Journal of Visualized Experiments

# Vitrification of in vitro matured oocytes collected from adult and prepubertal ovaries in sheep

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Collection - Author Produced Video |
| **Manuscript Number:** | JoVE62272R2 |
| **Full Title:** | Vitrification of in vitro matured oocytes collected from adult and prepubertal ovaries in sheep |
| **Corresponding Author:** | Fiammetta Berlinguer, Ph.D.<br>University of Sassari: Universita degli Studi di Sassari<br>ITALY |
| **Corresponding Author's Institution:** | University of Sassari: Universita degli Studi di Sassari |
| **Corresponding Author E-Mail:** | berling@uniss.it |
| **Order of Authors:** | Sara Succu |
| | Elisa Serra |
| | Sergio Gadau |
| | Antonio Varcasia |
| | Fiammetta Berlinguer, Ph.D. |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$1200) |
| Please specify the section of the submitted manuscript. | Developmental Biology |
| Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below: | I agree to the Author License Agreement |
| Please provide any comments to the journal here. | |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access ($1400) |

**TITLE:**

# Vitrification of in vitro matured oocytes collected from adult and prepubertal ovaries in sheep

**AUTHORS AND AFFILIATIONS:**

Sara Succu, Elisa Serra, Sergio Gadau, Antonio Varcasia, Fiammetta Berlinguer

Department of Veterinary Medicine, University of Sassari, Italy

Email address of co-authors:

Sara Succu (succus@uniss.it)
Elisa Serra (eliserra@uniss.it)
Sergio Gadau (sgadau@uniss.it)
Antonio Varcasia (varcasia@uniss.it)

Corresponding author:

Fiammetta Berlinguer (berling@uniss.it)

**KEYWORDS:**

cryopreservation, gamete, post-warming culture, viability, developmental competence, cryotop, mitochondria, ROS

**SUMMARY:**

The protocol aims at providing a standard method for the vitrification of adult and juvenile sheep oocytes. It includes all the steps from the preparation of the in vitro maturation media to the post-warming culture. Oocytes are vitrified at the MII stage using Cryotop to ensure the minimum essential volume.

**ABSTRACT:**

In livestock, in vitro embryo production systems can be developed and sustained thanks to the large number of ovaries and oocytes that can be easily obtained from a slaughterhouse. Adult ovaries always bear several antral follicles, while in pre-pubertal donors the maximal numbers of oocytes are available at 4 weeks of age, when ovaries bear peak numbers of antral follicles. Thus, 4 weeks old lambs are considered good donors, even if the developmental competence of prepubertal oocytes is lower compared to their adult counterpart.

Basic research and commercial applications would be boosted by the possibility of successfully cryopreserving vitrified oocytes obtained from both adult and prepubertal donors. The vitrification of oocyte collected from prepubertal donors would also allow shortening the generation interval and thus increasing the genetic gain in breeding programs. However, the loss of developmental potential after cryopreservation makes mammalian oocytes probably one of the most difficult cell types to cryopreserve. Among the available cryopreservation techniques, vitrification is widely applied to animal and human oocytes. Despite recent advancements in the technique, exposures to high concentrations of cryoprotective agents as well as chilling injury and osmotic stress still induce several structural and molecular alterations and reduce the

developmental potential of mammalian oocytes. Here, we describe a protocol for the vitrification of sheep oocytes collected from juvenile and adult donors and matured in vitro prior to cryopreservation. The protocol includes all the procedures from oocyte in vitro maturation to vitrification, warming and post-warming incubation period. Oocytes vitrified at the MII stage can indeed be fertilized following warming, but they need extra time prior to fertilization to restore damage due to cryopreservation procedures and to increase their developmental potential. Thus, post-warming culture conditions and timing are crucial steps for the restoration of oocyte developmental potential, especially when oocyte are collected from juvenile donors.

## INTRODUCTION:

Long-term storage of the female gametes can offer a wide range of applications, such as improving domestic animal breeding by genetic selection programs, contributing to preserve biodiversity through the ex-situ wildlife species conservation program, and boosting in vitro biotechnology research and applications thanks to the availability of stored oocytes to be incorporated in in vitro embryo production or nuclear transplantation programs[1–3]. Juvenile oocyte vitrification would also increase genetic gain by shortening the generation interval in breeding programs[4]. Vitrification by ultra-rapid cooling and warming of oocytes is currently considered a standard approach for livestock oocytes cryopreservation[5]. In ruminants, before vitrification, oocytes are usually matured in vitro, after retrieval from follicles obtained from abattoir-derived ovaries[2]. Adult, and especially prepubertal ovaries[4, 6], can indeed supply a virtually unlimited number of oocytes to be cryopreserved.

In cattle, after oocyte vitrification and warming, blastocyst yields at >10% have been commonly reported by several laboratories during the last decade[3]. However, in small ruminants oocyte vitrification is still considered relatively new for both juvenile and adult oocytes, and a standard method for sheep oocyte vitrification remains to be established[2, 5]. Despite recent advancements, the vitrified and warmed oocyte indeed presents several functional and structural alterations that limit their developmental potential[7–9]. Thus, few articles have reported blastocyst development at 10% or more in vitrified/warmed sheep oocytes[2]. Several approaches have been investigated to reduce the above-mentioned alterations: optimizing the composition of the vitrification and thawing solutions[10, 11]; experimenting with the use of different cryo-devices[8, 12, 13]; and applying specific treatments during in vitro maturation (IVM)[4, 14, 15] and/or during the recovery time after warming[6].

Here we describe a protocol for the vitrification of sheep oocytes collected from juvenile and adult donors and matured in vitro prior to cryopreservation. The protocol includes all the procedures from oocyte in vitro maturation to vitrification, warming and post-warming culture period.

## PROTOCOL:

The animal protocol and the implemented procedures described below are in accordance with the ethical guidelines in force at the University of Sassari, in compliance with the European Union Directive 86/609/EC and the recommendation of the Commission of the European Communities

2007/526/EC.

## 1. Preparation of media for oocyte manipulation

1.1. Prepare the medium for transport of collected ovaries by supplementing Dulbecco's phosphate buffered saline with 0.1 g/L penicillin and 0.1 g/L streptomycin (PBS).

1.2. Prepare the medium for oocyte collection and maturation by diluting 9.5 g of Tissue Culture Medium (TCM) 199 in powder with 1 L of Milli-Q water supplemented with penicillin (0.1%) and streptomycin (0.1%).

1.2.1. After dilution, filter 100 mL of medium and store it at 4 °C as Stock Maturation Medium (SMM) to be used for one week.

1.2.2. Prepare the collection medium (CM) by supplementing the remaining 900 mL with 25 mM HEPES, 0.36 g/L bicarbonate and 0.1% (w/v) polyvinyl alcohol (PVA) (pH 7.3, osmolality 290 mOsm/kg).

1.3. Prepare the maturation medium with SMM supplemented with 0.021 g/L bicarbonate, 10% heat-treated estrus sheep serum, 1 IU/mL FSH, 1 IU/mL LH, 100 µL of cysteamine and 8 mg/mL of pyruvate.

NOTE: The maturation medium in a volume of 10 mL must be incubated at standard conditions (in a maximum humidified atmosphere at 39 °C in 5% $CO_2$ in air) for at least 4 h before use.

1.4. Prepare the base medium (BM) for manipulation of oocyte after in vitro maturation, consisting in PBS without $Ca^{++}$ and $Mg^{++}$, supplemented with 20% fetal calf serum (FCS).

## 2. Oocyte collection and maturation

2.1. Recover the oocytes from juvenile (30-40 days of age, body weight 6-10 kg) and adult ovaries.

2.2. Transport the collected ovaries from the commercial slaughterhouse to the laboratory within 1-2 h in PBS at 27 °C.

2.3. After washing in PBS fresh medium, slice the ovaries in CM using a micro-blade to release the follicle content.

2.4. Under a stereomicroscope examination with 60x magnification, select cumulus-oocyte complexes (COCs) for in vitro maturation by choosing those with 4-10 layers of granulosa cells, oocyte with a uniform cytoplasm, homogeneous distribution of lipid droplets in the cytoplasm and with the outer diameter of about 90 µm (mean).

2.5. Wash the selected COCs three times in CM and finally transfer them in maturation medium.

NOTE: For juvenile oocytes, to improve survival after vitrification, supplement the maturation medium with 100 µM trehalose.

2.6. For in vitro maturation, transfer 30-35 COCs in 600 µL of maturation medium in four-well Petri dishes, covered with 300 µL of mineral oil and incubate them for 22 (adult oocytes)/24 (juvenile oocytes) h in 5% $CO_2$ in air at 39 °C.

2.7. After in vitro maturation, denude COCs of cumulus cells by gently pipetting. Following the examination under a stereomicroscope with 60x magnification, select only those showing the extrusion on the first polar body, and thus at metaphase II (MII) stage, for vitrification.

**3. Semen collection, freezing and thawing procedures**

3.1. Prepare the base medium for semen cryopreservation consisting in ram extender (200 mM Tris; 70 mM citric acid; 55 mM fructose; pH 7.2, osmolality 300 mOsm/kg) supplemented with egg yolk 20% (v/v).

3.2. Collect the semen only during sheep breeding season (October-November).

3.3. Obtain ejaculates by artificial vagina from adult rams (aged 2-5 years), maintained in an outdoor environment and fed a live-weight maintenance ration. Keep rams isolated in separate pens, but with visual contact between each other.

3.4. Repeat semen collection one a week during the entire breeding season to obtain at least 8 ejaculates from each male.

3.5. Transport the semen samples to the laboratory at environmental temperature within 5 min after collection and immediately process. Pool the ejaculates of two-three rams and evaluate sperm concentration spectrophotometry.

3.6. After pooling, dilute the ejaculates up to 400 x $10^6$ spermatozoa/mL with base medium for semen cryopreservation supplemented with 4% glycerol. Then cool the diluted semen to 4 °C over a period of 2 h and equilibrate it for 20 min before freezing.

3.7. Freeze the semen samples in pellet form (0.25 mL) on dry ice and then plunge them into liquid nitrogen.

3.8. For thawing, put the pellet in a sterilized glass falcon and plunge it in a water bath for 20 s at 39 °C.

**4. In vitro fertilization and embryo culture**

4.1. Prepare stocks for constitution of the synthetical oviductal fluid (SOF).

4.1.1. Prepare Stock A: 99.4 mL of MilliQ-water; 6.29 g of NaCl; 0.534 g of KCl; 0.161 g of $KH_2PO_4$; 0.182 g of $MgSO_4 \cdot 7H_2O$; and 0.6 mL of Sodium Lactate. Keep at 4 °C for up to 3 months.

