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Effect of lipid peroxidation on the immunocytochemical detection of a leukocyte antigenic determinant in fresh and cryopreserved bovine spermatozoa

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ABSTRACT

Studies on different species, including rats, monkeys and humans, have shown the presence of leukocyte differentiation antigens in the spermatozoa. In some case the expression of these molecules is related to a specific functional state of the sperm cell, as was found for the CD 46 antigen, that in humans can be used as a marker of the acrosome reaction. The aim of the present study was to assess wether promoted lipid peroxidation of the spermatozoa induces any variations in their immunoreactivity with ILA 147 antibody that, in bull spermatozoa, recognizes bovine leukocyte antigens. Freshly ejaculated bovine spermatozoa and cryopreserved semen were tested for ILA 147 reactivity by standard immunoperoxidase staining, before and after promoted lipid peroxidation. Staining intensity was assessed in the individual cells using the microdensitometric method to measure integrated optical density (IOD), overcoming the disadvantage of an operator's subjective interpretation of the results. After the lipid peroxidation there was significantly decreased staining intensity in the fresh spermatozoa, but not in the cryopreserved cells. Furthermore, in the preincubation conditions, the cryopreserved spermatozoa had a lower mean I.O.D. value than the fresh sperm, showing that the freezing and thawing processes induced an alteration in the antigen exposure. However the mean immunoreactivity of the cryopreserved cells was not significantly influenced by lipid peroxidation. The absorbance value maps, made following immunoperoxidase staining by the examined antibody, showed that the reaction sites in the fresh and cryopreserved spermatozoa fell mainly within the periacrosomal region. Moreover, after induced lipid peroxidation there were fewer reaction sites in this domain. The present research has confirmed the presence of the examined leukocyte antigenic determinant in the bull spermatozoa, and suggests that promoted lipid peroxidation and the freezing and thawing of spermatozoa can produce membrane damage, leading to reduced ILA 147 antigenic site exposure.

Key words: Bovine spermatozoa, Sperm antigens, Lipid peroxidation, Immunocytochemistry

RIASSUNTO

EFFETTO DELLA PEROSSIDAZIONE LIPIDICA INDOTTA SULLA RILEVAZIONE IMMUNOCITOCHIMICA DI UN DETERMINANTE ANTIGENICO LEUCOCITARIO IN SPERMATOZOI DI BOVINO FRESCHI E CRIOCONSERVATI

Indagini in diverse specie, come nel ratto, nella scimmia e nella specie umana, mostrano la presenza di antigeni di differenziazione leucocitaria negli spermatozoi. In qualche caso l'espressione di queste molecole è legata a specifici stadi funzionali dello spermatozoo, come nel caso dell'antigene CD 46, che nella specie umana può essere utilizzato come marcatore dell'avvenuta reazione acrosomale. Scopo di questo studio è stato quello di verificare se la perossidazione lipidi-

ca indotta negli spermatozoi provoca variazioni della loro reattività nei confronti dell'anticorpo ILA 147, che riconosce negli spermatozoi di toro un antigene leucocitario bovino. Spermatozoi bovini ottenuti da eiaculato fresco e da sperma scongelato sono stati sottoposti a reazione immunoperossidasica, utilizzando ILA 147 quale anticorpo primario, prima e dopo perossidazione lipidica indotta. E' stata poi valutata l'intensità della colorazione immunoperossidasica determinando la densità ottica integrata (IOD) in situ, in singole cellule, tramite microdensitometria, in modo da eliminare la soggettività della semplice valutazione microscopica. Dai risultati si rileva che in seguito alla perossidazione lipidica gli spermatozoi freschi, ma non quelli crioconservati, hanno mostrato una diminuzione significativa dell'intensità di colorazione. Inoltre nelle condizioni di preincubazione il valore medio della IOD degli spermatozoi crioconservati è risultato inferiore a quello degli spermatozoi ottenuti da eiaculato fresco. I processi di congelamento/scongelamento hanno quindi indotto un'alterazione dell'esposizione dei determinanti antigenici in esame. La perossidazione lipidica indotta, invece, non ha alterato significativamente la reattività media delle cellule crioconservate. L'analisi delle mappe di distribuzione dei valori di assorbanza, dopo colorazione immunoperossidasica con l'anticorpo in esame, ha mostrato che sia negli spermatozoi freschi che in quelli crioconservati i siti di reazione più intensa sono localizzati soprattutto nella regione periacrosomale. Inoltre, in seguito all'induzione della perossidazione lipidica, si osserva una riduzione dei siti di reazione in tale regione. In conclusione questi dati confermano la presenza negli spermatozoi di questo determinante antigenico e suggeriscono che sia la perossidazione lipidica che la crioconservazione possano causare negli spermatozoi danni di membrana che portano ad una riduzione dell'esposizione di determinanti antigenici, quali quello riconosciuto dall'anticorpo ILA 147.

