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Étude du mécanisme impliqué dans la protection des spermatozoïdes de mammifères par le jaune d'oeuf

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Université de Montréal
Faculté des études supérieures

Cette thèse intitulée:
Étude du mécanisme impliqué dans la protection des spermatozoïdes de mammifères par le jaune d'oeuf

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RÉSUMÉ

Le jaune d'œuf est utilisé depuis plus de 60 ans comme agent protecteur dans les diluants utilisés pour cryopréserver la semence bovine. Depuis quelques années, il y a une demande grandissante pour l'utilisation de diluants protecteurs exempts de produits d'origine animale. Cependant, il est difficile de trouver des substances de remplacement au jaune d'œuf car le mécanisme par lequel il protège les spermatozoïdes est encore inconnu.

Au moment de l'éjaculation, les spermatozoïdes entrent en contact avec le plasma séminal (PS). Les protéines majeures du PS bovin sont les protéines BSP (Bovine Seminal Plasma). Elles stimulent un efflux du cholestérol et des phospholipides des membranes des spermatozoïdes qui peut être néfaste pour les spermatozoïdes. Nous avons émis l'hypothèse qu'il est possible que le jaune d'œuf protège les spermatozoïdes en liant les protéines BSP et en empêchant ces dernières de se lier aux spermatozoïdes et de stimuler un efflux des lipides membranaires.

Premièrement, nous avons démontré que les protéines BSP ont la capacité de lier les lipoprotéines de faible densité (LDF) du jaune d'œuf. La liaison des protéines BSP aux LDF du jaune d'œuf est rapide, saturable, spécifique et stable après la cryopréservation. Deuxièmement, nous avons démontré que l'interaction des protéines BSP avec les LDF du jaune d'œuf empêche les protéines BSP de se lier à la membrane plasmique des spermatozoïdes et de provoquer un efflux des lipides membranaires, permettant ainsi de maintenir les fonctions spermatiques après les

étapes du procédé d'entreposage de la semence. Ces résultats indiquent que le jaune d'œuf protège les spermatozoïdes en empêchant les protéines BSP de stimuler un efflux des lipides des membranes des spermatozoïdes.

Nous avons ensuite voulu vérifier si le PS des autres espèces de mammifères contient des facteurs néfastes (protéines BSP) pour l'entreposage de la semence. Grâce à une chromatographie d'affinité à la gélatine et à l'héparine, nous avons isolé 4 homologues aux protéines BSP à partir du PS de bison (BiSV-16kDa, BiSV-17kDa, BiSV18kDa et BiSV-28kDa) et de bélier (RSP-15kDa, RSP-16kDa, RSP-22kDa et RSP-24kDa). De plus, nous avons démontré que les protéines BiSV et RSP ont la capacité de lier les LDF du jaune d'œuf. Ces résultats confirment que des protéines homologues aux protéines BSP sont présentes dans le PS des mammifères et que le mécanisme de protection des spermatozoïdes par le jaune d'œuf pourrait être le même pour les spermatozoïdes de ces espèces.

Nous croyons que les résultats décrits dans cette thèse aideront à développer de nouveaux diluants exempts de substances d'origine animale afin de cryopréserver la semence des mammifères.

Mots clés: cryopréservation, jaune d'œuf, protection des spermatozoïdes, lipoprotéines de faible densité.

ABSTRACT

Over the past 60 years, egg yolk has been included as a protective agent in extender used to freeze semen. Recently, there has been increased interest in using extender free of animal products to freeze semen. However, it is difficult to find suitable component in order to replace egg yolk in the extender because the mechanisms involved in sperm protection by egg yolk against damages caused by storage, cooling and freezing remain unclear.

Upon ejaculation, epididymal sperm are mixed with seminal plasma (SP). The BSP (Bovine Seminal Plasma) proteins are the major proteins of bull SP and they stimulate cholesterol and phospholipid efflux from the sperm membrane, which may be detrimental for sperm. We hypothesized that egg yolk protects sperm by binding BSP proteins and preventing them from stimulating lipid efflux from the sperm membrane.

Our studies showed that BSP proteins can bind the low-density lipoprotein fraction (LDF) from egg yolk. This binding of BSP proteins to LDF is rapid, saturable and specific and the binding capacity of LDF is very high. Furthermore, this interaction is stable even after freeze-thawing. Our studies also showed that the interaction between LDF and BSP proteins prevents BSP proteins from binding to sperm and stimulating lipid efflux from the sperm membrane while maintaining sperm motility during sperm storage. These results indicated that egg yolk protects sperm by preventing the stimulation of lipid efflux from the sperm membrane by BSP proteins.

In addition, we verified whether or not deleterious factors (BSP proteins) are present in the SP of other mammalian species. By gelatin- and heparin-affinity chromatography, we isolated four BSP protein homologs from bison SP (BiSV-16kDa, BiSV-17kDa, BiSV18kDa and BiSV-28kDa) and ram SP (RSP-15kDa, RSP-16kDa, RSP-22kDa and RSP-24kDa). Furthermore, we showed that BiSV and RSP proteins bind to egg yolk LDF. These results confirm that BSP protein homologs are ubiquitous among mammals and suggest that the mechanism of sperm protection by egg yolk is the same in those species.

We believe that the results described in this thesis will aid in the development of novel extenders that do not contain animal products for sperm preservation in liquid or frozen state.

Key words: cryopreservation, egg yolk, sperm protection, low-density lipoprotein

TABLE DES MATIÈRES

RÉSUMÉ i

ABSTRACT iii

TABLE DES MATIÈRES v

LISTE DES FIGURES viii

LISTE DES TABLEAUX ix

LISTE DES ABRÉVIATIONS x

REMERCIEMENTS xii

1. INTRODUCTION 1

1.1. Les spermatozoïdes 1

1.1.1. Morphologie des spermatozoïdes 1

1.1.2. Membrane plasmique des spermatozoïdes 3

1.1.3. Composition lipidique des membranes des spermatozoïdes 4

1.2. L'éjaculation 5

1.3. La capacitation et la réaction de l'acrosome 6

1.4. Le plasma séminal (PS) 7

1.4.1. Rôle du PS 8

1.4.2. Composition du PS 8

1.4.3. Effets du PS sur les spermatozoïdes 9

1.5. Les protéines majeures du PS bovin 11

1.5.1. Propriétés biochimiques des protéines BSP 12

1.5.2. Structure des protéines BSP 14

1.5.3. Propriétés de liaison des protéines BSP 14

1.5.4. Rôle biologique des protéines BSP 15

1.5.5. Les protéines homologues aux protéines BSP 17

1.6. La conservation des spermatozoïdes 18

1.6.1. Facteurs influençant le pouvoir fécondant des spermatozoïdes après la conservation 20

1.6.2. Les diluants protecteurs 25

1.6.3. Composition des agents protecteurs 27

1.6.4. Mécanismes impliqués dans la protection des spermatozoïdes 30

1.6.5. Amélioration de l'efficacité des diluants protecteurs 33

1.7. Problématique du projet de recherche 35

1.7.1. Hypothèses 37

1.7.2. Objectifs expérimentaux 37

2. ARTICLES 39

2.1 Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. Manjunath P., Nauc V., Bergeron A. et Ménard M. *Biology of Reproduction*, 67:1250-1258, 2002. 39

2.2 Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. Bergeron A., Crête M-H, Brindle Y. et Manjunath P. *Biology of Reproduction*, 70:708-717, 2004. 50

2.3. Isolation and characterization of gelatin-binding bison seminal vesicle secretory proteins. Boisvert M*, Bergeron A*, Lazure C., Majunath P. *Biology of Reproduction*, 70:656-661, 2004 61

2.4. Isolation and characterization of major proteins of ram seminal plasma. Bergeron A., Villemure M., Lazure C. and Manjunath P. Soumis pour publication dans *Biology of Reproduction*, Décembre, 2004. 69

2.5. The mechanism of sperm protection by egg yolk low density lipoprotein. Bergeron A. et Manjunath P. *Proceedings of the 20th technical conference on artificial insemination and reproduction*, NAAB, 2004, 68-75. 111

3. DISCUSSION 140

3.1. Pourquoi le PS est-il néfaste pour les spermatozoïdes bovins? 141

3.2. Caractérisation des facteurs néfastes présents dans le PS des mammifères 143

3.3. Les LDF du jaune d'œuf lient les facteurs néfastes contenus dans le PS 146

3.4 Nouveau mécanisme de protection des spermatozoïdes par le jaune d'œuf 147

3.5. Amélioration des protocoles de congélation de la semence bovine 151

3.6. Mise au point d'un diluant pour la conservation des spermatozoïdes de mammifères 154

3.7. Nouveau diluant protecteur exempt de substances d'origine animale 156

3.8. Mécanisme impliqué dans la protection des spermatozoïdes par le lait 159

3.9. Spermadhésines 160

4. CONCLUSION ET PERSPECTIVES 161

5. BIBLIOGRAPHIE 164

LISTE DES FIGURES

Figure 1: Structure du spermatozoïde. A) Schéma d'un spermatozoïde complet. B) Schéma de la tête d'un spermatozoïde. 2

Figure 2: Tractus génital du taureau. 7

Figure 3: Structure des protéines BSP. Les sites de glycosylation sont indiqués par les ronds noirs.[34] 13

Figure 4: Modèle de la capacitation des spermatozoïdes par les protéines BSP [21, 34]. 15

LISTE DES TABLEAUX

Tableau I. Caractéristiques des éjaculats de différentes espèces de mammifères (adapté de [9]). 5

Tableau II. Effet de la dilution de la semence afin d'obtenir une concentration de 80 X 10^6 spermatozoïdes/ml (20 X 10^6 spermatozoïdes/paillette). 153

Tableau III. Quantité moyenne de protéines BSP dans le PS de différentes espèces de mammifères. 155

LISTE DES ABRÉVIATIONS

°C	degré Celcius
ADN	acide désoxyribonucléique
ADNc	acide désoxyribonucléique complémentaire
AMPc	adénosine monophosphate cyclique
ATP	adénosine triphosphate
BSP	plasma séminal bovin (*Bovine Seminal Plasma*)
CTC	chlortétracycline
GSP	plasma séminal de bouc (*Goat Seminal Plasma*)
HDL	lipoprotéine de haute densité (*High-Density Lipoprotein*)
HSP	plasma séminal équin (*Horse Seminal Plasma*)
kDa	KiloDalton
LDF	fraction des lipoprotéines de faible densité (*Low Density Lipoprotein Fraction*)
mg	milligramme
min	minute
ml	millilitre
mM	millimolaire
mOsm	milliosmole
nm	nanomètre
pH	potentiel d’hydrogène
PS	plasma séminal

ROS	espèces réactives de l'oxygène
µg	microgramme
µl	microlitre

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1. INTRODUCTION

L'évènement clé de la reproduction sexuée est la fécondation, c'est à dire l'union d'une cellule reproductrice mâle haploïde et d'une cellule reproductrice femelle haploïde afin de donner un zygote diploïde. Ces cellules reproductrices sont appelées gamètes.

1.1. Les spermatozoïdes

Chez les mammifères, les gamètes mâles sont nommés spermatozoïdes et sont produits dans les tubules séminifères des testicules par un processus appelé spermatogenèse [1]. Ce processus implique une série de divisions mitotiques et deux divisions méiotiques suivies d'une série de modifications morphologiques nommée spermiogenèse. Cette chaîne d'évènements permet d'obtenir des cellules haploïdes (spermatozoïdes) à partir de cellules souches (spermatogonies).

1.1.1. Morphologie des spermatozoïdes

Les spermatozoïdes des mammifères sont tous formés de deux structures principales; la tête et le flagelle (figure 1). Ces deux structures ont chacune leur fonction particulière. La tête permet l'entreposage du matériel génétique à transférer à l'ovocyte et permet de digérer les enveloppes externes de l'ovocyte grâce au contenu enzymatique de l'acrosome. Quant à lui, le flagelle contient toute la machinerie nécessaire à la motilité spermatique. Par conséquent, le spermatozoïde est une cellule spécialisée pour se rendre au site de fécondation et pénétrer l'ovocyte.

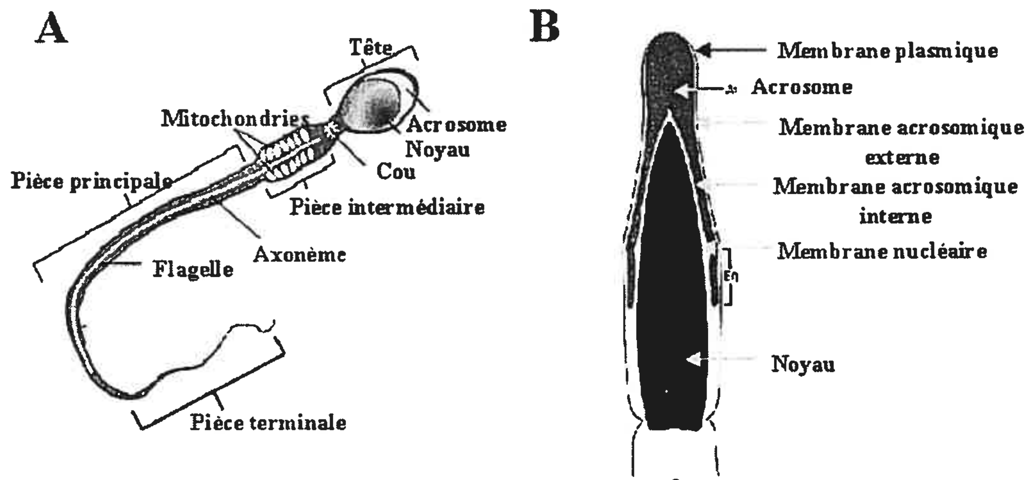


Figure 1: Structure du spermatozoïde. A) Schéma d'un spermatozoïde complet. B) Schéma de la tête d'un spermatozoïde.

La tête du spermatozoïde est principalement constituée du noyau, de l'acrosome et d'une quantité relativement faible de composantes cytosquelettiques et de cytoplasme [1]. Le noyau du spermatozoïde est haploïde et la chromatine qu'il contient est très condensée grâce à son association avec des petites protéines basiques appelées protamines. L'acrosome est formé de la membrane acrosomique interne qui est accolée à la membrane nucléaire et de la membrane acrosomique externe qui est accolée à la membrane plasmique du spermatozoïde (figure 1). Il origine de l'appareil de Golgi des spermatides et renferme les enzymes hydrolytiques nécessaires aux spermatozoïdes pour digérer les couches extérieures de l'ovocyte [1]. Les principaux enzymes présents dans l'acrosome sont la proacrosine qui est une protéase à sérine et l'hyaluronidase [2]. D'autres protéases, des phospholipases, des glycosidases et des estérases, sont aussi présentes dans l'acrosome [2].

Le flagelle des spermatozoïdes se divise en quatre segments: le cou qui relie la tête au flagelle, la pièce intermédiaire, la pièce principale et la pièce terminale (figure 1) [1]. L'axonème, se prolonge sur toute la longueur du flagelle et est la structure responsable de générer le mouvement du flagelle. La pièce intermédiaire contient les mitochondries, qui sont enroulées en spirale autour des fibres denses entourant l'axonème. Ces mitochondries fournissent l'énergie nécessaire au mouvement. Plus précisément, l'adénosine triphosphate (ATP) produite par les mitochondries active les molécules de dynéines liées aux microtubules et c'est cette activation qui induit le mouvement flagellaire.

1.1.2. Membrane plasmique des spermatozoïdes

La membrane plasmique des spermatozoïdes a pour particularité de posséder des régions spécialisées (domaines) qui diffèrent quant à leur composition et leur fonction [1]. Au niveau de la tête, les différents domaines sont l'acrosome antérieur, le segment équatorial et la région post-acrosomique. Au niveau du flagelle, les différents domaines sont la pièce intermédiaire et la queue distale (pièce principale et pièce terminale). La composition ainsi que l'organisation lipidique et protéique de la membrane plasmique varient d'un domaine à l'autre, ce qui reflète probablement les différentes fonctions des différentes parties du spermatozoïde. De plus, des modifications dans la composition et l'organisation de la membrane plasmique des spermatozoïdes ont lieu pendant le transit des spermatozoïdes dans l'épididyme, lors de l'éjaculation, ainsi que lors du passage des spermatozoïdes dans le tractus génital femelle. Certaines de ces modifications seront discutées dans les prochaines sections.

1.1.3. Composition lipidique des membranes des spermatozoïdes

Chez les spermatozoïdes éjaculés bovins, le seul stérol libre présent dans la membrane plasmique est le cholestérol, et le ratio molaire cholestérol:phospholipide est d'environ 1:4,55 [3]. La concentration en cholestérol dans les membranes des spermatozoïdes est de 270 $\mu g/10^9$ spermatozoïdes alors que celle des phospholipides est de 1377 $\mu g/10^9$ spermatozoïdes [4]. Plus de 50% des phospholipides présents dans les membranes des spermatozoïdes sont des phospholipides portant un groupement choline tels que la phosphatidyl-choline, le phosphatidyl-choline plasmalogène et la sphingomyéline [3, 4]. Les autres phospholipides présents dans la membrane sont les phospholipides portant un groupement éthanolamine ou sérine. La majorité des phospholipides portant un groupement choline sont situés dans le feuillet externe de la bicouche lipidique tandis que les phospholipides portant un groupement éthanolamine, sérine, ou inositol sont situés dans le feuillet interne (revu dans [5]). Il existe des différences au niveau de la composition lipidique de la membrane des spermatozoïdes de différentes espèces de mammifères. Par exemple, le ratio molaire cholestérol:phospholipide des membranes des spermatozoïdes est moins élevé chez le taureau et le bélier que chez le lapin et l'humain [6] et le ratio molaire acides gras insaturés:saturés est plus élevé dans les membranes des spermatozoïdes de porc, de taureau et de bélier que dans celles des spermatozoïdes d'humain, de lapin et de chien [7].

1.2. L'éjaculation

Après leur production dans les testicules, les spermatozoïdes sont entreposés dans l'épididyme et au moment de l'éjaculation, les spermatozoïdes épididymaires sont mélangés avec plasma séminal (PS) et sont propulsés à l'extérieur du tractus génital mâle [8]. Dans les centres d'insémination artificielle, la semence éjaculée (composée des spermatozoïdes et du PS) est récoltée dans un flacon, tandis que *in vivo*, la semence est déposée dans le tractus génital femelle, plus précisément au niveau du vagin ou du col de l'utérus selon l'espèce. Le volume et la concentration spermatique de l'éjaculat varient entre les différentes espèces de mammifères (voir tableau I).

Tableau I. Caractéristiques des éjaculats de différentes espèces de mammifères (adapté de [9]).

	Volume de l'éjaculat (ml)	Concentration spermatique (# X 10^6/ml)	Concentration protéique du PS (mg/ml)
Taureau	2 - 10	300 - 2 000	83
Bélier	0,7 - 2	2 000 - 5 000	50
Bouc	0,2 - 2,5	1 000 - 5 000	28
Porc	150 - 500	25 - 3 000	30
Étalon	30 - 300	30 - 800	10
Homme	2 - 6	50 - 150	43

1.3. La capacitation et la réaction de l'acrosome

Chez les mammifères, les spermatozoïdes émergeant du tractus reproducteur mâle doivent subir le processus de capacitation afin d'acquérir leur pouvoir fécondant [10-12]. Plus précisément, la capacitation est l'étape qui rend le spermatozoïde capable d'enclencher la réaction de l'acrosome qui est nécessaire à la fécondation. La capacitation est un processus complexe caractérisé par plusieurs modifications biochimiques du spermatozoïde: perte de certaines protéines présentes à la surface du spermatozoïde, diminution du ratio cholestérol:phospholipide de la membrane, augmentation de la perméabilité membranaire aux ions, production d'espèces réactives de l'oxygène (ROS), augmentation du pH, de la concentration du Ca^{2+} et de l'AMPc intracellulaire ainsi que l'augmentation de la phosphorylation sur tyrosine de certaines protéines spermatiques [13, 14]. Ces modifications sont induites par les agents capacitants présents dans le tractus génital femelle. Parmi ces agents capacitants, on retrouve les glycosaminoglycanes tels que l'héparane sulfate et la chondroitine sulfate ainsi que les accepteurs de cholestérol tels que les lipoprotéines de haute densité (HDL) [15-21]. *In vitro*, la capacitation peut être induite par l'héparine [22-24]. Une fois le processus de capacitation complété, le spermatozoïde peut enclencher la fusion de sa membrane acrosomique externe avec sa membrane plasmique. Cette fusion, appelée réaction de l'acrosome, permet au spermatozoïde de libérer les enzymes hydrolytiques contenus dans l'acrosome qui vont servir à la digestion de la zone pellucide entourant l'ovocyte [2]. Ensuite, le spermatozoïde pourra fusionner avec la membrane plasmique de l'ovocyte. Chez la plupart des espèces de mammifères,

seulement les spermatozoïdes possédant un acrosome intact peuvent interagir avec la zone pellucide de l'ovocyte grâce à des récepteurs spécifiques situés à la surface du spermatozoïde [25]. C'est la protéine ZP3 de la zone pellucide qui induit la réaction de l'acrosome chez les spermatozoïdes [26].

1.4. Le plasma séminal (PS)

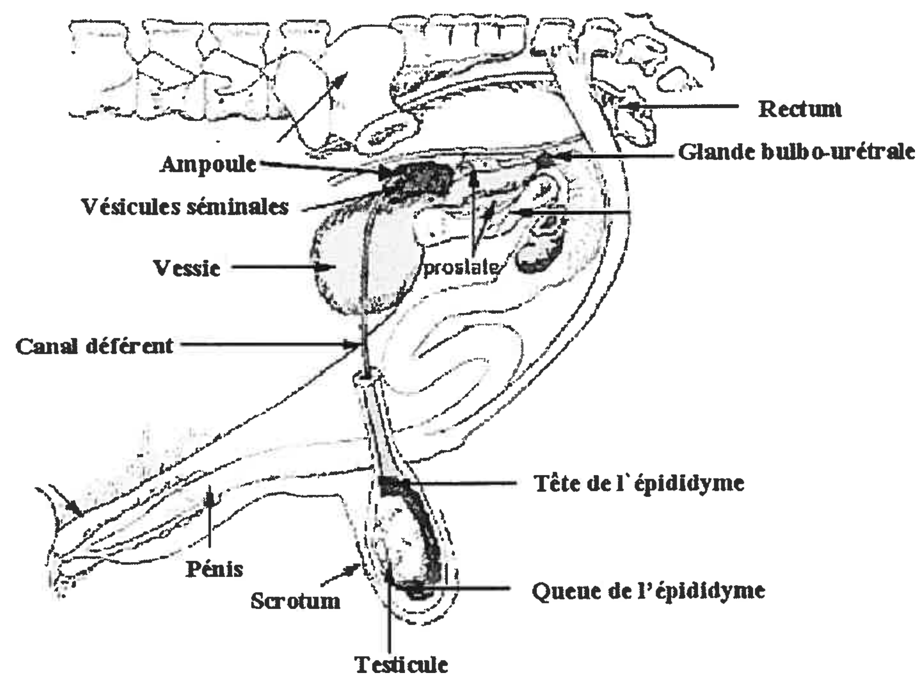


Figure 2: Tractus génital du taureau.

Le PS est constitué des sécrétions produites par les différentes glandes accessoires du tractus génital mâle (ampoules, vésicules séminales, prostate et glandes bulbo-uréthrales) (figure 2) [27]. C'est au moment de l'éjaculation que les spermatozoïdes entreposés dans l'épididyme entrent en contact avec le PS pour former

l'éjaculat. Ce sont les sécrétions des vésicules séminales qui composent majoritairement le PS (environ 60%) [27].

1.4.1. Rôle du PS

Le rôle du PS est de fournir un environnement optimal pour la survie et le transport des spermatozoïdes de l'épididyme jusqu'au tractus génital femelle [27]. De plus, le PS est connu pour activer la motilité des spermatozoïdes épididymaires [28] grâce à des facteurs qu'il contient [29] et au changement de concentration ionique que subissent les spermatozoïdes lorsqu'ils entrent en contact avec le PS [30]. Il a aussi été démontré que le PS joue un rôle dans l'initiation de la capacitation des spermatozoïdes [31-34].

1.4.2. Composition du PS

La composition du PS est variable entre les espèces et peut être variable chez un même individu où elle peut dépendre de la période de temps séparant les récoltes de semence [9]. Il existe aussi des variations saisonnières au niveau de la composition du PS au sein d'une même espèce [9].

Plusieurs ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, CO_3^-) sont présents dans le PS et servent à maintenir l'équilibre osmotique idéal pour le spermatozoïde [9]. Ce sont les ions Na^{2+} et K^+ qui sont majoritaires. Le principal substrat métabolique présent dans le PS est le fructose et il fournit l'énergie nécessaire à la motilité spermatique [27]. La présence d'inositol et de sorbitol a elle aussi été observée dans le PS [9]. De plus, une grande variété de protéines compose le PS telles que des protéines liant l'héparine ou

le zinc, des enzymes, des caltrines, des calmodulines, des inhibiteurs de protéases, des facteurs stabilisant l'acrosome, etc. [27]. Plusieurs de ces protéines se lient à la surface du spermatozoïde au moment de l'éjaculation. Chez le bovin, les protéines qui sont présentes en plus grande quantité dans le PS sont les protéines BSP (Bovine Seminal Plasma). Ces protéines seront décrites de façon détaillée à la section 1.5. Dans le PS de porc et de cheval, ce sont les protéines de la famille des spermadhésines qui sont présentes en plus grande quantité. La concentration protéique du PS est très variable entre les différentes espèces de mammifères (voir Tableau I). Le PS contient aussi les prostaglandines E et F mais leur rôle dans le PS est inconnu [35].

1.4.3. Effets du PS sur les spermatozoïdes

L'influence du PS sur les fonctions spermatiques est très complexe. D'ailleurs, les travaux tentant de démontrer les effets du PS sur les fonctions spermatiques donnent des résultats contradictoires. En effet, le PS ou ses composants ont souvent été décrits comme étant bénéfiques pour les spermatozoïdes [36-39] mais ont souvent aussi été décrits comme étant néfastes pour les fonctions spermatiques [40-42]. Ces différentes conclusions peuvent s'expliquer par le type de spermatozoïdes, épididymaires (n'a jamais été en contact avec le PS) ou éjaculés (a été en contact avec le PS), qui a été utilisé pour ces études ainsi que par la durée de l'incubation des spermatozoïdes avec le PS ou ses composants. Malgré le fait que le PS soit le milieu dans lequel les spermatozoïdes sont déposés dans le tractus génital femelle, il ne semble pas être le milieu idéal pour la survie des spermatozoïdes *in vitro*. *In vivo*, les spermatozoïdes entrent en contact avec le PS au moment de l'éjaculation et ce, pour

une courte période de temps. En effet, lorsque les spermatozoïdes entreprennent leur voyage dans le tractus génital femelle, le PS est laissé derrière eux.

1.4.3.1. Effets positifs du PS

Plusieurs études ont démontré que le PS peut avoir un effet positif sur les fonctions spermatiques telles que la motilité [36, 37, 43], la viabilité et le pouvoir fécondant [28, 38, 39]. Le PS a aussi été démontré comme facilitant la capacitation [32]. En effet, lorsque des spermatozoïdes épididymaires sont mis en contact avec du PS, le nombre de sites de liaison à l'héparine présents sur la surface des spermatozoïdes augmente [31] et il est connu que l'héparine, qui est un inducteur de la capacitation *in vitro*, doit se lier aux spermatozoïdes afin d'induire la capacitation. De plus, il a été démontré que pour enclencher la réaction de l'acrosome induite par la zone pellucide, les spermatozoïdes épididymaires bovins doivent être préalablement mis en contact avec le PS avant de subir la capacitation *in vitro* en présence d'héparine [33]. Par conséquent, le PS est bénéfique pour le pouvoir fécondant du spermatozoïde.

Plusieurs études avaient pour but de déterminer quels composants du PS sont responsables de ses effets positifs sur les spermatozoïdes. Par exemple, les protéines de faible poids moléculaire du PS rétablissent la motilité chez les spermatozoïdes éjaculés lavés [36]. De plus, chez le taureau, le PS contient une protéine appelée 'forward motility protein' qui induit la motilité chez les spermatozoïdes épididymaires [29]. Le PS de taureaux contient aussi des protéines dont la présence est associée à une fertilité élevée [38, 39] et la majorité des protéines qu'il contient potentialisent la capacitation des spermatozoïdes par l'héparine [24] et les HDL [44].

1.4.3.2. Effets négatifs du PS

Le PS peut aussi avoir des effets négatifs sur les fonctions spermatiques. Par exemple, le PS contient des facteurs inhibant la motilité spermatique [45, 46]. Il diminue le pourcentage de spermatozoïdes vivants lorsqu'il est ajouté à des spermatozoïdes épididymaires *in vitro* [40, 41] et les protéines ayant une masse moléculaire élevée qui sont présentes dans le PS diminuent le pourcentage de spermatozoïdes motiles lors qu'elles sont ajoutées à des spermatozoïdes éjaculés [36]. D'autres facteurs affectent négativement le pouvoir fécondant des spermatozoïdes [42]. Par exemple, le facteur de décapacitation qui prévient la capacitation des spermatozoïdes [47-50] et les protéines dont la présence dans le PS est associée à une faible fertilité des taureaux [28].

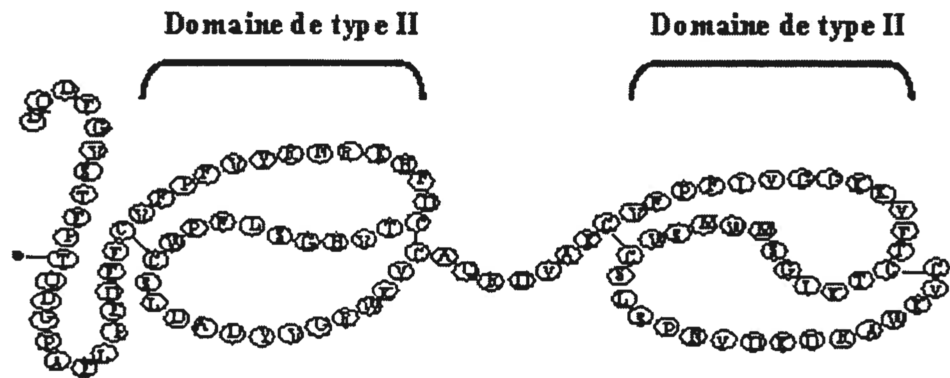
1.5. Les protéines majeures du PS bovin

Le PS contient une grande variété de protéines représentant une concentration totale moyenne de 68 mg/ml [27]. Les protéines majeures du PS bovin sont les protéines faisant partie de la famille des protéines BSP et sont sécrétées par les vésicules séminales. Cette famille est constituée de 4 protéines homologues (figure 3): BSP-A1, BSP-A2, BSP-A3 et BSP-30kDa [51, 52] et représente jusqu'à 65% des protéines totales du PS bovin. Les protéines BSP-A1 et BSP-A2 sont considérées comme étant la même protéine car elles sont constituées de la même chaîne de 109 acides aminés et elles diffèrent seulement au niveau de leur degré de glycosylation, BSP-A1 étant la plus glycosylée. Ces deux protéines sont souvent appelées BSP-A1/A2 ou PDC-109 (P désignant le mot protéine, D et C désignant l'abréviation des

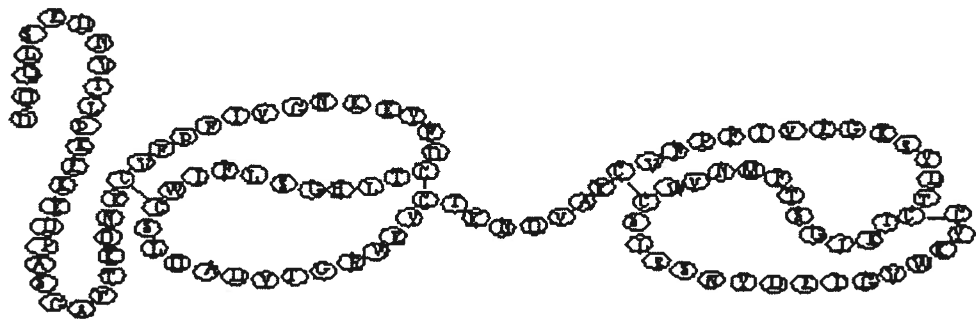
acides aminés retrouvés aux extrémités amino-terminale et carboxy-terminale respectivement et 109 pour le nombre d'acides aminés qui composent la protéine) [53].

1.5.1. Propriétés biochimiques des protéines BSP

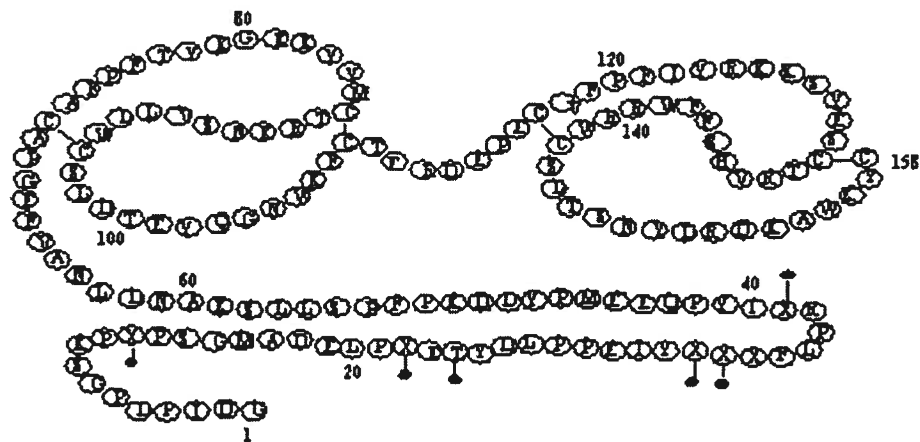
La masse moléculaire des protéines BSP-A1/A2 et BSP-A3 se situe entre 15 et 16,5 kDa alors que celle de la protéine BSP-30kDa est de 28-30 kDa [51, 52, 54, 55]. À l'exception de BSP-A3, toutes les protéines BSP sont des glycoprotéines. BSP-A1 et BSP-30kDa contiennent toutes les deux des sucres neutres, de la galactosamine et de l'acide sialique et c'est BSP-30kDa qui est la plus glycosylée. Quant à elle, BSP-A2 ne contient pas de sucres neutres. Elle contient seulement de la galactosamine et de l'acide sialique mais en plus faible quantité que la protéine BSP-A1 [52, 55]. BSP-A1 et BSP-A2 sont composées de la même séquence d'acides aminés tandis que la composition en acides aminés des protéines BSP-A3 et BSP-30kDa est différente de celle de BSP-A1/A2 [52, 55]. Les protéines BSP sont des protéines acides ayant un PI se situant entre 3,9 et 5,2 [56].



Protéines BSP-A1/-A2



Protéines BSP-A3



Protéines BSP-30-kDa

Figure 3: Structure des protéines BSP. Les sites de glycosylation sont indiqués par les ronds noirs.

1.5.2. Structure des protéines BSP

Tel qu'illustré à la figure 3, les protéines BSP possèdent une structure secondaire similaire. En effet, chacune des protéines BSP contient deux domaines de type II disposés en tandem [53, 57, 58]. Les domaines de type II sont des domaines de liaison au collagène et sont constitués de 38-41 acides aminés et de deux ponts disulfures [59]. Quant à elle, l'extrémité N-terminale est variable pour chaque protéine. D'ailleurs, la protéine BSP-30kDa se distingue des autres protéines BSP par sa longue extrémité N-terminale. L'ADNc correspondant à chacune des trois protéines BSP a aussi été cloné [60, 61].

1.5.3. Propriétés de liaison des protéines BSP

Grâce à leurs deux domaines de type II, les protéines BSP sont capables de s'associer avec plusieurs macromolécules. Les protéines BSP lient la gélatine [54], le collagène de type I, II, III et IV, le fibrinogène, le groupement choline des phospholipides [62], la calmoduline [63], et des facteurs capacitants tels que l'héparine [64], les HDL [65], l'héparane sulfate et la chondroitine sulfate B [21]. La protéine BSP-A1/A2 a aussi la capacité de lier le fucose [66] et de lier les phospholipides portant un groupement glycérol ou éthanolamine mais avec beaucoup moins d'affinité que les phospholipides portant un groupement choline [67, 68]. Ces diverses propriétés de liaison des protéines BSP permettent d'isoler les protéines BSP du PS par chromatographie d'affinité.

1.5.4. Rôle biologique des protéines BSP

Tel que démontré par des travaux de notre laboratoire, les protéines BSP seraient impliquées dans les modifications lipidiques des membranes des spermatozoïdes ayant lieu pendant la capacitation. Cette section décrit le mécanisme général de la capacitation par les protéines BSP tel que proposé par notre laboratoire (voir figure 4) [34].

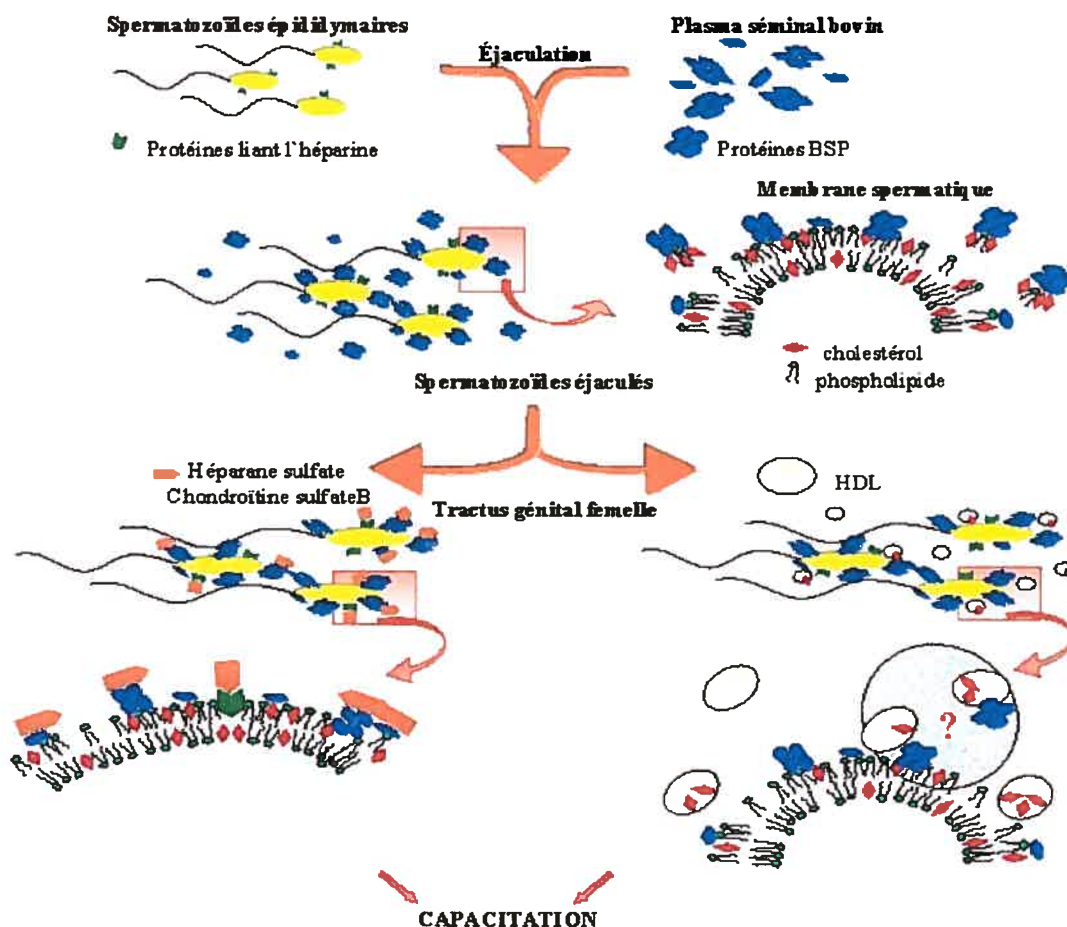


Figure 4: Modèle de la capacitation des spermatozoïdes par les protéines BSP [21, 34].

Premièrement, au moment de l'éjaculation, les spermatozoïdes épididymaires entrent en contact avec les protéines BSP qui sont contenues dans le PS. Les protéines BSP se lient alors à la membrane plasmique des spermatozoïdes grâce à leur interaction avec le groupement choline des phospholipides présents dans la membrane [62, 69]. Elles sont généralement présentes sur l'acrosome, la région post-acrosomique et la pièce intermédiaire du spermatozoïde [69]. Au même moment, des protéines BSP stimulent un efflux de cholestérol et de phospholipides portant un groupement choline des membranes des spermatozoïdes. En effet, il a été démontré que chacune des protéines BSP est capable de stimuler un efflux de lipides des spermatozoïdes épididymaires et que cet efflux lipidique dépend de la concentration des protéines BSP présentes dans le milieu d'incubation et du temps d'incubation des spermatozoïdes avec les protéines BSP [20, 70]. Ensemble, la liaison des protéines BSP à la membrane plasmique des spermatozoïdes et l'efflux de cholestérol peuvent induire une réorganisation de la membrane du spermatozoïde. De plus, il est possible que la présence de protéines BSP à la surface des spermatozoïdes empêche les phospholipides de se déplacer librement dans la membrane, stabilisant ainsi la membrane du spermatozoïde avant son parcours dans le tractus génital femelle. Pendant les 10 à 20 minutes suivant l'éjaculation, les spermatozoïdes nagent à travers le mucus cervical pour atteindre l'utérus, laissant derrière eux la majorité du PS. Cependant, les spermatozoïdes ont des protéines BSP liées à leur membrane et continuent leur voyage dans le tractus génital femelle où les HDL peuvent les capaciter. Ayant la capacité de lier les HDL, les protéines BSP liées aux spermatozoïdes peuvent interagir avec les HDL, ce qui pourrait stimuler un second

efflux de cholestérol. Cet efflux lipidique résulterait en une plus grande diminution du ratio cholestérol/phospholipides de la membrane et déclencherait la cascade de signaux menant à la capacitation. Chez le bovin, la capacitation peut être induite *in vitro* par l'héparine. Puisque les protéines BSP ont aussi la capacité de lier l'héparine, la liaison des protéines BSP sur les spermatozoïdes augmente le nombre de sites de liaison à l'héparine sur la surface des spermatozoïdes. L'héparine peut ainsi lier ces sites et induire la capacitation des spermatozoïdes [17, 22-24, 33]. De la même manière, la capacitation des spermatozoïdes bovins peut être induite par les glycosaminoglycanes semblables à l'héparine (chondroitine sulfate B et héparane sulftate) présents dans le tractus génital femelle qui ont eux aussi la capacité de lier les protéines BSP [21].

