COMPONENTS IN EGG YOLK WHICH PROTECT BOVINE SPERMATOZOA DURING FREEZING

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Summary

Bull spermatozoa were pellet frozen on dry ice in a TES buffer containing combinations of egg yolk and glycerol to determine which component of an extender afforded cryoprotection to the spermatozoa. Neither buffer nor buffer plus glycerol protected sperm cell motility in the absence of egg yolk, while sperm cells frozen in egg yolk buffer in the absence of glycerol had 24% postthaw motility (P < .05). Egg yolk was found to be the main cryoprotective agent, but there was a synergistic effect between glycerol and egg yolk in providing the greatest postthaw survival (40%) of sperm cells (P < .05).

Purification of egg yolk to determine which fractions were affording protection was conducted using ultracentrifugation, Biogel filtration and Sephadex filtration. Purification procedures and agar gel electrophoresis indicated that a large lipoprotein complex could be isolated free of contaminating migrating proteins. The lipoprotein complex was the low density fraction (LDF) of egg yolk. In the absence of glycerol this complex protected motility of sperm cells during the freezing process.

Introduction

The discovery that egg yolk has a beneficial effect on fertility in semen extenders (Phillips, 1939; Phillips and Lardy, 1940) led to its widespread use in bull semen extenders. Early researchers reported that egg yolk aids the sperm cell in resisting cold shock (Bogart and Mayer, 1950; Lasley, Easley and Bogart, 1942; Lasley and Mayer, 1944). However, the discovery by Polge, Smith and Parkes (1949) that bull semen could be successfully frozen in extenders containing glycerol diverted researchers from the study of the protective qualities of egg yolk. Recent evidence indicated that glycerol was not essential for freezing spermatozoa under certain conditions (Berndtson and Foote, 1972; Gibson and Graham, 1969; Nagase, Graham and Niwa, 1964; Nagase and Niwa, 1964). Glycerol has also been shown to decrease fertility in chickens, turkeys and boars (Polge, 1951; Neville 1971; Brown and Graham, 1971; Polge, 1956; Crabo et al., 1971; Graham et al., 1971).

The object of this research was to establish the contribution that egg yolk makes as a cryoprotective substance and to purify and characterize the protective egg yolk fraction.

Materials and Methods

General. Extenders were prepared by adding various fractions of egg yolk to TesNak₂PO₄F buffer (Graham, Crabo and Brown, 1972). All extenders were centrifuged at 20,000 g for 10 min. and the precipitate discarded. One part of neat semen from Holstein bulls was added to 20 parts of the various extenders and cooled to 5°C in 1 hour. Four hours after the semen reached 5°C, it was pellet frozen on solid CO₂ in .1 ml aliquots. Semen was thawed on either a teflon coated slide warmer or aluminum weigh boats floating on a 37°C water bath. Progressive motility estimations were made by viewing samples on closed circuit television attached to a microscope (Graham, Schmehl and Maki-Laurila, 1970).

Analysis of variance and Tukey's w procedure (Steele and Torrie, 1960) were used to determine which means were significantly different at P < .05.
Contributions of Egg Yolk to the Cryoprotective Mechanism of the Extender. TesNak₂PO₄F was used in a 2 x 2 factorial design with egg yolk (0 or 20%) as one factor and glycerol (0 or 4%) as the other. The percentage of progressive motility and amount of GOT (glutamic oxaloacetic transaminase E.C. 2.6.1.1.) remaining in the spermatozoa after freezing and thawing were used as monitors of cryoprotection afforded by egg yolk.

Purification and Characterization of the Protective Egg Yolk Fraction. A preliminary investigation was conducted using dialysis to determine whether large or small molecules were offering protection. TesNak₂PO₄F containing 20% egg yolk was dialyzed against TesNak₂PO₄F buffer and water at 5°C. After the osmotic pressure of the water-dialyzed samples was 10 to 20 mOsm/l, samples were freeze-dried. TesNak₂PO₄F buffer or water was added back to the freeze-dried samples to bring the samples back to the original volume and osmotic pressure.

The macromolecules of egg yolk were separated as outlined in figure 1. Whole egg yolk was separated into three components: 1) granules, 2) crude low density fraction (CLDF), and 3) crude water soluble fraction (CWSF) using a Spinco Model L preparatory ultracentrifuge (Burley and Cook, 1961). All samples were dialyzed to remove the sodium chloride used during centrifugation, and the nondialyzable portion was freeze-dried. Six grams of the freeze-dried material were reconstituted in 100 ml buffer.

Crude LDF was separated into LDF₁ and LDF₂ in a second centrifugation procedure using a Spinco Model L preparatory ultracentrifuge (Martin, Augustyniak and Cook, 1964). Samples were freeze-dried and 3 g were reconstituted in 100 ml of buffer.

