Liposomes of phospholipids, a promising approach for stallion sperm freezing

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Why freezing semen?

Cryopreservation of stallion semen is a very useful biotechnology for:

1° Patrimonial conservation of biological resources
2° Large diffusion of genetics within and between countries using artificial insemination

A lot of advantages

- Transport of semen is easier
- Storage can be unlimited
- Choice of stallion is wider for breeders
  ...
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Why freezing semen?

BUT

A few limits as:

- Fertility rate is lower than fresh semen because of sperm injury during freeze-thaw
- Freezing extenders:
  - to be optimized in their composition
  - composed of animal products
Impact of cryopreservation on sperm cells

Decrease of temperature:
- 37 °C - 4 °C: "Cold Shock"
- Then 4 °C to -196 °C

Osmotic stress solution effect ice crystals
Mazur, 1963; Amann et Pickett, 1987

Cellular damages can be reversible or not
Watson, 2000

Elodie Pillet, 2009

Key factor

Freezing extender
Impact of cryopreservation on sperm cells

Cellular damages (especially membranes)

Normal membrane

After decrease of temperature

Amann et Pickett, 1987
Impact of cryopreservation on sperm cells

To limit membrane damages induced by low temperatures (-196°C)

- Increase of membrane permeability
- Decrease of the fertility potential after artificial insemination

Very protective freezing extenders are needed
Our objective was to develop a new freezing extender:

- able to improve fertility rates after AI with frozen sperm
- easy to use
- able to avoid sanitary risks (without animal products)

3 different steps were conducted (in vitro and in vivo studies):

1. remove from the composition of the extender
2. replace whole (EY) by egg yolk plasma
3. identify the protective fraction in EY plasma: phospholipids
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Development of a new freezing extender in the equine species (Elodie PILLET PhD, 2006-2009)

Step 1: successful replacement of by INRA96* extender

*INRA96 contains only the purified fraction of native milk caseins

sterilized plasma + glycerol \rightarrow excellent freezing extender

Pillet et al., DST, 88 (2), 2008
Magistrini et al., 2007, Patent FR-07 09145

Step 2: successful replacement of by egg yolk plasma

Egg yolk (EY)

sterolized plasma + glycerol \rightarrow INRA Freeze extender ready to use

Pillet et al., Theriogenology, 75 (1), 2011
Development of a new freezing extender in the equine species (Elodie PILLET PhD, 2006-2009)

Step 3: phospholipids the protective fraction in EY plasma?

PC is the most representative phospholipid (PL) in egg yolk

PC : phosphatidylcholine
PE : phosphatidylethanolamine
SPH : sphingosine
PS : phosphatidylserine
PI : phosphatidylinositol
Phospholipids: the protective fraction in EY plasma?

- Which EY phospholipids: PC, PE, PS%?
  \[\text{Commercial EY phospholipids: PL E80 (Lipoïd)}\]
  - 83.6% PC
  - 9.1% PE
  - 2% SPH
  - 1.8 LPC
  - 0.5% LPE

- Which arrangement of phospholipids?
  \[\text{Liposomes were chosen as the "vehicle" to transport PL up to spermatozoa}\]

Double layer of PL
Phospholipids: the protective fraction in EY plasma?

1°/ Comparison of INRA96 + EY + G vs. INRA96 + PLE80 liposomes + G (2009)

NFS

In vitro parameters

Liposomes of egg yolk PL (E80) can replace egg yolk in the freezing extender

Pillet et al, Theriogenology, in press
Motility parameters analyzed by CASA (computer assisted analysis)

Membrane integrity evaluated by a range of osmotic pressures (330mOsm to 10mOsm)

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**Phospholipids : the protective fraction in EY plasma ?**

2% Comparison of EY plasma (INRA Freeze extender) vs. INRA96 +PLE80 liposomes + G (2010)

- **In vitro parameters**

  - **Fertility/cycle (%)**
    - INRA Freeze: 58% (15/26)
    - Liposomes: 54% (14/26)
    - NS

  - **Motility parameters**
    - VAP (µ/sec)
    - Rapid %
    - Prog%

- **In vitro parameters**

  - Liposomes of egg yolk PL (E80) can replace egg yolk plasma in the freezing extender

- **Fertility/cycle (%)**
  - INRA Freeze: 58% (15/26)
  - Liposomes: 54% (14/26)
  - NS

  - n = 52 cycles
  - 2 stallions

- **Motility parameters**
  - INRA Freeze: 58% (15/26)
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  - NS

  - VAP (µ/sec)
  - Rapid %
  - Prog%

**Membrane integrity evaluated by a range of osmotic pressures (330mOsm to 10mOsm)**

- Fluorescence intensity (rfu)
  - INRA Freeze
  - Liposomes

- **INRA Freeze**
  - 58% (15/26)

- **Liposomes**
  - 54% (14/26)

- **p < 0.05**

- **NS**

- n = 52 cycles

- 2 stallions

- **In vitro parameters**

- **Fertility/cycle (%)**
  - INRA Freeze: 58% (15/26)
  - Liposomes: 54% (14/26)
  - NS
In Summary

Our results demonstrate that liposomes of egg yolk phospholipids (commercial PL E80) can replace egg yolk or egg yolk plasma in stallion sperm freezing extender.

More liposomes are a very promising approach since it is possible:
- to modulate - the composition in phospholipids
- the diameter
- to sterilize them

These conditions are essential to optimize the freezing extender.
Thanks for attention!

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G. Duchamp et al.

Jean-Marie, Yvan, Thierry, Philippe etc.....

...and V. Beaumal & M. Anton (INRA, Nantes)