

Cryopreservation Alters the Levels of the Bull Sperm Surface Protein P25b

CARL LESSARD,* SOPHIE PARENT,* PIERRE LECLERC,* JANICE L. BAILEY,† AND ROBERT SULLIVAN*

From the **Département d'Obstétrique-Gynécologie, Faculté de Médecine, and the †Département des Sciences Animales, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Sainte-Foy, Quebec G1K 7P4, Canada.*

ABSTRACT: Fertility of frozen-thawed bull sperm is reduced by cryopreservation. Freezing-thawing procedures can result in as much as a sevenfold fertility decrease. Sperm mortality and loss of motility do not fully explain the reduced fertility of cryopreserved semen; they may be partially explained by the loss of sperm surface proteins, which are necessary for fertilization. We have previously identified P25b, a sperm surface protein, which is associated with the fertility index of bulls used for artificial insemination. Using Western blotting techniques, we have evaluated P25b levels before and after cryopreservation of bull spermatozoa in extenders based on either egg yolk or milk. Long storage periods (28 days) in liquid nitrogen results in a threefold decrease of P25b levels associated with cryopreserved versus fresh spermatozoa. Over a short storage period (3–7 days), a stable P25b level was observed on spermatozoa cryopreserved in

extender containing either egg yolk or milk. A decrease in P25b levels associated with spermatozoa was observed after 5 days of storage in egg yolk extender, whereas a significant decrease was observed after 14 days of sperm storage in milk extender ($P < .05$). Therefore, the loss of P25b may be responsible, at least in part, for the decrease in fertility following the freezing-thawing procedure of bull semen. Moreover, the cryopreservation extender used may have different effects on the loss of sperm surface proteins after even brief storage periods in liquid nitrogen. Considering that a sperm protein similar to P25b exists in humans (P34H), these results may have significant clinical applications in which frozen semen is used.

Key words: Spermatozoa, bovine sperm, sperm protein, fresh versus frozen semen.

J Androl 2000;21:700–707

Many aspects of sperm physiology and morphology are affected by cryopreservation procedures (Hammerstedt et al, 1990; De Leeuw et al, 1993; Foote and Parks, 1993; Watson, 1995). It is generally admitted that most damage to sperm occurs during the freezing and thawing procedures as opposed to dilution or the addition of glycerol. These steps have major consequences on sperm fertilizing ability. Generally, sperm viability is decreased by 50%, whereas fertilizing capacity is affected by a factor of sevenfold. Many factors contribute to sperm damage, including temperature and osmotic effects during freezing and thawing (Watson, 1995). These injuries affect sperm morphology (Gravance et al, 1998) and physiology, including regulation of intracellular Ca^{2+} , sperm plasma membrane fluidity, permeability, lipid composition, and mitochondrial activity (Hammerstedt et al, 1990; De Leeuw et al, 1993; Watson, 1995; Bailey et al, 2000). These parameters can be quantified and used to evaluate injuries associated with cryopreservation and their rela-

tive consequences on sperm fertilizing ability. More subtle sperm damages can also be induced by cryopreservation, resulting in reduced fertilizing ability (Lasso et al, 1994). These sublethal damages include injuries to sperm surface proteins that occur during cryopreservation procedures (Lasso et al, 1994). Segregation of membrane proteins as a result of lateral phase separation (De Leeuw et al, 1990), inactivation of membrane-bound enzymes, and decreased lateral protein diffusion within the membrane (Hazel, 1995) have been shown to occur at different steps of cryopreservation. These damages can be greatly reduced by using cryoprotectants such as glycerol (Polge, 1949) and sugars (Strauss, 1986; Crowe et al, 1987; De Leeuw et al, 1993). These reagents protect the plasma membrane from the disorganization that occurs during freezing; they also favor sperm motility (De Leeuw et al, 1993). The loss of sperm surface proteins also occurs during cryopreservation procedures (Lasso et al, 1994). Taken together, these biochemical events affect the ability of spermatozoa to efficiently interact with the female reproductive tract and oocytes. Even though many of the sperm surface modifications that occur during cryopreservation have been described, the relationship between these injuries and the decrease in fertility remains poorly understood.

Different sperm surface proteins have been identified

Supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Cattle Breeding Research Council (Canada).

Correspondence to: Dr Robert Sullivan, Unité d'Ontogénie-Reproduction, Centre de Recherche, Centre Hospitalier de l'Université Laval, 2705 Blvd Laurier, Ste-Foy, PQ, Canada, G1V 4G2.

Received for publication December 22, 1999; accepted for publication March 13, 2000.