De plus, un article récent démontre que la protéine BSP-A1/A2 joue un rôle dans la formation du réservoir de spermatozoïdes dans l'oviducte [71]. En effet, les protéines BSP-A1/A2 présentes à la surface des spermatozoïdes permettraient aux spermatozoïdes de se lier aux cellules épithéliales de l'oviducte via leur interaction avec le fucose présent sur ces dernières. Puisque les protéines BSP potentialisent la capacitation et semblent jouer un rôle dans la liaison des spermatozoïdes aux cellules épithéliales de l'oviducte, les protéines BSP sont bénéfiques pour le pouvoir fécondant des spermatozoïdes.

1.5.5. Les protéines homologues aux protéines BSP

L'utilisation d'anticorps polyclonaux dirigés contre chacune des protéines BSP a permis de détecter des protéines homologues aux protéines BSP dans le plasma

séminal de hamster, d'humain, de souris, de porc et de rat [72]. Des protéines homologues ont aussi été isolées et séquencées chez le cheval, le porc [73-75] et le bouc [76]. Trois protéines homologues ont été isolées à partir du plasma séminal de cheval. Les deux premières protéines à avoir été isolées se nomment HSP-1 et HSP-2 et leur séquence ainsi que leurs propriétés de liaison ressemblent à celle des protéines BSP-A1/A2 et BSP-A3 [73]. Récemment, une troisième protéine, nommée HSP-12kDa, a été isolée [77]. Ces trois protéines représentent environ 20% des protéines totales du PS de cheval. Chez le porc, seulement une protéine homologue aux protéines BSP a été isolée à partir du PS. Cette protéine a été nommée pB1 et elle se retrouve en beaucoup plus faible quantité dans le PS de porc comparativement aux protéines BSP dans le PS bovin [74, 75]. En effet, elle représente seulement environ 1% des protéines totales du PS de porc. Quatre protéines homologues aux protéines BSP ont été isolées à partir du PS de bouc et ont été nommées selon leur masse moléculaire; GSP-14kDa, GSP-15kDa, GSP-20kDa et GSP-22kDa [76]. Ces protéines représentent environ 20% des protéines totales du PS de bouc et seulement GSP-20kDa et GSP-22kDa ont la capacité de lier l'héparine.

1.6. La conservation des spermatozoïdes

Il est possible de conserver les spermatozoïdes sur une courte période de temps (quelques heures à quelques jours) en les entreposant à 4°C. Il est aussi possible de les conserver sur une période de temps pouvant s'étaler sur plusieurs années grâce à la cryopréservation (congélation et entreposage de la semence dans l'azote liquide à une température de -196°C).

L'entreposage de la semence afin d'utiliser les spermatozoïdes pour l'insémination artificielle est un des outils les plus importants dans la reproduction des animaux de ferme. La semence ainsi entreposée peut être utilisée au moment voulu pour l'insémination artificielle, facilite la sélection génétique et permet de diviser un éjaculat en plusieurs doses d'insémination maximisant ainsi l'utilisation des éjaculats [78, 79]. En plus d'être utile à l'industrie des animaux de ferme, la cryopréservation des spermatozoïdes est utile à la conservation des animaux en voie de disparition et au traitement de l'infertilité.

Afin d'être conservée, la semence doit être diluée avec un diluant protecteur et dans le cas de la semence bovine, la concentration spermatique de la semence diluée est généralement 80 X 10^6 spermatozoïdes/ml. La composition de ces diluants ainsi que les rôles de leurs composants seront décrits dans les sections suivantes. Une fois diluée, la semence est ensuite refroidie à 4°C sur une période de 2-4 heures pour être conservée ainsi ou pour être distribuée dans des paillettes de 0,25 ou 0,5 ml et être ensuite congelée et entreposée dans l'azote liquide. L'entreposage de la semence à 4°C a pour but de diminuer le métabolisme des spermatozoïdes afin de prolonger leur durée de vie. Ces étapes du processus de cryopréservation, soit la dilution, le refroidissement et la congélation, causent des dommages aux spermatozoïdes qui se traduisent par une diminution du pouvoir fécondant des spermatozoïdes [80]. Après la cryopréservation des spermatozoïdes bovins, seulement la moitié des spermatozoïdes survivent [80-82] et la majorité de ces derniers sont endommagés et ont de la difficulté à compléter leur voyage jusqu'à l'ovocyte dans le tractus génital femelle [83]. En

effet, la motilité des spermatozoïdes est affectée négativement par la cryopréservation car la vélocité des spermatozoïdes cryopréservés est réduite comparativement à celle des spermatozoïdes fraîchement éjaculés [84]. Il faut donc utiliser 8 fois plus de spermatozoïdes bovins cryopréservés que de spermatozoïdes frais lors de l'insémination artificielle afin d'atteindre un pourcentage de fécondation équivalent [85]. À cause de leur forme très effilée, de la présence d'un acrosome sur leur tête et de la nature hétérogène de leur membrane, les spermatozoïdes sont des cellules très vulnérables aux dommages causés par la congélation [5]. Comparativement aux autres types cellulaires, les spermatozoïdes doivent être dilués avec un diluant protecteur complexe au lieu de seulement du glycérol ou du diméthylsulfoxyde afin d'être cryopréservés.

1.6.1. Facteurs influençant le pouvoir fécondant des spermatozoïdes après la conservation

1.6.1.1. Le choc dû au froid

Le choc dû au froid, appelé 'cold shock' dans la littérature, réfère au choc thermique que subissent les spermatozoïdes lorsqu'ils sont refroidis à des températures plus faibles que 15°C (revue par Watson, [86]). Le choc dû au froid est néfaste pour le pouvoir fécondant des spermatozoïdes et ce sont les membranes des spermatozoïdes (membrane plasmique, acrosomique et mitochondriale) qui sont la cible principale des dommages causés par ce refroidissement [5, 84, 87]. Par exemple, il y a une diminution de la quantité du cholestérol et des phospholipides membranaires lorsque les spermatozoïdes subissent un choc dû au froid [88, 89]. Le choc dû au froid est

aussi caractérisé par une augmentation de la perméabilité membranaire ce qui indique que la membrane a été endommagée [5, 86]. En effet, les ions Na^+ et Ca^{2+} entrent dans la cellule tandis que les ions K^+ et Mg^{2+} sortent [86]. De plus, des composés généralement présents à l'intérieur des spermatozoïdes sont retrouvés dans le diluant protecteur après le refroidissement de la semence [86]. Par exemple, la présence d'hyaluronidase dans le diluant après le choc dû au froid [90] confirme que la membrane acrosomique a été endommagée par le refroidissement. Les membranes mitochondriales sont elles aussi endommagées par le refroidissement et il en résulte un ralentissement de la production d'ATP (revu dans Watson [84]).

Les changements de température modifient la fluidité des membranes cellulaires. Lors du refroidissement, les membranes des spermatozoïdes passent de la phase liquide-cristalline, qui est plus fluide, à la phase gel, résultant en une structure plus rigide qui est plus facile à casser. À cause de ce changement de phase membranaire, les membranes des spermatozoïdes deviennent moins fonctionnelles lors du refroidissement (revu dans [91]).

1.6.1.2. La congélation et le dégel

La congélation et le dégel induisent eux aussi des dommages aux spermatozoïdes (revue par [91]). Lorsque la semence est congelée, l'eau présente dans l'environnement extérieur des spermatozoïdes subit un changement de phase. Il y a formation de glace à l'extérieur de la cellule ce qui résulte en l'augmentation de la concentration des solutés dans le milieu extracellulaire [92]. Le spermatozoïde se déshydrate donc, provoquant ainsi des fluctuations du volume de la cellule qui causent

des bris membranaires. La congélation des spermatozoïdes peut aussi être accompagnée de la formation de glace intracellulaire qui est généralement létale pour la cellule. Afin de maximiser la survie des spermatozoïdes, le taux de refroidissement subi par les spermatozoïdes doit être assez rapide pour minimiser l'effet des solutés concentrés à l'extérieur de la cellule et assez lent pour empêcher la formation de glace intracellulaire [5]. Les taux de refroidissement optimaux utilisés pour la congélation des spermatozoïdes de différentes espèces de mammifères ont été déterminés [84, 93]. Chez le bovin, celui-ci se situe entre -50 et -100°C/min (revu dans [79]). La semence, préalablement refroidie à 4°C, est généralement congelée jusqu'à une température de -140°C pour ensuite être directement plongée dans l'azote liquide (-196°C).

Afin de décongeler la semence, les paillettes entreposées dans l'azote liquide sont généralement transférées dans un bain d'eau à 37°C pendant une minute. La semence ainsi décongelée peut être directement utilisée pour l'insémination artificielle. Lors de la décongélation de la semence, la glace présente dans le milieu extracellulaire fond, diminuant rapidement la concentration des solutés présents dans le milieu extracellulaire [5]. De l'eau va alors pénétrer dans les spermatozoïdes, ce qui provoque des fluctuations du volume cellulaire causant des dommages aux membranes des spermatozoïdes [5, 84]. Lors de leur réchauffement, les membranes des spermatozoïdes vont encore une fois subir un changement de phase (phase gel à la phase liquide-cristalline) ce qui provoque une réorganisation des lipides et des protéines membranaires rendant les membranes spermatiques moins fonctionnelles

[5]. De plus, le réchauffement des spermatozoïdes provoque des dommages au niveau de la structure de l'acrosome [84] et des ruptures des membranes spermatiques [94].

Les dommages membranaires causés par la cryopréservation influencent le pouvoir fécondant des spermatozoïdes. Par exemple, la cryopréservation induit des modifications chez les spermatozoïdes qui ressemblent aux caractéristiques de la capacitation. En effet, les spermatozoïdes qui ont étés refroidis et réchauffés se comportent comme s'ils avaient été capacités [84]. Ce phénomène est appelé cryocapacitation [95] et est caractérisé par une augmentation du Ca^{2+} intracellulaire [95] et une augmentation de la proportion de spermatozoïdes ayant le patron B (patron observé au microscope caractérisant les spermatozoïdes capacités lorsque déterminé avec une coloration au CTC) [96, 97]. De plus, la cryopréservation induit une diminution de la capacité des spermatozoïdes à lier les cellules épithéliales de l'oviducte [98] et induit la perte de protéines présentes à la surface des spermatozoïdes [99-101].

En résumé, lors de la cryopréservation de la semence, les membranes spermatiques subissent des stress thermiques, mécaniques, chimiques et osmotiques qui résultent en des dommages membranaires irréversibles qui sont néfastes pour le pouvoir fécondant des spermatozoïdes.

1.6.1.3. Effet de la composition lipidique des membranes des spermatozoïdes

Selon les espèces, il existe des différences de susceptibilité des spermatozoïdes aux dommages membranaires causés par la conservation entre. Ces différences

résulteraient des variations dans la composition lipidique des membranes des spermatozoïdes des différentes espèces. En effet, les spermatozoïdes de taureau et de bélier, reconnus pour leur faible taux de cholestérol membranaire, sont moins résistants au refroidissent que les spermatozoïdes de lapin et d'humain, lesquels ont un niveau de cholestérol membranaire plus élevé [102]. De plus, des études ont démontré qu'une diminution du contenu en cholestérol des membranes des spermatozoïdes diminue la résistance des spermatozoïdes au choc dû au froid [103]. Le ratio cholestérol:phospholipide de la membrane du spermatozoïde est important pour déterminer la fluidité membranaire et la susceptibilité de la membrane aux dommages causés par le refroidissement. Le cholestérol stabilise la membrane en interagissant avec les chaînes d'acide gras des phospholipides [104]. Lorsque la membrane contient moins de cholestérol, elle devient moins stable et plus susceptible à se briser sous l'effet du refroidissement. De la même manière, la susceptibilité au choc dû au froid est aussi corrélée avec un ratio élevé de lipides insaturés:saturés [7]. Les acides gras saturés sont plus rigides que les acides gras insaturés. Une quantité élevée d'acides gras saturés dans les membranes spermatiques les rend donc plus résistantes aux bris causés par les changements de température.

1.6.1.4. Les espèces réactives de l'oxygène (ROS)

Les ROS ont un effet bénéfique sur les spermatozoïdes car ils jouent un rôle physiologique dans la cascade d'évènements menant à la capacitation et à la réaction de l'acrosome [105]. Cependant, les ROS sont aussi connues pour causer des dommages oxydatifs à l'ADN et aux lipides membranaires ainsi que pour inactiver des

protéines. Pendant la cryopréservation, des ROS sont générées. Ces ROS peuvent causer des dommages oxydatifs aux spermatozoïdes. Les spermatozoïdes sont très susceptibles aux dommages causés par les ROS. En effet, les membranes des spermatozoïdes contiennent une grande proportion d'acides gras insaturés susceptibles d'être oxydés. De plus, les spermatozoïdes sont incapables de remplacer les acides gras ayant été oxydés car ils sont incapables de synthétiser des lipides (revu dans [79]). De plus, les spermatozoïdes cryopréservés ne peuvent pas se défendre convenablement contre les ROS car leurs niveaux de défenses antioxydantes sont diminués comparativement à ceux des spermatozoïdes fraîchement éjaculés [106, 107]. Les dommages oxydatifs causés par les ROS sont néfastes pour les fonctions spermatiques et peuvent résulter en une diminution du pouvoir fécondant. Par exemple, l'augmentation de la peroxydation des lipides membranaires des spermatozoïdes est associée à une perte de motilité.

1.6.2. Les diluants protecteurs

Afin de protéger les spermatozoïdes pour qu'ils survivent et demeurent fertiles pendant l'entreposage à 4°C et/ou la cryopréservation, la semence doit être diluée avec un diluant protecteur.

La cryopréservation des spermatozoïdes a débuté il y a plus de 60 ans avec la découverte du jaune d'œuf comme agent protecteur pendant le refroidissement [108] et du glycérol comme agent protecteur pendant la congélation [109]. Aujourd'hui, les diluants protecteurs sont généralement composés d'un tampon qui maintient le pH extracellulaire entre 6,7 et 7,0 et l'osmolalité entre 320 et 350 mOsm, d'antibiotiques

afin d'empêcher la croissance bactérienne pendant l'entreposage, d'un agent cryoprotecteur tel que le glycérol et d'un agent protégeant les spermatozoïdes contre le choc dû au froid tel que le jaune d'œuf. Les tampons entrant dans la composition des diluants protecteurs sont généralement des tampons à base de phosphate, de citrate de sodium ou de Tris [78]. Les diluants dont l'utilisation est la plus répandue sont: 1) le diluant Tris-glycérol (200 mM Tris, 5.6% glycérol) contenant 20% de jaune d'œuf et 2) le diluant à base de lait de vache (lait entier ou écrémé, 5-8% glycérol). Ces deux diluants sont utilisés pour conserver la semence de plusieurs espèces de mammifères telles que le bovin, l'humain, le mouton, la chèvre, le cheval, le chien, etc. [82, 84]. L'utilisation du lait comme diluant pour la conservation de la semence bovine a été décrit pour la première fois en 1940 par Phillips et Lardy [108]. Le lait joue le rôle de tampon et d'agent protégeant contre les chocs dûs au froid [110-112]. Avant d'être utilisé dans un diluant, le lait, écrémé ou non, doit être maintenu pendant 10 minutes à une température de 95°C afin d'inactiver la lacténine, une substance qui est toxique pour les spermatozoïdes [113]. Après avoir été chauffé, le lait est refroidi et ensuite filtré afin d'enlever le coagulum formé sous l'action de la chaleur. Les diluants protecteurs contenant du lait sont tout aussi efficaces que les diluant contenant du jaune d'œuf [114]. Cependant, les spermatozoïdes ayant étés dilués avec le diluant contenant du lait sont moins bien visibles aux microscopes que ceux ayant étés dilués avec le diluant contenant du jaune d'œuf rendant difficile l'évaluation de la semence au microscope [78, 91].

Le glycérol fait partie des agents cryoprotecteurs perméants (qui pénètrent à l'intérieur des cellules) et est l'agent cryoprotecteur le plus efficace et le plus utilisé jusqu'à aujourd'hui. D'autres agents cryoprotecteurs perméants tels que le diméthylsulfoxyde, l'éthylène glycol ou le 1,2 propanédiol ont été utilisés pour la congélation des spermatozoïdes [79, 91, 115].

1.6.3. Composition des agents protecteurs

1.6.3.1. Le jaune d'œuf

Le jaune d'œuf est composé d'eau, de protéines, de lipides, de glucides (principalement du glucose), de minéraux et de vitamines [116].

Le constituant majeur du jaune d'œuf est la fraction des lipoprotéines de faible densité (LDF). Les LDF représentent environ 2/3 de la matière solide du jaune d'œuf de poule et sont localisées dans le plasma, la fraction soluble du jaune d'œuf. La densité des LDF du jaune d'œuf est de 0,982 mg/ml. Les LDF du jaune d'œuf sont des molécules sphériques de 17-60 nm de diamètre composées de 83-89% de lipides et de 11-17% de protéines [117]. Les lipides retrouvés dans les LDF du jaune d'œuf sont les triglycérides (69%), les phospholipides (26%) et le cholestérol (5%) [117]. Les triglycérides sont situés au centre de la molécule de LDF et sont entourés d'une monocouche lipidique composée de cholestérol et des phospholipides (72% de phosphatidyl-choline, 19% de phosphatidyl-éthanolamine et 9% de lysophosphatidyl-choline, de lysophosphatidyl-éthanolamine et sphingomyéline) [118]. Les protéines, insérées dans la monocouche lipidique entourant les triglycérides, sont nommées

apovitellinine I, II, III, IV, V et VI [119]. L'apovitellinine I et II sont des glycoprotéines qui proviennent du clivage de la vitellogénine synthétisée dans le foie tandis que les apovitellinines III, IV, V et VI proviennent du clivage de l'apolipoprotéine B [119].

Le plasma du jaune d'œuf, qui est constitué d'eau, contient aussi d'autres particules appelées granules. Les granules sont composés de protéines appelées vitellines qui sont associées à des lipides et qui sont complexées avec des ions Ca^{2+} et Fe^{2+} ainsi que des glycophosphoprotéines appelées phosvitines [119]. Le jaune d'œuf contient aussi des protéines solubles dans son plasma, les livetines α, β et γ, qui ne sont pas associées à des lipides [119]. Ensemble, les granules et les livetines composent 1/3 de la matière solide du jaune d'œuf [119]. Le plasma du jaune d'œuf contient aussi des minéraux dont les principaux sont le PO_4^-, le Cl^-, le K^+, le Na^+ et le Mg^{2+} [116].

1.6.3.2. Le lait

Le lait entier est un fluide biologique complexe composé de cinq principaux constituants: l'eau (90%), les lipides (3,7%), les protéines (3,2%), les glucides (4,6%, principalement du lactose) et les minéraux (0,8%,) [120]. Le lait contient aussi quelques constituants en quantité moins importante tels que des enzymes, des vitamines, des gaz et des pigments. Le lait écrémé est composé des mêmes constituants que le lait entier à l'exception des lipides. Plus précisément, le lait écrémé contient 99,5% moins de lipides que le lait entier.

La matière grasse du lait de vache est principalement composée de triglycérides (98%), de phospholipides (1%) et de cholestérol (0.4%) [120]. Parmi les phospholipides, on retrouve des phosphatidyl-choline (33%), des phosphatidyl-éthanolamine (33%), des phosphatidyl-sérine (0.3%), des phosphatidyl-inositol (0.5%) et des shpingomyélines (25%). Ces lipides sont retrouvés dans le lait sous forme de petites gouttelettes sphériques de 0,1 à 20 μm de diamètre [120]. Chacune de ces gouttelettes est formée de triglycérides entourés d'une enveloppe de phospholipides et de protéines [121]. La réduction du diamètre des gouttelettes par l'homogénéisation prévient la séparation du gras du lait de la partie liquide du lait.

La concentration en protéines totales du lait est d'environ 30 mg/ml [120]. Les protéines majeures du lait sont des phosphoprotéines appelées caséines et représentent 80% des protéines totales. Dans le lait, les caséines sont retrouvées sous forme de grosses particules colloïdales appelées micelles. Ces agrégats de caséines sont gros avec un diamètre variant de 90 à 150 nm et sont complexés avec du calcium et du phosphate inorganique. Les quatre principales protéines composant les micelles sont les caséines αs1, αs2, β et κ et sont retrouvées dans les proportions respectives suivantes: 4:1:3,6:1,4 [122]. Le modèle le plus accepté afin d'expliquer la structure des micelles de caséines implique que les micelles soient formées de sous-micelles reliées ensemble par des ponts phosphate de calcium. Quant à elles, les sous-micelles sont formées d'un cœur hydrophobe de caséines α et β qui est majoritairement recouvert de caséines κ (revu dans [122]).

Le lait contient une autre catégorie de protéines appelée protéines du sérum qui représente environ 20% des protéines totales contenues dans le lait. Ces protéines sont moins hydrophobes que les caséines et ne sont pas retrouvées sous forme de micelle. Ces protéines sont l'α-lactalbumine, la β-lactoglobuline, l'albumine sérique bovine, les immunoglobulines et la lactoferrine [120]. De plus, le lait contient quelques enzymes [120].

1.6.4. Mécanismes impliqués dans la protection des spermatozoïdes

1.6.4.1. La protection par le glycérol

Le mécanisme d'action du glycérol dans la protection des spermatozoïdes lors de la congélation a été discuté par plusieurs auteurs [79, 84, 94]. Le glycérol est un agent cryoprotecteur perméant. Il diffuse à l'intérieur de la cellule et diminue la température de nucléation de l'eau intracellulaire, diminuant ainsi les chances de formation de glace intracellulaire connue pour être létale pour les cellules. Il diminue aussi la température à laquelle les membranes des spermatozoïdes subit son changement de phase (passage de la phase liquide-cristalline à la phase gel) lors du refroidissement. Ainsi, les membranes des spermatozoïdes demeurent fluide jusqu'à des températures plus faibles que lorsqu'elle n'est pas en présence de glycérol, diminuant ainsi les chances de cassures membranaires lors du refroidissement. De plus, il atténue les changements de volume cellulaire subis par les spermatozoïdes sous l'effet de la concentration croissante des solutés présents à l'extérieur des spermatozoïdes lors de la congélation de l'eau extracellulaire [92].

1.6.4.2. La protection par le jaune d'œuf

Comme mentionné précédemment, le jaune d'œuf protège les spermatozoïdes contre les chocs dus au froid [123]. Il est un agent protecteur non-perméant qui reste à l'extérieur de la cellule. Il prévient l'enroulement du flagelle du spermatozoïde et protège la motilité [124]. Le constituant du jaune d'œuf responsable de la protection des spermatozoïdes pendant la cryopréservation est la fraction des LDF [125-127]. Les LDF isolés à partir du jaune d'œuf sont plus efficaces [128] ou aussi efficaces [90, 126, 127] que le jaune d'œuf entier afin de protéger les spermatozoïdes bovins pendant le refroidissement et la congélation.

Malgré le fait que les LDF semblent être responsables de la protection offerte par le jaune d'œuf, le mécanisme reste inconnu. Une première hypothèse propose que ce soit l'association très étroite des lipides des LDF avec la membrane des spermatozoïdes qui stabiliserait la membrane et permettrait la protection des spermatozoïdes. En effet, les LDF pourraient adhérer à la membrane des spermatozoïdes pendant le processus de cryopréservation [90, 129]. Malgré qu'il y ait des études démontrant la liaison des LDF du jaune d'œuf sur les spermatozoïdes pendant l'entreposage de la semence, on ne sait pas comment les LDF liés aux spermatozoïdes protègent les spermatozoïdes. Cependant, il y a des résultats contradictoires concernant la stabilité de la liaison des LDF aux spermatozoïdes [90, 130, 131].

Une deuxième hypothèse est à l'effet que les LDF offriraient une protection aux spermatozoïdes via leurs phospholipides qui formeraient un film protecteur à la

surface des spermatozoïdes [132] ou qui répareraient les dommages membranaires en remplaçant les phospholipides perdus ou endommagés pendant le processus de cryopréservation [129, 133]. Cependant, l'utilisation de liposomes composés de phospholipides portant un groupement choline et de cholestérol ne mime pas l'effet cryoprotecteur du jaune d'œuf [123]. De plus, l'utilisation de diluants contenant des phospholipides d'origine végétale portant un groupement choline en remplacement du jaune d'œuf, par exemple le diluant Biociphos® qui contient des extraits de fève de soya, est moins efficace que les diluants contenant du jaune d'œuf pour la cryopréservation des spermatozoïdes bovins [134, 135]. Ainsi, le rôle protecteur des LDF ne résiderait pas seulement au niveau du remplacement des phospholipides portant un groupement choline perdus ou endommagés pendant la cryopréservation.

Une troisième hypothèse propose que le jaune d'œuf entrerait en compétition avec des agrégats de peptides cationiques (< 5 kDa) présents dans le PS et néfastes pour les spermatozoïdes [136]. En présence de jaune d'œuf, les sites de liaisons pour des facteurs cationiques présents à la surface des spermatozoïdes seraient occupés par des facteurs cationiques du jaune d'œuf, empêchant ainsi les peptides cationiques du PS connus pour inhiber la motilité de se lier aux spermatozoïdes [137].

1.6.4.3. La protection par le lait

Après le jaune d'œuf, le lait est l'agent protecteur le plus utilisé pour la congélation des spermatozoïdes. Il est à noter que le lait entier et le lait écrémé sont aussi efficaces l'un que l'autre lorsque utilisés comme diluant lors de l'entreposage à 4°C ou la cryopréservation des spermatozoïdes [111, 112, 114]. Ce ne sont donc pas

les lipides présents dans le lait entier qui sont responsables de la protection des spermatozoïdes pendant l'entreposage de la semence. En effet, il semble que ce soient les micelles de caséines présentes dans le lait qui protègent les spermatozoïdes contre les chocs dus au froid [138, 139]. Cependant, les mécanismes par lesquels les micelles de caséines protègent les spermatozoïdes ne sont pas connus et, contrairement au jaune d'œuf, aucune hypothèse n'a été émise quant à ces mécanismes.

1.6.5. Amélioration de l'efficacité des diluants protecteurs

Depuis la découverte du jaune d'œuf, du lait et du glycérol comme agents protecteurs, plusieurs tentatives ont été faites dans le but d'améliorer l'efficacité des diluants utilisés pour la cryopréservation. Cependant, ces tentatives ont été peu fructueuses et résultent seulement en une faible amélioration du pourcentage de spermatozoïdes motiles obtenus après congélation [79, 94].

Plusieurs polysaccharides (fructose, glucose, raffinose, galactose, etc.) ont été utilisés comme agents cryoprotecteurs non-perméants pour la congélation des spermatozoïdes (revue par [140]). Ils participent à l'équilibre osmotique du diluant et stabiliseraient les membranes des spermatozoïdes [78]. En effet, l'ajout de lactose (revue par [140]), de sucrose [141, 142], ou de tréhalose [142] dans le diluant protecteur utilisé pour la congélation de la semence bovine augmente le pourcentage de spermatozoïdes motiles obtenu après dégel de la semence. Cependant, l'amélioration du pourcentage de spermatozoïdes motiles étant faible, l'utilisation de polysaccharides dans les diluants protecteurs n'est pas généralisée. Chez le bouc, le jaune d'œuf peut même être substitué par du tréhalose lors de la congélation des

spermatozoïdes car il protège efficacement les spermatozoïdes en augmentant leur fluidité membranaire [143]. De la même manière, le lactose [144] et le tréhalose [145] ont été démontrés comme étant capables de protéger les spermatozoïdes épididymaires de souris pendant la cryopréservation.

L'incubation des spermatozoïdes bovins avec de la méthyl-β-cyclodextrine chargée de cholestérol avant la cryopréservation améliore le pourcentage de spermatozoïdes motiles obtenus après congélation [146]. Les cyclodextrines sont des molécules hydrophiles ayant la capacité de transporter du cholestérol dans leur structure. Lorsque des cellules sont mises en présence de cyclodextrines chargées de cholestérol, du cholestérol est transféré à la membrane cellulaire. Tel que décrit à la section 1.6.1.3, un niveau de cholestérol membranaire élevé augmente la résistance des spermatozoïdes au choc dû au froid. Les spermatozoïdes ayant été incubés avec la méthyl-β-cyclodextrine chargée de cholestérol sont donc plus résistants à la congélation.

L'ajout de substances anti-oxydantes dans les diluants contenant du jaune d'œuf et du glycérol améliore la qualité des spermatozoïdes ayant été congelés. En effet, l'ajout d'hydroxytoluène [147], de catalase [148], de vitamine E [149] et d'ascorbate de sodium [149] dans le diluant améliore le pourcentage de spermatozoïdes motiles obtenu après cryopréservation. L'ajout de substances antioxydantes dans le diluant protecteur empêcherait les ROS de causer des dommages oxydatifs aux acides nucléiques et aux membranes spermatiques améliorant ainsi la qualité de la semence cryopréservée. Cependant, l'ajout de substances antioxydantes

dans des diluants à base de lait n'améliore pas la qualité de la semence après cryopréservation [111, 148].

L'ajout d'acides aminés tels que la proline, la glutamine ou la bétaïne (un dérivé triméthylé de l'acide aminé glycine et portant un groupement pseudocholine) dans le diluant Tris-glycérol contenant du jaune d'œuf, améliore le pourcentage de spermatozoïdes motiles obtenus après cryopréservation [150, 151]. De plus, il a aussi été démontré que lorsque des spermatozoïdes fraîchement éjaculés sont incubés dans le diluant Tris-glycérol contenant du jaune d'œuf à des températures variant de 0 à 20°C, l'ajout de bétaïne améliore le pourcentage de spermatozoïdes motiles [152]. Cependant, le mécanisme d'action de ces acides aminés sur les spermatozoïdes est inconnu.

Il a aussi été démontré que le tampon Tris peut être substitué par de l'eau provenant de noix de coco lors de la congélation des spermatozoïdes de chien dans un diluant Tris-glycérol jaune d'œuf [153]. L'avantage à utiliser l'eau de coco comme tampon réside dans le fait qu'elle est peu coûteuse et que contrairement au tampon Tris, elle ne demande pas de préparation.

1.7. Problématique du projet de recherche

Depuis quelques années, il y a une demande grandissante pour l'utilisation de diluants cryoprotecteurs exempts de produits d'origine animale. En effet, l'utilisation de jaune d'œuf ou de lait dans la composition des diluants cryoprotecteurs utilisés pour la congélation de la semence de taureaux représente un risque potentiel de

contamination des doses d'insémination par des bactéries ou des mycoplasmes [154]. Les pays importateurs de semence se sentent donc concernés par le risque d'introduire dans leur pays des maladies provenant de produits contenant du jaune d'œuf et du lait [155]. De plus, plusieurs autres désavantages sont associés à l'utilisation du jaune d'œuf ou du lait dans les diluants protecteurs. La production d'endotoxines qui est causée par la contamination microbienne par le jaune d'œuf est néfaste pour le pouvoir fécondant des spermatozoïdes [154] et un facteur contenu dans le jaune d'œuf inhibe la respiration des spermatozoïdes [156]. De plus, la composition du jaune d'œuf et du lait est souvent variable, rendant difficile la comparaison des résultats obtenus dans différentes études voulant tester l'efficacité des diluants protecteurs. Il y aurait donc plusieurs avantages à trouver de nouveaux agents protecteurs dont la composition est plus uniforme et qui sont exempts d'agents pathogènes et de substances d'origine animale afin de les inclure dans les diluants utilisés pour la congélation de la semence. Cependant, les mécanismes par lesquels le jaune d'œuf et le lait protègent les spermatozoïdes de mammifère sont encore inconnus. Il est donc difficile de leur trouver des substances de remplacement. Des diluants contenant des phospholipides d'origine végétale en remplacement du jaune d'œuf ont été utilisés pour la cryopréservation des spermatozoïde mais ne sont pas aussi efficaces que le diluant Tris-glycérol jaune d'œuf [134, 135, 154].

Le but de cette étude est de trouver par quel mécanisme le jaune d'œuf protège les spermatozoïdes pendant le processus de cryopréservation.

1.7.1. Hypothèses

Des travaux de notre laboratoire ont démontré que les protéines BSP contenues dans le PS stimulent un efflux du cholestérol et des phospholipides de la membrane des spermatozoïdes épididymaires et que cet efflux lipidique est directement proportionnel à la concentration des protéines BSP et à la durée de l'incubation des spermatozoïdes en présence des protéines BSP. Nous avons donc proposé un nouveau mécanisme de protection des spermatozoïdes par le jaune d'œuf. Notre hypothèse était à l'effet qu'un contact continu entre le PS et les spermatozoïdes éjaculés peut être néfaste pour ces derniers car il provoque un efflux continu des lipides de la membranaires et que le jaune d'œuf protègerait les spermatozoïdes grâce à des facteurs qui peuvent lier les protéines BSP et ainsi les empêcher de se lier aux des spermatozoïdes et de provoquer l'efflux des lipides membranaires.

1.7.2. Objectifs expérimentaux

Les objectifs expérimentaux de la présente étude étaient de:

1) Déterminer si des substances présentes dans le jaune d'œuf peuvent lier les protéines BSP.

2) Déterminer si le jaune d'œuf protège les spermatozoïdes bovins en empêchant les protéines BSP de se lier aux spermatozoïdes et de provoquer un efflux des lipides membranaires.

3) Déterminer si les mécanismes impliqués dans la protection des spermatozoïdes par le jaune d'œuf est le même chez les mammifères autres que le bovin.

Ces travaux permettront une meilleure compréhension des mécanismes impliqués dans la protection des spermatozoïdes par le jaune d'œuf pendant l'entreposage de la semence. Par conséquent, il sera aussi plus facile de trouver des agents protecteurs d'origine non animale afin de remplacer le jaune d'œuf dans les diluants protecteurs et il sera plus facile d'améliorer l'efficacité des diluants protecteurs généralement utilisés pour la conservation des spermatozoïdes.

2. ARTICLES

2.1 Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk.

2.1 Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. Manjunath P., Nauc V., Bergeron A. et Ménard M. *Biology of Reproduction*, 67:1250-1258, 2002.

Préambule:

Malgré l'utilisation depuis plus de 60 ans du jaune d'œuf comme agent protecteur dans les diluants utilisés pour la conservation des spermatozoïdes de mammifères, le mécanisme par lequel le jaune d'œuf protège les spermatozoïdes est encore inconnu. Le jaune d'œuf est composé majoritairement de LDF et il a été démontré que ce sont ces LDF qui sont responsables de la protection apportée aux spermatozoïdes par le jaune d'œuf lors de l'entreposage de la semence. Les principales protéines du PS bovin sont les protéines BSP (BSP-A1/A2, BSP-A3 et BSP-30kDa). Ces protéines représentent environ 65% des protéines totales du PS bovin et stimulent un efflux des lipides membranaires qui peut être néfaste pour les spermatozoïdes. Dans l'étude présentée dans cette section, nous avons démontré pour la première fois que les protéines BSP lient les LDF du jaune d'œuf. Cette liaison est spécifique, saturable et stable pendant la congélation. Nous avons suggéré que la liaison des protéines BSP avec les LDF est bénéfique pour les fonctions spermatiques.

Ces travaux étaient en cours lors de mon arrivée dans l'équipe du Dr. Manjunath. Mes premiers travaux au sein du laboratoire ont démontré la spécificité de l'interaction des LDF du jaune d'œuf avec les protéines BSP ainsi que la saturabilité

de la liaison des protéines BSP aux LDF du jaune d'œuf. Ceci m'a permis de produire les figures 2, 3 et 4 de l'article présenté dans cette section. J'ai aussi participé à l'écriture de l'article ainsi qu'à l'interprétation des résultats. Nauc V. et Ménard M. ont participé aux travaux ayant généré les figures 1, 5, 6 et 7 ainsi qu'à l'écriture de la première ébauche de l'article.

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Major Proteins of Bovine Seminal Plasma Bind to the Low-Density Lipoprotein Fraction of Hen's Egg Yolk[1]

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ABSTRACT

Over the past 60 years, egg yolk (EY) has been routinely used in both liquid semen extenders and those used to cryopreserve sperm. However, the mechanism by which EY protects sperm during liquid storage or from freezing damage is unknown. Bovine seminal plasma contains a family of proteins designated BSP-A1/-A2, BSP-A3, and BSP-30-kDa (collectively called BSP proteins). These proteins are secretory products of seminal vesicles that are acquired by sperm at ejaculation, modifying the sperm membrane by inducing cholesterol efflux. Because cholesterol efflux is time and concentration dependent, continuous exposure to seminal plasma (SP) that contains BSP proteins may be detrimental to the sperm membrane, which may adversely affect the ability of sperm to be preserved. In this article, we show that the BSP proteins bind to the low-density fraction (LDF), a lipoprotein component of the EY extender. The binding is rapid, specific, saturable, and stable even after freeze-thawing of semen. Furthermore, LDF has a very high capacity for BSP protein binding. The binding of BSP proteins to LDF may prevent their detrimental effect on sperm membrane, and this may be crucial for sperm storage. Thus, we propose that the sequestration of BSP proteins of SP by LDF may represent the major mechanism of sperm protection by EY.

gamete biology, male reproductive tract, seminal vesicles, sperm, sperm maturation

INTRODUCTION

The cryopreservation of bull semen [1, 2] represents one of the most important achievements in dairy farming after the introduction of artificial insemination. These two approaches have enabled the worldwide distribution and use of desired genetic lines at a reasonable cost. Over the past 60 years, the cryoprotective media for sperm storage have been continuously revised, but the basic ingredients of the media remain unchanged. The egg yolk (EY) and glycerol represent the indispensable compounds of practically all media used for bull sperm preservation in liquid or frozen states. It is also clear that the interaction between the sperm and surrounding medium is a crucial factor affecting the preservation of sperm integrity and fertilizing ability.

The role of glycerol in cryopreservation is that it contributes to sperm integrity conservation [3, 4]; however, the protection afforded by EY is more complex. The EY has been shown to increase the sperm fertilizing ability when present in extenders for semen storage at ambient temperature [5–8] and appears to prevent sperm cell damage at cooling and freezing [9–11]. Various components of EY have been investigated to identify the most active component(s) responsible for the protective effect [11–19]. Evidence indicates that the low-density lipoprotein fraction (LDF), characterized biochemically by Banaszac et al. [20] and Kuksis [21], shows the highest protective ability; however, the mechanism by which this protection is provided to sperm remains elusive. It is speculated that the LDF associates with sperm membranes and provides protection against membrane damage, but there is contradictory evidence concerning the stability of this association [12, 22–24]. Vishwanath et al. [25] suggest that EY lipoproteins compete with detrimental seminal plasma (SP) cationic peptides (<5 kDa) in binding to the sperm membrane and thus protect the sperm.

Seminal plasma, which facilitates the transport of sperm in the female genital tract, also contains factors that influence sperm motility [26] and fertility [27, 28]. In addition, SP also appears to be detrimental for sperm storage [29–31]. Our studies indicate that the major protein fraction (50–70% or 35–50 mg/ml) of bovine seminal plasma is represented by a family of related proteins, designated BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa (collectively called BSP proteins) [32–34]. The biochemical characteristics of BSP proteins have been well described [34, 35]. They bind to sperm membrane choline phospholipids at ejaculation [36, 37]. They also bind to capacitation factors such as high-density lipoprotein (HDL) and heparin [34, 38, 39], and the BSP proteins potentiate sperm capacitation induced by HDL and heparin [40, 41]. Thus, BSP proteins are beneficial for sperm function. In contrast, our recent studies also show that the BSP proteins induce changes in sperm plasma membrane by stimulating cholesterol and phospholipid efflux [42, 43]. This lipid efflux by BSP proteins is time and concentration dependent. Continuous exposure of sperm to SP that contains BSP proteins is detrimental to the sperm membrane, and this may render the membrane very sensitive to sperm storage in liquid or frozen states (cryopreservation). Therefore, BSP proteins in SP have the potential to act as both beneficial and detrimental factors to sperm depending on the concentration of SP and exposure time.

In the present study, we show that the BSP proteins, the major proteins of SP, interact with the LDF, the major com-

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ponent of EY extender. We discuss how this interaction could protect sperm and influence sperm storage.