Egg yolk, CLDF and CWSF were further purified on a Biogel A-50m, 100-200 mesh (Biorad Laboratories, Richmond, California) column (5 x 80 cm) using Tris buffer, pH 8.9 (Kalab and Martin, 1968). Fifteen milligrams of sample/cm² surface area were added and eluted at 3 ml/cm² surface area/hour. Optical density of samples was continuously measured at 280 nm and collected in 10 ml volumes. The eluted samples were dialyzed against water, freeze-dried, and 2 g were reconstituted in 100 ml of buffer. The void volume of the column was determined by adding 2 mg salmon sperm DNA. The DNA was dissolved in 0.1 M NaCl containing 0.01 M sodium citrate.

Additional purification of CLDF and CWSF was conducted using Sephadex G-200 (Uppsala, Sweden) column size 2.5 x 45 centimeters. The gel was hydrated and washed with Tris buffer, pH 8.9. Sixteen milligrams of sample/cm² surface area were added and eluted at 2 ml/cm² surface area/hour. The column was standardized (Dostal, 1970). The optical density of the sample was continuously measured at 280 nm and collected in 3 ml volumes. The eluted samples were dialyzed against water, freeze-dried and then 2 g were reconstituted in 100 ml of buffer.

The purity of the various fractions obtained through preparatory ultracentrifugation, Biogel filtration or Sephadex filtration were monitored using agar gel electrophoresis (Wieme, 1959). The electrophoresis was conducted using barbital buffer, pH 8.4. Each sample was compared to an egg yolk control sample.

Results and Discussion

Various extender components had different effects on the postthaw motility of the sperm cells. There was a significant (P < .05) interaction between the glycerol levels used and the addition of egg yolk (table 1). Neither buffer nor buffer plus glycerol protected sperm cell motility in the absence of egg yolk, while sperm cells frozen in egg yolk buffer in the
TABLE 1. EFFECT OF EGG YOLK AND GLYCEROL ON POSTTHAW MOTILITY AND GOT RELEASE OF BULL SPERMATOZOA FROZEN IN TesNaK2PO4 BUFFER

<table>
<thead>
<tr>
<th>Percent glycerol</th>
<th>Percent egg yolk</th>
<th>Meana progressve post-thaw motility</th>
<th>Meanb IU GOT remaining in 109 spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1c</td>
<td>35c</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>3e</td>
<td>52e</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>24d</td>
<td>157d</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>40d</td>
<td>179d</td>
</tr>
</tbody>
</table>

a Each value is the mean of six ejaculates from different bulls.

b GOT remaining in the spermatozoa was calculated by subtracting the amount of GOT in the extracellular fluid after freezing and thawing from the total amount of GOT that could be released by 3X plunging the spermatozoa directly into liquid nitrogen (406 IU/109 spermatozoa).

c, d, e Column means with a common superscript are not significantly different (P > .05).

Absence of glycerol had substantial postthaw motility. Glycerol plus egg yolk resulted in a significantly higher (P < .05) percentage of motile sperm cells than the extender without glycerol.

Spermatozoa protected by egg yolk released significantly less (P < .05) GOT than spermatozoa in 0% egg yolk buffer upon freezing and thawing. Buffered spermatozoa which did not contain egg yolk or glycerol had only small amounts of GOT (34 IU/109 spermatozoa) remaining in the cells after the freeze-thaw cycle. Spermatozoa that went through the freeze-thaw cycle in complete extender maintained more GOT in the cells than spermatozoa which were frozen and thawed with egg yolk in the absence of glycerol (157 vs. 179 IU/109 spermatozoa), but the difference was not significant (P > .05).

The data in table 1 indicated that neither buffer alone nor buffer containing glycerol offered significant cryoprotection. The addition of egg yolk to buffer enhanced the survival of the sperm cells. These data were in agreement with Berndtson and Foote (1972), Gibson and Graham (1969), Nagase et al. (1964) and Nagase, Yamashita and Irie (1968), who have obtained similar postthaw sperm survival in the absence of glycerol. However, these researchers believed that the sugar component of their extender offered the protection, not the egg yolk. Analysis of data in this study indicated that egg yolk was the main cryoprotective agent, but there was a synergistic effect between glycerol and egg yolk in providing the greatest postthaw survival of sperm cells. Since egg yolk was found to be the main cryoprotective agent, all subsequent experiments to isolate the protective fraction in egg yolk were conducted in the absence of glycerol.

The postthaw motility of spermatozoa was not affected by dialyzing the extenders with water or TesNaK2PO4F to remove the low molecular weight material of egg yolk (table 2). The temporary denaturation of egg yolk by water dialysis did not affect cryoprotective action. It was concluded that the cryoprotective component in egg yolk can be found in the high molecular weight fraction and that the dialysate was not essential.