4.1.2. Prepare Stock B: 10 mL of MilliQ-water; 0.210 g of $NaHCO_3$; and 2-3 g of Phenol Red. Keep at 4 °C for 1 month.

4.1.3. Prepare Stock C: 10 mL of MilliQ-water; and 0.051 g of sodium pyruvate. Keep at 4 °C for 1 month.

4.1.4. Prepare Stock D: 10 mL of MilliQ-water; and 0.262 g of $CaCl_2$ $2H_2O$. Keep at 4 °C for 1 month.

4.1.5. Prepare 10 mL of SOF consisting of 7.630 mL of MilliQ water, 1 mL of Stock A, 1 mL of Stock B, 0.07 mL of Stock C and 0.7 mL of stock D.

4.1.6. Prepare in vitro fertilization (IVF) medium: SOF supplemented with 2% heat-treated estrous sheep serum, 10 µg/mL heparin and 1 µg/mL hypoutarine (osmolality 280-290 mOsm/kg).

NOTE: The IVF medium in a volume of 10 mL must be incubated at standard conditions (in a maximum humidified atmosphere at 39 °C in 5% $CO_2$, 5% $O_2$ and 90% $N_2$) at least 4 h before use.

4.2. Transfer frozen/thawed semen aliquots in a sterilized glass conical tube below 1.5 mL of warmed IVF medium and incubate them for 15 min at 39 °C in a humidified atmosphere at 5% $CO_2$ in air.

4.3. After incubation, the motile spermatozoa swim towards the apical portion of the liquid column. Collect the top layer and observe for sperm motility evaluation.

NOTE: Sperm motility parameters should be assessed using a computer-assisted sperm analysis (CASA) system with the following settings: 25 frames acquired to avoid sperm track overlapping, minimum contrast 10, minimum velocity of average path 30 µm/s, and progressive motility > 80% straightness. This system has a specific setup for ram sperm evaluation. For each sample, 5 µL subsample of sperm suspension are loaded into a pre-warmed analysis chamber with a depth of 10 µm. Sperm motility is assessed at 37 °C at 40x using a phase contrast microscope and a minimum of 500 sperm per subsample should be analyzed in at least four different microscopic fields. The percentage of total motile and progressive motile sperm were evaluated. For the IVF, the percentage of progressive motile spermatozoa should be ≥ 30%.

4.4. Dilute swim-up derived motile spermatozoa at 1 x $10^6$ spermatozoa/mL final concentration and co-incubate them with MII oocytes in 300 µL of IVF medium covered with

mineral oil in four-well Petri dishes.

4.5. After 22 h transfer the presumptive zygotes in four-well Petri dishes containing SOF supplemented with 0.4% bovine serum albumin and essential and non-essential amino acids at oviductal concentration as reported by[16] under mineral oil and culture them under standard conditions up to the blastocyst stage.

4.6. At 22-, 26- and 32- h post-insemination, record the number of cleaved oocytes, showing two distinct blastomeres, by the examination under a stereomicroscope with 60x magnification.

4.7. Observe the embryos daily starting from the fifth to the ninth day of culture and newly formed blastocysts should be recorded by the examination under a stereomicroscope with 60x magnification.

## 5. Oocyte vitrification and warming

NOTE: Perform vitrification following the method of minimum essential volume (MEV) using device cryotops[17].

5.1. Equilibrate a group of five oocytes at 38.5 °C for 2 min in BM. The use of BM guarantees a low calcium concentration ([$Ca^{2++}$] 2.2 mg/dL)[10].

5.2. Dehydrate the oocytes with a 3 min exposure to equilibration solution containing 7.5% (v/v) dimethyl sulfoxide (DMSO) and 7.5% (v/v) ethylene glycol (EG) in BM.

5.3. Transfer the oocytes to the vitrification solution containing 16.5% (v/v) DMSO, 16.5% (v/v) EG and 0.5 M trehalose in BM before loading them in a cryotop device and directly plunging them into liquid nitrogen within 30 s.

5.4. To warm to a biological temperature, transfer the content of each vitrification device from liquid nitrogen into 200 µL drops of 1.25 M trehalose in BM for 1 min at 38.5 °C, and gently stir to facilitate the mixing.

5.5. To promote removal of intracellular cryoprotectants, transfer oocytes stepwise into 200 µL drops of decreasing trehalose solutions (0.5 M, 0.25 M, 0.125 M trehalose in BM) for 30 s at 38.5 °C before being equilibrated for 10 min at 38.5 °C in BM.

## 6. Assessment of oocyte quality post-warming

6.1. After warming, incubate the oocytes for 6 h in PBS without $Ca^{++}$ and $Mg^{++}$ plus 20% FCS (BM) in 5% $CO_2$ in air at 38.5 °C.

NOTE: The oocyte ability to restore biological and structural features after vitrification is in relation to the species and classes of used oocytes.

6.2. Since the oocyte ability to recover cryopreservation damages is time-dependant, assess oocyte quality at different time points of in vitro culture (0 h, 2 h, 4 h, 6 h) after warming, to define the optimal time window for oocyte fertilization.

NOTE: In adult sheep oocyte, the optimal time is 4 h post-warming; for prepubertal oocyte, the optimal time is 2 h post-warming.

**7. Oocyte survival assessment**

7.1. Immediately after warming and for each time point of post-warming culture, morphologically evaluate oocytes using an inverted microscope with 100x magnification.

NOTE: Oocytes with structural alterations, such as faint cytoplasm, damage zona pellucida and/or membrane should be classified as degenerated.

7.2. Validate the membrane integrity evaluation using a double differential fluorescent staining.

7.3. Incubate the oocytes in 2 mL of BM containing propidium iodide (PI; 10 µg/mL) and Hoechst 33342 (10 µg/mL) for 5 min in 5% $CO_2$ in air at 38.5 °C.

7.4. After washing three times in fresh BM, observe the oocytes under a fluorescent microscope using an excitation filter of 350 nm and emission of 460 nm for Hoechst 33342 and an excitation filter of 535 nm and emission of 617 nm for PI.

NOTE: Oocytes with an intact membrane can be recognized by the blue fluorescence of colored DNA with Hoechst 33342. Oocyte with damaged membranes show a red fluorescence due to DNA staining with PI.

**8. Evaluation of mitochondrial activity and ROS intracellular levels by confocal laser scanning microscopy**

8.1. Prepare the MitoTracker Red CM-$H_2$XRos (MT-Red) probe.

8.1.1. Dilute the content of 1 vial (50 µg) with 1 mL of DMSO to obtain a 1 mM solution. Keep the diluted vial in liquid nitrogen.

8.1.2. Dilute the solution 1 mM with DMSO to obtain the 100 µM stock solution and store it at -80 °C in the dark.

8.2. Prepare 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) probe.

8.2.1. Dilute the $H_2$DCF-DA in 0.1% polyvinyl pyrrolidone (PVA)/PBS to obtain the first 100 mM

solution. Keep the solution at -80 °C in the dark .

8.2.2. Dilute the first solution in 0.1% PVA/PBS to obtain the 100 µM stock solution. Store it at -20 °C in the dark.

8.3. Prepare the mounting medium (MM): for 10 mL of MM, add 5 mL of glycerol, 5 mL of PBS and 250 mg of sodium azide. Store it at -20 °C until use.

8.4. Incubate the oocytes for 30 min at 38.5 °C in BM with 500 nM MT-Red (stock solution: 100 µM in DMSO).

8.5. After incubation with MT-Red probe, wash the oocytes three times in BM and incubate for 20 min in the same media containing 5 µM $H_2$DCF-DA (stock solution: 100 µM in BM).

8.6. After exposure to the probes, wash the oocytes in BM and fix in 2.5% glutaraldehyde/PBS for at least 15 min.

8.7. After fixation, wash the oocytes three times in BM and mount on glass slides in a 4 µL drop of MM with 1 mg/mL Hoechst 33342 using wax cushions to avoid compression of samples.

8.8. Store slides at 4 °C in the dark until evaluation.

8.9. Perform the analysis of immunolabelled sections with a confocal laser scanning microscope. The microscope is equipped with Ar/He/Ne lasers, using a 40/60x oil objective. Analyze the sections by sequential excitation.

8.10. For mitochondrial evaluation, observe the samples with a multiphoton laser to detect MT-Red (exposure: 579 nm; emission: 599 nm).

8.11. Use an argon ions laser ray at 488 nm and the B-2 A filter (495 nm exposure and 519 nm emission) to point out the dichlorofluorescein (DCF)[18].

8.12. In each individual oocyte, measure MT-Red and DCF fluorescence intensities at the equatorial plane[19].

8.13. Maintain parameters related to fluorescence intensity at constant values during all image acquisitions (laser energy 26%, Sequential Settings 1: PMT1 gain 649-PMT2 gain 482; Sequential Setting 2: PMT1 gain 625-PMT2 gain 589; offset 0; pinhole size: 68).

8.14. Perform quantitative analysis of fluorescence intensity using the Leica LAS AF Lite image analysis software package, following the procedures standardized by[20].

8.15. Capture the pictures once, moving on the Z axis, until reaching the equatorial plane.

8.16. For each photo, transform to gray scale and turn off channel 1 (related to Hoechst blue) was turned off. Then manually draw a region of interest (ROI) on a circumscribed area, that is around the meiotic spindle.

NOTE: The software can automatically read the pixel average value on the channel 2 (FITC), subtracting the value of the background from it.

8.17. Record the mean values of pixels and submit for statistical analysis.

## 9. Statistical Analyses

9.1. Analyze the following differences: survival rates in juvenile vs adult oocytes, survival rates and developmental competence in control and trehalose-treated juvenile oocytes, survival and parthenogenetic activation rates and developmental competence of adult oocytes vitrified with different calcium concentration media, fertilization rates and embryo production in juvenile oocytes vitrified with low or high calcium concentration, active mitochondria phenotypes in juvenile vitrified oocytes during different time points of post-warming culture and parthenogenetic activation rates between adult and juvenile vitrified oocytes using the chi square test.

9.2. Analyze the cleavage rate and embryo output in vitrified adult oocytes during different time points of post-warming culture, fluorescence intensity of mitochondrial activity and ROS intracellular levels in juvenile vitrified oocytes during different time points of post-warming culture by ANOVA after analysis for homogeneity of variance by Levene's test. Use a post-hoc test Tukey's test to highlight differences between and among groups.

9.3. Perform statistical analysis using the statistical software program and consider a probability of $P < 0.05$ to be the minimum level of significance. All results are expressed as mean ± S.E.M.

## REPRESENTATIVE RESULTS:

The cryotolerance of oocyte from juvenile donors is lower compared to adult ones. The first effect observed is a lower post-warming survival rate compared to adult oocytes (**Figure 1A**; $\chi^2$ test $P<0.001$). Juvenile oocytes showed a lower membrane integrity after warming (**Figure 1B**). The use of trehalose in the maturation medium was intended to verify whether this sugar could reduce cryoinjuries in juvenile oocytes. The data have demonstrated[23] that oocytes matured for 24 h with trehalose supplementation showed higher survival rates after vitrification/warming compared to the non-treated group (**Table 1**: 85.7% vs 75.3% respectively; $\chi^2$ test $P<0.05$). Trehalose supplementation was indeed associated with higher membrane integrity after warming (**Figure 2A**). Thus, the use of trehalose during the in vitro maturation of juvenile oocytes increased the rates of survival after vitrification (85.7%) to values comparable to adult ones (90.3%). However, cleavage, fertilization and developmental rates of juvenile oocytes were not increased by trehalose supplementation (**Table 1**).

