Coenzyme Q10 and soyphosphatidylcholine in EK extender on preservation of Rhode Island Red poultry semen

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ABSTRACT

The objective of this study was to evaluate the efficacy of EK extender alone or incorporation with CoenzymeQ10 (CoQ10) and/or soyphosphatidylcholine (SPC) in poultry semen and their effects on seminal traits during temporal storage at 4°C for different time intervals (12 h, 24 h, and 36 h). Heterospermic pooled semen samples diluted (1:4) with EK, EK + SPC, EK+ CoQ10 and EK + SPC + CoQ10 extenders separately, preserved and different spermiogram were assessed. Various seminal traits within the same extender differ significantly (p<0.05) among different groups and with different time intervals of storage. CoQ10 and SPC in the EK extender exhibited favorable synergistic effect on sperm quality and were able to protect the male gametes against cold-stress up to 36h at 4°C. In this study, we concluded that incorporation of SPC and CoQ10 together in EK extender possess novel potentiality to maintain seminal quality during liquid storage of poultry semen at 4°C and for their safe transportation and further use for Artificial Reproductive technologies (ARTs).

Keywords
CoQ10, SPC, EK Extender, Poultry Semen, Preservation

INTRODUCTION

The genetic diversity within Indian indigenous chicken breeds is decreasing due to their replacement by high-producing exotic commercial hybrids, as well as selection for heavy broad-breasted broiler breeder strains has led to a continuous decline in fertility using natural mating (Mc Daniel,1978). Hence, artificial insemination (AI) will have to be employed to protect the indigenous germ pool. As poultry semen is highly concentrated and is of low volume and spermatozoan motility and fertilizing ability of undiluted neat semen stored in vitro usually decreases within 1h of collection, therefore, to utilize AI successfully, evaluation of semen quality before and after storage as well as prior to insemination is very important (Reddy, 1995) including type of diluents used, storage techniques and temperature to avoid a reduction in sperm quality (Donoghue and Wishart, 2000; Dumpala et al., 2006). Moreover, assessment of semen quality characteristics of the poultry gives an excellent indicator of the reproductive potential and has been reported to be a major determinant of fertility and subsequent hatchability of eggs (Peters et al., 2004).

Several types of extenders have been formulated for temporal storage of poultry semen at 4°C and its suitable transportation; however, most of the researchers have reported maximum 24 h of preservation at 4°C. (Sexton, 1978, 1988). For the confirmation of long time in-vitro survival of poultry spermatozoa, use of low temperature along with buffered saline having glycolytic substrates and few intermediates of citric acid cycle are not sufficient. Lipids are considered to have a vital role in conserving structure and function of spermatozoa both in-vitro and in-vitro conditions (Parks and Lynch, 1992; Jones, 1998). Lipids appeared to be greatly involved in in-vitro semen storage by cooling without freezing; the membrane lipid moieties in a liquid-crystalline phase may affects both physical and biochemical properties of spermatozoa (Parks and Lynch, 1992). Endogenous phospholipids are metabolized during in-vitro storage.
of mammalian spermatozoa (Scoot and Dawson, 1968; Neill and Masters, 1972). Polyunsaturated fatty acids (PUFA) present in cell membrane may also be sensitive to lipid peroxidation (LPO) in-vitro. The susceptibility of Turkey and chicken spermatozoa to in-vitro LPO and positive effects of antioxidants on the success of semen preservation has been reported by many workers (Fujihara and Haworth, 1978; Blesbois et al., 1993; Wishart, 1984; Donoghue and Donoghue, 1997). However, the use of CoQ10 a potent antioxidant and sperm mitochondrial stabilizer along with SPC, the phospholipids and a good emulsifier and capable to maintain structural integrity of the cells have never been studied during hypothermic storage of poultry semen so far. Hence, the basic knowledge of using CoQ10 and SPC in the extender could be extremely useful for in vitro storage of poultry semen at 4°C.

**MATERIALS AND METHODS**

**Ethics:** Ethical consent was obtained from the Animal Ethics committee from the Faculty of Veterinary and Animal Sciences at the West Bengal University of Animal and Fishery Sciences before this study was conducted.

