1 **Title**

2 **Evaluating the use of piezo manipulator, laser, or their combination for blastocoel**
3 **cavity puncture to improve cryopreservation outcomes of large equine embryos**

4 **Running Head:** Improving cryopreservation of large equine embryos

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Abstract

The main difficulty of large equine embryo cryopreservation is the replacement of blastocoel fluid with cryoprotectant solution. The objective of this study was to improve the cryopreservation of large equine embryos with PMAP and/or LAP. Embryos were collected via non-surgical transcervical procedure and divided into three groups based on their size (A≤300 µm; 300 µm<B<700 µm; and C≥700 µm). Six embryos 233-1360 µm in diameter were punctured via piezo manipulator and/or laser pulse before cryopreservation. All embryos were cryopreserved on a Cryotop®. Frozen-thawed embryos were cultured for 3h and transferred to the recipient mares. After one week, pregnancy was diagnosed by ultrasonography. Two of six embryos resulted in a positive pregnancy, the result of pregnancy in group A and B was positive, but in group C was negative, and further investigation is necessary for ≥700 µm embryos. The results showed laser-assisted puncture could be helpful to extract blastocoel fluid and replace it with cryoprotectant. This is the first positive pregnancy report in laser puncture-assisted frozen-thawed equine embryo (>300 µm). However, more research is required to find the best method for embryos ≥700 µm.

KEYWORDS: Equine; Embryo; Cryopreservation; Blastocoel fluid; Cryoprotectant; Piezo manipulator; Laser pulse.

37 **1 INTRODUCTION**

38 Embryo cryopreservation is a valuable method for banking valuable equine genetics
39 (Allen & Stewart, 2001). The success of equine embryo cryopreservation is affected by
40 three main factors, which are: embryo size, blastocoel volume, and having an intact
41 capsule (Diaz et al., 2016). Large equine embryos (>300 μm) have unacceptably low post-
42 thaw pregnancy rates after transfer unless the blastocoel cavity is punctured. However,
43 embryos smaller than 300 μm diameter can be cryopreserved without puncturing the
44 blastocoel cavity and have pregnancy rates of 50–65% (Stout, 2012). Embryos bigger
45 than 300 μm show a reduced ability to shrink during cryopreservation and re-expand
46 during post-warming incubation (Stout, 2012).

47 Although the exact functions of the capsule are not known, its presence is crucial to
48 the survival of the early embryo (Stout et al., 2005). The presence of a capsule makes the
49 embryos less permeable to the cryoprotectants, and the large blastocoel volume is prone
50 to ice crystal formation (Eldridge-Panuska et al., 2005).

51 Several methods have been proposed to puncture the blastocoel cavity of large equine
52 embryos to improve cryopreservation survival. Laser technology is now being applied
53 across assisted reproductive technology (ART) to reduce procedure times and increase
54 the consistency and reproducibility of traditional ART techniques such as assisted

55 hatching, embryo biopsy, intracytoplasmic sperm injection, sperm
56 immobilization/selection, and cryopreservation (Davidson et al., 2019). In addition, the
57 use of microinjection needles has been evaluated for blastocoel puncture (with or without
58 aspiration). There are a few studies on equine laser-assisted cryopreservation but more
59 investigation in this area is needed to obtain the best method for cryopreservation of
60 equine embryos.

61 We evaluated piezo micromanipulator-assisted puncture (PMAP) and/or laser-
62 assisted puncture (LAP) for creating a hole in the capsule and trophoblast layer to improve
63 the exchange of blastocoel fluid with cryoprotectant solution for cryopreservation of large
64 equine embryos on Cryotop® (Kitazato, Shizuoka, Japan). The aim of this study was to
65 improve the cryopreservation of large equine embryos with PMAP and/or LAP.

66

67 **2 MATERIALS AND METHODS**

68 Animals belonging to the Horse Research Farm in the Obihiro University of
69 Agriculture and Veterinary Medicine were kept according to the university animal care
70 regulations. Also, all experimental procedures were approved by the Animal Experiment
71 Committee of Obihiro University of Agriculture and Veterinary Medicine.

