Alternative methods of semen preservation: A pig model for endangered species

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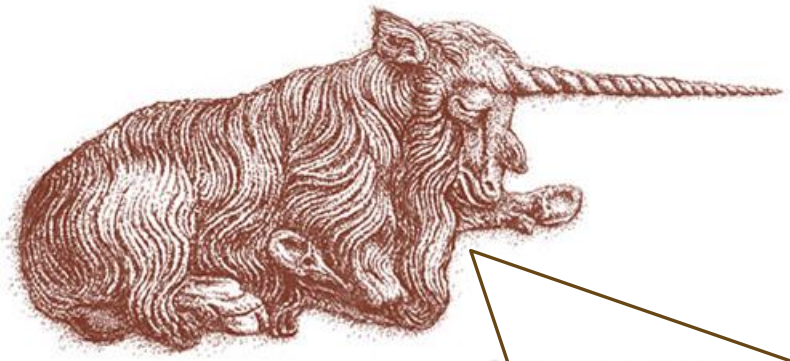
Contents

- Motivation
- Goal
- Materials and Methods
- Results
- Conclusions

Motivation

A high valuable animal suddenly died and had no preserved semen !



``Please, help me doctor!´´

``How can I get a descendant from my dead animal ?´´

Motivation

There are less than 250 wild Riverine rabbits left in South Africa

Bunolagus monticularis; *Red list (IUCN): critically endangered species*

http://www.edgeofexistence.org/mammals/species_info.php?id=3

Motivation

Tuaklom † 25.06.2010

Motivation

Some methods for desiccated semen preservation

- Freeze-drying
- Convective- or evaporative-drying
  (Bhowmick et al. 2003, McGinnis et al. 2005)
- Air-drying *(Imoedamhe 2005)*

Requirement: drying chamber, gas releasing regulator, electric fan and larminar flow chamber

Motivation

Some methods for desiccated semen preservation

- Heat-drying *(Lee and Niwa 2006, Rungroekrit et al. 2012, Lee et al. 2013)*
- Flame-drying *(Rungroekrit et al. 2013)*

Motivation

- Simple semen preservation method
  - Less equipment requirement
  - Portable stuffs
  - Preserved sperm can be stored at ambient temperature for short- and/or long- term

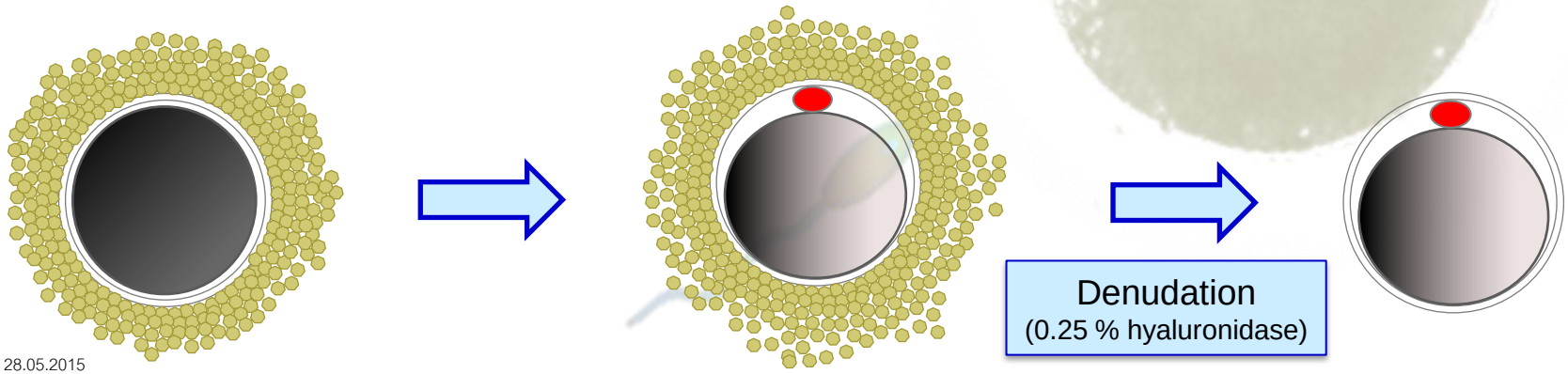
Goal

- **Heat- and Flame-drying** as an alternative methods (simple procedure and possible to use under extreme condition area)
- Fertilization ability investigation using ICSI
- Investigation of sperm DNA fragmentation using Halomax® test kit