and proposed to be involved in the steps leading to fertilization (McLesley et al, 1998). One of these proteins in hamster, P26h, has been identified as essential for fertilization (Sullivan and Robitaille, 1989; Bérubé and Sullivan, 1994). This protein is involved in zona pellucida binding, is associated with the plasma membrane covering the acrosome, and is acquired by spermatozoa during epididymal transit (Sullivan and Bleau 1985; Sullivan and Robitaille, 1989; Robitaille et al, 1991; Bérubé and Sullivan, 1994; Légaré et al, 1999a; Sullivan, 1999). Using a P26h antiserum, we have identified homologous proteins in human (Boué et al, 1996) and bull (Parent et al, 1999) sperm. These proteins have been named P34H and P25b, respectively. Like P26h, P34H is involved in a key event leading to fertilization in humans (Boué et al, 1994, 1996). P34H shows a 65% amino acid similarity with P26h (Gaudreault et al, 1999; Légaré et al, 1999b) and the absence of P34H correlates with some cases of male infertility (Boué and Sullivan, 1996; Guillemette et al, 1999). Similarly, the amount of P25b associated with spermatozoa shows interindividual variation, and low P25b levels are observed in spermatozoa from bulls of lower fertility, as evaluated by their nonreturn rates (NRRs; Parent et al, 1999). Therefore, it was suggested that P25b is a marker of bull fertility, as are P34H and P26h in the human and hamster, respectively (Sullivan, 1999). Considering the similarities between these sperm fertility markers and the fact that sperm cryopreservation is associated with the loss of surface proteins, we have hypothesized that P25b loss could be used to explain, at least in part, the fertility decrease associated with sperm cryopreservation. Our results show that the duration of storage in liquid nitrogen and the extender used to cryopreserve bull spermatozoa both affect the level of P25b present on sperm surface. Considering that proteins related to P25b are present in other species, including humans, loss of these proteins could also be associated with mammalian sperm cryodamage.

Materials and Methods

Animals

Semen samples used in this study were generously provided by the Canadian bovine artificial insemination industry (Semex, Guelph, ON, Canada). Eight bulls were selected to provide a range of high to low fertility levels on the basis of their 60- to 90-day NRR of artificially inseminated cows (ie, meaning that inseminated cows do not return to an estrus cycle between 60 and 90 days postinsemination). NRRs, which are the best evaluation of bull fertility in the bovine artificial insemination industry, were adjusted by a linear statistical model for the age of cows, month of insemination, technician, herd, and price of bull semen (Schaeffer, 1993). These fertility values were converted to a scale of 1 to 9, with 1 being the lowest fertility, 5 the

average for a given breed, and 9 the highest fertility. Semen was collected using an artificial vagina, diluted in the extender, and packaged to a final concentration of 15×10^6 spermatozoa per straw of 250 μ L. The extenders used to freeze the semen samples were based on either whole milk or egg yolk; each containing 6% glycerol prepared as previously described by Bailey and Buhr (1993) and Cormier et al. (1997), respectively. Immediately after collection, aliquots of unextended semen were either processed for P25b extraction, or frozen in each extender according to commercial procedures used in the industry.

Briefly, the freezing procedure included 4 steps. First, extended spermatozoa were cooled and incubated at 4°C for 2 h. The samples were then cooled in liquid nitrogen vapor to -40°C, -100°C, and -140°C at different speeds. Finally, the straws were plunged and stored in liquid nitrogen (-196°C) for different periods of time.

Extraction of P25b

Straws stored in liquid nitrogen for different periods of time were immediately thawed in a water bath at 37°C for 1 min. Fresh or frozen-thawed spermatozoa were washed twice by centrifugation at $300 \times g$ for 10 min with 4 to 9 volumes of Dulbecco's-phosphate buffered saline (D-PBS; GIBCO BRL, Grand Island, NY) pH 7.3. Sperm pellets were resuspended for 15 min at room temperature in 9 volumes of 0.2% (vol/vol) Triton X-100 (Fisher, Montréal, PQ, Canada) in D-PBS. Spermatozoa were pelleted by centrifugation at $300 \times g$ for 15 min, supernatants were collected, and proteins were precipitated overnight at -20°C with 9 volumes of ice cold acetone. Proteins were pelleted by centrifugation for 10 min at $4000 \times g$, air dried, and resuspended in sample buffer (2% [wt/vol] SDS, 75 mM Tris-HCl, pH 6.8).

Preparation of P26h Control

The rabbit polyclonal antiserum used to detect bovine P25b was previously produced against a hamster sperm protein, P26h (Bérubé and Sullivan, 1994), which was thus used as a positive control in Western blot experiments. P26h protein was obtained by Nonidet P-40 extraction on cauda epididymal spermatozoa as previously described by Sullivan and Bleau (1985). One μ g of hamster sperm protein extract was used in parallel with each SDS-PAGE performed on bull sperm extracts.

Western Blots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins extracted from 10^7 bull spermatozoa was performed in duplicate according to the procedure of Laemmli (1970). One gel was stained with Coomassie blue, the other was electrotransferred onto a nitrocellulose membrane (BioRad, Hercules, Calif). This transfer was performed using a Milliblot-Graphite electroblotter system (Millipore, Bedford, Mass) at 13 mA per gel for 45 minutes, according to the procedure of Towbin (Towbin et al, 1979). The nitrocellulose membrane was stained with Ponceau Red in order to identify molecular weight markers. The Western blots were blocked with 5% skim milk, 0.1% (vol/vol) Tween 20 (ICN Inc, Aurora, Ohio) in PBS (PBS-T) pH 7.3, for 1 hour at room temperature. The first antibody, a rabbit anti-P26h serum that cross-reacts with the

