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ANTISPERM ANTIBODIES OF THE Ig A CLASS IN THE CERVICAL MUCUS AND SERA OF ARTIFICIALLY INSEMINATED COWS

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The aim of this study was to investigate the level of anti-sperm antibodies of the Ig A class in the cervical mucus and sera of artificially inseminated cows. Cervical mucus and sera samples were collected on the day of artificial insemination and the animals were divided into four groups of 20 cows each according to the number of previous inseminations. The titer of antibodies was determined by the indirect immunofluorescence method using sperm cells suspended in Tris egg-yolk extender or the commercial extender «Biociphos +». Our results indicate that titers of antisperm antibodies of the Ig A class elevate with the number of artificial inseminations. Mean titer values were higher in the cervical mucus samples than in sera, indicating that the local immune response is more relevant for the immunological reactivity to sperm and extender antigens. In addition, the titer of antisperm antibodies was generally higher when sperm cells suspended in Tris egg-yolk extender were used for the test.

Key words: cows, subfertility, Ig A, cervical mucus, sera, sperm cells

INTRODUCTION

An understanding of the functions of the mucosal immune system in the female reproductive tract is essential for dealing with problems of infertility. One of the major problems, in both human and animal reproduction, is the sterility with no obvious symptoms (unexplained infertility) that can be caused by antisperm antibodies (ASA) in seminal plasma, cervical mucus (CM), uterine fluid, follicular fluid or sera. The role of ASA in infertility and sub fertility in mammalian species is still not clearly understood but it is well known that auto-immunization of the male and iso-immunization of the female with sperm cells can lead to significant impairment of fertility. Interference of ASA with reproductive processes may occur by impairment of sperm migration through the cervix, uterus and tubes and by blocking adherence of spermatozoa to the surface of the zona pellucida of the oocyte as postulated by Schumacher (1998). The same author stated that serum antibody levels do not reflect properly the immunological situation in secretions of the genital tract especially in females, and that the secretory immunological system may be operational mainly in the cervical compartment of the genital tract. Kim *et al*. (1999)

demonstrated recently that experimentally generated bull antisperm antibodies significantly reduce fertilization *in vitro*. Much earlier, Wright (1980) described the negative influence of antisperm antibodies raised by intensive immunization of adult animals on semen quality in the bull. This phenomenon has been recorded for numerous laboratory animals as reviewed by Hogarth (1982). Bratanov *et al*. (1980) showed that sera containing antisperm antibodies from infertile cows and women with unexplained infertility inhibit acrosomal proteolytic activity in vitro and therefore possibly may affect fertilization. Maas *et al*. (1998) demonstrated a close correlation between a positive sperm antibody test and a poor postcoital test in infertile couples. These authors concluded that determination of ASA in the CM must be regarded as an improvement in the diagnostic procedure in human infertility. We have also recently reviewed the possible negative influence of antisperm antibodies on reproduction processes (Jaćević and Lazarević, 2000).

There is strong evidence that in humans sperm-mucus interactions can be affected by local ASA, especially of the Ig A class, both under *in vitro* and *in vivo* conditions (Eggert-Kruse *et al*., 1991). However, the significance of ASA in sera of infertile patients was not established, while those in seminal plasma or CM impaired the ability of sperm cells to penetrate CM (Eggert-Kruse *et al*., 1995). Check *et al*. (1994) demonstrated that the antifertility effect of ASA may be mainly due to immobilization of sperm in the CM and thus intrauterine insemination may effectively correct the problem. The role of antisperm Ig A antibodies in man has also documented by Clarke *et al*. (1984) and Kremer and Jager (1988).

However, investigations in veterinary medicine regarding the influence of antispermatozoal antibodies in CM on fertility rates are very few. An elevation of sperm-agglutinating antibody titer in the CM and sera of artificially inseminated cows was clearly demonstrated by Jaćević (1998).

In this study we investigated the presence of ASA of the Ig A class in the sera and CM of artificially inseminated Holstein cows. In the technology of artificial insemination (AI), the antigenic structure of sperm cells is changed due to the addition of different extenders, freezing and thawing procedures and reduction of seminal plasma volume. In addition, the immunosuppressive activity of bull seminal plasma is significantly reduced during semen preparation for AI (Lazarević, 1991). Therefore we have investigated the presence of ASA in CM and sera using sperm cells previously suspended in two different extenders that underwent the complete procedure of deep freezing and subsequent thawing.

