Cytoskeletal and mitochondrial properties of bovine oocytes obtained by Ovum Pick-Up; the effects of follicle stimulation and *in vitro* maturation

Tamás SOMFAI,[1] Satoko MATOBA,[1] Yasushi INABA,[1,#] Michiko NAKAI,[2] Kei IMAI,[3,$] Takashi NAGAI[4] and Masaya GESHI[1*]

[1]*Animal Breeding and Reproduction Research Division, NARO Institute of Livestock and Grassland Science, Tsukuba, Ibaraki 305-0901, Japan,* [2]*National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-0901, Japan,* [3]*National Livestock Breeding Center, Nishigo, Fukushima 961-8511, Japan, and* [4]*Food and Fertilizer Technology Center, Taipei 10648, Taiwan*

[#]Present address: National Livestock Breeding Center Tokachi Station, Otohuke, Hokkaido 080-0572, Japan

[$]Present address: Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

*Correspondence:

Masaya GESHI, Ph.D.

Animal Breeding and Reproduction Division, NARO Institute of Livestock and Grassland Science

2 Ikenodai, Tsukuba, Ibaraki 905-0901, Japan

Tel/Fax: +81-29-838-8637 E-mail: geshi@affrc.go.jp

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ABSTRACT

Follicle stimulation by follicular stimulating hormone (FSH) is known to improve developmental competence of bovine oocytes obtained by Ovum Pick-Up (OPU); however, the exact factors in oocytes affected by this treatment have remained unclear. We compared *in vitro* matured (IVM) oocytes obtained at the immature stage from cows by OPU either without or with stimulation with FSH (non-stimulated and stimulated OPU, respectively) to those obtained by superstimulation and *in vivo* maturation in terms of cytoskeleton morphology, mitochondrial distribution, intracellular ATP content and H_2O_2 levels at the metaphase-II stage and intracellular Ca^{2+} levels after *in vitro* fertilization (IVF). Confocal microscopy after immunostaining revealed the reduced size of meiotic spindle, associated with increased tendencies of microfilament degradation and insufficient mitochondrial re-distribution in non-stimulated OPU-derived IVM oocytes compared with those collected by stimulated OPU, which in turn resembled to *in vivo* matured oocytes. However, there was no difference in mitochondrial functions between oocytes obtained by stimulated or non-stimulated OPU in terms of ATP content, cytoplasmic H_2O_2 levels, base Ca^{2+} levels and the frequencies and amplitudes of Ca^{2+} oscillations after IVF. Larger size of metaphase spindle in oocytes obtained by stimulated OPU may reflect and potentially contribute to their high developmental competence.

Key words: bovine oocyte, in vitro maturation, in vivo maturation, cytoskeleton, mitochondria

INTRODUCTION

Collection of immature oocytes by Ovum Pick-Up (OPU) for *in vitro* embryo production (IVP) offers a sustainable means for the utilization of genetic materials from individual cows of high genetic background. In last decades, IVP systems, applying *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF) and subsequent *in vitro* embryo culture (IVC) have been greatly improved for the efficient production of bovine embryos (reviewed by Machaty *et al.* 2012). Still, embryo development to the blastocyst stage in most of current IVP systems does not exceed 50 % of total oocytes subjected to the process, which underlines the necessity for further improvements of IVP systems. The developmental competence of IVM oocytes is still significantly lower than that of their *in vivo* matured counterparts (Matoba *et al.* 2012) which suggests that further improvements in efficacy of IVP systems can be achieved by the optimization of methods used for recovery of immature oocytes by OPU and subsequent IVM. In accordance, the method used for obtaining oocytes during OPU has been found to greatly affect the yield of transferable embryos after IVP as a superstimulation of follicles in cows with FSH before OPU (a method named Follicular Growth Treatment = FGT) has been proven to significantly improve the developmental competence of immature oocytes collected by OPU and subsequently matured by IVM (Imai *et al.* 2008; Sugimura *et al.* 2012). Nevertheless, the factors within the oocyte that are responsible for the high developmental competence of FGT-derived oocytes have remained unknown.