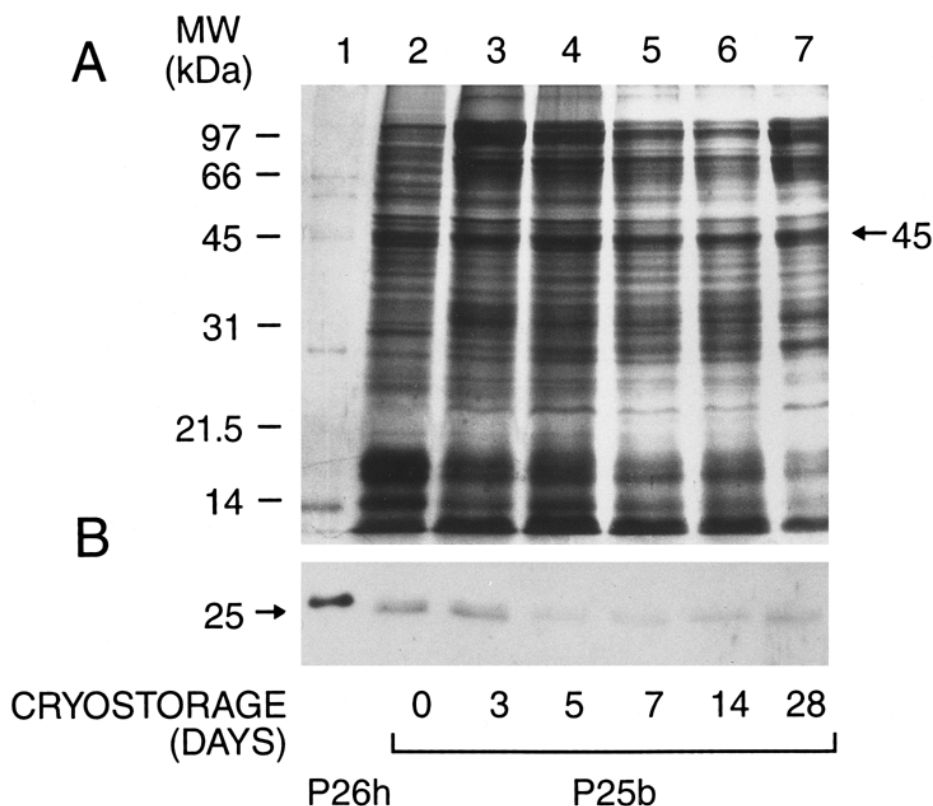


Figure 1. Example of SDS-PAGE (**A**) and corresponding Western blots (**B**) of cryopreserved spermatozoa cryostored in egg yolk extender for different periods of time. Lane 1 represents the positive control, 1 μ g of total protein detergent extracted from hamster spermatozoa; lane 2 shows proteins extracted from 10^7 fresh spermatozoa; lanes 3 to 7 represent proteins extracted from 10^7 spermatozoa cryostored for durations of 3, 5, 7, 14, and 28 days, which are indicated in **Panel B**. Molecular weight standards are indicated on the left of **Panel A** and 25 kd is indicated on the left of **Panel B**. The arrow on the right of **Panel A** indicates the 45-kd protein band quantified by densitometry.

analogous bull sperm P25b protein (Parent et al, 1999) was used throughout this study. The antiserum was preadsorbed on human keratin (Bérubé et al, 1994) and used at a dilution of 1:500 in PBS-T containing 5% skim milk. After 2 hours at room temperature, the blots were washed 3 times (10 min each) with PBS-T. The secondary antibody, a goat peroxidase-conjugated anti-rabbit immunoglobulin G (BioRad) was used at a dilution of 1:3000 in PBS-T containing 5% skim milk. After 45 minutes of incubation followed by 3 washes with PBS-T, the P25b was revealed by incubating the membrane with a peroxidase substrate (ECL Kit; Amersham, Buckinghamshire, United Kingdom) for 1 minute or with Super Signal Ultra (Pierce, Rockford, Ill) for 5 minutes. The ECL reaction was visualized on X-ray film, whereas the Super Signal Ultra was used for quantification using a camera (MultiImage Light Cabinet, Alpha Innotech Corporation, Canberra Packard, ON, Canada).

Quantification of P25b

In order to quantify P25b levels associated with 10^7 spermatozoa present in each sample, the Western blots were analyzed with Alpha Ease software, version 3.3d (1993; Alpha Innotech). Conditions were adjusted so that P25b measurements were performed in the linear part of the enzymatic reaction. Aliquots of fresh spermatozoa (Day 0) obtained prior to cryopreservation were considered as 100% of P25b.

In order to ascertain that the P25b loss was not a common phenomenon that could be generalized to all the proteins extracted by detergent treatment of frozen-thawed spermatozoa, different protein bands were quantitated by densitometry. As an example, quantities of a 45-kd protein extracted from 10^7 spermatozoa, as determined by its Coomassie blue density, are presented. As for P25b, the levels of 45-kd extracted from unfrozen spermatozoa was considered as 100%.

Statistical Analysis

The Scheffé test was used to analyze the P25b and 45 kd levels associated with spermatozoa that were thawed after different storage periods and in different cryopreservation media. P25b or 45-kd levels were considered significantly different at $P < .05$.

