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CUTANEOUS BASOPHIL HYPERSENSITIVITY REACTION TO PHYTOHEMAGGLUTININ IN REPEAT BREEDER COWS

LAZAREVIĆ M*, MILOVANOVIĆ A**, MILANOVIĆ SVETLANA*, KIROVSKI DANIJELA* and ILIĆ V*

** Faculty of Veterinary Medicine, Belgrade, ** Scientific Veterinary Institute, Novi Sad*

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In this study the relationship was explored between general immune reactivity, as estimated by the cutaneous basophil hypersensitivity reaction (CBHR) to phytohemagglutinin (PHA), and the level of anti-sperm antibodies of the Ig A and Ig G class in the cervical mucus and sera of cows with different reproductive results. The hypersensitivity test was performed on the day of artificial insemination when cervical mucus and sera samples were also collected. The animals were divided into groups according to the total number of inseminations, number of inseminations per calving and their age. The titer of antibodies was determined by the indirect immunofluorescence method using sperm cells suspended in Tris egg-yolk extender.

Our results indicate that the intensity of CBHR is lowest in cows inseminated once or twice and in younger animals, while, there were no differences between the groups when they were formed according to the number of inseminations per calving. Titers of antisperm antibodies (ASA) of the Ig A class in cervical mucus (CM) increased with the number of artificial inseminations, number of inseminations per calving and age of the cows.

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

INTRODUCTION

Repeat breeding (RB) is still a significant problem in the reproduction of dairy cows. It is usually defined as a lack of conception following three subsequent artificial inseminations (AI) with no obvious symptoms of genital tract disease. A repeat-breeder is a cow which shows a reduced probability of conception, while all other factors are optimal (Casida, 1961), and thus requires more AI attempts to achieve pregnancy. If mated naturally, RB cows or heifers very often show improved reproductive results (Vukotić *et al*., 1982). The possible causes of this phenomenon are numerous and include delicate nutritional misbalance, subtle hormonal disturbances and immunological alterations. It was postulated that enhanced immunological reactivity to sperm or semen extender antigens might be one of the reasons for RB (Park and Hunter, 1977).

In natural mating, an immune response to sperm antigens usually does not occur due to the low sperm and seminal plasma immunogenicity (Hogarth, 1982). Also, all body fluids important for fertilization (seminal plasma, cervical mucus, follicular and uterine fluid) have immunosuppressive properties (Landers *et al*., 1994). In AI the chances for immunization of females to sperm antigens are enhanced because seminal plasma is diluted several times, thus reducing its very strong immunosuppressive effects (Lazarević, 1991; Lazarević *et al*., 1992) and new antigens originating from semen extenders are present. We have documented (Lazarević *et al*,. 2003; Jaćević and Lazarević, 2000; Jaćević, 1998) by sperm agglutination and the indirect immunofluorescence method that bull spermatozoa differ in antigenicity if different semen extenders are used for semen preparation for AI. Therefore, it was possible to postulate that cows with high general immunoreactivity (high responders) might also react to sperm and extender antigens and become repeat breeders.

One of the methods that is widely used in veterinary medicine to examine general immunological reactivity is the cutaneous basophil hypersensitivity reaction to phytohemagglutinin (PHA). PHA is a herbal lectin with mitogenic properties and usually serves in laboratory practice to induce nonspecific proliferation of mononuclear cells (Ekkel *et al*., 1995). If administered intradermally, PHA provokes a hypersensitive reaction characterized by enhanced skin thickness, erythema, induration and swelling accompanied with mononuclear cell accumulation. Histological examination of the skin at the site of PHA injection reveals massive accumulation of basophil cells and therefore this phenomenon was described as the cutaneous basophil hypersensitivity reaction or, alternatively, the PHA skin test. Moreover, PHA leads to changes in permeability of high endothelial venules, enabling easier passage of lymphocytes in tissues (Stedecker *et al*., 1977). The same authors postulated that the CBHR is a consequence of lymphokine production by stimulated mononuclear cells. The degree of reaction is highest 24 hrs after application (Mc Corkie *et al*., 1980) This test has been used to investigate the influence of various stress factors on the immune response in various domestic animals (Ekkel *et al*., 1995; Wesley and Kelly, 1984; Kelley *et al*., 1982; Regnier and Kelley, 1981; Žikić, 2000; Lazarević *et al*., 2000). It was clearly documented that under intensive stress conditions the CBH reaction is significantly reduced. The PHA skin test is frequently used as an indicator of *in vivo* cellular immunity in calves (Kelley *et al*., 1982).

