EQUINE SEMEN FREEZING

State of the art and future developments

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1. Introduction

- Equine semen freezing:
  - Doses available worldwide
  - Any time available doses
  - Sanitary safe semen
- 20% of stallions with unfreezeable semen
  - Economic and genetic losses for owner and clients
- Aim of presentation:
  - Review of spermatozoa physiology with implications in semen freezing
  - Review of freezing process and future improvements

2. From spermatogenesis to ejaculation

- In the testicle: 57d to form a spermatozoon:
  - Proliferation (mitosis ➔ meiosis)
  - Differentiation (round cell ➔ differentiated cell)
- In epididymis: 12d to mature a spermatozoon:
  - From head to tail:
    - Motility & Fertility acquisition
    - Cytoplasmic droplet elimination
- Seminal plasma: prostate + vesicular + bulbo urethral grans
  - Volume of ejaculate: sexual arousal effect on volume & concentration
  - Total sperm number per ejaculate 8-10x10⁹ spz
3. Spermatozoon

Targets for ROS

Membrane proteins
Seminal proteins
Capacitation

Tail

Targets for freezing lesions

Nucleus: DNA
Acrosome
Mitochondria
4. Freezing procedures

- Extender 1
- Extender 2: with cryoprotector

Gel

| Smear | Dilution 1 | Concentration | Dilution 2 | Cooling 4°C straws | Freezing -140°C -196°C |

- Centrifugation
- Cooling 4°C
- Freezing straws
4. Freezing procedures

• New process: Cushioned centrifugation
  – g force and duration increased
    • No spermatozoa lesions
  – Recovery rate increased:
    • Conventional protocol 600g 10 min: RR=60-70%
    • Cushioned protocol 1000g 20min: RR=85-95%

• New process: Spermatozoa selection
  – Gradient density centrifugation:
    • Nidacon\textsuperscript{TM} Equipure\textsuperscript{®}
  – Morphology & specific gravity selection
    • Isopicnotic point
  – Selection of motile and normal spermatozoa but also selection of non fragmented DNA spermatozoa
4. Freezing procedures

Add 3ml density gradient medium (Nidacon)
Without drops on the wall!

Supernatant: - Non sperm cells
- Abnormal spermatozoas

Centrifugation: 30min 200g

1ml of semen 1ml pipette

When first drop is done, push it slowly on the top of Equipure Medium
Then, retrieve slowly the pipette, while continuing to add semen

Pellet

5. Quality control

• Requirements for frozen semen:
  – French National studs: 140x10^6 progressive spz
  – World Breeding Federation: 250x10^6 progressive spz
  – >35% progressive spz

• Concentration: according producer definition
  – For AI doses with 8 straws of 0.5ml:
    • 0.35 x 0.5 x 8 x 100 = 140
  – For AI doses with 4 straws of 0.5ml:
    • 0.35 x 0.5 x 4 x 200 = 140
5. Quality control

1. Motility

### Definition

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<tr>
<td>VCL</td>
<td>Velocity Curvilinear Path</td>
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<td>VSL</td>
<td>Velocity Straight Line Path</td>
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<td>VAP</td>
<td>Velocity Average Path</td>
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5. Quality control

1. Motility

5. Quality control

2. Stainings for microscopic exam
5. Quality control

3. New quality controls: flow cytometry

- Membrane integrity
- Tail
- Mitochondrial potential
- Acrosome integrity
- Membran proteins
- Seminal proteins
- Capacitation
- Nucleus: DNA
- DNA fragmentation
- Acrosome
6. Limits, results and future

1. Freezability prognosis
   - In the human:
     • Membrane fluidity
     • GSH concentration & GPX transcription
     • Membrane integrity & symmetry: Annexin V
   - In the equine:
     • Apoptotic factors (by flow cytometry)

2. Extender improvement
   - Cryoprotective agents
     • Reducing glycerol concentration (3.5%)
     • Lipids and Amides cryoprotective effects to replace glycerol (Botucryo®)
   - Physicochemicals and nutritive characteristics
     • Cholesterol
     • Isolation of LDL (INRA Freeze®)
     • Animal protein free media
6. Limits, results and future

3. Freezing process improvement
   – Cooling?
     • After centrifugation
     • Time can be increased
   – Freezing?
     • Curves?
     • Vitrification?

6. Limits, results and future

4. Reactive oxygen species and semen freezing
   – Effects on motility, membrane integrity, DNA fragmentation but necessary for some functions
   – Origin:
     • Intrinsic pathway
     • Extrinsic pathway
       – MPO?
   – Anti-oxidant therapy?
     • Broad spectrum anti-oxidants (vit E & C): +/-
     • Specific MPO inhibitors: ++
6. Conclusions

• Spermatozoa: motility, but also: acrosome, DNA, mitochondria,…
• Equine frozen semen: harmonization of thresholds
• Future:
  – Selection of good freezers on fresh semen basis
    • Treatment of bad freezer
    • Better media to freeze
    • New processes?

Questions?