To improve oocyte competence after vitrification we tested in adult ovine oocytes different vitrification media with calcium concentrations ranging from 9.9 to 0.4 mg/dL[10]. Obtained results showed that the use of media with calcium concentration equal to 2.2 mg/dL increased post-warming survival rates, improved developmental competence and reduced parthenogenetic activation of adult oocyte[10] (**Table 2**). We thus tested the low calcium vitrification media for the vitrification of juvenile oocytes. As shown in **Table 3**, juvenile oocytes vitrified with low calcium concentration evidenced higher fertilization rates compared to oocytes vitrified with high calcium concentration (44.35% vs 32.29 % respectively; $P<0.05$), but no differences were found in embryo production.

Vitrified/warmed oocytes need extra time prior to fertilization to restore damage due to cryopreservation procedures and to increase their developmental potential. A previous study has indeed demonstrated that ATP intracellular concentration, mitochondrial activity and in vitro developmental competence are reduced in vitrified/thawed oocytes, which also show high intracellular ROS concentrations[6]. These alterations are particularly marked immediately after warming. During the post-warming culture, both adult and juvenile oocytes are able to partially recover from the damages suffered during the vitrification procedures[6, 24]. By comparing post-warming culture of different durations (0, 2, 4, and 6 h), we showed that after 4 h of culture oocytes collected from adult ewes are able to recover the energetic balance[6] and microtubular setup[24] and to restore the developmental competence with higher cleavage (50.7 ± 3.9%; $P< 0.01$ ANOVA) and blastocyst rates (14.40 ± 1.3%; ANOVA $P< 0.01$) compared to other time points (0, 2 and 6 h; **Table 4**). Thus, 4 h of post-warming culture represents the ideal time window for fertilization of vitrified/warmed adult oocytes[6].

When the same experiment was repeated with oocytes collected from juvenile donors, these results were partially confirmed. Mitochondrial activity was higher in vitrified/warmed juvenile oocytes after 4 h of post-warming culture compared to other time points (0, 2, 6; **Figure 3**: ANOVA $P<0.01$). Several patterns of mitochondrial distribution are observed and classified into the following three groups (as reported by[21] ): Pattern A: homogeneous FINE with small granulations spread throughout the cytoplasm; Pattern B: homogeneous GRANULAR with large granulations spread throughout the cytoplasm; Pattern C: heterogeneous CLUSTERED when particularly large granulations were present, spread all over the cytoplasm or located in specific cytoplasmic domains. The different phenotypes in the cytoplasm distribution of active mitochondrial in MII can be related to oocyte developmental competence. A FINE homogeneous distribution is an indicator of poor developmental competence while a GRANULAR and CLUSTERED distribution are related to an increased mitochondria activity and consequently higher developmental competence[22]. Mitochondrial distribution patterns changed during 6 h of post-warming culture. **Figure 4** shows examples of juvenile oocytes having different patterns of mitochondrial distribution and their fluctuations during post-warming culture. The pattern A increase significantly during the prolonged incubation and reaches the higher value at 6 h post-warming (**Figure 4Aa**: $\chi^2$ $P<0.05$), the pattern B did not show significant changes during post-warming culture (**Figure 4Bb**), the pattern C was not found in any juvenile vitrified/warmed oocyte during the prolonged incubation (**Figure 4Cc**: $\chi^2$ $P<0.05$).

Moreover, ROS intracellular levels were significantly lower in juvenile oocytes at 2 h of post-warming culture compared to 0, 4 and 6 h (**Figure 5**: ANOVA P<0.001). However, and in contrast to what was found in adult oocytes, the rate of spontaneous parthenogenetic activation increased during the post-warming culture in juvenile oocytes (**Figure 6**). For this reason, the recommended time point for fertilization in juvenile oocytes would be 2 h after the post warming culture.

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Survival rates of vitrified/warmed ovine oocyte collected from juvenile and adult donors. (A)** Oocyte were vitrified after in vitro maturation. Survival rates were determined after vitrification and warming by fluorescent staining with propidium iodide (10 µg/mL) and Hoechst 33342 (10 µg/mL). N = 165 adult oocytes and 170 juvenile oocytes. Different letters indicate significant differences between adult and juvenile oocytes: $\chi^2$ test P<0.001. **(B-C)** Examples of vitrified/warmed juvenile oocytes with intact (B) and damaged plasma membrane (C) at the morphological evaluation (inverted microscope with 100x magnification). Scale bar = 10 µm.

**Figure 2. Juvenile ovine oocyte survival rates and in vitro developmental competence after maturation with (TRH) and without (CTR) trehalose (100 mM in maturation medium). (A-B)** Examples of juvenile oocytes vitrified after in vitro maturation in media supplemented (**A**) with or (**B**) without trehalose at the morphological evaluation (inverted microscope with 100x magnification). Scale bar = 30 µm.

**Figure 3. Quantification of active mitochondrial fluorescence intensity in vitrified/warmed juvenile oocytes at different time points (0, 2, 4, 6) during post-warming in vitro culture.** IVM oocytes were used as a control (CTR N = 77). In total 163 (0 h N = 45; 2 h N = 39; 4 h N = 40; 6 h N = 39) juvenile oocytes were vitrified and warmed in three independent experiments. Different letters indicate statistically significant differences (ANOVA P = 0.0000).

**Figure 4. Distribution of mitochondrial pattern in vitrified/warmed juvenile oocytes during 6 hours of post-warming in vitro culture.** Representative images of Fine **(A)**, Granular **(B)** and Clustered **(C)** mitochondrial distribution in vitrified/warmed juvenile oocytes. **(D)** Percentage of juvenile vitrified/warmed oocytes showing a fine mitochondrial distribution; **(E)** Percentage of juvenile vitrified/warmed oocytes showing a granular mitochondrial distribution; **(F)** Percentage of juvenile vitrified/warmed oocytes showing a clustered mitochondrial distribution. IVM juvenile oocytes were used as control (CTR; N = 77). In total 163 (0 h N = 45; 2 h N = 39; 4 h N = 40; 6 h N = 39) juvenile oocytes vitrified and warmed in three independent experiments were used. Different letters indicate statistically significant differences (Aa: $\chi^2$ P = 0.026; Bb: $\chi^2$ P = 0.097; Cc: $\chi^2$ P = 0.014). Scale bar = 30 µm.

**Figure 5. Quantification of intracellular ROS fluorescence intensity in vitrified juvenile oocytes during of 6 hours post-warming in vitro culture. (A-B)** Representative images of ROS fluorescence intensity in *in vitro* matured (**A**) and vitrified (**B**) juvenile oocytes. (**C**) Intracellular levels of ROS as determined by quantification of fluorescence intensity in vitrified juvenile oocytes at different time points (0 h N=45; 2 h N=39; 4 h N=40; 6 h N=39) during post-warming

in vitro culture. *In vitro* matured juvenile oocytes were used as a control (CTR N = 77). Different letters indicate statistically significant differences (ANOVA P = 0.0000). Scale bar = 50 μm.

**Figure 6. Parthenogenetic activation in vitrified juvenile and adult oocytes during 6 hours of post-warming *in vitro* culture.** (**A-B**) Representative images of oocyte parthenogenetic activation: (**A**) oocyte in metaphase II-telophase II transition and (**B**) pronucleus formation. (**C**) Percentages of parthenogenetic activated adult and juvenile oocytes at different time points (0, 2, 4, 6 h) during post-warming in vitro culture. Asterisks indicate statistical differences between juvenile and adult oocytes at each time point of incubation (ANOVA P = 0.000). This figure has been modified from Serra et al.[24] Scale bar = 50 μm.

**Table 1. Juvenile ovine oocytes survival rates and in vitro developmental competence after maturation with and without trehalose and vitrification.** TRH = juvenile oocytes matured with trehalose supplementation (100 mM in maturation medium). CTR = control juvenile oocytes matured without trehalose supplementation. Survival rates were determined after fluorescent staining with propidium iodide (10 μg/mL) and Hoechst 33342 (10 μg/mL) of vitrified/warmed oocytes. Oocyte developmental competence was determined after incorporation in an in vitro production system. [a] Percentages are calculated on IVF oocytes. [b] Percentages are calculated on fertilized oocytes. [c] Percentages are calculated on cleaved oocytes. * $\chi^2$ test P<0.5. This table has been modified from Berlinguer et al.[23]

**Table 2. Developmental competence of *in vitro* matured adult oocytes vitrified in vitrification media (16.5% ethylene glycol + 16.5% dimethyl sulfoxide) containing different calcium concentrations.** Survival and fertilization rates are calculated on vitrified oocytes; total cleavage and blastocyst rates are calculated on survived oocytes. Values with different subscript within the same column are significantly different: $\chi^2$ test P<0.05. This table has been modified from Succu et al.[10]

**Table 3. Fertilization and developmental rates after in vitro fertilization and culture of vitrified/warmed juvenile oocyte using high ([Ca 2++] = 9.9 mg/dL) and low ([Ca 2++] = 2.2 mg/dL) calcium concentration in vitrification media.** Different letters indicate statistical difference (a ≠ b P<0.05 $\chi^2$ test).

**Table 4. Cleavage rate and embryo output in vitrified/warmed adult oocytes fertilized at different time points of post-warming culture.** Different letters indicate statistical difference within the same column: ANOVA P<0.01.

**DISCUSSION:**

Oocyte cryopreservation in domestic animals can allow not only the long-term conservation of female genetic resources, but also advance the development of embryonic biotechnologies. Thus, the development of a standard method for oocyte vitrification would advantage both the livestock and the research sector. In this protocol, a complete method for adult sheep oocyte vitrification is presented and could represent a solid starting point for the development of an efficient vitrification system for juvenile oocyte.

One of the main advantages of the proposed method is that it includes all the steps from oocyte collection, in vitro maturation, vitrification, and warming. Moreover, it includes a post-warming culture period to allow oocytes to recover from the damages incurred during the vitrification procedure before being fertilized. The optimum time for fertilization should be tailored according to the method of cryopreservation, initial oocyte quality, patient age, and species, being essential to consider both aspects of time recovery and oocyte aging[25, 26]. Thus, choosing the duration of the post-warming incubation period is challenging and it may impact the outcome of oocyte vitrification programs. Based on the results obtained in terms of cleavage rates and embryo output, and under the conditions described in the presented protocol, the optimum time for fertilization of vitrified adult sheep oocytes is after 4 hours of post-warming incubation (**Table 4**)[6]. This information is crucial when designing an oocyte vitrification program.

