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THE PRESENCE OF NATURALLY OCCURRING ANTISPERM ANTIBODIES IN THE SERA OF PREPUBERTAL CALVES

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In this study we investigated the presence of antisperm antibodies in the sera of neonatal and young calves up to the age of 120 days by indirect immunofluorescence assay (IIF) and the sperm-agglutination method. By IIF assay, anti IgG and anti IgM antibodies were detected. In the sera of neonatal calves, before colostrum ingestion, no anti-sperm antibodies were detected due to physiological agammaglobulinemia. Titer obtained values in two day old and older calves were different for native sperm cells and sperm cells previously suspended in TRIS egg yolk extender or Biociphos plus extender (IMV, France) indicating antigenic differences between them. Their titer increased with age. This study confirmed the higher sensitivity of the IIF assay. We have also confirmed the hypothesis that in calves, antisperm antibodies naturally occurring before puberty, are most probably the result of cross reactivity with microbial antigens

Key words: bull, spermatozoa, antibodies, calf

INTRODUCTION

The significance of antisperm antibodies (ASA) for infertility and subfertility in mammalian species is still not clearly understood. One of the problems in both human and animal reproduction, is sterility with no obvious symptoms (unexplained infertility) that can be caused by ASA in seminal plasma, cervical mucus or sera. The antigenic structure of bull sperm cells still attracts much scientific attention and numerous authors have developed different tests in order to isolate and characterize sperm surface antigens important for fertilization (Lalancette *et al.* 2001, Ignotz *et al.* 2001, Tripathi *et al.* 1999). The significance of isolated sperm-specific antigens for fertilization differs and some of the monoclonal antibodies raised against them may completely block fertilization (Dubova-Mihailova *et al.* 1994). Kim *et al.* (1999) demonstrated recently that experimentally generated bull antisperm antibodies significantly reduce fertilization in vitro. This phenomenon was described much earlier for meny laboratory animals as reviewed by Hogarth (1982). Using monoclonal antibodies Ambrose *et al.* (1996) identified major antigenic domains of bull sperm but their role needs further evaluation. In 1980 Wright described the negative influence of antisperm antibodies raised by intensive immunization of adult animals on semen quality in the bull. However, he did not demonstrate any naturally occurring antisperm antibodies. 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. We have also recently reviewed the possible negative influence of antisperm antibodies on reproduction processes (Jaćević and Lazarević, 2000).

Among the numerous studies conducted in order to estimate the possible negative influence of ASA on reproductive processes, one surprisingly showed the presence of ASA in the sera of boys and girls that had never been previously exposed to sperm cells i.e. before puberty. This phenomenon was assigned to cross reactivity of antibodies directed against some bacterial and spermatozoal cells (Landers *et al.* 1994). As mentioned previously, it is well documented that under experimental conditions, following intensive immunization of females with washed sperm cells in Freunds adjuvant, ASA may affect successful conception. The significance of these antibodies in low titers is still unclear and further studies are needed to show if they can form a barrier to conception.

In this study we investigated the presence of ASA in the sera of neonatal calves and calves up to the four months of age. It is well known that the level of natural ASA is high in ruminant species due to their exposure to microbial flora in the rumen. On the other hand, in the technology of artificial insemination of cows, the antigenic structure of sperm cells may be changed due to the addition of different extenders, freezing and thawing procedures and reduction of seminal plasma volume. Therefore we have investigated the presence of ASA in calves using native sperm cells (directly from fresh ejaculate) and also sperm cells suspended in two different extenders that had undergone the complete procedure of deep freezing and subsequent thawing.

MATERIAL AND METHODS

Sera sampling: Sera were collected from 9 calves of both sexes (5 females and 4 males) by iugular vein puncture 1-2 h after birth (before colostrum ingestion), after 48 hours and at the age of 30 and 120 days. 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.