Parole chiave: *Spermatozoi bovini, Antigeni spermatici, Perossidazione lipidica, Immunocitochimica.*

Introduction

Studies in different species show the presence of leukocyte differentiation antigens, also called CD (cluster of differentiation) antigens, in male gamete or in germ cells. For example, in rat spermatocytes, Jeongwu *et al.* (1997) found the expression of CD 95, a membrane bound protein involved in apoptosis. In monkey and in human spermatozoa, Ching-Hei *et al.* (2000) and Focarelli *et al.*, (1999) observed CD52, that is secreted by the epididymis. In some case the expression of these molecules is related to a specific functional state of the sperm cell, as has been found for the CD 46 antigen (D'Cruz and Haas, 1992) that, in humans, can be used as a marker of the acrosome reaction. Leukocyte differentiation antigens are cell surface glycoproteins, and more than 190 CD molecules with many different functions are known for humans (Abbas *et al.*, 2000). It is generally recognised that the proteins of the plasma membrane surface mediates many dynamic cellular activities such as effector-receptor or cell-cell interactions, membrane permeability, transmembrane signalling, etc. In sperm cells the membrane components can overcome free random diffusion and restrict specific molecules, both lipids and proteins, to defined regions of the sperm surface. During sperm differentiation in spermatogenesis, epididymal maturation and capacitation the patterns of these "regionalizations" shift (Wolf and Voglmayr, 1984). Several techniques to show the regionalization of the sperm plasma membrane are based on the use of antibodies that react with cell surface antigenic determinants. The antigens of sperm plasma membrane have been investigated in several domestic animals like the rabbit (Shaha C., 1994), boar (Okamura *et al.*, 1992), ram (Weaver *et al.*, 1993) and bull (Spungin *et al.*, 1995) and, more generally, in mammals (Myles D., 1993). The findings have suggested that antigenic molecules in sperm plasma membrane are a dynamic assembly of proteins, probably responsive to developmental and environmental signals. In fact, as has been found in man (Naz *et al.*, 1991) and rats (Nehme *et al.*, 1993), some sperm plasma membrane proteins are also expressed in other tissues and cells.

In an earlier study (Meggiolaro *et al.*, 1998) the spermatozoa of five bulls were tested for the immune reactivity, using 25 monoclonal antibodies recognising several bovine leukocyte antigens; it was observed that only the antibody ILA 147 reacted with the sperm cells of all tested bulls. The aim of the present research was to confirm, using additional experimental data, the immunoreactivity of the above mentioned antibody with bull spermatozoa, and to make a semi-