Components from the separation of egg yolk macromolecules by preparatory ultracentrifugation were compared with unaltered egg yolk to determine their ability to protect the spermatozoa during the freezing process (table 3). The crude low density fraction (CLDF) was equal to egg yolk in protecting the sperm cells, while the crude water soluble fraction (CWSF) offered significantly less (P < .05) protection. The granular fraction offered the least (P < .05) protection to the sperm cells. Agar gel electrophoresis of the CLDF and CWSF (figure 2) indicated that both fractions contained the same number of protein bands as egg yolk and that further purification was needed.

Purification of CLDF by further ultracentrifugation did affect postthaw sperm motility, but the differences were small (table 3). No significant (P > .05) difference was found between CLDF and LDF2 nor between LDF1 and LDF2, but the difference between CLDF and LDF1 was significant (P < .05). Agar gel electrophoresis (figure 1) indicated that LDF1 was free of any contaminating migrating protein bands, but LDF2 had slight contamination by a protein moving into area three.
### Table 3. Cryoprotective Properties of Egg Yolk Fractions Separated by Ultracentrifugation

<table>
<thead>
<tr>
<th>Egg yolk fraction</th>
<th>Centrifugation I&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Centrifugation II&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg yolk (control)</td>
<td>41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CLDF</td>
<td>41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDF&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>32&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDF&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CWSF</td>
<td>21&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Granules</td>
<td>3&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Each value is the mean of six ejaculates from different bulls.

<sup>b</sup>Each value is the mean of five ejaculates from different bulls.

<sup>c,d,e</sup>Column means with a common superscript were not significantly different (P > .05).

Extensive studies by Martin et al. (1964) and Saarl, Powrie and Fennema (1964) using the ultracentrifugation procedure to isolate LDF<sub>1</sub> and LDF<sub>2</sub> showed the composition of this complex group of lipoproteins to be 80 to 90% lipid. The molecular weight ranged from 0.5 × 10<sup>6</sup> to 34.0 × 10<sup>6</sup>, with LDF<sub>1</sub> having an average molecular weight of 10.3 × 10<sup>6</sup> and LDF<sub>2</sub> having an average molecular weight of 3.3 × 10<sup>6</sup>. The small differences in postthaw motility between LDF<sub>1</sub> and LDF<sub>2</sub> in the present study indicated that the average size of the lipoprotein did not affect the protective qualities of the material.

Further purification of CLDF and CWSF was conducted using gel chromatography to see if it was possible to further separate the macromolecules. Samples were separated using Biogel A-50m, 100-200 mesh and compared with egg yolk separation (figure 3). All samples had a peak eluting at void volume. The major peak of egg yolk was eluted at 1,120 ml with another small diffuse peak between 1,400 to 1,500 ml. The main fraction of the CWSF had a maximum concentration at 1,300 ml with other small descending shoulders. The major peak of CLDF was eluted just after void volume at 810 milliliters. There was a long descending shoulder of CLDF that did not elute completely from the column until 1,400 ml of buffer had been removed. The second peaks of egg yolk and CLDF were similar in appearance, although eluting at different volumes. Evidence by Soliman and van den Berg (1971) that this lipoprotein aggregated when isolated under similar conditions could explain the difference in the elution volume between the second peaks of egg yolk and CLDF.

No protective component was found in the material eluting at void volume of egg yolk or CWSF from Biogel A-50m chromatography (table 4). Sperm cells frozen in the material eluting at void volume of CLDF had an average postthaw motility of 20%, but the separation of the component was not sufficient to distinguish whether it offered protection or was con-

![Diagram](image-url)

**Figure 2.** A schematic representation of the purification obtained through preparatory ultracentrifugation, Biogel filtration and Sephadex filtration as monitored by agar gel electrophoresis using barbital buffer pH 8.4. Samples were identified as follows: centrifugation, EY = egg yolk supernatant, CLDF = crude low density fraction, LDF<sub>1</sub> = low density fraction, LDF<sub>2</sub> = low density fraction; Biogel A-50m filtration (peaks from Figure 3), EY<sub>1</sub> & EY<sub>2</sub> = 1st and 2nd peaks of egg yolk supernatant, WSF<sub>1</sub> & WSF<sub>2</sub> = 1st and 2nd peaks of the CWSF, LDF<sub>1</sub> & LDF<sub>2</sub> = 1st and 2nd peaks of the CLDF; Sephadex G-200 filtration (peaks from Figure 4), LDF<sub>1</sub>, WSF<sub>1</sub>, WSF<sub>2</sub> and WSF<sub>3</sub> = peaks to come off the Sephadex column from crude CLDF and CWSF.
Figure 3. Elution patterns of egg yolk, CLDF and CWSF from a Biogel A-50m 100-200 mesh (5.0 x 80.0 cm) column. Samples were eluted with Tris buffer pH 8.9.

taminated by the following peak. Material from peak 2 of egg yolk maintained the highest sperm motility and was significantly different (P < .05) from either of the peaks from CWSF but was not significantly different (P > .05) from the first and second peaks of CLDF.