Semen sampling: Semen samples were collected from three 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 three equal portions (split technique). One third was immediately centrifuged to remove seminal plasma at 1800g for 10 min. Sperm cells were then washed two times in PBS (phosphate buffered saline, pH=7.2) under the same conditions and resuspended in the same buffer to reach a final concentration of 40 X 10^{6}/ml for the agglutination test. The same suspension served for the preparation of smears on microscopic slides and the IIF test. The rest of the ejaculate underwent the standard procedure of preparation for AI. One third was diluted with TRIS egg yolk extender as described elsewhere (Lazarevic *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 °C until use. After thawing the straws for AI (medium French straws 0.45 ml) sperm cells were separated and washed in PBS by the same procedure as for native ejaculates.

Indirect immunofluorescence assay: The IIF assay was performed according to Noel et al (1974). Basically, 50 μl of serum sample was placed on the microscopic slides with dried sperm cell smears, and incubated for 20 min at 37 °C in a wet chamber. Sera dilutions from 1:2 to 1:32 were used for the test. Following incubation, the slides were washed three times (5 min) in PBS and dried at room temperature. In a second step, 50 μl of secondary FITC conjugated antibody (anti -bovine IgG FITC conjugated, ICN, USA, (Cat No 672041) or anti bovine IgM FITC conjugated, Nordic Immunological Laboratories, Netherlands) was placed on the slide and incubated again under the same conditions. After incubation the same washing procedure was performed and slides were kept in the dark and wet chamber till examined. As a positive control we used rabbit antisera obtained by immunization with washed sperm cells, content of straws prepared with TRIS egg yolk extender and Biociphos plus extender as described elsewhere (Lazarevic *et al* 2000). As secondary antibody we used FITC conjugated antirabbit IgG (Cat. No. 55648) and FITC conjugated antirabbit IgM (Cat. No 55651, ICN, USA). Calf sera before colostrum ingestion served as the negative control. Microscopic examination was performed on the NIKON EFD - 3 microscope with B-2A filter and 1600 X magnification. The appearance of fluorescence on the head, tail or neck of the sperm cell was considered as a positive result. Titer values were expressed according to Sjurin *et al.* (1984) as $\log_2 n$ (1:2 = 1, 1:4 = 2 etc).

Sperm agglutination test (KBM - Kibrick, Belding and Merill): This test was performed according to the modification of Noel *et al* (1974) using all three different sources of sperm cells mentioned above. The rocedure of semen preparation was the same for both tests but for the KBM test the sperm cell suspension was adjusted to reach a final concentration of 40 x 109/ml . Details of this procedure are described elsewhere (Jaćević 1998)

RESULTS

Titers of the anti-sperm IgG antibodies are presented in Table 1. No antibodies were detected in the calves sera obtained before colostrum ingestion due to the physiological agammaglobulinemia. After 48 hours, when the concentration of colostral immunoglobulins reaches the maximal level, anti-sperm antibodies of the Ig G class were detected in the majority of sera samples. The highest titers were observed with sperm cells previously suspended in the TRIS - egg-yolk extender and only in this case in all samples tested. After 30 and 120 days these values were obviously elevated. Statistical significance of the observed differences were noticed between 30 vs. 120 day (TRIS – egg yolk extender) and 48h vs. 120 day ($p < 0.05$) as well as between days 30 and 120 ($p < 0.001$) when spermatozoa suspended in the "Biociphos plus" extender were used for the test

Similar findings were observed when anti-sperm antibodies of the IgM class were detected by IIF tests starting from 48 hours after colostrum ingestion. Interestingly, at 48 h post partum we noticed anti-sperm antibodies of this class only when sperm cells suspended in the TRIS egg yolk extender were used. In the sera

Table 1. Titers of Ig G anti - sperm antibodies in calf sera detected by IIF assay

