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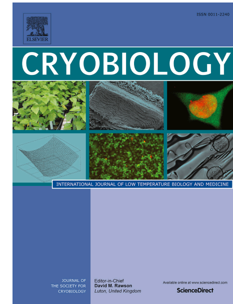

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Maturation and fertilization of African lion (*Panthera leo*) oocytes after vitrification

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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_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

46

47 ## 1. INTRODUCTION

48 Cryopreservation of oocytes has become common practice for human in-vitro fertilization
49 (IVF) treatments. In Germany about one third of all IVF cycles are performed with frozen-
50 thawed oocytes, leading to a pregnancy rate of 29.1% (pregnancy rate for fresh cycles: 32.1%;
51 German IVF Registry, Annual Report 2018). While transfer of frozen embryos in selected
52 farm animal species i.e. bovines is also a standard operation, cryopreservation of oocytes is
53 not. Mature and immature bovine oocytes as well as those of many other mammalian species
54 are susceptible to several cryoinjuries like zona hardening or meiotic spindle disassembly
55 [26]. Some improvement of survival and cleavage rates of oocytes was achieved by
56 vitrification using the "Cryotop" device. However, development to blastocyst stage is still low
57 [6,57].

58 Among felids, cryopreservation of domestic cat oocytes has been tried several times but led to
59 similar results as in bovine. With current protocols most of the oocytes survive vitrification,
60 but maturation, fertilization and embryo developmental competences are severely affected
61 [3,12,19,45].

62 The domestic cat is the only permanently available model species for assisted reproductive
63 technology (ART) in other felids, and conservation and reproduction in particular might
64 benefit from these techniques as well as biobanking of gametes. Many felid (sub)species are
65 threatened in the wild (The IUCN Red List of Threatened Species Version 2020-2). Ex-situ
66 populations are struggling with the dilemma to retain their limited and scattered number of
67 animals healthy and genetically diverse [35,46,50].

68 Currently, protocols exist for the electro-ejaculation or urethral catheterization [4,15] and the
69 cryopreservation of sperm [29]. Also IVF, artificial insemination [34,48] and even embryo
70 transfer (for review see [40,47]) have been performed successfully in selected felid species.

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71 The retrieval of immature oocytes by ovum-pickup or from ovaries of castrated or deceased
72 individuals combined with subsequent cryopreservation of oocytes is still taken into account
73 only on rare occasions, although gamete biobanking for conservation purpose gathers growing
74 attention [25,27,30].
75
76 Within the scope of the Felid-Gamete-Rescue-Project [20] we previously performed
77 successful cryopreservation of sperm, in-vitro maturation and fertilization of African lion
78 oocytes and were able to produce advanced stage embryos after intracytoplasmic sperm
79 injection (ICSI) [18]. The African lion is reproducing well in zoos and has a stable population
80 size of about 750 animals within European zoos with EAZA memberships. But even if the
81 production of IVF embryos has no practical application for the captive population, the African
82 lion is an excellent model for the highly endangered Asiatic lion and maybe also other
83 Panthera species. Moreover, the high number of African lions kept in captivity allows
84 occasional access to a larger amount of samples. Since we already showed that the production
85 of embryos is feasible but of minor practical value, we decided to investigate oocyte
86 cryopreservation for such an occasion.
87 The possibility of on-site vitrification directly after retrieval of oocytes from ovarian tissue
88 alleviates sample recovery from exotic animals in zoos. Transportation of fresh oocytes and
89 ovarian tissue over country`s borders to a suitable lab is complex and critical in terms of time,
90 whereas on-site vitrification of oocytes allows plannable and safe transportation within dry
91 shippers.
92
93 The aim of the present study was to evaluate the survival and developmental potential of
94 African lion vitrified oocytes. As a result, we could demonstrate a high survival and in-vitro
95 maturation rate of African lion oocytes after warming and we produced the first embryos
96 following fertilization via ICSI.