Results

Effect of the Storage Period on Sperm Cryopreserved in Egg Yolk

The quantity of P25b associated with a constant number of spermatozoa cryopreserved in egg yolk was evaluated after different periods of storage using straws prepared from the same semen sample. Coomassie blue-stained

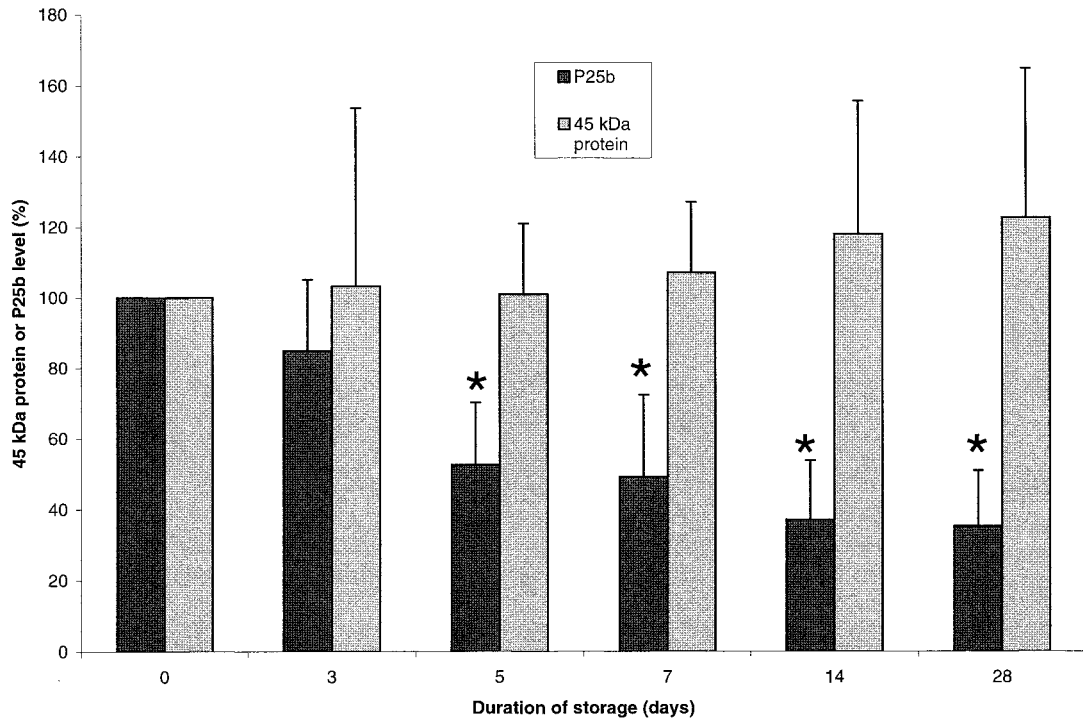


Figure 2. Average P25b and 45-kd protein levels associated with spermatozoa from 8 bulls cryostored in egg yolk-based extender for different storage durations. Results are expressed as a percentage of either P25b or 45-kd protein associated with the same number (10⁷) of fresh spermatozoa (storage Day 0). Vertical bars represent the standard deviation. * = P25b levels different from P25b level measured in fresh spermatozoa ($P < .05$).

gels of protein extracted from cryopreserved spermatozoa thawed after different storage periods showed quantitative variations in protein patterns (Figure 1A). These differences were observed when SDS-PAGE patterns of fresh versus frozen-thawed spermatozoa were compared. These quantitative protein variations were less apparent when comparing fresh spermatozoa to those stored for less than 5 days than when comparing fresh spermatozoa to those that had been stored for a period of 7 to 28 days. Compared with fresh spermatozoa, certain proteins show no variation during the storage period in liquid nitrogen. This is well-illustrated by the 45-kd protein bands (Figure 2). The P25b reduction during cryodamage is not associated with a general loss of all proteins. P25b registers with less intensity on Western blots than does hamster P26h (Figure 1B). This is due to a higher affinity of the antibody for P26h than for P25b (Bérubé and Sullivan, 1994; Parent et al, 1999).

Western blots of proteins extracted from spermatozoa cryopreserved in egg yolk for 3 days showed that P25b levels were not significantly different from those of fresh spermatozoa from the same semen sample (Figure 2). The effect of cryopreservation duration on P25b levels was evaluated on semen from 8 different bulls showing high interindividual variations (Figure 3). However, a longer cryostorage period was associated with a significant decrease in P25b levels ($P < .05$) after a minimum of 5

cryostorage days (Figure 2). No statistical difference was observed when P25b levels of fresh sperm were compared with those that had been cryostored for 3 days ($P > .05$). No further decrease in sperm P25b level was observed when semen samples were stored for more than 28 days ($P > .05$, data not shown). No correlation between the percentage of P25b lost on spermatozoa cryopreserved for all durations investigated and the NRR of these different bulls was observed ($P > .05$, data not shown).