MATERIAL AND METHODS

Cutaneous basophil hypersensitivity reaction (CBHR): A total of 60 Holstein cows was tested by intradermal application of 200 μg of PHA (INEP, Zemun). PHA was diluted in sterile phosphate buffered saline (PBS) pH 7.2, to reach a final concentration of 1 mg/mL and 200 μL of this solution was intradermally injected into the neck region. As a control, the same volume of sterile PBS was injected at a 15 cm distant site. Skin thickness (ST) was measured by cutimeter prior to administration of PHA or PBS and 24 hrs later. The magnitude of the reaction was calculated as the difference in double-fold skin thickness between the sites of PHA and PBS application according to the following formulas:

$$\Delta\ PHA = ST\ 0^h - ST\ 24^h$$
$$\Delta\ PBS = ST\ 0^h - ST\ 24^h$$

$$CBHR = \Delta\ PHA - \Delta\ PBS$$

Basically, 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. Furthermore, the results obtained were analysed considering the number of inseminations per calving and also the age of the cows. 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 as described earlier (Lazarević *et al*, 2003). All samples were kept frozen at - 20 ^oC 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 and underwent the standard procedure of preparation for AI. Ejaculates were diluted with Tris egg yolk extender at an average ratio of 1:10 and frozen at - 196 ^oC until requred.

Indirect immunofluorescence assay (IIF): The IIF assay was performed according to Noel *et al.* (1974) as described in detail earlier (Lazarević *et al.*, 2003). Briefly, after thawing the 12 (3 from each of 4 bulls) medium French straws for AI, sperm cells were washed twice in PBS (pH 7.2), resuspended by Vortex and used for smear preparations. On the dried sperm cell smears, 10 μl of CM sample (inactivated at 56 ^oC for 20 minutes) was placed and incubated for 20 min at 37 ^oC in a wet chamber. 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 (fluorescein isothiocyanate) conjugated antibodies (anti-bovine Ig A, ICN, USA, Cat No 641 751) was placed on the slide and incubated again under the same conditions. Anti Ig A antibodies were conjugated with FITC (ICN, USA, Cat No F 4274) according to The and Feltkamp (1970). After incubation, followed by the same washing procedure, the slides were kept in the dark and wet chamber till examined. As a positive control we used sera obtained from calves after immunization with the contents of straws prepared with Tris egg yolk extender as described in detail elsewhere (Lazarevic *et al*., 2000). Calf sera obtained before immunization served as the negative control. Using a 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

Our results indicated differences in the degree of cutaneous basophil reaction to PHA (CBHR) in cows with different total numbers of artificial inseminations (Table 1). These differences were statistically significant when CBHR results obtained on the animals inseminated 1-2 and 6-8 times were compared. We found the same level of significance when we compared the results obtained for the animals inseminated 1-2 and over 8 times (Table 1a).

Table 1. CBHR in cows with different numbers of artificial inseminations ($\bar{X} \pm SD$)

Total number of AI	N	CBHR (mm)
1 - 2	9	3.74 ± 2.148
3 - 5	12	6.06 ± 3.048
6 - 8	9	6.19 ± 1.861
over 8	30	7.54 ± 3.145

Table 1a. Statistical significance of differences in CBH reaction values between cows with different numbers of artificial inseminations

Total number of AI	1 - 2	3 - 5	6 - 8	over 8
1 - 2	–	NS	P < 0.05	P < 0.05
3 - 5	–	–	NS	NS
6 - 8	–	–	–	NS

NS – not significant

When we sorted the results obtained for CBHR on the same 60 cows, according to the number of AI needed for successful pregnancy we found no statistical differences between the experimental groups (Tables 2, 2a).