This protocol, however, while giving acceptable results in terms of embryo output from vitrified/warmed adult oocytes, still leads to low to zero embryos if applied to juvenile oocytes. Several structural and functional limitations impair prepubertal oocyte developmental competence, such as small size, defective coupling between cumulus cells and oocytes, decrease in amino acid uptake, reduced protein synthesis and energy metabolism [27–29]. In a previous study we reported that prepubertal oocytes show high sensitivity to the vitrification procedure[30]. The low developmental competence shown after vitrification and warming is probably the result of damages to cytoplasmic factors involved in the reorganization of the cytoskeleton and (or) in the activation of maturation promoting factor[30]. As shown in **Table 1**, the supplementation of the maturation medium with trehalose, a non-permeable cryoprotectant, was able to increase survival rates after vitrification and warming to values comparable to those of adult oocytes[4]. In the same way, the use of vitrification solution with low calcium concentrations increases fertilization rates of juvenile oocytes after vitrification and warming, as shown in **Table 3**. Thus, both the optimization on culture conditions during in vitro maturation and of the vitrification media composition may help in increasing the quality of the juvenile oocyte after vitrification and warming. Juvenile oocytes show some ability to recover from the damages induced by the vitrification procedure, as shown in **Figure 3, 4 and 5**.

However, the high rates of spontaneous parthenogenetic activation during post-warming culture still limit their developmental potential. Ethylene glycol and DMSO, which are commonly used cryoprotective agents, may artificially activate the oocyte before the actual fertilization, thereby limiting fertilization success and embryo development. They can indeed cause a transient increase in intracellular $Ca^{2+}$ concentration[31], thus triggering cortical granule exocytosis, pronuclei formation, and meiotic resumption[32]. In fact, vitrification may artificially activate the oocyte before the actual fertilization, thereby limiting fertilization success and embryo development. Calcium chelator may thus be used to further limit calcium availability during the vitrification process with the aim of limiting the rate of spontaneous activation in juvenile oocytes.

It should also be considered that, unlike slow-freezing, vitrification is an exclusively manual technique and it is thus operator dependent[33, 34]. Thus, the availability of trained personnel is a

key factor for the success of this method. First of all, the operator has to properly select the oocytes to be vitrified. After IVM, oocytes are gently denuded of cumulus cells and evaluated under a stereomicroscope to select for cryopreservation only those with a uniform cytoplasm, homogeneous distribution of lipid droplets in the cytoplasm and with the outer diameter of about 90 µm. Moreover, only oocytes showing the extrusion on the first polar body, and thus at the MII stage, must be selected.

The morphological evaluation of the oocytes must be completed in a few minutes and being operator-dependant, it is very sensitive to variations in its proper implementation. To help standardize the selection procedure, the method suggests limiting the culture time for in vitro maturation to 22 h for adult oocytes. At this time point, sheep oocytes of high quality have already completed the first meiotic division[22] and can be selected for cryopreservation. This way the elimination of low-quality oocytes, which are the slowest ones in the completion of the first meiotic division, should be simpler.

The operator must also strictly respect the timing set for the vitrification procedure, from the first exposure to the cryoprotectant to the immersion in liquid nitrogen. Another critical step is the loading of the oocyte in the vitrification device used. The procedure must use minimum sample volumes to increase the cooling rate and to help cells pass through the phase transition temperature rapidly, thereby decreasing cryoinjuries[35]. Cryotop uses a polypropylene strip attached to a holder. In this method, the oocytes in the vitrification solution (<0.1 µL) are rapidly loaded with a glass capillary on top of the film strip. Then, the solution must be removed, leaving behind a thin layer sufficient to cover the cells to be cryopreserved. Once again, this step must be completed as fast as possible to limit the exposure of the oocytes to the high cryoprotectant concentrations of the vitrification solution, which can cause osmotic shock and are toxic to the cells.

For these reasons, a major challenge associated with this method is the need for manual handling and skilled technician. Other authors reported that the oocyte vitrification outcome appears influenced by a "learning curve" effect, as the acquisition of manual skills can significantly reduce the biological damage induce by the vitrification procedure[34]. Researchers should thus take into consideration the “operator effect” in the evaluation of the outcome of the vitrification procedure.

Further studies will focus both on standardizing the oocyte selection procedure and better tailoring media composition and culture conditions to the needs of the juvenile oocytes. At this regard, both the use of calcium chelator and antioxidants may offer promising opportunities. Similarly, the optimization of the protocol will allow increasing the developmental competence of vitrified/warmed adult oocytes.

**ACKNOWLEDGMENTS:**

The authors received no specific funding for this work. Professor Maria Grazia Cappai and Dr. Valeria Pasciu are gratefully acknowledged for the video voiceover and for setting up the lab during the video making.

**DISCLOSURES:**

The authors declare they have no competing financial interests.

**REFERENCES:**


1. Arav, A. Cryopreservation of oocytes and embryos. *Theriogenology*. **81** (1), 96–102, doi: 10.1016/j.theriogenology.2013.09.011 (2014).
2. Mullen, S.F., Fahy, G.M. A chronologic review of mature oocyte vitrification research in cattle, pigs, and sheep. *Theriogenology*. **78** (8), 1709–1719, doi: 10.1016/j.theriogenology.2012.06.008 (2012).
3. Hwang, I.S., Hochi, S. Recent progress in cryopreservation of bovine oocytes. *BioMed Research International*. **2014**, doi: 10.1155/2014/570647 (2014).
4. Berlinguer, F. *et al.* Effects of trehalose co-incubation on in vitro matured prepubertal ovine oocyte vitrification. *Cryobiology*. **55** (1), doi: 10.1016/j.cryobiol.2007.04.004 (2007).
5. Quan, G., Wu, G., Hong, Q. Oocyte Cryopreservation Based in Sheep: The Current Status and Future Perspective. *Biopreservation and Biobanking*. **15** (6), 535–547, doi: 10.1089/bio.2017.0074 (2017).
6. Succu, S. *et al.* A recovery time after warming restores mitochondrial function and improves developmental competence of vitrified ovine oocytes. *Theriogenology*. **110**, doi: 10.1016/j.theriogenology.2017.12.031 (2018).
7. Succu, S. *et al.* Vitrification of in vitro matured ovine oocytes affects in vitro pre-implantation development and mRNA abundance. *Molecular Reproduction and Development*. **75** (3), doi: 10.1002/mrd.20784 (2008).
8. Succu, S. *et al.* Vitrification Devices Affect Structural and Molecular Status of In Vitro Matured Ovine Oocytes. *Molecular Reproduction and Development*. **74**, 1337–1344, doi: 10.1002/mrd (2007).
9. Hosseini, S.M., Asgari, V., Hajian, M., Nasr-Esfahani, M.H. Cytoplasmic, rather than nuclear-DNA, insufficiencies as the major cause of poor competence of vitrified oocytes. *Reproductive BioMedicine Online*. doi: 10.1016/j.rbmo.2015.01.003 (2015).
10. Succu, S. *et al.* Calcium concentration in vitrification medium affects the developmental competence of in vitro matured ovine oocytes. *Theriogenology*. **75** (4), doi: 10.1016/j.theriogenology.2010.10.012 (2011).
11. Sanaei, B. *et al.* An improved method for vitrification of in vitro matured ovine oocytes; beneficial effects of Ethylene Glycol Tetraacetic acid, an intracellular calcium chelator. *Cryobiology*. **84**, 82–90, doi: 10.1016/j.cryobiol.2018.07.001 (2018).
12. Quan, G.B., Wu, G.Q., Wang, Y.J., Ma, Y., Lv, C.R., Hong, Q.H. Meiotic maturation and developmental capability of ovine oocytes at germinal vesicle stage following vitrification using different cryodevices. *Cryobiology*. **72** (1), 33–40, doi: 10.1016/j.cryobiol.2015.11.007 (2016).
13. Fernández-Reyez, F. et al. Viability, maturation and embryo development in vitro of immature porcine and ovine oocytes vitrified in different devices. *Cryobiology*. **64** (3), 261–266, doi: 10.1016/j.cryobiol.2012.02.009 (2012).
14. Ahmadi, E., Shirazi, A., Shams-Esfandabadi, N., Nazari, H. Antioxidants and glycine can improve the developmental competence of vitrified/warmed ovine immature oocytes. *Reproduction in Domestic Animals*. **54** (3), 595–603, doi: 10.1111/rda.13402 (2019).

15. Barrera, N. *et al.* Impact of delipidated estrous sheep serum supplementation on in vitro maturation, cryotolerance and endoplasmic reticulum stress gene expression of sheep oocytes. *PLoS ONE*. **13** (6), doi: 10.1371/journal.pone.0198742 (2018).
16. Walker, S.K., Hill, J.L., Kleemann, D.O., Nancarrow, C.D. Development of Ovine Embryos in Synthetic Oviductal Fluid Containing Amino Acids at Oviductal Fluid Concentrations. *Biology of Reproduction*. **55** (3), 703–708, doi: 10.1095/biolreprod55.3.703 (1996).
17. Kuwayama, M., Vajta, G., Kato, O., Leibo, S.P. Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive BioMedicine Online*. **11** (3), 300–308, doi: 10.1016/S1472-6483(10)60837-1 (2005).
18. Wu, X., Jin, X., Wang, Y., Mei, Q., Li, J., Shi, Z. Synthesis and spectral properties of novel chlorinated pH fluorescent probes. *Journal of Luminescence*. **131** (4), 776–780, doi: https://doi.org/10.1016/j.jlumin.2010.12.001 (2011).
19. Dell'Aquila, M.E. *et al.* Prooxidant effects of verbascoside, a bioactive compound from olive oil mill wastewater, on in vitro developmental potential of ovine prepubertal oocytes and bioenergetic/oxidative stress parameters of fresh and vitrified oocytes. *BioMed Research International*. **2014**, doi: 10.1155/2014/878062 (2014).
20. Gadau, S.D. Morphological and quantitative analysis on α-tubulin modifications in glioblastoma cells. *Neuroscience Letters*. **687**, 111–118, doi: 10.1016/j.neulet.2018.09.044 (2018).
21. Reyes, M.D. los, Palomino, J., Parraguez, V.H., Hidalgo, M., Saffie, P. Mitochondrial distribution and meiotic progression in canine oocytes during in vivo and in vitro maturation. *Theriogenology*. doi: 10.1016/j.theriogenology.2010.09.005 (2011).
22. Leoni, G.G. *et al.* Differences in the kinetic of the first meiotic division and in active mitochondrial distribution between prepubertal and adult oocytes mirror differences in their developmental competence in a sheep model. *PLoS ONE*. **10** (4), doi: 10.1371/journal.pone.0124911 (2015).
23. Berlinguer, F. *et al.* Effects of trehalose co-incubation on in vitro matured prepubertal ovine oocyte vitrification. *Cryobiology*. **55** (1), 27–34, doi: 10.1016/j.cryobiol.2007.04.004 (2007).
24. Serra, E., Gadau, S.D., Berlinguer, F., Naitana, S., Succu, S. Morphological features and microtubular changes in vitrified ovine oocytes. *Theriogenology*. **148** (xxxx), 216–224, doi: 10.1016/j.theriogenology.2019.11.007 (2020).
25. Asgari, V., Hosseini, S.M., Ostadhosseini, S., Hajian, M., Nasr-Esfahani, M.H. Time dependent effect of post warming interval on microtubule organization, meiotic status, and parthenogenetic activation of vitrified in vitro matured sheep oocytes. *Theriogenology*. **75** (5), 904–910, doi: https://doi.org/10.1016/j.theriogenology.2010.10.033 (2011).
26. Ciotti, P.M. et al. Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. *Fertility and Sterility*. **91** (6), 2399–2407, doi: https://doi.org/10.1016/j.fertnstert.2008.03.013 (2009).
27. Ledda, S., Bogliolo, L., Leoni, G., Naitana, S. Cell Coupling and Maturation-Promoting Factor Activity in In Vitro-Matured Prepubertal and Adult Sheep Oocytes1. *Biology of Reproduction*. **65** (1), 247–252, doi: 10.1095/biolreprod65.1.247 (2001).
28. Palmerini, M.G. *et al.* In vitro maturation is slowed in prepubertal lamb oocytes: ultrastructural evidences. *Reproductive Biology and Endocrinology*. **12**, doi: 10.1186/1477-7827-12-115 (2014).