MATERIALS AND METHODS

Materials

BSA (fraction V), myoglobin, and lactoperoxidase were from Sigma (St. Louis, MO). $Na^{125}I$ was bought from Amersham (Oakville, ON, Canada). Anti-rabbit gamma globulin (goat anti-RGG) was from Medicorp Inc. (Montréal, PQ, Canada). Sephadex G-25, Sepharose CL-4B, lysozyme (egg white), and the low molecular weight (LMW) electrophoresis calibration kit were from Pharmacia Biotech Inc. (Baie d'Urfé, PQ, Canada). Acrylamide, bisacrylamide, SDS, and other electrophoresis products were obtained from Bio-Rad (Mississauga, ON, Canada). Polyethylene glycol (PEG) was from ICN Biomedicals, Inc. (Cleveland, OH). Centriflo CF-25 cones and PM-10 and YM-3 ultrafiltration membranes were from Millipore (Bedford, MA). The immobilon-P membrane and enhanced chemiluminescence (ECL) reagent kit were purchased from Mandel Scientific (Boston, MA). Tween-20 (enzyme grade) and EDTA were from Fisher Scientific (Nepean, ON, Canada). All other chemicals used were of analytical grade and were obtained from commercial suppliers.

Bull semen collected with an artificial vagina, EY Tris-glycerol (EYTG) extender (20% EY, 6% glycerol, 200 mM Tris, pH 6.7), and semen cryopreserved in EYTG extender were obtained from the Centre d'Insémination Artificielle du Québec Inc. (St. Hyacinthe, PQ, Canada). Crude seminal plasma proteins (cBSP) were prepared by ethanol precipitation of bovine seminal plasma followed by lyophilization. This preparation consists of 50–70% BSP proteins. The purification of the BSP-A1/-A2, -A3, and -30-kDa was done as described previously [33], and their purity was assessed by SDS-PAGE [44].

Preparation of BSP Protein-Depleted Seminal Plasma

To prepare the BSP protein-depleted bovine seminal plasma, 100 mg of cBSP was dissolved in 5 ml of 50 mM phosphate-buffered saline (PBS) and loaded onto a gelatin-agarose column (2.5 × 27 cm). The column was washed with the same buffer, and the unbound material (designated dBSP), consisting of all seminal plasma proteins but depleted with the BSP proteins, was dialyzed against ammonium bicarbonate and was lyophilized. This fraction generally contains less than 1% of the BSP proteins. The gelatin-agarose adsorbed proteins (designated GA-BSP) were eluted with 7 M urea, dialyzed against ammonium bicarbonate, and lyophilized. This fraction consists of a mixture of the BSP-A1/-A2, -A3, and -30-kDa proteins in the proportion (~8:1:1) that is generally present in the SP.

Preparation of Seminal Plasma

Fresh semen was diluted with nine volumes of 50 mM PBS, pH 7.4, and centrifuged in a microcentrifuge (Eppendorf, model 5415C) at low speed (5000 × *g*, 5 min). The supernatants were transferred into Eppendorf tubes, recentrifuged (10 000 × *g*, 10 min) to eliminate the remaining cells, and stored at −20°C until further analysis of SP proteins.

Isolation of Egg Yolk Lipoprotein

Egg yolk was separated from egg white and any adhering albumin was removed by blotting on filter paper. The yolk membrane was broken and the liquid yolk was collected. The liquid yolk was diluted 10-fold with 10 mM Tris-HCl (pH 7.4), the density was raised to 1.21 g/ml by adding solid potassium bromide, and the substance transferred into 11.5-ml Quick Seal tubes (Mandel Scientific Co., Guelph, ON, Canada). The tubes were centrifuged (Sorvall Ultracentrifuge; Rotor T-865) for 20 h at 60 000 rpm at 20°C. After centrifugation, the lipoproteins (designated low-density fraction, LDF) concentrated at the top of the tube were retrieved. The fraction was extensively dialyzed against 10 mM Tris-HCl (pH 7.4) and preserved at 4°C. The LDF was also isolated from 10× diluted (in 10 mM Tris-HCl buffer, pH 7.4) EYTG extender used routinely for cryopreservation of bull sperm by the same ultracentrifugation procedure. The protein concentration in LDF was determined by the modified Lowry procedure [45].

Agarose-Gel Electrophoresis

The interaction between LDF and BSP proteins or other proteins was studied using the Paragon electrophoresis kit (Beckman Instruments, Fullerton, CA). Lipo gels (0.5% agarose) and SPE (serum protein electrophoresis) gels (1% agarose) were used for lipoprotein and protein analysis, respectively. LDF was incubated in the presence or absence of cBSP or purified BSP proteins or other control proteins (BSA, myoglobin, lysozyme, heat-denatured BSP-A1/-A2, dBSP). After 3 min, 3–4 µl of incubation mixture were applied to each template slot. Agarose gels were subjected to electrophoresis for 30 min at 100 V, then immersed in fixative solution and dried. The LDF was visualized by lipid staining in Sudan Black B solution. The protein bands were revealed by staining in Paragon Blue solution.

Gel Filtration Chromatography

Gel filtration chromatography was carried out on a Sepharose CL-4B column (70 × 2.5 cm) equilibrated with PBS at a flow rate of 80 ml/h. After a 40-min wait, fractions of 3 ml were collected and the absorbance was determined at 280 nm. Elution profiles of SP proteins, purified BSP-A1/-A2, and dBSP were analyzed before and after incubation with LDF. The column was calibrated using blue dextran (M_r 2×10^6), thyroglobulin (M_r 669 000), BSA (M_r 66 000), and soy bean trypsin inhibitor (M_r 20 000).

Immunoblot Analysis of BSP Proteins

The chromatography fractions under elution profile corresponding to peaks a, b, and c (Fig. 5) were pooled separately and concentrated using Centriflo CF-25, PM-10, and YM-3 membranes, respectively. Aliquots of each concentrated fraction were delipidated using *n*-butanol/di-isopropyl ether (15:85 v/v) solvent. The proteins were separated on SDS polyacrylamide gels and were transferred onto Immobilon-P membrane according to the method of Towbin et al. [46]. Immunodetection was done with specific polyclonal antibodies against each BSP protein as described previously [35, 47] by using an ECL reagent kit.

Radioimmunoassay of BSP Proteins

The total content of each BSP protein in peaks a, b, and c (Fig. 5) were determined by radioimmunoassay (RIA) as described recently [47]. All reagents for RIA were diluted in the immunoassay buffer (50 mM phosphate buffer, pH 7.4, containing 5 mM EDTA, 0.45% NaCl, 0.25 mg/ml sodium azide, 0.5 ml/l Tween-20, and 0.1% BSA). The primary antibodies were used in the following dilutions: 1:1000 for anti-BSP-A1/-A2 and anti-BSP-30-kDa and 1:10 000 for anti-BSP-A3. After overnight (20-h) incubation, 50 µl of secondary antibodies (10%) were added, followed by another incubation for 12–16 h. At the end of the second incubation, 500 µl of 10% PEG-8000 were added to each tube (except total counts) and vortexed, and tubes were centrifuged at 2200 × *g* for 15 min. The supernatant was aspirated and the radioactivity associated with the pellet was determined in a gamma counter (1272 CliniGamma, Pharmacia Wallac, Finland).

Isolation of LDF-BSP Complex from Cryopreserved Semen

Cryopreserved semen in straws was subjected to a thawing procedure (40°C water bath, 1 min). The SP along with cryoprotective extender were separated from sperm by centrifugation at 3000 × *g* for 10 min and were then recentrifuged at 10 000 × *g* for 10 min to remove any remaining cell debris. The supernatant was subjected to ultracentrifugation as described previously to separate LDF-BSP protein complex (top 2 ml, yellow fraction) and the bottom opalescent fraction (~9–10 ml). Some white precipitates settled at the bottom of the tube were also recovered as a separate fraction. The same batch of EYTG extender (control) was also centrifuged to separate top (LDF) and bottom fractions. All fractions were extensively dialyzed against 10 mM Tris-HCl (pH 7.4) and were delipidated using *n*-butanol/di-isopropyl ether (15:85 v/v) solvent. The protein concentration was determined in each delipidated fraction by the modified Lowry procedure [45] and adjusted to 1 mg/ml for protein separation by SDS-PAGE.

RESULTS

Isolation and Characterization of the LDF

The LDF from the EYTG extender and the EY were obtained as described in *Materials and Methods.* Two fractions were obtained from both sources. The top yellow fraction contained the LDF. The bottom fraction was slightly

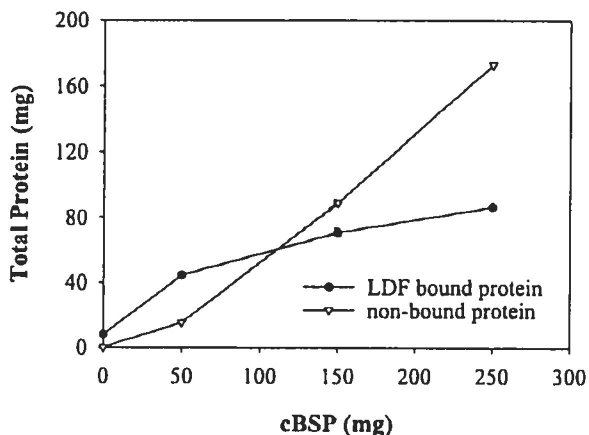

FIG. 1. Interaction between LDF and cBSP proteins. Increasing amounts of cBSP were added to 10 mg LDF protein. The mixtures were transferred into quick-seal tubes and subjected to ultracentrifugation as described under *Materials and Methods*. After ultracentrifugation, the top fraction, i.e., LDF-protein complex (●) and the bottom fraction (▽) were separated, dialyzed, and analyzed for total protein content by the modified Lowry procedure [45]. The data shown are representative of two experiments performed separately.

opalescent and corresponded probably to the water-soluble fraction described earlier [12, 48]. The LDF isolated either from the EYTG extender or EY had neutral surface charge as evaluated by agarose-gel electrophoresis (see later). The size-exclusion chromatography separated the LDF into two peaks: a minor peak, a (LDF-I), and a major peak, b (LDF-II; see later).

Interaction of cBSP Proteins with LDF

First, we investigated the interaction of cBSP proteins with the LDF. A constant amount of LDF was incubated (15 min) with increasing concentrations of cBSP and the LDF was reisolated along with the associated proteins. The addition of cBSP to LDF increased the total protein content of the reisolated fraction. Ten milligrams of LDF bound ~35 mg (70%) of proteins present in 50 mg of cBSP (Fig. 1). When 150 and 250 mg of cBSP was added to the same amount of LDF, ~65 mg (43%) and ~75 mg (30%) of cBSP proteins were associated with reisolated LDF.

Interaction of BSP Proteins with LDF

The binding of purified BSP-A1/-A2, -A3, and -30-kDa proteins to LDF was examined by electrophoresis. The LDF alone, when subjected to agarose-gel electrophoresis, remained at the point of application (Fig. 2A, lane 2), thus indicating a neutral surface charge. However, when LDF was mixed with either cBSP (Fig. 2A, lane 3) or each of the purified BSP proteins (Fig. 2A, lanes 4–9) and then subjected to the agarose-gel electrophoresis, a significant increase in migration of LDF particles was observed, indicating association of BSP proteins with LDF. The migration of the LDF-BSP protein complex was dependent on the ratio of LDF and BSP proteins. The complex migrated faster at a 1:4 LDF:BSP protein ratio than at a 1:1 ratio (lane 4 vs. 5, 6 vs. 7, and 8 vs. 9).

Specificity of Interaction of BSP Proteins with LDF

In order to establish the specificity of the interaction between the BSP proteins and LDF, we studied the migration

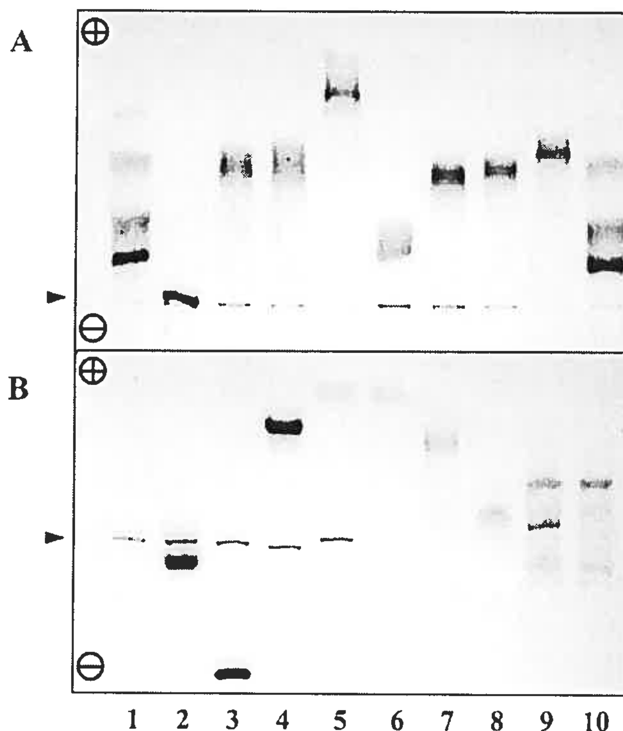

FIG. 2. Specificity of interaction of BSP proteins with LDF. **A)** Interaction of LDF with BSP proteins. LDF (5.6 μg) and cBSP (22.4 μg) or purified BSP proteins (5.6 and 22.4 μg) were incubated in 15 μl of Tris-HCl buffer, and 4 μl of each sample was applied to the lipo gel slots. The electrophoresis and lipid staining were performed as described under *Materials and Methods*. Lanes 1 and 10: human serum (2 μl); lane 2: LDF (1.5 μg); lane 3: LDF (1.5 μg) + cBSP (6 μg); lanes 4 and 5: LDF (1.5 μg) + BSP-A1/-A2 (1.5 and 6 μg, respectively); lanes 6 and 7: LDF (1.5 μg) + BSP-A3 (1.5 and 6 μg, respectively); lanes 8 and 9: LDF (1.5 μg) + BSP-30-kDa (1.5 and 6 μg, respectively). The amount of each protein in a 4-μl sample is shown in parentheses. **B)** Interaction of LDF with control proteins. LDF (4 μg) and control proteins (16–48 μg) were incubated in 16 μl of Tris-HCl buffer, and 4 μl of each sample were applied to SPE gel slots. The electrophoresis and protein staining were performed as described under *Materials and Methods*. Lane 1: LDF (1 μg); lanes 2–4: LDF (1 μg) with myoglobin (6 μg), lysozyme (6 μg), and BSA (6 μg), respectively; lane 5: LDF (1 μg) + heat denatured BSP-A1/-A2 (4 μg); lane 6: heat denatured BSP-A1/-A2 (4 μg); lane 7: LDF (1 μg) + GA-BSP (4 μg); lane 8: GA-BSP (4 μg); lane 9: LDF (1 μg) + dBSP (12 μg); lane 10: dBSP (12 μg). BSP-A1/-A2 proteins were denatured by holding the tube containing protein solution (2 mg/ml in barbital buffer, pH 8.6) in boiling water for 20 min. The arrowheads indicate the point of sample application.

pattern of LDF with several control proteins (myoglobin, lysozyme, and BSA), heat denatured BSP-A1/-A2, dBSP (BSP depleted SP), and GA-BSP (gelatin-agarose adsorbed fraction, i.e., mixture of all BSP proteins). Incubation with control proteins did not change the surface charge of LDF particles, and the LDF band remained at the point of application (Fig. 2B, lanes 1–4). All control proteins migrated away from LDF. The heat denaturation of BSP-A1/-A2 inhibited its LDF-binding ability; thus, the denatured protein and LDF migrated separately (lane 5). The dBSP also did not interact with LDF (lane 9), whereas LDF with GA-BSP (lane 7) migrated as a single band and showed an increased migration compared with the GA-BSP alone (lane 8), indicating the formation of a complex between GA-BSP and LDF.

The specificity of binding of BSP proteins to LDF was

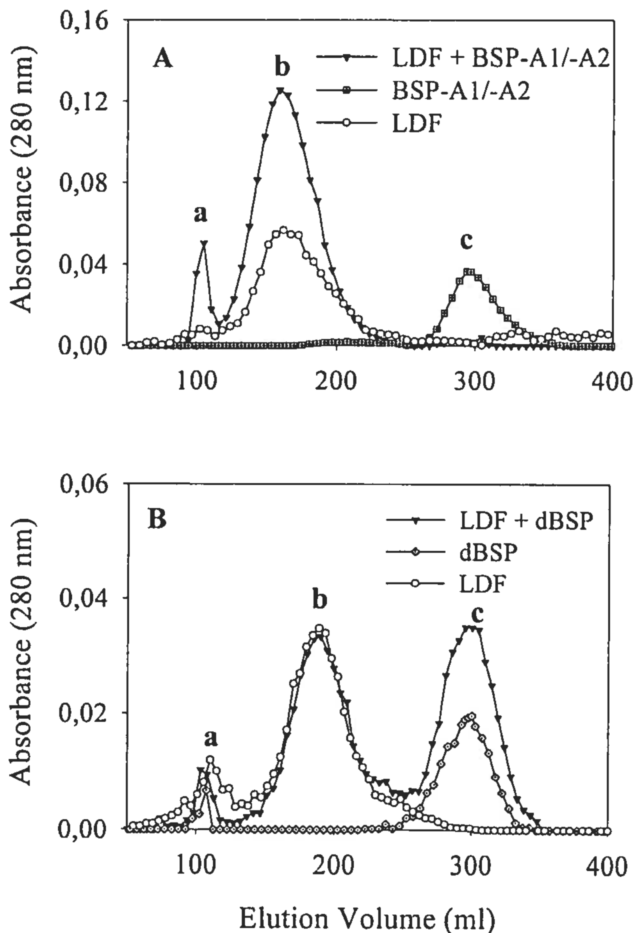

FIG. 3. Gel-filtration analysis of LDF interaction with BSP-A1/-A2 and dBSP. A) Interaction of LDF with BSP-A1/-A2. Solutions of 1.1 mg LDF protein, 1.1 mg BSP-A1/-A2, and a mixture of LDF (1.1 mg) and BSP-A1/-A2 (2.2 mg) in a final volume of 1 ml were prepared and subjected to chromatography on a Sepharose CL-4B column. B) Interaction of LDF with dBSP. Solutions of 1.1 mg LDF protein, 1.1 mg dBSP proteins, and a mixture of LDF (1.1 mg) and dBSP proteins (2.2 mg) in a final volume of 1 ml were prepared and subjected to chromatography on a Sepharose CL-4B column. Chromatography was performed as described under *Materials and Methods*. The data shown are representative of five experiments performed separately.

further established by size-exclusion chromatography. The LDF was resolved into two lipoprotein peaks (Fig. 3; peak a, or LDF-I, 100–130 ml; and peak b, or LDF-II, 131–250 ml). BSP-A1/-A2 alone was eluted as a single peak (270–340 ml, designated peak c). When the mixture of LDF and BSP-A1/-A2 was subjected to size-exclusion chromatography, peak c corresponding to the BSP-A1/-A2 alone disappeared, whereas the absorbance under peaks a and b of the LDF showed a considerable increase, indicating an interaction between BSP-A1/-A2 proteins and LDF. In contrast, when the mixture of LDF and dBSP proteins was analyzed by size-exclusion chromatography (Fig. 3B), the elution profile did not differ from that of LDF alone in the area under peaks a and b, whereas peak c (270–340 ml), corresponding to the dBSP, was also present, indicating that the dBSP did not bind to LDF.

Saturation of the Binding of BSP Proteins to LDF

In order to establish the saturation of the binding sites on LDF, we incubated a constant amount of LDF with vary-

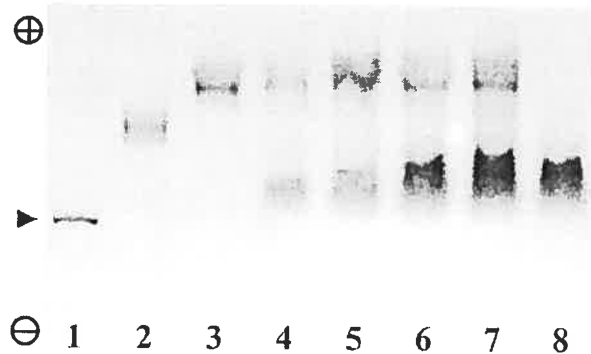

FIG. 4. Saturation of the binding of BSP proteins to LDF. LDF (4 μg) and BSP-A1/-A2 proteins (10–60 μg) were incubated in 12 μl of Tris-HCl buffer, and 3 μl of each sample was applied to SPE-gel slots. Electrophoresis and protein staining were performed as described under *Materials and Methods*. Lane 1: LDF (1 μg); lanes 2–7: LDF (1 μg) + BSP-A1/-A2 protein (2.5, 5, 7.5, 10, 12.5, and 15 μg, respectively); lane 8: BSP-A1/-A2 (10 μg). The amount of each protein present in 3-μl sample is shown in parentheses. The arrowhead indicates the point of sample application.

ing concentrations of BSP-A1/-A2 (Fig. 4) and analyzed their migration on agarose gel. At LDF and BSP-A1/-A2 ratios of 1:2.5 and 1:5, only the bands corresponding to LDF-BSP-A1/-A2 complex were visualized (lanes 2 and 3). Further increases in protein ratio (1:7.5, 1:10, 1:12.5, and 1:15) resulted in the appearance of the BSP-A1/-A2 band (lanes 4–7), whereas all LDF particles migrated with the complex. We chose BSP-A1/-A2 for this and the previous (Fig. 3A) experiments because it represents almost 80% of total BSP proteins [47]. Moreover, BSP-A1/-A2, BSP-A3, and BSP-30-kDa exhibit similar binding properties and biological activity.

Interaction of SP Proteins with the LDF

To verify whether the LDF and BSP proteins in SP form stable complexes, the LDF was incubated with SP and subjected to size-exclusion chromatography (Fig. 5). The chromatography of SP alone showed a minor peak (volume 100–130 ml, designated peak a) and a major peak (280–360 ml, designated peak c). The chromatography of the mixture of LDF and SP resulted in a considerable decrease in the area under the major protein peak of the SP (peak c) and a considerable increase in the area under peak a and b of the LDF, indicating association of large amounts of SP proteins with LDF.

Identification and Quantification of BSP Proteins in the Chromatography Fractions

In order to confirm the specific interaction of BSP proteins with LDF, fractions under peaks a, b, and c (Fig. 5) were pooled, concentrated, and subjected to immunoblotting using the specific antibodies against the BSP-A1/-A2 (Fig. 6A), BSP-A3 (Fig. 6B), and BSP-30-kDa (Fig. 6C). The results indicated the presence of BSP-A1/-A2, BSP-A3, and BSP-30-kDa in peaks a and b derived from the LDF-SP mixture. Because the polyclonal antibodies against BSP-A1/-A2, -A3, and -30-kDa are specific [47], the bands shown with the arrows in lane 5 of Figure 6 correspond to the degradation product of the respective BSP proteins.

BSP-A1/-A2, BSP-A3, and BSP-30-kDa protein content in pooled fractions under peaks a, b, and c of respective chromatography (SP, LDF, and SP+LDF) were further

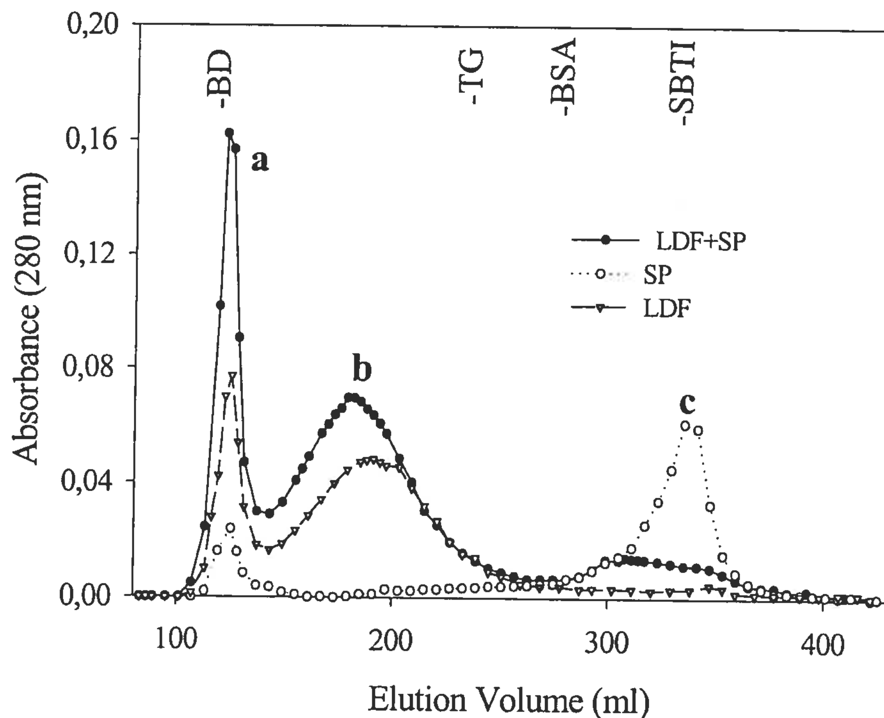

FIG. 5. Interaction of SP proteins with LDF. Solutions of 1.1 mg LDF protein, 2.2 mg total protein of SP, and mixture of LDF (1.1 mg) and SP (2.2 mg) in a final volume of l ml were prepared and subjected to chromatography on a Sepharose CL-4B column. The elution position of BD (blue dextran), TG (thyroglobulin), BSA (bovine serum albumin), and SBTI (soy bean trypsin inhibitor) are indicated. The data shown are representative of five experiments performed separately.

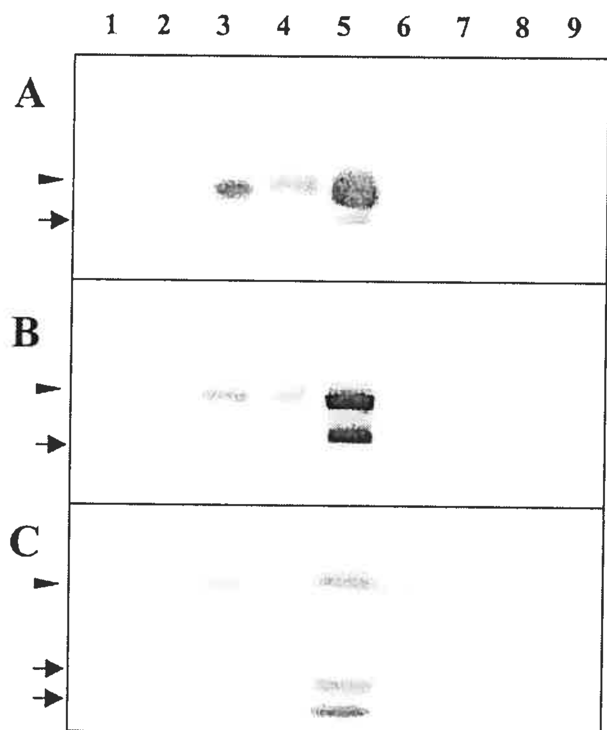

FIG. 6. Immunoblot analysis of chromatography peaks. Aliquots (200 ng total protein) corresponding to each pooled peak (Fig. 5) were reduced, denaturated, and then subjected to SDS-PAGE. The separated proteins were transferred to Immobilon-P and probed with antibodies directed against BSP proteins. A) Anti-BSP-A1/-A2. B) Anti-BSP-A3. C) Anti-BSP-30 kDa. Lanes 1–3: Correspond to peaks a–c derived from SP; lanes 4–6: correspond to peaks a–c derived from LDF and SP mixture; lanes 7–9: correspond to peaks a–c derived from LDF. The arrowheads in **A**, **B**, and **C** indicate the position of BSP-A1/A2, BSP-A3, and BSP-30-kDa, respectively. Arrows in **A**, **B**, and **C** correspond to degraded fragments of BSP proteins.

quantified by specific RIA and expressed in percent distribution in each peak. The results (Table 1) show that ~80% of BSP proteins were present in peak c when SP alone was chromatographed, whereas ~90% of these proteins were found in peak b upon SP preincubation with LDF and followed by chromatographic separation of the mixture.

Stability of LDF-BSP Protein Complex Following Freeze-Thaw of the Extended Semen

An experiment was carried out to verify whether the interaction between the BSP proteins and EY-LDF was stable during semen freezing and thawing. We isolated the LDF from frozen/thawed semen and analyzed the protein pattern by SDS-PAGE. Figure 7 shows the presence of the BSP proteins in the LDF (lane 5). The bottom fraction (lane 6) and the precipitates (lane 7) contained very insignificant amounts of BSP proteins. These results indicate that the

TABLE 1. The distribution of BSP proteins in pooled fractions (peaks a, b, and c) obtained by gel filtration chromatography of SP, LDF, and SP+LDF.[a]

Sample	Peak	BSP-A1/-A2 (%)	BSP-A3 (%)	BSP-30-kDa (%)
SP	a	0.8	5.0	1.3
	b	18.0	12.5	16.4
	c	81.2	82.5	82.3
SP + LDF	a	6.7	5.9	4.6
	b	89.3	93.1	87.0
	c	4.0	0.9	8.4
LDF	a	—	—	—
	b	—	—	—
	c	—	—	—

[a] BSP-A1/-A2, BSP-A3, and BSP-30-kDa content in pooled fractions peaks a, b, and c of respective chromatography (SP, LDF, and SP+LDF) were determined by specific RIA and expressed in percent distribution in each peak. Data are means of three independent experiments performed in duplicate.

EY-LDF-BSP protein complex remains stable during the freeze/thaw procedures.

DISCUSSION

In the current study, we have shown for the first time that the LDF isolated from EY interacts with the BSP proteins, factors in SP, which are detrimental to sperm. The binding of BSP proteins to LDF is rapid, specific, and saturable and the complexes formed (LDF-BSP proteins) are stable. This interaction may be critical for sperm protection and storage in the liquid or frozen state.

The isolated EY-LDF showed similarities with those reported by earlier workers [48–50]. The chromatographic separation of the isolated LDF resulted in two lipoprotein fractions (LDF-I and LDF-II; Figs. 3 and 5). Foulkes [12] reported the separation of LDF into three lipoprotein fractions, the second fraction (lipoprotein II) eluting as the shoulder in front of the third lipoprotein fraction (lipoprotein III). The lipoprotein fractions I, II, and III differed by their lipid:protein ratio (11.7, 6.6, and 2.8, respectively), and lipoprotein III was responsible for protecting bovine sperm against damage during freezing and thawing [12]. It is possible that our separation procedure did not resolve the lipoproteins II and III and that both these lipoproteins appeared in peak b (LDF-II; Figs. 3 and 5). In addition, differences were also noted from batch to batch in the proportion of LDF-I and LDF-II (Figs. 3 and 5). These differences may be attributed to LDF isolation techniques used or the EY sources.

We investigated first the interaction between the isolated LDF and cBSP proteins. Our results (Fig. 1) show a dose-dependent binding of cBSP proteins to LDF. At a LDF: cBSP ratio of 1:5, ~70% of cBSP proteins associated with reisolated LDF. Because ~70% of cBSP proteins correspond to BSP proteins, it is logical to assume that all the protein bound to LDF may be BSP proteins. Indeed, the SDS-PAGE indicated the presence of large amounts of the BSP proteins in the reisolated fraction (data not shown).

The specific binding of the BSP proteins to LDF was established by using purified BSP-A1/-A2, -A3, and -30-kDa proteins. The LDF-BSP protein complex was analyzed by agarose-gel electrophoresis and gel filtration chromatography. Agarose-gel electrophoresis (Fig. 2A) showed an increased electrophoretic mobility after LDF incubation with each purified BSP protein. This is due to the binding of the negatively charged BSP proteins [32, 34, 35] to the surface of LDF. In contrast, the control proteins (myoglobin, lysozyme, BSA) migrated either to cathode or anode, depending on their charge, and the LDF remained at the point of application (Fig. 2B). Similarly, the heat-denatured BSP-A1/-A2 or the dBSP (BSP protein-depleted fraction of seminal plasma) did not change the mobility of LDF, indicating no association of these proteins with the LDF (Fig. 2B). In addition, during gel filtration, the BSP-A1/-A2, but not dBSP, proteins eluted at the LDF region when coincubated (Fig. 3). The specificity of interaction between LDF and the BSP proteins was further confirmed by revealing the presence of the BSP proteins in peaks a and b derived from the LDF and SP chromatography (Fig. 6). The evaluation of BSP proteins and their percentages in each chromatographic peak (Table 1) of SP alone and LDF along with SP indicate that peak b of the LDF fraction bound most of the BSP proteins.

A higher mobility of particles noted in the presence of BSP-A1/-A2 may indicate a higher negative charge associated with BSP-A1/-A2 than with either BSP-A3 or BSP-30-kDa (Fig. 2A). Furthermore, the LDF-BSP protein complex had a higher negative charge than the purified BSP protein alone (Fig. 4, lane 8). This suggests changes in conformation of BSP proteins upon binding to LDF.

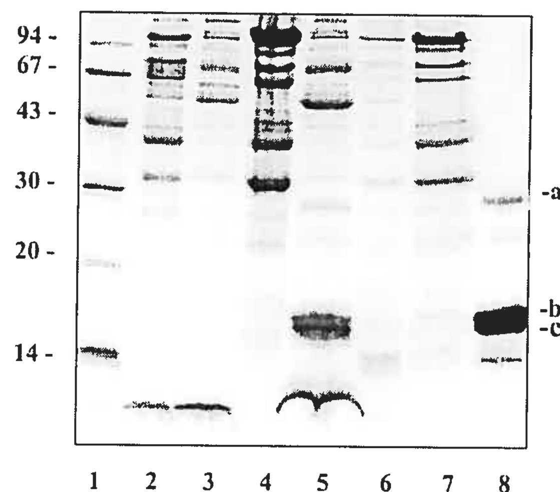

FIG. 7. Analysis of proteins in LDF isolated from frozen-thawed EYTG extended semen. Fractions obtained from EYTG extender (control) and from frozen/thawed extended semen by ultracentrifugation were dialyzed and delipidated. The proteins in these fractions were separated by SDS-PAGE and stained with Coomassie Blue. Lane 1: LMW standard; lane 2: whole EY proteins; lane 3: LDF from EYTG; lane 4: bottom fraction from EYTG; lanes 5 and 6, respectively: top (LDF) and bottom fractions from frozen/thawed semen; lane 7: precipitates from frozen/thawed semen; lane 8: cBSP proteins. a, b, and c indicate the positions of BSP-30-kDa, BSP-A1/-A2, and BSP-A3, respectively.

Our previous studies show that the BSP proteins bind specifically to choline phospholipid liposomes [36] and this binding occurs in less than a second [51]. In the current study, agarose-gel electrophoresis and gel filtration analysis were performed immediately after mixing BSP proteins with LDF, and in all instances, complex was observed. Therefore, it is reasonable to conclude that the LDF association with BSP proteins is rapid. Because the LDF-BSP complex is stable during ultracentrifugation, electrophoresis, and gel filtration, it is likely that the binding constant is in the μM range (high affinity binding).

The binding of the BSP proteins to the LDF is saturable. Based on the data shown in Figure 1, at saturation, 10 mg of LDF appeared to bind ~70 mg of cBSP proteins. The saturation of the BSP proteins binding to the LDF was also demonstrated by analyzing the complex at various ratios of LDF:BSP-A1/-A2 protein (Fig. 4). The saturation of LDF binding sites occurred between LDF:BSP-A1/-A2 at ratio of 1:5 and 1:7.5. This value is similar to that established by ultracentrifugation (i.e., 1:7 ratio).

The binding capacity of the LDF appears to be very high. LDF-I and LDF-II have an average M_r of 1.4×10^6 Da and 0.6×10^6 Da, respectively, as determined by gel filtration (calibrated with proteins of known molecular weight). BSP-A1/-A2 (M_r 16 500 Da), -A3 (M_r 15 500 Da), and -30-kDa (M_r 28 000 Da) proteins are present in an approximate ratio of 8:1:1 in SP [33, 47]. Assuming an average M_r of BSP proteins in SP to be 18 000, at saturation (1:7 ratio), nearly 555 and 243 moles of the BSP proteins can bind to a mole of LDF-I and LDF-II, respectively. The high capacity binding of LDF is important for sequestration

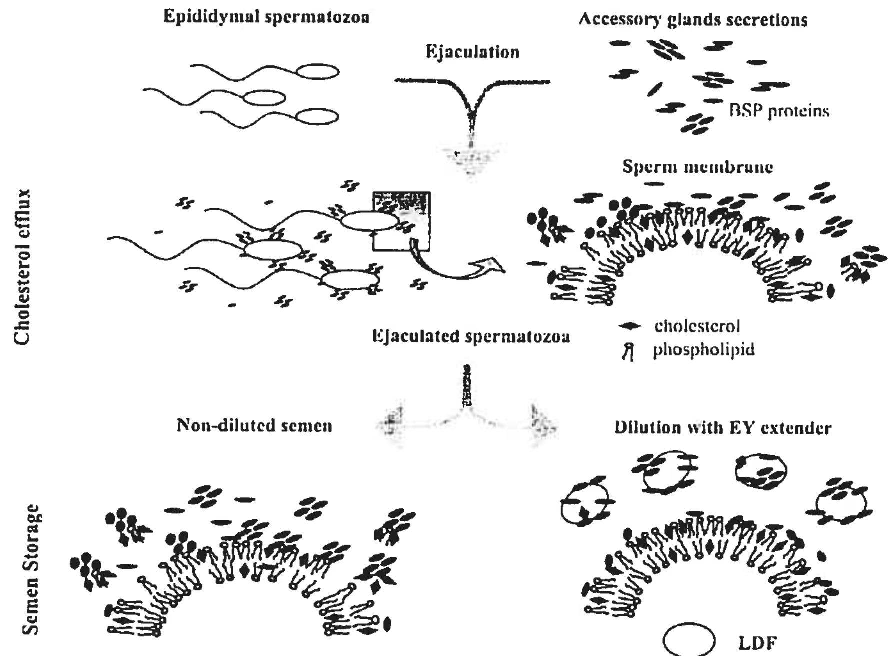

FIG. 8. The proposed mechanism of sperm protection by EY-LDF.

of large amounts of BSP proteins present in semen. The average concentration of BSP proteins in semen is 35–50 mg/ml [47] and of LDF in 20% egg yolk is 10–15 mg/ml (current study). Semen is normally diluted 10 times and higher with 20% EY-containing medium (EYTG extender) prior to cryopreservation. At this dilution, most of the BSP proteins may be associated with LDF.

In the artificial insemination industry, semen is diluted with EYTG extender within minutes after collection, cooled to 4°C, packed in straws, frozen, and stored in liquid nitrogen. The LDF isolated from frozen-thawed semen also contained BSP proteins (Fig. 7). This indicates that the LDF-BSP protein complex remains stable even after semen cooling, freezing, and preservation in liquid nitrogen and after thawing.

In view of our discovery that the BSP proteins specifically bind to LDF and form a stable complex, we suggest a novel mechanism of sperm protection by EY lipoproteins (Fig. 8). Our previous studies have shown that the BSP proteins are added to sperm at ejaculation [34]. The BSP proteins coat the sperm membrane [36, 37] and induce cholesterol [42] and phospholipid efflux [43]. The lipid efflux is time and concentration dependent [42, 43]. At higher concentrations of the BSP proteins (i.e., SP) and/or at longer exposure (as in nondiluted semen), more cholesterol and phospholipids are removed. The removal of lipids, particularly cholesterol, results in sperm membrane destabilization. Evidence shows that the decrease in cholesterol content in plasma membrane also appears to decrease sperm resistance to cold shock and freezing [52, 53]. Therefore, prolonged exposure of sperm to SP that contains BSP proteins is deleterious to sperm. Because ejaculates are diluted with EY extenders within minutes after collection, the lipoproteins (LDF) may sequester most of the BSP proteins present in semen. This may result in a minimum modification of the sperm plasma membrane and allow better sperm storage. Thus, EY lipoproteins may offer protection to sperm by reducing the deleterious effect of SP proteins on sperm membranes. During natural mating, a mechanism may also exist to eliminate the detrimental effect of BSP proteins on sperm. After being ejaculated into the vagina, sperm swim through cervical mucus and enter the uterus within minutes. Cervical mucus acts as a barrier for SP. In the artificial insemination industry, the BSP proteins (i.e., SP proteins) are not removed from semen, but their effect is eliminated probably by the rapid formation of a stable complex with egg yolk lipoproteins.

Our model can provide an explanation for the various effects of SP. For instance, it is reported that sperm become more sensitive to cold shock and freeze-thaw following exposure to SP [7, 54, 55]. A recent report also indicates that the exposure of sperm to accessory sex gland fluid (AGF) is toxic and that rapid removal of sperm from SP or AGF is critical for maximal viability [56]. These deleterious effects on sperm could be essentially due to BSP proteins that modify the sperm membrane extensively by removing cholesterol. In ejaculates, the activity of BSP proteins on sperm membrane continues, whereas in extended semen, it is likely inhibited by EY-LDF. Consequently, extended sperm could better resist cold shock and/or freeze-thaw effects, as observed with epididymal sperm that are not exposed to SP [54].

The toxic effect of SP or AGF on sperm is concentration and time dependent [55, 56]. Studies have also suggested that the toxic effect is immediate and persists even after washing sperm [57]. Furthermore, the amount of EY required in semen extenders to provide protection against SP is proportional to the amount of SP in diluted semen [58]. The toxic effects of SP could be minimized by dilution with EY extenders within minutes of semen collection [9, 13]. All these effects can be explained with our model (Fig. 8). The modification of sperm surface (removal of cholesterol from sperm membrane or toxic effect) by BSP proteins is a concentration- and time-dependent phenomenon [42, 43]. Because of the magnitude of the BSP protein concentration (40–60 mg/ml) and the proteins' affinity to sperm membrane, the toxic effect could be caused within minutes and prolong even after washing the sperm. BSP proteins form

a complex with LDF rapidly, and therefore their toxic effect on sperm could be minimized or inhibited within minutes. Because the LDF binding sites can be saturated by BSP proteins, the protection provided by the EY is proportional to its capacity to bind the BSP proteins.

