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Maturation and fertilization of African lion (Panthera leo) oocytes after vitrification

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Abstract

The African lion is an excellent model species for the highly endangered Asiatic lion. African lions reproduce well in zoos, leading to the fact that occasionally ovaries and testis are available for in-vitro experiments. We previously performed in-vitro maturation (IVM) and fertilization of lion oocytes and were able to produce advanced embryos after intracytoplasmic sperm injection (ICSI) with cryopreserved sperm. Here we examined whether our in-vitro method is also applicable after vitrification of immature oocytes. Oocytes of four lionesses (5-7 years old) were obtained after euthanasia and immediately processed on site. Half of the oocytes (n = 60) were subjected to IVM for a total of 32-34 hours at 39ºC, 5% CO\textsubscript{2} and humidified air atmosphere. The second group (59 oocytes) was vitrified instantly using the Cryotop method. Following 6 days of storage in liquid nitrogen, oocytes were warmed and subjected to IVM as well. Mature oocytes of both groups were fertilized with frozen-thawed African lion sperm using ICSI. Maturation rate was 55% and 49.2% for the control and vitrified group, respectively. In the control group, three oocytes cleaved and another three were arrested at the pronuclei stage. Due to the low fertilization result, a sperm sample of another male was used for the vitrified group. Of the vitrified oocytes 7 cleaved and 9 more oocytes stopped at pronuclei stage. All embryos of the vitrified group did not develop beyond 4 cell stage. This is the first time that African lion in-vitro-derived embryos have been produced following oocyte vitrification.

Keywords: biobanking, cryopreservation, embryo, feline, gamete, germinal vesicle, ICSI, oocyte vitrification
1. INTRODUCTION

Cryopreservation of oocytes has become common practice for human in-vitro fertilization (IVF) treatments. In Germany about one third of all IVF cycles are performed with frozen-thawed oocytes, leading to a pregnancy rate of 29.1% (pregnancy rate for fresh cycles: 32.1%; German IVF Registry, Annual Report 2018). While transfer of frozen embryos in selected farm animal species i.e. bovines is also a standard operation, cryopreservation of oocytes is not. Mature and immature bovine oocytes as well as those of many other mammalian species are susceptible to several cryoinjuries like zona hardening or meiotic spindle disassembly [26]. Some improvement of survival and cleavage rates of oocytes was achieved by vitrification using the “Cryotop” device. However, development to blastocyst stage is still low [6,57].

Among felids, cryopreservation of domestic cat oocytes has been tried several times but led to similar results as in bovine. With current protocols most of the oocytes survive vitrification, but maturation, fertilization and embryo developmental competences are severely affected [3,12,19,45]. The domestic cat is the only permanently available model species for assisted reproductive technology (ART) in other felids, and conservation and reproduction in particular might benefit from these techniques as well as biobanking of gametes. Many felid (sub)species are threatened in the wild (The IUCN Red List of Threatened Species Version 2020-2). Ex-situ populations are struggling with the dilemma to retain their limited and scattered number of animals healthy and genetically diverse [35,46,50].

Currently, protocols exist for the electro-ejaculation or urethral catheterization [4,15] and the cryopreservation of sperm [29]. Also IVF, artificial insemination [34,48] and even embryo transfer (for review see [40,47]) have been performed successfully in selected felid species.
The retrieval of immature oocytes by ovum-pickup or from ovaries of castrated or deceased individuals combined with subsequent cryopreservation of oocytes is still taken into account only on rare occasions, although gamete biobanking for conservation purpose gathers growing attention [25,27,30].

Within the scope of the Felid-Gamete-Rescue-Project [20] we previously performed successful cryopreservation of sperm, in-vitro maturation and fertilization of African lion oocytes and were able to produce advanced stage embryos after intracytoplasmic sperm injection (ICSI) [18]. The African lion is reproducing well in zoos and has a stable population size of about 750 animals within European zoos with EAZA memberships. But even if the production of IVF embryos has no practical application for the captive population, the African lion is an excellent model for the highly endangered Asiatic lion and maybe also other Panthera species. Moreover, the high number of African lions kept in captivity allows occasional access to a larger amount of samples. Since we already showed that the production of embryos is feasible but of minor practical value, we decided to investigate oocyte cryopreservation for such an occasion.