Effect of Storage Period on Cryopreserved Sperm in Milk

Coomassie blue-stained SDS-PAGE of proteins extracted from spermatozoa that had been cryopreserved for different periods of time in milk-based extender showed similar results to those of spermatozoa that had been cryopreserved in egg yolk-based extender (data not shown). Semen from 3 different bulls was evaluated and the average levels of P25b after cryopreservation in milk or egg yolk extender are shown in Figure 4. Western blot analysis revealed that no decrease of sperm P25b level was observed prior to 7 days of cryostorage in milk-based extender. Less P25b was associated with spermatozoa after a 14 days of storage in milk-based extender compared with only 5 days in egg yolk-based extender (Figure 4). After 14 days of storage, P25b levels showed a greater interindividual variability on spermatozoa that had been

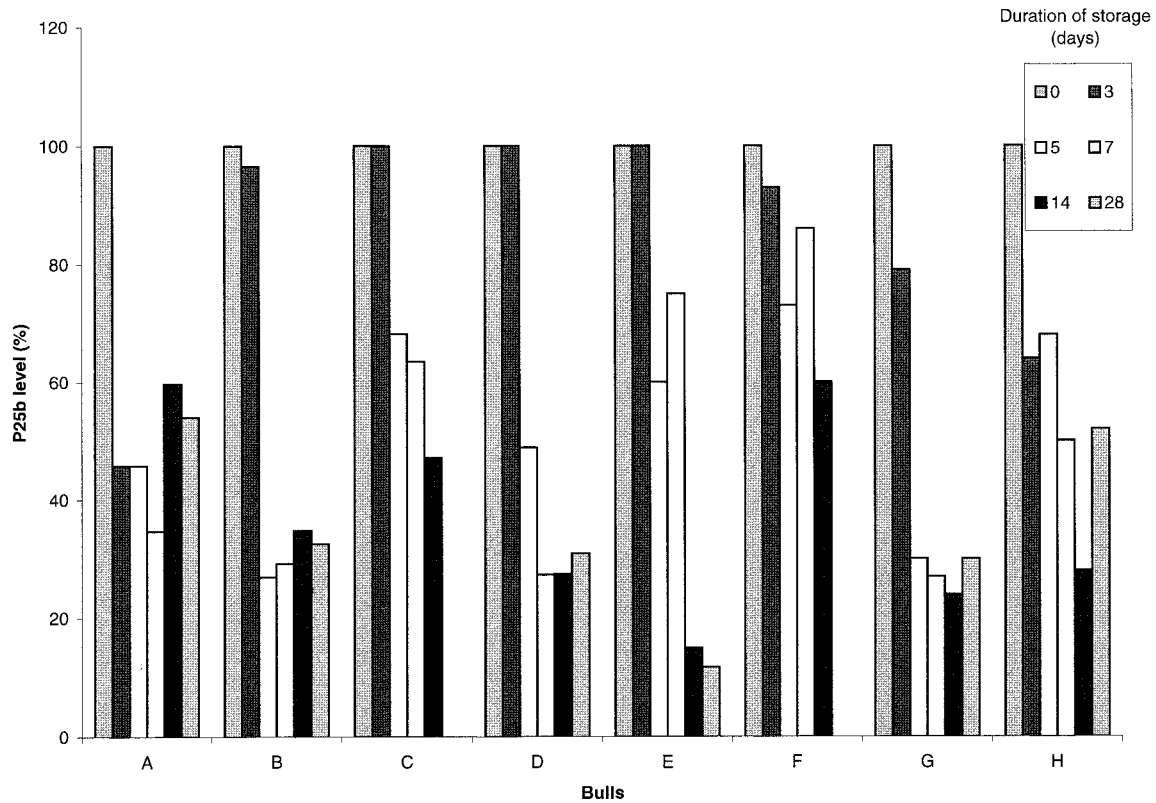


Figure 3. P25b level associated with 10^7 spermatozoa cryostored in egg yolk-based extender for 3 to 28 days for each individual bull investigated. P25b is expressed as a percentage of P25b associated with the same number of fresh spermatozoa (Day 0 of storage). Semen from 8 (A to H) bulls was evaluated. P25b level was quantified in comparison with the P25b level of fresh spermatozoa (storage Day 0).

cryopreserved in milk extender than the P25b levels on spermatozoa that had been cryopreserved in egg yolk extender. Spermatozoa that had been cryopreserved in egg yolk extender exhibited no further decrease in P25b levels when cryopreservation was tested for longer durations.

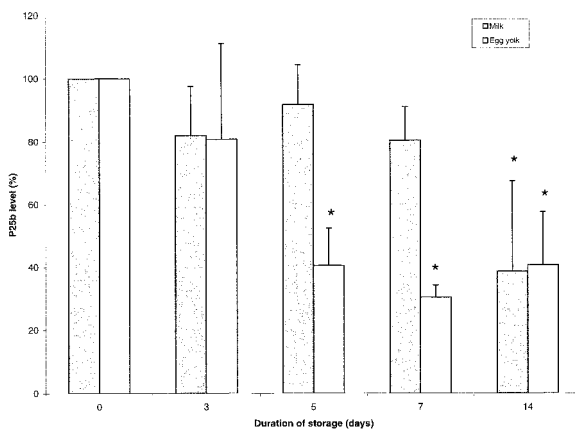


Figure 4. Average P25b level associated with spermatozoa of 3 bulls cryostored in milk (gray bar) or egg yolk-based (white bar) extenders for different storage durations. Results are expressed as a percentage of P25b associated with the same number (10^7) of fresh spermatozoa (storage Day 0). Vertical bars represent standard deviations. *P25b level is different from P25b level measured in fresh spermatozoa ($P < .05$).

Again, no correlation between the percentage of P25b lost during cryostorage and bull NRR was observed ($P > .05$).

