Laparoscopic insemination method in sheep allows the use of an animal protein-free and inexpensive freezing medium

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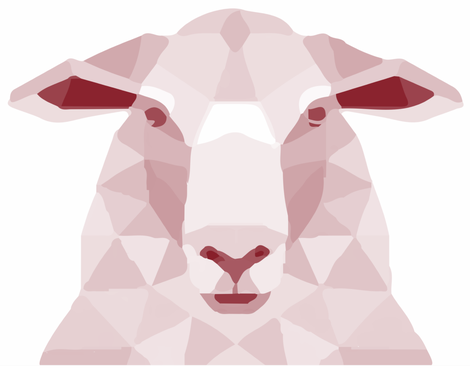

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Laparoscopic insemination method in sheep allows the use of an animal protein-free and inexpensive freezing medium

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CONTEXT

In breed societies managing ovine species, sperm is cryopreserved according to a method adapted from Colas [1], using egg yolk and milk in extenders. Using such media is acceptable within a short-term commercial strategy. However, it sounds necessary to develop the use of stable, synthetic and chemically defined medium in biobank to overcome any update of the regulations by the future. STEMALPHA.CRYO3 (Ref 5617, Stem Alpha, Saint-Genis-l'Argentiere, France) called 'CRYO3' is a chemically defined preservation medium currently used for freezing human tissue and adult stem cells.

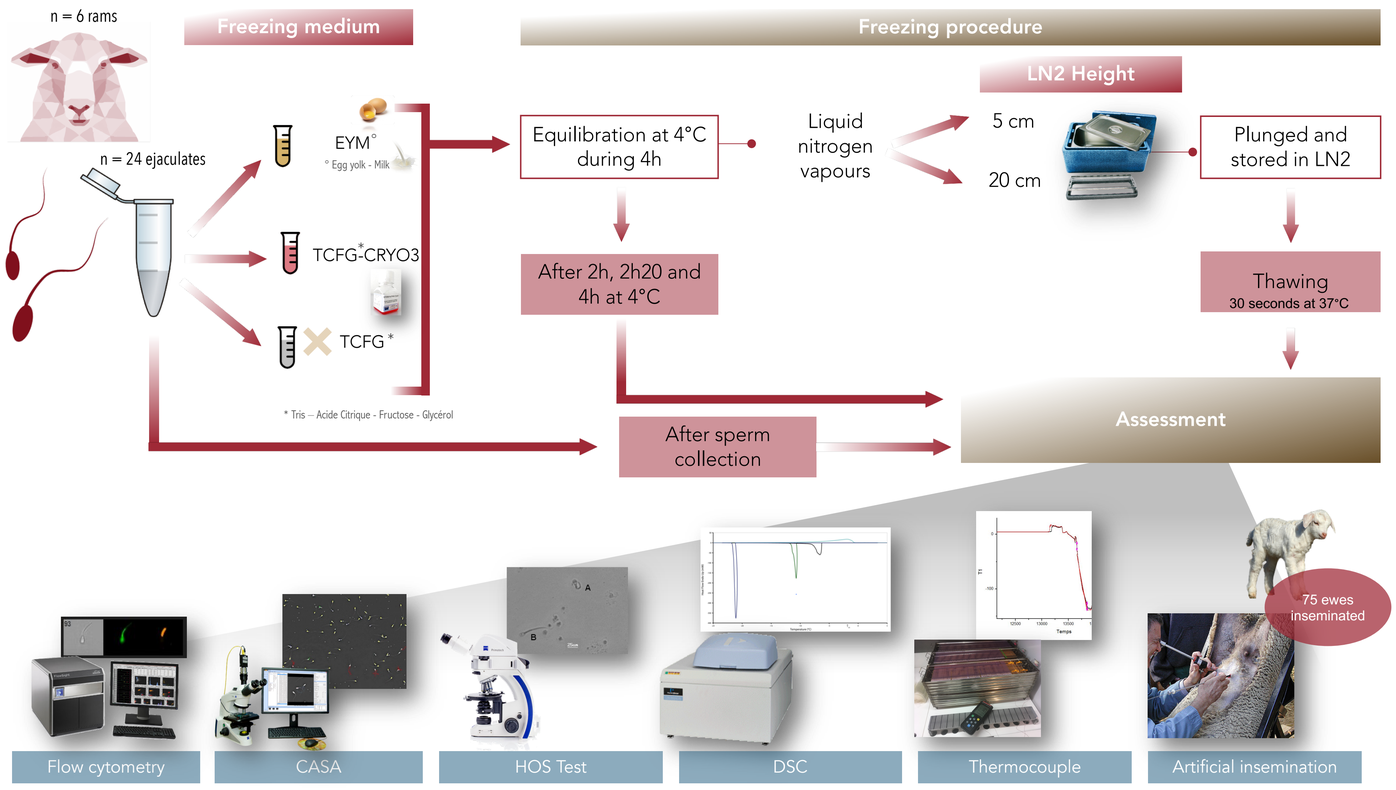
[1] Colas G. Effect of initial freezing temperature addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. Journal of the Society for Reproduction and Fertility. 1975;277-85.