Materials and Methods

In vitro maturation

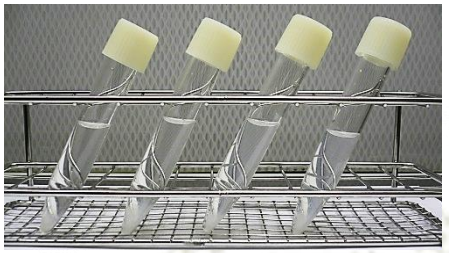
- Cumulus oocyte complexes (COCs) ≥ 3 layers
- IVM in mTCM 199 (insulin, L-glutamine, FCS, gentamicin; stock medium) and eCG
- Maturation period: 44 - 46 h (39 °C, 5 % CO_2, humidified atmosphere)



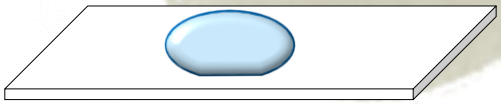
Materials and Methods

Sperm preparation: Heat-drying

Semen collection
(Sperm rich fraction)



Swim up
(Androhep®)



50 µl

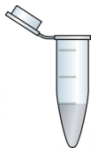
Heat-drying
(50, 56, 90 °C for 45 min,
120 °C for 20 min)

Materials and Methods

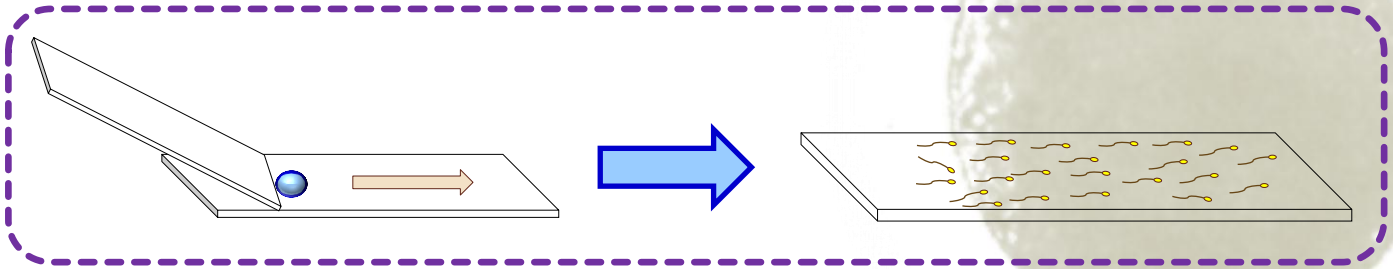
Sperm preparation: Flame-drying

Semen collection

Semen collection
(Sperm rich fraction)



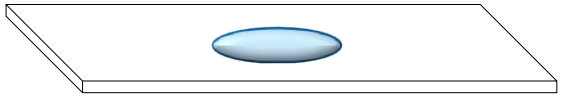
Swim up
(Androhep®)



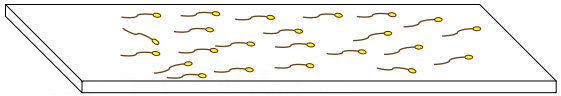
Flame-drying for 2 s
(Campingaz®)

Materials and Methods

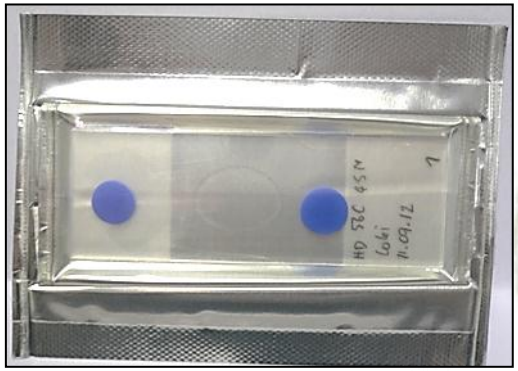
Dried sperm samples packaging and storage



