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Cryopreservation of okapi (*Okapia johnstoni*) oocytes following in vitro maturation

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ABSTRACT

Okapi (*Okapia johnstoni*) belong to the Giraffidae family and is the only representative of the *Okapia* genus. The species has been declared as endangered and due to its very elusive nature, little is known about its reproductive behavior. The wild population has continued to decrease over the last decades despite the different conservation programs developed worldwide. Captive breeding is difficult and the European population is not self-sustaining. An alternative tool to prevent the extinction of this charismatic species is the use of assisted reproductive technologies (ART). Specifically, we introduce a novel protocol for the in vitro maturation (IVM) and cryopreservation of matured oocytes obtained post-mortem. Out of sixteen cumulus-oocytes complexes isolated from one deceased individual, four matured metaphase II (MII) oocytes were successfully obtained following IVM. Furthermore, these matured oocytes demonstrated resilience during the vitrification/warming process and underwent sperm injection. Although no embryos were obtained, this study represents the first step towards the application of ART in okapi, providing a foundation for future reproductive interventions aimed at preserving self-sustaining captive populations.

1. Introduction

Okapi (*Okapia johnstoni*), also known as forest giraffes, Congolese giraffes and Zebra giraffes, are the only representative of the *Okapia* genus, in the Giraffidae family. Their geographic distribution ranges from central, to Northern and Eastern parts of the Democratic Republic of the Congo [1,2]. They live in rainforests, where they can easily camouflage with vegetation, thanks to their characteristic skin pattern [3]. Even though the first written report of this charismatic species was a little over a century ago [4], since 2013 it has been listed as "endangered" by the International Union for Conservation of Nature (IUCN) [5]. Major threats to the survival of this elusive Giraffidae are progressive habitat loss and fragmentation [6], hunting for meat consumption and skin collection [7]. Moreover, Congolese political instability [2] and extractive industries [8] play a role in the continuous decrease of the wild population.

Starting from 1987, the Okapi Conservation Project (OCP) was established, focused on rain forests protection and of the culture of local indigenous people, with the construction of capacity buildings, education on agroforestry and community assistance. In 1985, the Wildlife Conservation Society (WCS) initiated a field programme in Ituri forest, including the first field-collared okapi, which led to the establishment in 1992 of the okapi wildlife reserve. The main conservation efforts are oriented toward habitat protection, and several international organizations, included OCP and WCS are working with the Institut Congolais pour la Conservation de la Nature (ICCN) trying to re-established a sustainable wild population. The first-ever species-wide Okapi conservation strategy was developed at a workshop organized by the ICCN in 2013 in the DRC, but despite the commitment and the conservation actions taken to slow down the continuous wild population decline, the trend is still not reversed, with a rate of decline estimated to exceed 50% in 24 years [1]. The extinction of a species from the ecosystem may have

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unpredictable effect on a larger scale, due to a phenomenon known as vortex effect [9]. Therefore, it is crucial to use all our knowledge to prevent the extinction of wild species. In order to do so, different conventional conservation strategies, such as hunting bans, habitat protection [10], translocation of individuals [11], and tailored breeding programs can be applied. For example, thanks to the implementation of such actions, it was possible to rescue, the southern white rhinoceros (*Ceratotherium simum simum*), which was on the brink of the extinction at the beginning of the 1900 s. Sometimes, though, the implementation of aforementioned conservation strategies is not always effective, especially when the number of founder individuals is low [12], or the reproductive behaviors of the targeted species are not well known, like in the okapi.

Our knowledge of the okapi's reproductive physiology comes mainly from studies done in the captive population. The length of the oestrous cycle has been determined through the monitoring of urinary hormones, and it has been estimated to be approximately 15 days, with 7–10 days of follicular phase and 6–8 days of luteal phase [13]. These data are also supported by O`Hanlon and colleagues [14] in a more recent study. However, the monitoring of hormones from faeces suggested a shorter follicular phase, about 5.3 days [15]. Gestation duration is ca. 14 months [16], at the end of which females give birth to one calf. Twins were observed in extremely rare occasions [6].