**Management and grouping of birds:** Twenty eight healthy RIR cocks of forty-one weeks of age were divided into four groups (Gr I, II, III and IV) having seven cocks in each group. Individual groups of cock were reared separately under deep liter system. All the birds were kept in uniform husbandry practices, water was supplied ad libitum and received 14 h of light/day throughout the experiment.

**Preparation of the extender:**

*Extender 1:* EK extender was prepared as developed by Lukaszewicz (2002).

*Extender 2:* EK extender with CoQ10: EK extender 100 mL +CoQ10 30 mg.

*Extender 3:* EK extender with SPC: EK extender 100 mL +SPC 10 mg.

*Extender 4:* EK extender with CoQ10 and SPC: EK extender 100 mL +CoQ10 30 mg +SPC 10 mg.

All the prepared media were passed through Millipore filter separately to free the microbial contamination. Osmomolality and pH were adjusted to 330mOsm and 7.1, respectively. Prepared extenders were kept in sterile amber coloured coded bottle stored at 4°C and used within a week.

**Semen collection, dilution and evaluation:** The cocks were adapted for semen collection within two weeks using abdominal sexual massage technique (Burrows and Quinn, 1937). Semen samples were collected in every five days interval in the afternoon (1 pm - 2 pm) for a period of five months. The birds responded to massage by partial aversion of cloaca, and semen samples were collected from the Ventral lip of the vent in sterile 15 mL graduated tubes in a thermo flask. Collected semen samples from each individual group was pooled as one sample and divided into two parts. Evaluations of the seminal traits were performed with one part of the heterospermic pooled neat semen samples collected from each group. Observation of spermatozoan progressive motility were assessed by standard subjective ranking method (WHO, 1997) using a phase-contrast ranking method (×400).

Motility was expressed as the % of motile sperm with moderate to rapid progressive movement (Cheng et al., 2002). Only samples showing highest motility (≥70%) were considered. Percentage of live spermatozoa was evaluated with 10 μL of fresh semen and 200 μL of Eosin-Nigrosin stain and smears were made with the semen-stain mixture on microscopic slides and examined under the microscope (×1000) (Lukaszewicz et al., 2008). Spermatozoan abnormalities were determined by 3% Rose Bengal stain prepared in 3.55% sodium citrate solution. Smears of semen samples were prepared on dry microscopic glass slides and allowed to air dry. Smears were placed on the staining rack, flooded with 3% Rose Bengal stain and allowed for 10 min. After staining, the stained Smears were washed by dipping into distilled water twice into two beakers for 3 sec each. Excess water was blotted carefully and the slides were washed and allowed to air dry. Gross observations of different types of sperm abnormalities in respect of head, neck, midpiece and tail were made under the microscope(x200; x400) (Tomar, 1976). Hypo-osmotic swelling test (HOST) was conducted and different types of sperm tail coiling were evaluated following the method of Ravell and Mordoe (1994). HOS solution was prepared with fructose (1.351 gm) and trisodium citrate (0.735 gm) in 100 mL of double distilled water. Osmomolality was adjusted to 150 mOsm. Each 100 μL semen sample was mixed with 1 mL of HOS solution into separate round bottom test tubes and incubated at 37°C for 60 min providing 5% CO2 in the air. After incubation, 20 mL of sample from each test tube was placed on clean glass slides and covered with cover slips. Swelling of different parts of sperm specially tail portion as positive response to HOS solution was observed under phase-contrast microscope (x200; x400). For each observation, 200 individual sperm cells from each sample in triplicate
were considered and their mean values were expressed as %.

The other part of the semen samples from individual groups was split again into 4 equal parts (total 16 samples). The semen samples and extenders were kept at 35±2°C prior to dilution. The dilution of each semen sample from the 4 groups were performed with prepared extenders separately at 1:4 ratio (0.25 mL semen: 1 mL of extender) in different 10 mL sterile coded glass tubes (Lake, 1960). Spermatozoan characters were evaluated again as above with 100 µL of extended semen samples immediately after dilution. Rest of the diluted samples stored at 4ºC in a refrigerator were used to determine the effects of different semen extenders on the male gametes at 12 h, 24 h and 36 h of storage respectively.

Statistical Analysis: Data were analyzed using SPSS package following standard procedures (Snedecor and Cochran, 1994) with Duncan's multiple range tests (Duncan, 1955).