quantitative evaluation of the reaction intensity to improve observation accuracy. Furthermore, among exogenous and endogenous factors considered able to morphofunctionally alter spermatozoon membrane, the major ones are oxidative stress (Alvarez and Storey, 1982; Alvarez and Storey, 1984; Ferrandi *et al.*, 1989; Ferrandi *et al.*,1990; Ferrandi *et al.*, 1992; Ferrandi *et al.*, 1995; Ferrandi and Porcelli, 1999) and cryopreservation (Royere *et al.*, 1996; Valcarcel *et al.*, 1997). The former gives rise to deleterious reaction products, like those resulting from the lipid peroxidation of the membrane polyunsaturated fatty acids. Such lipid peroxidation of the biological membranes is a free-radical-mediated chain reaction, involving a series of rearrangements, cleavages and oxidations, with the formation of free radical intermediates and carbonyl products. The interaction of such products with cellular macromolecules can seriously alter several functions of the male gamete (Mammoto *et al.*, 1996; Golapalakrishnan and Shaha, 1998). Furthermore Bilodeau *et al.* (2000) reported a general decrease of antioxidant defence in bovine spermatozoa after a freezing / thawing cycle. Many studies have come to the conclusion that the sperm membrane overlying the head, rich in unsaturated fatty acids on account of its fusogenic nature, is higly susceptible to peroxidation, due to these fatty acids. In addition, the lack of cytoplasmic enzymes in sperm cells means there is poor protection from peroxidation damage. The addition of ascorbate and catalytic amounts of certain heavy metal cations like Fe^{2+}, Co^{2+} or Cu^{2+} considerably accelerates lipid peroxidation, and it has long been believed that these metal cations act by initiating free radical formation or increasing the rate of peroxide breakdown to short-chain carbon fragments. Since substantial information indicates that domains of loosely packed lipids, the principal target of lipoperoxidation, are involved in the regionalization of sperm membrane proteins we decided to assess the effect of promoted lipid peroxidation on the regional expression of the antigenic determinant ILA 147 in bovine spermatozoa.

The present study also investigated whether ILA 147 antigen immunoreactivity of bull spermatozoa plasma membrane was modified in two experimental conditions: promoted lipoperoxidation and freezing - thawing.

Material and methods

Animals

Semen was obtained from three 3-4 years old Holstein Friesian bulls of proven fertility. Sperm motility and morphology were within normal range.

Sampling and semen processing

The ejaculate was collected with an artificial vagina, pooled and used for the fresh semen study, and to prepare 105 straws, each of 0.2 ml diluted semen (80 x 10^6 spermatozoa/ml, in Tris-citrate-egg yolk extender, containing glycerol as cryoprotective agent), that were kept at 5 °C for 3 h and then were frozen in liquid nitrogen vapour. This frozen semen was stored under liquid nitrogen for three months. To promote endogenous phospholipid peroxidation, the fresh and frozen semen was processed according to the method proposed by Mennella Faraone and Jones (1980). The semen was centrifuged at 1000 g for 10 min, seminal plasma and cryoprotectant medium were discarded and the sperm pellet was resuspended in phosphate-buffered saline (PBS, 138 mM NaCl - Sigma Aldrich, Milan, Italy - 7 mM Na_2HPO_4 - Farmitalia Carlo Erba, Milan, pH 7.2) to twice the original volume of semen. This was then centrifuged at 100 g for 10 min, the supernatant removed and the spermatozoa resuspended in PBS containing 0.5 mM sodium ascorbate (Sigma Aldrich, Milano) and 0.05 mM $FeSO_4$ (Sigma Aldrich, Milano), to a final concentration of $5x10^8$ cells/ml. The fresh and frozen-thawed sperm suspensions were incubated in aerobic conditions at 37 °C for 3h. Not incubated fresh and thawed semen (controls) and spermatozoa collected at 90 and 180 min after the beginning of aerobic incubation were fixed onto slides by cytospin. Although the present study did not assess the actual membrane lipoperoxidative damage due to oxidative stress, such damage had already been extensively demonstrated by researchers in past investigations (Jones and Mann, 1973, 1976, 1977a, b; Jones at al., 1978; Alvarez and Storey,

1984) and, more recently, by Fraga *et al.*, (1992) and our group (Ferrandi *et al.*, 1992a, 1995; Ferrandi and Porcelli, 1999). This paper does not include data on the extent of lipid peroxidation as we relied on the tested production of malonaldehyde as the index of lipid peroxidation, measured by thiobarbituric acid assay by Mennella Faraone (1980) in an identical experimental protocol (see Material and methods section) and by us (Ferrandi and Porcelli, 1999).