Since immature bovine oocytes can reach the matured metaphase-II (MII) stage during IVM at high rates, it seems evident that the lack or irregular action of cytoplasmic factors is to accuse for the low competence of IVM oocytes. Indeed, the

72 developmental competence of IVM oocytes is known to be dependent on several
73 cytoplasmic factors such as the proper distribution and function of cytoplasmic
74 organelles during IVM and the proper accumulation of certain proteins, glutathione and
75 maternal mRNA in the cytoplasm during oocyte growth (reviewed by Ferreira *et al.*
76 2009). Normal function of cytoplasmic organelles, such as that of mitochondria is
77 essential for the ability of oocytes to undergo fertilization and embryo development.
78 Besides their pivotal role in ATP production during energy metabolism, mitochondria
79 play important roles in the regulation of intracellular levels of reactive oxygen species
80 (ROS) and cytosolic free Ca^{2+} ions (Dumollard *et al.* 2007), which in proper amounts
81 are required for signal transduction processes during fertilization (Fissore *et al.* 1995;
82 Dumollard *et al.* 2007), but at constant high levels trigger apoptotic events and therefore
83 are detrimental to oocytes (Guerin *et al.* 2001; Dumollard *et al.* 2007). The distribution,
84 anchoring and, to some extent even the function of cytoplasmic organelles (including
85 mitochondria) are conducted by cytoskeletal elements such as microtubules (Sun *et al.*
86 2001) and microfilaments (Yu *et al.* 2010) which in turn are greatly dependent on ATP
87 production and are easily compromised by irregularly high ROS levels (Hinshaw *et al.*
88 1988; Kuhne *et al.* 1993; Zhang *et al.* 2006) demonstrating the complexity of
89 interactions among cytoplasmic factors in oocytes.

90 The aim of the present study was to compare cytoplasmic characteristics in
91 cattle oocytes that had been collected by OPU conducted on cows both without and
92 after FGT and subjected to IVM to test if differences in cytoskeletal and mitochondrial
93 properties could possibly be responsible for the high developmental competence of
94 oocytes obtained after FGT treatment. In this respect, we compared OPU-derived
95 oocytes obtained without and after FGT and matured *in vitro* (FGT–/IVM and

FGT+/IVM groups, respectively) with oocytes collected from cows with FGT treatment, but followed by *in vivo* maturation (FGT+/ *in vivo* matured group) in terms of cytoskeleton morphology, mitochondrial distribution, intracellular ATP content and H_2O_2 levels at the MII stage and Ca^{2+} regulation after IVF.

MATERIALS AND METHODS

1 Animal care and use

The use of animals was subject to the regulations set out by the Law for the Humane Treatment and Management of Animals (Law No. 105, 1973) and notification no. 6 and no. 22 of the Japanese Guidelines for Animal Care and Use. All experimental procedures involving animals were approved by the Ethics Committees for Care and Use of Experimental Animals of NARO Institute of Livestock and Grassland Science (NILGS) and National Livestock Breeding Center (NLBC), Japan. Lactating Holstein cows serving as oocyte donors were individually fed a based on a total mixed ration according to the Japanese Feeding Standard for Dairy Cattle (2006) in the NILGS. Lactating Holstein cows serving as oocyte donors were reared under the same feeding according to the Nutrient Requirements of Dairy Cattle (2001) and environmental conditions in the NLBC.

2 Superstimulation of donor cows and collection of in vivo *matured oocytes*

Supersimulation of donor cows was conducted according to the previous report of Matoba *et al.* (2014). Briefly, cows received an intravaginal progesterone-releasing device [controlled internal drug release, (CIDR); Pfizer, Tokyo, Japan; CIDR insertion = day 0].

Before FSH administration, all follicles ≥8 mm in diameter were aspirated (dominant follicle ablation, DFA) on day 5. A total of 30 armour units (AU) of FSH (Antrin R10; Kyoritsu Seiyaku Co., Tokyo, Japan) was administered twice daily for 4 days in decreasing doses (6, 6, 4, 4, 3, 3, 2, and 2 AU, respectively) from the evening of day 6 to the morning of day 10 to stimulate follicular growth. All cows received 0.225 mg of $PGF_{2\alpha}$ (D-cloprostenol, Dalmazin; Kyoritsu Seiyaku Co.) on the evening of day 8 and the CIDR was removed on the morning of day 9. Gonadotropin-releasing hormone analogue (GnRH) (Spornen; Kyoritsu Seiyaku Co., 200 μg of fertirelin acetate; 0 h) was administered to induce the LH surge of growing follicles on the morning of day 10. All drugs were administered i.m. The number of antral follicles >2 mm was monitored and their size was measured ultrasonically (SSD-900 or SSD-1700; Hitachi Aloka Medical Ltd., Tokyo, Japan) on days 5 and 10, and 11.