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97

98 ## 2. MATERIALS AND METHODS

99 ### 2.1. Chemicals and Reagents

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

102

103 ### 2.2. Animals and oocyte retrieval

104 Ovaries were obtained from four lionesses (Leo1-4; 5-7 years old) after euthanasia for
105 population management in Givskud Zoo - Zootopia (Givskud, Denmark) and immediately
106 processed in a field laboratory temporarily set up in the Zoo premises. Three of the females
107 were of unknown fertility; Leo 2 had one litter in the past with one cub. Each animal was
108 anesthetized with 400 mg Ketamine and 5 mg Medetomidine. Euthanasia was performed by
109 intracardial injection of 3500 mg Pentobarbital. Ovaries of each animal were processed
110 separately from the others. All ovaries were in a follicular state with many small but clearly
111 visible follicles. Additionally, ovaries of Leo 2 exhibited one small corpus albicans. To
112 retrieve the oocytes, ovaries were sliced in washing medium (WM), prepared from Medium
113 199 with Earle's salts, supplemented with 3 mg/mL bovine serum albumin (BSA), 0.1 mg/mL
114 cysteine, 1.4 mg/mL HEPES, 0.25 mg/mL sodium pyruvate, 0.6 mg/mL sodium lactate, 0.15
115 mg/mL L-glutamine, and 0.055 mg/mL gentamicin, and good quality oocytes (with dark,
116 homogeneous cytoplasm and several granulosa cell layers) were selected under a
117 stereomicroscope for further processing (Fig.1-A). For each animal, half of the oocytes were
118 in-vitro matured and half were vitrified on site (*in-loco* VO).

119

120 ### 2.3. Vitrification and warming

121 Oocytes were vitrified by the Cryotop method [8,31] as previously described [9]. Briefly,
122 groups of 4-8 oocytes were equilibrated at room temperature in an equilibration solution (ES)

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123 containing 7.5% (v/v) ethylene glycol (EG) and 7.5% dimethylsulfoxide (Me2SO) in Medium
124 199, with 20% fetal bovine serum (FBS) for 15 minutes. Within the next 60-90 seconds, they
125 were transferred into a vitrification solution (VS: 15% (v/v) EG, 15% Me2SO and 0.5 M
126 sucrose in Medium 199 with 20% FBS), placed on Cryotop polypropylene strip, removing the
127 excess of liquid to reduce the volume as much as possible and finally immersed into liquid
128 nitrogen. Vitrified oocytes were transported to the laboratory in a dry shipper (Air Liquide –
129 healthcare, Duesseldorf, Germany) and moved to a liquid nitrogen tank on arrival.
130 At warming, the Cryotop strip was immersed for 1 minute in a thawing solution (TS) at 38ºC
131 containing 1 M sucrose in Medium 199, with 20% FBS. Vitrified oocytes were retrieved and
132 transferred for 3 minutes to a solution containing 0.5 M sucrose in Medium 199, with 20%
133 FBS and then for 5 minutes to a solution without sucrose. Finally, they were washed again in
134 the same solution (Medium 199 with 20% FBS) and transferred into fresh culture medium for
135 in-vitro maturation (Fig. 1-B).
136
137 2.4. In-vitro maturation (IVM) of fresh and vitrified oocytes
138 In-vitro maturation was performed in Quinn's Advantage Protein Plus™ Blastocyst Medium
139 (SAGE In Vitro Fertilization Inc., CooperSurgical, Trumbull, CT, USA; kindly donated by
140 Pei-Chih Lee, Smithsonian Conservation Biology Institute's (SCBI) Center for Species
141 Survival, Washington D.C., USA) supplemented with 0.2 IU /mL human luteinizing hormone
142 (LH) and 0.5 IU /mL human pituitary follicle-stimulating hormone (FSH), for a total of 32-34
143 hours at 39ºC in 5% CO_2 in a humidified air atmosphere [18]. Fresh oocytes started IVM in a
144 transport incubator (CellTrans+, Labotect Labor-Technik-Göttingen GmbH, Rosdorf,
145 Germany) which was able to maintain temperature, CO_2 concentration and humidity for the
146 length of the trip (8 hours). Oocytes were transferred to a conventional incubator on arrival at
147 the laboratory. Vitrified oocytes were warmed after 6 days of storage and in-vitro matured as
148 described for fresh oocytes.

