A new promising approach for donkey (Equus asinus) sperm freezing

CRB-Anim project :
Network of Biological Resources Centers for domestic animals

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INTRODUCTION

Traditional use of asses:

- Animal tract for agriculture before mechanization,
- Mule production.

↳ Since 30 years ass population decreases

In France, we have 7 breeds:

- Âne Bourbonnais (male headcount= 8);
- Âne du Cotentin (male headcount = 48);
- Âne Grand Noir du Berry (male headcount= 23);
- Âne Normand (male headcount = 26);
- Âne de Provence (male headcount = 30);
- Âne des Pyrénées (male headcount = 36)
- Baudet du Poitou (male headcount = 85).

} Male headcount in reproduction activity is very low!

↳ In order to maintain genetic diversity in ass breeds and to create a genetic bank of donkey frozen sperm in each breed.

↳CRB-Anim supports research project to find an effective freezing method

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State of the art in donkey sperm freezing

Short review of bibliography

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State of the art in donkey sperm freezing

In bibliography, very few papers (± 15) on donkey sperm freezing (from 1992 to 2013)

10 freezing methods were tested…
They differ in several points:

- Based-extender (composition in sugar and/or salts),
- With or without egg yolk as non-permeant cryoprotectant,
- Different permeant cryoprotectants (glycerol, ethylene glycol, DMSO, etc…),
- Rate of temperature decrease (from 4°C to -196°C),
- Final concentration of sperm cells (from 60.10^6/mL to $400.\ 10^6$/mL) in straws

But the authors obtained variable *in vitro* results after freezing and thawing process :

- Motility parameters :
  - 18% < total motility< 70%
  - 37µm/s < mean velocity < 110 µm/s
  - 22% < progressive sperm < 57%
- Viability (Propidium Iodide staining)
  - 27% to 65% viable spermatozoa

State of the art in donkey sperm freezing

But there is no efficient method to ensure adequate fertility…

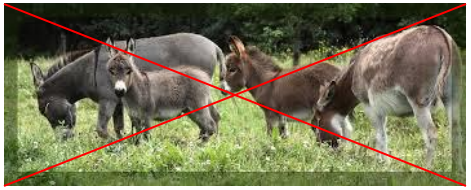
Fertility trials after AI with frozen sperm were conducted in only few papers (less than 10).

2 different fertility tests were done:

- **Cross breeding → donkey x mare**
- **Pure breed → donkey x jenny**

- 36% < donkey x mare< 60%;

- 0% < donkey x jenny< 13%



Cryoprotectants are toxics for jenny's tract? For spermatozoa?
Artificial Insemination method is not adapted to jenny?

Only one paper (Rota *et al., 2012):* results from 20% to 60% (pure breed)

- They used INRA96® as based-extender
- Their freezing method is closed to ours

Previously in our lab :

Vidament *et al.*, 2006 and 2009.

Glycerol was suspected as toxic ➢ Vidament *et al.* tested DMF (di-methyl formamide) instead of glycerol as permeant cryoprotectant

3 experiments:

1 – *In vitro* study : Spermatozoa motility was assessed by their response to a range of DMF concentration (from 0% to 6%)

⇒ Spermatozoa motility was higher with DMF 1% and 2%

2 – *In vitro* study : Donkey sperm and stallion sperm were compared before and after freezing and thawing process

⇒ Similar motility BUT donkey spermatozoa were more damaged than stallion spermatozoa (membrane integrity donkey sperm 20% less than stallion sperm)

3 – *In vivo* study: Artificial insemination of frozen semen with or without DMF compared to glycerol

⇒ No difference in fertility rate per cycle (# 10%) between DMF 0%, DMF1%, DMF 2% and glycerol 2%

↳ Toxicity or not ?
Glycerol, DMF? egg yolk?
Interactions components of extenders / sperm cells, genital tract?

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First data of CRB Anim project on donkey sperm freezing

In vitro study : which extender(s) to limit interactions between components and sperm cells

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Materials and methods

It has been previously demonstrated that:

in stallion freezing extender :

INRA96® can be a good based-extender (Pillet et al., 2007; Fayrer-Hosken *et al.* 2008),
EY can be replaced by EY plasma in the extender (Pillet et al. 2009),

in donkey freezing extender :

INRA96® can be a good based-extender for freezing extenders (Rota *et al.* 2012)

- Two donkeys Grand Noir du Berry were used (Kanji & Nirus).