Heat-dried sperm



Flame-dried sperm

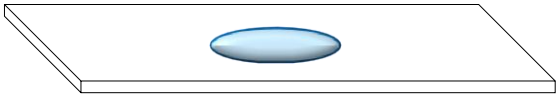


Packaging
(Vacuum seal in ESCAL film and aluminium film bag)

Storage
[4 °C in household refrigerator, short-term (1-5 days) and long-term (90-100 days)]

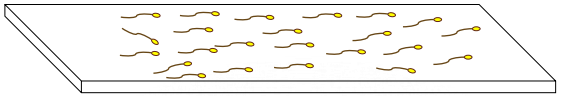
Materials and Methods

Dried sperm sample rehydration



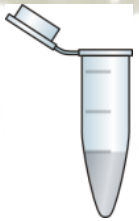
Heat-dried sperm

Drop of 50 µl ultrapure water



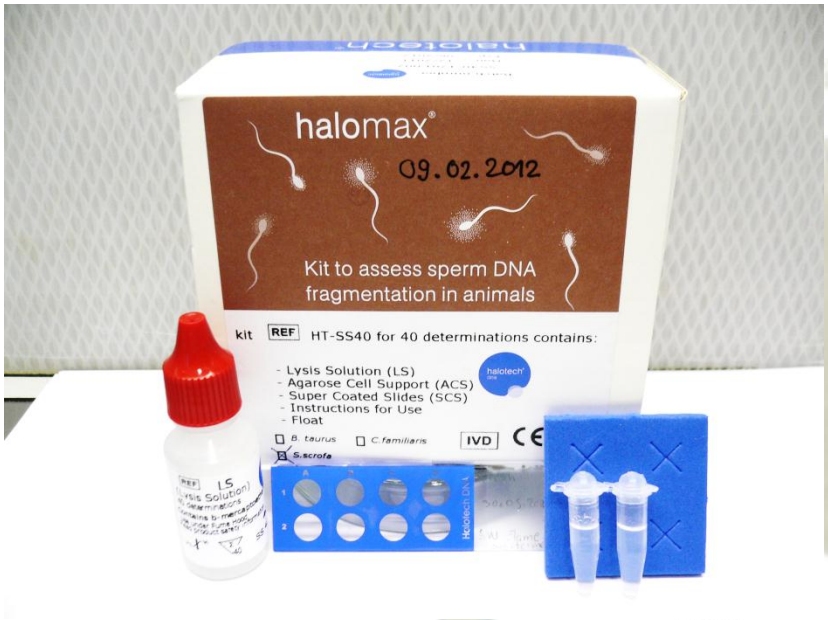
Flame-dried sperm

Drop of 10 µl ultrapure water



Materials and Methods

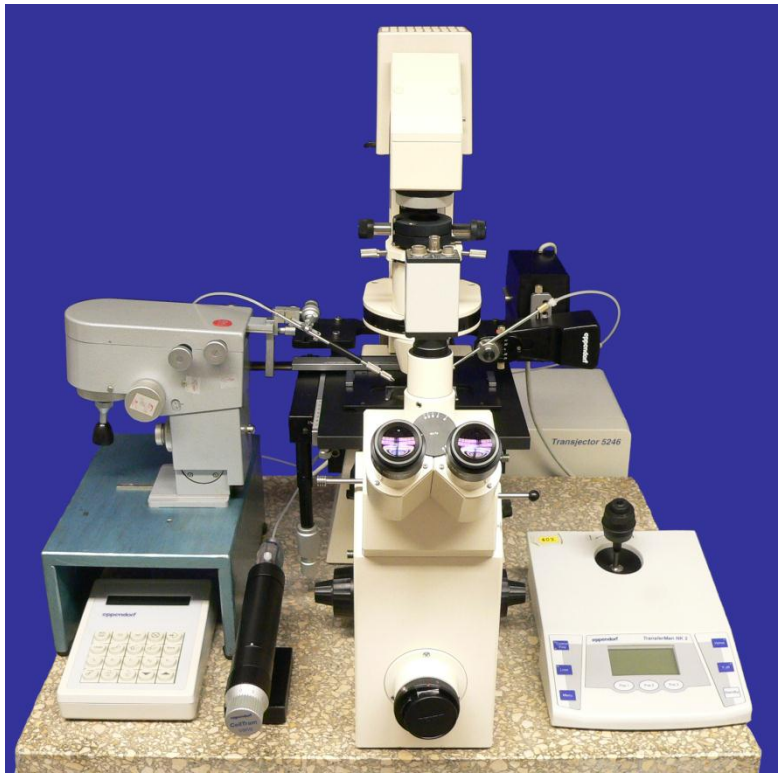
Sperm DNA fragmentation assessment



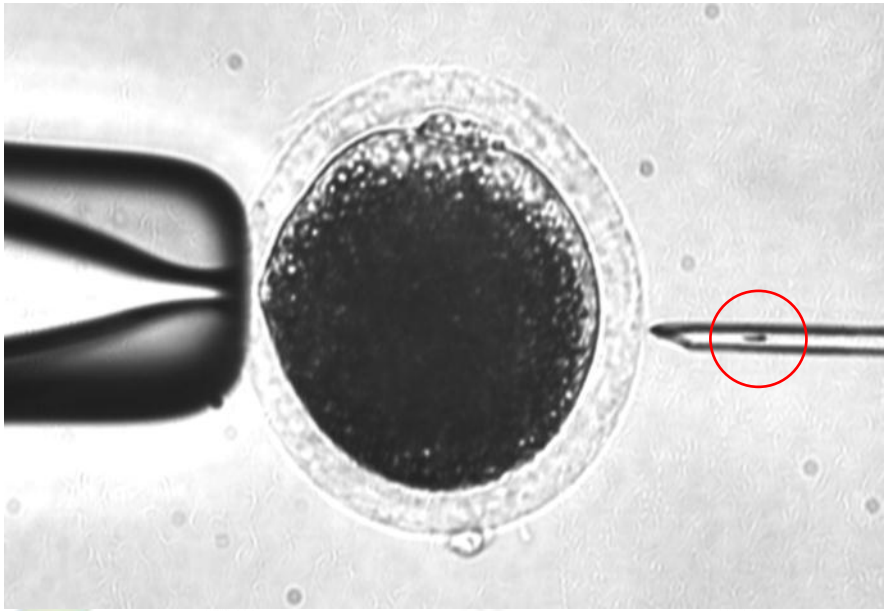
Halomax® (Halotech DNA SL, Spain)