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149

150 2.5. Intracytoplasmic sperm injection and embryo culture

151 Oocytes were fertilized by ICSI using frozen lion spermatozoa. The epididymal lion sperm
152 used for fertilization of fresh oocytes originated from a 12-year old male, which was
153 euthanized in 2012 for management reasons and that was already stored in our felid gametes
154 bank. Before cryopreservation, the estimated fresh motility was 30%, and freezing was
155 performed according to a modified protocol from Lengwinat and Blottner [33]. Briefly, a
156 cryotube was rapidly plunged into a warm bath at 38 °C for thawing and the spermatozoa
157 were washed in WM to remove the cryoprotectant. Finally, the sperm cells were resuspended
158 in fresh WM. Motility at thawing was 10%. The lion sperm used for fertilization of vitrified
159 oocytes was obtained *post mortem* from the epididymis of a 16 year old male. Before
160 cryopreservation, the estimated fresh motility was 55%; at thawing, it was 30%.
161 For ICSI, a 6-cm Petri dish (Nunc) was prepared with two 3 μL droplets of
162 polyvinylpyrrolidone (Gynemed, GM501 PVP). One of the drops was diluted 1:2 (v:v) in
163 ICSI-medium (WM with 3mg/ml HEPES). Less than 1 μL of sperm solution was placed in
164 each drop. Further nine 5 μL droplets of ICSI-Medium were added, where the oocytes were
165 transferred to after being stripped of cumulus cells by gently pipetting with a micropipette
166 (The Stripper, BioTipp, Waterford, Ireland). All drops were covered with mineral oil
167 (Reproline medical GmbH). Each oocyte was assessed for morphology and extrusion of the
168 first polar body as a sign of metaphase II under an inverted microscope at 200X magnification
169 (Axiovert 100; Carl Zeiss, Jena, Germany). Intracytoplasmic sperm injection was performed
170 as previously described for the domestic cat [43,54]. In brief, under the inverted microscope, a
171 sperm cell was immobilized and caught with an injection pipette (BioMedical Instruments,
172 Zoellnitz, Germany); the oocyte was held in place with a holding pipette with the polar body
173 between 11 and 1 or 5 and 7 o' clock, and the spermatozoon was injected head first from the 3
174 o' clock position (Fig. 1-C). In the lioness with the highest number of vitrified oocytes (i.e.

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175 Lion 3, n=27, injected n=12), half of them (n=6) were activated with 7% ethanol in WM for 5
176 minutes after ICSI [5] to assess whether this could improve embryo development.
177 After ICSI, all oocytes were placed in embryo culture medium, consisting of Ham's F-10
178 supplemented with 5% FBS, 0.11 mg/mL sodium pyruvate, 0.075 mg/mL L-glutamine, 0.06
179 mg/ml Gentamicin, in 20 µL microdrops covered by mineral oil in Petri dishes or in a time-
180 lapse system (Primovision, Vitrolife, Sweden) at 39ºC in 5% CO_2 and 5% O_2. The medium
181 was not changed during embryo culture. Assessment of embryo development was performed
182 every 24 hours. Non-cleaved oocytes and embryos arresting their development for 32 hours
183 were fixed in 96% ethanol overnight and then stained with propidium iodide (PI, 1.0 mg/mL,
184 1:100 in PBS; Thermo Fisher Scientific) to confirm their nuclear status or developmental
185 stage.
186
187 2.6. Statistical analysis
188 Data for maturation rates were analyzed by Chi-Square test. Significance was set at $p<0.05$.
189
190 3. RESULTS
191 From the ovaries of 4 lionesses aged 5 to 7 years, 119 oocytes could be isolated altogether
192 (Table1). The number of oocytes per lioness varied from 18 to 55 depending on the stage of
193 ovarian cycle. Half of all recovered oocytes of each lioness were directly subjected to IVM,
194 while the other half was immediately vitrified. With the exception of the fresh group of Leo2,
195 where only one of 9 oocytes was able to mature, all other fresh groups reached a maturation
196 rate between 55.6 to 71.4%. Maturation of the vitrified oocytes was very consistent between
197 all four lionesses ranging from 44.4 to 64.3%. The overall maturation rate of vitrified oocytes
198 (49.2%; n=59) did not differ (p=0.523) from that of fresh oocytes (55%; n=60). The number
199 of dividing embryos was particularly low in all fresh oocytes with no divisions in Leo1 and
200 Leo2 and one and two cleavages out of 17 and 10 (11.1%) matured oocytes in Leo3 and Leo4,