Effect of Extender on Spermatozoa Cryopreserved During a Long Period of Time

After 2 months of storage, P25b levels were evaluated on frozen-thawed spermatozoa that had been cryopreserved in either milk or egg yolk extender. P25b levels were not statistically different between spermatozoa in the two groups (Figure 5). However, a significant difference was observed between P25b levels of cryopreserved spermatozoa in either extender and those of fresh spermatozoa; a threefold decrease in sperm P25b was measured after 2 months of cryostorage ($P > .05$). Greater interindividual P25b level variations were observed with spermatozoa that had been cryopreserved in milk-based extender compared with those in egg yolk.

Discussion

Recently, bull subfertility has been shown to be associated with low levels of P25b, a sperm surface protein (Parent et al, 1999). P25b belongs to a family of proteins acquired by spermatozoa during epididymal transit (Sul-

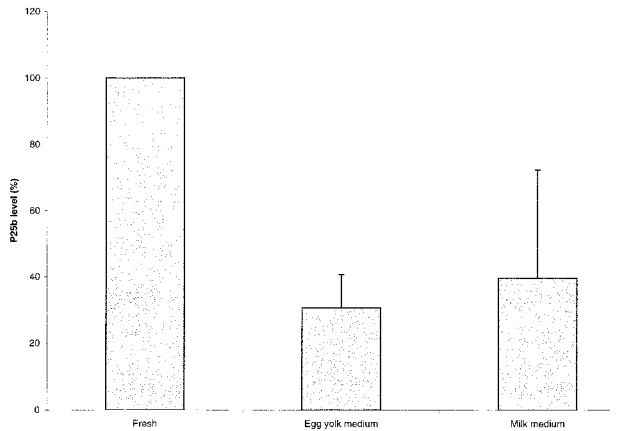


Figure 5. Average P25b level associated with fresh spermatozoa or spermatozoa cryostored for 2 months in egg yolk or milk-based extenders. Results are expressed as a percentage of P25b associated with the same number (10^7) of fresh spermatozoa. Three samples from each bull were evaluated. Vertical bars represent standard deviations. The P25b levels of spermatozoa cryostored in milk or egg yolk were statistically different ($P < .05$) from that of fresh spermatozoa. The P25b levels of spermatozoa cryostored in the 2 extenders were not statistically different ($P > .05$).

livan, 1999). These proteins are involved in binding spermatozoa to the surface of the egg vestment, the zona pellucida (Bérubé and Sullivan, 1994; Boué et al, 1994). Considering that spermatozoa acquire this property during epididymal transit, these proteins have been considered as markers of epididymal maturation of male gametes. Following a long storage period in liquid nitrogen (ie, more than 30 days), a decrease in P25b level was observed on spermatozoa that had been cryopreserved in egg yolk as well as in milk-based extenders (Figure 5). Compared with fresh spermatozoa, the amount of P25b that remained associated with the same number of frozen-thawed spermatozoa is in the order of 30%. Such a difference in P25b amounts associated with a constant number of spermatozoa is sufficient to discriminate between fertile and subfertile bulls (Parent et al, 1999). The loss of P25b occurring during cryoprotection can thus be sufficient to decrease frozen-thawed semen fertilizing ability, a decrease that cannot be evaluated by conventional andrological tests that are used to evaluate the injury of frozen-thawed spermatozoa.

P25b is associated with the plasma membrane that covers the acrosomal cap of spermatozoa (Parent et al, 1999). This surface protein is thus exposed to ice-formation injuries during the cryopreservation process (Hammerstedt et al, 1990; Watson, 1995). It is conceivable that P25b is partly cryoeluted from the surface of frozen-thawed sperm. Cryoelution of superoxide dismutase, an enzyme localized on the surface of human spermatozoa, has also been described (Lasso et al, 1994). Considering the importance of the P25b protein during key steps leading to fertilization, P25b loss is probably one of the cryoinjuries

causing the fertility decrease of frozen-thawed semen. Impaired zona pellucida binding ability after freezing-thawing procedures has been described in human sperm as the result of cryoelution of an "essential ligand" from the sperm surface (Amann et al, 1999c). This is true for human as for well as boar and bull cryopreserved spermatozoa (Amann et al, 1999a,b,d). Loss of surface proteins mediating sperm function appears to be an important mechanism of the cryoinjury that lowers sperm fertility. In bulls, it appears that P25b is one of these proteins. Loss of P25b does not appear to be the consequence of a general loss of some fraction of all proteins (Figure 2). The mechanism of P25b anchoring to the sperm surface is presently unknown and will eventually give some indication of the cause of its loss during cryopreservation of bull sperm.

Cryoinjuries are generally considered to occur during the freezing and thawing procedures, not during the storage period in liquid nitrogen (Hammerstedt et al, 1990; De Leeuw et al, 1993; Watson, 1995). If this is the case for cryoelution of P25b from the sperm surface, differences in P25b levels between fresh and cryopreserved sperm would be apparent after the shorter storage period evaluated (3 days) and not after 5 days and more than 7 days, as was observed when egg yolk and milk, respectively, were used as extenders (Figure 4). Analysis of P25b levels performed on each bull shows, however, that sperm P25b from 3 out of 8 individuals decreases significantly after 3 days of cryopreservation (Figure 3). It thus appears that sperm sensitivity to protein cryoelution, which occurs during cryostorage, varies from one individual to another.