The effect of egg yolk on the success of sperm storage and the mechanisms involved have been studied extensively [12, 22–25]. It is suggested that the EY lipoproteins associate with sperm membranes and shield against the toxic effect of SP. This hypothesis simply cannot explain all the effects of SP and why it requires large amounts of EY to protect sperm although a very small fraction of it may bind to sperm membrane. In fact, the specific binding of EY lipoproteins to the sperm membrane itself is controversial [22–25]. The current study suggests that the beneficial role of semen dilution with extenders containing EY is not limited to the direct binding of EY lipoproteins to the sperm plasma membrane but may involve interplay among BSP proteins (SP), EY-LDF, and the sperm membrane (i.e., how LDF and BSP association could effect the sperm membrane lipid composition). In this context, it is interesting to note that the extended washed sperm (unpublished data) and extended frozen-thawed washed sperm contain 80–85% less BSP proteins than washed ejaculated sperm [47]. This may result in less damage to sperm in extended semen than in unextended semen.

Our recent studies show that the BSP-like proteins present in stallion [59, 60], boar [60], ram, water buffalo, and human semen also bind to LDF (unpublished), suggesting that the mechanism of sperm protection by EY is similar in all these species. Furthermore, milk extender is also used for sperm storage, and it also contains lipoproteins and phospholipids. These milk components also interact with the BSP proteins (will be reported elsewhere), suggesting that the mechanism of sperm protection by milk is similar to that proposed for EY-LDF.

In summary, we have shown for the first time that the LDF isolated from EY and EYTG extenders interacts with the BSP proteins. By using a number of biochemical approaches, we conclude that this binding is rapid, specific, and saturable. In addition, LDF has a very high capacity for BSP proteins and the complex formed is stable even after freeze-thaw. BSP proteins destabilize the sperm membrane by removing cholesterol and phospholipids. However, it may be possible that this effect is abolished or minimized by the association of BSP proteins with EY lipoproteins, the major component of extenders used in sperm storage. Therefore, we suggest that the scavenging of the BSP proteins by EY lipoproteins may represent the major mechanisms of sperm protection by EY. Further studies on how LDF-BSP protein interaction affects a) BSP proteins binding to sperm membrane, b) sperm membrane lipid composition (cholesterol and phospholipid), and c) sperm functions (motility, viability, acrosomal integrity) will clarify the beneficial effects of LDF and BSP protein interaction on sperm storage. These studies may also aid in improving current protocols for sperm processing and developing better extenders for commercial application. In addition, the current discovery should aid in developing novel extenders for preservation of semen (liquid or frozen) from several farm animals, domestic animals, wild animals, and endangered species for which protocols do not exist presently.

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2.2 Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane.

Bergeron A., Crête M-H, Brindle Y. et Manjunath P. *Biology of Reproduction*, 70:708-717, 2004.

Préambule:

Dans l'article précédent, nous avons démontré que les protéines BSP lient les LDF (le constituant majeur du jaune d'œuf) et suggéré que cette liaison est bénéfique pour les fonctions spermatiques. Dans l'article présenté ici, nous avons voulu vérifier si la liaison des protéines BSP aux LDF avait un effet sur: 1) la liaison des protéines BSP aux spermatozoïdes, 2) la composition lipidique des membranes des spermatozoïdes et 3) les fonctions spermatiques (motilité, viabilité, réaction de l'acrosome). Nous avons démontré que les LDF empêchent les protéines BSP de se lier aux spermatozoïdes et de stimuler un efflux des lipides membranaires permettant ainsi de maintenir les fonctions spermatiques pendant l'entreposage de la semence.

J'ai planifié toutes les expériences et réalisé tous les travaux qui ont mené à la rédaction de cet article. J'ai fait l'interprétation des résultats et rédigé l'article présenté dans cette section. Crête M-H a aidé à la préparation des échantillons de semence bovine. Brindle Y. nous a fourni les échantillons de semence bovine.

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Low-Density Lipoprotein Fraction from Hen's Egg Yolk Decreases the Binding of the Major Proteins of Bovine Seminal Plasma to Sperm and Prevents Lipid Efflux from the Sperm Membrane[1]

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ABSTRACT

For sperm preservation, semen is generally diluted with extender containing egg yolk (EY), but the mechanisms of sperm protection by EY are unclear. The major proteins of bull seminal plasma (BSP proteins: BSP-A1/A2, BSP-A3, and BSP-30-kDa) bind to sperm surface at ejaculation and stimulate cholesterol and phospholipid efflux from the sperm membrane. Since EY low-density lipoprotein fraction (LDF) interacts specifically with BSP proteins, it is proposed that the sequestration of BSP proteins in seminal plasma by EY-LDF represents the major mechanism of sperm protection by EY. In order to gain further insight into this mechanism, we investigated the effect of seminal plasma, EY, and EY-LDF on the binding of BSP proteins to sperm and the lipid efflux from the sperm membrane. As shown by immunodetection, radioimmunoassays, and lipid analysis, when semen was incubated undiluted or diluted with control extender (without EY or EY-LDF), BSP proteins bound to sperm in a time-dependent manner, and there is a continuous cholesterol and phospholipid efflux from the sperm membrane. In contrast, when semen was diluted with extender containing EY or EY-LDF, there was 50%–80% fewer BSP proteins associated with sperm and a significant amount of lipid added to sperm membrane during incubation. In addition, sperm function analysis showed that the presence of EY or EY-LDF in the extender preserved sperm motility. These results show that LDF is the constituent of EY that prevents binding of the BSP proteins to sperm and lipid efflux from the sperm membrane and is beneficial to sperm functions during sperm preservation.

assisted reproductive technology, gamete biology, male reproductive tract, seminal vesicles, sperm

INTRODUCTION

Seminal plasma, which is mixed with epididymal sperm at ejaculation, serves as the carrier of sperm to the female genital tract and has been described as both beneficial and detrimental for sperm. More precisely, several workers have described seminal plasma factors that support sperm functions, such as motility [1] and viability and fertility [2, 3], and that facilitate capacitation [4]. However, seminal plasma is also known to contain factors that are detrimental for sperm fertilizing ability [5], such as decapacitation factor [6–8] and motility inhibiting factor [9]. In addition, seminal plasma is detrimental to sperm storage [10] because it contains factors that negatively affect sperm viability [11–13].

The major protein fraction of bovine seminal plasma is represented by a family of closely related proteins designated BSP-A1/A2, BSP-A3, and BSP-30-kDa (collectively called BSP proteins) [14, 15]. The BSP proteins represent ~65% of seminal plasma total protein, and their biochemical characteristics have been extensively described (for a review, see [16]). BSP proteins bind to sperm membrane choline phospholipids at sperm ejaculation and potentiate sperm capacitation induced by high-density lipoprotein and heparin [17, 18] by stimulating cholesterol and phospholipid efflux from the sperm membrane. Therefore, BSP proteins are beneficial to sperm. In contrast, the same BSP proteins may be deleterious for the sperm membrane in vitro. The lipid efflux stimulated by BSP proteins is time and concentration dependent [19, 20], and therefore a continuous exposure of sperm to seminal plasma that contains BSP proteins may damage the sperm membrane [16].

Hen's egg yolk (EY) is the most effective agent to protect sperm against cold shock and has been shown to improve sperm functions and preserve sperm fertility after storage in liquid [21–24] or frozen state [25–28]. Despite the use of extender containing EY for more then 60 yr, the mechanisms involved in sperm protection by EY against storage, cooling, and freezing damages remain unclear. The low-density lipoprotein fraction (LDF) of EY appears to be the constituent responsible for sperm protection against cold shock and freezing damages [29–32]. Several mechanisms of sperm protection by EY-LDF have been proposed. It is suggested that EY-LDF provides protection by associating with sperm membrane [28, 30, 31, 33, 34]. Another hypothesis is that EY-LDF prevents the loss of membrane phospholipids, thus increasing the sperm tolerance to the cold shock [35], and previous studies that indicate that EY phospholipids protect sperm from cold shock support this speculation [31, 36]. Studies from our laboratory indicate that the EY-LDF interacts specifically with the BSP proteins [37]. The binding of the BSP proteins to LDF is rapid and saturable, and the binding capacity of the LDF is very high. Furthermore, this interaction is stable even after freeze-thawing. In view of this, we have proposed that the scavenging of the BSP proteins by EY lipoproteins on dilution of semen with extender containing EY protects sperm from deleterious effects of BSP proteins present in seminal plasma.

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In order to gain further insight into the mechanisms of sperm protection by EY, it is essential to study further the interplay among BSP proteins, extender constituents, and sperm. In the present study, it is hypothesized that dilution of semen with EY containing extender prevents the binding of BSP proteins to sperm and prevents a lipid efflux from sperm membrane. First, we investigated the effect of semen dilution in EY Tris-glycerol (EY-TG) extender on 1) the binding of BSP proteins to the sperm membrane, 2) cholesterol and phospholipid efflux from the sperm membrane, and 3) sperm functions (motility, viability, acrosomal integrity). The second objective of this study was to test the hypothesis that the EY-LDF is the extender constituent responsible for preventing BSP proteins from binding to sperm and stimulating sperm lipid gain during storage.

MATERIALS AND METHODS

Materials

BSA (fraction V), lactoperoxidase, leupeptin, erythrosin B, and flavianic acid (naphthol) were from Sigma (St. Louis, MO). Anti-Rabbit immunoglobulin G (H + L), acrylamide, bisacrylamide, SDS, and other electrophoresis products were obtained from Bio-Rad (Mississauga, ON, Canada). Low-molecular-weight (LMW) electrophoresis calibration kit was from Pharmacia Biotech Inc. (Baie d'Urfé, PQ, Canada). Polyethylene glycol (PEG) was obtained from ICN Biomedicals Inc. (Cleveland, OH). Immobilon-P membrane and enhanced chemiluminescence (ECL) reagent kit were purchased from Mandel Scientific (Boston, MA). ^{125}I was purchased from Perkin-Elmer Life Science (Boston, MA). Goat anti-rabbit gamma globulin (RGG) was from Medicorp Inc. (Montréal, PQ, Canada). All other chemicals used were analytical grade and obtained from commercial suppliers.

Freshly ejaculated semen was collected with an artificial vagina from bulls at the Centre d'Insémination Artificielle du Québec (St-Hyacinthe, PQ, Canada). Bulls were handled by qualified technicians according to the Guide for the Care and Use of Agricultural Animals established by the Ministry of Agriculture and Fisheries, Québec. EY-TG extender (200 mM Tris, pH 6.7; 20% EY, 5.6% glycerol) was added to the semen. The extender was prepared by adding fresh egg yolk to Tris-glycerol base.

Isolation of EY-LDF Lipoprotein Fraction

EY used to isolate EY-LDF was from the same batch used to prepare 20% EY extender. EY was diluted three times with 10 mM Tris-HCl (pH 7.4), and the density was raised to 1.21 mg/ml by adding solid potassium bromide and centrifuged for 20 h at 366 257 × g at 20°C as described previously [37]. The floating lipoproteins (designated low-density fraction [LDF]) were retrieved and extensively dialyzed against 10 mM Tris-HCl (pH 7.4). The volume of dialyzed LDF was adjusted with 10 mM Tris-HCl to original volume (EY used for isolation).

Preparation of Semen Samples

The same four bulls were used to conduct this study. In each experiment, three separate ejaculates from four different bulls were used. In the first experiment, one ejaculate from each bull was undiluted or immediately diluted after collection with TG (200 mM Tris, 5.6% glycerol, pH 6.7) or EY-TG (TG, 20% EY) extender to reach a sperm concentration of 80 × 10^6/ml as routinely done in the artificial insemination center and incubated at 37°C for 24 h. After dilution, final concentration of seminal plasma in diluted ejaculates was between 4.2% and 9.8% (v/v). In the second experiment, one ejaculate from each bull was diluted right after collection with TG, EY-TG, or LDF-TG extender to reach a sperm concentration of 80 × 10^6/ml and were incubated at 4°C to mimic the cooling procedure in the artificial insemination center. After dilution, final concentration of seminal plasma in diluted ejaculates was between 4.7% and 10.0% (v/v).

Final pH of the extenders was 6.7, and extenders were kept at 37°C prior to semen dilution. At 0, 1, 2, 4, 6, 8, and 24 h of incubation, samples of semen were taken of which one aliquot was used to assess sperm functions and two aliquots were used for sperm protein and lipid analysis. Time "zero" is the time at which the semen was mixed with EYTG or LDF or TG extender, and an aliquot was removed immediately and diluted with phosphate-buffered saline (PBS) for sperm washing. All samples were treated in the same manner at time zero in both experiment 1 and experiment 2. When the sample was undiluted, the time zero is considered to be the start of the incubation, which is usually within 5–6 min (time normally elapsed before mixing with extender) of semen collection. For protein and lipid analysis, semen samples were diluted (1:20) with 50 mM PBS in 15-ml plastic tubes and centrifuged at 1840 × g for 10 min. This washing procedure was repeated five times to remove extender or seminal plasma from sperm. Then the pellets were resuspended in 900 μl PBS, transferred into 1.5-ml tubes, and centrifuged at 15 800 × g for 10 min. The supernatants were discarded, and the sperm pellets were stored at −20°C until used for protein solubilization or lipid extraction.

Preparation of Protein Extracts from Sperm Membrane

The protein extracts from sperm membrane were prepared as described previously [38] and were stored at −20°C until used for sperm protein analysis. The protein contents of seminal plasma or sperm extracts were determined by the modified Lowry procedure [39].

SDS-PAGE and Immunoblot Analyses of BSP Proteins

Seminal plasma proteins or sperm membrane proteins were reduced, denatured, and separated in 15% polyacrylamide gels. For immunoblotting, the proteins in the gel were transferred on to Immobilon-P membrane as described by Towbin et al. [40], and the immunodetection was done with specific polyclonal antibodies against each BSP protein as described previously [38, 41].

Quantification of BSP Proteins on Sperm by Radioimmunoassays

Iodination of the BSP proteins was performed by the lactoperoxidase method as described previously [42]. Radioimmunoassays (RIAs) for each BSP protein were performed on sperm protein samples as described in Nauc and Manjunath [38]. Briefly, the assay tubes containing the ^{125}I-labeled and unlabeled antigen, the primary antibodies (anti-BSP-A1/A2, anti-BSP-A3, or anti-BSP-30-kDa), and normal rabbit serum (1.5% v/v) were incubated. After 20 h, 50 μl of 10% goat anti-RGG were added, and assay tubes were incubated for 16 h. Then 500 μl of 10% polyethylene glycol were added, the antibody-antigen complex was separated by centrifugation (2200 × g, 20 min), and the radioactivity associated with the pellet was determined in a gamma counter (1272 CliniGamma, Pharmacia Wallac, Finland).

Sperm Cholesterol and Choline Phospholipid Determination

Sperm lipids were extracted from pellets kept at −20°C using chloroform/methanol as described previously [19]. After solvent evaporation under N_2, the lipids were resuspended in isopropanol. The amount of cholesterol and choline phospholipids was determined following the protocol described in the cholesterol determination kit (catalog no. 139 050, Boehringer Mannheim, Roche Applied Science, Indianapolis, IN) and the choline phospholipid determination kit (catalog no. 691 844, Boehringer Mannheim), respectively.

Sperm Function Analysis

Sperm motility in each sample was assessed subjectively in duplicate by estimating the percentage of motile sperm in a drop of Tris-citrate buffer (200 mM Tris, 73 mM citric acid) on a warm slide using light microscopy. Three fields per drop were examined under 100× magnification. The viability was assessed by staining of sperm according to the protocol of Dott and Foster [43]. Acrosomal integrity was assessed by determining the percentage of sperm acrosome-intact on air-dried sperm smears stained according to a naphthol yellow-erythrosin B-staining procedure [44]. For viability and acrosomal integrity, two samples by treatments and 200 sperm by sample were assayed.

Statistical Analysis

The data for sperm functions, RIAs data, and sperm cholesterol and phospholipid loss or gain were analyzed for significant difference by ANOVA. The significant differences among treatments were determined with the protected Fisher least significant difference (LSD) test within each incubation time. A value of $P < 0.05$ was considered statistically signif-

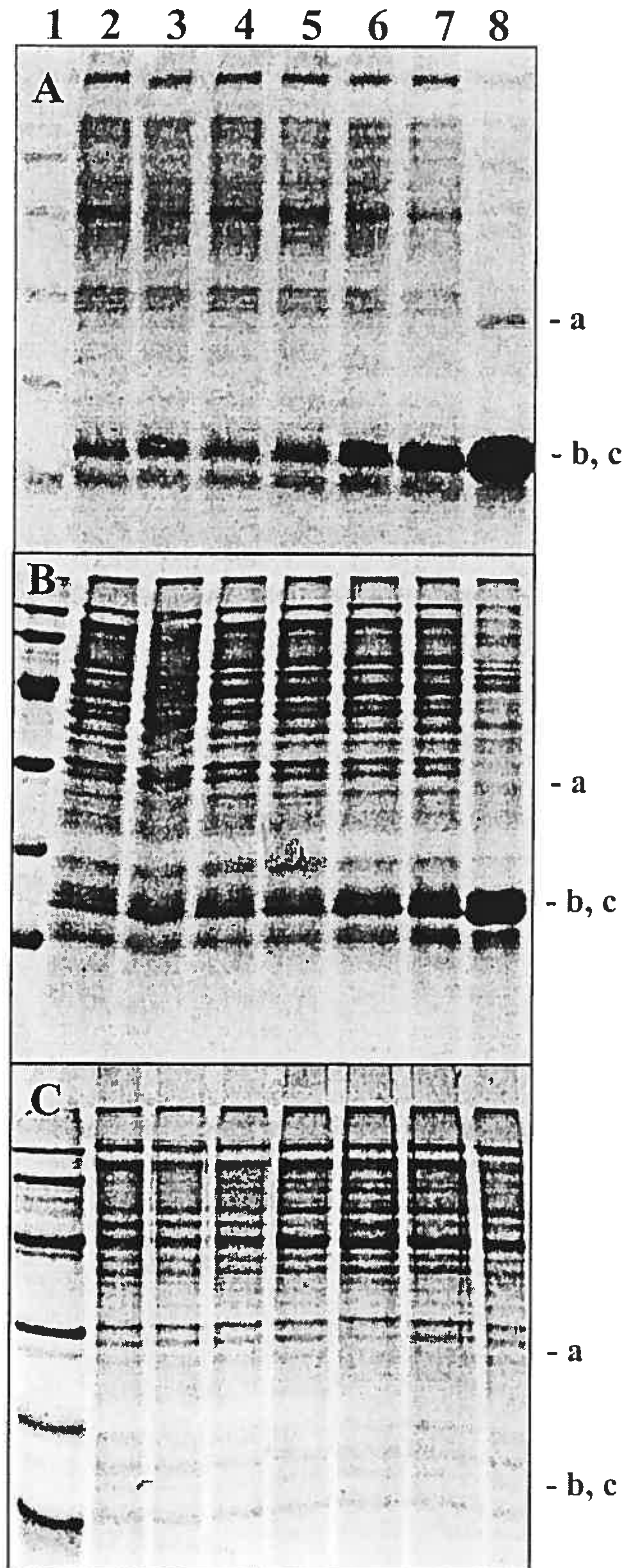

FIG. 1. SDS-PAGE pattern of sperm proteins from semen incubated undiluted or diluted with TG or EY-TG extender and incubated at 37°C. Aliquots of sperm proteins (15 μg) from semen incubated for 0 to 24 h were reduced, denatured, and separated on 15% polyacrylamide gel and stained with Coomassie Blue R-250 dye. **A**) Sperm proteins from undiluted semen. **B**) Sperm proteins from semen diluted with TG extender. **C**) Sperm proteins from semen diluted with EY-TG extender. In **A–C**, lane 1 corresponds to LMW standard (phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa) and lanes 2–8 correspond to samples at 0, 1, 2, 4, 6, 8, and 24 h of incubation at 37°C. a, b, and c indicate the position of BSP-30-kDa, BSP-A1/A2, and BSP-A3, respectively. The experiment was performed on four ejaculates, and a representative experiment is shown.

icant. The data for sperm function analysis were transformed as a percentage of motility, viability, or acrosomal integrity at time 0 h (time 0 h being 100%) before analysis.

RESULTS

SDS-PAGE Patterns of Sperm Protein

SDS-PAGE analyses were performed to verify whether modifications occurred in the sperm protein pattern during incubation of semen. In undiluted semen and semen diluted with TG extender and incubated at 37°C, electrophoresis of sperm protein samples corresponding to each incubation time (0, 1, 2, 4, 6, 8, and 24 h) revealed changes in protein pattern (Fig. 1, A and B, respectively; lanes 2–8). The intensity of 15–16.5-kDa and 28-kDa bands (corresponding to BSP proteins) increased during incubation of sperm to reach a maximal intensity at 24 h incubation (lane 8). In contrast, in sperm protein extracts from semen diluted with EY-TG extender (Fig. 1C), intensity of the bands corresponding to BSP proteins decreased significantly.

In a similar manner, in the second experiment, electrophoresis of sperm protein extracts from semen diluted with TG extender and incubated at 4°C revealed that the intensity of 15–16.5-kDa and 28-kDa bands (corresponding to BSP proteins) increased during the incubation to reach a maximal intensity at 24 h incubation (Fig. 2A, lanes 2–8). In sperm protein extracts from semen diluted with EY-TG or LDF-TG extender (Fig. 2, B and C, respectively), the intensity of the bands corresponding to BSP proteins decreased significantly.

Immunoblot Analysis of BSP Proteins in Sperm Protein Extract

In order to confirm SDS-PAGE results, we subjected sperm protein extracts from each incubation time (0, 1, 2, 4, 6, 8, and 24 h) to immunoblotting using antibodies against BSP-A1/A2-, BSP-A3-, and BSP-30-kDa proteins. In the first experiment, in semen incubated undiluted or diluted with TG extender, intensity of the bands corresponding to BSP-A1/A2, BSP-A3, and BSP-30-kDa (Fig. 3, A–C, respectively) at each incubation time (0, 1, 2, 4, 6, 8, and 24 h) increased in a time-dependent manner to reach maximal intensity at 24 h of incubation. In contrast, when the semen was diluted with EY-TG extender and subjected to immunoblotting, it revealed that the intensity of the bands corresponding to BSP-A1/A2 and BSP-A3 proteins at each incubation time remained the same, and the intensity of the bands corresponding to BSP-30-kDa increased slightly (Fig. 3C, lanes 6 and 7).

In a similar manner, in the second experiment, intensity of the bands corresponding to BSP-A1/A2, BSP-A3, and BSP-30-kDa (Fig. 4, A–C, respectively) at each incubation time (0, 1, 2, 4, 6, 8, and 24 h) increased in a time-dependent manner to reach maximal intensity at 24 h of incubation in semen diluted with TG extender and incubated at 4°C. Moreover, when the semen was diluted with EY-TG or LDF-TG extender and subjected to immunoblotting, it

revealed that the intensity of the bands corresponding to BSP-A1/A2 and BSP-A3 proteins at each incubation time seemed to be the same or slightly decreased. Immunoblotting using antibodies against BSP-30-kDa revealed that the intensity of the bands corresponding to each incubation time increased slightly (Fig. 4C).

Amount of BSP Proteins Associated with Sperm During Incubation of Semen

To confirm the immunoblot results, the concentration of BSP proteins in sperm protein extracts was assessed by RIAs. In the first experiment, during the incubation, the quantity of BSP-A1/A2, BSP-A3, and BSP-30-kDa associated with sperm increased in a time-dependent manner in undiluted semen or semen diluted with TG extender and remained the same in semen diluted with EY-TG extender (Fig. 5, A–C, respectively). Furthermore, in semen incubated undiluted or diluted with TG extender, there is no significant difference between the amounts of each BSP protein associated with sperm. However, at the start of the incubation, a decrease in the quantity of BSP proteins associated with sperm was evident in semen diluted with EY-TG extender as compared to undiluted semen or semen diluted with TG extender, and it represented an average of 79% for BSP-A1/A2 and BSP-A3 and 55% for BSP-30-kDa. In the presence of EY, the average amount of BSP-A1/A2, BSP-A3, and BSP-30-kDa associated with sperm at the start of the incubation was 29.4 ± 7.3, 8.7 ± 3.0, and 47.1 ± 7.5 ng/10^6 sperm, respectively.

In the second experiment, the quantity of BSP-A1/A2, BSP-A3, and BSP-30-kDa associated with sperm increased in a time-dependent manner during the incubation of semen diluted with TG extender and remained the same during the incubation of semen diluted with EY-TG or LDF-TG extender (Fig. 6, A–C, respectively). Moreover, there is no significant difference in the amount of BSP proteins associated with sperm in semen diluted with EY-TG or LDF-TG extender. Furthermore, at the start of the incubation, 70% less BSP-A1/A2 and 50% less BSP-A3 and BSP-30-kDa was bound to sperm in semen diluted with EY-TG or LDF-TG extender as compared to sperm incubated in TG extender. In semen diluted with TG extender, the average amount of BSP-A1/A2, BSP-A3, and BSP-30-kDa associated with sperm at the start of incubation was 102.5 ± 10.2, 11.9 ± 1.0, and 36.7 ± 4.1 ng/10^6 sperm, respectively.

Sperm Cholesterol and Choline Phospholipid Analysis

Lipid analyses were used to verify if there is a continuous cholesterol and choline phospholipid efflux from the sperm membrane caused by BSP proteins present in seminal plasma during incubation of undiluted semen or semen diluted with TG extender and if the presence of EY in the extender protects sperm against cholesterol and choline phospholipid loss. In the first experiment, the average amount of cholesterol and choline phospholipid associated with sperm at the start of the incubation was 213.1 ± 10.4 μg/10^9 sperm and 816.2 ± 27.8 μg/10^9 sperm, respectively. Right after dilution of semen with EY-TG extender, no differences were observed in the amount of cholesterol and choline phospholipids associated with sperm as compared to undiluted semen or semen diluted with TG extender. As shown in Figure 7, a gradual loss of sperm cholesterol and choline phospholipids was observed during incubation of undiluted semen and semen diluted with TG extender. After 24 h of incubation, sperm from semen incubated undiluted

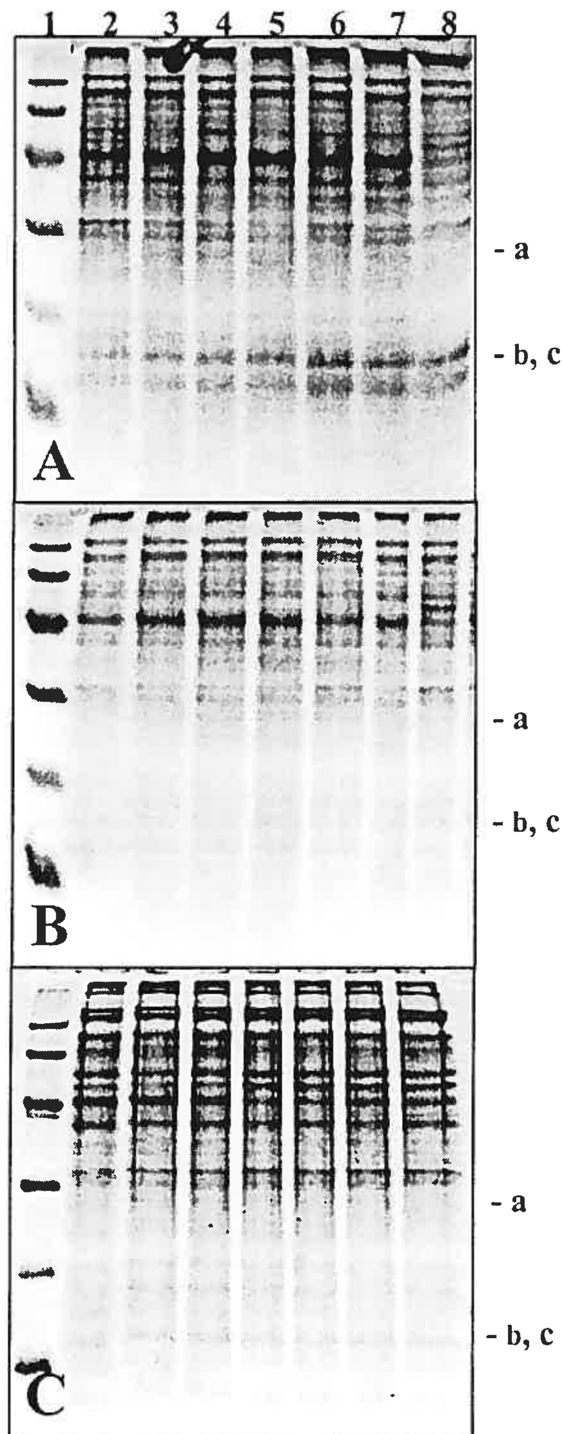

FIG. 2. SDS-PAGE pattern of sperm proteins from semen diluted with TG, EY-TG, or LDF-TG extender and incubated at 4°C. Aliquots of sperm proteins (15 μg) from semen incubated for 0 to 24 h were reduced, denatured, and separated on 15% polyacrylamide gel and stained with Coomassie Blue R-250 dye. **A**) Sperm proteins from semen diluted with TG extender. **B**) Sperm proteins from semen diluted with EY-TG extender. **C**) Sperm proteins from semen diluted with LDF-TG extender. In **A–C**, lane 1 corresponds to LMW standard (see Fig. 1). Lanes 2–8 correspond to samples at 0, 1, 2, 4, 6, 8, and 24 h of incubation, respectively. a, b, and c indicate the positions of BSP-30-kDa, BSP-A1/A2, and BSP-A3, respectively. The experiment was performed on four ejaculates, and a representative experiment is shown.

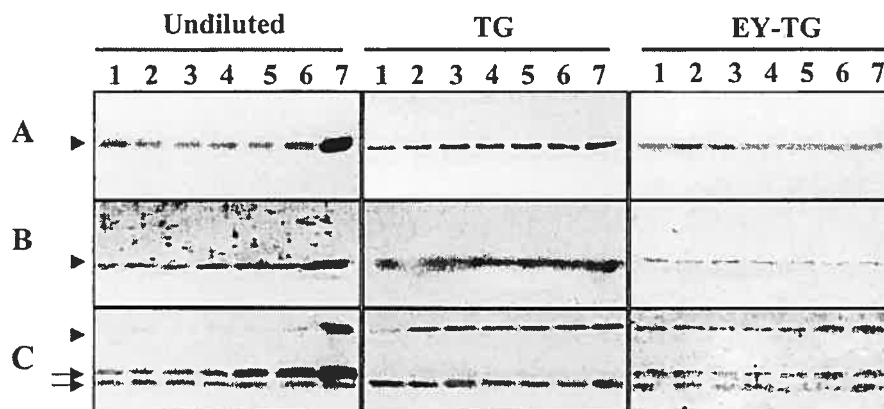

FIG. 3. Immunoblot analysis of BSP proteins associated with sperm during incubation at 37°C of semen undiluted or diluted with TG or EY-TG extender. Aliquots of sperm protein samples (100 ng) corresponding to each incubation time (0, 1, 2, 4, 6, 8, and 24 h) in undiluted semen or semen diluted with TG or EY-TG extender were separated by SDS-PAGE. The separated proteins were transferred to Immobilon-P and probed with purified antibodies directed against BSP proteins. **A**) Anti-BSP-A1/A2. **B**) Anti-BSPA3. **C**) Anti-BSP-30-kDa. Lanes 1–7 correspond to 0, 1, 2, 4, 6, 8, and 24 h of incubation at 37°C. The arrowheads in **A–C** correspond to the position of BSP-A1/A2, BSP-A3, and BSP-30-kDa, respectively. Arrows in **C** correspond to BSP-30-kDa immunoreactive proteins. The experiment was performed on four ejaculates, and a representative experiment is shown.

or diluted with TG extender lost 51.0% ± 2.1% and 35.1% ± 7.5% of their cholesterol, respectively ($P < 0.001$; Fig. 7A), and 42.2% ± 4.0% and 40.2% ± 7.1% of their choline phospholipids, respectively ($P < 0.001$; Fig. 7B). However, after 24 h of incubation, sperm diluted with EY-TG extender gained 47.9% ± 10.3% of cholesterol ($P < 0.001$; Fig. 7A) and 61.1% ± 9.7% of choline phospholipids ($P < 0.001$; Fig. 7B).

In the second experiment, the amount of cholesterol and choline phospholipids associated with sperm at the start of incubation was 185.9 ± 11.6 $\mu g/10^9$ sperm and 733.9 ± 60.7 $\mu g/10^9$ sperm, respectively. No significant differences were observed in the average amount of cholesterol and choline phospholipids associated with sperm in semen diluted with EY-TG or LDF-TG extender as compared to semen diluted with TG extender (control). As shown in Figure 8, a gradual loss of sperm cholesterol and choline phospholipids was observed over 8 h of incubation, and then it reached a plateau in semen diluted with TG extender. After 24 h of incubation, sperm from semen diluted with TG extender lost 15.6% ± 4.2% of their cholesterol ($P < 0.01$; Fig. 8A) and 12.4% ± 3.5% of their choline phospholipids ($P < 0.001$; Fig. 8B). However, during incubation of semen diluted with EY-TG or LDF-TG, a gradual gain of cholesterol and choline phospholipids was observed during 8 h, and then it reached a plateau. After 24 h of incubation, sperm diluted with EY-TG or LDF-TG extender gained 35.7% ± 17.1% or 21.6% ± 6.1% of cholesterol respectively ($P < 0.01$; Fig. 8A), and 48.1% ± 17.8% and 33.6% ± 2.6% of choline phospholipids, respectively ($P < 0.01$; Fig. 8B).

Seminal Plasma and EY Effect on Sperm Functions

The sperm function analyses were performed in order to determine the deleterious effects of seminal plasma in undiluted semen or semen diluted with TG extender and the protective effect of the presence of EY or EY-LDF in the extender on sperm viability, motility, and acrosomal integrity during semen incubation. Table 1 shows that during the

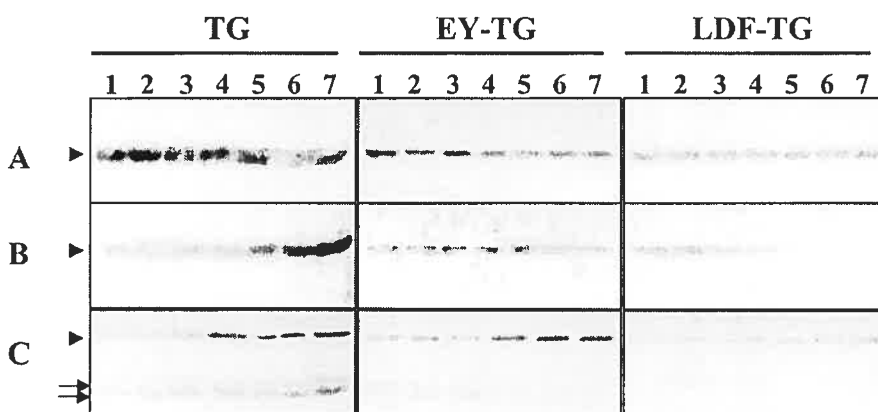

FIG. 4. Immunoblot analysis of BSP proteins associated with sperm during incubation at 4°C of semen diluted with TG, EY-TG or LDF-TG extender. Aliquots of sperm protein samples (100 ng) corresponding to each incubation time (0, 1, 2, 4, 6, 8, and 24 h) in semen diluted with TG, EY-TG, and LDF-TG extender were separated by SDS-PAGE. The separated proteins were transferred to Immobilon-P and probed with purified antibodies directed against BSP proteins. **A**) Anti-BSP-A1/A2. **B**) Anti-BSPA3. **C**) Anti-BSP-30-kDa. Lanes 1–7 correspond to 0, 1, 2, 4, 6, 8, and 24 h of incubation at 4°C. The arrowheads in **A–C** correspond to the position of BSP-A1/A2, BSP-A3, and BSP-30-kDa, respectively. Arrows in **C** correspond to BSP-30-kDa immunoreactive proteins. The experiment was performed on four ejaculates, and a representative experiment is shown.

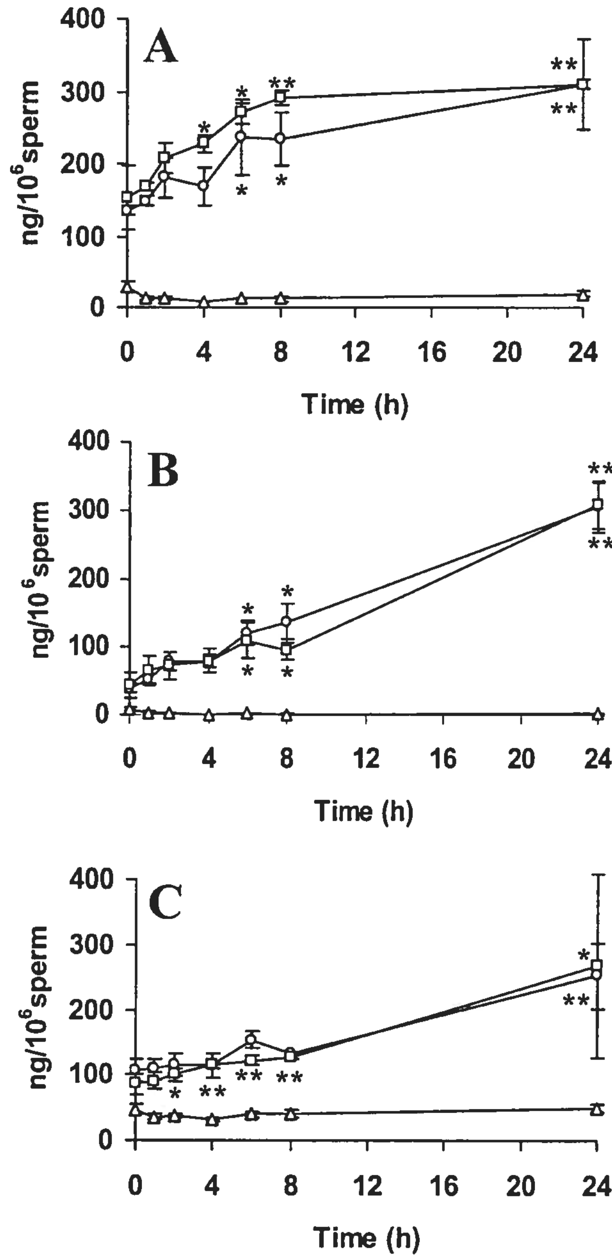

FIG. 5. Amount of BSP proteins associated with sperm during incubation at 37°C of semen undiluted or diluted with TG or EY-TG extender. Each BSP protein was quantified by RIAs in sperm protein extracts from semen incubated 24 h at 37°C undiluted (circles) or diluted with TG (squares) or EY-TG extender (triangles). **A**) BSP-A1/A2. **B**) BSP-A3. **C**) BSP-30-kDa. Results represent the mean ± SEM of four ejaculates performed in triplicate. Significant differences vs. control (0 h): * $P < 0.01$; ** $P < 0.001$. Within each incubation time, amount of BSP proteins associated with sperm in undiluted semen and semen diluted with TG extender is significantly different as compared to semen diluted with EY-TG extender according to the protected least significant difference (LSD) test ($P < 0.05$).

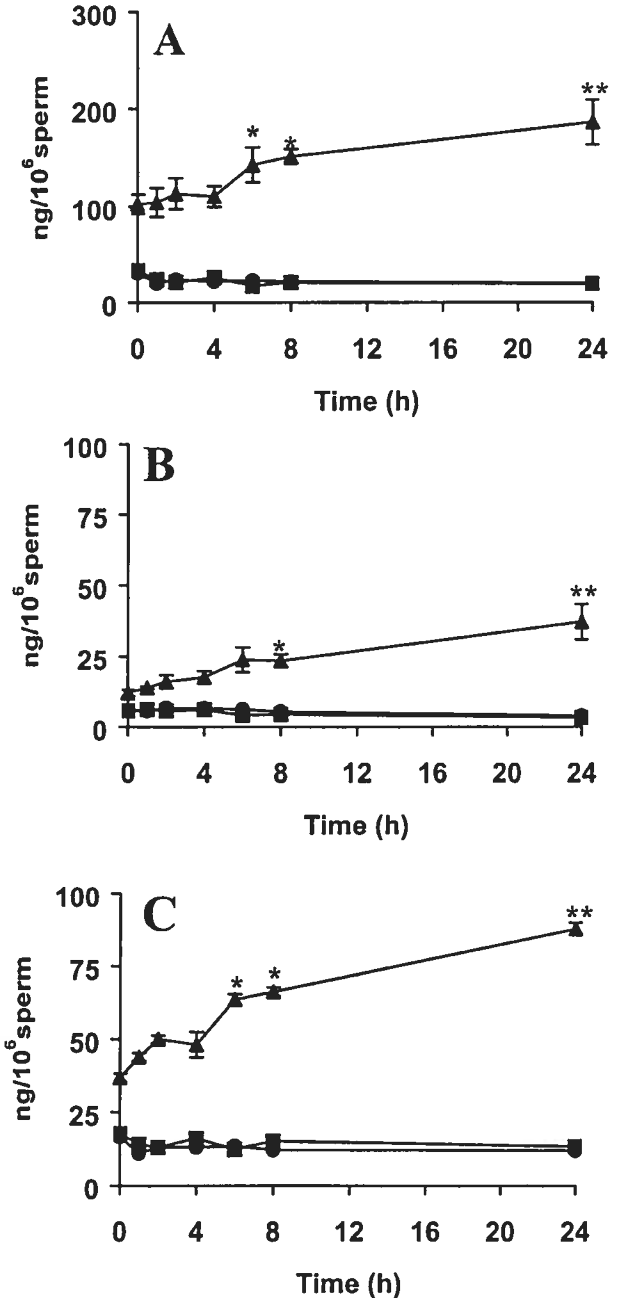

FIG. 6. Amount of BSP proteins associated with sperm during incubation at 4°C of semen diluted with TG, EY-TG or LDF-TG extender. Each BSP protein was quantified by RIAs in sperm protein extracts from semen incubated 24 h at 4°C diluted with TG (solid triangles), EY-TG (solid circles), or LDF-TG (solid squares) extender. **A**) BSP-A1/A. **B**) BSP-A3. **C**) BSP-30-kDa. Results represent the mean ± SEM of four ejaculates performed in duplicate. Significant differences vs. control (0 h): * $P < 0.01$; ** $P < 0.001$. Amount of BSP proteins associated with sperm diluted with TG extender is significantly different as compared to semen diluted with EY-TG and LDF-TG extender within each incubation time according to the protected least significant difference (LSD) test ($P < 0.05$).