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201 respectively. Division of the *in-loco* VO group was higher, ranging from 1 out of 9 matured
202 oocytes (11.1% cleavage rate, Leo4) up to 2 out of 4 oocytes (50% cleavage rate, Leo1) and a
203 total number of 7 early embryos out of 29 matured oocytes (total cleavage rate 24.1%). All
204 fresh embryos were vitrified at 2-6 cell stage in a framework of an ongoing study on feline
205 early stage embryo vitrification. Embryos of all *in-loco* VO groups arrested at a 2-4 cell stage
206 (Fig. 1-D).
207 There was a three times higher number of arrested pronuclei (PN) stages (Fig. 2-A) in the *in-*
208 *loco* VO compared to fresh control (9 versus 3 oocytes). Three of these 9 stages were 3PN
209 stages (Fig. 2-B). Arrested 3PN stages were distributed equally between three lionesses and
210 were only detected in the vitrified group.
211 Six out of 12 warmed *in-loco* VO of Leo3 were activated with ethanol following ICSI (Table
212 2). One embryo cleaved in the activation group and two in the control group. Other 3 oocytes
213 arrested at PN stage (one of them was a 3 PN), while 2 of the un-activated oocytes arrested at
214 PN stage.
215

216 4. DISCUSSION

217 Storage of frozen biomaterials represents an important tool to preserve the diversity of genetic
218 information and variety of endangered species [13]. Gamete banking in particular offers the
219 immediate potential to use these cells directly after thawing to produce offspring with the help
220 of assisted reproductive techniques [20]. In contrast to oocytes, cryopreservation of sperm
221 cells is an established method. Velocity results after thawing are acceptable and pregnancies
222 can be achieved via artificial insemination, though with a lower success rate compared to
223 fresh sperm or connected to complex surgical techniques [48,52]. However, many
224 (epididymal) sperm samples of aged felids are not suitable for artificial insemination due to
225 low concentration or motility after thawing [20]. Therefore, it is important to improve the
226 alternative method panel of IVF and embryo transfer while additionally strengthening the

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227 cryopreservation of feline oocytes for biobanks. IVF protocols for domestic cat including in-
228 vitro maturation have been published several times [21,23,41,44]. Rarely have they been
229 transferred to other felid species. Compared to domestic cats, success was more inconsistent
230 probably also owed to the age of available donors, necessity of transportation and delayed
231 processing [28,42,56].

232 In the present study, oocytes of four African lion females were used to investigate survival,
233 maturation and fertilization potential and embryo developmental competence. Of particular
234 interest was to study the impact of immediate processing after euthanasia, thus half of the
235 oocytes were directly subjected to IVM (fresh), whereas the other half was vitrified *in-loco*.
236 The amount of isolated oocytes per individual varied between 18 and 55, which is in the range
237 of our previous experience with lions [20] and also of other groups [1,28,42]. The overall
238 maturation rate of fresh oocytes was 55% (33/60 oocytes) which is almost exactly the same
239 percentage as the 53.8% (14/26 oocytes) reported in [42] for 3 Asiatic lionesses. Merlo et al.
240 [36] reported an even higher rate of 73.1% but only for one African lioness (19/26 oocytes).