Materials and Methods

Fertilization ability testing using ICSI



Micromanipulation unit

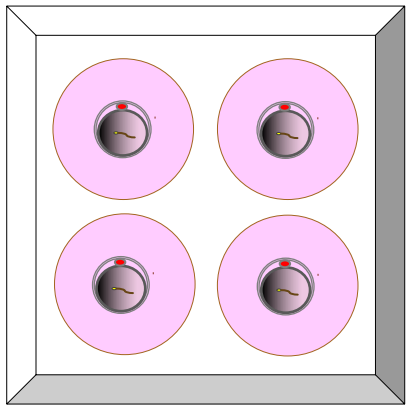


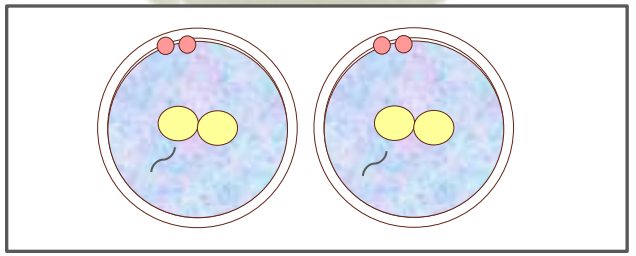
Intracytoplasmic sperm injection

Materials and Methods

Sperm-injected oocytes activation and *in vitro* culture

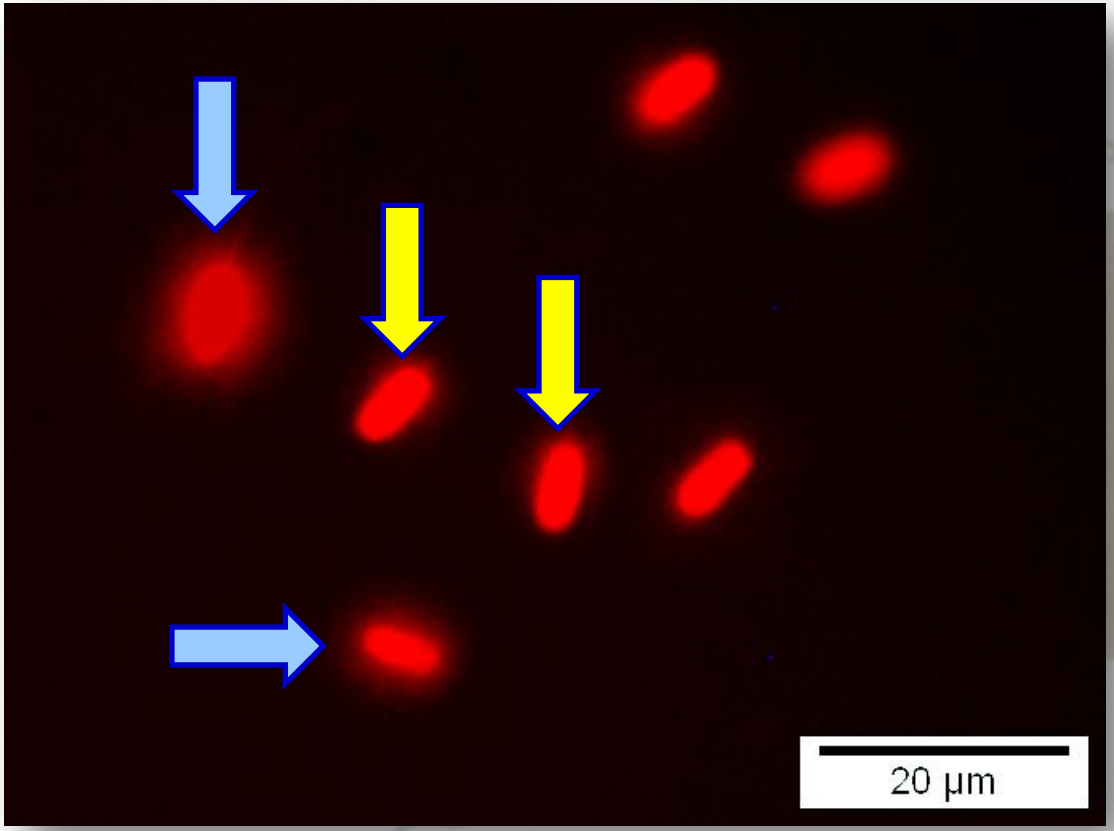
- 10 % ethanol (2 min) in mTCM 199
- mTCM 199 + 10 µg/ml CHX (24 h)
- staining with aceto-orcein