The possibility of on-site vitrification directly after retrieval of oocytes from ovarian tissue alleviates sample recovery from exotic animals in zoos. Transportation of fresh oocytes and ovarian tissue over country’s borders to a suitable lab is complex and critical in terms of time, whereas on-site vitrification of oocytes allows plannable and safe transportation within dry shippers.

The aim of the present study was to evaluate the survival and developmental potential of African lion vitrified oocytes. As a result, we could demonstrate a high survival and in-vitro maturation rate of African lion oocytes after warming and we produced the first embryos following fertilization via ICSI.
2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

All chemicals were purchased from Sigma–Aldrich (Taufkirchen, Germany) unless stated otherwise.

2.2. Animals and oocyte retrieval

Ovaries were obtained from four lionesses (Leo1-4; 5-7 years old) after euthanasia for population management in Givskud Zoo - Zootopia (Givskud, Denmark) and immediately processed in a field laboratory temporarily set up in the Zoo premises. Three of the females were of unknown fertility; Leo 2 had one litter in the past with one cub. Each animal was anesthetized with 400 mg Ketamine and 5 mg Medetomidine. Euthanasia was performed by intracardial injection of 3500 mg Pentobarbital. Ovaries of each animal were processed separately from the others. All ovaries were in a follicular state with many small but clearly visible follicles. Additionally, ovaries of Leo 2 exhibited one small corpus albicans. To retrieve the oocytes, ovaries were sliced in washing medium (WM), prepared from Medium 199 with Earle’s salts, supplemented with 3 mg/mL bovine serum albumin (BSA), 0.1 mg/mL cysteine, 1.4 mg/mL HEPES, 0.25 mg/mL sodium pyruvate, 0.6 mg/mL sodium lactate, 0.15 mg/mL L-glutamine, and 0.055 mg/mL gentamicin, and good quality oocytes (with dark, homogeneous cytoplasm and several granulosa cell layers) were selected under a stereomicroscope for further processing (Fig.1-A). For each animal, half of the oocytes were in-vitro matured and half were vitrified on site (in-loco VO).

2.3. Vitrification and warming

Oocytes were vitrified by the Cryotop method [8,31] as previously described [9]. Briefly, groups of 4-8 oocytes were equilibrated at room temperature in an equilibration solution (ES)
containing 7.5% (v/v) ethylene glycol (EG) and 7.5% dimethylsulfoxide (Me2SO) in Medium 199, with 20% fetal bovine serum (FBS) for 15 minutes. Within the next 60-90 seconds, they were transferred into a vitrification solution (VS: 15% (v/v) EG, 15% Me2SO and 0.5 M sucrose in Medium 199 with 20% FBS), placed on Cryo top polypropylene strip, removing the excess of liquid to reduce the volume as much as possible and finally immersed into liquid nitrogen. Vitrified oocytes were transported to the laboratory in a dry shipper (Air Liquide – healthcare, Duesseldorf, Germany) and moved to a liquid nitrogen tank on arrival.

At warming, the Cryotop strip was immersed for 1 minute in a thawing solution (TS) at 38°C containing 1 M sucrose in Medium 199, with 20% FBS. Vitrified oocytes were retrieved and transferred for 3 minutes to a solution containing 0.5 M sucrose in Medium 199, with 20% FBS and then for 5 minutes to a solution without sucrose. Finally, they were washed again in the same solution (Medium 199 with 20% FBS) and transferred into fresh culture medium for in-vitro maturation (Fig. 1-B).

2.4. In-vitro maturation (IVM) of fresh and vitrified oocytes

In-vitro maturation was performed in Quinn’s Advantage Protein Plus™ Blastocyst Medium (SAGE In Vitro Fertilization Inc., CooperSurgical, Trumbull, CT, USA; kindly donated by Pei-Chih Lee, Smithsonian Conservation Biology Institute's (SCBI) Center for Species Survival, Washington D.C., USA) supplemented with 0.2 IU/mL human luteinizing hormone (LH) and 0.5 IU/mL human pituitary follicle-stimulating hormone (FSH), for a total of 32-34 hours at 39°C in 5% CO₂ in a humidified air atmosphere [18]. Fresh oocytes started IVM in a transport incubator (CellTrans+, Labotect Labor-Technik-Göttingen GmbH, Rosdorf, Germany) which was able to maintain temperature, CO₂ concentration and humidity for the length of the trip (8 hours). Oocytes were transferred to a conventional incubator on arrival at the laboratory. Vitrified oocytes were warmed after 6 days of storage and in-vitro matured as described for fresh oocytes.
2.5. Intracytoplasmic sperm injection and embryo culture