	0 h			48 h			30 day			120 day		
Calve No	N	T	B	N	T	B	N	T	B	N	T	B
1	0	0	0	1	2	0	2	3	2	2	3	2
2	0	0	0	2	2	0	2	2	0	2	2	3
3	0	0	0	0	2	0	0	3	0	2	4	4
4	0	0	0	2	2	0	2	2	0	2	2	2
5	0	0	0	2	2	2	2	2	2	2	2	4
6	0	0	0	2	3	2	2	3	2	2	4	4
7	0	0	0	2	1	0	2	2	0	2	3	2
8	0	0	0	0	2	0	0	2	0	0	2	3
9	0	0	0	0	2	0	0	2	0	0	2	2
X ± SD*	0	0	0	1.22	2 ± 0.5	0.44	1.33	2.33 ± 0.5	0.67	1.55	2.67 ± 0.86	2.89 ± 0.93

Legend: N - native spermatozoa, T - spermatozoa suspended in the TRIS - egg yolk extender,
B - spermatozosuspended in Biociphos plus extender (IMV, France
* Standard deviation was calculated only when all samples were positive for antisperm antibodies

Table 2. Titers of Ig M anti - sperm antibodies in calf sera detected by IIF assay

	0 h			48h			30 day			120 day		
Calve No	N	T	B	N	T	B	N	T	B	N	T	B
1	0	0	0	0	0	0	0	2	2	0	2	0
2	0	0	0	0	2	0	0	3	0	2	3	2
3	0	0	0	0	2	0	0	2	2	0	2	0
4	0	0	0	0	2	0	0	2	0	0	2	0
5	0	0	0	0	3	0	0	3	2	0	3	3
6	0	0	0	0	2	0	0	2	0	0	2	0
7	0	0	0	0	3	0	0	3	0	0	3	0
8	0	0	0	0	2	0	0	2	0	0	2	0
9	0	0	0	0	0	0	0	0	0	0	2	2
X ± SD*	0	0	0	0	1.78	0	0	2.11	0.67	0.22	2.33	0.78 ± 0.5

Legend: N - native spermatozoa, T - spermatozoa suspended in the TRIS - egg yolk extender,
B - spermatozoa suspended in Biociphos + extender (IMV, France)
* Standard deviation was calculated only when all samples were positive for antisperm antibodies

of calves aged 30 days no positive reactions occurred with native spermatozoa, while at the age of 120 days positive titers were found in only a few serum samples when native sperm cells and sperm cells suspended in Biociphos plus extender were used. Differences between the observed values were documented only when samples from calves aged 48 h vs. 120 day ($p < 0.05$) and 30 vs. 120 day ($p < 0,01$) were compared.

Spermagglutinating antibodies were not detected in the sera obtained before colostrum ingestion and only when "Biociphos plus" suspended sperm cells were used for the test after 48 hours and 30 days. At the age of 120 days sperm agglutinins were present in the majority of samples with the highest values for "Biociphos plus" suspended sperm cells. Statistical significance occurred only when 48h vs. 120 day ($p < 0.001$) and 30 vs. 120 day ($p < 0.05$) samples were compared.

Table 3. Titers of sperm agglutinating antibodies in calf sera detected by the KBM method

	0 h			48 h			30 day			120 day		
Calve No	N	T	B	N	T	B	N	T	B	N	T	B
1	0	0	0	0	0	0	0	0	0	0	0	4
2	0	0	0	0	0	0	0	0	0	2	2	4
3	0	0	0	0	0	0	0	0	0	2	0	4
4	0	0	0	0	0	2	0	0	3	2	2	2
5	0	0	0	0	0	3	0	0	4	2	0	4
6	0	0	0	0	0	3	0	0	4	2	0	3
7	0	0	0	0	0	0	0	0	3	2	0	3
8	0	0	0	0	0	0	0	0	0	0	0	2
9	0	0	0	0	0	0	0	0	0	0	0	2
X ± SD*	0	0	0	0	0	0.89	0	0	1.55	1.33	0.44	3.11± 0.93

Legend: N - native spermatozoa, T - spermatozoa suspended in the TRIS - egg yolk extender, B - spermatozoa suspended in Biociphos plus extender (IMV, France)