A great interindividual variability is observed when P25b is determined on a constant number of thawed spermatozoa. The difference in P25b level between the 8 bulls used in this study can be as high as a threefold difference (data not shown). Using frozen-thawed spermatozoa, we have previously shown that low levels of P25b are observed only on spermatozoa from poor-fertility bulls (low NRR; Parent et al, 1999). One hypothesis would be that this interindividual variability in P25b levels associated with thawed spermatozoa is due to a variation in sperm sensitivity to P25b cryoelution from one bull to another. However, this is not likely because cryopreservation-associated loss of P25b does not correlate with bull NRR fertility scores. In fact, it is the resting levels of P25b associated with spermatozoa that are correlated with bull fertility. It thus appears that cryodamage associated with P25b affects thawed sperm fertilizing ability of all bulls in a similar way. In humans, the counterpart of P25b is a protein named P34H (Boué et al, 1994). When determined on fresh spermatozoa, P34H shows similar interindividual variability, with low values associated with male infertility (Boué and Sullivan, 1996). Freezing-thaw-

ing procedures decrease sperm P25b levels of all bulls in a similar manner, the interindividual variability being a consequence of the differing efficiency of sperm maturation during epididymal transit (Parent et al, 1999; Sullivan, 1999).

When the effects of the cryostorage period were evaluated on bull sperm P25b levels, differences were observed among egg yolk- and milk-containing extenders. Compared with fresh semen, a P25b decrease was statistically different after a 5-day cryostorage of sperm diluted in egg yolk-based extender, whereas it took more than 7 days for spermatozoa prepared in milk-containing extenders to show a significant decrease in P25b levels (Figure 4). It thus appears that a milk-based extender is more efficient than egg yolk in protecting sperm against the protein cryoelution that occurs during cryopreservation. This discrepancy between the 2 extenders is significant only after a short period of cryostorage. However, in long-term cryostorage using extenders such as egg yolk and milk, similar effects on P25b loss in bull semen were observed (Figure 5). This is in agreement with most studies comparing the efficiency of these 2 extenders (Almquist, 1954; Foote and Arriola, 1987).

The cryoelution of P25b during a short duration of cryostorage at -196°C is puzzling. To our knowledge, the potential injury of such brief periods of cryostorage on sperm surface proteins has not been investigated until now. Below -120°C , no chemical reaction can occur and thermally driven reactions are impossible at -196°C (Gao, 1997). Physical effects could be a possible cause of P25b cryoelution. The semen samples used in this study were prepared and cryostored in research facilities in which cell culture is routinely performed. It appears that the physical vibrations of the building are sufficient to interfere with in vitro cell attachment and growth of particularly sensitive cell lines (Nalge Nunc International, 1999). If these vibrations can interfere with cell culture, they may also cause a degree of physical cryoelution of surface proteins at the interface between the membrane bilayer phospholipids and extracellular ice. The effect of continuous vibrations on cryoelution of sperm surface proteins during short-period storage in liquid nitrogen is under investigation. Different levels of liquid nitrogen in the container had no effect on motility of thawed bull spermatozoa (Arbeiter et al, 1980) and we suppose that this variable also does not affect P25b levels in cryopreserved bull spermatozoa. Over a longer storage period, our results showed that P25b is cryoeluted from bull spermatozoa during cryopreservation and that 2 commonly used extenders have similar effects after such durations.

The bull sperm protein, P25b, belongs to a family of proteins that are evolutionarily conserved between different mammalian species, including humans. The loss of P34H, the human counterpart of P25b, on cryopreserva-

tion of human sperm samples could be a useful clinical evaluation mechanism of the ability to cryopreserve sperm from different individuals, especially in an artificial insemination with donor program.

Acknowledgments

We thank Ms Christine Légaré, Ms Nathalie Lamontagne, and Mr Christian Gaudreault for their advice and assistance; Mr Tom Kroetsch of GENCOR; and Mr Yves Brindle, Centre d'Insémination Artificielle du Québec; and the technicians who provided us with bull semen samples.