MATERIAL AND METHODS

Sera sampling: Sera were collected from 80 Holstein cows (four groups of 20) at the regional dairy farm by jugular vein puncture on the day of artificial insemination. Sera were obtained following coagulation at room temperature and centrifugation at 3000 rpm for 20 min. All samples were kept frozen at - 20 ^{o}C until use. The animals were divided into four groups according to the total number of previous artificial inseminations as follows: cows and heifers inseminated 1-2 times, cows inseminated 3-5, 6-8 and over 8 times. All cows were inseminated with bull semen prepared for AI with Tris egg-yolk extender.

Cervical mucus sampling: Prior to AI, CM samples were collected by placing a sterile sponge swab in the near vicinity of the external cervix portion. Sponges were placed with a sterile plastic tube (1.5 cm width, 45 cm long) and the tube was removed while the swab remained inside for the next 5 minutes. The swab was then removed and placed in an opened sterile syringe (20 ccm). The syringe was closed and the cervical mucus extracted by gentle pushing into a sterile plastic polystyrene tube. We were able to collect approx. 3 ccm of CM. All samples were kept frozen at - 20 ^{o}C until use.

Semen sampling: Semen samples were collected from four black and white spotted bulls (Holstein breed) by means of an artificial vagina in the Regional Center for Artificial Insemination. The semen possessed normal characteristics of motility, morphology and concentration. Ejaculates were pooled and then divided into two equal portions (split technique). The ejaculates underwent the standard procedure of preparation for AI. One half of each ejaculate was diluted with Tris egg yolk extender as described elsewhere (Lazarević *et al*., 1992) and one with Biociphos plus extender (IMV, France). Ejaculates were diluted at an average ratio of 1:10 and kept frozen at - 196 ^{o}C before use.

Indirect immunofluorescence assay (IIF): The IIF assay was performed according to Noel *et al.* (1974). After thawing the straws for AI (medium French straws 0.45 ml), sperm cells were separated and washed twice in PBS (pH 7.2) with centrifugation at 2000 rpm for 10 minutes. We always used straws originating from the same four bulls (3 from each bull). When the last supernatant was discarded the remaining cells were resuspended by Vortex and used for smear preparations. On the microscopic slides with dried sperm cell smears, 10 μl of sera or CM sample (inactivated at 56 ^{o}C for 20 minutes) was placed and incubated for 20 min at 37 ^{o}C in a wet chamber. Sera and CM dilutions from 1:4 to 1:2048 (highest positive titer) were used for the test. Following incubation, the slides were washed three times (5 min) in PBS and dried at room temperature. In the second step, 10 μl of secondary FITC (florescein isothiocyanate) conjugated antibody (anti -bovine Ig A, ICN, USA, Cat No 641 751) was placed on the slide and incubated again under the same conditions. Conjugation with FITC (ICN, USA, Cat No F 4274) was performed according to Hudson and Hay (1976). After incubation, followed by the same washing procedure the slides were kept in dark and wet chamber till examined. As a positive control we used sera obtained by immunization of calves with content of straws prepared with Tris egg yolk extender and Biociphos plus extender as described in detail elsewhere (Lazarevic *et al*., 2000). Two 5 month-old calves were immunized for the first time with straw content mixed with complete Freunds adjuvant and for the second time (after two weeks) with straw content mixed with incomplete adjuvant. Calf sera before immunization served as the negative control. Microscopic examination was performed on the NIKON EFD - 3 microscope with the B-2A filter at 1600 X magnification. The appearance of fluorescence on the head, tail or neck of the sperm cell was considered as a positive result and the last dilution giving a positive reaction was taken into account. Titer values were expressed according to Sjurin *et al*. (1984) as $\log_2$ n (1:2 = 1, 1:4 = 2 etc).

Statistical analyses were performed after calculating mean values and standard deviations. The significance of the differences between mean values was estimated by Student's t test.