241 The overall maturation rate for African lion of 55% in our lab is clearly below the rate for
242 domestic cat of 75% under the same conditions [11]. Lion oocytes obviously need more hours
243 to mature in-vitro than domestic cat. As Adamiak and Bartels demonstrated before [1], the
244 maturation rate increased from zero to 80.4% (70/87 oocytes) when maturation time was
245 extended from 26 to 38 hours. Based on these findings and our own experience we also
246 performed prolonged maturation duration of 32-34 hours, compared to the usual 24-28 hours
247 for domestic cat.

248 Fertilization rate after ICSI in terms of first cleavage was unexplainable low for fresh oocytes
249 (3/33 oocytes; 9.1%) though the frozen epididymal sperm sample chosen had proven
250 successful at fertilizing in the past (11/25; 44% fertilization rate) and embryos developed to
251 blastocysts [18]. Therefore, the sperm sample used for the fertilization of fresh oocytes has

252 been substituted to a different male with former in-vitro fertilization success for the ICSI of
253 vitrified oocytes.

254 Current fertilization rates of domestic cat in terms of cleavage were 77% for fresh and 73%
255 for vitrified oocytes in our lab but were generated with IVF and fresh epididymal sperm [11].
256 In a previous study [19] fertilization rate of vitrified domestic cat oocytes with ICSI ranged
257 between 21-29%, depending on the utilized vitrification method. To our knowledge this is the
258 first study demonstrating cleavage of vitrified African lion or any other non-domestic cat
259 oocytes subsequent to cryopreservation, in-vitro maturation and fertilization via ICSI. Lion
260 oocytes exhibit a high cytoplasmic lipid content and these types of oocytes are presumed to be
261 very sensitive to chilling, resulting in poor revival following slow cooling [22,32].
262 Vitrification can circumvent that problem, but at least the warming procedure could be a
263 source of cryodamage of organelles adjacent to cytoplasmic lipid droplets.

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

269 Fasano et al. reported a drop of maturation rate from 46% for fresh to 24% for human vitrified
270 oocytes despite a high survival rate of 87% [17]. Similarly, Hochi et al. observed a drop of
271 maturation rate in horse from 56% for fresh oocytes to 17% after vitrification [24]. Better
272 results were shown by Ezoe et al. in bovine, where 90% of the oocytes survived vitrification
273 and 70% matured, though maturation rate of the fresh control was again significantly higher
274 (84%) [16].

275 One of the minor cryoinjuries of vitrified oocytes being discussed in several species is zona
276 hardening [38,55]. Therefore, ICSI is considered to be the fertilization method of choice for

277 vitrified oocytes. In this study ICSI has also been used, but mainly because of the lack of lion
278 sperm in a suitable quality for IVF. We currently demonstrated again [12], as others before,
279 that at least in domestic cat IVF of vitrified oocytes is possible and zona hardening is not an
280 issue.

281 Activation of oocytes following ICSI is another aspect of discussion not only for vitrified
282 oocytes, but for ICSI in general. It is necessary for example in bovine species to accomplish
283 acceptable fertilization results and embryo development [7]. Bogliolo et al. reported a
284 significantly higher cleavage frequency of ICSI-activated oocytes also in the domestic cat [5].

285 To our own experience with domestic cat and several wild cat species, including the African
286 lion, it is not beneficial when using fresh or frozen ejaculated or epididymal sperm. However,
287 it is necessary when using immotile freeze-dried sperm, or testicular sperm cells [14,43,51].
288 Since this was our first experience with vitrified non-domestic cat oocytes, we performed
289 activation with half of the fertilized oocytes of Leo3. Activation of these 6 oocytes with
290 ethanol did not support further development or a higher cleavage rate.
291 In this study 7 out of 29 (24.1 %) matured vitrified oocytes got fertilized in terms of reaching
292 first cleavage. Another 7 oocytes arrested at pronuclei stage (PN) and noticeably 3 of these
293 were 3-PN stages (Fig. 2-B). Thus, fertilization rate for vitrified lion oocytes is comparable to
294 results for domestic cat, ranging between 20.0 – 32.2% in several studies [19,37,39,49] and
295 even better than our overall fertilization rate of 12.9% for 37 female lions of the Felid-
296 Gamete-Rescue-Project (individual fertilization rate of 0 – 58.3 % [20]).

