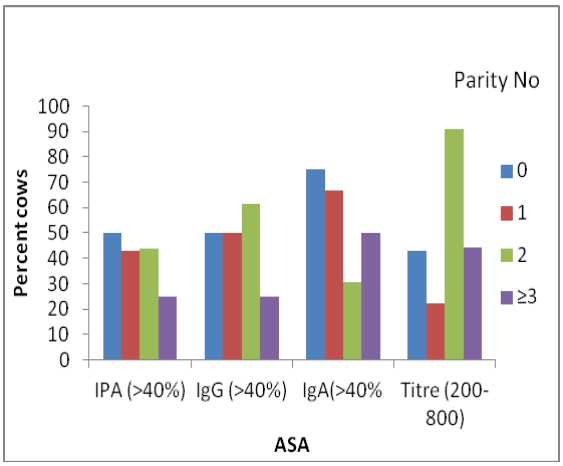

Figure 2b: *Occurrence of significant level of ASA in cervical mucus of cows according to parity number*

3.3. ASA in blood serum & cervical mucus of cows according to num. of inseminations (n=53)

ASA, detected by IPA showed a significant (p<0.05) increase in blood serum of cows from GI to GIII (Table 3). There was not any significant (p<0.05) difference in IgG in serum among the groups. However, IgA class antibodies and ASA titre in blood serum of cows showed an increase with increase in number of inseminations i.e. G-I to G-III. Results of Lazarevic et al. [16] indicated that titers of antisperm antibodies of the IgA class elevated with the number of artificial inseminations. Sarna et al. [11] observed the presence of ASA in serum of 100% cows repeating 3-5 times with a titre of 1:3120. It has been proposed that ASA positive rate varies according to the mating number and reproductive status. In the sera and cervical mucus of cows, high levels of ASA were found in animals with longer open day's period [17].

Table 3: *Presence of ASA (evaluated by IPA, SpermMar test and ELISA, Mean ± SE) in blood serum and cervical mucus of cross-bred cattle according to number of inseminations*

Number of AI (G. No.)	IPA (%)		SpermMar Test				ELISA titre (%)	
			IgG (%)		IgA (%)			
	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus	Serum	Cervical mucus
0 (G-I) N=3	27±3.7[a] (19.7-31.1)		35.4±3.1[a] (30.1-40.8)		31.5±0.4[a] (30.8-32.2)		333±233a (100-800)	
1-3 (G-II) N=25	45.4±2.9[b] (13.5-70.3)	32.6±3.1[a] (11.4-70.4)	34.8±2.6[a] (17.6-57.5)	36.6±2.7[a] (13-57.8)	38.3±3.5[b] (13.2-63.7)	43.1±3.7[a] (17-68.4)	1616±184[b] (400-3200)	205±45[a] (25-800)
4-6 (G-III) N=18	51.6±3.3[b] (28.9-79.5)	43.8±16.7[a] (28-72.8)	37.8±4.3[a] (15.7-65.6)	48.4±4.9[a] (27.1-88.1)	44.6±3.2[c] (19.4-55.8)	47±3.5[a] (29.1-69.4)	4222±876[c] (800-12800)	279±68[a] (25-800)
>6 (G-IV) N=7	49.2±6.5[b] (23.9-68.1)	34.8±6.1[b] (17.9-65.1)	38±6.9[a] (13.9-57.7)	37.6±5.8[a] (17.9-57.4)	40.1±7.2[c] (17.8-73.8)	42±3.3[a] (30.9-49.4)	4114±1501[c] (1600-12800)	133±26[a] (100-200)

- Figures in parentheses represent range of values
- Values with different superscripts are significant ($p<0.05$)

Percentage of cows with significant level of IPA, IgG and ASA titre in BS was maximum in G-IV, whereas that with IgA in G-III in comparison to other groups (Figure 3a). Percentage of cows with significant level of IPA, IgG, IgA and ELISA-titre were higher in G-III as compared to G-I, G-II and G-IV (Figure 3b). Tripathi et al. [9] revealed variable occurrence of ASA in repeat breeders. In our study percentage of cows that repeated 4-6 times were higher with significant level of ASA. Wang and Xie [8] studied ASA by ELISA in Chinese black and white dairy cows and found that prevalence of ASA was greatest (17.65%) amongst the cows that had three or more inseminations.

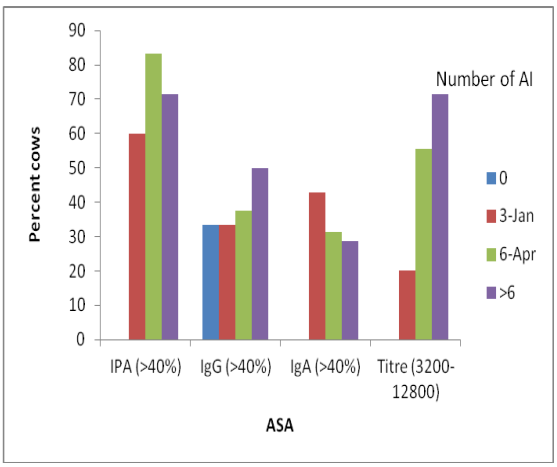

Figure 3a: *Occurrence of significant level of ASA in blood serum of cows according to number of inseminations*

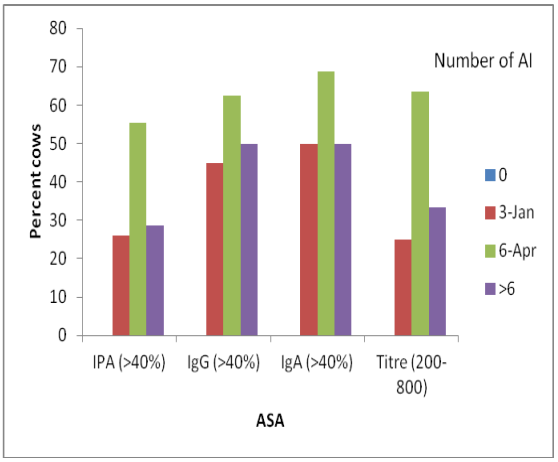

Figure 3b: *Occurrence of significant level of ASA in cervical mucus of cows according to number of inseminations*

Milovanović et al. [18] stated that the critical titre value of the antisperm IgA from CM in cows is 1:64, as of AI in the last oestrus and number of AI per pregnancy were significantly higher in cows that had IgA - ASA titer above this value. During the present study, ELISA titre in CM first showed an increase upto 3 - 4.5 years of age, second parity and 4-6 inseminations and then a decrease after 4.5 years of age, second parity and > 6 inseminations. During the period of estrus CM contains different amounts of water according to the hormonal status and therefore samples can be diluted several times. Consequently the titers of ASA will be lower but since this phenomenon is an individual characteristic,

the degree of dilution will not be the same in all animals. This is probably one of the causes for the high individual variability and along with the individual immune response, this result in large standard deviations and the lack of statistical significance for the obtained differences.

4. Conclusions

It can be concluded from our study that ASA were present in blood serum and cervical mucus of cross-bred cows irrespective of age, parity and number of inseminations. A significant increase in ASA in serum/cervical mucus was observed with increase in number of inseminations, but no significant difference was observed according to age and parity.

Acknowledgements

Authors are thankful to UGC, New Delhi for providing grant under major research projects. Authors are also thankful to semen freezing lab, GADVASU, Ludhiana, India for providing semen samples.

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