* Standard deviation was calculated only when all samples were positive for antisperm antibodies

DISCUSSION

The results of our investigations reveal that the titer of antisperm antibodies increases with the age of the calf. In this study, sera collected before colostrum ingestion could serve as a negative control, since no antibodies were found after applying both methods. This is in agreement with Jimanez *et al* (1986) who did not find agglutinating factors in foetal calf sera. The same authors reported naturally occurring antisperm antibodies in sera of cows and bulls. The first positive reactions occurred in samples tested for the presence of antisperm IgG antibodies collected 48 hours after colostrum ingestion (Table 1). Their titer was highest when sperm cells previously suspended in TRIS egg yolk extender were used in the test and these antibodies are of colostral origin. Later, at the age of 30 days we found more positive samples and at the age of 120 days almost all sera had antisperm antibodies of the IgG class but native sperm cells showed a weaker reaction. Titers of antisperm antibodies of the IgM class were lower and even on day 120 the majority of samples showed no reactivity. However, at this age, when sperm cells previously suspended in TRIS egg yolk extender were used for the test immunoreactivity occurred in all samples The same samples showed a very weak agglutination reaction except when sperm cells suspended in Biociphos plus extender were used (days 30 and 120). We may thus conclude that antisperm antibodies from colostrum give very weak immunofluorescence and with few exceptions almost no agglutination. Immunoreactivity against sperm cells increases with age but generally remains at a low level . Most probably it is only the consequence of cross reactivity with microbial antigens. It is our opinion that the results obtained in this study can be useful in estimating possible causes of unexplained infertility, because naturally occurring antibodies should always be considered as basic values for adult animals. The highest titer values in this study were 1: 16. This is in agreement with the results of a field study (Jaćević, 1998) when sera and cervical mucus of heifers and cows were tested for the presence of sperm-agglutinating antibodies. In virgin heifers very low titers were demonstrated both in sera and cervical mucus but they increased with the number of artificial inseminations (AI) up to the 1:1024. The majority of cows with unexplained infertility had antisperm antibodies in titres above 1:64. Also, in this field trial the existence of antigenic differences between sperm cells obtained from the same bulls (if they were prepared for AI with different extenders) was clearly demonstrated. Similar findings were reported by Leipute (2002) who stated that serum titer levels above 1:32 may impair successful fertilization in cows. Interestingly Richards and Witkin (1984) described nonspecific binding of IgG molecules to sperm cells via the Fc portion while IgA amd IgM were completely unreactive. The physological significance of this phenomenon still needs an explanation.

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PRIRODNA ANTITELA PROTIV SPERMATOZOIDA U SERUMU TELADI PRE PUBERTETA

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SADRŽAJ

U ovom radu su izneti rezultati ispitivanja prisustva antitela protiv spermatozoida bika u serumu neonatalne teladi i teladi uzrasta do 120 dana metodama indirektne imunofluorescencije i aglutinacije u želatinu. Za izvođenje testova su korišćeni nativni spermatozoidi iz svežih ejakulata kao i spermatozoidi iz pajeta za veštačko osemenjavanje prethodno suspendovani u TRIS-žumanjčanom razređivaču ili razređivaču Biociphos plus (IMV, France). U serumu teladi pre ingestije kolostruma nisu dokazana antitela što je u skladu sa fiziološkom agamaglobulinemijom kod ove životinjske vrste. Kod teladi stare dva dana dokazano je prisustvo antitela protiv spermatozoida poreklom iz kolostruma i to u različitom titru u odnosu na vrstu korišćenih spermatozoida što ukazuje na njihovu antigensku različitost. Titar antitela se povećavao sa starošću teladi. Ova ispitivanja su potvrdila veću osetljivost indirektne imunofluorescence u odnosu na aglutinaciju. Na osnovu dobijenih rezultata se može pretpostaviti da su kod teladi, prirodna antitela na antigene spermatozoida nastala kao posledica unakrsne reaktivnosti sa mikrobijalnim antigenima.