RESULTS AND DISCUSSION

Seminal traits immediately after collection and just after dilution (0 h) are depicted in Table 1, where percentage of progressive motility, viability and HOS reactive spermatozoa differed significantly (p<0.05) among different groups, however, morphologically abnormal sperm remained non-significant among the groups when compared with the control.

Values (%) of sperm progressive motility (Table 2) viability (Table 3) and HOST reactive cells (Table 5) at 12 h, 24 h and 36 h exhibited significant differences (p<0.05) among different groups of extenders. The mean values (%) of morphological abnormalities (Figure 1) from different extender groups at 12 h, 24 h and 36 h had significant differences (p<0.05). The morphological abnormalities of spermatozoon were found highest in group I that contained EK extender only (Table 4).

Observations revealed that the Extender-4 which contained both the SPC and CoQ10 exerts maximum protective effect on spermatozoan motility, viability and HOS reactivity as well as able to reduce the number of spermatozoan abnormality significantly (p<0.05) at 24h and even up to 36 h of storage when compared with extender 1,2 and 3. Though the spermiogram were not optimum up to 36 h, however, extender-4 maintained its good protective effect.

Over the past 50 years, ARTs including preservation technologies have been developed for mammalian gametes and embryo which enables to run programmes to preserve genetic materials. Because of anatomical and physiological differences, preservation techniques of fowl sperm are less developed, specially as conditions vary between avian species and breeds within species and require advanced method of preservation. Additionally, semen has to be kept fertile in the female genital tract over several days or weeks after A.I requiring a higher vitality of stored/frozen-thawed semen as compared to mammalian spermatozoa. Hence, search for an optimal extender-composition for fowl semen is still in progress, because, differences in the extenders, additives and environmental conditions make it difficult to get variable estimations of the efficiency of various producers.

Figure 1. Microphotograph of RIR Cock sperm (normal A, abnormal B; Rose Bengal Stain, x 400).

Till date, in spite of using any extender, seminal characters diminished gradually with time during liquid storage of poultry semen, however, some important outcome from the present study have been achieved which could be explained by that the lipids are the main structural and functional components of the sperm and their compositions may undergo a series of modifications in relation to either physiologic events or from any stresses. Loss of phospholipids from sperm plasma membrane (SPM) occurs during cold shock (Parker and Graham, 1992). Chilling/freezing and thawing causes increase in reactive oxygen species (ROS) responsible for destruction of all cellular structure including membrane lipid and / or membrane bound PUFA resulting impairment of
cellular structure (Leinz, 2000). Chicken and turkey sperm contain high amount of polyunsaturated fatty acyl groups (Ravie and lake, 1985) and spontaneous peroxidation occurs in vitro storage in both species (Cecil and Bakst, 1993). There are considerable evidences that such fatty acids play an important role in sperm function since causes of impaired fertility have been associated with reduced amount of these polyunsaturates in sperm cells (Kelso et al., 1996). However, the high degree of polyunsaturation typical to sperm lipids renders gametes highly susceptible to LPO, with the consequent risk of damage to cellular structures (Niki et al., 1993). Evidences indicate that the fatty acid composition of SPM, specially PUFA determine their biophysical characteristics, such as fluidity and flexibility both of which are necessary to fulfill specific functions including motility and fertilizing capacity of the sperm cells. Moreover, the high levels of PUFA render avian spermatozoa predispose to LPO and it seems reasonable that antioxidant protection plays a very crucial role in the maintenance of SPM integrity and thereby fertilizing

Table 1. Evaluation of pooled fresh semen samples (control) of RIR cocks and just after dilution with different extenders.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Extenders</th>
<th>Control</th>
<th>Group-I (EK)</th>
<th>Group-II (EK+COQ10)</th>
<th>Group-III (EK+SPC)</th>
<th>Group-IV (EK+COQ10+SPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive motility (%)</td>
<td></td>
<td>82.2±0.41a</td>
<td>81.3±0.36ab</td>
<td>80.8±0.38b</td>
<td>80.7±0.36b</td>
<td>81.2±0.32ab</td>
</tr>
<tr>
<td>Viability (%)</td>
<td></td>
<td>90.8±0.62a</td>
<td>87.3±0.51b</td>
<td>86.1±0.65b</td>
<td>87.1±0.70b</td>
<td>88.2±0.74ab</td>
</tr>
<tr>
<td>Morphological abnormality (%)</td>
<td></td>
<td>6.5±0.30</td>
<td>7.0±0.25</td>
<td>6.9±0.23</td>
<td>6.8±0.24</td>
<td>6.9±0.23</td>
</tr>
<tr>
<td>HOST (%)</td>
<td></td>
<td>69.6±0.30a</td>
<td>64.3±0.53c</td>
<td>67.2±0.32b</td>
<td>65.2±0.57c</td>
<td>67.1±0.31b</td>
</tr>
</tbody>
</table>