- 3 freezing extenders were compared :
  INRA Freeze® (INF) control (INRA96® + EY plasma + glycerol),
  INRA96® + 2,5% of glycerol (IN2,5)
  INRA96® + 4% of glycerol (IN4).

- 4 ejaculates/donkey were frozen and analysed

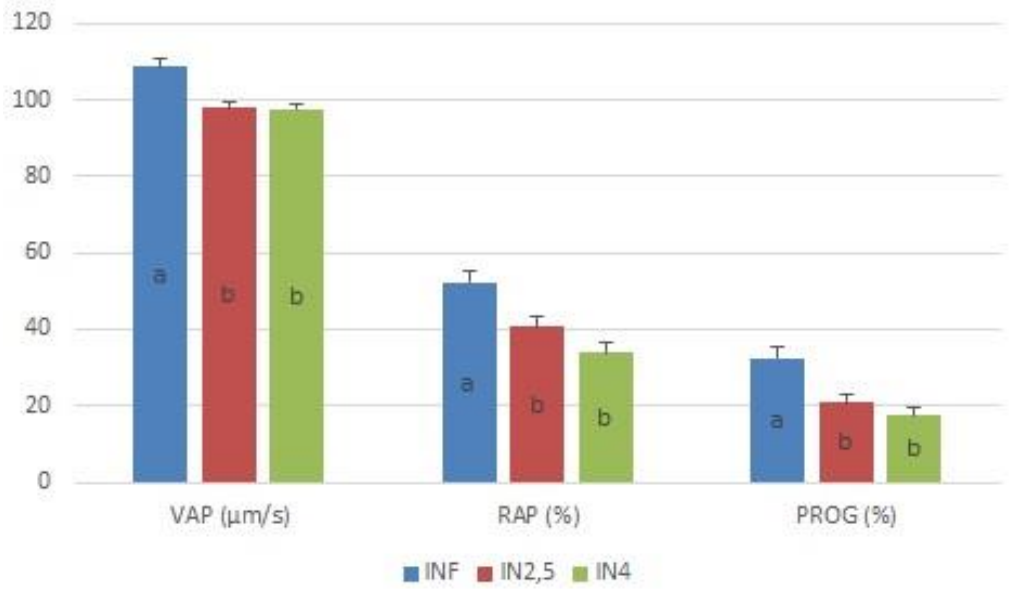
Results

Motility parameters analysis

➢ Motility was assessed by Computer Assisted Sperm Analysis (CASA) .

The parameters analysed were:

- VAP (µm/sec) = average path velocity
- PROG % = progressive cells (VAP>40µm/sec; Straightness > 80%)
- RAP% = rapid cells (VAP > 40µm/sec)



N = 8 (2 donkeys X 4 ejaculates)

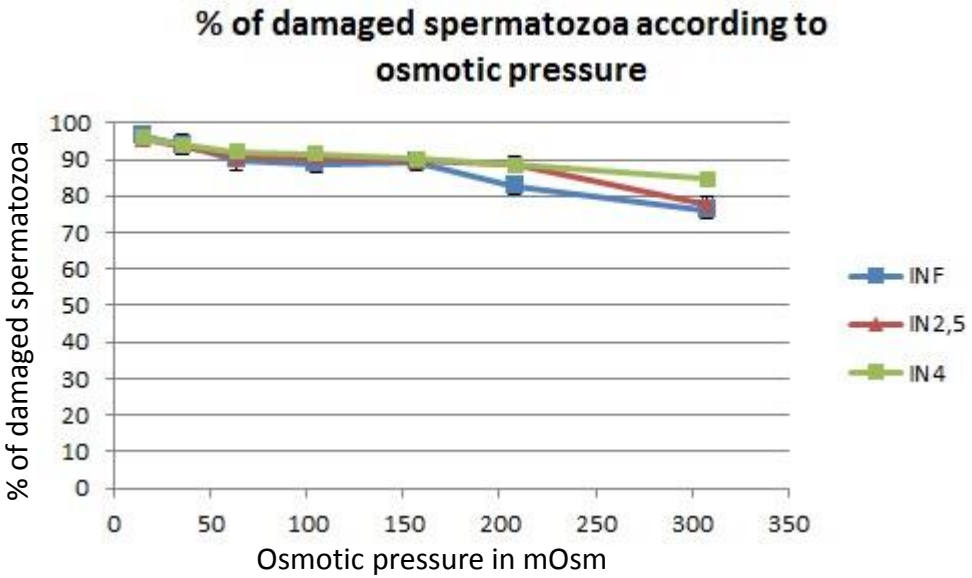
INRA Freeze® > INRA96 + 2,5% and INRA96 + 4% ($p<0.05$)

INRA96 + 2,5% ~ INRA96 + 4% ($p>0.05$)

Results

Membrane integrity analysis

➢ Membrane integrity of spermatozoa was assessed by their response to a range of hypotonic steps (from 303mOsm to 12 mOsm). Propidium Iodide was used to evaluate membrane integrity by Flow Cytometry.