Taking into account the limited data on okapi's reproductive behaviour, their big body size (180–356 kg, [6]), and the unknown effect that stress could have on their behaviour, the relocation of individuals for tailored mating programs is technically challenging.

In order to avoid these problems, an alternative conservational strategy is offered by assisted reproductive technologies (ART). This approach could be applied as conservational tool in those cases in which conventional conservation strategies are technically and/or practically challenging [17]. Among ART, cryopreservation of gametes and embryos has been proven to be a vital tool for long storage and transport of genetic material [9]. Moreover, preserving genetic material from deceased individuals is important since it allows to safeguard and increase the genetic diversity of endangered species. If oocytes or spermatozoa are not available at the same moment to allow immediate fertilization, cryopreservation techniques play a crucial role.

Here, for the first time, we present a protocol for in vitro maturation (IVM) of okapi's cumulus-oocyte-complexes (COCs), opportunistically retrieved from ovaries post-mortem. Since at the time of obtaining the ovaries, no sperm was available, matured oocytes were vitrified and stored in liquid nitrogen. When a sperm sample became accessible, oocytes were successfully warmed and used for fertilization via piezo driven intracytoplasmic sperm injection (piezo-ICSI).

2. Materials and methods

2.1. Ethics statement

No prior ethical approval was required for this study, since both ovaries and testes from okapis were opportunistically obtained post-mortem.

2.2. Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Missouri, USA). COCs and oocytes outside the incubator were handled in manipulation medium based on TCM 199 supplemented with 20 mM HEPES and 10% (v/v) fetal calf serum (FCS).

2.3. Retrieval of ovaries

The 12-year-old female okapi was kept at ZooParc de Beauval (France), as part of the European captive breeding program (EEP). After a miscarriage, she underwent a check-up for reproductive diseases and/or malformations. The analysis revealed the presence of four large endometrial cysts within the corpus uteri, derived most likely from her abortion, which needed to be removed by aspiration. To allow the aspiration without any danger for the animal, her estrous cycle was down-regulated with two injections of 480 mg each of degarelix (Firmagon®, Ferring Pharmaceutical, Denmark), a GnRH antagonist (tot 960 mg) over a two-month period. During the medical procedure, the animal accidentally died under anesthesia due to the aspiration of ruminal content. Ovaries were immediately retrieved post-mortem and transported to the laboratory located in Berlin in Euroflush medium (IMV technologies, France) at room temperature within eight hours from excision.

2.4. COCs collection and IVM

Once in the lab, the ovaries were washed twice in pre-warmed phosphate buffer saline (PBS - Biowest, France) and they underwent immediate and repeated slicing with the use of a disposable sterile scalpel blade; follicular fluid with COCs was released in a Petri dish filled with pre-warmed Euroflush medium. COCs were analyzed under a stereomicroscope on a heating plate (37℃). Only oocytes with regular ooplasm, intact oolema and with several layers of granulosa cells, were selected for IVM, which was performed in commercial BO-IVM medium (IVF bioscience, UK) under mineral oil, 38.8℃ at 6% CO_2 in humidified air. BO-IVM was chosen because for this study the bovine was the livestock species used as model. After 24 h of culture, COCs were mechanically and enzymatically denuded with fine glass pipette in hyaluronidase solution (80 U/ml in TCM199 + 20 mM HEPES) to check for the presence of polar body (PB). Since no PB was observed, denuded oocytes were put again in the IVM culture and assessed every 6 h for the presence of PB. After 48 h, visible PB in 4 oocytes were clearly recognized (MII stage). Images of denuded oocytes were analyzed via ImageJ software for measuring oocytes' diameter, comprehensive of the zona pellucida (ZP), and thickness of the same.

2.5. Vitrification and warming of oocytes

Oocytes in MII stage were cryopreserved using Chian et al., 2004 [18] protocol for bovine oocytes vitrification. The base solution (BS) was composed of PBS supplemented with 1 mg/ml glucose, 0.11 mg/ml sodium pyruvate, 20% (v/v) FCS and 5 mg/ml gentamicin. The equilibration solution (ES) was prepared with 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethyl sulfoxide (DMSO) in BS. The vitrification solution (VS) was composed of 15% (v/v) ethylene glycol, 15% (v/v) DMSO and 0.5 M sucrose in BS. All steps were performed at room temperature. Denuded oocytes with visible PB were equilibrated for 5 min in ES and then transferred to VS for 45–60 seconds. Within this last step, oocytes were individually loaded on Cryotops (Kitazato, Japan) and directly plunged into liquid nitrogen.