Oocytes were fertilized by ICSI using frozen lion spermatozoa. The epididymal lion sperm used for fertilization of fresh oocytes originated from a 12-year old male, which was euthanized in 2012 for management reasons and that was already stored in our felid gametes bank. Before cryopreservation, the estimated fresh motility was 30%, and freezing was performed according to a modified protocol from Lengwinat and Blottner [33]. Briefly, a cryotube was rapidly plunged into a warm bath at 38 °C for thawing and the spermatozoa were washed in WM to remove the cryoprotectant. Finally, the sperm cells were resuspended in fresh WM. Motility at thawing was 10%. The lion sperm used for fertilization of vitrified oocytes was obtained post mortem from the epididymis of a 16 year old male. Before cryopreservation, the estimated fresh motility was 55%; at thawing, it was 30%.

For ICSI, a 6-cm Petri dish (Nunc) was prepared with two 3 µL droplets of polyvinylpyrrolidone (Gynemed, GM501 PVP). One of the drops was diluted 1:2 (v:v) in ICSI-medium (WM with 3mg/ml HEPES). Less than 1 µL of sperm solution was placed in each drop. Further nine 5 µL droplets of ICSI-Medium were added, where the oocytes were transferred to after being stripped of cumulus cells by gently pipetting with a micropipette (The Stripper, BioTipp, Waterford, Ireland). All drops were covered with mineral oil (Reproline medical GmbH). Each oocyte was assessed for morphology and extrusion of the first polar body as a sign of metaphase II under an inverted microscope at 200X magnification (Axiovert 100; Carl Zeiss, Jena, Germany). Intracytoplasmic sperm injection was performed as previously described for the domestic cat [43,54]. In brief, under the inverted microscope, a sperm cell was immobilized and caught with an injection pipette (BioMedical Instruments, Zoellnitz, Germany); the oocyte was held in place with a holding pipette with the polar body between 11 and 1 or 5 and 7 o’clock, and the spermatozoon was injected head first from the 3 o’clock position (Fig. 1-C). In the lioness with the highest number of vitrified oocytes (i.e.
Lion 3, n=27, injected n=12), half of them (n=6) were activated with 7% ethanol in WM for 5 minutes after ICSI [5] to assess whether this could improve embryo development. After ICSI, all oocytes were placed in embryo culture medium, consisting of Ham’s F-10 supplemented with 5% FBS, 0.11 mg/mL sodium pyruvate, 0.075 mg/mL L-glutamine, 0.06 mg/ml Gentamicin, in 20 µL microdrops covered by mineral oil in Petri dishes or in a time-lapse system (Primovision, Vitrolife, Sweden) at 39°C in 5% CO₂ and 5% O₂. The medium was not changed during embryo culture. Assessment of embryo development was performed every 24 hours. Non-cleaved oocytes and embryos arresting their development for 32 hours were fixed in 96% ethanol overnight and then stained with propidium iodide (PI, 1.0 mg/mL, 1:100 in PBS; Thermo Fisher Scientific) to confirm their nuclear status or developmental stage.

2.6. Statistical analysis
Data for maturation rates were analyzed by Chi-Square test. Significance was set at p<0.05.

3. RESULTS
From the ovaries of 4 lionesses aged 5 to 7 years, 119 oocytes could be isolated altogether (Table1). The number of oocytes per lioness varied from 18 to 55 depending on the stage of ovarian cycle. Half of all recovered oocytes of each lioness were directly subjected to IVM, while the other half was immediately vitrified. With the exception of the fresh group of Leo2, where only one of 9 oocytes was able to mature, all other fresh groups reached a maturation rate between 55.6 to 71.4%. Maturation of the vitrified oocytes was very consistent between all four lionesses ranging from 44.4 to 64.3%. The overall maturation rate of vitrified oocytes (49.2%; n=59) did not differ (p=0.523) from that of fresh oocytes (55%; n=60). The number of dividing embryos was particularly low in all fresh oocytes with no divisions in Leo1 and Leo2 and one and two cleavages out of 17 and 10 (11.1%) matured oocytes in Leo3 and Leo4,
respectively. Division of the in-loco VO group was higher, ranging from 1 out of 9 matured oocytes (11.1% cleavage rate, Leo4) up to 2 out of 4 oocytes (50% cleavage rate, Leo1) and a total number of 7 early embryos out of 29 matured oocytes (total cleavage rate 24.1%). All fresh embryos were vitrified at 2-6 cell stage in a framework of an ongoing study on feline early stage embryo vitrification. Embryos of all in-loco VO groups arrested at a 2-4 cell stage (Fig. 1-D).