72 Two Hokkaido native pony and two crossbreed (Hokkaido native pony × Haflinger)

73 donor mares and four Hokkaido native pony and two crossbreed (Hokkaido native pony
74 × Haflinger) recipient mares were used in this study. Artificial insemination and embryo
75 collection were done as described previously (Hannan et al., 2019, 2020). Briefly,
76 artificial inseminations using frozen semen (200×10^6 spermatozoa/AI) from a Connemara
77 pony stallion were performed approximately 32 h after hCG injections. Embryo
78 collections were done via non-surgical transcervical procedure, a sterile uterine catheter
79 with a cuff attached to a sterile Y Junction W/Folly Connector with clamps to regulate the
80 inflow and outflow of a commercially prepared complete flush solution (VIGRO®,
81 Vetoquinol N. -A. Inc., Princeville, QC, Canada) in one end and with a sterile embryo
82 filter (EZ-Way Filter, SPI-MFG) on the other. After uterine flushing the mesh of embryo
83 filter was rinsed several times and the filter dish was examined by means of a dissecting
84 microscope (Olympus Corp., Tokyo, Japan) to identify the embryo. Embryo was washed
85 in holding media (VIGRO®, Vetoquinol N. -A. Inc.,) and transferred to the laboratory in
86 the 4-well cell culture dish.

87 In the laboratory, embryos were sized and graded morphologically on a scale of 1-4
88 (Tremoleda et al., 2003) and were categorized into three groups A≤300 µm, 300
89 µm<B<700 µm, and C≥700 µm. Embryos were transferred to Medium 199 (Sigma
90 M7528; with Earle's salts, 25 mM HEPES and sodium bicarbonate) and punctured 20 µm

91 with a laser system (LYKOS-DTS: Hamilton Thorne, Beverly, MA, USA) and/or piezo
92 micromanipulator with 20 µm needle (PMM-150FU: Prime Tech, Ibaraki, Japan).
93 Punctured embryos were transferred in an equilibration medium containing 7.5%
94 ethylene glycol and 7.5% Dimethyl Sulfoxide (Me_2SO) in Medium 199 plus 20% Fetal
95 Bovine Serum (FBS) for a maximum of 15 min at room temperature. After that, embryos
96 were transferred to the vitrification solution containing 15% ethylene glycol and 15%
97 Me_2SO in Medium 199 plus 20% FBS, loaded onto the Cryotop®, and soaked into the
98 liquid nitrogen within 1 min.

99 Embryos were thawed in Medium 199 containing 1M sucrose and 20% FBS for 1 min
100 at 37 °C then transferred to Medium 199 containing 0.5 M sucrose and 20% FBS for 3
101 min at room temperature. After that, embryos were washed in Medium 199 with 20%
102 FBS for 5 min at room temperature and transferred to fresh Medium 199 with 20% FBS
103 and turned on the warm plate (37 °C) and kept for 5 min. Embryos were cultured in
104 Medium 199 with 20% FBS for 3h. After 3h of culture, embryos and capsule size were
105 measured, and the quality of the embryo were assessed before embryo transfer. Embryo
106 transfers were performed non-surgically as described previously (Hannan et al., 2019,
107 2020). One week after embryo transfer, a spherical embryonic vesicle was diagnosed by
108 ultrasonographic examination of the uterus and pregnancy was confirmed at the heartbeat

109 stage, on days 26 (Case 1) and 28 (Case 3). Pregnant mares were checked twice a week

110 for normal embryonic development and a heartbeat in the early pregnancy stage and once

111 a week after that (Hannan et al., 2020).

112

113 **3 RESULTS**

114 In this study, six embryos were collected from eight uterine flushes (75%) from four

115 mares on day 7 and 8. All embryos were in the blastocyst stage, and their size were

116 between 233 and 1360 μm. All collected embryos were evaluated in grade 1 and

117 categorized into 3 groups A≤300 μm (n=1), 300 μm<B<700 μm (n=3) and C≥700 μm

118 (n=2) (Table 1). Three out of six embryos (50%) remained a grade 1 after thawing, and

119 both trophoblast and capsule diameters were similar to the pre-cryopreservation size.

120 PMAP treatment was effective in group A, and embryo quality was good (Fig. 1 A &

121 B), and the result of pregnancy was positive for case number 1 (Fig. 2). After thawing and

122 3h of culture, trophoblast size was 186 and 242 μm, and capsule size was 241 and 258

123 μm respectively (Case number 1; Table 1). In group B, PMAP was not a successful

124 method for removing blastocoel fluid, and trophoblast size was 476 and 453 μm

125 respectively after thawing and culture (Case number 2; Table 1). The pregnancy result

126 was positive (Fig. 2) when we used LAP in an embryo in group B case number 3 (Fig. 1.