24 h of incubation at 37°C, sperm in undiluted semen and semen diluted with TG or EY-TG extender underwent a time-related decrease of viability as compared to control (0 h) ($P < 0.01$). After 24 h of incubation, more sperm were alive in semen diluted with EY-TG extender as compared to undiluted semen or semen diluted with TG extender ($P < 0.01$). However, after 24 h of incubation, sperm from undiluted semen and semen diluted with TG or EY-TG extender were immotile. A gradual decrease in sperm motility was observed during the first 8 h of incubation of undiluted semen and semen diluted with TG or EY-TG extenders

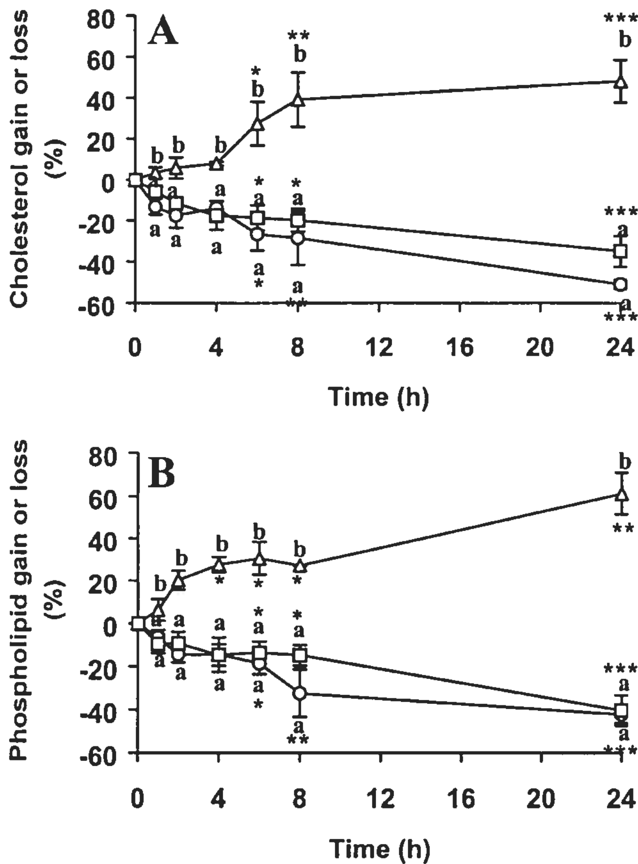

FIG. 7. Sperm cholesterol and choline phospholipids loss or gain in undiluted semen or semen diluted with TG or EY-TG extender and incubated at 37°C. Fresh semen samples were collected and incubated undiluted (circles) or diluted with TG (squares) or EY-TG (triangles) extender. At 0, 1, 2, 4, 6, 8, and 24 h of incubation at 37°C, semen samples were taken, and the cholesterol and choline phospholipid amounts associated with sperm were determined as described in *Materials and Methods* and expressed as the loss or gain in sperm cholesterol and choline phospholipids. A) Cholesterol. B) Choline phospholipids. Results represent the mean ± SEM of four ejaculates performed in duplicate. Significant differences vs. control (0 h): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Different letters within each incubation time denote significant differences between treatments according to the protected least significant difference (LSD) test ($P < 0.05$).

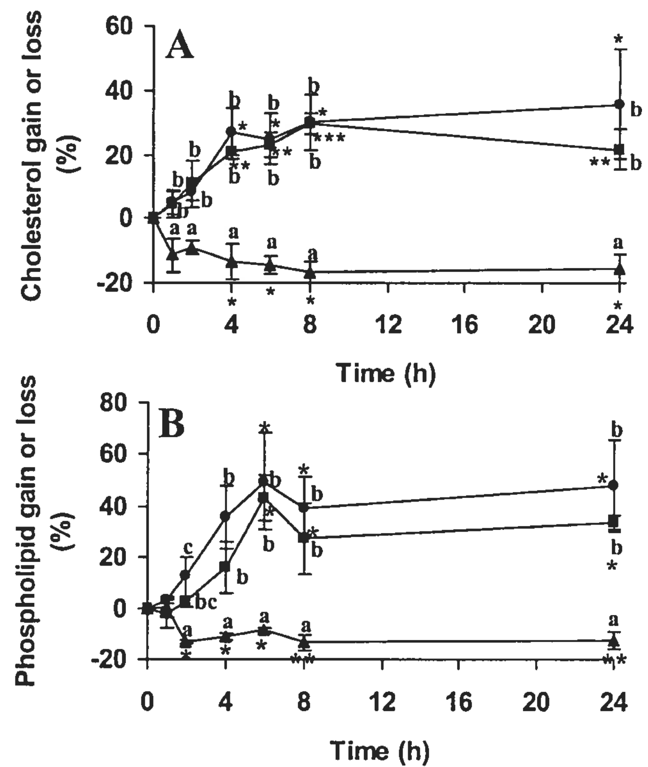

FIG. 8. Sperm cholesterol and choline phospholipids loss or gain in semen diluted with TG, EY-TG, or LDF-TG extender and incubated at 4°C. Fresh semen samples were collected and diluted with TG (solid triangles), EY-TG (solid circles), or LDF-TG (solid squares) extender. At 0, 1, 2, 4, 6, 8, and 24 h of incubation at 4°C, semen samples were taken, and the cholesterol and choline phospholipid amounts associated with sperm were determined as described in *Materials and Methods* and expressed as the loss or gain of sperm cholesterol and choline phospholipids. A) Cholesterol. B) Choline phospholipids. Results represent the mean ± SEM of four ejaculates performed in duplicate. Significant differences vs. control (0 h): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Different letters within each incubation time denote significant differences between treatments according to the protected least significant difference (LSD) test ($P < 0.05$).

(data not shown). The acrosomal integrity remained the same during 24 h of incubation in undiluted semen or in semen diluted with TG or EY-TG extender.

Effect of the Presence of LDF or EY in the Extender on Sperm Functions During Incubation at 4°C

In the second experiment (Table 2), percentage of live sperm remained the same, and sperm acrosome remained intact during incubation of semen in the absence or presence of EY or LDF in the extender. However, there was a gradual decrease in motility during the first 8 h of incubation, reaching a plateau during 24 h when semen was diluted with TG or EY-TG extender (data not shown), while motility in semen diluted with LDF-TG extender remained the same. After 24 h of incubation, percentage motility was better in semen diluted with LDF-TG extender as compared to semen diluted with EY-TG extender, which was better than semen diluted with TG extender ($P < 0.05$).

DISCUSSION

For sperm preservation, the semen of several species is generally diluted with extender containing EY, and on dilution, sperm have fewer interactions with seminal plasma and more interactions with the surrounding extender. A previous study shows that EY-LDF binds BSP proteins, the major proteins of seminal plasma (35–50 mg/ml), and it is suggested that this interaction plays a key role in sperm protection by EY lipoproteins [37]. At ejaculation, the BSP proteins bind to sperm and stimulate cholesterol and choline phospholipid efflux from the sperm membrane. The lipid efflux is time and concentration dependent. If the ejaculate is undiluted, sperm are exposed to a high concentration of seminal plasma (BSP proteins), and there is a continuous lipid efflux that could be detrimental to sperm membrane. Since ejaculates are diluted with extender containing EY for sperm preservation, the EY-LDF may sequestrate BSP proteins and prevent them from binding to sperm and cause extensive modifications to the sperm membrane. The current study is focused on gaining more insight into this proposed mechanism of sperm protection by EY.

BSP proteins bind to choline phospholipids of the sperm

TABLE 1. Effect of seminal plasma, TG, and EY-TG extenders on sperm functions during 24-h of incubation at 37°C.

Treatments	Viability* (%)		Motility* (%)		Acrosomal integrity* (%)	
	0 h	24 h	0 h	24 h	0 h	24 h
Undiluted	61.7 ± 8.9	0.0 ± 0.0†a	65.0 ± 5.8	0.0 ± 0.0†	94.2 ± 1.5	90.0 ± 2.2
TG	73.8 ± 6.8	7.8 ± 6.5†a	62.5 ± 6.3	0.0 ± 0.0†	91.9 ± 3.0	87.9 ± 3.3
EY-TG	79.2 ± 0.5	56.9 ± 9.6†b	62.0 ± 5.0	0.0 ± 0.0†	95.1 ± 1.3	90.7 ± 3.3

* Mean ± SEM of four ejaculates that included two samples per experiment and 200 sperm assayed per sample.
† $P < 0.01$ as compared with value at time 0 h.
a,b Different letters within incubation time denote significant differences according to the protected least significant difference (LSD) test ($P < 0.05$).

membrane right after ejaculation [45, 46], but the current data show that there is a time-related increase in the BSP protein binding to sperm during the incubation of undiluted semen (Figs. 1, 3, and 5) or semen diluted (>10 times) with TG extender (Figs. 1–6). Thus, during a continuous contact of sperm with seminal plasma (diluted or not), there is a continuous binding of BSP proteins to sperm. In contrast, the presence of EY in the extender prevented the increased binding of BSP proteins to sperm during the incubation of semen (Fig. 5), and it is EY-LDF that is responsible for this effect (Fig. 6). In addition, on dilution of semen with EY-TG or LDF-TG extenders, there is 50%–80% less BSP proteins bound to the sperm surface (Figs. 5 and 6). In a previous study, it was shown that sperm diluted with EY-TG extender and frozen-thawed contained almost 80% fewer BSP proteins than sperm from fresh ejaculates [38]. EY-LDF is the only component of EY that binds specifically the BSP proteins [37]. Thus, EY-LDF is responsible for preventing the binding of BSP proteins to sperm on dilution in extender containing EY. Since the polyclonal antibodies against BSP-A1/A2, BSP-A3, and BSP-30-kDa are specific [38], the arrows in Figures 3C and 4C may correspond to the proteolytic fragments of BSP-30-kDa. Alternatively, it is also possible that the 12–14-kDa immunoreactive bands detected in the present study (Figs. 3C and 4C) may correspond to a novel BSP-30-kDa-like protein reported recently [47].

BSP proteins are the factors in seminal plasma that stimulate lipid efflux from epidydimal sperm membrane [19, 20]. The present study shows that a continuous contact of ejaculated sperm with seminal plasma that contains BSP proteins stimulates a continuous efflux of sperm cholesterol and choline phospholipids (Fig. 7, undiluted semen). This effect was observed even after dilution of the semen ~10 times with TG extender. Thus, the dilution of semen did not prevent the effect of seminal plasma on the sperm membrane. This result corroborates a study by Parks showing that in the absence of EY in the extender, there is a decrease in sperm cholesterol and phospholipid content during incubation [48]. Interestingly, the presence of EY in the extender stimulated a sperm lipid gain during incubation, and it is EY-LDF that is responsible for this lipid gain (Fig. 8). At each incubation time, no significant differences were observed between lipid gain in semen diluted with EY-TG and LDF-TG extenders. Therefore, cholesterol and choline phospholipids from LDF are added to sperm, or whole molecules of LDF bound to sperm during incubation of semen diluted with EY-TG or LDF-TG extender. Some studies suggest that EY-LDF binding occurs when semen is stored in EY extender, and this binding is important for sperm protection [30, 49].

In the presence of EY in the extender, sperm cholesterol and choline phospholipids gain were two times less in semen incubated at 4°C (Fig. 8) than in semen incubated at 37°C (Fig. 7). This can be explained by the phase transition that occurs in biological membranes during cooling. During cooling, biological membranes become less fluid and are less susceptible for exchange of lipids. It is possible that the change in the lipid phase of the sperm membrane and the phospholipid film of LDF molecule during cooling prevents partially the exchange of lipid from LDF to sperm membrane or the binding of LDF to sperm. In a similar manner, in semen diluted with TG extender, the lipid loss from sperm membrane was two to three times less in semen incubated at 4°C (Fig. 8) than semen incubated at 37°C (Fig. 7).

The presence of EY or LDF in the extender maintained sperm motility during 24 h of incubation at 4°C (Table 2). It has been shown that sperm motility could be maintained more than 8 days when semen is diluted with EY-TG extender and cooled [50] and that EY is the extender constituent that is responsible for maintaining motility [25, 48, 51]. Furthermore, after 24 h of incubation, motility was better in semen diluted with extender containing LDF than whole EY. This can be explained by the fact that EY is known to protect sperm, but it is also known to contain factors that inhibit sperm respiration [52] and decrease motility [29], and it may be possible that those factors are not present in the LDF. Whether semen was diluted with TG or EY-TG extender, sperm viability and motility were better at 4°C (Table 2) than at 37°C (Table 1). When the semen is stored at 4°C, the metabolic rate of sperm is lowered,

TABLE 2. Effect of TG, EY-TG, and LDF-TG extenders on sperm functions during 24 h of incubation at 4°C.

Extender	Viability* (%)		Motility* (%)		Acrosomal integrity* (%)	
	0 h	24 h	0 h	24 h	0 h	24 h
TG	74.8 ± 4.4	74.8 ± 3.3	67.5 ± 2.5	30.3 ± 3.5†a	95.7 ± 0.7	91.1 ± 0.7
EY-TG	77.8 ± 2.0	74.4 ± 3.4	60.0 ± 5.0	47.5 ± 5.0b	95.7 ± 0.2	90.2 ± 1.6
LDF-TG	71.6 ± 4.9	75.8 ± 3.3	66.3 ± 4.8	62.5 ± 4.8c	95.3 ± 1.3	94.3 ± 0.9

* Mean ± SEM of four ejaculates that included two samples per experiment and 200 sperm assayed per sample.
† $P < 0.001$ as compared with value at time 0 h.
a,b,c Different letters within incubation time denote significant differences according to the protected least significant difference (LSD) test ($P < 0.05$).

and this could contribute to the extension of sperm survival. Seminal plasma has been shown to be deleterious to sperm functions by several workers, and the present study supports this notion.

Extensive modification of the lipid content of the sperm membrane, such as removing cholesterol and phospholipids, can compromise the sperm ability to fertilize an oocyte. In the present study, seminal plasma stimulates continuous cholesterol and choline phospholipid efflux in undiluted semen (Fig. 7) or in semen diluted with extender containing neither EY nor LDF (Figs. 7 and 8). However, a continuous exposure of sperm to seminal plasma did not stimulate acrosome reaction (Tables 1 and 2). Furthermore, neither the cooling procedure (second experiment) nor lipid efflux from sperm membrane affected sperm acrosomal integrity during incubation of semen. Seminal plasma contains factors that prevent premature capacitation, such as decapacitation factor and acrosome stabilizing factor [5, 8]. Those factors may contribute to maintaining acrosomal integrity during incubation of undiluted or semen diluted with TG extender. However, some studies indicate that premature capacitation of sperm occurs when extended semen is cooled and incubated at 4°C [53, 54]. In the current study, the capacitation status of sperm was not investigated.

In summary, the current studies revealed that sperm contact with seminal plasma results in gradual loss of choline phospholipids and cholesterol from the sperm plasma membrane. This effect was persistent even when semen was diluted 10 times. Interestingly, when semen was diluted with EY, the opposite effect was observed with sperm (cholesterol and phospholipid gain and decreased association of BSP proteins to the sperm surface). Since the same effect was mimicked by the LDF, it is obvious that it is the LDF that is responsible for this effect. Moreover, the prevention of BSP binding to sperm and lipid efflux from sperm membrane resulted in the increase in semen quality (motility). In view of the present study, we suggest that the EY in extender protects sperm in two ways. First, the association of LDF with BSP proteins protects sperm by preventing BSP proteins in seminal plasma to bind to sperm and intrinsically damage sperm membrane by removing lipid. Second, the lipid from LDF or the whole molecule of LDF could associate with the sperm membrane and preserve the integrity of the plasma membrane during sperm preservation.

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2.3. Isolation and characterization of gelatin-binding bison seminal vesicle secretory proteins.

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*Bergeron A. et Boisvert M. ont contribué de façon équivalente à ces travaux.

Préambule:

Dans les travaux précédents, nous avons démontré que les LDF du jaune d'œuf protègent les spermatozoïdes bovins en séquestrant les protéines BSP présentes dans le plasma séminal lors de la dilution de la semence avec le diluant protecteur. Nous avons voulu vérifier si le mécanisme de protection des spermatozoïdes par le jaune d'œuf était le même chez les autres espèces de mammifères importantes pour l'agriculture. Pour ce faire, nous avons vérifié si le plasma séminal de ces espèces contenait des protéines homologues aux protéines BSP et si ces homologues avaient la capacité de lier les LDF du jaune d'œuf. Dans la présente étude, nous avons isolé des protéines homologues aux protéines BSP à partir des sécrétions de vésicules séminales de bison et confirmé leur capacité à lier les LDF du jaune d'œuf.

Boisvert M. a fait un stage dans le laboratoire du Dr. Manjunath au cours de l'été 2003. J'ai supervisé Boisvert M. au cours de son stage. Boisvert M. et moi avons contribué également à la réalisation des travaux qui ont mené à la rédaction de cet article à l'exception du séquençage des protéines qui a été réalisé par Lazure C. Boisvert M. a écrit la première ébauche de l'article selon mon interprétation des

résultats. J'ai révisé son manuscrit et complété la rédaction de l'article afin de le soumettre pour publication dans *Biology of Reproduction*.

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Isolation and Characterization of Gelatin-Binding Bison Seminal Vesicle Secretory Proteins[1]

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ABSTRACT

Bovine seminal plasma (BSP) contains a family of major proteins designated BSP-A1/A2, BSP-A3, and BSP-30kDa (collectively called BSP proteins) that bind to sperm at ejaculation and potentiate sperm capacitation. Homologous proteins have been identified in stallion, boar, goat, and ram seminal plasma. We report here the isolation and characterization of homologous proteins from bison seminal vesicle secretions. Seminal vesicle secretory proteins were precipitated by adding cold ethanol and recovered by centrifugation. The precipitates were resuspended in ammonium bicarbonate, dialyzed, and lyophilized. Lyophilized proteins were dissolved in 0.05 M phosphate buffer (PB) and loaded onto a gelatin-agarose column. The unadsorbed proteins and adsorbed proteins were eluted with PB and 5 M urea in PB, respectively. The gelatin-adsorbed fraction was analyzed by SDS-PAGE and revealed the presence of four major proteins designated BiSV-16kDa, BiSV-17kDa, BiSV-18kDa, and BiSV-28kDa (BiSV: bison seminal vesicle proteins). Heparin-Sepharose chromatography allowed the separation of BiSV-16kDa, which did not bind heparin from other BiSV proteins, which bound heparin. Immunoblotting revealed that BiSV-16kDa cross-reacted with BSP-A3 antibodies, BiSV-17kDa and BiSV-18kDa cross-reacted with BSP-A1/-A2 antibodies, and BiSV-28kDa cross-reacted with BSP-30kDa antibodies. Radioimmunoassays indicated that ~25% of bison seminal vesicle total proteins are related to BSP proteins. The amino-terminal sequencing indicated that BiSV proteins share almost 100% sequence identity with BSP proteins. In addition, BiSV proteins bind to low-density lipoproteins isolated from hen's egg yolk. These results confirm that BSP protein homologs are present in mammalian seminal plasma and they may share the same biological role.

male reproductive tract, sperm, sperm capacitation, sperm maturation

INTRODUCTION

Bovine seminal vesicles secrete a family of acidic proteins designated BSP-A1, BSP-A2, BSP-A3, and BSP-30kDa (collectively called bovine seminal plasma [BSP] proteins). BSP-A1, -A2, and -A3 have apparent molecular masses ranging from 15 to 17 kDa and the BSP-30kDa protein has a molecular mass of 28 to 30 kDa [1–6]. BSP-A1 and -A2, also designated PDC-109 [7] or BSP-A1/-A2, have an identical amino acid sequence but their difference resides in their degree of glycosylation. BSP-30kDa is also a glycoprotein, whereas BSP-A3 does not contain any carbohydrate [4]. The structure of each BSP protein contains two tandemly arranged homologous domains that are similar to the type II structures present in the collagen-binding domain of fibronectin [3, 7–10]. This structural property allows BSP protein to bind gelatin, heparin [11], high-density lipoprotein (HDL), choline phospholipids, and low-density lipoprotein fraction (LDF) from hen's egg yolk [12–15].

Seminal plasma contains BSP-homologous proteins in a variety of mammalian species; such as boar (pB1 [16–18]), stallion (HSP-1, HSP-2, and HSP-12kDa [16, 19, 20]), and goat (GSP-14kDa, GSP-15kDa, GSP-20kDa, and GSP-22kDa [21]). BSP-like antigens are also present in rat, mouse, hamster, and human seminal plasma [22]. The biological properties of BSP proteins have been extensively studied [23]. They play a role in fertilization, more specifically in sperm capacitation [13, 14, 24, 25]. A recent report demonstrated that BSP-A1/-A2 (PDC-109) protein enable sperm to bind the oviductal epithelium and plays a major role in the formation of the oviductal sperm reservoir [26].

The general mechanism of capacitation proposed by our laboratory includes modification of the sperm membrane by BSP proteins [23]. At ejaculation, sperm are exposed to seminal plasma, which contains BSP proteins. BSP proteins bind to sperm membrane choline phospholipids [12] and stimulate cholesterol efflux from the sperm membrane (first cholesterol efflux) [24]. When sperm reach the oviduct, HDL present in oviductal and/or follicular fluid interact with the sperm membrane, which results in further removal of cholesterol (second cholesterol efflux). This cholesterol efflux destabilizes the sperm membrane, increases permeability to certain ions such as Ca^{2+}, changes internal pH, and increases membrane fluidity and metabolism, leading to the capacitation state [27–29]. Moreover, heparin-like glycosaminoglycans (GAGs) in the female genital tract could also play a role in this capacitation mechanism because GAGs also interact with BSP proteins [25, 30].

Converse to this positive role in fertility, BSP proteins may also have negative effects on sperm in the context of sperm storage. Because lipid efflux induced by BSP proteins is time and concentration dependant, continuous exposure of sperm to seminal plasma that contains BSP proteins can be detrimental to the sperm membrane and renders the membrane very sensitive to storage in liquid or frozen states [15]. Recent results indicate that BSP proteins form stable complexes with LDF from hen's egg yolk used in

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sperm preservation media [15]. This interaction appears to play a key role in protecting sperm against the deleterious effect of BSP proteins, thereby permitting sperm preservation in a liquid or frozen state.

The growth of the bison industry over the past 10 yr has created the urgent need of novel reproductive technologies to optimize the quality and rapidity of bison production. However, to date, bison reproductive mechanisms are not well understood. In the present study, we took advantage of the affinity of BSP proteins for gelatin to isolate BSP-like homologs from bison seminal vesicle secretions. We also tested their binding ability to heparin and LDF from hen's egg yolk as well as their cross-reactivity with anti-BSP antibodies to confirm their structure-function relationship to BSP proteins. We quantified the amount of BSP-related proteins in bison seminal vesicle secretions using radioimmunoassays. In addition, N-terminal sequences of bison seminal vesicle (BiSV) were determined to establish the structural relationship with BSP proteins.

MATERIALS AND METHODS

Materials

Gelatin was purchased from Eastman Kodak Company (Rochester, NY). Affi-Gel 15, SDS, and other electrophoresis products were from Bio-Rad (Mississauga, ON, Canada). Sephadex G-50 (super fine) and heparin-Sepharose CL-6B were from Amersham Biosciences (Baie d'Urfé, QC, Canada). Acrylamide and bisacrylamide were purchased from ICN (Mississauga, ON, Canada). Immobilon-P polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Nepean, ON, Canada). HPLC-grade trifluoroacetic acid (TFA) was from Fisher Scientific (St-Laurent, PQ, Canada). All other chemicals used were of ultrapure grade and were obtained from local suppliers, mostly Fisher Scientific and Sigma-Aldrich (Oakville, ON, Canada).

Bovine seminal plasma was obtained from the Centre d'Insémination Artificielle du Québec (St-Hyacinthe, PQ, Canada). Crude bovine seminal plasma (cBSP) proteins were prepared by ethanol precipitation of bovine seminal plasma followed by dialysis of the precipitates against 50 mM ammonium bicarbonate and lyophilization. This preparation consisted of 60–70% BSP proteins. Bison seminal vesicles were provided by Abattoir Lefaivre (Lefaivre, ON, Canada) and Abattoir Louis Lafrance & Fils Ltée, (St-Séverin de Proulxville, PQ, Canada).

Isolation of Bison Seminal Vesicle Secretory Proteins

Bison seminal vesicle secretions were obtained by gently squeezing seminal vesicle tissues and collecting fluid in a plastic tube. Approximately 1 ml of seminal vesicle secretions were collected, diluted in 4 ml of 0.05 M phosphate buffer (PB), and centrifuged (10 000 × g, 10 min) to remove cellular debris. Nine volumes of cold (−20°C) ethanol were added and left with constant stirring for 90 min at 4°C to precipitate the proteins. Proteins were then recovered by centrifugation at 10 000 × g for 10 min. The precipitates were washed three times with cold ethanol. The precipitates were then solubilized and dialyzed against 50 mM ammonium bicarbonate and lyophilized. Approximately 225 mg of dried powder, designated crude bison seminal vesicle secretory proteins (starting material), was recovered and stored at 4°C until further purification.

Gelatin-Agarose Affinity Chromatography

Gelatin was coupled to Affi-Gel 15 as described previously [3]. All purification steps were conducted at 4°C. One hundred milligrams of lyophilized crude bison seminal vesicle secretory proteins were dissolved in 3 ml PB and loaded on a gelatin-agarose column at a flow rate of 30 ml/h, which was previously equilibrated with the same buffer. Once the sample entered the column, the flow was stopped for 30 min to allow proteins to interact with the gel. After unadsorbed proteins were washed out with PB, the bound protein fraction was eluted with 5 M urea in PB. Four-milliliter fractions were collected with a fraction collector (FRAC-100) and their absorbance at 280 nm was measured. Then the fractions corresponding to each peak were pooled. Fraction A was dialyzed against ammonium bicarbonate 50 mM and lyophilized. Half of fraction B was dialyzed against 50 mM ammonium bicarbonate and lyophilized whereas the other half of fraction B was dialyzed against PB and used in the next step.

Heparin-Sepharose Affinity Chromatography

All steps were conducted at 4°C at a flow rate of 20 ml/h. The gelatin-agarose-adsorbed proteins (fraction B) dialyzed against PB were concentrated to 2 ml and loaded on a heparin-Sepharose CL-6B column, which was previously equilibrated with the same buffer. Once the sample entered the column, the flow was stopped for 30 min to allow proteins to interact with the gel. The column was then washed with PB to remove unadsorbed proteins and the bound proteins were eluted with 1 M NaCl in PB. Four-milliliter fractions were collected and their absorbance at 280 nm was measured. Fractions corresponding to each protein peak were pooled, dialyzed against 50 mM ammonium bicarbonate, and lyophilized.

SDS-PAGE and Immunodetection of BSP-Related Proteins

Starting material, gelatin-agarose, and heparin-Sepharose fractions were denatured and separated in 15% polyacrylamide gel. The apparent molecular mass was estimated using the Low Molecular Weight Calibration Kit from Amersham Biosciences. Proteins in the gel were either stained with Coomassie Blue or transferred electrophoretically to immobilon-P PVDF membranes, as described previously [31], using Trans-Blot Cell apparatus from Bio-Rad. Transferred proteins were stained with Coomassie Blue for N-terminal sequence analysis or used for immunodetection using specific antibodies against each BSP protein (BSP-A1/A2, BSP-A3, and BSP-30kDa) as described previously [6].

Quantification of BSP-Related Proteins by Radioimmunoassays

Iodination of each BSP protein was performed by the lactoperoxidase method as described previously [32]. The quantity of BSP-related proteins present in starting material, gelatin, and heparin-unadsorbed and -adsorbed peaks A, B, B1, and B2 were determined by RIAs as described recently [33]. The assay tubes containing the ^{125}I-labeled and unlabeled antigen (BSP-A1/A2, BSP-A3, and BSP-30kDa) or bison seminal vesicle proteins, the primary antibodies (anti-BSP-A1/A2, anti-BSP-A3, and anti-BSP-30kDa), and normal rabbit serum (1.5%, v/v) were incubated for 20 h at 37°C. After incubation, 500 μl of 10% polyethylene glycol was added, the antibody-antigen complex was separated by centrifugation (2200 × g, 20 min) and the radioactivity associated with the pellet was determined in a gamma counter (1272 CliniGamma; Pharmacia, Wallac, Finland).

RP-HPLC

Reverse phase-high performance liquid chromatography (RP-HPLC) was performed on bison seminal vesicle secretory proteins as described previously [21]. Dialyzed and lyophilized starting material, gelatin-agarose-unadsorbed and -adsorbed fractions, and heparin-Sepharose-unadsorbed and -adsorbed fractions were dissolved in 0.1% TFA and loaded on the column and eluted using a gradient of acetonitrile in 0.1% TFA. Fractions of 1 ml were collected at a flow rate of 1 ml/min. The eluting proteins were monitored at 235 nm and dried under vacuum.

Sequencing

Following transfer of the proteins to Immobilon-P membrane, the Coomassie Blue-stained protein bands were cut and placed in the sequenator reactor. Sequencing was carried out according to the manufacturer's protocol using an Applied Biosystems Procise sequencer (model 494; Foster City, CA).

Interaction of BiSV Proteins with Hen's Egg Yolk Lipoproteins

The binding of heparin-adsorbed fractions to LDF isolated from hen's egg yolk was studied using a Paragon electrophoresis kit (Beckman Coulter, Fullerton, CA). LDF from hen's egg yolk was isolated as described previously [15]. Fraction B1 containing BiSV-16kDa, fraction B2 containing BiSV-17kDa, BiSV-18kDa, and BiSV-28kDa, and cBSP proteins were incubated with LDF in Tris-HCl buffer in a total volume of 8 μl for 15 min. Four microliters of each sample were loaded on the lipogel (0.5% Agarose gel) and subjected to electrophoresis. The electrophoresis was run at 100 V for 30 min, then the gel was dipped in fixative solution and dried. The lipid bands were subsequently stained with Sudan black B.

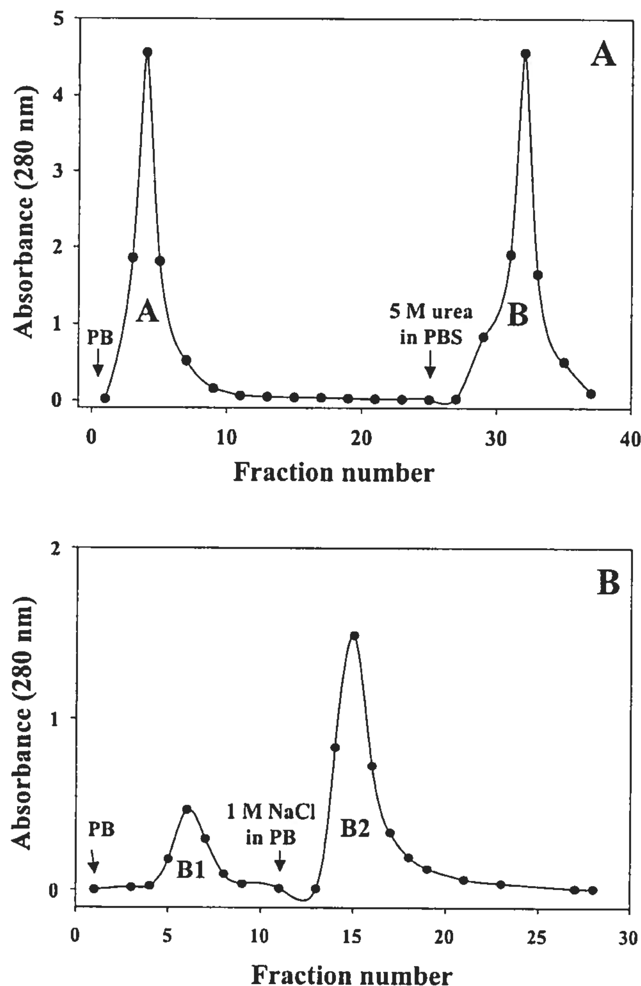

FIG. 1. Isolation of bison seminal plasma proteins. A) Gelatin-agarose affinity chromatography elution profile of crude bison seminal vesicle secretory proteins. B) Heparin-Sepharose affinity chromatography pattern of gelatin-adsorbed proteins (fraction B).

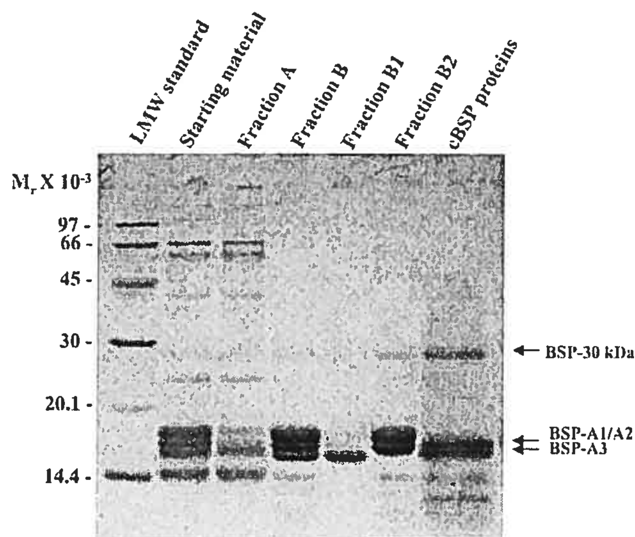

FIG. 2. SDS-PAGE pattern of gelatin-agarose- and heparin-Sepharose-eluted fractions. Twenty micrograms of crude bison seminal vesicle secretory proteins (starting material) and 10 μg of gelatin-agarose- and heparin-Sepharose-adsorbed (B and B2) and -unadsorbed (A and B1) fractions were reduced, denatured, and subjected to electrophoresis. Proteins were stained with Coomassie brilliant Blue R-250. The electrophoresis pattern of cBSP proteins was used for comparison.

RESULTS

Isolation and Purification of Gelatin-Binding Proteins

Figure 1A shows the gelatin-agarose chromatography pattern of crude bison seminal vesicles secretory proteins (starting material). Fraction A represented the unadsorbed proteins and was eluted with PB. The adsorbed proteins were eluted with 5 M urea in PB and correspond to fraction B. The total weight of proteins recovered from the gelatin-agarose column was approximately 85% of that of starting material and the adsorbed proteins constituted approximately 28% of the recovered proteins. Fraction B was analyzed by SDS-PAGE (Fig. 2), which revealed the presence of four different proteins of apparent molecular masses of 16, 17, 18, and 28 kDa. We designated these proteins bison seminal vesicle secretory proteins, or BiSV proteins (BiSV-16kDa, BiSV-17kDa, BiSV-18kDa, and BiSV-28kDa). Furthermore, the SDS-PAGE showed that fraction A contained a small amount of proteins having a molecular mass similar to BiSV-16kDa that did not bind gelatin-agarose.

Gelatin-adsorbed proteins (fraction B) were loaded onto a heparin-Sepharose affinity column. Unadsorbed proteins were eluted with PB (Fig. 1B, fraction B1) and proteins bound to the column were eluted with buffer containing 1 M NaCl in PB (fraction B2). Approximately 72% of total proteins were recovered in fraction B after this chromatography step, of which fractions B1 and B2 represented 22% and 78%, respectively. The SDS-PAGE pattern (Fig. 2) showed that BiSV-16kDa did not bind heparin (unadsorbed fraction, B1) while BiSV-17kDa, BiSV-18kDa, and BiSV-28kDa proteins bound heparin because they were contained in fraction B2.

RP-HPLC

Figure 3 A, B, and C, shows the HPLC pattern of fractions B, B1, and B2, respectively. The elution pattern of fraction B showed the presence of two peaks. Peak I corresponded to BiSV-17kDa, BiSV-18kDa, and BiSV-28kDa. Peak II corresponded to BiSV-16kDa. The elution of fraction B1 (Fig. 3B, peak II) generated one peak, which corresponded to BiSV-16kDa. Proteins in fraction B2 did not separate and the elution pattern showed only one peak (peak I) containing BiSV-17kDa, BiSV-18kDa, and BiSV-28kDa (Fig. 3C). The identity of each peak was determined by SDS-PAGE analysis (data not shown).

Comparison of Amino-terminal Sequences of the BiSV Proteins and BSP Proteins

The N-terminal sequence of the first few residues (Fig. 4) of protein bands in fractions B1 and B2 (Fig. 2) showed that the sequences of BiSV-17kDa and -18kDa are identical to BSP-A1/A2 except for a reported glutamine at position 4 [7]. The sequence of BiSV-16kDa was identical to BSP-A3 sequence [9] and the N-terminal sequence of BiSV-28kDa was identical to BSP-30kDa deduced by cDNA cloning [34].

Cross-Reactivity of BiSV Proteins with BSP Antibodies

The presence of BSP-related proteins in starting material and gelatin-agarose and heparin-Sepharose fractions were

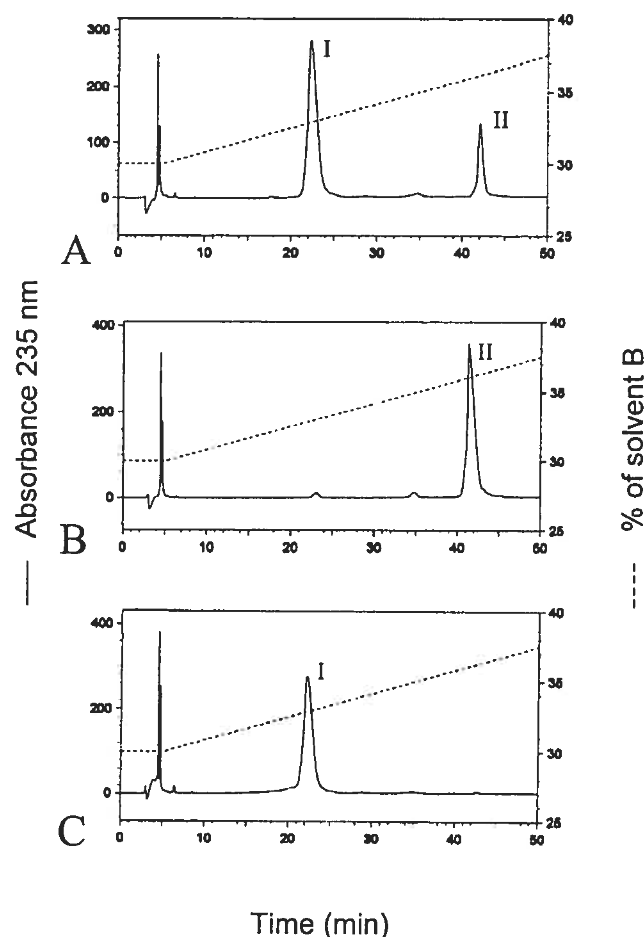

FIG. 3. RP-HPLC pattern of Gelatin-agarose- and Heparin-Sepharose-eluted fractions. Approximately 100 μg of proteins were dissolved in 1 ml of 0.1% (v/v) TFA in water and injected into a Vydac C18 column. Proteins were eluted using a 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) buffer system. Elution was isocratic at 30% B for the first 5 min, followed by a 90-min gradient of 30–45% B. The eluting proteins were monitored at 235 nm. A) Gelatin-agarose-adsorbed fraction; (B) heparin-Sepharose-unadsorbed fraction; (C) heparin-Sepharose-adsorbed fraction. Peak I was identified as BiSV-17kDa/18kDa and BiSV-30kDa, and peak II as BiSV-16kDa.

detected by probing with purified antibodies raised against each of the BSP proteins (BSP-A1/A2, BSP-A3, and BSP-30kDa) (Fig. 5 A, B, and C, respectively). All three antibodies (anti-BSP-A1/A2, -A3, and -30kDa) showed cross-reacting proteins in starting material and the gelatin-adsorbed fraction (fraction B). In the gelatin-unadsorbed fraction (fraction A), an immunoreactive band was detected when probed with anti-BSP-A3. In addition, immunoreactive bands having the same molecular mass as BiSV-17/18kDa and BiSV-28kDa proteins were detected in the heparin-adsorbed fraction (fraction B2) when probed with anti-BSP-A1/A2 and anti-BSP-30kDa antibodies, respectively, while an immunoreactive band having the same molecular mass as BiSV-16kDa was detected in fraction B1 when probed with anti-BSP-A3. Anti-BSP-30kDa antibodies cross-reacted with three different proteins (Fig. 5C): band 1 (strong reaction), corresponding to the 28-kDa protein present in starting material; fractions B and B2, band 2 (weak reaction), with a molecular mass around 27 kDa found in fraction B; and band 3 (weak reaction), with a

	1	6	11	16	21
BiSV-17kDa	DQD**_K_**G	VSTEP	TQDGP	....	
BiSV-18kDa	DQD**_K_**G	VSTEP	TQDGP	....	
BSP-A1A2:	DQD**_E_**G	VSTEP	TQDGP	AELPE	....
BiSV-16kDa	DQQLS	EDNVI	LPKEK	....	
BSP-A3:	DQQLS	EDNVI	LPKEK	KDPAS	....
BiSV-28kDa	GDIPD	PGSKP	TPPGM	....	
BSP-30 kDa:	GDIPD	PGSKP	TPPGM	ADELP	

FIG. 4. The N-terminal sequence comparison between BiSV proteins and BSP proteins. Residue numbering is indicated for BiSV-16kDa, -17kDa, -18kDa, and -28kDa and the other sequences were aligned to show the homology. The "..." indicates continuation of the protein sequence at C-terminal. The different amino acids are presented in bold and underlined.

molecular mass around 16 kDa present in fraction B and B1.