References

- Almquist JO, Flipse RJ, Thackers DL. Diluters for bovine semen. IV. Fertility of bovine spermatozoa in heated homogenized milk and skim milk. *J Dairy Sci.* 1954;37:1303-1307.
- Amann RP, Hammerstedt RH, Shabanowitz RB. Exposure of human, boar, or bull sperm to a synthetic peptide increases binding to an egg-membrane substrate. *J Androl.* 1999a;20:34-41.
- Amann RP, Seidel GE Jr, Brink ZA. Exposure of thawed frozen bull sperm to a synthetic peptide before artificial insemination increases fertility. *J Androl.* 1999b;20:42-46.
- Amann RP, Shabanowitz RB, Huszar G, Broder SJ. In vitro sperm-binding assay to distinguish differences in populations of human sperm or damage to sperm resulting from cryopreservation. *J Androl.* 1999c; 20:648-654.
- Amann RP, Shabanowitz RB, Huszar G, Broder SJ. Increased in vitro binding of fresh and frozen-thawed human sperm exposed to a synthetic peptide. *J Androl.* 1999d;20:655-660.
- Arbeiter K, Barsch E, Arbeiter E. Deleterious effects on frozen bull semen resulting from storage and handling in the field [in German]. *Zuchthygiene.* 1980;15:60-66.
- Bailey JL, Buhr MM. The calcium control of fresh and cryopreserved bovine spermatozoa in response to A23187. *Cryobiology.* 1993;30: 470-481.
- Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl.* 2000; 21:1-7.
- Bérubé B, Coutu L, Lefevre L, Begin S, Dupont H, Sullivan R. The elimination of keratin artifacts in immunoblots probed with polyclonal antibodies. *Anal Biochem.* 1994;217:331-333.
- Bérubé B, Sullivan R. Inhibition of in vivo fertilization by active immunization of male hamsters against a 26-kDa sperm glycoprotein. *Biol Reprod.* 1994;51:1255-1263.
- Boué F, Bérubé B, De Lamirande E, Gagnon C, Sullivan R. Human sperm-zona pellucida interaction is inhibited by an antiserum against a hamster sperm protein. *Biol Reprod.* 1994;51:577-587.
- Boué F, Blais J, Sullivan R. Surface localization of P34H an epididymal protein, during maturation, capacitation, and acrosome reaction of human spermatozoa. *Biol Reprod.* 1996;54:1009-1017.
- Boué F, Sullivan R. Cases of human infertility are associated with the absence of P34H, an epididymal sperm antigen. *Biol Reprod.* 1996; 54:1018-1024.
- Cormier N, Sirard MA, Bailey JL. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *J Androl.* 1997;18:461-468.
- Crowe JH, Crowe LM, Carpenter JF, Aurell Wistrom C. Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem J.* 1987; 242:1-10.
- De Leeuw FE, Chen HC, Colenbrander B, Verkleij AJ. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. *Cryobiology.* 1990;27:171-183.

- De Leeuw FE, De Leeuw AM, Den Daas JH, Colenbrander B, Verkleij AJ. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology*. 1993;30:32–44.
- Foote RH, Arriola J. Motility and fertility of bull sperm frozen-thawed differently in egg yolk and milk extenders containing detergent. *J Dairy Sci*. 1987;70:2642–2647.
- Foote RH, Parks JE. Factors affecting preservation and fertility of bull sperm: a brief review. *Reprod Fertil Dev*. 1993;5:665–673.
- Gaudreault C, L egar  C, B erub  B, Sullivan R. Hamster sperm protein, P26h: a member of the short-chain dehydrogenase/reductase superfamily. *Biol Reprod*. 1999;61:264–273.
- Gao D, Mazur P, Crister JK. Fundamental cryobiology of mammalian spermatozoa. In: Karow AM, Crister JK, eds. *Reproductive Tissue Banking*. New York: Academic Press; 1997:263–328.
- Gravance CG, Vishwanath R, Pitt C, Garner DL, Casey PJ. Effects of cryopreservation on bull sperm head morphometry. *J Androl*. 1998;19:704–709.
- Guillemette C, Thabet M, Dompierre L, Sullivan R. Some vasovasostomized men are characterized by low levels of P34H, an epididymal sperm protein. *J Androl*. 1999;20:214–219.
- Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl*. 1990;11:73–88.
- Hazel JR. Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Ann Rev Physiol*. 1995;57:19–42.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.
- Lasso JL, Noiles EE, Alvarez JG, Storey BT. Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation. *J Androl*. 1994;15:255–265.
- L egar  C, B erub  B, Bou  F, Lefi vre L, Morales CR, El-Alfy M, Sullivan R. Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. *Mol Reprod Dev*. 1999a;52:225–233.
- L egar  C, Gaudreault C, St-Jacques S, Sullivan R. P34H sperm protein is preferentially expressed by the human corpus epididymidis. *Endocrinology*. 1999b;140:3318–3327.
- McLesley SB, Dowds C, Carballada R, White RR, Saling PM. Molecules involved in mammalian sperm-egg interaction. *Int Rev Cytol*. 1998;177:57–101.
- Nalge Nunc International. Vibration patterns in tissue culture vessels. Technical information. Available at: <http://nunc.nalgenunc.com/resource/bulletins/02-vibrations.html>. 1999.
- Parent S, Lefi vre L, Brindle Y, Sullivan R. Bull subfertility is associated with low levels of a sperm membrane antigen. *Mol Reprod Dev*. 1999;52:57–65.
- Polge CS, Smith AU, Parks AS. Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature*. 1949;164:666.
- Robitaille G, Sullivan R, Bleau G. Identification of epididymal proteins associated with hamster sperm. *J Exp Zool*. 1991;258:69–74.
- Schaeffer L. Evaluation of bulls for nonreturn rates within artificial insemination organizations. *J Dairy Sci*. 1993;76:837–842.
- Strauss GH, Hausser H. Stabilization of lipid bilayer vesicles by sucrose during freezing. *Proc Natl Acad Sci USA*. 1986;83:2422–2426.
- Sullivan R, Bleau G. Interaction of isolated components from mammalian sperm and egg. *Gamete Res*. 1985;12:101–116.
- Sullivan R, Robitaille G. Heterogeneity of epididymal spermatozoa of the hamster. *Gamete Res*. 1989;24:229–236.
- Sullivan R. Interaction between sperm and epididymal secretory proteins. In: Gagnon C, ed. *The Male Gamete: From Basic Science to Clinical Applications*. Vienna, Austria: Cache River Press; 1999:93–104.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA*. 1979;76:4350–4354.
- Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*. 1995;7:871–891.