There was a three times higher number of arrested pronuclei (PN) stages (Fig. 2-A) in the in-loco VO compared to fresh control (9 versus 3 oocytes). Three of these 9 stages were 3PN stages (Fig. 2-B). Arrested 3PN stages were distributed equally between three lionesses and were only detected in the vitrified group.

Six out of 12 warmed in-loco VO of Leo3 were activated with ethanol following ICSI (Table 2). One embryo cleaved in the activation group and two in the control group. Other 3 oocytes arrested at PN stage (one of them was a 3 PN), while 2 of the un-activated oocytes arrested at PN stage.

4. DISCUSSION

Storage of frozen biomaterials represents an important tool to preserve the diversity of genetic information and variety of endangered species [13]. Gamete banking in particular offers the immediate potential to use these cells directly after thawing to produce offspring with the help of assisted reproductive techniques [20]. In contrast to oocytes, cryopreservation of sperm cells is an established method. Velocity results after thawing are acceptable and pregnancies can be achieved via artificial insemination, though with a lower success rate compared to fresh sperm or connected to complex surgical techniques [48,52]. However, many (epididymal) sperm samples of aged felids are not suitable for artificial insemination due to low concentration or motility after thawing [20]. Therefore, it is important to improve the alternative method panel of IVF and embryo transfer while additionally strengthening the
cryopreservation of feline oocytes for biobanks. IVF protocols for domestic cat including in-vitro maturation have been published several times [21,23,41,44]. Rarely have they been transferred to other felid species. Compared to domestic cats, success was more inconsistent probably also owed to the age of available donors, necessity of transportation and delayed processing [28,42,56].

In the present study, oocytes of four African lion females were used to investigate survival, maturation and fertilization potential and embryo developmental competence. Of particular interest was to study the impact of immediate processing after euthanasia, thus half of the oocytes were directly subjected to IVM (fresh), whereas the other half was vitrified *in-loco*. The amount of isolated oocytes per individual varied between 18 and 55, which is in the range of our previous experience with lions [20] and also of other groups [1,28,42]. The overall maturation rate of fresh oocytes was 55% (33/60 oocytes) which is almost exactly the same percentage as the 53.8% (14/26 oocytes) reported in [42] for 3 Asiatic lionesses. Merlo et al. [36] reported an even higher rate of 73.1% but only for one African lioness (19/26 oocytes). The overall maturation rate for African lion of 55% in our lab is clearly below the rate for domestic cat of 75% under the same conditions [11]. Lion oocytes obviously need more hours to mature in-vitro than domestic cat. As Adamiak and Bartels demonstrated before [1], the maturation rate increased from zero to 80.4% (70/87 oocytes) when maturation time was extended from 26 to 38 hours. Based on these findings and our own experience we also performed prolonged maturation duration of 32-34 hours, compared to the usual 24-28 hours for domestic cat.

Fertilization rate after ICSI in terms of first cleavage was unexplainable low for fresh oocytes (3/33 oocytes; 9.1%) though the frozen epididymal sperm sample chosen had proven successful at fertilizing in the past (11/25; 44% fertilization rate) and embryos developed to blastocysts [18]. Therefore, the sperm sample used for the fertilization of fresh oocytes has
been substituted to a different male with former in-vitro fertilization success for the ICSI of vitrified oocytes.

Current fertilization rates of domestic cat in terms of cleavage were 77% for fresh and 73% for vitrified oocytes in our lab but were generated with IVF and fresh epididymal sperm [11]. In a previous study [19] fertilization rate of vitrified domestic cat oocytes with ICSI ranged between 21-29%, depending on the utilized vitrification method. To our knowledge this is the first study demonstrating cleavage of vitrified African lion or any other non-domestic cat oocytes subsequent to cryopreservation, in-vitro maturation and fertilization via ICSI. Lion oocytes exhibit a high cytoplasmic lipid content and these types of oocytes are presumed to be very sensitive to chilling, resulting in poor revival following slow cooling [22,32].