OBJECTIVE

The objectives of this study were firstly to avoid all forms of derived (plant or animal origin), or unstable and variable products, and secondly to test a faster cooling rate. The aim of the present study was to evaluate the effects of a CRYO3-based medium and of two cooling rates on *in vitro* parameters and *in vivo* fertility of ram sperm.

1 MATERIALS & METHODS

Experimental study



Ram sperm was frozen either in a control medium (EYM), containing egg yolk and milk; or in a CRYO3-based medium, called "TCFG-CRYO3", or in a negative control, called "TCFG", consisted of a Tris buffer and glycerol. T corresponds to Tris buffer; C to citric acid; F to Fructose; G to Glycerol. After collection, sperm was assessed by subjective motility and HOS Test, while equilibrated sperm at 4°C after 2h, 2h20 and 4h was only assessed by HOS Test. Frozen-thawed sperm quality was evaluated by flow cytometry, CASA and HOS Test. Freezing media were thermodynamically characterized using a Differential Scanning Calorimeter. The cooling rates associated to the two levels of LN2 heights were recorded. Artificial inseminations on 75 ewes were performed to evaluate sperm fertility. CASA: Computer-Assisted Sperm analysis; DSC : differential scanning calorimeter; LN2 : Liquid Nitrogen; TCF : Tris buffer supplemented with citric acid and fructose.

Medium composition

Table 1. Composition of extender 1 and extender 2 for each freezing medium.

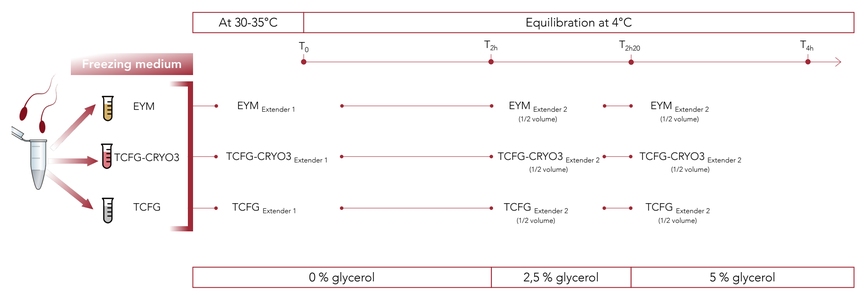
		EYM1]	TCFG-CRYO3	TCFG
Extender 1 (at 30°C)	Sugar	Lactose (102.96 g/L)	-	-
	Additive	20 % (*v/v*) hen egg yolk	20 % CRYO3	-
	Buffer	Gentamicin (Gibco, 10 mg/mL) in sterile water	TCF	TCF
Extender 2 (at 4°C)	Sugar	-	0.2 M trehalose	0.2 M trehalose
	Glycerol	10 % (*v/v*) glycerol	10 % (*v/v*) glycerol	10 % (*v/v*) glycerol
	Buffer	90 % (*w/v*) of milk powder diluted in sterile water (Regilait, 40 g/L of semi-skimmed milk) and gentamicin (Gibco, 10 mg/mL)	TCF	TCF
pH		Adjusted at 6.8	7.0	7.0

TCF: Tris buffer supplemented with citric acid and fructose (Tris-hydroxymethyl-aminomethane, 27 g/L; citric acid, 14 g/L; fructose, 10 g/L; pH = 7.0).