RESULTS

The results obtained by analyzing CM samples for the presence of antisperm Ig A antibodies are presented in Table 1. When the IIF test was performed using sperm cells previously suspended in the "Biociphos +" extender the titer of antibodies directed against sperm cells increased with the number of inseminations. The same phenomenon was noted when sperm cells suspended in Tris-egg yolk extender were used for the test. Moreover, titer values were always higher in the latter case. Due to the high standard deviations these differences were not statistically significant except in the animals inseminated one or two times (Table 1c)

Table 1. Mean titer of Ig A anti-sperm antibodies (log_2) in the cervical mucus of inseminated cows ($X \pm SD$, $n = 20$)

Number of AI	Sperm cells suspended in "Biociphos +" extender	Sperm cells suspended in Tris-egg yolk extender
1-2	3.00 ± 1.29	3.85 ± 0.87
3-5	5.20 ± 1.60	5.75 ± 2.63
6-8	5.45 ± 2.11	6.65 ± 2.77
over 8	5.45 ± 2.39	6.45 ± 2.39

When we compared titer values between cows inseminated one or two times with those inseminated more than twice (3-5, 6-8, over 8), we were able to demonstrate significant differences between groups. These differences were evident both with sperm cells suspended in "Biociphos +" extender and Tris-egg yolk extender. No differences were found when the groups of animals inseminated 3-5, 6-8 and over 8 were compared (Tables 1a and 1b)

Table 1a. Statistical significance of differences between titers of antisperm Ig A antibodies in the CM of cows when the IIF test was performed with cells previously suspended in "Biociphos +" extender

No of AI	3-5 B	6-8 B	over 8 B
1-2 B	$p < 0.001$	$p < 0.001$	$p < 0.001$
3-5 B	–	NS	NS
6-8 B	–	–	NS

Table 1b. Statistical significance of differences between titers of antisperm Ig A antibodies in the CM of cows when the IIF test was performed with cells previously suspended in Tris egg-yolk extender

No of AI	3-5 T	6-8 T	over 8 T
1-2 T	$p < 0.01$	$p < 0.001$	$p < 0.01$
3-5 T	-	NS	NS
6-8 T	-	-	NS

Table 1c. Statistical significance of differences between titers of antisperm Ig A antibodies in the CM of cows when the IIF test was performed with cells previously suspended in Tris egg-yolk extender or "Biociphos +" extender

Number of AI	Level of significance
1-2 T vs. 1-2 B	$p < 0.05$
3-5 T vs. 3-5 B	NS
6-8 T vs. 6-8 B	NS
over 8 T vs. over 8 B	NS

Similar results were obtained when sera of the cows were analyzed for the presence of anti-sperm Ig A antibodies. Titers of antibodies increased with the number of inseminations and this phenomenon was especially evident when sperm cells suspended in Biociphos + extender were used for the test (Tables 1a and 1b). The statistical significance of differences in titar values when different extenders were used for the test was again evident only when sera samples originating from animals inseminated 1-2 times were compared (Table 1c).

Table 2. Mean titer of Ig A anti-sperm antibodies (log_2) in the sera of inseminated cows ($X \pm SD$, $n = 20$)

Number of AI	Sperm cells suspended in "Biociphos +" extender	Sperm cells suspended in Tris-egg yolk extender
1-2	1.90 ± 1.19	2.50 ± 1.10
3-5	2.85 ± 1.46	3.00 ± 1.45
6-8	3.50 ± 1.57	3.15 ± 1.66
over 8	3.65 ± 1.42	3.90 ± 1.52

Table 2a. Statistical significance of differences between titers of antisperm Ig A antibodies in the sera of cows when the IIF test was performed with cells previously suspended in "Biociphos +" extender

No of AI	3-5 B	6-8 B	over 8 B
1-2 B	$p < 0.05$	$p < 0.01$	$p < 0.01$
3-5 B	–	NS	NS
6-8 B	–	–	NS

Table 2b: Statistical significance of differences between titers of antisperm Ig A antibodies in the sera of cows when the IIF test was performed with cells previously suspended in Tris egg-yolk extender