Vitrification can circumvent that problem, but at least the warming procedure could be a source of cryodamage of organelles adjacent to cytoplasmic lipid droplets.

The lion oocytes in this experiment survived vitrification to a percentage of 74.6% and exhibited a developmental competence to perform maturation comparable to our fresh control group (49.2 vs. 55%). This result is unique not only for lions. Vitrification of oocytes has been performed to some extent in domestic cat, but was linked to a significant decline of maturation rate from 50-75% for fresh to 20-39% for vitrified oocytes [2,10,19,53].

Fasano et al. reported a drop of maturation rate from 46% for fresh to 24% for human vitrified oocytes despite a high survival rate of 87% [17]. Similarly, Hochi et al. observed a drop of maturation rate in horse from 56% for fresh oocytes to 17% after vitrification [24]. Better results were shown by Ezoe et al. in bovine, where 90% of the oocytes survived vitrification and 70% matured, though maturation rate of the fresh control was again significantly higher (84%) [16].

One of the minor cryoinjuries of vitrified oocytes being discussed in several species is zona hardening [38,55]. Therefore, ICSI is considered to be the fertilization method of choice for
vitrified oocytes. In this study ICSI has also been used, but mainly because of the lack of lion sperm in a suitable quality for IVF. We currently demonstrated again [12], as others before, that at least in domestic cat IVF of vitrified oocytes is possible and zona hardening is not an issue.

Activation of oocytes following ICSI is another aspect of discussion not only for vitrified oocytes, but for ICSI in general. It is necessary for example in bovine species to accomplish acceptable fertilization results and embryo development [7]. Bogliolo et al. reported a significantly higher cleavage frequency of ICSI-activated oocytes also in the domestic cat [5]. To our own experience with domestic cat and several wild cat species, including the African lion, it is not beneficial when using fresh or frozen ejaculated or epididymal sperm. However, it is necessary when using immotile freeze-dried sperm, or testicular sperm cells [14,43,51]. Since this was our first experience with vitrified non-domestic cat oocytes, we performed activation with half of the fertilized oocytes of Leo3. Activation of these 6 oocytes with ethanol did not support further development or a higher cleavage rate.

In this study 7 out of 29 (24.1 %) matured vitrified oocytes got fertilized in terms of reaching first cleavage. Another 7 oocytes arrested at pronuclei stage (PN) and noticeably 3 of these were 3-PN stages (Fig. 2-B). Thus, fertilization rate for vitrified lion oocytes is comparable to results for domestic cat, ranging between 20.0 – 32.2% in several studies [19,37,39,49] and even better than our overall fertilization rate of 12.9% for 37 female lions of the Felid-Gamete-Rescue-Project (individual fertilization rate of 0 – 58.3 % [20]).

None of the early lion embryos produced from vitrified oocytes developed beyond the 4-cell stage. In this study the arrest of oocytes at PN stage and in particular the genetic dysregulation reflected by the occurrence of 3PN after ICSI was conspicuous. Embryonic development up to morula or blastocyst stage and even birth of live kittens after embryo transfer are described in the literature for domestic cat [22,37], but developmental stop at early stages is frequently
observed. Since we were able to produce blastocysts from fresh lion oocytes in another study we suppose that the vitrification process itself must have detrimental influence on the developmental integrity, even though in-vitro maturation did not seem to be affected.

Clarifying the pathways that are affected or dysregulated by vitrification would be important to get a profound understanding of the specific needs vitrified oocytes may have after warming. Specifically adapted embryo culture media might be able to balance negligible defects caused by vitrification.

5. CONCLUSIONS

The present study demonstrates successful vitrification, in-vitro maturation and fertilization of African lion oocytes for genetic resource banking. Though embryo development was impaired, these results give hope that not only felid sperm but also oocytes can be cryopreserved and stored in biobanks in the future.

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DECLARATIONS OF INTEREST
We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

FIGURES

Fig. 1: African lion oocytes and embryo. A – Freshly isolated oocytes; B – Vitrified oocytes directly after warming; C – Vitrified oocyte, in vitro matured after warming and subjected to subsequent intracytoplasmic sperm cell injection; arrow indicates sperm head inside the injection pipette; D – embryo generated from vitrified oocyte, 3-4 cell stage. Magnification of A and B 100X; C and D 200X.