127 C & D). However, the result of pregnancy for the combination of PMAP and LAP method
128 was not positive, the quality of embryo was good, and the embryo was expanded after
129 culture (grade 1). The trophoblast size was 431 and 547 μm after thawing and culture
130 (Case number 4; Table 1). All embryos in groups A and B re-expanded after thawing and
131 culture with case number 2 being the only exception. Embryos ≥700 μm in group C did
132 not re-expand with trophoblast diameter of 953 and 807 μm for case number 5, and 1153
133 and 752 μm for case number 6, respectively after thawing and 3h of culture.
134

135 **4 DISCUSSION**

136 Embryos with a diameter of >300μm have been reported to have a 44% (4/9)
137 pregnancy rate post-thaw between day 11 and 14, but in the second pregnancy check on
138 day 23, none of the recipient mares remained pregnant (Scherzer et al., 2011). In the
139 current study, embryos treated with LAP or LAP and PMAP showed two out of three
140 embryos maintaining grade 1 morphology after cryopreservation (Case number 3 and 4).
141 One of the embryos resulted in a successful pregnancy (case number 3; Fig. 1. C & D).
142 In the PMAP treated embryos, one out of three embryos was assessed as grade 1 and
143 transfer of that embryo resulted in a pregnancy (case number 1; Fig. 1 A & B). Heartbeat
144 confirms both foals are alive till now (170-day of pregnancy; Fig. 2).

Several different methods were used to induce blastocoel collapse for the successful equine embryo cryopreservation including direct puncture with a glass biopsy needle, acupuncture needle, tungsten microneedle (Wilsher et al., 2020), piezo manipulator (Choi et al., 2011) and laser pulse puncture (Scherzer et al., 2011). The previous results showed microneedle or Laser pulse puncture could be helpful in human cryopreservation and pregnancy rate (60% and 62%, respectively) (Mukaida et al., 2006). To the best of our knowledge, it is the first report on the LAP cryopreservation method with a positive and stable pregnancy in >300 µm equine embryo. It seems LAP is a quicker and more precise method with less damage to the embryo than other puncture methods.

We had a positive pregnancy in the piezo group. Case number 1 was in the small embryo group but the two other embryos in the medium and big size groups did not result in pregnancy. On the other hand, we could get a positive pregnancy result in a medium sized embryo cryopreserved after blastocoel puncture with LAP technology. In conclusion, using LAP can be helpful for equine medium size embryos; however, the current methodology is not suitable for big embryos (≥700 µm), and it needs more investigation to improve LAP technology for large equine embryos.

163 **Acknowledgments**

164 The authors are thankful to M. A Hannan and Shiori Takeuchi for technical assistance.

165

166 **Conflict of interest**

167 None of the authors have any conflict of interest to declare.

168

169 **Funding**

170 This study was supported by the Livestock Promotional Subsidy from the Japan

171 Racing Association.

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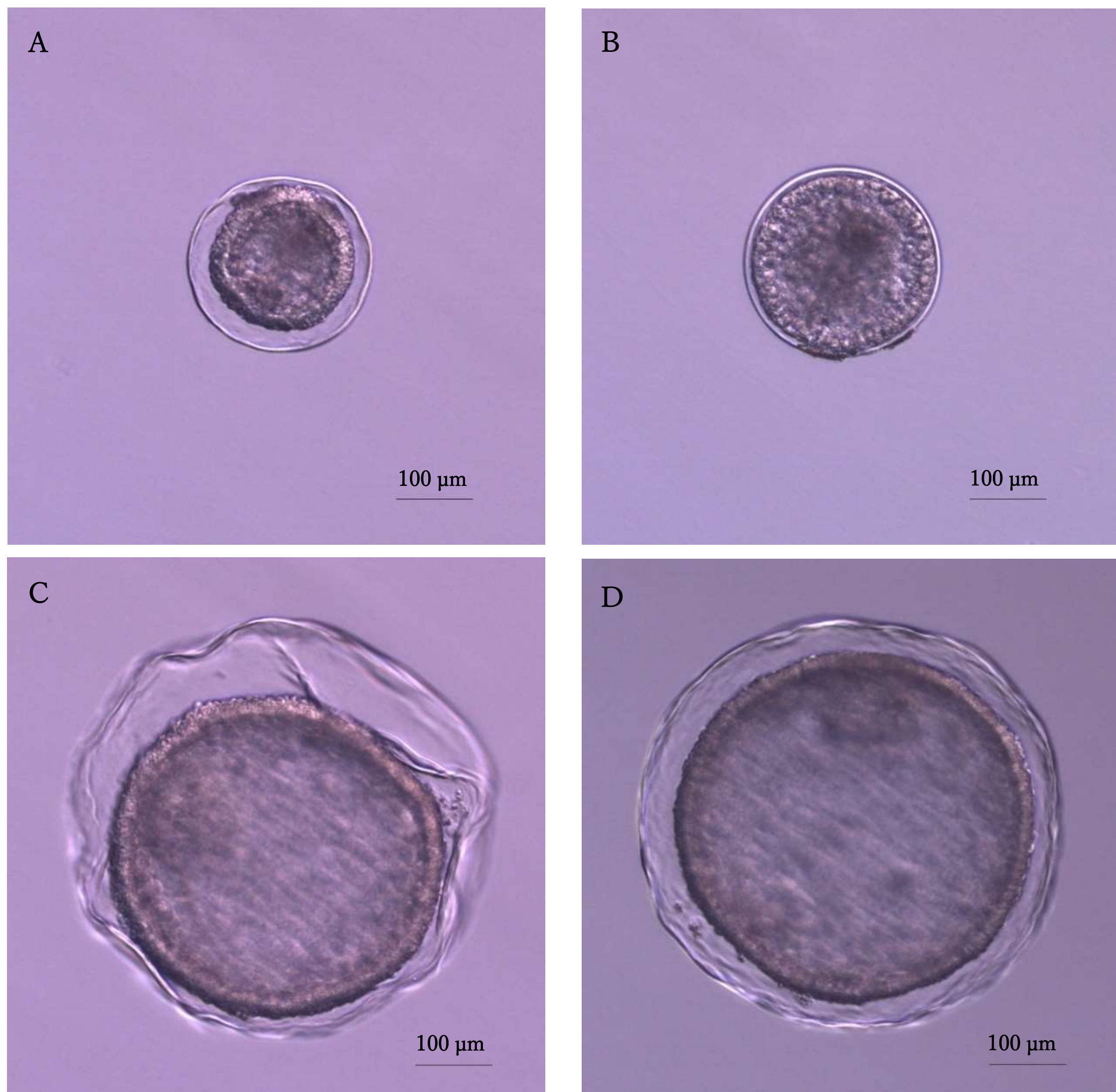