29. Leoni, G.G. *et al.* Relations between relative mRNA abundance and developmental competence of ovine oocytes. *Molecular Reproduction and Development*. **74** (2), 249–257, doi: 10.1002/mrd.20442 (2007).
30. Succu, S. *et al.* Effect of vitrification solutions and cooling upon in vitro matured prepubertal ovine oocytes. *Theriogenology*. **68** (1), 107–114, doi: 10.1016/j.theriogenology.2007.04.035 (2007).
31. Larman, M.G., Sheehan, C.B., Gardner, D.K. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. *Reproduction*. **131** (1), 53–61, doi: 10.1530/rep.1.00878 (2006).
32. Yeste, M., Jones, C., Amdani, S.N., Patel, S., Coward, K. Oocyte activation deficiency: a role for an oocyte contribution? *Human Reproduction Update*. **22** (1), 23–47, doi: 10.1093/humupd/dmv040 (2016).
33. Rienzi, L. *et al.* Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Human Reproduction Update*. **23** (2), 139–155, doi: 10.1093/humupd/dmw038 (2017).
34. De Santis, L. et al. Oocyte vitrification: influence of operator and learning time on survival and development parameters. *Placenta*. **32**, S280–S281, doi: https://doi.org/10.1016/j.placenta.2011.07.025 (2011).
35. Zhang, X., Catalano, P.N., Gurkan, U.A., Khimji, I., Demirci, U. Emerging technologies in medical applications of minimum volume vitrification. *Nanomedicine (London, England)*. **6** (6), 1115–1129, doi: 10.2217/nnm.11.71 (2011).