Upon sperm availability, oocytes were warmed. Used solutions for warming were thawing solution (TS), 1 M sucrose in BS and diluent solution (DS), 0.5 M sucrose in BS. The warming procedure was performed at 37°C. Cryotops were directly submerged into TS for one minute. Warmed oocytes were then transferred to DS for 3 min, washed twice in BS for 5 min and cultured for 2 h in BO-IVC medium (IVF Bioscience, UK) before injection.

2.6. Sperm preparation and fertilization

Epididymal sperm was collected from a euthanized 15-year-old male from Wilhelma Zoologisch-Botanischer Garten (Stuttgart, Germany). Cauda epididymis were minced in petri dish using a sterile scalpel. 5 ml of semen-extender (Botucrio, Nidacon, Sweden), pre-warmed to 37°C were added to each cauda. The epididymal sperm was allowed to swim out of the cauda epididymidis on a 37°C warm stage for 15 min before the sample was packaged in 2 ml vials within 3 h from collection. The

extracted semen was transported in cooled container at 4°C for 6 h. Once in the laboratory, the sperm sample was centrifuged in pre-warmed HEPES-TALP medium [19], in order to remove the extender. Fertilization was performed via piezo-ICSI, which results in a more gentle penetration of the oocyte thanks to the drilling of the ZP and of the oolemma, on an inverted microscope Olympus IX73 (Olympus, Japan) coupled with Transferman micromanipulators, Cell Tram 4e injectors and PiezoXpert (Eppendorf, Germany) on a heated plate at 37C. A blunt needle filled with Fluorinert FC-770 with the inner diameter of 5 μm and an angle of 15° (Biomedical instruments, Germany) was used for injection. The holding needle had an inner diameter of 25 μm and an angle of 30° (Biomedical instruments, Germany) and it was used for the holding of the oocytes. ICSI manipulation dish was prepared with one drop of HEPES-TALP medium, one drop of 10% (w/v) polyvinylpyrrolidone (PVP) in HEPES-TALP, and 3 drops of manipulation medium under light mineral oil (Reproline Medical GmbH, Rheinbach, Germany). A small amount of sperm suspension was transferred to the HEPES-TALP drop and single motile sperm cell with normal morphology was moved into the PVP drop, where it was immobilized with multiple pulses applied on the tail region.

2.7. *In vitro embryo-culture*

After the injection, presumptive zygotes were transferred into a GERI dish (Genea BiomedX, Australia) with equilibrated BO-IVC medium and cultured at 38.8C in 5% CO_2, 5% O_2 in GERI time-lapse incubator (Genea BiomedX, Australia). After 7 days in culture, all the presumptive zygotes were fixed with 4% (w/v) paraformaldehyde in PBS and stained with Hoechst 33258 for nuclear chromatin pattern analysis.

3. Results

Among 16 COCs retrieved after slicing (Fig. 1 A), 10 were selected for IVM (Fig. 1 B). After 24 h of IVM, all the COCs were denuded (Fig. 1 C), but no visible polar body was observed. Two degenerated oocytes were excluded. Therefore, eight denuded oocytes were put back into the incubator for the continuing of the culture for additional 24 h. After 48 h of IVM (Table 1), four oocytes showed visible PBs (Fig. 1 D).

The average size of the oocytes was 157.58 ± 14.78 μm (mean ± SD), the thickness of zona pellucida was 8.52 ± 0.97 μm (mean ± SD).

Oocytes at MII stage were vitrified separately and stored in liquid nitrogen for three weeks until the sperm sample was obtained. All four oocytes survived vitrification/warming process with intact oolema.

The quality of semen sample was very poor with low concentration of motile spermatozoa (Supplementary video 1 shows one motile spermatozoon in HEPES-TALP). Single motile spermatozoa were transferred to the PVP drop (Fig. 2 A) and one by one immobilized and injected singly into each oocyte (Fig. 2 B). No hardening of zona pellucida was observed after vitrification, which allowed easy penetration.