Antibodies

ILA 147 is a monoclonal Ig G_1 antibody produced by Dr. J. Naessens (International Livestock Research Institute, Nairobi, Kenya) by immunizing mice with bovine cells. This antibody was submitted to the third Workshop on Ruminant Leukocyte Antigens (Naessens and Hopkins, 1996) and tested by flow cytometry, together with 301 other monoclonals, against a panel of 80 bovine or ovine target cells, including *Bos taurus* and *Bos indicus* spermatozoa. The Workshop data showed ILA 147 to be the only reagent that gave an unambiguous positive reaction with bovine *(Bos taurus)* sperm cells; it was also found to react strongly with bovine erythrocytes and granulocytes, bone marrow cells and *Theileria annulata*-infected mononuclear cells, but showed very poor or no reaction at all with platelets, peripheral blood lymphocytes and peripheral blood monocytes. Thus it is believed that ILA 147 recognizes a bovine panleukocyte antigen, of which the precise structure and characteristics are still unknown and need studying. As already mentioned, other data have been obtained on ILA 147 immunoreactivity with spermatozoa: Meggiolaro *et al.* (1998) tested this antibody and 25 other monoclonals on the ejaculated spermatozoa of five bulls. Only ILA 147 gave intense immunoperoxidase staining of the sperm cells in all five animals.

In the present study the BAQ 92 monoclonal, kindly provided by Dr. W.C. Davis (Department of Veterinary Microbiology & Pathology, Washington State University, Pullmann, Washington), was used as the primary antibody in evaluating non-specific reaction intensity. The BAQ 92 monoclonal recognizes an epitope of the bovine CD62L antigen, corresponding to the L-selectin molecule (Howard, 1996) that is expressed on granulocytes and monocytes in bovine blood. In a previous study (Meggiolaro *et al.*, 1998) BAQ 92 did not appear to give any consistently positive reaction with sperm.

Immunocytochemistry

We detected the immunoreactivity of bull spermatozoa with ILA 147 and BAQ 92 antibodies using a standard immunoperoxidase method (Vectastain® Elite ABC kit, Vector Laboratories, Burlingame, CA). After blocking non-specific binding sites (20 min in normal horse serum: 15 µl/ml Tris-HCl 0.05 M, pH 7.6), spermatozoa were incubated overnight with ILA 147 (1: 30,000 in Tris-HCl 0.05 M) at 5°C. Controls were run in parallel using BAQ 92 as primary antibody, at 1:1000 dilution in Tris-HCl 0.05 M. After washing in Tris-HCl 0.05 M buffer, biotinylated horse anti-mouse Ig G (Vector) was added as the secondary antibody (5 µl/ml Tris-HCl 0.05 M) and the cells were incubated for another 30 min at room temperature. In the third step the avidin-biotin-peroxidase conjugated reagent (Vectastain® ABC reagent, Vector Laboratories) was added (9 µl reagent A and 9 µl reagent B in 1ml Tris-HCl 0.05 M), incubating at room temperature for 45 min. Last, the enzymatic substrate 3, 3'-diaminobenzidine tetrahydrochloride (prepared by the DAB substrate kit, Vector Laboratories, SK-4100, following kit instructions) was added and the reaction was developed for 10 min. The cells were counterstained with Mayer.

Detection of immunoperoxidase staining

Microdensitometric measurements (integrated optical density, IOD expressed in arbitrary units, a.u.) were taken with a Vickers M85a scanning microdensitometer at λ=480 nm; this wavelength was employed as the final product of the immunoperoxidase reaction is based on the oxidative polymerization of 3,3'-diaminobenzidine. The microdensitometer was used in the following conditions: 100x oil immersion objective, 10x eyepiece, dry condenser, 0.4 µm diameter flying spot, 16 sec scan time, 40 nm band width, 0.04 threshold and rectangular, external adjustable mask.

Statistical analysis

The data were evaluated by analysis of vari-

ance, using the JMP® 3.2.2 statistical package (1989-1997; SAS Institute, Cary NC, USA).