Transvaginal recovery of *in vivo* matured oocytes was carried out as previously described by Matoba *et al.* (2014) at 25 to 26 h after GnRH administration to cows on day 11. All visible follicles ≥5 mm in diameter were aspirated from cows using a 7.5 MHz convex array transducer (UST-9106P-7.5, Hitachi Aloka Medical Ltd.) with a 17 gauge × 500 mm disposable needle attached to a 1,500-mm polyvinyl chloride tube (COVA Needle; Misawa Medical Industry Co. Ltd., Tokyo, Japan) connected to an ultrasound scanner using a relatively high vacuum pressure (130 mm of Hg; aspiration rate approximately 24 mL/min). The follicular contents were aspirated into a 50-mL conical tube containing approximately 5 mL of lactate Ringer's solution supplemented with 1% (v/v) bovine serum (16170-078; Gibco Invitrogen, Life Technologies, Auckland, New Zealand) and 10 IU/mL heparin (Novo-Heparin Injection 1000; Aventis Pharma Ltd., Tokyo, Japan). Sedimented materials in the conical tubes were transferred to 90-mm Petri

144 dishes and the remaining solution was filtered through an EmCon filter (Immuno System
145 Inc., Spring Valley, WI, USA). Cumulus-oocyte complexes (COCs) with expanded
146 cumulus were collected under a stereomicroscope and stored in a collection medium until
147 the end of the entire collection procedure. The collection medium ("TCM 199 Air") was a
148 tissue culture medium 199 (TCM 199; 31100-035, Medium 199 powder; Gibco Invitrogen)
149 buffered with 11 mmol/L of HEPES, 9 mmol/L of Na-HEPES, and supplemented with 5
150 mmol/L of sodium bicarbonate and 5% (v/v) newborn calf serum (NBS; S0750-500;
151 Biowest SAS, Nuaillé, France; pH 7.3). Oocytes surrounded were collected under a
152 stereomicroscope and excessive cumulus layers were trimmed with a blade in "TCM 199
153 Air" medium. Then oocytes covered by a few layers of expanded cumulus were cultured in
154 IVM medium as described below, for approximately 3 h (i.e., until 30 h after GnRH
155 administration on day 11) to ensure the finalization of nuclear maturation.
156
157 *3 Collection of immature oocytes by OPU conducted on cows without and after FGT*
158 *treatment*
159 To obtain immature oocytes from donor cows without FGT treatment (FGT–
160 group), follicles larger than 2 mm in diameter (along with dominant follicles) were
161 aspirated with OPU (first OPU session) conducted with vacuum (120 mmHg and 22
162 mL/min aspiration rate) on arbitrary days of estrus (day 0). The DFA of all follicles ≥8
163 mm in diameter was conducted 5 days after first OPU and CIDR was inserted on day 5.
164 Then, FSH was administered twice a day, for 4 days in decreasing doses (6, 6, 4, 4, 3, 3,
165 2, and 2 AU, respectively) from the morning of day 7 to the evening of day 10 to
166 stimulate follicular growth. All cows received 0.225 mg of $PGF_{2\alpha}$ in the morning of day
167 9. The second OPU session was performed 48 h after PGF administration (day 11), and

only follicles larger than 5 mm in diameter were aspirated (FGT+ group). The CIDR was removed from the cows just before OPU on the same day. The sediment of the aspirated follicular fluid was filtered and placed in a collection medium until the end of the entire collection procedure. Fully grown immature oocytes characterized by a compact cumulus compartment were collected under a stereo microscope on both day 0 (FGT– group) and day 11 (FGT+ group) and were subjected to IVM (FGT–/IVM and FGT+/IVM treatment groups, respectively).

4 IVM of immature oocytes

The medium used for IVM was TCM 199 (12340-030, Medium 199, GIBCO Invitrogen) supplemented with 5% (v/v) NBS, 0.02 AU/mL FSH and 100 IU/mL penicillin G potassium (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and 100 μg/mL streptomycin sulfate (Meiji Seika Pharma Co., Ltd.). Oocytes with homogenous ooplasm, surrounded by compact multiple layers of cumulus cells were submitted to IVM. After washing twice in pre-incubated IVM medium, groups of 5-20 COCs were cultured in 50 μL droplets of IVM medium covered by paraffin oil (Paraffin Liquid; Nacalai Tesque Inc., Kyoto, Japan) in 35-mm Petri dishes (Nunclon Multidishes, Nalge Nunc International, Roskilde, Denmark) for 22 h at 38.5 C in 5% CO_2 in air with saturated humidity.