Figure 1



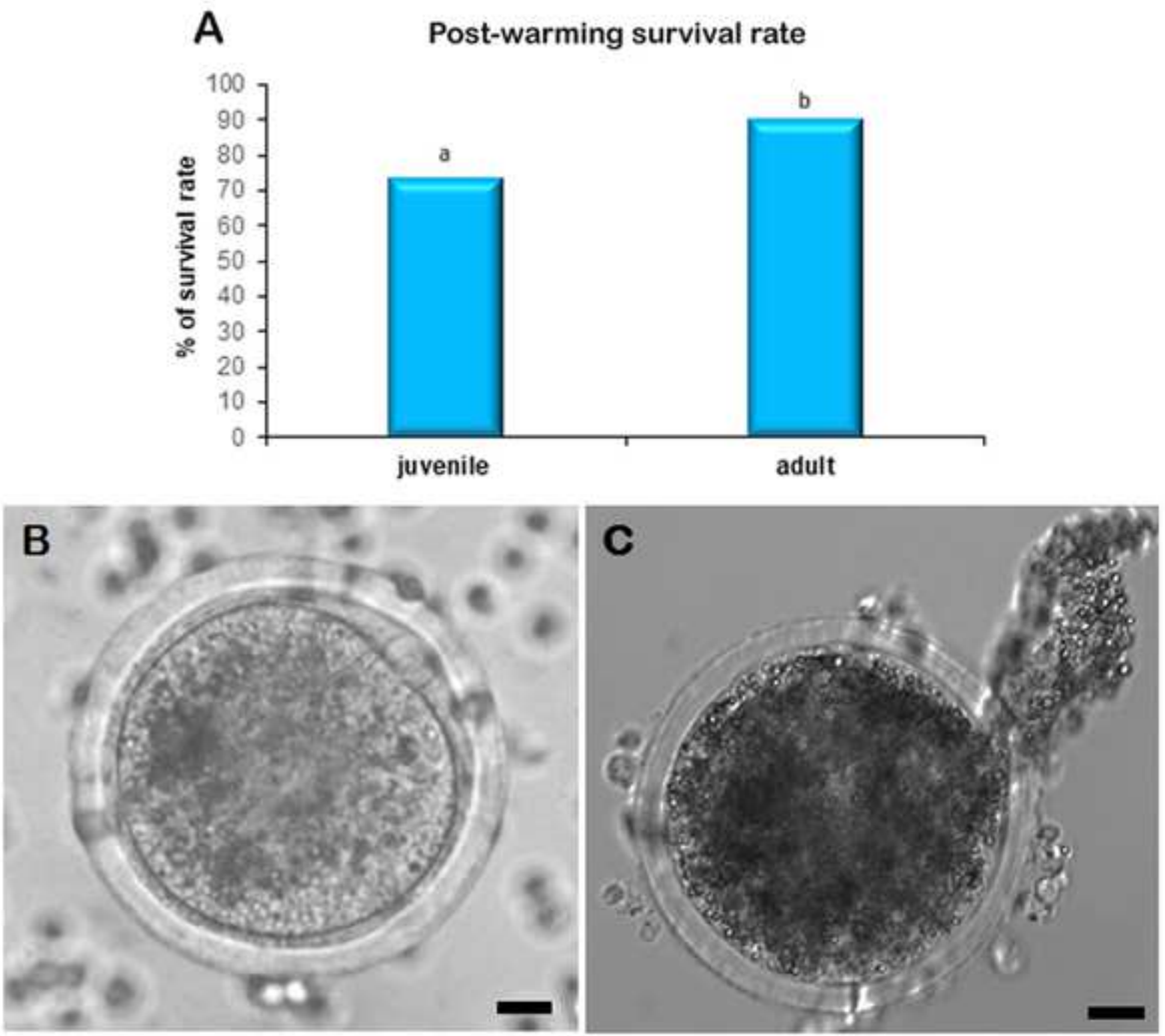


Figure 2



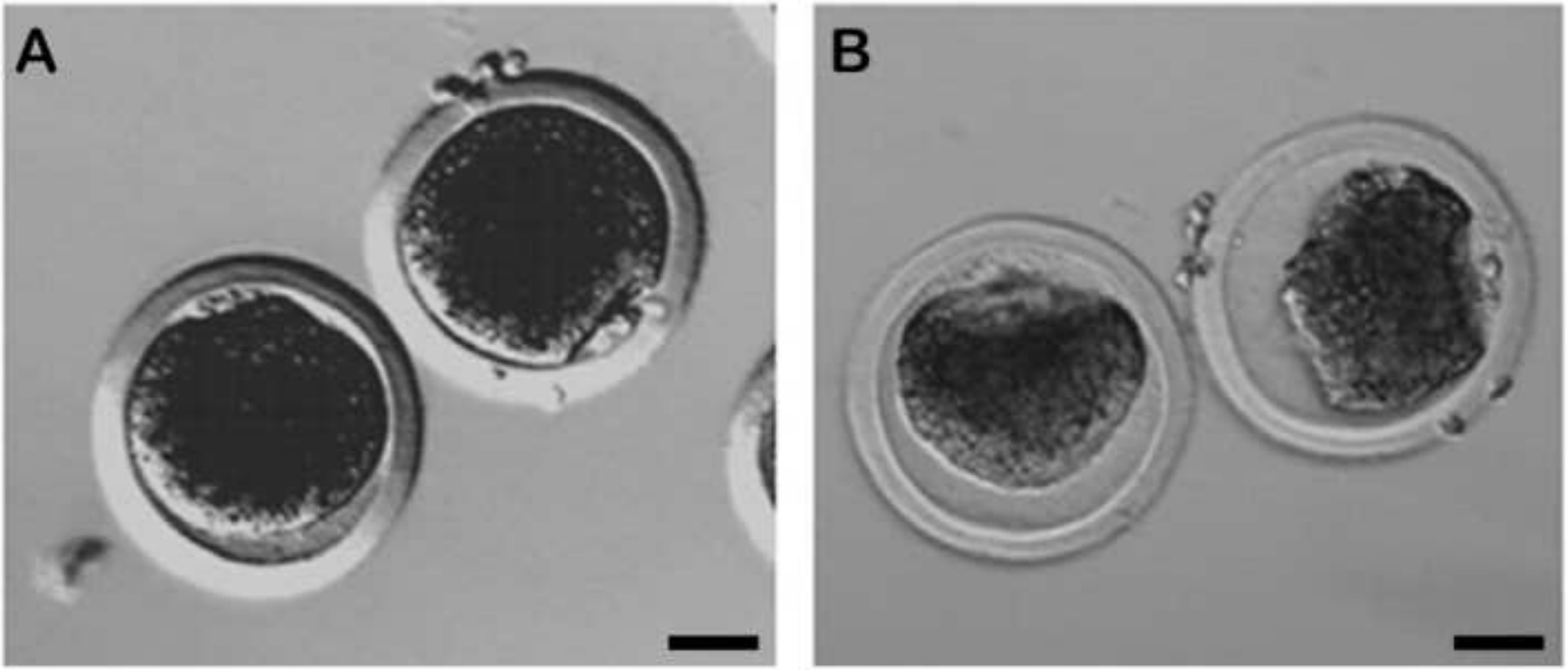


Figure 3

Click here to access/download;Figure;Fig 3.psd

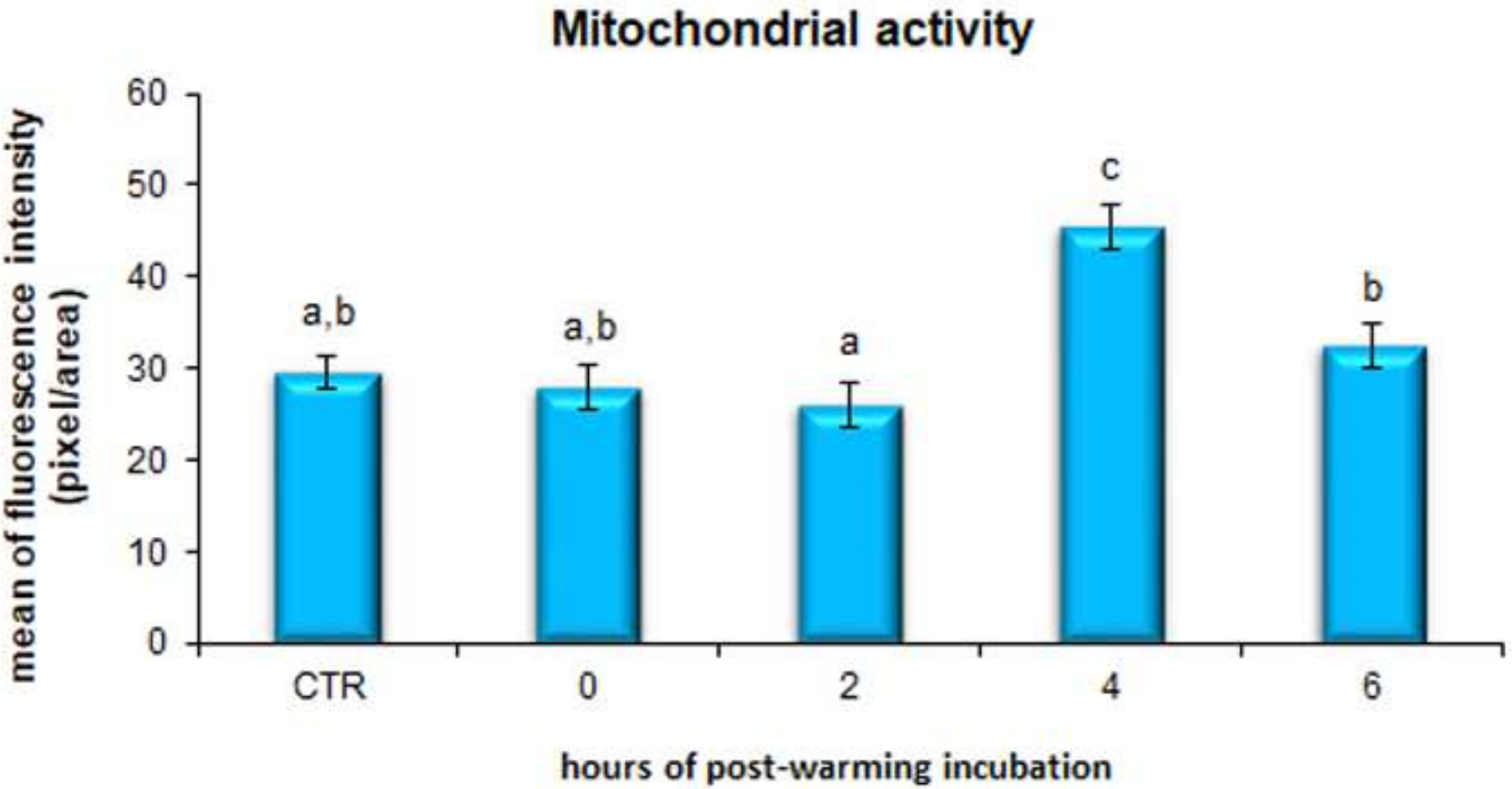


Figure 4

Click here to access/download;Figure;Fig 4.psd

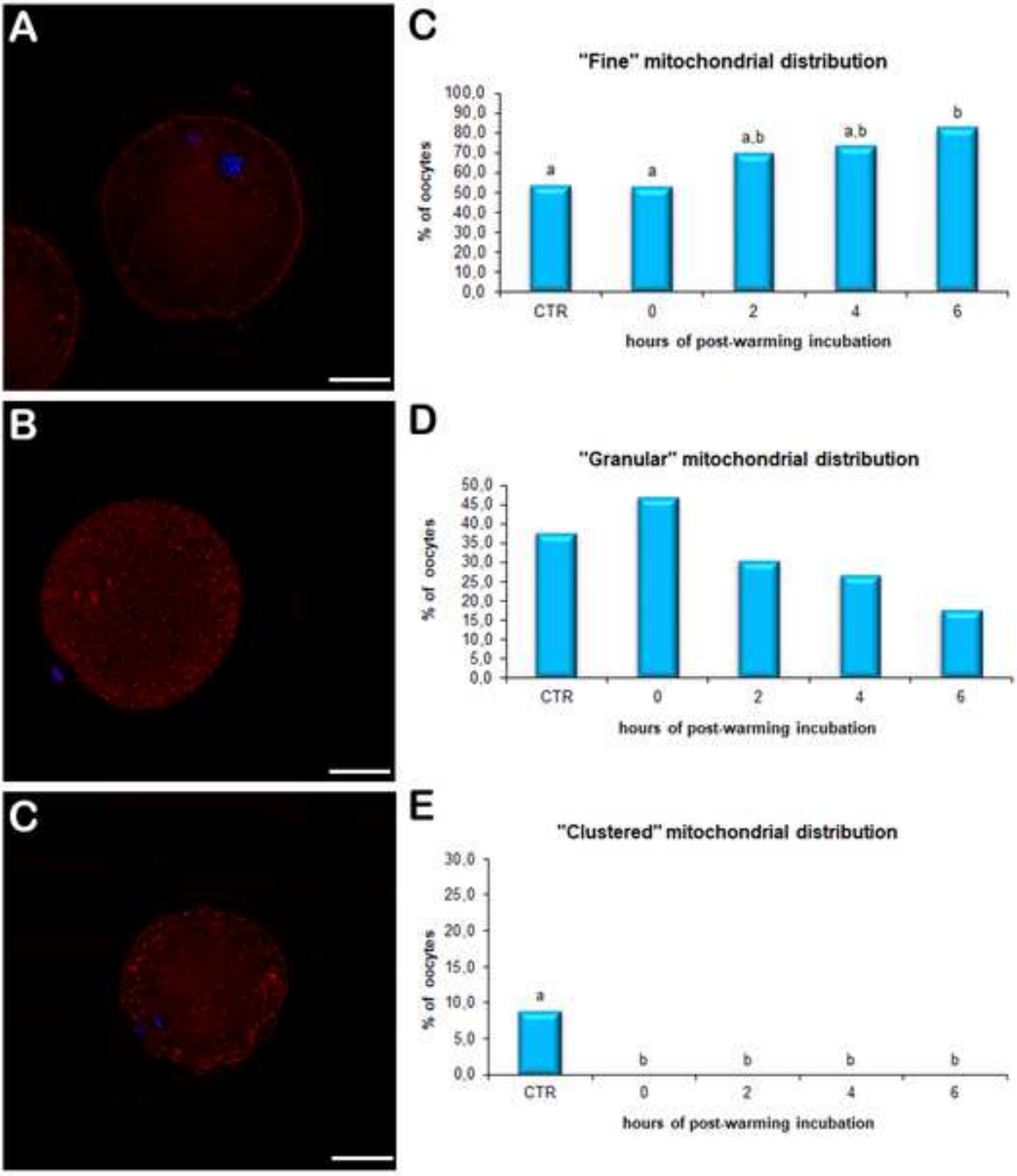
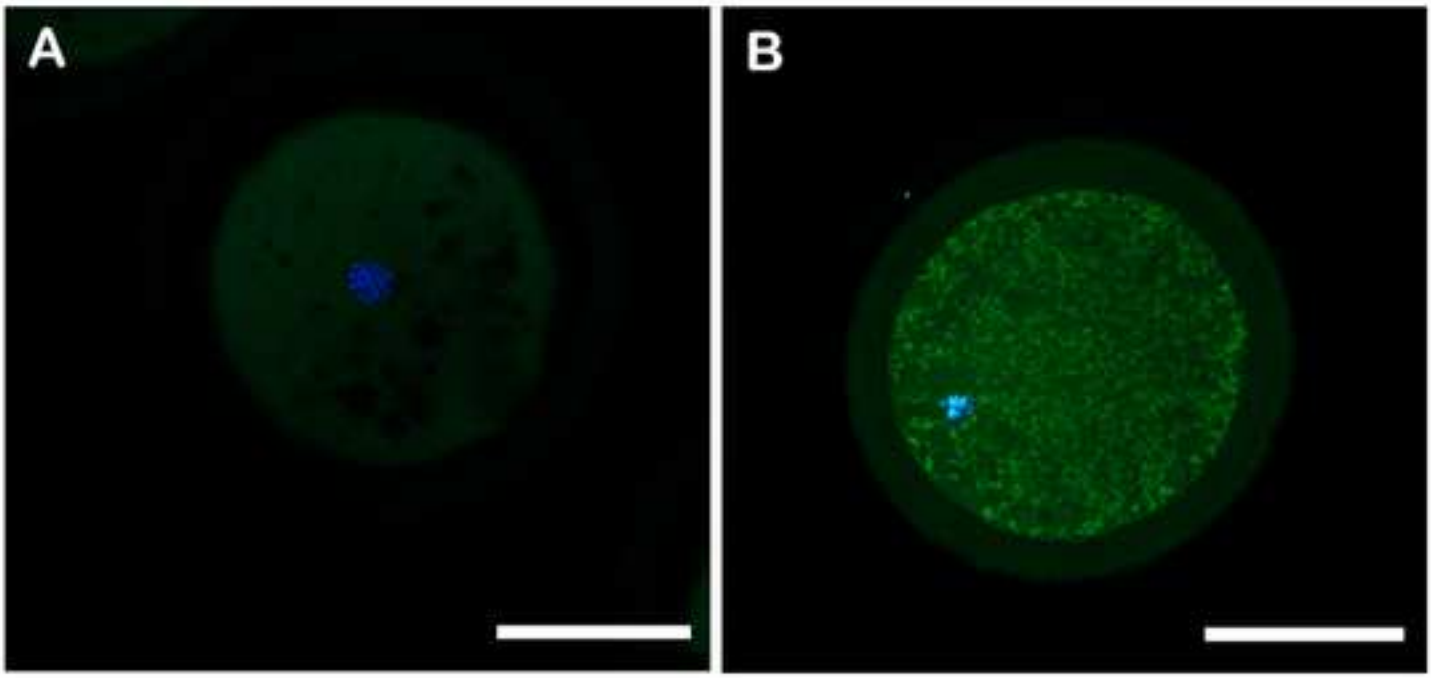