Supplementary material related to this article can be found online at doi:10.1016/j.therwi.2024.100088.

All presumptive zygotes were monitored in a time-lapse system for 168 hours post injection and no cleavage was observed. Up to 1 hpi, two of the four injected oocytes were degenerated with pale ooplasm. In one oocyte, the probable extrusion of the second polar body was observed at 35.5 hpi. For the rest of the culture, movement in the ooplasm was observed and the oolema was still intact. The chromatin staining after culture showed no recognisable pattern, i.e. pronuclear formation, syngamy etc. (Fig. 3).

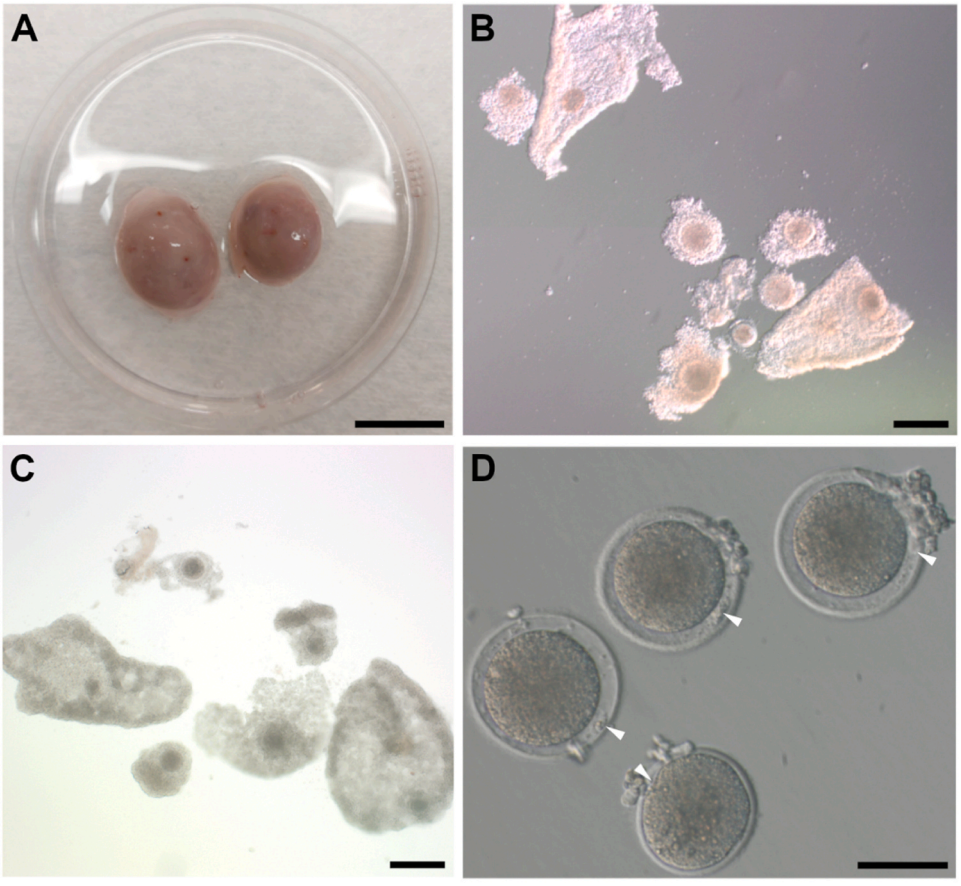

Fig. 1. Collection of cumulus-oocyte complexes (COCs) from ovaries obtained post-mortem. **A:** Ovaries of okapi after transport and washing. Scale bar = 1 cm; **B:** COCs before IVM. Scale bar = 200 μm; **C:** COCs after 24 h of in vitro maturation. Scale bar = 200 μm; **D:** Denuded oocytes after 48 h of IVM. Scale bar = 100 μm.

Table 1
Processing of collected COCs from okapi.