Values expressed as Mean±SE
Mean values bearing different superscripts (a,b,c ) in a row differ significantly (p<0.05)

Table 2. Comparison of progressive motility (%) among different groups (extenders) in different time intervals at 4°C.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Extenders</th>
<th>Group-I (EK)</th>
<th>Group-II (EK+COQ10)</th>
<th>Group-III (EK+SPC)</th>
<th>Group-IV (EK+COQ10+SPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td></td>
<td>69.8±0.20ab</td>
<td>72.5±1.54a</td>
<td>63.9±0.50b</td>
<td>75.5±1.17a</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>51.5±0.76b</td>
<td>55.9±1.95ab</td>
<td>51.4±0.80b</td>
<td>60.5±1.74a</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>9.5±0.89c</td>
<td>16.1±1.24b</td>
<td>9.2±0.29c</td>
<td>21.5±1.30a</td>
</tr>
</tbody>
</table>

Values expressed as Mean±SE
Mean values bearing different superscripts (a,b,c ) in a row differ significantly (p<0.05)

Table 3. Comparison of spermatozoan viability (%) among different groups (extenders) in different time intervals at 4°C.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Extenders</th>
<th>Group-I (EK)</th>
<th>Group-II (EK+COQ10)</th>
<th>Group-III (EK+SPC)</th>
<th>Group-IV (EK+COQ10+SPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td></td>
<td>71.1±0.58c</td>
<td>76.1±0.54b</td>
<td>70.8±0.97c</td>
<td>78.1±0.69a</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>52.2±0.89b</td>
<td>58.5±0.71a</td>
<td>55.8±1.40ab</td>
<td>59.8±0.96a</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>20.2±0.90b</td>
<td>25.8±2.15a</td>
<td>25.2±0.95a</td>
<td>24.5±1.38a</td>
</tr>
</tbody>
</table>

Values expressed as Mean±SE
Mean values bearing different superscripts (a,b,c ) in a row differ significantly (p<0.05)

Table 4. Comparison of morphological abnormalities (%) among different groups (extenders) in different time intervals at 4°C.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Extenders</th>
<th>Group-I (EK)</th>
<th>Group-II (EK+COQ10)</th>
<th>Group-III (EK+SPC)</th>
<th>Group-IV (EK+COQ10+SPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td></td>
<td>9.2±0.24a</td>
<td>8.4±0.16b</td>
<td>8.9±0.27b</td>
<td>8.5±0.16b</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>29.3±1.17a</td>
<td>16.4±0.63c</td>
<td>19.3±0.51b</td>
<td>16.7±0.73c</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>44.9±0.99a</td>
<td>27.9±0.79bc</td>
<td>29.5±1.17b</td>
<td>26.5±0.76c</td>
</tr>
</tbody>
</table>

Values expressed as Mean±SE
Mean values bearing different superscripts (a,b,c ) in a row differ significantly (p<0.05)
ability (Ladha, 1998). Hence, the Viability, storage efficiency and fertilizing ability of spermatozoa are highly dependent on the expression of an effective antioxidant capacity to these cells and in the surrounding seminal plasma. Exogenous lipids from the diluents have been shown strongly associated with the SPM and can prevent membrane damage during cold storage/cryopreservation and able to preserve successfully the spermatozoan motility, viability and fertility (Ricker et al., 2005).

The SPC contains three types of phospholipids namely phosphatidylcholine, Phosphatidylethanolamine and phosphatidylinositol, of which phosphatidylcholine plays a rate limiting role in the activation of numerous enzymes located in the membrane, for example, super oxide dismutase (SOD) and glutathione content (GSH). The SOD or GSH are important antioxidant in protecting cell membranes from damage elicted by reactive oxygen species (ROS). The ROS damages mitochondrial DNA causing reduced function of mitochondria. Lecithin may protect mitochondrial DNA ensuring the normal activity of mitochondria (Ulkowski et al., 2005). Hence, it is speculated that SPC is able to maintain the cellular structural integrity by protecting fatty acids from damages caused by ROS and lowering LPO to the cell membrane and stabilize SPM against adverse effects of cooling, freezing and thawing (Thun et al., 2002).