5 Analysis of nuclear stage and cytoskeleton morphology in matured oocytes

Matured oocytes were denuded from cumulus cells after a brief treatment with 0.5 mg/mL hyaluronidase (Sigma-Aldrich Co., St Louis, MO, USA) by gentle repeated pipetting through a fine glass capillary. The denuded oocytes were fixed and stored in a

192 microtubule stabilization buffer as described by Ju *et al.* (2003) for up to one week.
193 Then, oocytes were incubated in a blocking solution which was Dulbecco`s Phosphate
194 Buffered Saline (PBS) containing 20 mg/mL BSA (Sigma-Aldrich Co.) and 150 mM
195 glycine for 1 h at room temperature (RT). After washing 2–3 times in PBS with 0.2%
196 (v/v) Triton X-100 (PBS-T) oocytes were incubated with an anti-α-tubulin primary
197 antibody (T-5168; Sigma-Aldrich) at a dilution of 1:100 for 2 days at 4°C in PBS-T. The
198 oocytes were then washed 2 times in PBS-T and then they were incubated with Alexa
199 Fluor^{TM}488 conjugated anti tubulin monoclonal secondary antibody (A21121,
200 Molecular Probes Inc., Eugene, OR, USA) (1:2000) for 1 h at 37 °C. After two
201 consecutive washings in PBS-T, the oocytes were incubated with 1 IU/mL rhodamine
202 phalloidin (R415, Molecular Probes) in PBS-T for 30 min at RT and washed again twice.
203 The oocytes were then mounted on glass slides with anti-phade solution (Component A;
204 S2828, Molecular Probes) supplemented with 10 µg/mL Hoechst 33342 (Calbiochem,
205 San Diego, CA, USA). To prevent the possibility for spindle distortion caused by
206 excessive pressure on oocytes during mounting, two lines of a vaseline-paraffin mixture
207 were applied between the slide glass and cover glass. The normality of the chromosome
208 alignment (blue) and meiotic spindles (green) and microfilaments (red) was evaluated
209 by a confocal laser scanning microscope (D-eclipse C1; Nikon, Tokyo, Japan) according
210 to Somfai *et al.* (2011). In oocytes where the spindle was located in a lateral position
211 (characterized by the appearance of the aligned metaphase chromosomes as a straight
212 line accompanied by microtubules from its two sides forming a barrel-shaped structure),
213 spindle length between poles (Fig. 1 A'-C') was measured using the ImageJ software
214 (Abramoff *et al.* 2004).
215

6 Evaluation of the distribution of mitochondria in matured oocytes

Oocytes were denuded as described previously and then incubated with 350 nmol/L MitoTracker Red CMXRos (MTR) dye (Molecular Probes) for 30 min in PBS containing 3 mg/mL BSA (Sigma-Aldrich Co.) under culture conditions. The oocytes were washed three times (10 min each) in pre-warmed PBS and then fixed for 20 min in 2% paraformaldehyde in PBS. The oocytes were then washed twice in PBS, mounted carefully with PBS- supplemented with 10 μg/mL Hoechst 33342 (Calbiochem) on glass slides under cover slips and examined immediately at RT in a dark room. The nuclear status and the distribution of active mitochondria were investigated in oocytes (confirmed by H33342 staining) by a laser-scanning confocal microscope (Nikon) using 405 nm and 543 nm excitation barrier filters, respectively. Mitochondrial distribution in matured oocytes (having the first polar body [1PB]) was classified as either "peripheral", "semi-peripheral" or "diffused" according to Brevini *et al.* (2005).

7 Measurement of intracellular ATP levels in matured oocytes

The ATP content of matured oocytes was measured using a commercial assay (FL-ASC; Sigma-Aldrich Co.) according to the method of Stojkovic *et al.* (2001) with modifications. Briefly, oocytes were completely denuded from cumulus cells. Pooled samples of 5 oocytes with the 1PB in each treatment group were rinsed three times in PBS- supplemented with 3 mg/mL polyvinyl pyroridone (PVP, P0930; Sigma-Aldrich Co.), then placed in plastic tubes with 50 μL of PBS- on ice/water and the samples were stored at -20 C until analysis. To measure ATP contents sample tubes were thawed and kept on ice. Then, 100 μL of ice-cold somatic cell reagent (FL-SAR) was added to all tubes that were incubated for 5 min on ice/water. Subsequently, 100 μL of ice cold

assay mix solution (dilution 1:25 with ATP assay mix dilution buffer, FL-AAB) was added, and the tubes were kept for 5 min at room temperature in the dark. The ATP content of the samples was measured using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA) with high sensitivity (0.01 pmol/tube). A seven-point standard curve (0–30 pmol/tube) was routinely included in each assay. The ATP content in samples was determined from the formula for the standard curve (linear regression).