Semen diluted up to **800 × 10⁶ spz/mL**

Final concentration of **400 × 10⁶ spz/mL**

Dilution procedure of ram sperm

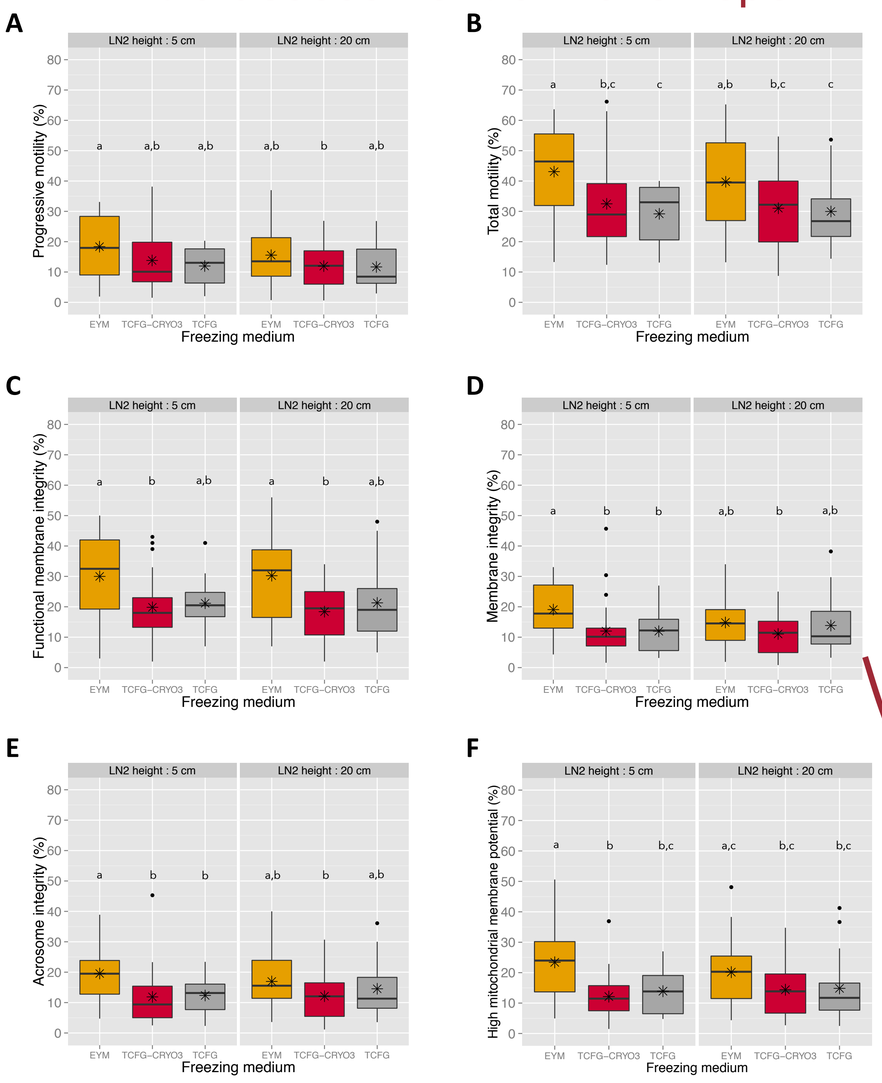


Statistics

- **R software**
- *in vitro* parameters : a **mixed model** including the ram as a random effect and the medium and the distance above the surface of liquid nitrogen as fixed effects with three and two levels respectively.
- Pregnancy and parturition rates (binomial distribution) & prolificacy (Poisson distribution) : **generalized linear models,** including the medium as a fixed effect and the ram as a random effect.
- Differences with $p < 0.05$ were considered statistically significant. Thermodynamic values were analysed using descriptive statistics.