	24 h of IVM			48 h of IVM			ICSI	
Tot COCs	MII	not matured[a]	Degenerated	MII	not matured[a]	Degenerated	Tot Injected	Lysed
10	0	8	2	4	3	1	4	2

Acronyms: COCs = cumulus-oocyte complexes; IVM = in vitro maturation; MII – metaphase II oocytes
[a] Oocytes with no visible polar bodies

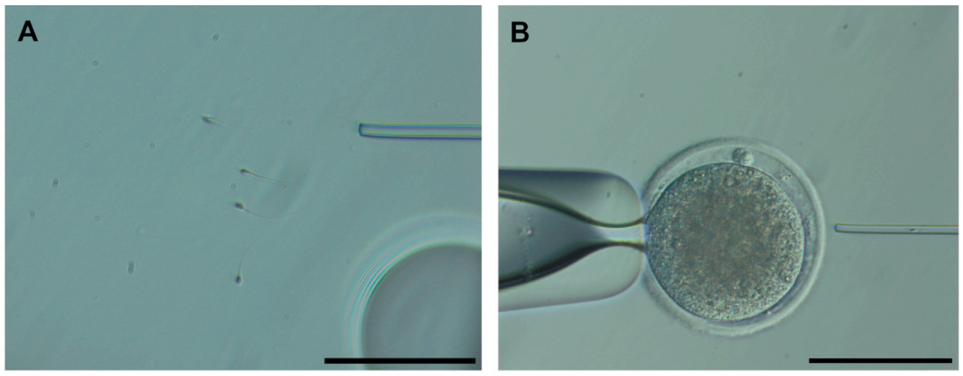

Fig. 2. In vitro fertilization of okapi oocytes via piezo-driven ICSI. **A**: Sperm collection in 10% PVP; **B:** Piezo ICSI; Scale bar = 100 μm.

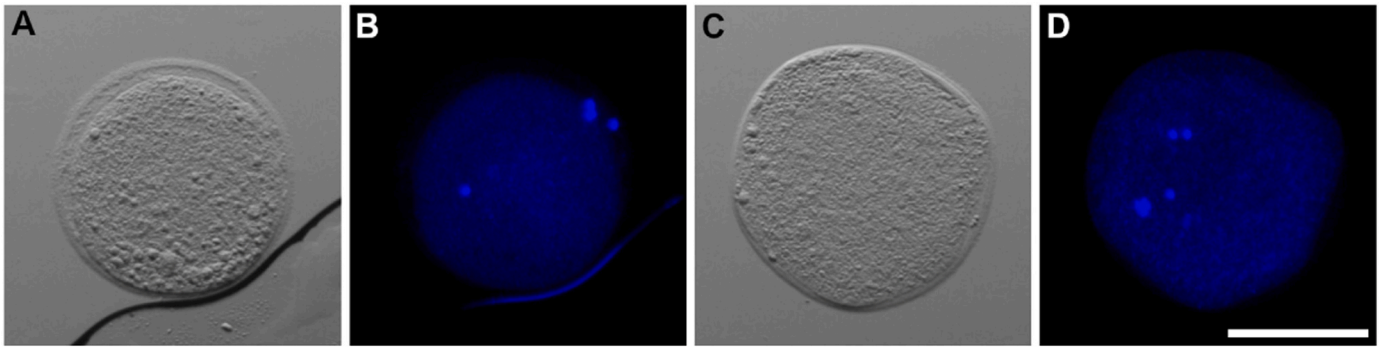

Fig. 3. Staining of chromatin of presumptive zygotes 168 h after sperm injection in bright and dark field. Scale bar = 100 μm.

4. Discussion

To the best of our knowledge, this is the first report of successfully IVM of okapi oocytes retrieved from ovaries post-mortem.

Obtaining biological material from females and males post-mortem is a unique opportunity to preserve genetic variability in endangered species [9]. The IVF success using post-mortem collected gametes may vary due to many factors: (i) age of the animal [20], (ii) health condition (in euthanized individuals or in ones which died by natural causes it may be suboptimal), and (iii) unsuitable stage of estrous cycle in females at the moment of death.

Moreover, we have to take into account that, by definition, there is a deficiency of biological material from endangered species, and this makes the optimization of in vitro embryo production (IVEP) protocols for endangered mammals very challenging.