8 Measurement of intracellular H_2O_2 levels in matured oocytes

The intracellular H_2O_2 concentration in the oocytes was quantified by the method of (Nasr-Esfahani *et al.* 1990) with modifications. Matured oocytes with 1PB were incubated for 15 min in 50 μL of Medium 199 supplemented with 3 mg/mL PVP containing 10 μmol/L 20,70-dichlorodihydro-fluorescein diacetate (DCHFDA). Then, they were rinsed twice in the medium without DCHFDA and carefully mounted on a glass slide with a cover slip. After mounting, the space between the glass slide and cover slip was rinsed through with PBS. The fluorescence intensity in groups of 4-6 oocytes in each treatment groups was measured by taking grayscale images under an epifluorescence microscope (Eclipse E-600; Nikon) equipped with a digital camera (Pixera Penguin 150 CL; Pixera Corp., Los Gatos, CA, USA) at 200 × magnification at an excitation wavelength of 480 nm and emission at 510 nm using the same exposition interval at 20 s after exposure to UV light. Mean fluorescence intensity and the surface area of each oocyte were measured in images using the NIH ImageJ. Total fluorescence/oocyte was calculated as mean fluorescence (“mean gray value”) multiplied with oocyte surface (“area”).

9 In vitro *fertilization (IVF) and the measurement of intracellular Ca^{2+} levels*

Frozen-thawed semen of a Japanese black bull was thawed at 37 °C for 30 sec, then layered on a Percoll (Sigma-Aldrich Co.) density gradient (45 and 60%) and centrifuged at 740 x g for 10 min at 37 °C. Then the sperm pellet was washed once with 6 mL IVF 100 medium (Research Institute for Functional Peptides Co., Ltd., Yamagata, Japan) at 540 x g for 5 min at 37 °C. The final sperm concentration was adjusted to 3 x 10^6 sperm/mL. Twenty COCs were fertilized with frozen-thawed sperm in a 100 μL droplet of IVF 100 medium under paraffin oil overlay. The COCs and sperm were co-incubated for 5 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. Excessive cumulus cells and sperm were then removed by repeat pipetting in TCM199 Air. Presumptive zygotes were incubated in the presence of 2 mmol/L fura-2 acetoxymethyl ester, the Ca^{2+} indicator dye, and 0.02% pluronic F-127 (both from Molecular Probes) in TCM199 Air for 40-50 min. After incubation the oocytes were rinsed in a HEPES-buffered TCM-199 (12340-030, Medium 199; GIBCO Invitrogen) and changes in the $[Ca^{2+}]_i$ were measured by an AQUACOSMOS fluorescence microscope system (Hamamatsu Photonics, Hamamatsu, Japan). A75 watt xenon arc lamp was used as the light source. Dual excitation at 340 and 380nm was achieved with a rotating chopper disk and appropriate bandpass filters. The emitted fluorescence intensity was measured after background subtraction at 510 nm with a photomultiplier tube. Intracellular free Ca^{2+} levels are presented as fluorescence ratio (R) values of the 340/380nm excitation intensities. The ratio of 1.2 and 6.5 represents about 65 and 602nM Ca^{2+}, respectively (Machaty *et al.* 1997). Ca^{2+} levels in individual oocytes were recorded in every 30 sec during a 3 h interval. The frequency of oscillations, the base Ca^{2+} level and mean the deviation of Ca^{2+} level from the base level during oscillations were recorded.

10 Statistical analysis

All data were analyzed by one way ANOVA at a $P<0.05$ significance level using the KyPlot package (Ver. 4.0, Kyens Lab. Inc., Tokyo, Japan) followed by Tukey`s multiple comparison test. Percentage data were arcsine transformed before analysis.

RESULTS

1 Nuclear stage and cytoskeleton morphology in oocytes

There was no significant difference ($P>0.05$) in the percentage of matured (MII) stage oocytes among *in vivo* and *in vitro* matured oocytes irrespective of follicular stimulation prior to OPU. (Table 1). Regarding the cytoskeleton status in matured oocytes there were no significant differences among groups in frequencies of oocytes showing abnormal spindle or microfilament integrities. (Table 2, Fig. 1). In the FGT–/IVM group high rates of oocytes showed the lack of internal microfilaments (Fig. 1A) compared with other groups; however, the differences in frequencies were not statistically significant due to low numbers. The measurement of spindle length between poles of meiotic spindle in lateral position revealed significantly lower spindle length in FGT–/IVM oocytes compared with both FGT+/IVM and FGT+/ in vivo oocytes (Table 2, Fig. 1A`-C`).