Quantification of BiSV Proteins

The cross-reactivity between anti-BSP antibodies with BiSV proteins allowed determination of the quantity of BSP-related proteins present in starting material (crude bison seminal vesicle secretory proteins), fractions A, B, B1, and B2 by radioimmunoassays (data not shown). The results showed that BiSV proteins represented approximately 25% of the crude bison seminal vesicle secretion proteins. BiSV-17kDa and BiSV-18kDa were the major BSP-related proteins and represented ~16% of the total crude bison seminal vesicle secretory proteins while BiSV-16kDa and BiSV-28kDa represented ~7% and ~2.5%, respectively. Heparin-unadsorbed proteins contained >95% of BiSV-16kDa and heparin-adsorbed proteins contained ~82% of BiSV-17/18kDa and ~8% of BiSV-28kDa.

Interaction of BiSV Proteins with LDF

The gelatin-adsorbed fraction from bison seminal vesicle secretory proteins was used to test the binding of these proteins to LDF isolated from hen's egg yolk. As shown in Figure 6, LDF incubated with heparin-unadsorbed and -adsorbed fractions migrated to the anode (lanes 3–6), as did the positive control containing cBSP proteins (lane 7). This indicated the formation of a complex between BiSV proteins and hen's egg yolk, changing the overall charge of the molecule. LDF alone (lane 2) had a neutral charge and remained at the point of application.

DISCUSSION

In the present study, BSP protein homologs from crude bison seminal vesicle secretory proteins were isolated using gelatin-agarose column followed by a heparin-Sepharose column. This method allowed the identification of four BiSV proteins that we named BiSV-16kDa, BiSV-17kDa,

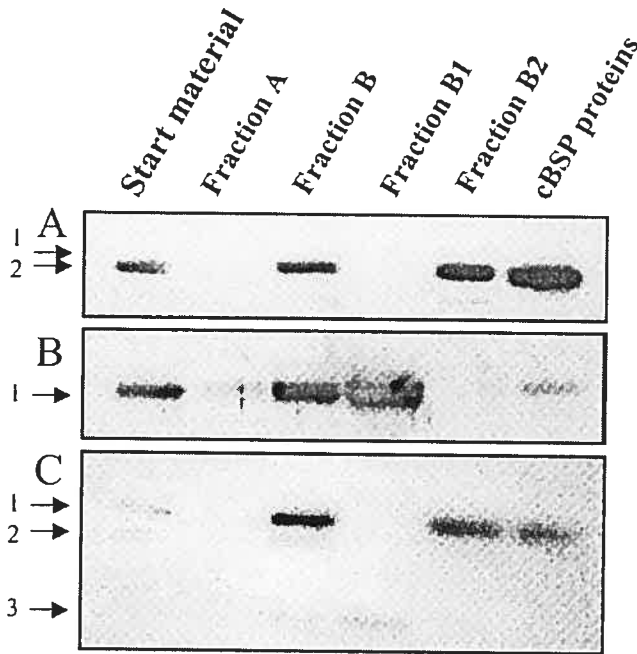

FIG. 5. Cross-reactivity of BiSV proteins with BSP antibodies. Proteins (~50 ng) from crude bison seminal vesicle secretory proteins (starting material), gelatin-agarose- and heparin-Sepharose-adsorbed (B and B2, respectively) and -unadsorbed (A and B1, respectively) fractions obtained were separated by SDS-PAGE, transblotted to Immobilon PVDF-P membrane, and probed with antibodies directed against BSP proteins. A) Anti-BSP-A1/A2: band 1, BiSV-18kDa; band 2, BiSV-17kDa; (B) anti-BSP-A3: band 1, BiSV-16kDa; and (C) anti-BSP-30kDa: band 1, BiSV-28kDa; band 2, BiSV-27kDa; band 3, BiSV-16kDa.

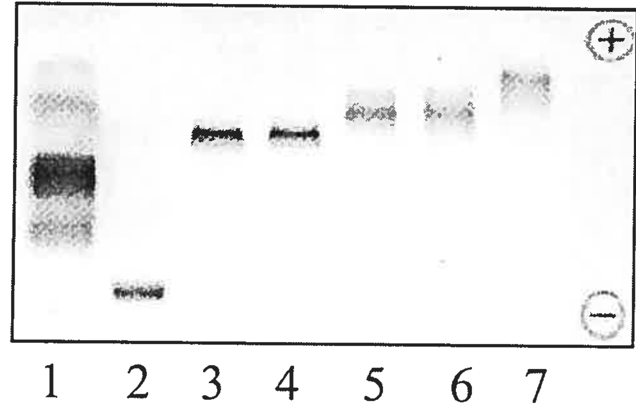

FIG. 6. Electrophoretic analysis of the interaction of BiSV proteins with LDF. Four microliters of each sample (except the human serum used as a positive control of the migration) were applied on the lipogel. Lane 1, human serum (3 μl); lane 2, LDF (1.5 μg); lanes 3 and 4, LDF (1.5 μg) + fraction B1 containing BiSV-16kDa (6 μg and 12 μg, respectively); lanes 5 and 6, LDF (1.5 μg) + fraction B2 containing BiSV-17kDa, BiSV-18kDa, and BiSV-28kDa (6 μg and 12 μg, respectively); lane 7, LDF (1.5 μg) + cBSP proteins (6 μg) as a positive control.

BiSV-18kDa, and BiSV-28kDa according to their apparent molecular mass.

The capacity of BSP proteins to bind gelatin, a denatured collagen, was used to isolate BSP homologous proteins from bison seminal vesicle secretion. The ability to bind gelatin is conferred by the presence of two fibronectin type II domains [3, 7–10]. We can deduce that BiSV proteins, by their gelatin-binding property, are also constituted of similar type II structures. In view of their molecular masses, we also suggest that they contain two type II domains. The complete amino acid sequence, along with disulfide bridge assignment, should confirm their structural similarity.

BSP proteins and their homologs can bind GAGs, such as heparin, and play a role in sperm capacitation [13, 30]. The heparin-binding regions are characterized by clusters of basic amino acids [11] of high positive-charge density that electrostatically interact with the acidic groups of GAGs such as heparin. The present study shows that all BiSV proteins except BiSV-16kDa bind to heparin. Another important property exhibited by BSP proteins is that they form stable complexes with LDF [15]. The present study revealed that BiSV proteins also share this property.

BiSV-17kDa and BiSV-18kDa could be identical proteins that may differ principally by their degree of glycosylation, as in BSP-A1 and BSP-A2. This would explain the fact that these two proteins migrated as doublets on SDS-PAGE (Fig. 2) as in the case of BSP-A1/A2 [3]. Furthermore, BSP antibodies cross-reacted with BiSV proteins as well as with BSP proteins. Thus, BiSV proteins are homologs of BSP proteins. The gelatin-unadsorbed fraction contained a small amount of anti-BSP-A3 cross-reacting protein with a molecular mass similar to BSP-A3-related protein. This protein may be a new protein contained in bison seminal vesicle secretion with a high affinity for BSP-A3 antibodies or it could be the same BiSV-16kDa protein associated with choline phospholipids thus incapable of binding to the column. As shown in Figure 5C, anti-BSP-30kDa cross-reacted with three proteins. Band 1 corresponds to BiSV-28kDa, as determined by N-terminal sequencing. It may be possible that band 2 correspond to a BiSV-28kDa degradation fragment [34] or to a novel homolog of BSP-30kDa [35]. On the other hand, band 3 could indicate a weak cross-reaction of BiSV-16kDa with anti-BSP-30kDa.

The high cross-reaction between anti-BSP antibodies and BiSV proteins allowed the quantification of each BiSV protein in seminal vesicle secretions by radioimmunoassay. Almost 25% of the total protein in bison seminal vesicle secretions represent BSP-related proteins. BiSV-17/18kDa, BiSV-16kDa, and BiSV-28kDa were present in 7:3:1 ratio, respectively. In bull seminal plasma, BSP proteins represent approximately 45% of total seminal plasma proteins and BSP-A1/A2, BSP-A3, and BSP-30kDa are present in the ratio of 11:1:1, respectively [33]. Given the strong sequence homology between BiSV and BSP proteins, it is probable that BiSV proteins play a similar physiological role in bison sperm capacitation. BSP protein homologs have previously been found in boar (pB1 [16–18]), stallion (HSP-1, HSP-2, and HSP-12kDa [16, 20]), and goat seminal plasma (GSP-14kDa, GSP-15kDa, GSP-20kDa, and GSP-22kDa [21]). However, BiSV proteins are the only proteins that share complete sequence identity at the amino terminus with BSP proteins. The N-terminal sequence of BSP proteins shows no identity with the N-terminal sequence of boar, goat, and stallion homologs. However, BSP proteins and their homologs show identity in the type II domains.

In conclusion, we have shown that bison seminal vesicle secretions contain a group of four major proteins that are structurally related to the BSP family of proteins found in bull, boar, stallion, and goat seminal plasma. The bison homologs have the same properties as BSP proteins. They bound to gelatin and heparin and formed complexes with LDF from hen's egg yolk. Moreover, antibodies against BSP proteins cross-reacted with BiSV proteins with high

affinity and the amino-terminal sequence showed almost 100% identity with BSP proteins. These results clearly demonstrate that proteins of the BSP family are ubiquitous in mammals and are meant to play the same biological role. Moreover, as with BSP proteins, BiSV proteins formed complexes with LDF from hen's egg yolk. Thus, hen's egg yolk could be used for bison semen preservation in liquid or frozen state for use in artificial insemination. Further investigations on the effect of BiSV proteins on bison sperm capacitation will help to understand the molecular basis of bison sperm capacitation.

AKNOWLEDGMENTS

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2.4. Isolation and characterization of major proteins of ram seminal plasma.

Bergeron A., Villemure M., Lazure C. and Manjunath P. Soumis pour publication dans *Molecular Reproduction and Development*, janvier, 2005.

Préambule:

Le mouton est une autre espèce importante en agriculture. Afin d'être conservée, la semence de bélier doit être diluée avec un diluant contenant du jaune d'œuf. Nous avons donc voulu vérifier si le plasma séminal de bélier contenait des protéines homologues aux protéines BSP et si ces homologues avaient la capacité de lier les LDF du jaune d'œuf. Dans la présente étude, nous avons isolé des protéines homologues aux protéines BSP à partir du plasma séminal de bélier et démontré leur capacité à lier les LDF du jaune d'œuf. Ces travaux supportent notre hypothèse voulant que le mécanisme de protection des spermatozoïdes par le jaune d'œuf soit le même chez les autres espèces de mammifères utilisés en agriculture. De plus, dans cette étude, nous avons isolé la protéine majeure du plasma séminal de bélier (~40% des protéines totales) qui est une protéine faisant partie de la famille des spermadhésines.

J'ai réalisé tous les travaux qui ont mené à la rédaction de cet article à l'exception du séquençage des protéines qui a été réalisé par Lazure C. Villemure M. a réalisé des travaux préliminaires concernant la purification des protéines du plasma séminal de bélier. J'ai fait l'interprétation des résultats et rédigé l'article.

ABSTRACT

Mammalian seminal plasma contains, among others, two major families of proteins, namely spermadhesins and those proteins that contain fibronectin type II domains. Spermadhesins are the major proteins of boar and stallion seminal plasma and homologous proteins have been identified in the bull. These proteins appear to be involved in capacitation and sperm-egg interaction. In bovine seminal plasma, proteins containing fibronectin type II domains are the major proteins and are designated BSP proteins. These proteins play a role in sperm capacitation. Both families display heparin-binding activities and bind to the sperm membrane at ejaculation. In this study, we present the isolation and characterization of the major proteins of ram seminal plasma. Precipitated proteins from Suffolk ram seminal plasma were loaded onto a gelatin-Agarose column. The unadsorbed (fraction A) and retarded proteins (fraction B) were removed by washing the column with phosphate buffered-saline and the adsorbed proteins (fraction C) were eluted with 5 M urea. SDS-PAGE of fraction B indicated the presence of a 15.5 kDa protein, which is the major protein of ram seminal plasma (~45% of total protein by weight) and was identified as a spermadhesin by N-terminal sequencing. SDS-PAGE analysis of fraction C revealed the presence of four proteins, which represented ~20% of total ram seminal plasma proteins by weight, and were identified as proteins of the BSP family and named RSP proteins. These RSP proteins were designated RSP-15kDa, RSP-16kDa, RSP-22kDa and RSP-24kDa. Only RSP-15kDa and -16kDa proteins cross-reacted with antibodies against BSP proteins. Ram spermadhesin and RSP proteins

interact with heparin but only RSP proteins bind to hen's egg yolk low-density lipoprotein. In conclusion, spermadhesin is the major protein of ram seminal plasma and other major proteins belong to the BSP protein family. These proteins possibly play a role in sperm-egg interaction or capacitation.

INTRODUCTION

The major proteins of mammalian seminal plasma are members of two main families of proteins namely spermadhesins, which are the major proteins of boar and stallion seminal plasma and those proteins that contain fibronectin type II domains, which are the major proteins of bull seminal plasma.

Six spermadhesins have been identified in boar seminal plasma (AWN-1, AWN-2, AQN-1, AQN-3, PSP-I and PSP-II [1, 2]) representing up to 30% of total seminal plasma proteins by weight. In stallion seminal plasma, only one spermadhesin has been identified (HSP-7) [3]. Homologous proteins of spermadhesin have also been identified in bovine seminal plasma (aSFP [4] and Z13 [5]). Spermadhesins are secretory products of the seminal vesicles with the exception of AWN-1 which is present on epididymal sperm and also synthesized by the rete testis and tubuli recti (reviewed in [6]). Members of the spermadhesin family are closely related proteins of 12-16 kDa which contain a single CUB domain named after three proteins where it was first identified (C1r, Uegf and Bmp1) [7]. HSP-7, AQN-1, Z13 and aSFP proteins are not glycosylated. PSP-I and PSP-II are glycosylated whereas AQN-3 and AWN spermadhesins are found as both non- and glycosylated forms (reviewed in [1]).

Porcine and equine spermadhesins display carbohydrate-binding activity [1]. With the exception of PSP-I, they also show interaction with heparin and zona pellucida glycoconjugates, and bind to the sperm surface upon ejaculation. However, each spermadhesin exhibits a different set of affinities depending on their

glycosylation and aggregation state [8]. Spermadhesins adhere to sperm by binding to phosphatidyl ethanolamine present in the sperm membrane [1]. Members of the spermadhesin family share 60-98% sequence identity but they are not functionally equivalent. Since they bind to the sperm surface at ejaculation and can bind to the zona pellucida, sperm-bound spermadhesins may serve as receptors for sperm-egg recognition [6]. Porcine spermadhesin AWN-1 and its equine homolog (HSP-7) are thought to play a role in gamete recognition and sperm-egg binding at fertilization [6]. Since spermadhesins can also bind heparin and are released from the sperm membrane during capacitation, a role for spermadhesins in sperm capacitation is suggested [1]. However, PSP-I and PSP-II do not seem to play a role in sperm capacitation or binding to the oocyte. In fact, the PSP-I/PSP-II heterodimer displays a proinflammatory effect and may modulate immune responses in the porcine uterine environment [9]. Despite the fact that bovine spermadhesins present a very high sequence similarity with porcine spermadhesins, they do not bind to the sperm surface nor do they display carbohydrate, heparin or zona pellucida binding activity [4]. This suggests that these proteins play different roles in various steps which lead to fertilization.

Proteins that contain fibronectin type II domains have been found first in bovine seminal plasma and designated BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa (collectively called BSP proteins) [10, 11]. They are secretory products of the seminal vesicles. BSP-A1, A-2 and -A3 have molecular masses between 15 and 17 kDa while BSP-30 kDa has a molecular mass of 28-30 kDa. BSP-A3 is not glycosylated, whereas BSP-A1, -A2 and -30 kDa are glycosylated. BSP-A1 and -A2 are considered

to be glycoforms of the same protein named BSP-A1/-A2 [10] and also known as PDC-109 [12]. Homologs of these BSP proteins have been found in boar (pB1; [13, 14]), stallion (HSP-1, HSP-2 and HSP-12 kDa; [14-16]), goat (GSP-14 kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa; [17]) and bison (BiSV-16kDa, BiSV-17kDa, BiSV-18kDa and BiSV-28kDa; [18]) seminal plasma. BSP-like antigens are also present in rat, mouse, hamster, and human seminal vesicle fluid or seminal plasma [19].

BSP proteins have the property to interact with choline phospholipids on the sperm membrane, with high- and low-density lipoproteins (HDL and LDL), and with heparin (reviewed in [20]). These binding properties confer upon BSP proteins their biological role in sperm membrane stabilization (decapacitation) and destabilization (capacitation) during the fertilization process. Briefly, BSP proteins are mixed with sperm upon ejaculation, and induce a cholesterol efflux from the sperm membrane, which results in reorganization of its components. BSP proteins also bind to the choline phospholipids of the sperm membrane, which may sterically hinder phospholipid movements and thereby stabilize the membrane. As sperm reach the oviduct they encounter follicular and oviductal fluids which contain HDL and heparin-like glycosaminoglycans, the physiological capacitation factors. HDL could interact with sperm-bound BSP proteins and induce capacitation that does not involve protein tyrosine phosphorylation [21-23]. Alternatively, glycosaminoglycans could interact with sperm-bound BSP proteins and promote capacitation, which involves protein tyrosine phosphorylation [21, 22, 24, 25]. The exact transduction mechanism of these

two pathways is under investigation. A recent report demonstrates that the BSP-A1/-A2 (PDC-109) proteins enable sperm to bind the oviductal epithelium and play a major role in the formation of the oviductal sperm reservoir [26]. Therefore, BSP proteins appear to be multifunctional.

Conversely to this positive role in fertility, BSP proteins may be detrimental in the context of sperm storage [27]. It has been shown that the cholesterol and choline phospholipid efflux induced by BSP proteins is time and concentration dependent [20, 21, 23], thus long exposure of sperm to these proteins or exposure to large concentrations of them could be deleterious to the sperm membrane. We have shown that BSP proteins form stable complexes with the low-density lipoprotein fraction (LDF) of hen's egg-yolk, which is present in extenders used for sperm preservation [28]. Consequently, the scavenging of BSP proteins by LDF protects sperm by preventing lipid loss (cholesterol and choline phospholipid) from the sperm membrane [29].

In ram seminal plasma, certain proteins bind to the sperm membrane and increase the resistance of cells to cold-shock or restore the live cell properties when added to washed sperm after cold-shock treatment [30-33]. However, these proteins have never been characterized and in fact, none of the proteins in ram seminal plasma has been characterized. The objective of this study was to isolate and characterize the major proteins of ram seminal plasma. Their structure-function relationship to spermadhesin and the BSP protein family was also investigated by N-terminal sequencing and by binding experiments with heparin and with LDF from egg-yolk.

MATERIALS AND METHODS

Materials

Gelatin was purchased from Eastman Kodak Company (Rochester, New-York, USA). Heparin-Sepharose CL-6B was from Amersham Biosciences (Baie d'Urfé, QC, Canada). Acrylamide and bisacrylamide were purchased from ICN (Mississauga, ON, Canada). Affi-Gel 15, Sodium dodecyl sulfate (SDS) and other electrophoresis products were from Bio-Rad (Mississauga, ON, Canada). Immobilon-P (PVDF) membranes were purchased from Millipore (Nepean, ON, Canada). HPLC grade trifluoroacetic acid (TFA) was from Fisher Scientific (St-Laurent, QC, Canada). All other chemicals used were of ultra pure grade and obtained from local suppliers, mostly Fisher Scientific and Sigma-Aldrich (Oakville, ON, Canada).

Bovine seminal plasma was obtained from the Centre d'Insémination Artificielle du Québec (St-Hyacinthe, QC, Canada). Crude bovine seminal plasma (cBSP) proteins were prepared by ethanol precipitation of bovine seminal plasma followed by dialysis of the precipitates against 50 mM ammonium bicarbonate and lyophilization [34]. This preparation resulted in 60-70% BSP proteins. Suffolk ram semen was provided by the Centre d'Insémination Ovine du Quebec (LaPocatière, QC, Canada). Bulls and rams were handled by qualified technicians, according to the Guide for the Care and Use of Agricultural Animals established by the Quebec Ministry of Agriculture and Fisheries.

Alcohol precipitation of the ram seminal plasma proteins

Eighty ml of pooled frozen ram semen (-80°C) was thawed and centrifuged at 1 000 X g for 10 min to remove sperm. The supernatant was further centrifuged at 10 000 X g for 10 min to obtain clear seminal plasma. Nine volumes of cold ethanol were added to the seminal plasma and left with stirring for 90 min at 4°C to precipitate the proteins which were then recovered by centrifugation at 10 000 X g for 10 min. After three subsequent ethanol washes, the precipitates were solubilized in 50 mM ammonium bicarbonate and lyophilized. Approximately 900 mg of dried powder was recovered.

Gelatin-Agarose affinity chromatography

Gelatin was previously coupled to Affi-Gel 15 as described earlier [11]. All purification steps were conducted at 4°C at a flow rate of 30 ml/h. Lyophilized proteins (130 mg) were dissolved in phosphate buffered-saline (PBS) and loaded onto a gelatin-Agarose column (1.5 X 28 cm), which was previously equilibrated with the same buffer. Once the sample entered the column, the flow was stopped for 15 min to allow proteins to bind. Following this step, the column was washed with PBS to eliminate unadsorbed and retarded proteins, the bound proteins were eluted with PBS containing 5 M urea. Four ml fractions were collected and their absorbance at 280 nm was measured. Fractions corresponding to each protein peak were pooled, dialyzed against 50 mM ammonium-bicarbonate and lyophilized.

Heparin-Sepharose affinity chromatography

The following steps were conducted at 4°C at a flow rate of 30 ml/h. The gelatin-retarded or adsorbed proteins (5 mg) were dissolved in 1 ml phosphate buffer (PB) and loaded onto a heparin-Sepharose CL-6B column (1 X 12.5 cm), which was previously equilibrated with the same buffer. Once the sample entered the column, the flow was stopped for 15 min to allow proteins to bind. The column was washed extensively with PB and the bound proteins were eluted with 1 M NaCl in PB. Fractions of 0.5 ml were collected and their absorbance at 280 nm was measured. Fractions corresponding to each protein peak were pooled, dialyzed against 50 mM ammonium bicarbonate and lyophilized.

SDS-PAGE and blotting

SDS-PAGE was performed on 15% polyacrylamide gels according to Laemmli [35], using the Mini protean III apparatus from Bio-Rad. Molecular mass was estimated by comparison with the Low Molecular Weight Calibration Kit from Amersham Biosciences. Proteins from the SDS-PAGE were then either stained with Coomassie Blue or transferred electrophoretically to PVDF membranes as described previously [36], using a Trans-Blot apparatus from Bio-Rad. Transferred proteins were also stained with Coomassie Blue for N-terminal sequence analysis or used for immunodetection using specific antibodies against each BSP protein (BSP-A1/A2, BSP-A3 and BSP 30 kDa) as described previously [37].

RP-HPLC

Reversed phase-high performance liquid chromatography (RP-HPLC) was performed as described previously [17] with the exception of using a Vydac diphenyl column (250 X 4.6 mm, 5 µm, 300 Å; Mandel Scientific, Guelph, ON, Canada). Lyophilized proteins (100 µg) from the gelatin-Agarose and heparin-Sepharose chromatography steps were dissolved in 0.1% TFA or 0.1% TFA containing 6 M guanidine-HCl in the case of heparin-Sepharose fraction C3. Protein solutions were then loaded onto the column and eluted using a gradient of 0.1% TFA in acetonitrile. Fractions of 0.5 ml were collected at a flow rate of 1 ml/min. The eluted proteins were monitored at 235 nm and dried under vacuum for SDS-PAGE and sequence analysis.

Sequencing

Following the transfer of those proteins that were separated by SDS-PAGE to an Immobilon-P membrane, the Coomassie Blue stained protein bands were cut and placed in the sequenator reactor. Sequencing was carried out according to the manufacturer's protocol using an Applied Biosystems Procise sequencer (model 494).

Interaction of ram seminal plasma proteins with hen's egg yolk lipoproteins

The binding of RSP proteins to LDF was studied using the Paragon electrophoresis kit from Beckman. Hen's egg yolk LDF isolated on a KBr solution (1.21 g/ml) as described previously [28], was incubated with gelatin-Agarose chromatography fractions (fraction B and C), heparin-Sepharose chromatography fractions (fraction C1 and C3) or cBSP proteins in a total volume of 15 µl of 10 mM Tris-HCl buffer (pH 7.4). After a 15 min incubation, 4 µl of each sample were applied

to Lipogel (0.5% Agarose gel) and subjected to electrophoresis. The electrophoresis was run at 100 V for 30 min, the gel was dipped in fixative solution and dried. The lipid bands were subsequently stained with Sudan black B.

Protein determination

The protein content of each purification step was measured by a modified Lowry method [38] or by weighing on a Cahn microbalance (Model C-31, Fisher Scientific, St-Laurent, QC, Canada).

RESULTS

Isolation of ram seminal plasma proteins by gelatin-Agarose chromatography

The solubilized alcohol-precipitated proteins from ram seminal plasma were chromatographed on a gelatin-Agarose affinity column (Fig. 1A). The proteins were eluted in three peaks: unadsorbed (fraction A), retarded (fraction B) and adsorbed (fraction C). The proportion of proteins found in fractions A, B and C was approximately, 30:50:20 respectively. The recovery of the total proteins was about 85% of the starting material following gelatin-Agarose chromatography. SDS-PAGE analysis of alcohol-precipitated ram seminal proteins indicated the presence of about 25 proteins with molecular masses from 14 kDa to 120 kDa (Fig. 1B). A group of proteins with a molecular mass of 15-16 kDa and 22-24 kDa was more predominant. Fraction A contained all ram seminal proteins but was depleted of the group of proteins of molecular mass 15-16 kDa and 22-24 kDa (lane 3). The fraction B contained one major protein (15.5 kDa) and several minor proteins (lane 4), which were also found in fraction A. This was expected since peak A and B, overlap slightly. In fraction C we observed the presence of four protein bands of molecular mass 15, 16, 22 and 24 kDa (lane 5), that were named RSP-15kDa, RSP-16kDa, RSP-22kDa and RSP-24kDa, respectively.

Characterization of the 15.5 kDa protein (gelatin-retarded protein)

The 15.5 kDa protein in fraction B was further purified by HPLC (Fig. 2) and the first 25 amino acids at the N-terminal end were identified. A sequence search in

protein data bank indicated a significant sequence identity to the one of spermadhesin AQN-1 purified from boar seminal plasma (Fig. 3). Furthermore, heparin-Sepharose chromatography of fraction B showed that the 15.5 kDa ram spermadhesin binds to heparin (data not shown).

Characterization of gelatin-bound proteins

The gelatin-bound proteins (fraction C) were loaded onto a heparin-Sepharose affinity column (Fig. 4A). All proteins were either retarded or adsorbed on the column. The first peak (fraction C1) was eluted after more than two bed volumes of PB were passed through the column. The second peak (fraction C2) was eluted following extensive washing (~8 bed volumes) of the column with PB. The third peak (fraction C3) was eluted with 1 M NaCl in PB. SDS-PAGE analysis of proteins in these peaks (Fig. 4B) showed that RSP-15kDa and RSP-16kDa proteins were present in fraction C1 (lane 3), whereas fraction C2 (lane 4) contained mostly RSP-24kDa and small amounts of RSP-15kDa and RSP-16kDa proteins. Fraction C3 (lane 5) contained RSP-22kDa and RSP-24kDa proteins.

The RP-HPLC elution pattern of gelatin-adsorbed proteins (fraction C) is shown in Figure 5A. Proteins were separated into four peaks designated I to IV. Peak I contained RSP-22kDa and RSP-24kDa (Fig. 5A) and by analyzing each HPLC fraction from peak I on SDS-PAGE, it was found that they were actually eluted in decreasing order of molecular weight respectively (data not shown). The heparin-Sepharose fraction C1 upon HPLC showed peaks II, III and IV (Fig. 5B). Peak II corresponds to RSP-16kDa protein; Peak III and IV correspond to RSP-15kDa protein

(see later). Peak I obtained by running the heparin-Sepharose fraction C2 on HPLC contained RSP-24kDa protein (Fig. 5C), whereas peak I obtained by running heparin-Sepharose fraction C3 contained RSP-22kDa and RSP-24kDa (Fig. 5D).

N-terminal sequencing of the 24 kDa protein obtained by HPLC and SDS-PAGE of fraction C2 or C3 (Fig. 5C and D) showed that both bands have an identical N-terminal sequence indicating that they are one and the same protein (RSP-24kDa). The comparison of the N-terminal sequence of RSP-22kDa and RSP-24kDa indicated that they share a high degree of similarity with each other (Fig. 6). In addition, comparison of the RSP-22kDa and RSP-24kDa with GSP-20kDa and GSP-22kDA proteins of goat seminal plasma indicated that they also share a high degree of similarity with each other. The single protein contained in peak III and IV (Fig. 5B) has a molecular mass of 15 kDa (RSP-15kDa) and the sequence of amino acids 1-32 of either peak III or peak IV were identical. The single protein in peak II with molecular mass of 16 kDa (Fig. 5B; RSP-16kDa) differed from that in peak III/IV (RSP-15kDa) in one amino acid (position 12). These two proteins also exhibited a high degree of similarity with the N-terminal sequence of GSP-14kDa and GSP-15kDa of goat seminal plasma.

Cross-reactivity of RSP proteins with BSP antibodies

The RSP proteins present in starting material, gelatin-Agarose adsorbed peak (fraction C) and heparin-Sepharose fractions (peak C1, C2 and C3) were probed with purified antibodies raised against each of the BSP proteins (BSP-A1/A2, BSP-A3 and BSP-30kDa) (Fig. 7A, B and C respectively). Anti-BSPA1/A2 and anti-BSP-A3

antibodies showed cross-reacting proteins (M_r 15-16 kDa) in the starting material as well as in fractions C and C1. However, the quantity of RSP proteins required to observe similar intensities of immunoreactive bands was 10-20 times higher than that of BSP proteins. Anti-BSP-30kD antibodies on the other hand showed no cross-reaction with any of the RSP proteins.

Interaction of major proteins of ram seminal plasma with LDF

In the present study, we tested whether or not ram 15.5 kDa spermadhesin and RSP proteins bind to LDF (Fig. 8). LDF preincubated with fraction C (containing RSP-15kDa, -16kDa, -22kDa and –24kDa), fraction C1 (containing RSP-15kDa and –16kDa) or fraction C3 (containing RSP-22kDa and -24kDa), and submitted to Agarose gel electrophoresis migrated towards the anode (lanes 4-6), as did the positive control containing cBSP proteins (lane 7). LDF alone has no charge and remained at the point of application (lane 2). These results indicate the formation of a complex between RSP proteins and LDF, changing the overall charge of the LDF molecule and hence its electrophoretic mobility. LDF preincubated with the ram spermadhesin of 15.5 kDa (lane 3) remained at the point of application indicating that there is no interaction between LDF and the spermadhesin.

DISCUSSION

The major proteins of most mammalian seminal plasmas appear to belong to two families of proteins: spermadhesins, which are the major proteins of boar and stallion seminal plasma and BSP proteins which are the major proteins of bull seminal plasma [1, 6, 20]. In the present study, we isolated and characterized the major proteins of ram seminal plasma. The major protein of ram seminal plasma is a spermadhesin of 15.5 kDa which represents ~45% of the total seminal plasma proteins. The other major proteins of ram seminal plasma belong to the BSP protein family (RSP-15kDa, RSP-16kDa, RSP-22kDa and RSP-24kDa) and they represent ~20% of ram seminal plasma total proteins.

The spermadhesin AQN-1 binds to the acrosomal region of sperm at ejaculation and most of the sperm bound AQN-1 is released during *in vitro* capacitation suggesting a role of AQN-1 in boar sperm capacitation [6]. In addition, AQN-1 can bind to the zona pellucida suggesting that sperm-egg binding might be mediated by this spermadhesin [6]. The 15.5 kDa spermadhesin that we have isolated from ram seminal plasma shares sequence identity with boar spermadhesin AQN-1 (Figure 3). Furthermore, as described for the spermadhesin AQN-1, the 15.5 kDa ram spermadhesin showed heparin-binding activity. This result suggests that the 15.5 kDa ram spermadhesin may play a similar role in ram sperm capacitation or binding to the zona pellucida.

As found for other members of the spermadhesin family, the new member (15.5 kDa) isolated from in ram seminal plasma has a molecular mass between 12 and 16 kDa. Moreover, the ram 15.5 kDa spermadhesin is expected to contain a single CUB domain because this structure is highly conserved in all members of the spermadhesin family [6]. Further structural analysis of ram spermadhesin should confirm this.

Proteins in seminal plasma containing a type-II domain are phospholipid-binding proteins and play an important role in sperm capacitation [21-25]. Since these proteins were originally characterized in bovine, they are generally designated as the BSP family of proteins [20]. They exist in several molecular forms and glycoforms. In this study, we showed the presence of homologous proteins in ram seminal plasma. We used their gelatin-binding property to isolate them by affinity chromatography. Four proteins were discovered that were named according to their molecular masses: RSP-15kDa, RSP-16kDa, RSP-22kDa and RSP-24kDa.

The gelatin-binding property of BSP proteins is due to the presence of two type II domains in their structure [11, 12, 39-41]. It has been shown that BSP proteins and their homologs in boar, stallion, goat and bison all bind gelatin [11, 16-18, 42], thus gelatin-affinity chromatography represents a good strategy for the isolation of homologs in other species. Since these RSP proteins also bind to gelatin, it indicates that they also contain type II domains.

BSP proteins are also known to bind heparin [43]. The binding is made through stretches of basic amino acids within the protein sequence with the acidic groups on

heparin-like glycosaminoglycans [44]. This interaction is thought to be important for the sperm capacitation process [24]. In the goat, only GSP-20 and GSP-22 kDa bound strongly to a heparin-affinity column, whereas GSP-14 and GSP-15 kDa interacted weakly [17]. In bison, all BiSV proteins bind to heparin except BiSV-16 kDa [18]. In the present study, all four RSP proteins bound to the heparin column but with different affinities. RSP-22kDa and RSP-24kDa appear to have higher affinity for heparin compared to the RSP-15kDa and RSP-16kDa proteins.

N-terminal sequencing of gelatin-binding proteins indicated that RSP proteins share sequence identity with BSP proteins (Fig. 6). In this figure, X represents likely half-cystine residues whose PTH-derivatives are not stable enough to be detected. RSP-15kDa and RSP-16kDa differ only by one residue (position 12). GSP-14 kDa and GSP-15 kDa proteins, which are goat homologs also differ in a few residues at the N-terminal end and show a high degree of sequence similarity with RSP-15kDa and RSP-16kDa proteins. RSP-22kDa and RSP-24kDa proteins showed several differences along the N-terminal sequence but they show a high degree of structural relatedness between them as well as with GSP-20kDa and GSP-22kDa proteins of goat homologs [17]. In ram seminal plasma, BSP homologs are more closely related to goat than to bovine or bison. BSP protein homologs present in bison seminal plasma showed almost 100% sequence identity with BSP proteins and complete immunoreactivity with antibodies raised against each BSP protein [18]. Immunoreactive bands having a molecular weight of 15-16 kDa were detected when probed with antibodies raised against BSP-A1/A2 and BSP-A3 proteins. However,

cross-reactivity of anti-BSP antibodies was higher with homologs found in bison seminal plasma than homologs found in ram seminal plasma. No immunoreactive bands having a molecular weight of 22-24 kDa were detected among ram seminal plasma proteins when probed with antibodies directed against each BSP protein (Figure 7). This corroborates our results obtained when goat seminal plasma proteins were probed with anti-BSP protein antibodies. GSP-20kDa and GSP-22kDa that share significant degree of similarity with RSP-22kDa and RSP-24kDa (Figure 6), do not cross-react with antibodies directed against each BSP protein (unpublished data). No immunoractive bands were detected in ram seminal plasma proteins when probed with antibodies directed against BSP-30kDa suggesting the absence of a BSP-30kDa protein homolog in ram seminal plasma. Conversely, it may be possible that RSP-22kDa and RSP-24kDa are homologs of BSP-30kDa but lack antibody binding sites. In this context, it should be noted that despite the fact that BSP-proteins show 50-70% structural identity, the antibodies directed against each of the proteins are specific and they do not cross-react with other antigens present in bovine seminal plasma. Further structural analysis should resolve this issue.

The N-terminal sequence of RSP-15kDa and RSP-16kDa proteins contains the characteristic motif, which constitute the type II domain (Fig. 4, bold letters). In the BSP proteins and homologs present in boar and stallion, this motif contained a CVFPFXY(R/K)X(R/K)(R/K)(H/Y)F sequence (where X represents a variable amino acid) [42]. This motif is also present in GSP-20 and GSP-22 kDa proteins from goat seminal plasma [17], except that the FPF segment was changed to an FAF segment. In

two attempts, we were unable to obtain the N-terminal sequence of RSP-22 and RSP-24kDa proteins longer than 23 and 35 amino acids respectively. The specific motif (CVFPF) may be present further down the sequence since both of these proteins exhibit all of the binding characteristics shown by the BSP proteins. In view of their gelatin-binding property, we can deduce that RSP proteins are also constituted of a similar type II structure.

RSP-15kDa and RSP-16kDa proteins appeared as distinct bands when low amounts of protein were loaded onto the gel, however when large amounts (>5 μg) were loaded (Figure 4, lane 3) they appeared as smears or diffuse bands of 14.5-16.5 kDa. Furthermore, using RP-HPLC to separate proteins contained in fraction C1 (Figure 5B), we obtained 3 clearly defined peaks (peak II, III and IV). However, N-terminal sequencing showed that these 3 peaks contained only 2 different proteins. Peak II contained RSP-16kDa while peak III and IV contained RSP-15kDa. The fact that RSP-15kDa is found in two peaks during HPLC may suggest that two differently glycosylated forms of this protein are present in ram seminal plasma. Various glycoforms of a protein can display different retention times during HPLC [34]. Similarly, RSP-24kDa is present in the retarded heparin-Sepharose fraction C2 and also present in the heparin-adsorbed fraction C3 (Figure 4, lane 4 and 5). Following SDS-PAGE, RSP-22kDa and RSP-24kDa also appeared as 22-25 kDa bands (Figure 4, lane 5) and both RSP-24kDa found in fraction C2 and C3 have the same N-terminal sequence. This would also suggest the existence of different glycoforms of RSP-24kDa in ram seminal plasma.

Proteins present in fraction C3 (RSP-22kDa and RSP-24kDa) appeared to interact with each other, because the aggregation of these proteins in this sample was observed during storage. This aggregation was time-dependent and the sample C3 (RSP-22kDa and RSP-24kDa) was insoluble in buffer A (0.1% TFA). However, addition of guanidine HCl (6 M) solubilized the proteins which facilitated HPLC analysis. No aggregation was noticed in fraction C2, which contained only RSP-24kDa. Thus, RSP-22kDa and RSP-24kDa appear to bind to each other leading to a decreased solubility. The aggregation of BSP proteins has been reported previously [1, 34].

Egg-yolk is widely used in preservation media employed for storage of semen from domestic, farm and zoo animals including endangered species. The mechanism by which egg yolk protects sperm during storage in the liquid or frozen state is not known. Our previous studies showed that exposure of sperm to seminal plasma which contains BSP proteins is detrimental to sperm [29]. Interestingly, LDF isolated from egg-yolk binds to BSP proteins [28] and this interaction protects sperm by preventing lipid loss (cholesterol and choline phospholipid) from the sperm membrane during storage [29]. In view of this, it was of interest to study whether or not RSP proteins bind to LDF. The binding of BSP proteins to LDF can be demonstrated by Agarose gel electrophoresis [28]. LDF alone does not migrate from the point of application; however, when it binds BSP proteins, the complex migrates towards the anode and the migration distance of the complex depends on the charge of the BSP proteins and relative concentration of LDF and BSP proteins [28]. Similar to BSP proteins [28],

RSP proteins also bind to LDF (Figure 8). Up to the present, it has not been established whether or not RSP proteins are detrimental to sperm, but it is important to note that these proteins are present in high concentration (15-20 mg/ml) in ram seminal fluid and that they interact with LDF. This information may be useful in developing appropriate preservation media for ram sperm. The 15.5 kDa spermadhesin found in ram seminal plasma does not bind to LDF. This result was expected since previous studies have shown that LDF bind specifically proteins of the BSP family and do not bind other proteins present in bovine seminal plasma [28].