297 None of the early lion embryos produced from vitrified oocytes developed beyond the 4-cell
298 stage. In this study the arrest of oocytes at PN stage and in particular the genetic dysregulation
299 reflected by the occurrence of 3PN after ICSI was conspicuous. Embryonic development up
300 to morula or blastocyst stage and even birth of live kittens after embryo transfer are described
301 in the literature for domestic cat [22,37], but developmental stop at early stages is frequently

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302 observed. Since we were able to produce blastocysts from fresh lion oocytes in another study
303 we suppose that the vitrification process itself must have detrimental influence on the
304 developmental integrity, even though in-vitro maturation did not seem to be affected.

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

309

310 5. CONCLUSIONS

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

315

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320

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324

325 DECLARATIONS OF INTEREST

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

329

330 FIGURES

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

336

337 Fig. 2: Vitrified African lion oocytes arrested at pronuclei stage after ICSI. A – 2PN; B –
338 3PN.

339

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516 TABLES

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

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

521 * All fresh embryos were vitrified at 2-4 or 5-6 cell stage
522 # PN – pronuclear stage

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

Lioness No.	Treatment	Activation	No. of oocytes subjected to ICSI	No. of oocytes arrested at PN stage (%)	No. of cleaved embryos	Final stage
Leo 3	*in-loco* VO	-	6	2 (33.3)	2	2-4 cells
	in-loco VO	Ethanol	6	3 (50.0)	1	2-4 cells

528 PN – pronuclear stage

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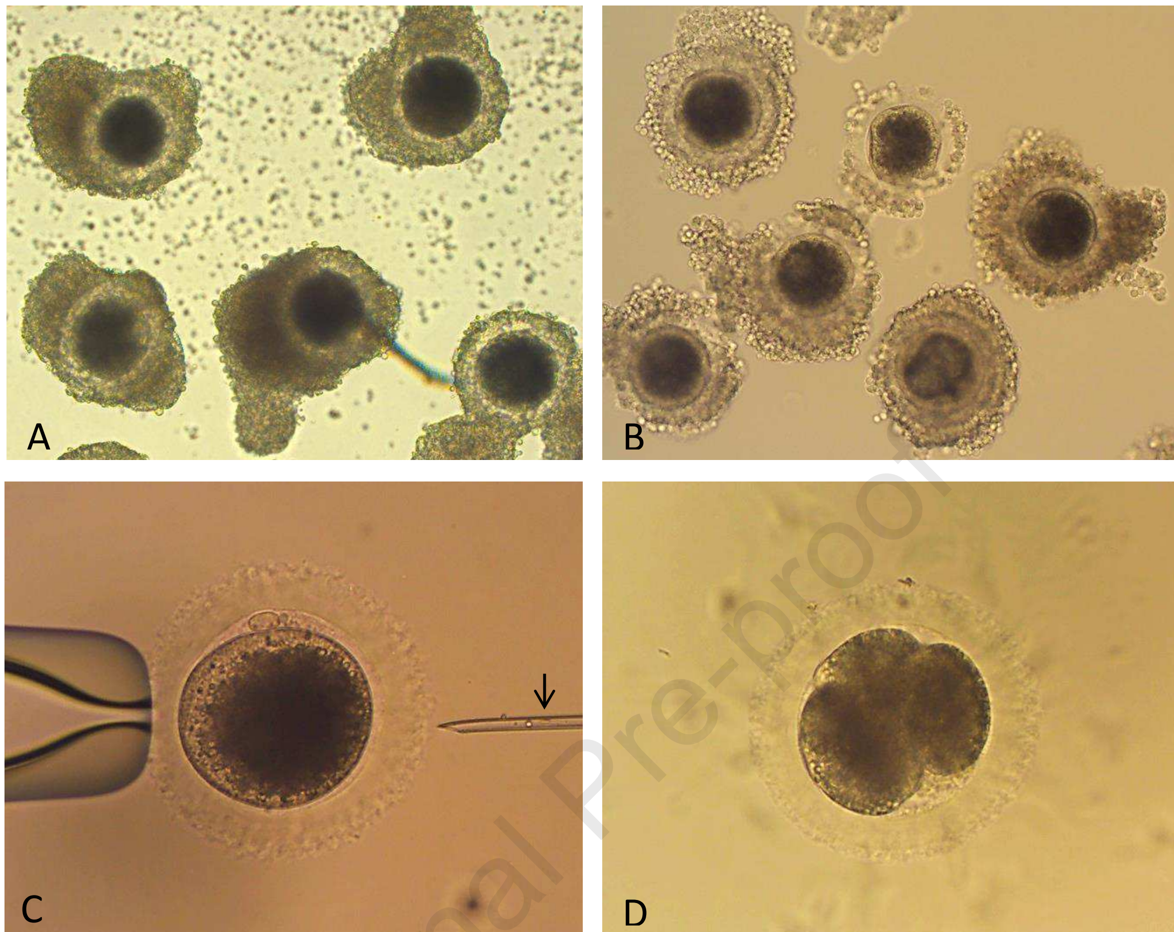