No of AI	3-5 T	6-8 T	over 8 T
1-2 T	NS	NS	$p < 0.01$
3-5 T	–	NS	NS
6-8 T	–	–	NS

Table 2c. Statistical significance of differences between titers of antisperm Ig A antibodies in the sera of cows when the IIF test was performed with cells previously suspended in Tris egg-yolk extender or "Biociphos +" extender

Number of AI	Level of significance
1-2 T vs. 1-2 B	$p < 0.05$
3-5 T vs. 3-5 B	NS
6-8 T vs. 6-8 B	NS
over 8 T vs. over 8 B	NS

DISCUSSION

One of the major difficulties in this type of investigation lies in fact that 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 anti-sperm antibodies 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. Along with the individual immune response this results in large standard deviations and the lack of statistical significance for the obtained differences. Despite that, we demonstrated a constant rise in titer values in animals inseminated three or more times.

The low titer values obtained in cows inseminated once or twice are in agreement of our previous results regarding the presence of naturally occurring

ASA in the sera of calves. In a recent study we low titar of ASA but of the Ig G and Ig M class in the sera of prepubertal calves by IIF method (Lazarević *et al*., 2002). We were also able to demonstrate differences in the immune response when different extenders were used for the semen dilution and this is also in agreement with our previous results (Lazarević *et al*., 2000).

Stern *et al.* (1993) also stated that the ASA level in serum did not correlate with that in CM. Moreover, according to their results, the level of ASA in women's sera did not change during the follicular phase of the cycle. This is obviously not the case with ASA in CM. Kohl *et al*. (1992) did not confirm the clinical significance of ASA in the sera of infertile women. In one survey (Shulman, 1977) it was found that in a large group of infertile women who were seronegative, 28% were positive in their CM. In our study we were also able to demonstrate that some sera samples had no detectable antisperm antibodies (data not presented here). Moghissi *et al*. (1980) found antibody activity in the mucus of 26% of a group of infertile women and none in the control group. They detected such activity in serum in only 13% of the infertile women. On the contrary, Vukotić *et al*. (1982), showed that reactivity of female blood serum towards sperm cells increases with repeated AIs and in parallel to that, the probability of conception declines. This is also partly in agreement with our results. Wang *et al*. (1985) confirmed that attachment of Ig A antibodies to the sperm surface lowers sperm penetration ability, while Ig G antibodies did not have the same effect. These findings were confirmed by Menge and Natz (1993) who demonstrated the presence of Ig A1 and Ig A2 subclasses in the CM of women. Since Ig A1 antibodies do not activate the complement system, the authors postulated that these antibodies have a protective role.

As stated by Schumacher (1988), demonstrations of sperm antibodies in serum or even in genital secretions are not necessarily indicative of permanent sterility but the chances of conception may be reduced. Immunity to spermatozoa does not seem to be an all-or-nothing phenomenon and should be considered as a relative rather than an absolute cause of infertility.

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Ig A ANTITITELA PROTIV SPERMATOZOIDA U CERVIKALNOJ SLUZI I SERUMU KRAVA KOJE SE VEŠTAČKI OSEMENJAVAJU

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Cilj ovih ispitivanja je bio da se utvrdi nivo antispermatozoalnih antitela Ig A klase u krvnom serumu i cervikalnoj sluzi krava koje se veštački osemenjavaju. Uzorci krvi i cervikalne sluzi su prikupljani na dan veštačkog osemenjavanja a životinje su bile podeljebne u četiri grupe na osnovu broja prethodnih osemenjavanja. Prisustvo antitspermatozoalnih antitela je određivano metodom indirektne imunofluorescence korišćenjem spermatozoida suspendovanih u Tris-žumanjčanom razređivaču i komercijalnom razređivaču «Biociphos +». Postignuti rezulati ukazuju da titar antispermatozoalnih Ig A antitela raste sa brojem osemenjavanja i ima veće vrednosti u cervikalnoj sluzi nego u serumu. Ovi rezultati potvrđuju hipotezu o značaju lokalnog imunskog odgovora na antigene spermatozoida i razređivača za spermu bika. Osim toga titar antitela je imao veće vrednosti kada su za izvođenje testa korišćeni spermatozoidi suspendovani u Tris-žumanjčanom razređivaču.