2 Mitochondrial distribution, ATP and ROS levels in matured oocytes

Among the 3 experimental groups significant difference was not observed in the frequencies of oocytes showing peripheral, semi-peripheral or diffuse mitochondrial distribution (Fig. 2). Nevertheless, the overall patterns for the frequencies of the 3

312 mitochondrial distribution types were similar in FGT+/IVM and FGT+/in vivo groups
313 (Fig. 2) whereas the frequency of peripheral distribution type appeared to be notably
314 high and that of the diffuse distribution type was very low in the FGT–/IVM group.
315 There was no significant difference in ATP levels between IVM oocytes obtained
316 without or after FGT treatment. However, the ATP content in *in vivo* matured oocytes
317 appeared significantly lower ($P<0.05$) than that in FGT–/IVM oocytes whereas ATP
318 levels in FGT+/IVM oocytes showed intermediate values (Table 3). Significant
319 difference was not detected in intracellular H_2O_2 levels in matured oocytes among the 3
320 treatment groups (Table 4, Fig. S1).
321
322 *3 Ca^{2+} oscillations after fertilization*
323 After measurement of intracellular free Ca^{2+} levels in oocytes, 3 types of Ca^{2+} level
324 patterns were distinguished: 1) normal regular oscillations, where oscillation peaks were
325 clearly characterized, distinguishable from the base Ca^{2+} levels and re-appeared in
326 regular intervals; 2) irregular oscillations, where the presence of oscillation peaks is
327 inevitable; however, their amplitude compared to the base levels and/or their numbers
328 were uncertain and/or their frequency was irregular; 3) the lack of oscillation peaks
329 (indicating the lack of fertilization) (Fig. S2). In the FGT–/IVM group 100% (22/22) of
330 oocytes showed type 1 oscillations whereas in FGT+/IVM group the frequencies of type
331 1, 2 and 3 oscillations were 80.8 % (25/31), 19.3 % (6/31) and 0 % (0/31), respectively.
332 In *in vivo* matured oocytes the frequencies of type 1, 2 and 3 Ca^{2+} level patterns were
333 52.9 % (9/17), 41.1% (7/17) and 5.8 % (1/17), respectively. Apparent differences were
334 not observed between IVM oocytes obtained without or after FGT treatment in terms of
335 the basic levels of cytosolic Ca^{2+}, oscillation frequencies and oscillation amplitudes. In

336 *in vivo* matured oocytes, oscillation frequency appeared to be higher than that in
337 FGT–/IVM oocytes; however, it was not different from that of FGT+/IVM oocytes.
338
339 **DISCUSSION**
340 In this study, we compared oocytes obtained from the same group of donor
341 cows by OPU without or after FGT treatment. This setup allowed us to control the
342 source of oocytes (such as the age, health, reproductive nutritional status of cows from
343 which they are obtained), and to minimalize individual effects and damages from
344 unknown stresses that may occur when slaughterhouse-derived oocytes of unknown
345 origin are used. The main differences between IVM oocytes collected from cows
346 without or after follicle superstimulation found in the present study was the reduced
347 length of meiotic spindle after maturation which was associated with a slight
348 degradation of internal actin microfilaments and a slightly reduced ability for
349 mitochondrial redistribution in oocytes obtained without FGT treatment.
350 In this study, IVM oocytes obtained from non-stimulated cows had
351 significantly shorter meiotic spindles compared with those obtained from stimulated
352 animals, which in turn resembled to *in vivo* matured oocytes. A positive correlation
353 between the spindle length in oocytes observed by live imaging and their developmental
354 competence to the blastocyst stage has been reported in humans (Rama Raju *et al.* 2007).
355 In oocytes of farm animals high content of intercellular lipid blocks the visibility of the
356 meiotic spindles by live imaging, therefore, direct evidence for the correlation between
357 spindle length and developmental competence has been lacking in cattle and pigs.
358 However, similarly to our results in bovine, *in vitro* matured porcine oocytes have been
359 reported to possess significantly shorter meiotic spindles compared with their *in vivo*