The present study indicates that ram seminal plasma contains proteins that are structurally related to proteins of the spermadhesin and the BSP family. These proteins can be isolated by gelatin-affinity chromatography, since the 15.5 kDa spermadhesin has less affinity for gelatin than RSP proteins. This study confirms that the spermadhesins and the BSP protein family are ubiquitous among mammals and are probably meant to play the same biological role(s). Further investigation of these proteins would aid in our understanding of capacitation, sperm-egg binding and cold-shock reversion process in ram sperm. Furthermore, the understanding of how the interaction of LDF and RSP proteins would affect sperm function would aid in the development of new extenders for ram semen storage.

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FIGURE LEGENDS

FIG. 1. Isolation and characterization of ram seminal plasma proteins. **A)** Elution profile of alcohol precipitated ram seminal plasma proteins on gelatin-Agarose. **B)** SDS-PAGE pattern of gelatin-Agarose eluted fractions. Protein samples were reduced, denatured, and separated on 15% polyacrylamide gel and stained with Coomassie Blue R-250. Lane 1, low molecular weight standard; lane 2, alcohol precipitated ram seminal plasma proteins (22 μg); lane 3-5, fraction A, B and C respectively (10 μg); lane 6, alcohol precipitated bovine seminal plasma proteins (cBSP proteins; 10 μg). a, b and c indicate the position of BSP-30-kDa, BSP-A1/A2 and BSP-A3 respectively.

FIG. 2. RP-HPLC pattern of gelatin-Agarose retarded fraction (fraction B). Proteins were eluted using 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) buffer system: first isocratically at 5% solvent B for 1 min, followed by a gradient of 5 to 90% B in 42 min. Inset represents the SDS-PAGE pattern of the protein peak (5 μg).

FIG. 3. N-terminal sequence comparison of the ram 15.5 kDa protein and boar spermadhesin AQN-1. The identical amino acids are shown in bold. X denotes an unidentified residue assumed to be by similarity an half-cystine residue.

FIG. 4. Fractionation and characterization of ram seminal plasma proteins. **A)** Elution profile of gelatin-bound ram seminal plasma proteins on heparin-Sepharose column. **B)** SDS-PAGE pattern of heparin-Sepharose eluted fractions. Protein

samples were reduced, denatured, and separated on 15% polyacrylamide gel and stained with Coomassie Blue R-250. Lane 1, low molecular weight standard; lane 2, gelatin-bound proteins (fraction C, 10 μg); lane 3-5, heparin-Sepharose retarded fractions (C1 and C2, 10 μg) and adsorbed fraction (C3, 10 μg) respectively; lane 6, alcohol precipitated bovine seminal plasma proteins (cBSP proteins; 10 μg). a, b and c indicate the position of BSP-30-kDa, BSP-A1/A2 and BSP-A3 respectively.

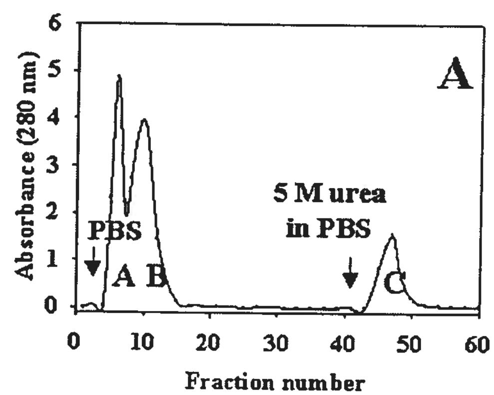
FIG. 5. RP-HPLC pattern of gelatin-Agarose and heparin-Sepharose eluted fractions. Proteins were eluted using 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) buffer system: first isocratically at 30% solvent B for 1 min, followed by a gradient of 30 to 37% B in 42 min. **A)** Gelatin-Agarose fraction C, inset represents the SDS-PAGE pattern of protein peak I (10 μg). **B)** Heparin-Sepharose fraction C1, inset represents the SDS-PAGE pattern of protein peaks II, III and IV (5 μg). **C)** Heparin-Sepharose fraction C2, inset represents the SDS-PAGE pattern of protein peak I (10 μg). **D)** Heparin-Sepharose fraction C3, inset represents the SDS-PAGE pattern of protein peak I (10 μg).

FIG. 6. N-terminal sequence comparison of RSP, GSP and BSP proteins. The identical amino acids are shown in bold. X denotes an unidentified residue assumed to be, by similarity, an half-cystine residue.

FIG. 7. Immunoblot analysis of RSP proteins with anti-BSP antibodies. Alcohol precipitated ram seminal plasma proteins (2 μg; lane 1), Gelatin-Agarose fraction C (1 μg; lane 3), heparin-Sepharose fraction C1, C2 and C3 (1 μg; lane 3-5) and cBSP (50

ng, lane 6) as a positive control were separated by SDS-PAGE, transferred to the membrane and probed with antibodies directed against BSP proteins. **A)** anti-BSP-A1/A2, B) anti-BSP-A3 and C) anti-BSP30kDa.

FIG. 8. Electrophoretic analysis of the interaction of major proteins of ram seminal plasma with LDF. Lane 1, human serum (3 μl) as a positive control of the migration; lane 2, LDF (1.5 μg); lane 3, LDF (1.5 μg) + 15.5 kDa ram spermadhesin (5.6 μg); lane 4, LDF (1.5 μg) + fraction C (5.6 μg) containing all RSP proteins; lane 5, LDF (1.5 μg) + fraction C1 (5.6 μg) containing RSP-15kDa and -16kDa; lane 6, LDF (1.5 μg) + fraction C3 (5.6 μg) containing RSP-22kDa and -24kDa and lane 7, LDF (1.5 μg) + cBSP proteins (5.6 μg) as a positive control.



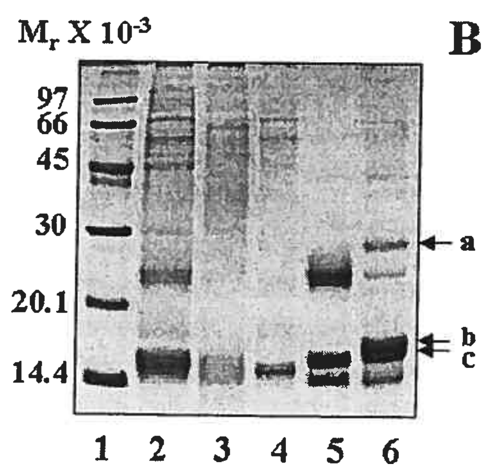


Figure 1

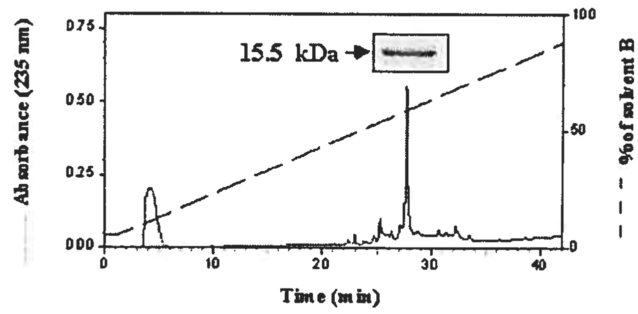
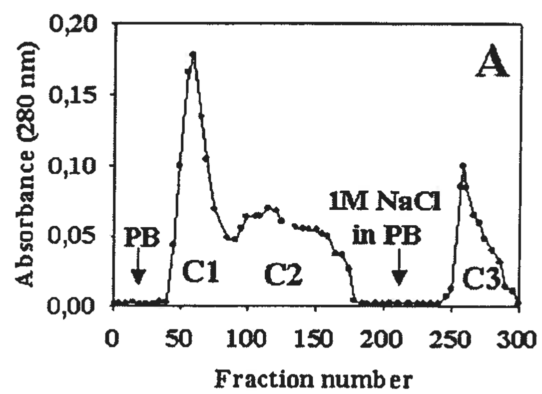


Figure 2

```
                1           11          21
RSP-15.5kDa:    ESDEDTRKXG  GVHRNFSGRI  SSSF
AQN-1      :    AQNKGPHKCG  GVLRNYSGRI  STYEGPKTDC …
```

Figure 3



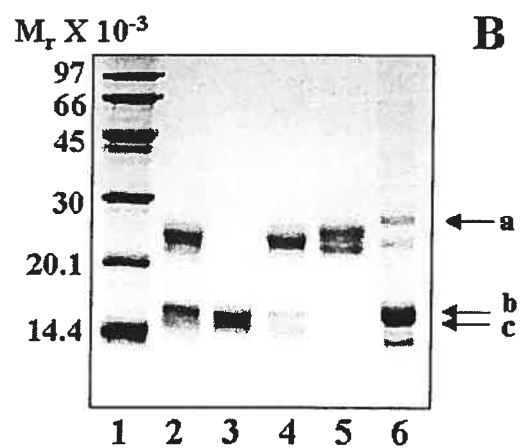


Figure 4

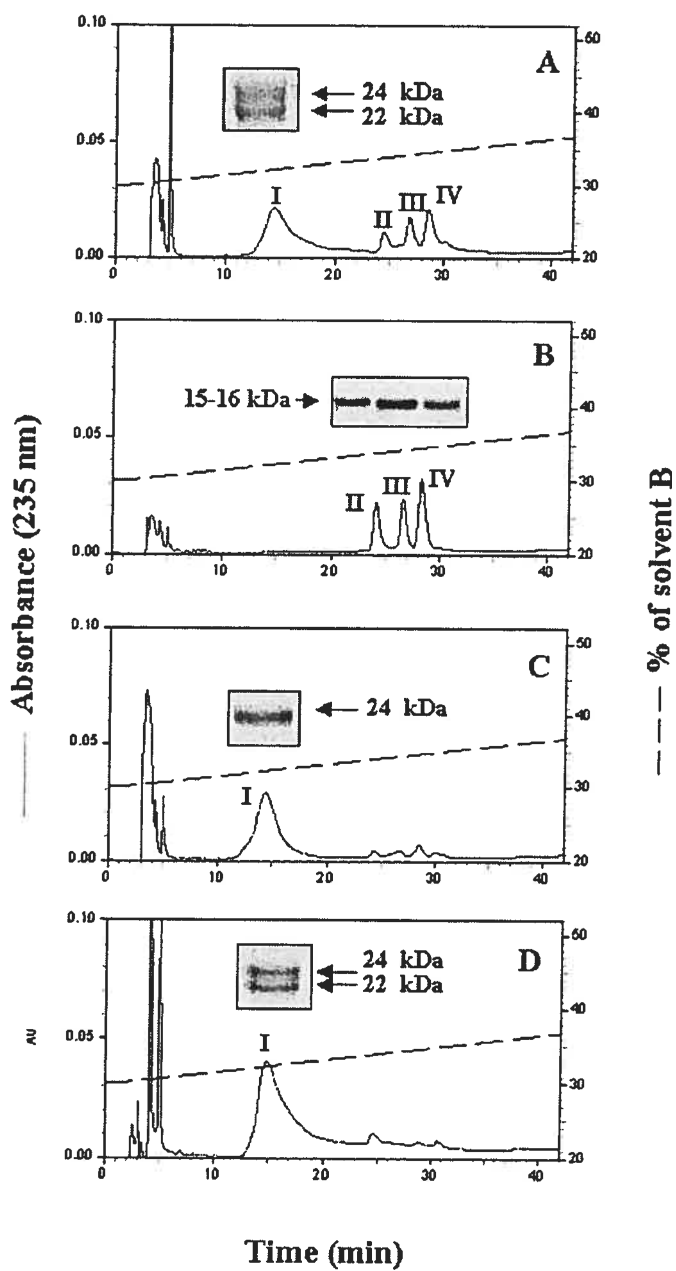


Figure 5

```
                1           11          21          31          41
RSP-24 kDa:     DEPLPAVYDV  LGMLSSTPSS  YIA...
RSP-22 kDa:     DEPLPDVYNL  PEMPRSTPSY  YIAGAQVEKP  HPXDR...

GSP-22 kDa:     DEPPPYVYNL  PEM-PTSIPY  YSADVQGP--  ------PXAF  AFTYRRKI Y IY
GSP-20 kDa:     DEPPPYVYNL  PETPPTTSSY  YIPGFQVEKP  HEDDSXPRAF  AFTYK ...

RSP-16 kDa:                           D DELTRDKSSE  VSHEDEEXVF  PFTYKDKRHF  D...
RSP-15 kDa:                           D DELTRDKSSE  ESHEDEEXVF  PFTYKDKRHF  D...

GSP-14 kDa:                           D DELTRDKS-E  ESHEDEAXVF  PFTYKDKKHF  ...
GSP-15 kDa:                           D DELTRDKSSE  ESHEEANXVF  PFTYKDK-HK  ...

BSP-A1/-A2: ... LDPVNGDQ--  -DEGVSTEPT  QDGPA---EL  PE--DEECVF  PFVYRNRKHF  ...
BSP-A3:               DQLL  SEDNVILPKE  KKDPA---SG  AETKDNKCVF  PFIYGNKKYF  D...
BSP-30 kDa: ... LPRTIYPQEE  MPYDDKPFPS  LLSKANDLN-  AVFEGPACAF  PFTYKGKKYY  M...
```

Figure 6

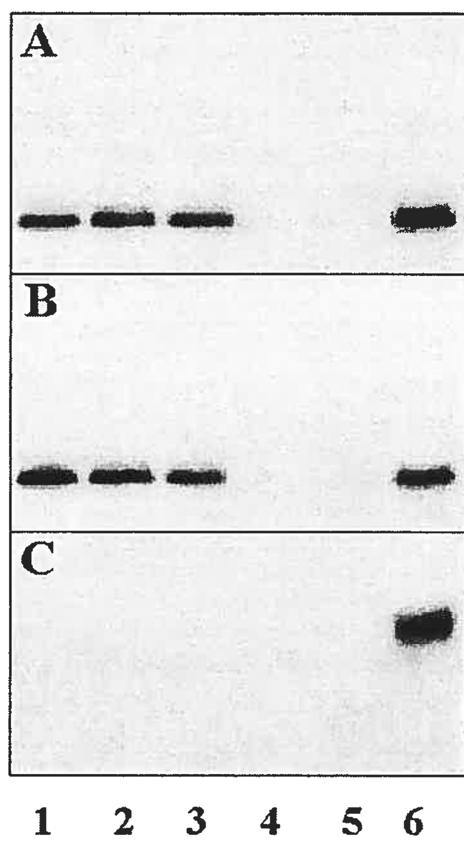


Figure 7

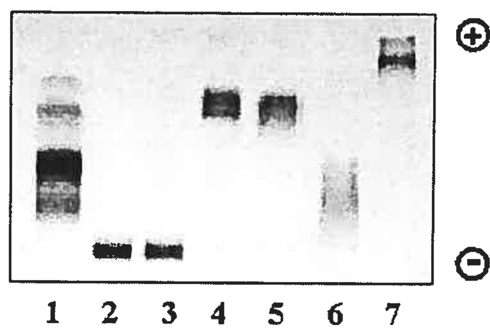


Figure 8

2.5. The mechanism of sperm protection by egg yolk low density lipoprotein.

Bergeron A. et Manjunath P. *Proceedings of the 20th technical conference on artificial insemination and reproduction*, NAAB, 2004, 68-75.

Préambule:

Dans cet article, nous avons rassemblé tous les résultats des 4 articles précédents et proposé un nouveau mécanisme de protection des spermatozoïdes par le jaune d'œuf, un agent protecteur utilisé partout dans le monde en tant que composant des diluants protecteurs. Nous avons aussi expliqué les effets de la liaison des protéines BSP sur les spermatozoïdes et comment la liaison des protéines BSP aux LDF du jaune d'œuf protège les spermatozoïdes. De plus, plusieurs effets du PS sur les spermatozoïdes sont expliqués.

J'ai rédigé l'article présenté dans cette section.

Abstract

Over the past 60 years, egg-yolk (EY) has been routinely used in both liquid semen extenders and those used to cryopreserve sperm. However, the mechanism by which EY protects sperm during liquid storage or from freezing damage is unknown. Bovine seminal plasma contains a family of lipid-binding proteins collectively called BSP proteins (BSP-A1/A2, BSP-A3 and BSP-30kDa). These proteins are secretory products of seminal vesicles that bind to sperm at ejaculation and modify the sperm membrane by removing cholesterol and phospholipids, which may adversely affect the ability of sperm to be preserved. We recently discovered that BSP proteins bind to the low-density lipoprotein fraction (LDF) of the EY present in extender. The binding is rapid, specific, saturable and stable even after freeze-thawing of semen. LDF has a very high capacity for BSP proteins binding. Furthermore, LDF prevents lipid efflux from the sperm membrane and maintains sperm motility. Thus, we propose that the sequestration of BSP proteins present in SP by LDF may represent the major mechanism of sperm protection by EY.

Sperm protection by egg yolk

The cryopreservation of bull semen (Polge and Rowson, 1952; Smith and Polge, 1950) represents one of the most important achievements in dairy farming after the introduction of artificial insemination. These two approaches have enabled the worldwide distribution and use of desired genetic lines at a reasonable cost. Over the past 60 years, the cryoprotective media for sperm storage have been continuously

revised but the basic ingredients of the media remain unchanged. The EY and glycerol represent the indispensable compounds of practically all media used for bull sperm preservation in liquid or frozen state.

The role of glycerol in cryopreservation is that it allows sperm survival during freezing (Anchordoguy et al., 1987; Farrant et al., 1977); however, the protection afforded by EY is more complex. The EY has been shown to increase the sperm fertilizing ability when present in extenders for semen storage at ambient temperature (Barak et al., 1992; Dunn et al., 1950; Shannon and Curson, 1983) and appears to prevent sperm cell damage during cooling and freezing (De Leeuw et al., 1993; Lasley and Mayer, 1944; Phillips and Lardy, 1940). Various components of EY have been investigated to identify the most active component(s) responsible for the protective effect (Foulkes, 1977; Kampschmidt et al., 1953; Watson, 1976). Evidence indicates that the low-density lipoprotein fraction (LDF) shows the highest protective ability (Pace and Graham, 1974; Watson, 1981); however, the mechanism by which this protection is provided to sperm remains elusive. It is speculated that the LDF associates with sperm membranes and provides protection against membrane damage, but there is contradictory evidence concerning the stability of this association (Foulkes, 1977; MacDonald and Foulkes, 1981; Watson, 1975). Another study suggests that EY lipoproteins compete with detrimental SP cationic peptides (< 5 kDa) in binding to the sperm membrane and thus protect the sperm (Vishwanath et al., 1992). In a recent study, we discovered that the major proteins of bull SP interact with

LDF and this interaction appears to have significant effect on the preservation of sperm integrity and fertilizing ability (Bergeron et al., 2004a; Manjunath et al., 2002).

BSP proteins

Seminal plasma (SP), which is mixed with epididymal sperm at ejaculation, serves as the carrier of sperm to the female genital tract and has been described as both beneficial and detrimental to sperm (Acott and Hoskins, 1978; Baas et al., 1983; Bellin et al., 1998; Dott, 1974; Killian et al., 1993; Shivaji and Bhargava, 1987; Vishwanath and Shannon, 1997). However, the factors responsible for these activities have not been characterized. Our laboratory has extensively investigated a family of closely related lipid-binding proteins of bull SP, collectively called BSP proteins (BSP-A1/A2, BSP-A3 and BSP-30kDa) (Desnoyers and Manjunath, 1994; Desnoyers et al., 1994; Manjunath, 1984; Manjunath et al., 1988; Manjunath and Sairam, 1987; Manjunath et al., 1987). The BSP proteins are secreted by seminal vesicles. BSP-A1, -A2, and –A3 have apparent molecular masses ranging from 15 to 17 kDa and the BSP-30kDa protein has a molecular mass of 28 to 30 kDa. BSP-A1 and –A2, have an identical amino acid sequence but their difference resides in their degree of glycosylation. Therefore, they are glycoforms and are considered as the same chemical entity designated BSP-A1/-A2. BSP-30kDa is also a glycoprotein while BSP-A3 is not (Manjunath et al., 1988). The BSP proteins contain two tandemly arranged homologous domains that are similar to the type II structures present in the collagen-binding domain of fibronectin (reviewed in reference Manjunath and Thérien, 2002).

The homologs of BSP proteins have been isolated and characterized in other mammalian SP or seminal vesicle secretions (Table 1), namely boar and stallion (Calvete et al., 1995; Calvete et al., 1997; Ménard et al., 2003; Sanz et al., 1993), bison (Boisvert et al., 2004), goat (Villemure et al., 2003) and ram (Bergeron et al., 2004b). It is interesting to note that boar has one form, while all other species contain 3-4 BSP homologs. BSP-like antigens are also present in rat, mouse, hamster, and human SP (Leblond et al., 1993) indicating that they are ubiquitous in mammals.

The concentration of BSP homologs in SP varies from species to species. In bull, the BSP proteins represent ~ 50% of total proteins. In boar, BSP homologs represent ~ 1% of the boar total total proteins. In man, BSP homologs represent < 0.02% of total SP proteins. In ram, goat and stallion, the BSP homologs represent ~ 20-30% of total proteins present in SP. This difference in the amount of BSP protein homologs in ejaculates may have an implication in sperm storage (discussed later).

BSP proteins are beneficial to sperm

The role of these novel lipid-binding proteins in the sperm membrane lipid modification that occurs during sperm capacitation, which is a prerequisite to fertilization, has been described in a series of studies (Thérien et al., 1995; Thérien et al., 1999; Thérien et al., 1998; Thérien et al., 1997). The general mechanism of capacitation proposed by our laboratory includes the sperm membrane stabilization and destabilization by BSP proteins (reviewed in reference Manjunath and Thérien, 2002). At ejaculation, sperm are exposed to SP, which contains BSP proteins. The BSP proteins then bind to the sperm surface and stabilize the sperm membrane. When

BSP–bound sperm reach the oviduct, HDL present in oviductal and/or follicular fluid interacts with the sperm membrane, which results in removal of cholesterol. This lipid efflux destabilizes the sperm membrane, increases permeability to ions such as Ca^{2+}, changes internal pH and increases membrane fluidity and metabolism, leading to the capacitation state. Moreover, heparin-like glycosaminoglycans present in the female genital tract could also play a role in capacitation, since they interact with BSP proteins (reviewed in reference Manjunath and Thérien, 2002). Consequently, BSP proteins are beneficial to sperm fertility.

BSP proteins are detrimental to sperm

In contrast to their positive role in fertility, BSP proteins may be detrimental to sperm in the context of sperm storage. BSP proteins stimulate cholesterol and choline phospholipid efflux from the epididymal sperm membrane in a dose and time dependent manner (Thérien et al., 1999; Thérien et al., 1998). When epididymal sperm were incubated in the presence of 0.5% SP, almost 20% of sperm cholesterol was removed in 1 hr (Fig. 1). The same amount of cholesterol was removed in 30 min if the SP concentration was 2% and it has been shown that it is the BSP proteins present in SP which stimulate this efflux (Thérien et al., 1998). Furthermore, when undiluted bovine ejaculates were stored at 37°C for 24 h, there was a loss of ~ 40% cholesterol and ~ 40% choline phospholipids (Bergeron et al., 2004a). Similar loss of cholesterol and phospholipids was observed even when semen was diluted 10 times or more with media devoid of EY (Bergeron et al., 2004a). Therefore, continuous exposure of sperm to SP that contains BSP proteins may damage the sperm membrane

and render the membrane very sensitive to storage in liquid or frozen states. Thus, BSP proteins in SP have the potential to act as both beneficial and detrimental factors to sperm depending on the concentration of SP and exposure time.

Interaction of BSP proteins with EY-LDF

Since BSP proteins are phospholipid-binding proteins and LDF contains phospholipids, we investigated whether or not the BSP proteins, the detrimental factors of SP, interact with LDF, the EY component responsible for sperm protection. Interestingly, the BSP proteins bind to LDF isolated from EY. First we subjected EY to ultracentrifugation in Tris-buffer containing potassium bromide (density 1.21 kg/l). The yellow viscous solution floating at the top of the centrifuge tube, which corresponds to LDF, was recovered, dialysed against Tris-buffer and used to study the interaction of LDF and BSP proteins by several techniques (Manjunath et al., 2002).

We incubated SP (50 mg protein) with LDF (10 mg) and reisolated floating lipoproteins by ultracentrifugation. SDS-PAGE analysis of reisolated LDF demonstrated the presence of all the BSP proteins, indicating that BSP proteins bind to LDF. Furthermore, we have calculated that a molecule of LDF can bind 243-555 molecules of BSP proteins (Manjunath et al., 2002). Thus, LDF has a high capacity to bind BSP proteins. When we submitted either BSP-A1/A2 or LDF to size exclusion chromatography, BSPA1/A2 alone eluted at 300 ml and the LDF alone eluted at 175 ml (Fig. 2). However, when a mixture of BSP-A1/-A2 protein and LDF were run on the same column, they co-eluted at the LDF position, indicating that BSP proteins bind to LDF. The binding of BSP proteins to LDF can also be demonstrated by lipogel

(agarose) electrophoresis (Fig. 3). LDF has a neutral charge and does not migrate from the origin. However, when mixed with SP or purified BSP proteins (BSP-A1/-A2, BSP-A3 and BSP-30 kDa), it forms a complex that migrates towards the positive electrode, and suggesting the association of BSP proteins with LDF. Moreover, the migration distance is concentration dependent. In addition, the LDF-BSP proteins interaction is specific because, when the mixture of LDF with proteins such as myoglobin, lysozyme or BSA were subjected to agarose gel electrophoresis, LDF remained at the point of application whereas the control proteins migrated towards the electrode opposite to their net charge (Fig. 4) (Manjunath et al., 2002). Furthermore, the binding of BSP proteins to LDF is saturable (Fig. 5). LDF was incubated with different concentrations of BSP proteins and subjected to agarose gel electrophoresis. At LDF and BSP protein ratio of 1:2.5 and 1:5, all BSP proteins bound to LDF. However, at a ratio of 1:7.5 and above, some BSP proteins did not bind to LDF. The LDF-BSP protein interaction is stable during freezing and thawing. In order to demonstrate this, we reisolated the LDF-BSP protein complex by ultracentrifugation of SP obtained from EY diluted semen, which was frozen and thawed (Manjunath et al., 2002).

Novel mechanism of sperm protection by EY-LDF

In view of the discovery that BSP proteins specifically bind to LDF and form a stable complex, we suggest a novel mechanism of sperm protection by EY lipoproteins (Fig. 6). The BSP proteins are secreted by seminal vesicles and are added to sperm at ejaculation (Manjunath et al., 1988). The BSP proteins coat the sperm membrane

(Desnoyers and Manjunath, 1992; Manjunath et al., 1994), and induce cholesterol (Thérien et al., 1998) and phospholipid efflux (Thérien et al., 1999). If semen is not diluted, the lipid efflux continues resulting in decreased sperm resistance to cold shock and freezing. Since ejaculates are diluted with EY extenders within minutes after collection, the LDF may sequestrate most of the BSP proteins present in semen. This could result in a minimal modification of the sperm plasma membrane and allow better sperm storage. Thus, EY lipoproteins may offer protection to sperm by reducing the deleterious effect of SP proteins on sperm membranes. During natural mating, a mechanism may also exist to eliminate the detrimental effect of BSP proteins on sperm. After being ejaculated into the vagina, sperm swim through cervical mucous and enter the uterus within minutes. Cervical mucous acts as a barrier for SP. In the artificial insemination industry, the BSP proteins (i.e SP proteins) are not removed from semen, but their effect is probably eliminated by the formation of rapid and stable complex with EY lipoproteins.

Our recent studies support the proposed mechanism (Bergeron et al., 2004a). In the presence of LDF or EY in the extender, there is about 50-80% less BSP proteins bound to sperm as compared to extender without EY or LDF (Fig. 7). Furthermore, in the absence of EY or LDF in the extender there was a continuous efflux of cholesterol and phospholipid (~20% and 15% respectively) from the sperm membrane during 24 h storage at 4°C (Fig. 8A and B). However, when LDF or EY was present in the extender, no loss of cholesterol and phospholipid was observed, but in fact sperm gained cholesterol and phospholipid (21-35% and 35-48% respectively; Fig 8A and

B). Interestingly, the presence of EY or LDF in the extender preserved sperm motility. The presence of LDF in the extender mimicked the effect of whole EY in the extender used to dilute semen. Therefore, LDF is the factor from EY, that protects sperm against the detrimental effect of BSP proteins during storage. Thus, the interaction between BSP proteins and LDF plays a key role in sperm protection by EY.

Conclusions and perspectives

The current study suggests that the beneficial role of semen dilution with extenders containing EY involve interplay among BSP proteins present in SP, EY-LDF and the sperm membrane. A continuous contact of sperm with BSP proteins or SP is detrimental to sperm function. Interestingly, the LDF presents in the EY extender decreases the association of BSP proteins to sperm and prevents the loss of lipids (cholesterol and phospholipid) from the sperm membrane while maintaining sperm motility. Thus, the association of LDF with BSP proteins protects sperm by preventing BSP proteins in SP to bind to sperm and intrinsically damage the sperm membrane by removing lipid. Since BSP protein homologs of stallion, boar, bison, goat and ram also bind to LDF, the mechanism of sperm protection by EY may be similar in all these species. The amount of BSP homologs in semen from these species is less than that found in bull semen. Consequently, the quantity of EY required to sequestrate all the BSP homologs should be less than that required for bull semen. This hypothesis remains to be investigated. The current study should aid in developing extenders for preservation of semen (liquid or frozen) from several farm animals, domestic animals, zoo animals, wild animals and endangered species for

which protocols do not exist presently. In addition, it will help us to develop novel extenders for commercial application that do not contain animal products for sperm preservation.

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FIGURE LEGENDS

Figure 1. Cholesterol efflux from bovine epididymal sperm in the presence of SP. Epididymal sperm were incubated for 8 h with: 0% (control, solid squares), 0.05% (open circles), 0.1% (solid triangles), 0.25% (inverted triangles), 0.5% (diamonds), 1% (open squares) or 2% (solid circles) SP. The cholesterol efflux in the presence of more than 0.1% SP was significantly different from control ($P < 0.05$). From Thérien et al. (1998).

Figure 2. Gel filtration analysis of LDF interaction with BSP-A1/A2. LDF, BSP-A1/A2 and a mixture of LDF and BSP-A1/A2 were submitted to chromatography on a Sepharose CL-4B column. From Manjunath et al (2002).

Figure 3. Electrophoretic analysis of the interaction of LDF with BSP proteins. Samples were incubated 15 min at room temperature, then submitted to agarose gel electrophoresis and stained for lipids. Lanes 1 and 10: human serum (positive control of the migration, 2 µl); lane 2: LDF (1.5 µg); lane 3: LDF (1.5 µg) + bovine SP total proteins (6 µg), lanes 4 and 5: LDF (1.5 µg) + BSP-A1/A2 (1.5 and 6 µg, respectively); lanes 6 and 7: LDF (1.5 µg) + BSP-A3 (1.5 and 6 µg, respectively); lanes 8 and 9: LDF (1.5 µg) + BSP-30kDa (1.5 and 6 µg, respectively). The arrow indicates the point of sample application. From Manjunath et al. (2002).

Figure 4. Electrophoretic analysis of the interaction of LDF with control proteins. Samples were incubated 15 min at room temperature, then submitted to agarose gel electrophoresis and stained for proteins. Lane 1: LDF (1 µg); lane 2: LDF (1 µg) +

myoglobin (6 μg); lane 3: LDF (1 μg) + lysozyme (6 μg); lane 4: LDF (1 μg) + BSA (6 ug). The arrow indicates the point of sample application. From Manjunath et al. (2002).

Figure 5. Electrophoretic analysis of the saturation of the binding of BSP proteins to LDF. Samples were incubated 15 min at room temperature, then submitted to agarose gel electrophoresis and stained for proteins. Lane 1: LDF (1 μg); lanes 2-7: LDF (1μg) + BSP-A1/A2 protein (2.5, 5, 7.5, 10, 12.5 and 15 μg, respectively); lane 8: BSP-A1/A2 (10 μg). The arrow indicates the point of sample application. From Manjunath et al. (2002).

Figure 6. Mechanism of sperm protection by EY. From Manjunath et al. (2002).

Figure 7. Amount of BSP-A1/A2 proteins associated with sperm during incubation at 4°C of bull sperm diluted with control extender (without EY or LDF; triangles), or extender containing EY (circles) or LDF (squares). BSP-A1/A2 was quantified by RIAs. The amount of BSP-A1/A2 proteins associated with bull sperm diluted with extender containing EY or LDF is significantly different from control ($P < 0.05$). From Bergeron et al. (2004).

Figure 8. Sperm lipid loss or gain during incubation at 4°C of bull semen diluted with control extender (without EY or LDF; triangles), or extender containing EY (circles) or LDF (squares). A) Cholesterol. B) Choline phospholipids. Significant difference versus 0 h: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Different letters within each

incubation time denote significant differences between treatments ($P < 0.05$). From Bergeron et al. (2004).

TABLE 1. BSP homologs isolated from SP of different species.

Species	BSP protein homologs
Bull	BSP-A1, BSP-A2, BSP-A3, BSP-30kDa
Boar	pB1
Stallion	HSP-1, HSP-2, HSP-12kDa
Goat	GSP-14kDa, GSP-15kDa, GSP-20kDa, GSP-22kDa
Ram	RSP-15kDa, RSP-16kDa, RSP-22kDa, RSP-24kDa
Bison	BiSv-16kDa, BiSV-17kDa, BiSV-18kDa, BiSV-28kDa

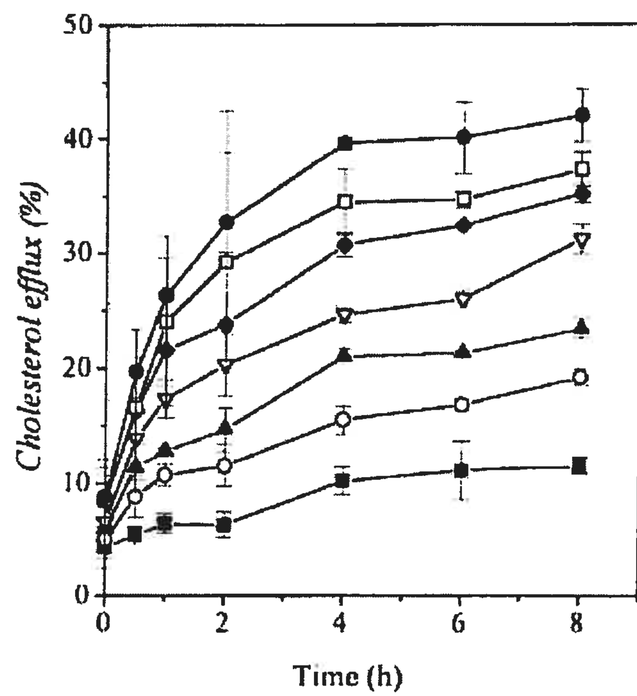
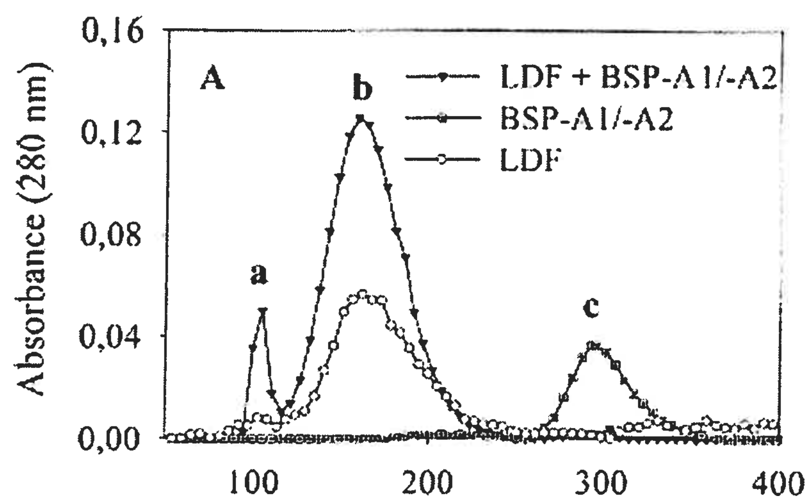


Figure 1



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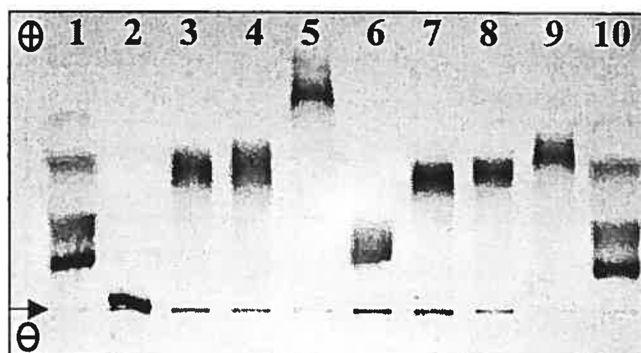

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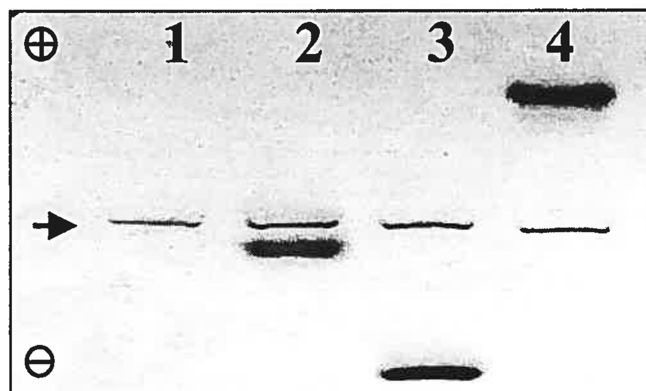

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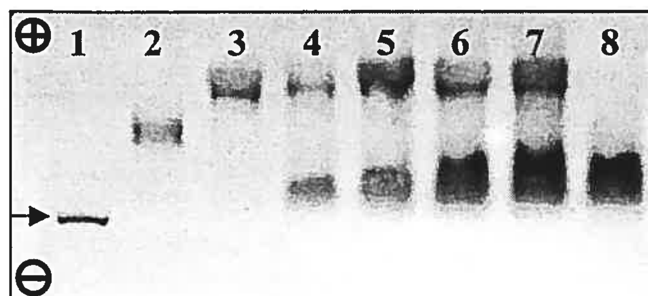

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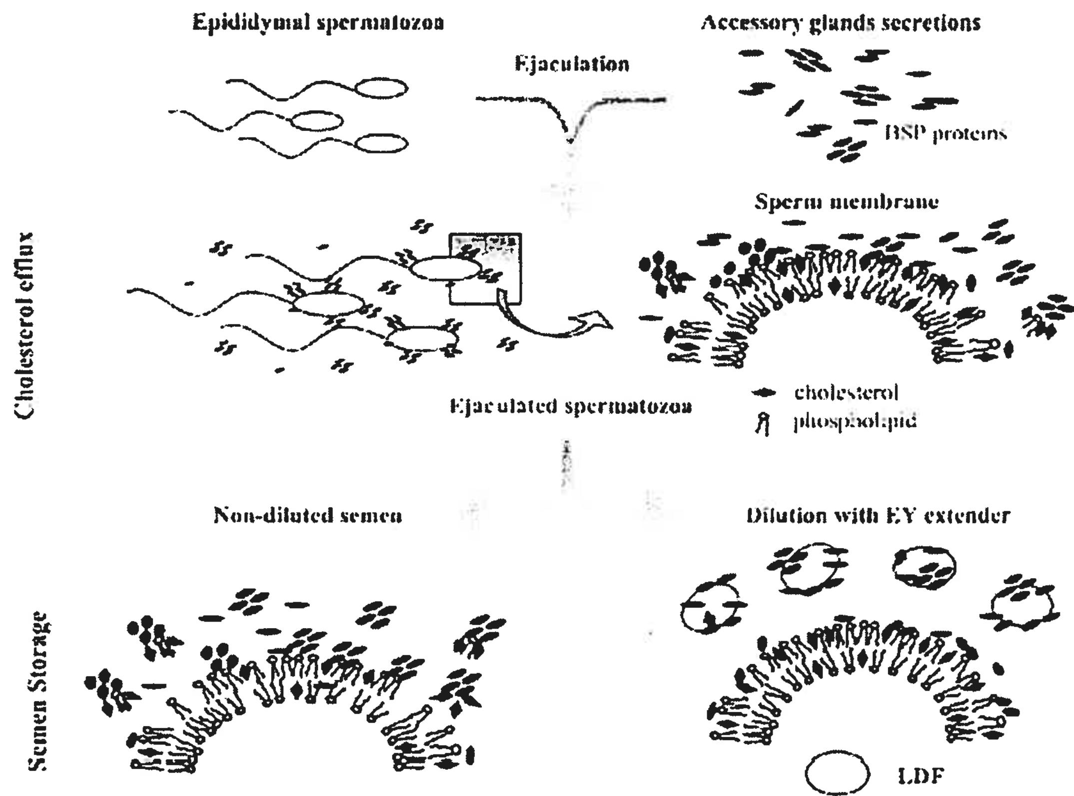

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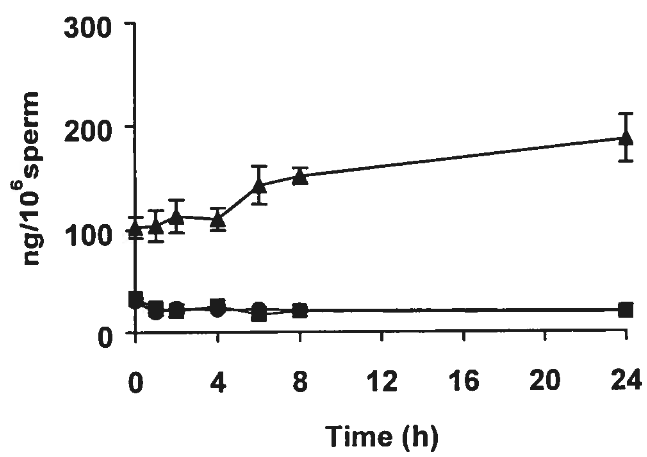

Figure 7

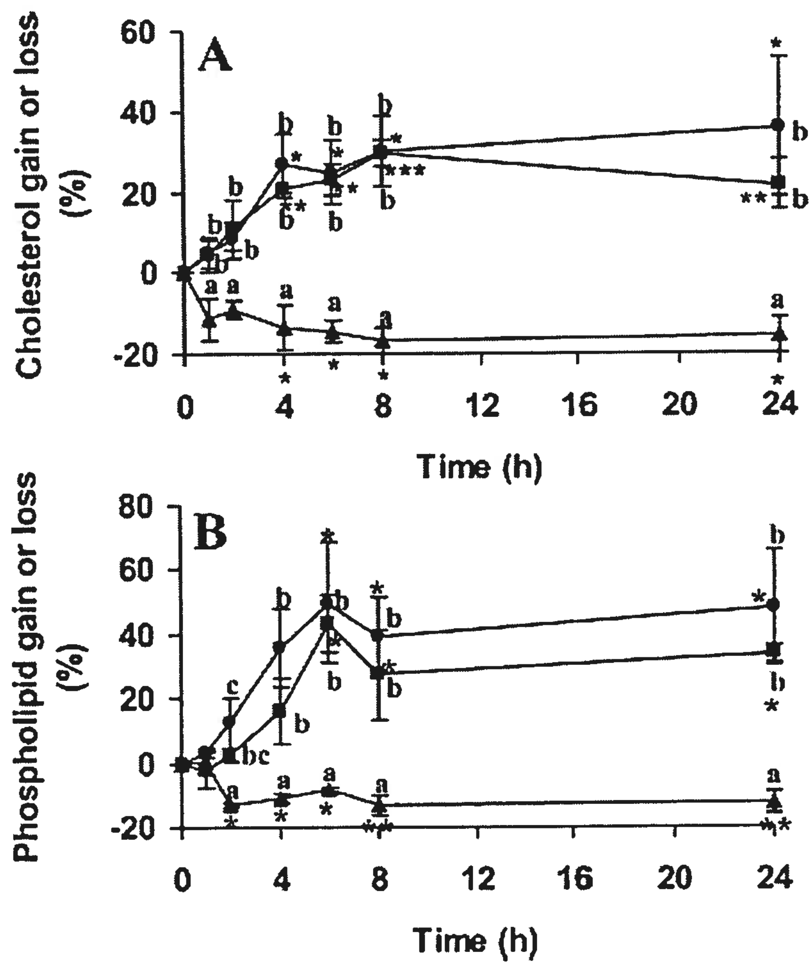

Figure 8

3. DISCUSSION

Afin d'être conservée à 4°C ou cryopréservée, la semence de mammifères doit être préalablement diluée avec un diluant contenant du jaune d'œuf ou du lait comme agent protecteur. Ces agents protecteurs permettent aux spermatozoïdes de résister aux changements de température qu'ils subissent lors de la cryopréservation. Grâce à la présence de ces agents dans les diluants protecteurs, on obtient des spermatozoïdes vivants et motiles après la cryopréservation. Malgré l'utilisation de ces agents protecteurs depuis plus de 60 ans, le mécanisme par lequel ils protègent les spermatozoïdes est encore inconnu. La protection offerte par le jaune d'œuf est attribuée aux LDF qu'il contient. Cependant, on ne connaît pas de quelle manière ces LDF protègent les spermatozoïdes pendant l'entreposage de la semence. L'hypothèse la plus acceptée est à l'effet que les LDF du jaune d'œuf s'associeraient aux spermatozoïdes pendant l'entreposage et fourniraient des lipides pour la réparation des membranes des spermatozoïdes. En ce qui concerne le lait, ce sont les micelles de caséines qu'il contient qui semblent être responsables de l'effet protecteur du lait sur les spermatozoïdes. Aucune hypothèse n'a été émise quant à leur mécanisme d'action sur les spermatozoïdes. Puisque les mécanismes par lesquels les spermatozoïdes sont protégés par le jaune d'œuf et le lait sont inconnus, il est difficile d'apporter des améliorations aux diluants utilisés pour la conservation de la semence et de trouver des sources de remplacements au jaune d'œuf et au lait qui ne sont pas d'origine animale.