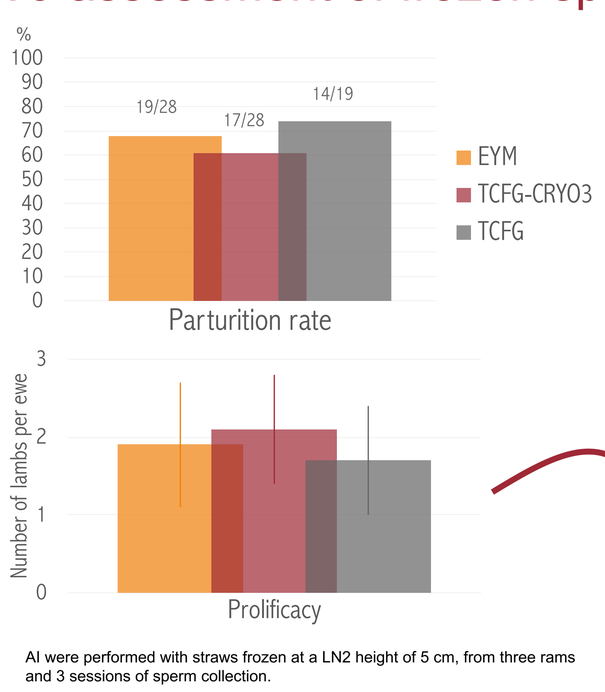
2 RESULTS

in vitro assessment of frozen sperm



(A) Progressive motility, (B) Total motility, (C) Functional membrane integrity (HOS test), (D) Membrane integrity, (E) Acrosome integrity, (F) High mitochondrial membrane potential.
The symbol * within the graphs corresponds to the mean. The median is indicated by a horizontal line.
[a, b, c] Values within a graph with different superscripts differ significantly at $P < 0.05$.

in vivo assessment of frozen sperm

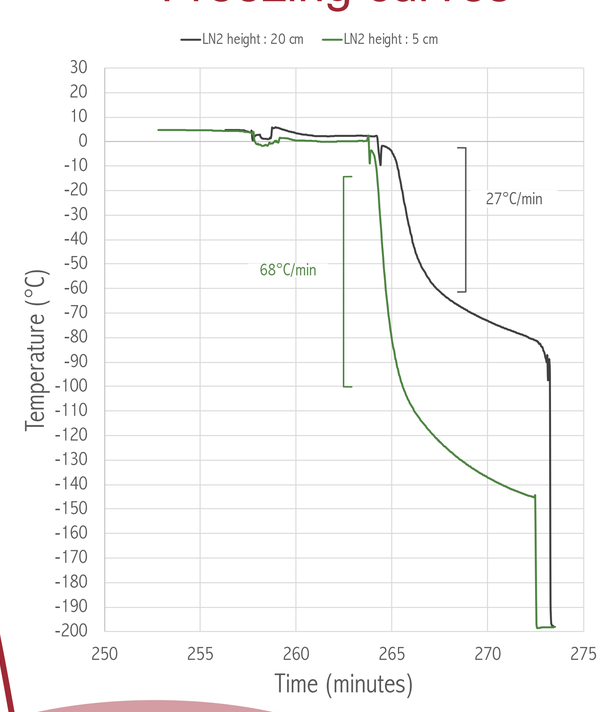


AI were performed with straws frozen at a LN2 height of 5 cm, from three rams and 3 sessions of sperm collection.

The cooling rates : no significant effect (except on the membrane integrity)
Media : significant differences

EYM showed the highest values. TCFG-CRYO3 and TCFG were not significantly different.

Freezing curves



No significant difference was observed. However, the **negative control TCFG** showed the **best parturition rate with 74 % of delivered ewes**.

Thermodynamic properties of the media

Table 2. Thermodynamic characteristics of the freezing media.

Medium	T_m (°C)	ΔH_{max} (J/g)	$T_{c2.5°C/min}$ (°C)	$\Delta H_{(-50°C/min)}$ (J/g)	$T_{c50°C/min}$ (°C)
EYM	-1.27 ± 0.16	-215.54 ± 5.70	-18.66 ± 2.33	-197.94 ± 0.91	-20.72 ± 1.21
TCFG-CRYO3	-1.44 ± 0.34	-223.14 ± 5.45	-15.42 ± 1.87	-217.74 ± 4.77	-16.84 ± 1.29

Results are presented as the means ± standard deviation of 3 replicates of each medium. Tc(2.5°C/min) : Crystallization temperature at a cooling rate of 2.5°C/min; Tc(50°C/min) : Crystallization temperature at a cooling rate of 50°C/min Tm : Melting temperature ; ΔHmax : maximum crystallization enthalpy variation; ΔH50°C/min : crystallization enthalpy variation at a cooling rate of 50°C/min.

The two media are quite similar in terms of thermodynamic properties. Surprisingly, TCFG-CRYO3 was found to show more variability than EYM.

3 CONCLUSION

This study showed that the product, CRYO3, cannot replace egg yolk and milk in freezing extenders. Moreover, we showed that laparoscopic inseminations allowed a 74 % parturition rate thanks to an easy and inexpensive medium composed only of a Tris buffer and glycerol. This medium, although could not be used in a large scale, remains an option for international transport or long-term storage of genetic diversity.