Okapis, together with giraffes (*Giraffa camelopardalis*), are the only living members of the *Giraffidae* family. In this group of ruminants, which diverged about 21.5 million years ago as a sister taxon from the *Cervidae* family [21], no previous attempts to culture COCs and embryos in vitro have been conducted. The only protocol developed for this family is for the cryopreservation of giraffes' epididymal spermatozoa [22]. Since no data are available for IVM protocols, we decided to use a commercial media for bovine IVM because it is a well-established system.

Another crucial parameter is the time frame between the moment of death and the moment of the ovaries' processing. Since it is not always feasible to minimize the transportation duration of gametes, it becomes essential to use a suitable medium and temperature to preserve the developmental potential of collected oocytes. In domestic cows, a moderate temperature (20–25℃) is routinely used during ovarian transport [19], and it has been shown that storage at 10℃ for 24 h does not affect oocyte maturation and blastocyst rate [23]. Similar results have been obtained also from another ungulate, the Iberian red deer (*Cervus elaphus hispanicus*) [24]). On the contrary, transport and storage at low temperatures in domestic pig has a detrimental effect on the oocyte's capacity to reach MII stage [25]. In our case, a moderate temperature had been maintained during the air transport of the ovaries. Due to the very limited biological material, we did not have the opportunity to test different conditions to establish the best transport environment for okapi ovaries.

IVM length varies among domestic ungulates, from a minimum of 22 – 24 h for cattle, sheep and goats [26–28], to a maximum of 36 – 44 h for horses and pigs [29,30]. For this specific case, since bovines were used as domestic animal model, COCs were initially checked and denuded after 24 h. As no MII oocytes were observed after denudation, IVM was prolonged up to 48 h.

The duration of IVM can be experimentally established with subsequential nuclear chromatin staining: the disadvantage of this

method is the large number of fixed oocytes, which cannot be used for fertilization [31]. Therefore, in this study, it was not possible to apply this protocol, given the low number of COCs available.

Direct oocyte-granulosa cell communication through the gap junctions is important for meiotic progression and high developmental capacity of oocytes [32]. Even though this connection was interrupted by removal of cumulus cells, MII-stage oocytes were achieved, but still the interruption of cell communication could had an effect of the later developmental competence.

Another important factor to consider in IVEP of wild animal is the availability and the quality of sperm used for fertilization. In this case, seminal fluid was flushed from the epididymides retrieved post mortem from an ill animal, refrigerated and transported to the laboratory. Both motility and morphology were suboptimal and the sample was not suitable for a conventional IVF procedure, therefore piezo-ICSI was chosen. With this technique, it is impossible to predict if a single spermatozoon has the capacity of fertilize an oocyte, even though only motile cells with normal morphology were selected for injection.

Since no cleavage occurred and the nuclear staining showed no pronuclear formation, we hypothesized that the fertilization process did not start after ICSI. Unfortunately, due to the low number and the poor motility of the sperm sample used, fertilization via piezo-ICSI was the only option. Probably, an electric activation of the oocyte right after injection, would have increased the chances of embryo development, as it happens in other big mammals, such as rhinoceros [33] and bovine [34].

A limiting factor of this study is that IVEP with fresh oocytes were not included, so a comparison wheatear the vitrification itself or the fertilization method affected the oocyte potential to support the embryo development was not possible.

5. Conclusions

In this study we are presenting a case report, in which we applied, for the first time, ART on gametes obtained post-mortem from okapi. Even though we were not able to produce fertilized oocytes and developing embryos, we showed that IVM in this species is possible by utilizing commercial bovine IVM medium.

CRediT authorship contribution statement

Tobias Knauf-Witzens: Writing – review & editing, Resources. **Benjamin Lamglait:** Writing – review & editing, Resources. **Baptiste Mulot:** Writing – review & editing, Resources. **Susanne Holtze:** Writing – review & editing, Conceptualization. **Daniel Čižmár:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Raffaella Simone:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Thomas Bernd Hildebrandt:** Writing – review & editing, Conceptualization. **Robert Hermes:** Writing – review & editing, Resources. **Annika Weigold:** Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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