No difference between extenders
Very high percentage of damaged spermatozoa

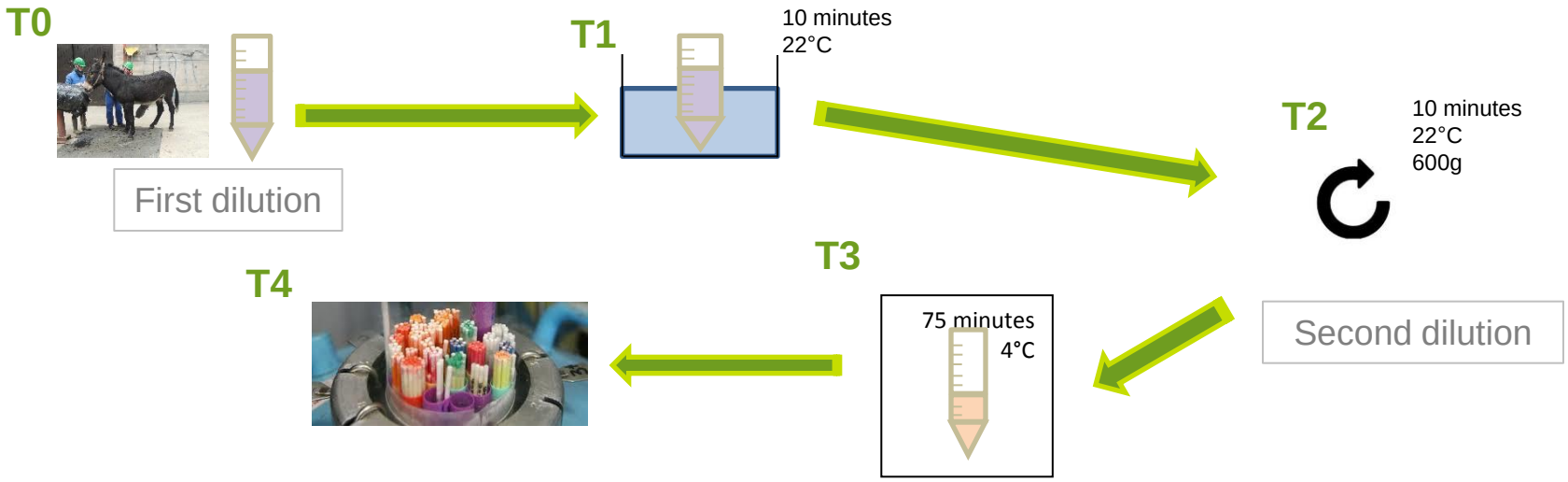
Same results as Vidament *et al.* (2006 and 2009)

N = 8 (2 donkeys X 4 ejaculates)

No relation between motility and membrane integrity in the donkey while there is high correlation in the stallion