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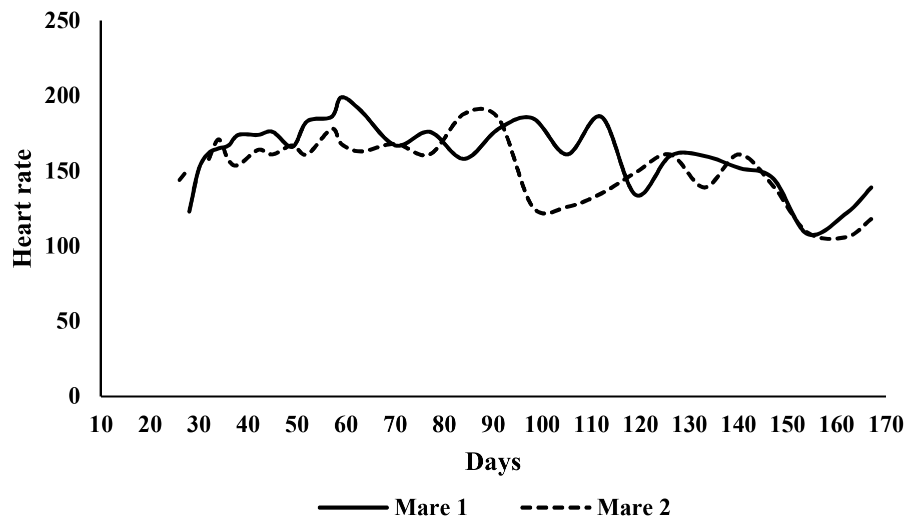
224 **Fig. 1.** Two transferred embryos with positive pregnancy, after thawing (A and C) and 3

225 h culture (B and D). A and B, C and D are the same embryo.

226



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228 **Fig. 2.** Heart rate of two positive pregnant mares from the day of ovulation until 170-day

229 of pregnancy.

230

231 **Table 1.** Embryo grade and diameter after collection, trophoblast and capsule size after thawing and culture, method of puncture, and the

232 result of pregnancy.

Case number	Group	Embryo diameter after collection (μm)	Diameter (μm) after thawing		Diameter (μm) after culture		Embryo grade after culture	Method of puncture	Result of pregnancy
			Trophoblast	Capsule	Trophoblast	Capsule			
1	A	233	186	241	242	258	1	PMAP	+
2	B	684	476	691	453	727	4	PMAP	−
3	B	598	414	546	468	548	1	LAP	+
4	B	571	431	530	547	558	1	LAP*2, PMAP	−
5	C	1235	953	1112	807	1103	3	PMAP*2	−
6	C	1360	1153	1244	752	1223	4	LAP, PMAP	−

233 *2: two times.