Agar gel electrophoresis of the samples from Biogel A-50m chromatography (figure 2) illustrated that material eluting at void volume from all samples, and from the second peaks of CLDF and egg yolk samples did not migrate. The main peak of WSF had proteins migrating

TABLE 4. CRYOPROTECTIVE PROPERTIES OF EGG YOLK, CLDF AND CWSF FRACTIONS WHICH WERE PURIFIED ON A BIOGEL A-50m COLUMN 100-200 MESH (5.0 x 80.0 cm) ELUTED WITH TRIS BUFFER pH 8.9a

<table>
<thead>
<tr>
<th>Item</th>
<th>Peak</th>
<th>Elution volume (ml)</th>
<th>Meanb post-thaw motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg yolk</td>
<td>Peak 1</td>
<td>600—800</td>
<td>1e</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>Peak 2</td>
<td>900—1300</td>
<td>28c</td>
</tr>
<tr>
<td>WSF</td>
<td>Peak 1</td>
<td>600—800</td>
<td>1e</td>
</tr>
<tr>
<td>WSF</td>
<td>Peak 3</td>
<td>1200—1400</td>
<td>16d</td>
</tr>
<tr>
<td>LDF</td>
<td>Peak 1</td>
<td>600—800</td>
<td>20cd</td>
</tr>
<tr>
<td>LDF</td>
<td>Peak 2</td>
<td>900—1300</td>
<td>20cd</td>
</tr>
</tbody>
</table>

aElution patterns of the fractions analyzed are found in figure 3.
bEach value is the mean of six ejaculates from different bulls.
c, d, eMeans with a common superscript were not significantly different (P > .05).

in all four areas. It was concluded that LDF material was isolated; free of contaminating migrating proteins and still had cryoprotective action, but separation of WSF into various fractions was ineffective.

Further purification of CWSF with Sephadex G-200 filtration was compared to CLDF separation (figure 4). Most LDF eluted at void volume (61 ml) while only small amounts of the WSF eluted at void volume. Two other minor LDF peaks were observed while the majority of the WSF was found in the second and third peaks.

The cryoprotective properties of CWSF were lost by separation with Sephadex G-200 (table 5). The first peak of CLDF still afforded the sperm cells cryoprotection. Insufficient material was obtained for motility analysis of the second and third peaks of CLDF and the first peak of CWSF.

Agar gel electrophoresis of material from Sephadex filtration (figure 2) indicated that the separation of LDF was not as complete as Biogel filtration or ultracentrifugation. Since the first peaks of LDF and WSF came off at the same volume in Sephadex filtration, it was concluded that the CWSF was really contaminated with LDF and removal destroyed any cryoprotective action of the latter two peaks of WSF.

The fact that the large lipoprotein (LDF) is protecting the sperm cell in the absence of glycerol during the pellet freezing process opens a new area for consideration on cryoprotective mechanisms for sperm cells. The mechanism used in this study to protect the sperm cell must be related to freezing rate. It has been reported (Larson and Graham, 1973) that the
TABLE 5. CRYOPROTECTIVE PROPERTIES OF CLDF AND CWSF FRACTIONS WHICH WERE FURTHER PURIFIED ON A SEPHADEX G-200 (2.5 × 45cm) COLUMN ELUTED WITH TRIS BUFFER pH 8.9

<table>
<thead>
<tr>
<th>Item</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean b post thaw motility (%)</td>
<td>15 c</td>
<td>0 d</td>
<td>0 d</td>
</tr>
</tbody>
</table>

a Elution patterns of the fractions analyzed are found in figure 4.

b Each value is the mean from five ejaculates from different bulls.

c, d Means with a common superscript were not significantly different (P > .05).

center of a .1 ml pellet frozen on solid CO2 has a freezing rate of 60 to 90°C/minute. Survival is very low if egg yolk samples are frozen by the slower ampule method (1 to 5°C/min) in the absence of glycerol (unpublished observation). The post-freeze motility of spermatozoa appeared to decrease as the purity of the fraction increased. Whether the decrease in motility was due to denatured lipoprotein, aggregated lipoprotein, or the removal of an essential component for motility needs to be further investigated.

Literature Cited