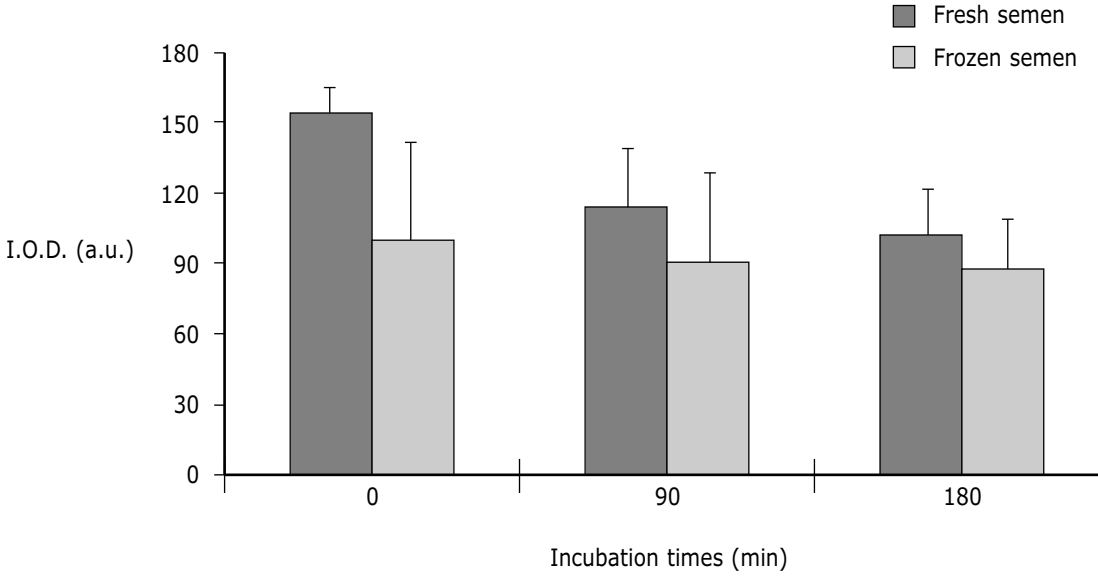
Results and discussion

The microdensitometric evaluations of the immunoperoxidase staining are shown in Fig. 1. Both fresh and cryopreserved sperm showed a decrease in the I.O.D. values after lipid peroxidation. However, the immunocytochemical behaviour of the two sperm populations showed differences: for the fresh semen, the spermatozoa that had undergone oxidative stress for 90 min showed, compared to the untreated sperm, a significant decrease in I.O.D. values; for the longer incubation time (180 min) there was no further significant decrease. Note that the cryopreserved spermatozoa at time 0 of incubation showed a lower staining intensity than the fresh sperm ($P<0.01$), but in this case the incubation in lipoperoxidative medium led to no significant variation in staining intensity.

Using I.O.D. microdensitometric evaluation it was also possible to describe the distribution pattern of the immunoperoxidase staining in the different sperm cell domains. Figure 2 shows four significant distribution patterns of I.O.D. in single cells: the highest I.O.D. values at time 0, both in fresh and cryopreserved spermatozoa were mostly localized in the periacrosomal domain. However at 180 min incubation in lipoperoxidative medium such high values were less frequent, and were more dispersed throughout the region, rarely being present in the postacrosomal domain.

Considering the microdensitometric evaluation of the staining intensity, the marked decrease in ILA 147 immunoreactivity in fresh spermatozoa that underwent the oxidative stress of 90 min incubation in lipoperoxidative medium supports the idea that the presumed alteration of membrane fluidity induced by the oxidative stress could have caused partial loss, or the masking, of surface macromolecules carrying the considered antigenic determinant. The decrease in immunocytochemical detectability of antigen ILA 147 is more marked in the acrosomal region. It therefore seems reasonable to assume that vulnerability to lipid peroxidation could differ with membrane regionalization. Studies using electron microscopy in conjunction with several lipid-specific probes have shown that lipids are, in fact, heterogeneous-