matured counterparts (Ueno *et al.* 2005). Both in cattle and pigs *in vivo* matured oocytes show higher developmental ability to the blastocyst stage than those matured *in vitro* (Nagashima *et al.* 1996; Matoba *et al.* 2012). Based on these, it can be concluded that the longer spindle attributed to follicle stimulation may be a marker of a higher competence. A possible reason for reduced spindle length could be excess time after reaching the MII stage without fertilization which can cause a variety of cytoplasmic changes in mammalian oocytes reducing developmental competence. This process is often called "oocyte ageing" and it includes the impairment of the meiotic spindle and other elements of the cytoskeleton (reviewed by Miao *et al.* 2009). Reduced spindle length has been reported previously in human oocytes as a result of ageing (Wang *et al.* 2001). In the present study, *in vivo* and *in vitro* matured oocytes obtained from super stimulated animals had tendentiously higher frequencies of oocytes at the AI or TI stage compared to IVM oocytes obtained from non-stimulated animals. This suggests that IVM oocytes from non-stimulated animals may reach the MII stage earlier, and therefore may be subjected to a higher degree of aging during IVM compared to the other groups, which may be a possible explanation for shorter spindle length in them. The suggestion for enhanced ageing in the FGT–/IVM group corresponds with the tendentious reduction of microfilaments in the central areas of oocytes - also known to be sensitive to the aging process (Miao *et al.* 2009) - and the increased ATP levels (Koyama *et al.* 2014) in this group. Besides aging, other factors such as stress or abnormal expression or regulatory proteins might possibly affect the morphology of the meiotic spindle and cytoskeleton. For instance, high intracellular ROS levels and ATP depletion caused by oxidative stress can also reduce spindle length and impair microfilaments (Hinshaw *et al.* 1988; Kuhne *et al.* 1993; Zhang *et al.* 2006). However,

384 our results did not reveal abnormally high levels of ROS and low levels of ATP in
385 FGT–/IVM oocytes therefore, this explanation can be ruled out. Furthermore, the
386 difference in follicle size between the FGT–/IVM and FGT+/IVM groups (2-8 mm and
387 5-8mm, respectively) may raise concerns for the possible effects of the actual follicle
388 size on spindle morphology and oocyte competence since small oocytes at the growth
389 phase collected from follicles less than 3 mm in diameter are known to have low
390 competence as accumulation of proteins and mRNA necessary for further maturation
391 and development has not been finalized in them (Fair *et al.* 1995; Hyttel *et al.* 2001;
392 Ferreira *et al.* 2009). However, in our system, oocytes smaller than 110 μm in diameter
393 are omitted from experiments and the oocytes used in this study appeared similar in
394 terms of their size (fully grown) and nuclear status (GV stage) both in the FGT–/IVM
395 and FGT+/IVM groups. Previous studies have shown similar metabolic activities and
396 transcript profiles for genes including those involved in spindle assembly in fully grown
397 oocytes obtained from small and large antral follicles (Lequarre *et al.* 2005; Mourot *et*
398 *al.* 2006). This suggests that within the size range used in this study, the size of the
399 antral follicle might not affect basic properties of spindle formation and mitochondrial
400 metabolism in oocytes during IVM. Nevertheless, the possibility that follicle size might
401 affect the function of these organelles cannot be ruled out. Further research will be
402 necessary to clarify this point.

403 In mammalian oocytes, mitochondria play pivotal roles in energy production,
404 ROS regulation and Ca^{2+} regulation processes; therefore, their distribution and activity
405 potentially determine their developmental competence (Dumollard *et al.* 2007). In
406 immature oocytes mitochondria are mainly located in the peripheral areas and their
407 redistribution towards the oocyte center seems to be an important process for energy