Figure 5





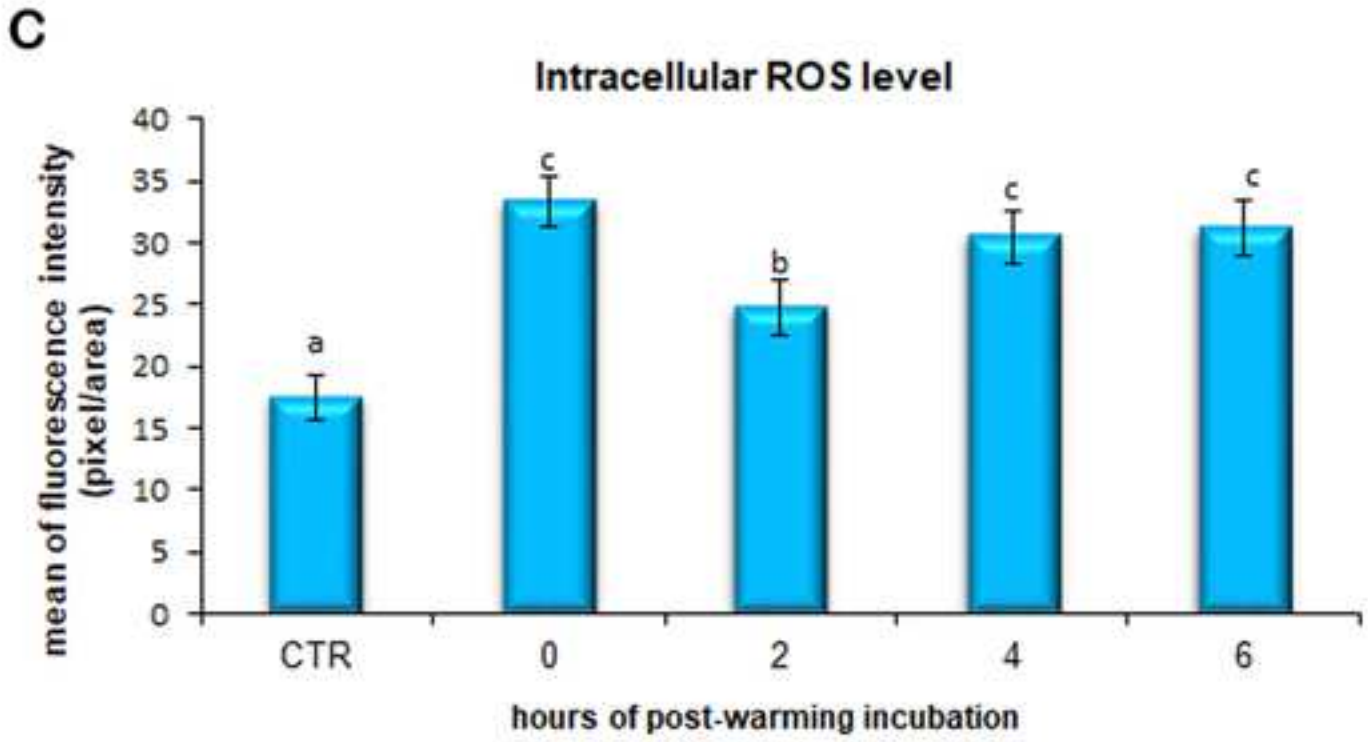
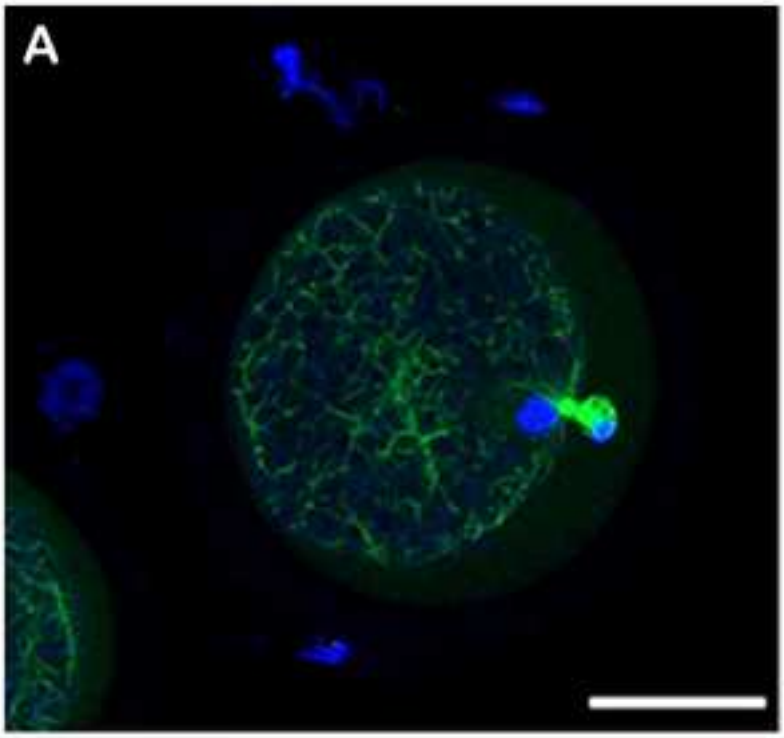
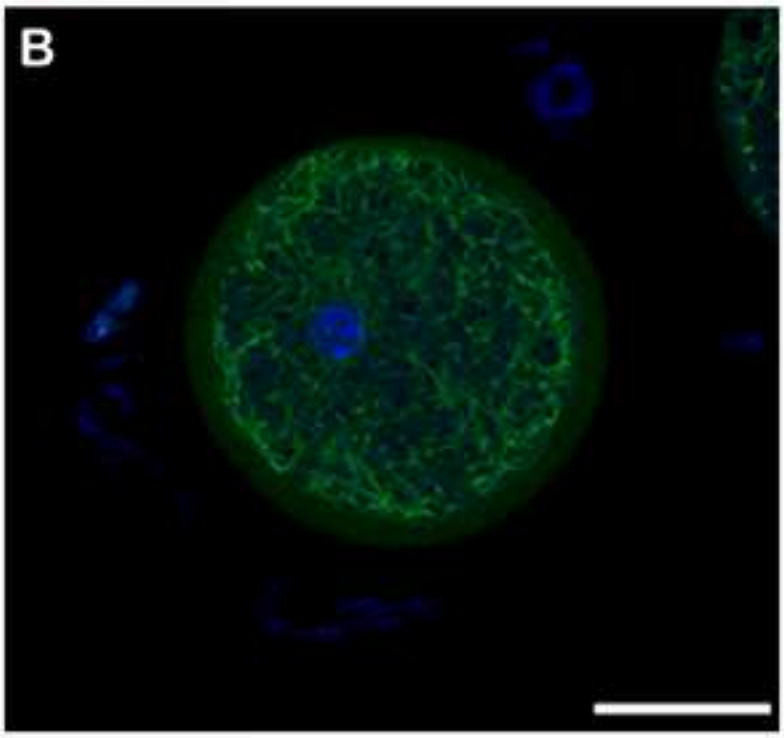
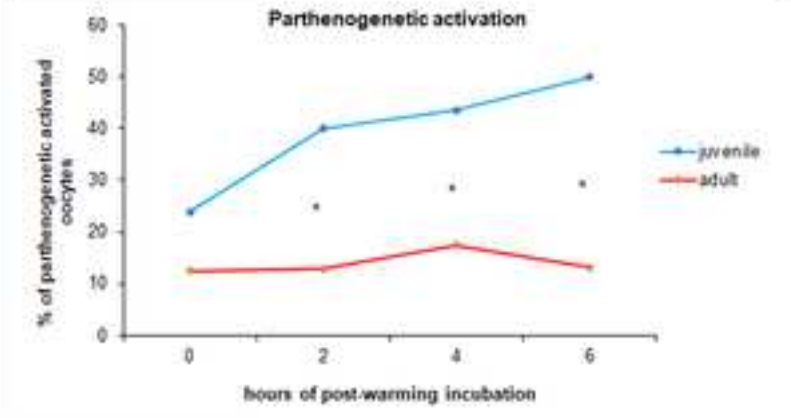



Figure 6

Click here to access/download;Figure;Fig 6.psd



Table



| | Oocytes (n) | Survival rate (%) | IVF (n) | Fertilized[a] (%) | Cleaved[b] (%) | Blastocysts[c] (%) |
|---|---|---|---|---|---|---|
| CTR | 73 | 75.3* | 452 | 91.1 | 96.1 | 14.3 |
| TRH | 77 | 85.7 | 470 | 92.5 | 95.4 | 13 |

* Chi square test $p<0.5$

[a] Percentages are calculated on IVF oocytes

[b] Percentages are calculated on fertilized oocytes

[c] Percentages are calculated on cleaved oocytes

| Groups | $[Ca^{++}]$ mg/dL | N oocyte | Spontaneous parthenogenetic activation (%) | Vitrified oocytes |
|---|---|---|---|---|
| TCM/FCS | 9.9 | 80 | 33 (41.2)[a] | 150 |
| PBS/FCS | 4.4 | 82 | 29 (35.3)[ac] | 115 |
| $PBS^{CaMg\ free}$/FCS | 2.2 | 86 | 11 (12.7)[b] | 126 |
| PBS/BSA | 3.2 | 83 | 21 (25.3)[c] | 110 |
| $PBS^{CaMg\ free}$/BSA | 0.4 | 87 | 10 (11.5)[b] | 149 |

| Survived and IVF oocytes (%) | Cleavage (%) | Blastocysts output (%) |
|---|---|---|
| 124 (82.7)[a] | 40 (32.5)[a] | 2 (1.6)[a] |
| 88 (76.5)[a] | 33 (37.5)[ae] | 1 (1.1)[a] |
| 115 (91.3)[b] | 74 (64.3)[b] | 12 (10.4)[b] |
| 90 (81.8)[a] | 18 (20)[c] | 0 (0)[a] |
| 123 (82.5)[a] | 57 (46.3)[de] | 3 (2.4)[b] |

| [$Ca^{2++}$] in vitrification media | No. Oocytes | Post-vitrification survival rate (%) | Fertilization rate (%) | Cleavage (%) | Blastocyst output (%) |
|---|---|---|---|---|---|
| High [9.9 mg/dL] | 190 | 161 (84.73) | 52/161 (32.29)[a] | 43/161 (26.7) | 0 |
| Low [2.2 mg/dL] | 150 | 124 (82.66) | 55/124 (44.35)[b] | 41/124 (33.1) | 0 |

| Hours of post-warming incubation | Cleavage rate (*n* ) | Embryo output (*n* ) |
|---|---|---|
| 0 | 19.2 ± 3%[a] (*82* ) | 0%[a] (*17* ) |
| 2 | 41.8 ± 3%[b] (*100* ) | 6.5 ± 1.3%[b] (*42* ) |
| 4 | 50.7 ± 3%[b] (*92* ) | 14.4 ± 1.3%[c] (*48* ) |
| 6 | 26 ± 3%[a] (*92* ) | 0%[a] (*23* ) |