Fig. 2: Vitrified African lion oocytes arrested at pronuclei stage after ICSI. A – 2PN; B – 3PN.

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TABLES

Table 1: Maturation rate and fertilization rates of African lion oocytes subjected to IVM (fresh) or vitrification (*in-loco* VO) directly after euthanasia. Warming and subsequent IVM of *in-loco* VO was performed one week later. Matured oocytes were fertilized via ICSI.

<table>
<thead>
<tr>
<th>Lioness No.</th>
<th>Treatment</th>
<th>No. of oocytes</th>
<th>No. of matured oocytes (%)</th>
<th>No. of oocytes arrested at PN# stage</th>
<th>No. of cleaved embryos</th>
<th>Final stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leo1</td>
<td>fresh</td>
<td>9</td>
<td>5 (55.6)</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>in-loco</em> VO</td>
<td>9</td>
<td>4 (44.4)</td>
<td>0</td>
<td>2</td>
<td>2-4 cells</td>
</tr>
<tr>
<td>Leo2</td>
<td>fresh</td>
<td>9</td>
<td>1 (11.1)</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>in-loco</em> VO</td>
<td>9</td>
<td>4 (44.4)</td>
<td>1 x 3PN</td>
<td>1</td>
<td>2-4 cells</td>
</tr>
<tr>
<td>Leo3</td>
<td>fresh</td>
<td>28</td>
<td>17 (60.7)</td>
<td>2 x 2PN</td>
<td>1</td>
<td>5-6 cells*</td>
</tr>
<tr>
<td></td>
<td><em>in-loco</em> VO</td>
<td>27</td>
<td>12 (44.4)</td>
<td>4 x 2PN</td>
<td>3</td>
<td>2-4 cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 x 3PN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leo4</td>
<td>fresh</td>
<td>14</td>
<td>10 (71.4)</td>
<td>1 x 2PN</td>
<td>2</td>
<td>2-4 cells*</td>
</tr>
<tr>
<td></td>
<td><em>in-loco</em> VO</td>
<td>14</td>
<td>9 (64.3)</td>
<td>2 x 2PN</td>
<td>1</td>
<td>2-4 cells</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1 x 3PN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum</td>
<td>fresh</td>
<td>60</td>
<td>33 (55)</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>in-loco</em> VO</td>
<td>59</td>
<td>29 (49.2)</td>
<td>9</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

* All fresh embryos were vitrified at 2-4 or 5-6 cell stage

# PN – pronuclear stage

Table 2: Influence of ethanol activation on the fertilization competence of vitrified African lion oocytes (*in-loco* VO) after ICSI.

<table>
<thead>
<tr>
<th>Lioness No.</th>
<th>Treatment</th>
<th>Activation</th>
<th>No. of oocytes subjected to ICSI</th>
<th>No. of oocytes arrested at PN stage (%)</th>
<th>No. of cleaved embryos</th>
<th>Final stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leo3</td>
<td><em>in-loco</em> VO</td>
<td>-</td>
<td>6</td>
<td>2 (33.3)</td>
<td>2</td>
<td>2-4 cells</td>
</tr>
<tr>
<td></td>
<td><em>in-loco</em> VO</td>
<td>Ethanol</td>
<td>6</td>
<td>3 (50.0)</td>
<td>1</td>
<td>2-4 cells</td>
</tr>
</tbody>
</table>

PN – pronuclear stage
Fig. 1: African lion oocytes and embryo. A – Freshly isolated; B – Vitrified oocytes directly after warming; C – matured oocyte after vitrification subjected to ICSI, arrow indicates sperm head inside of the injection pipette; D – embryo generated of vitrified oocyte, 3-4 cell stage. Magnification of A and B 100X; C and D 200X.
Fig. 2: Vitrified African lion oocytes arrested at pronuclei stage. A – 2PN; B – 3PN.
Highlights

- Vitrification with Cryotop device was suitable for African lion immature oocytes
- Lion vitrified and fresh oocytes matured at similar proportions in vitro
- Lion vitrified oocytes produced embryos after intracytoplasmic sperm injection
- Embryos derived from vitrified oocytes did not progress beyond cleavage stage