Le PS est constitué des sécrétions des glandes accessoires et entre en contact avec les spermatozoïdes au moment de l'éjaculation. Le PS est donc le milieu

environnant des spermatozoïdes lors de la récolte de la semence dans les centres d'insémination artificielle. L'influence du PS sur les fonctions spermatiques est très complexe. Les études ayant tenté de démontrer son effet sur les fonctions spermatiques donnent des résultats contradictoires. Plusieurs études décrivent le PS comme étant néfaste pour les spermatozoïdes et suggèrent qu'un facteur qu'il contient serait responsable de ses effets négatifs sur les spermatozoïdes. Cependant, ce facteur négatif du PS n'a pas été caractérisé.

De la même manière, peu d'information est disponible quant à l'effet des composants des agents protecteurs (jaune d'œuf ou lait) présents dans les diluants sur les fonctions spermatiques. On sait seulement que ces agents permettent d'obtenir des spermatozoïdes motiles après le refroidissement et la congélation de la semence. Quant à eux, les effets combinés du PS et des agents protecteurs sur les fonctions spermatiques sont inconnus.

La présente thèse avait pour but de clarifier les points ci-haut mentionnés.

3.1. Pourquoi le PS est-il néfaste pour les spermatozoïdes bovins?

Nos études permettent d'expliquer pourquoi le PS a souvent été décrit comme ayant un effet négatif sur les spermatozoïdes. *In vitro*, le PS peut être néfaste pour les spermatozoïdes car il provoque un efflux des lipides des membranes des spermatozoïdes. En effet, nous avons démontré qu'un contact continu des spermatozoïdes bovins avec le PS stimule un efflux continu du cholestérol et des phospholipides des membranes des spermatozoïdes et que cet efflux de lipides est

accompagné d'une diminution de la motilité spermatique (article 2.2). Des études de notre laboratoire ont identifié les facteurs présents dans le PS qui sont responsables de l'efflux des lipides des membranes des spermatozoïdes. Ces facteurs sont les protéines BSP. Le PS contient donc des facteurs néfastes à l'intégrité des membranes des spermatozoïdes et par conséquent, défavorables au maintien des fonctions spermatiques.

L'efflux des lipides membranaires est directement proportionnel aux temps d'incubation des spermatozoïdes avec le PS contenant les protéines BSP ainsi qu'à la concentration des protéines BSP dans le milieu environnant les spermatozoïdes. [20, 70]. Les résultats contradictoires obtenus dans les études voulant élucider l'effet du PS sur les spermatozoïdes peuvent donc être expliqués par des différences au niveau de la concentration de PS et de la durée de l'incubation des spermatozoïdes avec le PS ayant été utilisées dans ces études.

La quantité de lipides enlevée des membranes des spermatozoïdes pendant l'incubation de la semence a été la même pour la semence non-diluée et la semence ayant été diluée par au moins un facteur dix avec un diluant ne contenant pas de jaune d'œuf (article 2.2., figure 7). Ceci peut être expliqué par le fait que malgré que le PS soit dilué au moins dix fois dans la semence diluée, la concentration en protéines BSP dans le milieu environnant les spermatozoïdes est encore trop élevée pour amener une différence dans la quantité de lipides enlevés des membranes des spermatozoïdes. À une certaine concentration de protéines BSP, il est possible que les sites de liaison des protéines BSP sur la membrane des spermatozoïdes soient saturés. Lors des études

précédentes visant à démontrer que l'efflux lipidique observé en présence de protéines BSP dépendait de la concentration des protéines BSP présentes dans le milieu environnant les spermatozoïdes, les concentrations de protéines BSP variaient entre 20 et 120 μg/ml [20, 70] tandis que dans l'étude présentée dans la section 2.2., la concentration en protéines BSP était jusqu'à 50 fois plus élevée et ce, même si la semence avait été préalablement diluée par un facteur dix.

Le PS a donc un effet négatif sur les spermatozoïdes bovins dans un contexte d'entreposage de la semence. Il provoque un efflux des lipides membranaires qui endommage les membranes des spermatozoïdes et affecte négativement les fonctions spermatiques. De plus, le PS peut avoir un effet négatif sur la survie des spermatozoïdes pendant la cryopréservation. En effet, la diminution de la quantité de cholestérol membranaire fragilise les membranes des spermatozoïdes et rend les spermatozoïdes plus susceptibles d'être endommagés par le refroidissement [103]. Il est aussi possible que l'efflux du cholestérol membranaire contribue à la capacitation prématurée des spermatozoïdes, ce qui est néfaste pour leur survie.

3.2. Caractérisation des facteurs néfastes présents dans le PS des mammifères

Les protéines BSP peuvent être néfastes pour les membranes spermatiques dans un contexte où les spermatozoïdes bovins sont en contact continu avec le PS. Nous avons voulu vérifier si des facteurs négatifs pour les fonctions spermatiques étaient aussi présents dans le PS de mammifères autres que le bovin, espèce chez laquelle ces facteurs négatifs ont été initialement caractérisés. Des membres de la famille des protéines BSP avaient déjà été identifiés dans le PS de porc, d'étalon, de

bouc, d'humain, et de hamster. Dans cette thèse, nous avons démontré pour la première fois la présence de protéines homologues aux protéines BSP dans le PS de bison (protéines BiSV : BiSV-16kDa, BiSV-17kDa, BiSV-18kDa et BiSV-28kDa) et de bélier (protéines RSP : RSP-15kDa, RSP-16kDa, RSP-22kDa et RSP-24kDa), indiquant que les protéines de la famille des protéines BSP sont probablement présentes dans le PS de tous les mammifères. Le PS bovin contient quatre protéines BSP (BSP-A1, BSP-A2, BSP-A3 et BSP-30kDa). Nos résultats démontrent que le PS de bison et de bélier contiennent eux aussi, quatre formes de protéines homologues aux protéines BSP.

Malgré que la séquence complète des protéines homologues aux protéines BSP présentes dans le PS de bison et de bélier est inconnue, leur séquence N-terminale, leur masse moléculaire et leurs propriétés de liaison nous permettent de prédire que ces protéines homologues contiennent 2 domaines de type II. De plus, leur extrémité C-terminale est probablement très similaire à celle des protéines BSP. En effet, l'anticorps dirigé contre les 15 acides aminés correspondant à l'extrémité C-terminale de la protéine pB1 (protéine homologue aux protéines BSP du PS porcin) reconnaît les protéines homologues retrouvées dans le PS de bison (protéines BiSV), de bélier (protéines RSP), de cheval (protéines HSP), de bouc (protéines GSP), d'humain et de buffle ainsi que les protéines BSP [157]. Ces résultats indiquent que les protéines homologues aux protéines BSP partagent une forte identité avec l'extrémité C-terminale de la protéine pB1. Il existe donc une forte identité au niveau de la séquence des protéines homologues aux protéines BSP. Quant à elle, la séquence N-terminale

des protéines homologues retrouvées dans le PS de bison partage environ 100% d'identité avec l'extrémité N-terminale des protéines BSP (figure 4, article 2.3). De plus, les anticorps polyclonaux dirigés contre chacune des protéines BSP détectent spécifiquement les protéines BiSV (figure 5, article 2.4). Ces résultats indiquent que la séquence entière des protéines BiSV partage une forte identité avec les protéines BSP. Quant à elles, les protéines RSP partagent une plus forte identité de séquence avec les protéines homologues aux protéines BSP présentes dans le PS de bouc (protéines GSP) qu'avec les protéines BSP.

Il a été démontré que ce sont les 2 domaines de type II qui sont responsables de la liaison des protéines BSP aux groupements choline des phospholipides de la membrane plasmique des spermatozoïdes et de la stimulation de l'efflux du cholestérol membranaire [158]. Puisque les protéines homologues aux protéines BSP isolées à partir du PS des autres espèces de mammifères semblent contenir deux domaines de type II, il est probable qu'elles jouent un rôle biologique similaire à celui des protéines BSP (capacitation). Ces aspects devront être vérifiés afin de déterminer les mécanismes impliqués dans la capacitation des spermatozoïdes des autres espèces de mammifères. Ces nouveaux membres de la famille des protéines BSP sont des facteurs potentiellement néfastes pour les fonctions spermatiques dans un contexte d'entreposage de la semence à cause de leur capacité à stimuler un efflux des lipides membranaires.

3.3. Les LDF du jaune d'œuf lient les facteurs néfastes contenus dans le PS

Nous avons démontré que les facteurs néfastes contenus dans le PS bovin (protéines BSP) ont la capacité de lier les LDF du jaune d'œuf (article 2.1). Nos études précédentes ont démontré que les protéines BSP lient la surface des spermatozoïdes en se liant aux phospholipides portant un groupement choline de la membrane plasmique des spermatozoïdes [62]. Les molécules de LDF du jaune d'œuf sont composées de triacylglycérols qui sont entourés d'une monocouche de lipides contenant principalement des phospholipides portant un groupement choline [118]. Il est donc probable que la liaison des protéines BSP aux LDF du jaune d'œuf (article 2.1) se fait grâce à l'interaction des protéines BSP avec le groupement choline des phospholipides présents dans la monocouche lipidique des LDF du jaune d'œuf. La liaison des protéines BSP aux molécules de LDF serait donc proportionnelle à la quantité de phospholipides portant un groupement choline présents dans leur monocouche lipidique. Nous avons calculé qu'une molécule de LDF du jaune d'œuf peut lier entre 243 et 555 moles de protéines BSP. Cette liaison est spécifique aux protéines BSP. En effet, les LDF du jaune d'œuf ne lient pas les autres protéines contenues dans le PS bovin. Nous avons aussi démontré que les protéines homologues aux protéines BSP présentes dans le PS de mammifères ont, elles aussi, la capacité de lier les LDF du jaune d'œuf.

Nous avons démontré que la présence de jaune d'œuf ou de LDF du jaune d'œuf dans le diluant protecteur empêche la liaison des protéines BSP aux spermatozoïdes. Par conséquent, les spermatozoïdes cryopréservés ont moins de

protéines BSP liées à leur membrane que les spermatozoïdes fraîchement éjaculés. De plus, lorsque la semence est entreposée dans un diluant qui ne contient pas de jaune d'œuf ou de LDF du jaune d'œuf, les spermatozoïdes perdent des lipides membranaires (cholestérol et phospholipides portant un groupement choline). La diminution de la quantité de cholestérol des membranes des spermatozoïdes diminue la résistance des spermatozoïdes aux dommages causés par le refroidissement [103]. Par conséquent, les spermatozoïdes ayant perdu du cholestérol sont moins résistants au processus de cryopréservation. Les LDF du jaune d'œuf présentes dans les diluants protecteurs empêchent la perte de lipides membranaires. De plus, en présence de jaune d'œuf ou de LDF du jaune d'œuf, la quantité de lipides associés aux spermatozoïdes augmente. Il semble donc que des lipides des LDF du jaune d'œuf ou les molécules entières de LDF s'associent aux spermatozoïdes pendant l'entreposage de la semence.

Ces effets des LDF du jaune d'œuf sur la liaison des protéines BSP aux spermatozoïdes et sur les lipides des membranes des spermatozoïdes nous ont permis de proposer un nouveau mécanisme pour expliquer l'effet protecteur du jaune d'œuf pendant l'entreposage de la semence (article 2.5).

3.4 Nouveau mécanisme de protection des spermatozoïdes par le jaune d'œuf

Nos travaux ont confirmé que ce sont les LDF contenues dans le jaune d'œuf qui protègent les spermatozoïdes contre les dommages causés par la cryopréservation. Cependant, ils démontrent pour la première fois de quelle façon ils protègent les spermatozoïdes. L'élément clé du nouveau mécanisme de protection par le jaune

d'œuf que nous proposons est la séquestration des facteurs néfastes présents dans le PS (protéines BSP) par le jaune d'œuf. Lors de la dilution de la semence bovine avec un diluant protecteur contenant du jaune d'œuf, les LDF contenues dans le jaune d'œuf lient les protéines BSP empêchant ces dernières de stimuler un efflux des lipides membranaires des spermatozoïdes. Puisque les membres de la famille des protéines BSP présents chez l'étalon, le porc, le bison, le bouc, et le bélier ont eux aussi la capacité de lier les LDF du jaune d'œuf, le mécanisme impliqué dans la protection des spermatozoïdes par le jaune d'œuf est probablement le même pour toutes les espèces de mammifères ayant des protéines homologues aux protéines BSP dans leur PS. Nous avons démontré que la liaison des protéines BSP aux LDF du jaune d'œuf demeure stable même après le dégel de la semence (article 2.1). Ceci indique que les LDF peuvent exercer leur effet protecteur avant, pendant et après la décongélation de la semence.

Notre nouveau modèle permet d'expliquer les effets du PS ayant été rapportés dans certaines études. Des études ont démontré qu'au contact du PS, les spermatozoïdes deviennent plus susceptibles aux dommages occasionnés par la cryopréservation [159, 160]. Nos résultats sont en accord avec ces études et fournissent une explication à ces conclusions (article 2.2). L'effet négatif du PS sur les spermatozoïdes observé dans ces études est dû à l'efflux lipidique provoqué par les protéines BSP présentes dans le PS.

De plus, ce nouveau mécanisme de protection des spermatozoïdes par le jaune d'œuf, qui implique la séquestration des protéines BSP, permet d'expliquer la

controverse quant à l'effet (bénéfique ou néfaste?) du PS sur les spermatozoïdes. Les protéines majeures du PS bovin, les protéines BSP, stimulent la capacitation des spermatozoïdes via la stimulation d'un efflux du cholestérol membranaire [34]. Le PS est donc bénéfique pour les spermatozoïdes car il contribue à leur pouvoir fécondant *in vivo*. Cependant, un contact continu avec le PS qui contient les protéines BSP est néfaste pour les spermatozoïdes car l'efflux continu des lipides des membranes des spermatozoïdes cause des dommages membranaires et est associé à une diminution de la motilité spermatique. *In vivo*, le PS n'a pas le temps d'être néfaste pour les spermatozoïdes car après l'éjaculation, les spermatozoïdes nagent dans le tractus génital femelle et laissent le PS derrière eux. L'effet du PS sur les spermatozoïdes peut donc être positif ou négatif selon le contexte (*in vivo* ou *in vitro*) dans lequel les travaux ont été réalisés ainsi que le temps d'incubation et la concentration de PS à laquelle les spermatozoïdes ont été exposés.

Lors de l'entreposage de la semence diluée avec un diluant contenant du jaune d'œuf, la quantité de cholestérol et de phospholipides associée aux spermatozoïdes augmente. Cette augmentation de la quantité des lipides associés aux spermatozoïdes est due aux LDF contenues dans le jaune d'œuf. Il est possible que l'effet protecteur des LDF du jaune d'œuf soit non seulement apporté par la prévention de l'efflux lipidique stimulé par les protéines BSP mais aussi par le gain de lipides qu'il provoque au niveau des spermatozoïdes (article 2.2, figure 8). Cependant, le lait écrémé, qui est exempt de lipides, protège les spermatozoïdes aussi efficacement que le jaune d'œuf [114]. L'effet protecteur des LDF du jaune d'œuf semble donc résider dans la

séquestration des protéines BSP plutôt que dans le gain de lipides associés aux spermatozoïdes. Cette hypothèse devra être vérifiée.

La liaison des protéines BSP aux LDF du jaune d'œuf est spécifique et cette caractéristique peut avoir d'importants avantages pour les spermatozoïdes. En effet, les autres protéines qui sont contenues dans le PS et qui sont bénéfiques pour les spermatozoïdes ne sont pas séquestrées par le jaune d'œuf et peuvent donc exercer leur rôle sur les spermatozoïdes.

Les spermatozoïdes cryopréservés ont 50 à 80% moins de protéines BSP liées à leur membrane comparativement à ceux de la semence fraîchement éjaculée [161]. Les protéines BSP liées aux spermatozoïdes sont impliquées dans la stimulation de la capacitation des spermatozoïdes par les glycosaminoglycanes du tractus génital femelle. Il est donc possible que la capacitation des spermatozoïdes cryopréservés avec un diluant contenant du jaune d'œuf soit compromise. Cette diminution de la quantité de protéines BSP liées à la surface des spermatozoïdes pourrait être éliminée en incubant la semence en présence de protéines BSP avant de l'utiliser pour l'insémination artificielle. Ceci pourrait peut-être augmenter le pouvoir fécondant des spermatozoïdes cryopréservés. *In vivo*, les protéines BSP liées aux spermatozoïdes stabiliseraient les membranes des spermatozoïdes pendant leur voyage jusqu'à l'oviducte [34]. Cependant, lors de l'insémination artificielle, la semence cryopréservée est déposée directement dans l'utérus. La distance que les spermatozoïdes cryopréservés doivent parcourir pour atteindre l'oviducte est donc plus courte que celle parcourue par les spermatozoïdes fraîchement éjaculés. Les

spermatozoïdes cryopréservés nécessitent peut-être une plus faible quantité de protéines BSP pour la stabilisation de leur membrane étant donné la plus courte distance qu'ils ont à parcourir dans le tractus génital femelle.

3.5. Amélioration des protocoles de congélation de la semence bovine

À la lumière de nos résultats, plusieurs améliorations pourraient être apportées aux protocoles utilisés lors de la congélation de la semence bovine. Premièrement, la semence bovine devrait être diluée avec le diluant protecteur aussitôt que possible après la récolte de la semence. Dans les centres d'insémination artificielle, la semence est analysée (pourcentage de motilité, concentration spermatique et viabilité) avant d'être diluée. Pendant cette période d'analyses, les membranes des spermatozoïdes peuvent être endommagées à cause de l'efflux de lipides provoqué par le PS ([20, 70], article présenté dans la section 2.2). Cet efflux des lipides membranaires est directement proportionnel aux temps d'incubation des spermatozoïdes avec le PS contenant les protéines BSP ainsi qu'à la concentration des protéines BSP présentes dans le milieu environnant les spermatozoïdes. Pendant l'analyse de la qualité de la semence, cette dernière n'est pas diluée et les spermatozoïdes sont exposés à une concentration élevée de protéines BSP (35-60 mg/ml) qui peuvent enlever une grande quantité de lipides membranaires. En effet, lorsqu'un éjaculat non-dilué est placé à 4°C, la quantité de cholestérol des membranes des spermatozoïdes diminue d'environ 10% après seulement 1 h d'incubation (article 2.2, figure 7). Afin d'assurer une résistance maximale des spermatozoïdes au refroidissement, il faut assurer une perte minimale de lipides des membranes des spermatozoïdes. Pour ce faire, les protéines

BSP doivent être séquestrées aussitôt que possible après la récolte de la semence. De plus, il serait intéressant de récolter la semence dans un tube préalablement rempli de diluant protecteur contenant du jaune d'œuf. Ainsi, les protéines BSP seraient séquestrées immédiatement par les LDF présentes dans le diluant lors de la récolte de la semence, empêchant complètement les protéines BSP de venir endommager les membranes des spermatozoïdes avant le refroidissement et la congélation.

Deuxièmement, les éjaculats bovins devraient être dilués selon leur volume. Dans les centres d'insémination artificielle, la semence est généralement diluée de façon à obtenir une concentration de 80 X 10^6 spermatozoïdes/ml afin d'obtenir 20 X 10^6 spermatozoïdes par paillette de 250 µl (une paillette correspond à une dose d'insémination). Cependant, ce n'est peut-être pas la meilleure façon de protéger efficacement les spermatozoïdes contre l'effet néfaste des protéines BSP sur les membranes des spermatozoïdes. La concentration spermatique des éjaculats bovins est très variable et peu importe le volume de l'éjaculat, il sera dilué pour atteindre une concentration fixe de 80 X 10^6 spermatozoïdes/ml. Ainsi, le facteur de dilution utilisé pour diluer les éjaculats de faible concentration spermatique (300 X 10^6 spermatozoïdes/ml) est moins élevé que les éjaculats ayant une concentration élevée de spermatozoïdes (2000 X 10^6 spermatozoïdes/ml) (voir Tableau II). Par conséquent, plus la concentration spermatique de l'éjaculat est faible, plus la concentration du PS dans la semence diluée est élevée (voir Tableau II). Une concentration élevée de PS dans la semence diluée peut affecter négativement la qualité des spermatozoïdes entreposés. En diluant l'éjaculat selon son volume, la concentration de PS retrouvée

dans la semence diluée sera constante, peu importe la concentration spermatique des éjaculats. De plus, le volume des éjaculats bovins est aussi très variable (2-10 ml). Si le volume de l'éjaculat ayant une faible concentration spermatique est élevé, la quantité de jaune d'œuf présente dans le diluant n'est peut être pas assez élevée afin de fournir un nombre suffisant de molécules de LDF afin de séquestrer toutes les protéines BSP présentes dans le PS de l'éjaculat. Selon nos estimations, les centres d'insémination devraient s'assurer de diluer tous les éjaculats bovins par un facteur d'au moins dix avec le diluant contenant 20% de jaune d'œuf (v/v). À ce facteur de dilution, la quantité de jaune d'œuf présente dans le milieu environnant les spermatozoïdes est suffisante pour séquestrer la grande quantité de protéines BSP présentes dans le PS bovin.

Tableau II. Effet de la dilution de la semence afin d'obtenir une concentration de 80 X 10^6 spermatozoïdes/ml (20 X 10^6 spermatozoïdes/paillette).

Concentration spermatique de l'éjaculat (# X 10^9/ml)	Facteur de dilution (pour obtenir 80 X 10^6 sperm/ml)	% de PS dans la semence diluée (v/v)
0,3	1:3,75	26,7
0,5	1:6,25	16,0
1,0	1:12,5	8,0
1,5	1:18,8	5,3
2,0	1:25	4,0

Il est possible que le facteur de dilution avec lequel la semence bovine est diluée soit important pour la conservation de la semence car il permettrait d'expliquer

les différences obtenues dans la tolérance des spermatozoïdes de différents taureaux à la congélation. Les éjaculats bovins ayant une faible concentration spermatique sont moins dilués (surplus de BSP libres) et sont moins résistants à la congélation car les membranes des spermatozoïdes ont été modifiées par les protéines BSP libres.

3.6. Mise au point d'un diluant pour la conservation des spermatozoïdes de mammifères

Selon nos résultats, il sera difficile de trouver un diluant efficace pour cryopréserver la semence de tous les mammifères. En effet, la quantité de protéines homologues aux protéines BSP présente dans le PS est très variable entre les différentes espèces de mammifères (voir Tableau III). Par conséquent, la quantité de jaune d'œuf présente dans le diluant protecteur doit être adaptée pour chaque espèce. Par exemple, la semence de bélier, qui contient une quantité de protéines homologues aux protéines BSP plus faible que la quantité de protéines BSP présentes dans la semence bovine, devrait nécessiter une proportion de jaune d'œuf plus faible dans le diluant protecteur que celle requise pour la congélation de la semence bovine. Plus la quantité de protéines BSP à séquestrer est élevée, plus le diluant doit contenir une quantité élevée de jaune d'œuf. Il est aussi possible d'utiliser le diluant contenant 20% de jaune d'œuf pour chaque espèce mais en utilisant un facteur de dilution adapté pour chacune d'elle. Chez le bélier, la semence est généralement diluée 2 à 5 fois avec un diluant contenant 2-15% (v/v) de jaune d'œuf avant d'être congelée [162]. Ce faible facteur de dilution et la quantité de jaune d'œuf insuffisante dans le diluant expliquent probablement la faible efficacité des méthodes de cryopréservation chez le bélier. En

effet, les centres d'insémination artificielle doivent utiliser jusqu'à 200 X 10^6 spermatozoïdes pour inséminer une seule brebis. En diluant la semence de bélier avec suffisamment de jaune d'œuf, les spermatozoïdes seront protégés efficacement contre les protéines homologues aux protéines BSP et moins de spermatozoïdes pourront être utilisés par dose d'insémination.

Tableau III. Quantité moyenne de protéines BSP dans le PS de différentes espèces de mammifères.

	Protéines BSP dans le PS		Protéines BSP (mg/éjaculat)
	(mg/ml)	(% des protéines totales)	
Taureau	39,1	46,9	234,3
Bison	3*	25*	-
Bélier	15	30	20,3
Bouc	5,6	20	7,6
Étalon	2	20	330
Porc	0,32	1,1	104
Humain	0,007	0,02	0,03

* protéines BSP dans les sécrétions des vésicules séminales.

L'identification ainsi que la quantification des facteurs néfastes (famille des protéines BSP) présents dans le PS des différentes espèces de mammifères permettra la mise au point de diluants efficaces (contenant assez de jaune d'œuf) pour la conservation des spermatozoïdes de chacune des espèces. Par conséquent, il sera

possible de conserver la semence des animaux domestiques, de ferme, de zoo ou bien d'espèces menacées. De plus, les méthodes de cryopréservation des spermatozoïdes pourront être standardisées pour chaque espèce (facteur de dilution et nombre de spermatozoïdes à utiliser pour l'insémination) afin d'obtenir un taux de fécondation optimal avec la semence cryopréservée.

3.7. Nouveau diluant protecteur exempt de substances d'origine animale

Depuis plusieurs années, l'industrie de l'insémination artificielle est intéressée à éliminer tout facteur d'origine animale de la composition des diluants protecteurs et à utiliser des agents protecteurs ayant une composition plus uniforme. Afin de trouver des agents de remplacement capables de protéger efficacement les spermatozoïdes, il faut comprendre de quelle façon le jaune d'œuf protège les spermatozoïdes contre les dommages causés par la cryopréservation. Selon les résultats décrits dans cette thèse, il est clair que la séquestration des protéines BSP est le mécanisme par lequel le jaune d'œuf protège les spermatozoïdes. Afin de remplacer le jaune d'œuf, les produits d'origine non-animale choisis devront avoir les propriétés suivantes: 1) être capable de lier les protéines BSP rapidement et spécifiquement, 2) avoir une capacité élevée à lier les protéines BSP, 3) former un complexe avec les protéines BSP qui est stable pendant la congélation et le dégel, et 4) ne pas changer l'osmolalité du diluant protecteur. Cette dernière propriété a pour rôle d'empêcher la mort cellulaire causée par la sortie de l'eau intracellulaire à l'extérieur du spermatozoïde lors de la dilution de la semence.

Plusieurs polysaccharides (fructose, glucose, raffinose, galactose, etc.) ont été utilisés comme agents protecteurs non-perméants pour la congélation des spermatozoïdes (revue par Watson [140]). Il est intéressant de noter que les protéines BSP ont la propriété de lier des polysaccharides tels que le mannose [163] et le fucose [66]. Grâce à leur pouvoir cryoprotecteur et leur capacité à lier les protéines BSP, ces polysaccharides seraient des composés de choix pouvant être inclus dans un diluant protecteur. Cependant, jusqu'à aujourd'hui, aucune étude n'a démontré que les protéines BSP ont la capacité de lier ces sucres de façon stable. De plus, l'ajout d'une quantité élevée de sucres dans le diluant pourrait aussi contribuer à un changement important dans l'osmolalité du diluant protecteur.

Il a aussi été démontré que les protéines BSP lient le groupement choline des phospholipides [62]. La choline est vendue sous forme de sel et l'ajout de ce sel dans le diluant protecteur augmenterait l'osmolalité du diluant. Cependant, l'ajout de lipides portant un groupement choline dans les diluants protecteurs ne change pas leur osmolalité. La lécithine (phosphatidyl-choline végétale) provenant des fèves de soya, des graines de tournesol ou de canola est donc une avenue intéressante afin de remplacer le jaune d'œuf. Ce type de diluant (par exemple, Biociphos®) est utilisé dans l'industrie de l'insémination artificielle bovine mais n'est pas aussi efficace que les diluants contenant du jaune d'œuf [134, 135]. Cette faible efficacité est probablement dûe à une quantité de lécithines dans le diluant protecteur insuffisante pour séquestrer toutes les protéines BSP présentes dans le PS des éjaculats. La

quantité de phospholipides portant un groupement choline à ajouter dans le diluant afin de protéger efficacement les spermatozoïdes est à déterminer.

L'utilisation de liposomes composés de lipides végétaux ou synthétiques serait une bonne alternative à l'utilisation du jaune d'œuf. La composition de ces liposomes devrait refléter la composition de la monocouche lipidique entourant les LDF i.e. 16% de cholestérol et 84% de phospholipides (72% de phosphatidyl-choline, 19% de phosphatidyl-éthanolamine et 9% de lysophosphatidyl-choline, de lysophosphatidyl-éthanolamine et de sphingomyéline). La présence de cholestérol dans les liposomes les rend plus stables que des liposomes composés seulement de phospholipides et permet de fabriquer des grosses vésicules de lipides. Ces grosses vésicules peuvent séquestrer rapidement une plus grande quantité de protéines BSP et ne changent pas l'osmolalité du diluant. L'utilisation de liposomes de phospholipides et de cholestérol d'origine animale afin de protéger les spermatozoïdes pendant l'entreposage et le refroidissement de la semence est bien documentée [129, 164]. Cependant, les diluants contenant ces liposomes ne sont pas aussi efficaces que le jaune d'œuf pour conserver la semence. La faible efficacité de ces liposomes semble résider dans la quantité insuffisante de lipides présents dans le diluant protecteur afin de séquestrer toutes les protéines BSP présentes dans le PS des éjaculats. La concentration de phospholipides portant un groupement choline était de seulement 5 mg/ml dans ces diluants alors que dans un diluant contenant 20% de jaune d'œuf, elle est de 8 mg/ml.

La lécithine et les liposomes de lipides végétaux ou synthétiques sont les sources de remplacement du jaune d'œuf les plus susceptibles de protéger efficacement les spermatozoïdes.

3.8. Mécanisme impliqué dans la protection des spermatozoïdes par le lait

Le mécanisme par lequel le lait utilisé dans les diluants protecteurs protège les spermatozoïdes semble être le même que celui démontré pour les LDF du jaune d'œuf. Des études ont démontré que ce sont les micelles de caséines présentes dans le lait qui sont responsables de la protection offerte par le lait aux spermatozoïdes pendant le processus de cryopréservation [138, 139]. En effet, l'utilisation d'une solution de micelles de caséines dans le diluant protecteur est aussi efficace que le lait pour la cryopréservation des spermatozoïdes bovins. Nos études préliminaires suggèrent que les micelles de caséines présentes dans le lait ont la capacité de lier les protéines BSP. Il semble donc que le lait protège lui aussi les spermatozoïdes en empêchant les protéines BSP présentes dans le PS de venir se lier à la membrane plasmique des spermatozoïdes et de stimuler un efflux de lipides. Cette hypothèse devra être vérifiée. L'effet protecteur du lait ne semble pas résider dans les lipides qu'il contient puisque le lait écrémé, qui ne contient pas de lipides, protège les spermatozoïdes contre les dommages causés par la cryopréservation aussi efficacement que le lait contenant 3,25% de lipides.

3.9. Spermadhésines

En voulant isoler les protéines homologues aux protéines BSP présentes dans le PS de bélier (article 2.4), nous avons isolé une protéine homologue à la spermadhésine AQN-1 présente dans le PS de porc. Les spermadhésines sont une famille importante de protéines du PS de mammifères. Elles sont les protéines majeures du PS de porc et de cheval et deux spermadhésines ont aussi été identifiées dans le PS de bœuf [165]. Les spermadhésines sont caractérisées par la présence, dans leur structure secondaire, d'un domaine CUB, nommé selon la première lettre du nom de chacune des trois premières protéines dans lesquelles un tel domaine a été identifié (C1r, Uegf and Bmp1) [166]. Les spermadhésines ont la capacité de lier la gélatine, l'héparine et les sucres et lient la membrane plasmique des spermatozoïdes au moment de l'éjaculation [167]. Les membres de cette famille de protéines semblent jouer un rôle dans la capacitation des spermatozoïdes et dans la liaison des spermatozoïdes à la zone pellucide. Nous avons pour la première fois isolé une protéine de la famille des spermadhésines à partir du PS de bélier. Cette protéine (15,5 kDa) est la principale protéine retrouvée dans le PS de bélier, représentant environ 45% des protéines totales du PS. Le PS de porc contient sept spermadhésines différentes et celle retrouvée en plus grande quantité est la spermadhésine AQN-1. AQN-1 semblent jouer un rôle dans la capacitation des spermatozoïdes porcins. Il est possible que la spermadhésine identifiée chez le bélier joue un rôle similaire à AQN-1. Cette hypothèse devra être vérifiée.

4. CONCLUSION ET PERSPECTIVES

Nos études ont démontré que le PS peut être néfaste pour les spermatozoïdes bovins dans un contexte d'entreposage de la semence à cause des protéines BSP qu'il contient. Ces protéines BSP stimulent un efflux des lipides membranaires qui est accompagné d'une diminution du pourcentage de spermatozoïdes motiles retrouvés dans la semence. Ces travaux nous ont permis de proposer un nouveau mécanisme pour expliquer l'effet protecteur du jaune d'œuf présent dans les diluants utilisés pour la cryopréservation de la semence. L'élément clé de ce mécanisme est la séquestration des facteurs néfastes pour les spermatozoïdes présents dans le PS par le jaune d'œuf contenu dans les diluants protecteurs. En effet, nous avons démontré pour la première fois que les LDF du jaune d'œuf lient spécifiquement les protéines BSP présentes dans le PS, que leur capacité à lier les protéines BSP est élevée et que cette liaison est stable pendant la cryopréservation. De plus, au moment de la dilution de la semence bovine avec un diluant contenant du jaune d'œuf, les LDF contenues dans le jaune d'œuf lient les protéines BSP présentes dans le PS, empêchant ces dernières de se lier à la membrane plasmique des spermatozoïdes et de stimuler un efflux lipidique (cholestérol et phospholipide) pendant l'entreposage de la semence. De plus, nous avons démontré que ce sont les LDF du jaune d'œuf qui permettent de maintenir la motilité spermatique pendant l'entreposage de la semence. Pendant l'entreposage de la semence, les LDF du jaune d'œuf causent aussi une augmentation de la quantité des lipides associés aux spermatozoïdes. Le rôle de ce gain de lipides dans la protection des spermatozoïdes devra être déterminé. Aussi, il serait intéressant de déterminer si

ce gain de lipides est dû à l'incorporation de lipides dans la membrane plasmique des spermatozoïdes ou à l'association de molécules de LDF avec les spermatozoïdes.

Nous avons démontré que les facteurs néfastes de PS bovin (protéines BSP) lors de l'entreposage de la semence sont aussi présents dans le PS de bison (protéines BiSV: BiSV-16kDa, BiSV-17kDa, BiSV18kDa et BiSV-28kDa) et de bélier (protéines RSP: RSP-15kDa, RSP-16kDa, RSP-22kDa et RSP-24kDa). Les protéines BSP semblent donc être présentes dans le PS de tous les mammifères étudiés. De plus, nous avons démontré que les protéines BiSV et RSP lient les LDF du jaune d'œuf. Il serait intéressant de vérifier si le mécanisme de protection par le jaune d'œuf est le même chez toutes les espèces de mammifères. On pourrait par exemple préparer des diluants dans lesquels la quantité de jaune d'œuf est adaptée à la quantité de protéines homologues aux protéines BSP retrouvées dans le PS de chaque espèce. La compréhension des mécanismes impliqués dans la protection des spermatozoïdes de mammifères permettra la mise au point de nouveaux diluants efficaces adaptés pour chaque espèce et de standardiser les méthodes de cryopréservation des spermatozoïdes de chaque espèce.

L'industrie de l'insémination artificielle bovine étant un secteur très développé au Québec, toutes les études visant à améliorer la qualité de la semence cryopréservée pourraient être intéressantes pour cette industrie. Nos travaux vont permettre de développer de nouvelles stratégies afin d'améliorer les méthodes de cryopréservation de la semence bovine. Afin d'améliorer la qualité des spermatozoïdes cryopréservés, il serait intéressant de récolter la semence bovine dans un tube préalablement rempli

de diluant protecteur. De cette manière, le temps de contact des spermatozoïdes avec une concentration élevée de PS serait réduit, évitant ainsi la fragilisation des membranes des spermatozoïdes par un trop grand efflux lipidique avant la congélation. De plus, des études pourraient être entreprises afin d'étudier l'effet de la dilution de la semence bovine avec le diluant protecteur selon le volume des éjaculats plutôt que selon leur concentration spermatique. De cette manière, on s'assurerait que tous les éjaculats soient dilués avec une quantité suffisante de diluant protecteur afin de séquestrer toutes les protéines BSP qu'ils contiennent.

L'avancement des connaissances au niveau des mécanismes impliqués dans la protection des spermatozoïdes pendant la cryopréservation va aussi permettre d'élaborer de nouveaux diluants protecteurs exempts de substances d'origine animale. Afin de remplacer le jaune d'œuf ou le lait, des études pourraient être effectuées dans le but de déterminer l'efficacité de nouveaux agents protecteurs d'origine non-animale capables de séquestrer efficacement les protéines BSP présentes dans le PS des éjaculats.

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