CoQ10, the ubiquinone, is a highly lipophilic component and is found in highest amount in the mitochondria and a powerful antioxidant that protects the body from free radicals (Weber et al., 1994). CoQ10 plays an important role in scavenging reactive oxygen intermediates and other free radicals and able to protect spermatoza from membrane damage by inhibiting LPO (Lewin and Lavon, 1997; Duttan et al., 2000).

In sperm cells, the majority of CoQ10, an energy promoting agent could be concentrated in the mitochondria of the mid-piece; as a result, the energy for movement and all other energy dependent processes in the sperm cell depended on the availability of CoQ10. Lewin and Lavon (1997) reported an in-vitro study on 22 semen samples of asthenozoospermic men incubated with 50 μm of CoQ10 which significantly increased the sperm motility.

CoQ10 is the only lipid stable electron carrier in the mitochondrial electron transport system. The supplied energy is available in the form of ATP that was synthesized either by glycolysis in the cytoplasm (Ford and Rees, 1990), or through oxidative phosphorylation (OXPHOS) in the mitochondria (Mahadevan et al., 1997). ATP generation by OXPHOS in the inner mitochondrial membrane is transferred to the microtubules to derive motility. Hence, reduced motility may be associated with mitochondrial damage. Stress factors capable of uncoupling oxidation and phosphorylation in mitochondria, can be considered as possible effectors stimulating electron leakage and superoxide radical formation responsible for loss of spermatozoal membrane integrity, motility, livability and fertility. As CoQ10 is an integral redox and proton translocating components of the mitochondrial respiratory chain, plays a key role in energy metabolism and has potent antioxidant properties to protect cellular membrane integrity (Ernster and Dallner, 1995). Moreover, as CoQ10 is a lipophilic molecule, it is reasonably hypothesized that CoQ10 can diffuse the phospholipids bi-layer of cellular membrane and thus protect the SPM.

Importantly, CoQ10 helps to preserve/recycle Vitamin E one of the major antioxidant of the cell membrane (Thomas et al., 1997) which also naturally present in, chicken sperm (Blesbois et al., 1993). Both CoQ10 and Vitamin C have been found capable to oxidize Vitamin E into a strong antioxidant (Beyer, 1994).

Developing a defense system against lipid peroxide damage is of practical importance in improving the extended liquid storage of RIR semen. Present study demonstrated improved survivability, motility, membrane integrity, concomitantly suitable reduction

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**Table 5. Comparison of HOST positive sperm (%) among different groups (extenders) in different time intervals at 4°C**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Extenders</th>
<th>Group-I (EK)</th>
<th>Group-II (EK+COQ10)</th>
<th>Group-III (EK+SPC)</th>
<th>Group-IV (EK+COQ10+SPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td></td>
<td>52.2±0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.7±1.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.4±0.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60.2±0.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>30.5±1.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.5±1.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.9±0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.4±1.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>10.9±0.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.5±0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.4±0.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.5±0.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values expressed as Mean±SE.

Mean values bearing different superscripts (a,b,c) in a row differ significantly (p<0.05).

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in the percentage of abnormal sperm after cold storage of RIR semen with CoQ10 and SPC where both the component act as biological stabilizer of SPM and were able to scavenge ROS and LPO successfully from the SPM during hypothermic storage of semen at 4°C and could be useful in the media across species.

**CONCLUSION**

The results of the present study provide a new approach that outlines a basic idea in the development for preservation of poultry semen at refrigerated temperature with modified EK extender containing CoQ10 and SPC for the first time as initial step. Based on the above findings, it is hypothesized that low temperature (4°C) would be better for long term storage of RIR poultry spermatozoa for more than 24 h when both CoQ10 and SPC were added together in EK extender. However, as the percentage of motility, viability, HOST positivity of spermatozoa was found declining with the extended period of holding time in the present experiment, it is now suggested that further study with different concentrations of CoQ10 and SPC may be conducted in order to get the best results.

**REFERENCES**


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