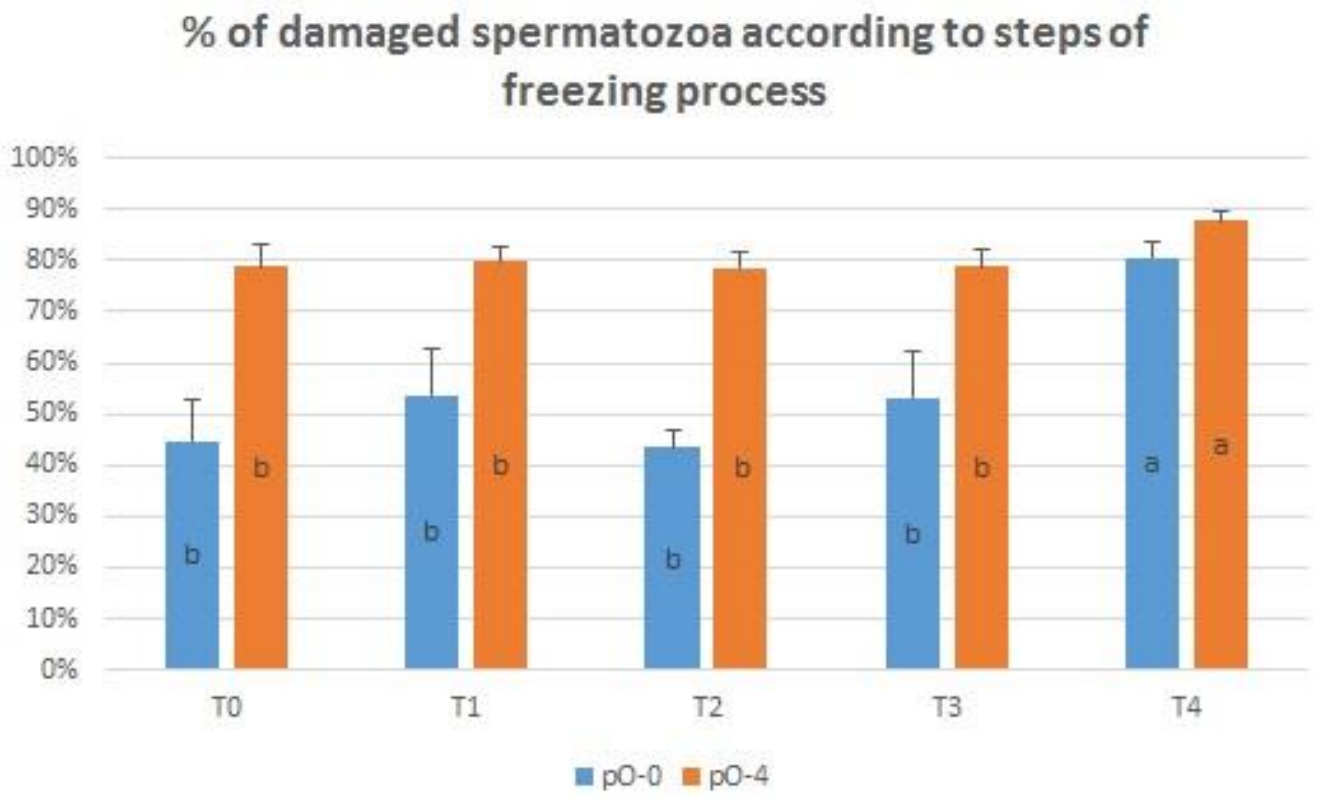
Motility and membrane integrity of donkey frozen sperm: A paradox…

Assessment of membrane integrity at each step of freezing process:



Membrane integrity was evaluated by fluorescence microscopy at 2 different osmotic pressures (303mOsm and 63mOsm) using 2 fluorescent probes : SyBR-14 and Propidium Iodide.

Plasma membrane damages occur only at the freezing and/or thawing step!



↳ significant effect of freezing and thawing step (T4).

To summarize…

➢ Motility parameters are significantly higher in INRA Freeze and no difference were observed between INRA + 2,5% and INRA + 4% of glycerol.

BUT…

➢A very high percentage of damaged sperm cells was observed, whatever the extender (around 80%).

→ Is the freezing and thawing process responsible of this high percent of damaged sperm cells?

↳The percentage of damaged sperm cells increases significantly only after the last step of freezing process (freezing and thawing).

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Perspectives

Perspectives

Keep in mind the need to be free of animal products in freezing extenders…

Our objectives for future:

- Understand high percentage of damaged spermatozoa after freezing and thawing process → we will analyse other parameters (mitochondrial activity and esterase activity by Flow Cytometry, etc.…)

- Assess fertility of donkey sperm frozen in INRA Freeze®, INRA96® + 2,5% of glycerol and INRA96® + 4% of glycerol → awaiting the arrival of jennies

- Assessment of liposomes, composed of egg yolk phospholipids, in freezing extenders for donkey sperm (stallions: Pillet *et al.* 2012). A promising approach to develop a donkey sperm freezing extender free of animal products.

Thanks to EAAP horse commission!

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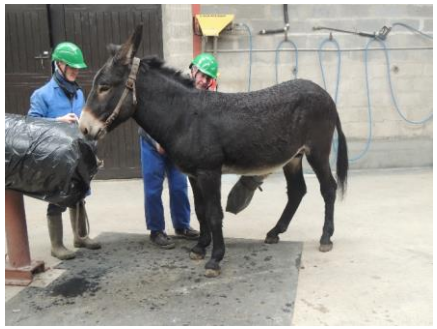

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Thanks to INRA for management !

Thanks for your attention!