Fig. 1: African lion oocytes and embryo. A – Freshly isolated; B – Vitrified oocytes directly after warming; C – matured oocyte after vitrification subjected to ICSI, arrow indicates sperm head inside of the injection pipette; D – embryo generated of vitrified oocyte, 3-4 cell stage. Magnification of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

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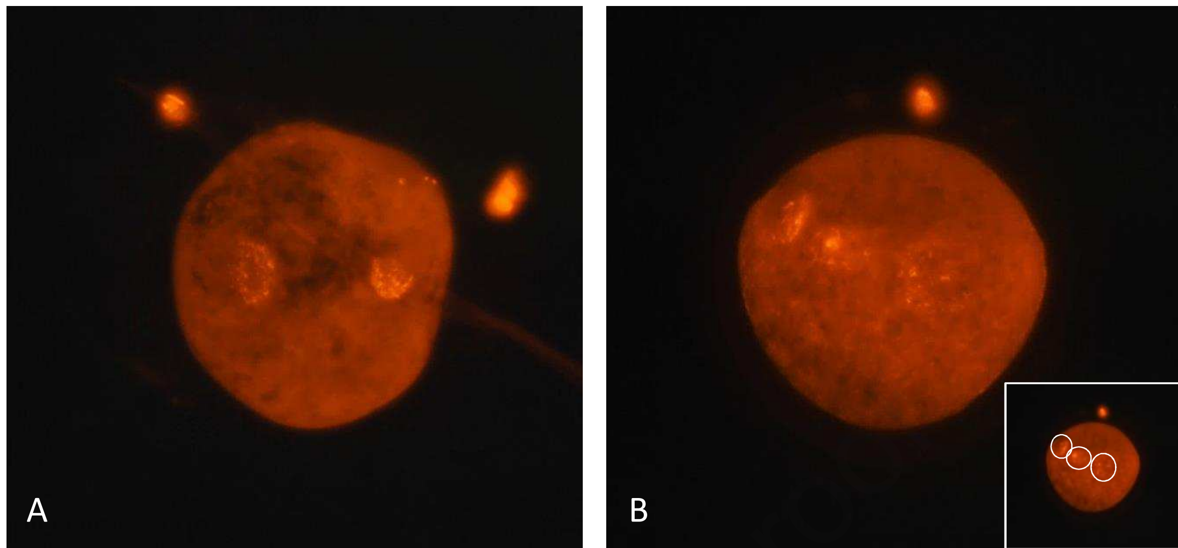

Fig. 2: Vitrified African lion oocytes arrested at pronuclei stage. A – 2PN; B – 3PN.

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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

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