Table 2. CBHR in cows with different numbers of artificial inseminations per calving ($\bar{X} \pm SD$)

Number of AI per calving	N	CBHR (mm)
1 - 2	24	6.22 ± 4.810
3 - 6	23	5.10 ± 2.505
over 6	13	6.25 ± 2.135

Table 2a. Statistical significance of differences in CBH reaction values between cows with different numbers of artificial inseminations per calving

Number of AI per calving	1 - 3	3 - 6	over 6
1 - 2	–	NS	NS
3 - 6	–	–	NS

NS – not significant

Finally, all cows included in this study were divided into three groups according to their age. It was documented that the younger animals (up to three years of age) had the lowest CBHR (Table 3) but we found no statistically significant differences between animals aged 3-5 and those older than 5 years (Table 3a).

Table 3. CBHR in cows of different age

Age (years)	N	CBHR (mm)
up to 3	15	3.12 ± 1.820
3 - 5	15	6.54 ± 1.770
over 5	30	6.86 ± 2.545

Table 3a. Statistical significance of differences in CBHR between cows of different age

Age (years)	up to 3	3 - 5	over 5
up to 3	–	$p < 0.01$	$p < 0.01$
3 - 5	–	–	NS

NS – not significant

The titer of Ig A anti-sperm antibodies in the cervical mucus of cows with different numbers of AI are presented in Table 4. The lowest values were recorded in cows with 1-2 artificial inseminations and the highest in cows inseminated more than 8 times. Statistically significant differences were found only when titer values in cows inseminated 1-2 times were compared to those for the other groups (Table 4a).

Table 4. Titer of Ig A anti-sperm antibodies in cervical mucus of cows with different numbers of artificial inseminations ($\overline{X}\pm SD$)

Total number of AI	N	Titer of CM anti-sperm Ig A
1 - 2	9	4.21 ± 0.782
3 - 5	112	6.76 ± 2.386
6 - 8	9	6.65 ± 1.509
over 8	30	7.10 ± 2.259

Table 4a. Statistical significance of differences in CM anti-sperm Ig A titer between cows with different number of artificial inseminations

Total number of AI	1 - 2	3 - 5	6 - 8	over 8
1 - 2	–	$p < 0.05$	$p < 0.01$	$p < 0.01$
3 - 5	–	–	NS	NS
6 - 8	–	–	–	NS

NS – not significant

Moreover, anti-sperm Ig A titers were the lowest in the group of cows that achieved successful pregnancy following 1-2 AI (Table 5). As in the previous case, we were able to note statistically significant differences between this group and the other two (3-6 and over 6 AI) groups of animals (Table 5a).

Table 5. Titer of Ig A anti-sperm antibodies in CM of cows with different numbers of artificial inseminations per calving ($\overline{X} \pm SD$)

Number of AI per calving	N	Titer of CM anti-sperm Ig A
1 - 2	24	5.66 ± 1.376
3 - 6	23	7.36 ± 2.597
over 6	13	7.40 ± 1.843

Table 5a. Statistical significance of differences in titer of CM anti-sperm antibodies between cows with different numbers of artificial inseminations per calving

Number of AI per calving	1 - 2	3 - 6	over 6
1 - 2	–	$p < 0.05$	$p < 0.05$
3 - 6	–	–	NS

NS – not significant

Finally, the titer of Ig A anti-sperm antibodies in the younger category of cows (up to 3 years) had the lowest values (Table 6) and there were statistically significant differences between this and the other two groups of animals (Table 6a).