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 2',7'-Dichlorofluorescin diacetate | Sigma-Aldrich | D-6883 | |
| Albumin bovine fraction V, protease free | Sigma-Aldrich | A3059 | |
| Bisbenzimide H 33342 trihydrochloride (Hoechst 33342) | Sigma-Aldrich | 14533 | |
| Calcium chloride (CaCl2 2H20) | Sigma-Aldrich | C8106 | |
| Citric acid | Sigma-Aldrich | C2404 | |
| Confocal laser scanning microscope | Leica Microsystems GmbH,Wetzlar | TCS SP5 DMI 6000CS | |
| Cryotop Kitazato | Medical Biological Technologies | | |
| Cysteamine | Sigma-Aldrich | M9768 | |
| D- (-) Fructose | Sigma-Aldrich | F0127 | |
| D(+)Trehalose dehydrate | Sigma-Aldrich | T0167 | |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D2438 | |
| Dulbecco Phosphate Buffered Saline | Sigma-Aldrich | D8537 | |
| Egg yolk | Sigma-Aldrich | P3556 | |
| Ethylene glycol (EG) | Sigma-Aldrich | 324558 | |
| FSH | Sigma-Aldrich | F4021 | |
| Glutamic Acid | Sigma-Aldrich | G5638 | |
| Glutaraldehyde | Sigma-Aldrich | G5882 | |
| Glycerol | Sigma-Aldrich | G5516 | |
| Glycine | Sigma-Aldrich | G8790 | |
| Heparin | Sigma-Aldrich | H4149 | |
| HEPES | Sigma-Aldrich | H4034 | |
| Hypoutarine | Sigma-Aldrich | H1384 | |
| Inverted microscope | Diaphot, Nikon | | |
| L-Alanine | Sigma-Aldrich | A3534 | |
| L-Arginine | Sigma-Aldrich | A3784 | |
| L-Asparagine | Sigma-Aldrich | A4284 | |
| L-Aspartic Acid | Sigma-Aldrich | A4534 | |
| L-Cysteine | Sigma-Aldrich | C7352 | |
| L-Cystine | Sigma-Aldrich | C8786 | |

| | | |
|---|---|---|
| L-Glutamine | Sigma-Aldrich | G3126 |
| LH | Sigma-Aldrich | L6420 |
| L-Histidine | Sigma-Aldrich | H9511 |
| L-Isoleucine | Sigma-Aldrich | I7383 |
| L-Leucine | Sigma-Aldrich | L1512 |
| L-Lysine | Sigma-Aldrich | L1137 |
| L-Methionine | Sigma-Aldrich | M2893 |
| L-Ornithine | Sigma-Aldrich | O6503 |
| L-Phenylalanine | Sigma-Aldrich | P5030 |
| L-Proline | Sigma-Aldrich | P4655 |
| L-Serine | Sigma-Aldrich | S5511 |
| L-Tyrosine | Sigma-Aldrich | T1020 |
| L-Valine | Sigma-Aldrich | V6504 |
| Magnesium chloride heptahydrate ($MgSO_4.7H_2O$) | Sigma-Aldrich | M2393 |
| Makler Counting Chamber | Sefi-Medical Instruments ltd.Biosigma S.r.l. | |
| Medium 199 | Sigma-Aldrich | M5017 |
| Mineral oil | Sigma-Aldrich | M8410 |
| MitoTracker Red CM-$H_2$XRos | ThermoFisher | M7512 |
| New born calf serum heat inactivated (FCS) | Sigma-Aldrich | N4762 |
| Penicillin G sodium salt | Sigma-Aldrich | P3032 |
| Phenol Red | Sigma-Aldrich | P3532 |
| Polyvinyl alcohol (87-90% hydrolyzed, average mol wt 30,000-70,000) | Sigma-Aldrich | P8136 |
| Potassium Chloride (KCl) | Sigma-Aldrich | P5405 |
| Potassium phosphate monobasic ($KH_2PO_4$) | Sigma-Aldrich | P5655 |
| Propidium iodide | Sigma-Aldrich | P4170 |
| Sheep serum | Sigma-Aldrich | S2263 |
| Sodium azide | Sigma-Aldrich | S2202 |
| Sodium bicarbonate ($NaHCO_{3)}$ | Sigma-Aldrich | S5761 |
| Sodium chloride (NaCl) | Sigma-Aldrich | S9888 |

| | | |
|---|---|---|
| Sodium dl-lactate solution syrup | Sigma-Aldrich | L4263 |
| Sodium pyruvate | Sigma-Aldrich | P2256 |
| Sperm Class Analyzer | Microptic S.L. | S.C.A. v 3.2.0 |
| Statistical software Minitab 18.1 | 2017 Minitab | |
| Stereo microscope | Olimpus | SZ61 |
| Streptomycin sulfate | Sigma-Aldrich | S9137 |
| Taurine | Sigma-Aldrich | T7146 |
| TRIS | Sigma-Aldrich | 15,456-3 |

Dear Editor,

Thank you for considering our manuscript “Vitrification of adult and juvenile sheep oocytes: challenges and opportunities” for a possible publication in JOVE. We have modified the manuscript according to your suggestions, as below explained.

Yours sincerely,

Fiammetta Berlinguer

**Editorial comments:**

Changes for the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: we proofread the manuscript and checked grammar and spelling

2. Please revise the following lines to avoid previously published work: 29-30, 32-34, 38-42.

Answer: we revised the above-listed lines as suggested.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 16, 2020

---

This Agreement between University of Sassari -- Fiammetta Berlinguer ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

| | |
|---|---|
| License Number | 4950801172343 |
| License date | Nov 16, 2020 |
| Licensed Content Publisher | Elsevier |
| Licensed Content Publication | Theriogenology |
| Licensed Content Title | Morphological features and microtubular changes in vitrified ovine oocytes |
| Licensed Content Author | Elisa Serra,Sergio Domenico Gadau,Fiammetta Berlinguer,Salvatore Naitana,Sara Succu |
| Licensed Content Date | May 1, 2020 |
| Licensed Content Volume | 148 |
| Licensed Content Issue | n/a |
| Licensed Content Pages | 9 |
| Start Page | 216 |
| End Page | 224 |
| Type of Use | reuse in a journal/magazine |
| Requestor type | academic/educational institute |

| | |
|---|---|
| Portion | figures/tables/illustrations |
| Number of figures/tables/illustrations | 1 |
| Format | electronic |
| Are you the author of this Elsevier article? | Yes |
| Will you be translating? | No |
| Title of new article | Vitrification of adult and juvenile sheep oocytes: challenges and opportunities |
| Lead author | Sara Succu |
| Title of targeted journal | Journal of Visualized Experiments |
| Publisher | United States MY JoVE Corporation |
| Expected publication date | Jan 2021 |
| Portions | figure 3 |
| Requestor Location | University of Sassari<br>via Vienna, 2<br><br>Sassari, 07100<br>Italy<br>Attn: University of Sassari |
| Publisher Tax ID | GB 494 6272 12 |
| Customer VAT ID | IT00196350904 |
| Total | 0.00 USD |

Terms and Conditions

## INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

## GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

## LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

**Posting licensed content on Electronic reserve**: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

**Preprints:**

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

**Accepted Author Manuscripts:** An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

**Published journal article (JPA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

## Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

**Terms & Conditions applicable to all Open Access articles published with Elsevier:**

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

**Additional Terms & Conditions applicable to each Creative Commons user license:**

**CC BY:** The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant

DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

**CC BY NC SA:** The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

**CC BY NC ND:** The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.10

**Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.**

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 16, 2020

---

This Agreement between University of Sassari -- Fiammetta Berlinguer ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

| | |
|---|---|
| License Number | 4950800617252 |
| License date | Nov 16, 2020 |
| Licensed Content Publisher | Elsevier |
| Licensed Content Publication | Cryobiology |
| Licensed Content Title | Effects of trehalose co-incubation on in vitro matured prepubertal ovine oocyte vitrification |
| Licensed Content Author | F. Berlinguer,S. Succu,F. Mossa,M. Madeddu,D. Bebbere,G.G. Leoni,S. Naitana |
| Licensed Content Date | Aug 1, 2007 |
| Licensed Content Volume | 55 |
| Licensed Content Issue | 1 |
| Licensed Content Pages | 8 |
| Start Page | 27 |
| End Page | 34 |
| Type of Use | reuse in a journal/magazine |

| | |
|---|---|
| Requestor type | academic/educational institute |
| Portion | figures/tables/illustrations |
| Number of figures/tables/illustrations | 2 |
| Format | electronic |
| Are you the author of this Elsevier article? | Yes |
| Will you be translating? | No |
| Title of new article | Vitrification of adult and juvenile sheep oocytes: challenges and opportunities |
| Lead author | Sara Succu |
| Title of targeted journal | Journal of Visualized Experiments |
| Publisher | United States MY JoVE Corporation |
| Expected publication date | Jan 2021 |
| Portions | table 1, figure 2 |
| Requestor Location | University of Sassari<br>via Vienna, 2<br><br>Sassari, 07100<br>Italy<br>Attn: University of Sassari |
| Publisher Tax ID | GB 494 6272 12 |
| Customer VAT ID | IT00196350904 |
| Total | 0.00 EUR |

Terms and Conditions

## INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

## GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

## LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

**Posting licensed content on Electronic reserve**: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to

bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

**Preprints:**

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

**Accepted Author Manuscripts:** An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

**Published journal article (JPA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

## Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

**Terms & Conditions applicable to all Open Access articles published with Elsevier:**

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

**Additional Terms & Conditions applicable to each Creative Commons user license:**

**CC BY:** The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

**CC BY NC SA:** The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

**CC BY NC ND:** The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.10

**Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.**

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 16, 2020

---

This Agreement between University of Sassari -- Fiammetta Berlinguer ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

| | |
|---|---|
| License Number | 4950800980736 |
| License date | Nov 16, 2020 |
| Licensed Content Publisher | Elsevier |
| Licensed Content Publication | Theriogenology |
| Licensed Content Title | Calcium concentration in vitrification medium affects the developmental competence of in vitro matured ovine oocytes |
| Licensed Content Author | Sara Succu,Fiammetta Berlinguer,Giovanni G. Leoni,Daniela Bebbere,Valentina Satta,Francisco Marco-Jimenez,Valeria Pasciu,Salvatore Naitana |
| Licensed Content Date | Mar 1, 2011 |
| Licensed Content Volume | 75 |
| Licensed Content Issue | 4 |
| Licensed Content Pages | 7 |
| Start Page | 715 |
| End Page | 721 |
| Type of Use | reuse in a journal/magazine |

| | |
|---|---|
| Requestor type | academic/educational institute |
| Portion | figures/tables/illustrations |
| Number of figures/tables/illustrations | 2 |
| Format | electronic |
| Are you the author of this Elsevier article? | Yes |
| Will you be translating? | No |
| Title of new article | Vitrification of adult and juvenile sheep oocytes: challenges and opportunities |
| Lead author | Sara Succu |
| Title of targeted journal | Journal of Visualized Experiments |
| Publisher | United States MY JoVE Corporation |
| Expected publication date | Jan 2021 |
| Portions | table 2, table 3 |
| Requestor Location | University of Sassari<br>via Vienna, 2<br><br>Sassari, 07100<br>Italy<br>Attn: University of Sassari |
| Publisher Tax ID | GB 494 6272 12 |
| Customer VAT ID | IT00196350904 |
| Total | 0.00 USD |

Terms and Conditions

## INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

## GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

## LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

**Posting licensed content on Electronic reserve**: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to

bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

**Preprints:**

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

**Accepted Author Manuscripts:** An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

**Published journal article (JPA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

## Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

**Terms & Conditions applicable to all Open Access articles published with Elsevier:**

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

**Additional Terms & Conditions applicable to each Creative Commons user license:**

**CC BY:** The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

**CC BY NC SA:** The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

**CC BY NC ND:** The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.10

**Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.**