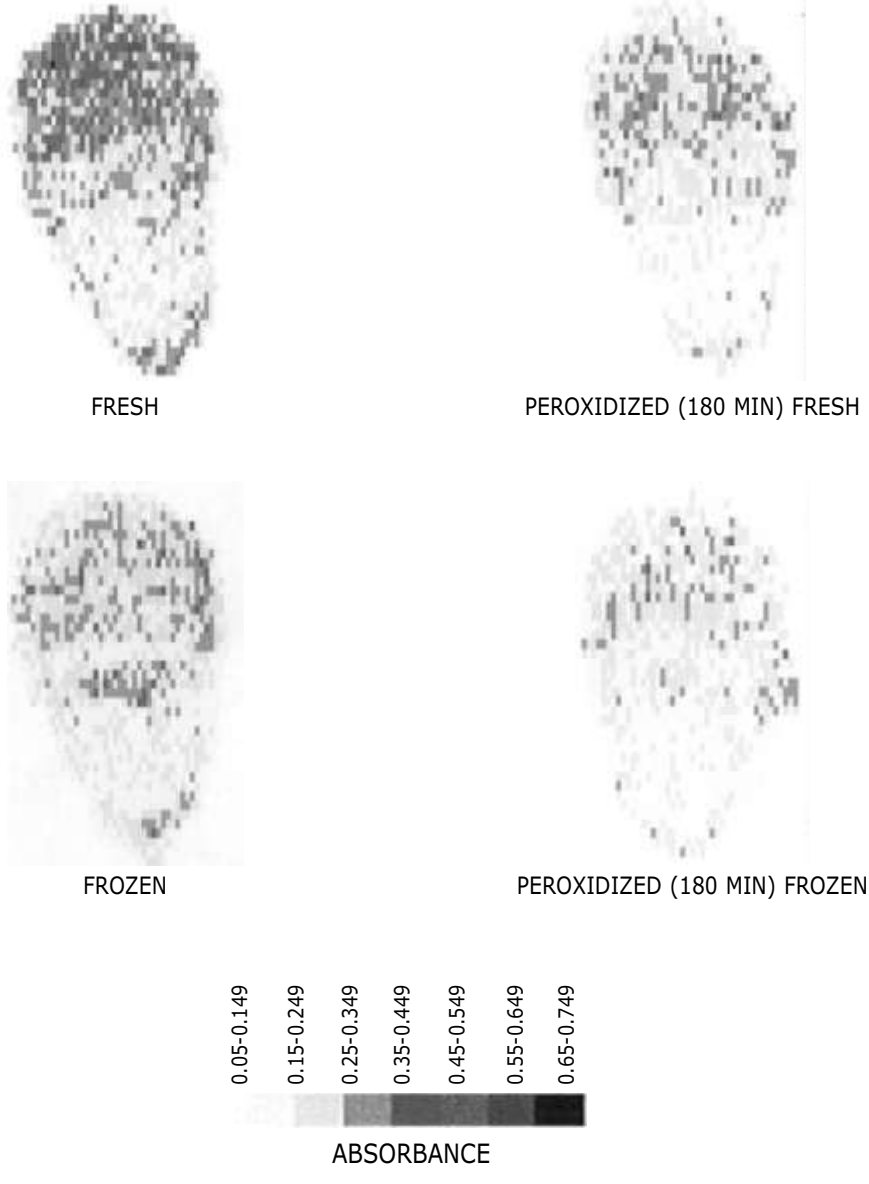
Figure 1. Microdensitometric measurements (I.O.D. mean values - expressed in arbitrary units ± SD; n=50) of the immunoperoxidase staining by ILA 147 monoclonal antibody of bull spermatozoa during incubation in lipoperoxidative medium.



ly distributed within the sperm plasma membrane, as emphasized by Schlegel *et al.* (1986).