Table 6. Titer of Ig A anti-sperm antibodies in CM of cows of different age ($\overline{X} \pm SD$)

Age (years)	N	Titer of CM anti-sperm Ig A
up to 3	15	4.76 ± 1.951
3 - 5	15	7.03 ± 1.907
over 5	30	7.10 ± 2.299

Table 6a. Statistical significances of difference in titer of CM anti-sperm antibodies between cows of different age

Age (years)	up to 3	3 - 5	over 5
up to 3	–	$p < 0.05$	$p < 0.05$
3 - 5	–	–	NS

NS – not significant

DISCUSSION

After years of implementing artificial insemination procedures in cattle breeding it seems that some cows “resist” AI. Since one of the causes of this phenomenon might be an immunological barrier to conception, it was possible to postulate that cows with high general immunoreactivity (high responders) might also react to sperm and extender antigens and become repeat breeders. In order to check this hypothesis we have investigated the relationship one of the tests for estimating general cellular immune reactivity (CBHR) with the reproductive results of cows and titers of anti-sperm Ig A antibodies in their cervical mucus.

We have shown that the intensity of CBHR is significantly lower in cows inseminated 1-2 times compared to that in animals inseminated more than 3 times. However, we found no differences between the other experimental groups and therefore it is our opinion that the degree of reaction is probably more related to the age of the animals. This statement is confirmed by analyzing the same results sorted according to the age of the cows. In this case, we were able to demonstrate that younger cows (up to 3 years of age) also have a significantly lower intensity of skin hypersensitivity reaction to PHA. Moreover, when we sorted data according to the number of inseminations needed for successful pregnancy, we found no differences in CBHR among the experimental groups. It is, then, possible to conclude that there are differences in CBHR related to age.

In our previous study on cows, we showed that mean titers of anti-sperm Ig A antibodies (ASA) 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 (Lazarević *et al*., 2003). An elevation of sperm-agglutinating antibody titer in the CM and sera of artificially inseminated cows was clearly demonstrated by Jaćević (1998). For that reason we have tried to correlate this parameter with CBHR and the reproductive results of artificially inseminated cows.

It is well known that ASA may interfere with reproductive processes 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 (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. 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).

As documented earlier (Lazarević *et al*., 2003), the titers of Ig A ASA were significantly lower in the group of animals inseminated once or twice when compared to results obtained for cows inseminated 3 or more times. These cows also had a lower intensity of CBHR. In this study we showed that titers of Ig A ASA in CM were lower in cows that need 1-2 inseminations per calving when compared to the other groups but there were no differences in CBHR. Finally, older cows had higher titers of Ig A ASA in CM and higher CBHR results but these were animals that had been inseminated more times as well.

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Address for correspondence:
Prof. Dr Miodrag Lazarević
Faculty of veterinary medicine
Department of physiology and biochemistry
Bul JNA 18, Belgrade
Serbia & Montenegro
e-mail: misha@vet.bg.ac.yu

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TEST KOŽNE PREOSETLJIVOSTI NA FITOHEMAGLUTININ (PHA) KOD KRAVA KOJE POVAĐAJU

LAZAREVIĆ M, MILOVANOVIĆ A, MILANOVIĆ SVETLANA, KIROVSKI DANIJELA i ILIĆ V

SADRŽAJ

Cilj ovih ispitivanja je bio da se utvrdi odnos između rezultata testa kožne preosteljivosti na fitohemaglutinin, nekih reproduktivnih pokazatelja i koncentracije antispermatozoalnih antitela Ig A klase u cervikalnoj sluzi krava koje se

veštački osemenjavaju. Test kožne preosetljivosti je izvođen na dan veštačkog osemenjavanja a istog dana su prikupljani i uzorci cervikalne sluzi. Krave su bile podeljene u grupe na osnovu broja ukupnih prethodnih osemenjavanja, prosečnog broja osemenjavanja po graviditetu i na osnovu starosti. Prisustvo antispermatozoalnih antitela je određivano metodom indirektne imunofluorescence korišćenjem spermatozoida suspendovanih u Tris-žumanjčanom razređivaču.

Postignuti rezulati ukazuju da je intenzitet kožne preosetljivosti na PHA manji kod krava koje su osemenjene 1-2 puta kao i kod mlađih jedinki. Intenzitet ove reakcije se nije razlikovao kod krava koje zahtevaju manje ili više pokušaja VO po teljenju. Takođe je dokazano da se titar antispermatozoalnih Ig A antitela u cervikalnoj sluzi povećava sa ukupnim brojem osemenjavanja, brojem osemenjavanja po teljenju i starošću plotkinja.