Results

Sperm DNA fragmentation by Halomax® test kit



Fluorescence microscope, 800x magnification

Results

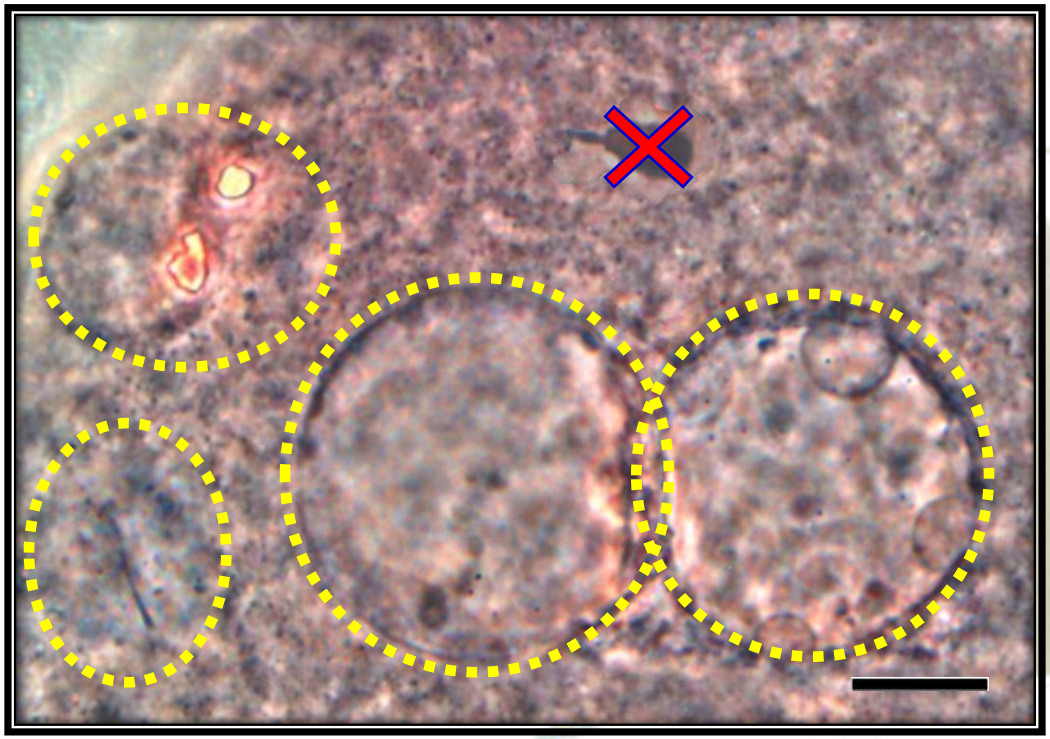
Percent of DNA fragmentation index (% DFI)

Spermatozoa	Storage duration	No. of replicates	Total no. of examined spermatozoa	% DFI (mean± SD)
Ejaculated semen (control gr.)		4	1,200	1.5 ± 0.4^{a}
HD 50 °C 45 min	short-term	4	1,200	59.66 ± 2.0^{c}
	long-term	4	1,200	68.66 ± 1.9^{c*}
HD 56 °C 45 min	short-term	4	1,200	58.17 ± 3.8^{c}
	long-term	4	1,200	64.58 ± 3.1^{c*}
HD 90 °C 45 min	short-term	4	1,200	75.0 ± 4.0^{d}
	long-term	4	1,200	82.66 ± 2.8^{d}
HD 120 °C 20 min	short-term	4	1,200	**1.25 ± 0.7^{a}**
	long-term	4	1,200	**2.33 ± 0.2^{a*}**
FF	short-term	4	1,200	38.0 ± 4.0^{E}
	long-term	4	1,200	50.58 ± 4.3^{e*}
FSW	short-term	4	1,200	30.25 ± 3.5^{F}
	long-term	4	1,200	34.0 ± 3.2^{f}

HD: Heat-dried semen samples, FF: Flame-dried sperm rich fraction, FSW: Flame-dried swim up semen, *P<0.05*

Results

Fertilization criteria



Two polar bodies, formation of 2 pronuclei, sperm tail and disappearence of sperm head, bar = 20 µm

Results

Percent of fertilized oocytes after ICSI

Experimental group	Storage duration	No. of examined oocytes	No. (%) of fertilized oocytes
Ejaculated semen (control gr.)		140	**26 (18.6)**
HD 50 °C 45 min	short-term	140	20 (14.3)
	long-term	124	7 (5.7)
HD 56 °C 45 min	short-term	122	**21 (17.2)**
	long-term	103	9 (8.7)
HD 90 °C 45 min	short-term	135	15 (11.1)
	long-term	142	22 (15.5)
HD 120 °C 20 min	short-term	102	0 (0)
	long-term	122	0 (0)
FF	short-term	128	14 (10.9)
	long-term	126	10 (7.9)
FSW	short-term	115	12 (10.4)
	long-term	130	**15 (11.5)**

HD: Heat-dried semen samples, FF: Flame-dried sperm rich fraction, FSW: Flame-dried swim up semen, 5-7 replications per sample

Conclusions

- Heat- and Flame-dried boar semen have an ability to fertilize porcine oocytes by using ICSI technique
- Long-term storage increased % DFI of dried samples
- Further experiments need to optimize the protocols in term of sperm DNA damage protection, storage method, and rehydration procedure to improve the fertilization rate

Thank you for your attention