The importance of this study lies in the fact that lipids, the major constituent of plasma membrane, play an important role in membrane structure, modulating the fluidity of the biomembranes. This means that lipid constituents have a profound influence on the regulation of membrane functions (Rana *et al.*, 1991); thus spermatozoa are highly susceptible to lipid peroxidation because of their high content of unsaturated fatty acids which, in the presence of oxygen can spontaneous-

Figure 2. Absorbance patterns of the immunoperoxidase staining by ILA 147 monoclonal antibody of bull spermatozoa (untreated and collected at the end of incubation in lipoperoxidative medium).



ly undergo autoxidation (Alvarez and Storey, 1982, 1984, 1985; Ferrandi *et al.*, 1989, 1990, 1992a, 1995, 1999). In general, plasma membrane disruption is a consequence of freezing injury, and freezing reduces the percentage of motile spermatozoa, as reported by McLaughlin and collegues (1992). Furthermore, reactive oxygen substances (ROS) are important mediators of sperm function, and should be considered that superoxide anions stimulate spermatozoa hyperactivation. Experimental data reveal the role of ROS in activating the acrosome reaction in some species such as the hamster (Lapointe *et al.*, 1998). Thus ROS play an important role in sperm physiology, but high concentrations can cause sperm pathology. This occurs when the fine balance between the production (de Lamirande *et al.*, 1997; Conte *et al.*, 1999) and scavenging of ROS is lost, and sperm functions and viability may be compromised. In this case enhanced lipid peroxidation, its extent depending on the antioxidant strategies employed by the spermatozoa, appears to be the reason for the disruption of membrane integrity (Golapalakrishnan and Shaha, 1998).

Given the present data, one particularly interesting finding was that cryopreservation alone caused a significant decrease in the ILA 147 immunoreactivity in the sperm. The freezing and thawing of the spermatozoa of several species leads to severe impairment of cellular functions, resulting in reduced fertility. Several papers have suggested cryopreservation to be a possible cause of morphofunctional damage to sperm plasma membrane (for a review see Royere *et al.*, 1996). In fact membrane integrity is drastically reduced after thawing (Valcarcel *et al.*, 1994), and freezing and thawing are reported to result in ultrastructural damage to the plasma membrane, acrosome, mid-piece and flagella of spermatozoa (for references see Valcarcel *et al.*, 1997). Furthermore, functions related to membrane integrity appear to be greatly affected by freezing and thawing, and this could be critical to the survival of spermatozoa in the female genital tract (Valcarcel *et al.*, 1994). During the freeze/thaw cycle, plasma membrane is subjected to a multitude of stresses including thermal, mechanical, chemical, osmotic and possibly even electrical perturbations (for references see Steponkus and Lynch, 1989). Therefore, it appears reasonable to suggest that cryopreservation could be involved in the different antigenic behaviour of the spermatozoa in the present study.

Conclusions

This research has confirmed the presence of ILA 147 immunoreactivity in bull spermatozoa and has shown that such immunoreactivity is mainly localised in the periacrosomal domain. It is known from the literature that the ILA 147 antibody recognizes a panleukocyte antigen, but the identity of this antigen is not known. Further research is needed to detect the nature of this antigen and its precise subcellular localisation. The present research using ILA 147 has demonstrated the modification of the antigenic properties of spermatozoa after promoted peroxidation of the membrane lipids, and has shown a reduction in the availability of ILA 147 antigenic sites in the periacrosomal domain. Furthermore, as the immunoreactivity of the untreated cryopreserved cells with ILA 147 was significantly lower than that of the untreated fresh spermatozoa, the data suggest that the cryopreservation process itself can cause alterations in the exposure of sperm cell antigenic molecules. This experimental model is of practical and scientific interest with regard to semen storage for both artificial insemination, especially where there are no precautions to exclude oxygen, and in vitro fertilization. These findings suggest that lipid peroxidation leads to a significant decline in immunoreaction with ILA 147, and that freezing-thawing enhances this decline in bovine sperm membrane. The data presented here are encouraging towards a systematic study of the physiological role of the antigenic determinant ILA 147 in the acquisition of properties essential to sperm function, especially in specific gamete recognition.

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