production during meiotic progression (Yu *et al.* 2010). Bovine IVM oocytes require a level of ATP higher than 0.6 pmol to maintain their ability to undergo fertilization (Somfai *et al.* 2012). In accordance with the above mentioned, IVM bovine oocytes with low developmental competence have been characterized with a peripheral mitochondrial distribution and reduced ATP contents (Stojkovic *et al.* 2001). In the present study, the frequency of MII stage oocytes with peripheral mitochondrial distribution was tendentiously higher in the FGT–/IVM group compared with FGT+/IVM and FGT+/*in vivo* matured groups, which in turn showed almost identical patterns for the frequencies of the three mitochondrial distribution. Higher grades of mitochondrial redistribution in FGT+/IVM and FGT+/*in vivo* matured groups correspond with the better morphology of cytoskeletal elements, which play important roles for moving and anchoring mitochondria in oocytes. On the other hand, mitochondrial activities in cytoplasm as measured by total ATP contents, ROS levels were not different between matured oocytes obtained from cows without and after follicle stimulation, irrespective of the way of maturation. In the present study, in all treatment groups, ATP levels measured in matured oocytes were comparable to those of previous reports using the same assay either on slaughterhouse-derived (Stojkovic *et al.* 2001; Iwata *et al.* 2011) or OPU-derived bovine oocytes (Tamassia *et al.* 2004). Nevertheless, the literature is lacking information on ATP levels in *in vivo* matured bovine oocytes. In pigs, ATP levels in *in vivo* matured oocytes were found to be similar to that in IVM oocytes (Brad *et al.* 2003). In our study, the intracellular ATP content in *in vivo* matured oocytes appeared to be slightly but significantly lower than that of IVM oocytes obtained from non-stimulated oocytes and did not differ significantly from IVM oocytes obtained after FGT. Nevertheless, even in *in vivo* matured oocytes the ATP

content reached 1.98 pmol which in bovine is high enough to enable the processes of fertilization and subsequent embryo development to the blastocyst stage (Somfai *et al.* 2012). The actual ATP levels in matured oocytes may reflect their ageing status since aging have been found to increase ATP levels in bovine oocytes (Koyama *et al.* 2014). In this respect, higher ATP levels in non-stimulated IVM oocytes may refer to their increased ageing status as suggested above. Furthermore, the possible presence of TI stage oocytes in ATP samples might have also contributed to low ATP levels in *in vivo* matured group. In mice, the approximate timing of the AI/TI stage is associated with a transient drop of cytosolic ATP in oocytes (Yu *et al.* 2010). In the present study, selection of matured oocytes was based on the presence of the first polar body which appears from the TI stage. Since in the FGT+/*in vivo* group 17.6% of the selected oocytes were still at the TI stage, there is a high probability that the ATP samples (each consisting of 5 oocytes/sample) could include an oocyte at the TI stage in this group.

Mitochondria play important roles in the regulation of Ca^{2+} ions during signal transduction in oocytes. Mitochondrial ATP production is required for maintaining a low base level of Ca^{2+} and also for sustaining sperm-triggered Ca^{2+} oscillations after penetration (Dumollard *et al.* 2004). Mitochondria also act as Ca^{2+} stores, thus during sperm-induced Ca^{2+} oscillations they play pivotal roles for returning Ca^{2+} levels to the base after each efflux thus preventing apoptosis triggered by excessively high levels of cytosolic free Ca^{2+} (Dumollard *et al.* 2007). The Ca^{2+} oscillation patterns obtained in this study were comparable to those reported previously in bovine oocytes after IVF (Fissore *et al.* 1995; Malcuit *et al.* 2006). Although the Ca^{2+} oscillations in fertilized oocytes seemed to be more frequent in FGT+/*in vivo* group compared with IVM groups, there was no obvious difference caused by FGT treatment. Taken together, we found no

difference in mitochondrial functions among oocytes obtained by stimulated or non-stimulated OPU in terms of ATP content, cytoplasmic H_2O_2 levels, base Ca^{2+} levels and the frequencies and amplitudes of Ca^{2+} oscillations after IVF. This suggest that follicle stimulation by FSH (FGT treatment) and *in vivo* maturation provides high developmental competence for oocytes by other ways than affecting mitochondrial functions. Slight differences in mitochondrial distribution among groups may only refer to differences in cytoskeletal organizations. This suggestion is confirmed by previous results demonstrating that a diffused mitochondrial distribution is not a pre-requisite for achieving developmental competence to the blastocyst stage for oocytes during IVM (Somfai *et al.* 2012).

In conclusion, this study revealed the reduced size of meiotic spindle, accompanied with a slight degradation of internal actin filaments and low degrees of mitochondrial redistribution in IVM oocytes obtained by OPU conducted on cows without FSH treatment compared with those collected from the same cows after stimulation with FSH. However, there was no evidence for different mitochondrial functions between oocytes obtained without or after follicle stimulation irrespective of the manner of maturation. Among the studied features only the greater spindle length was clearly attributed to follicle stimulation with FSH. Our results are in accordance with a recent report on global transcript analysis in bovine oocytes which revealed chromosome segregation control as the one of the most prominent cytoplasmic functions that were differentially expressed at the mRNA level as a result of follicle stimulation by FSH (Labrecque *et al.* 2013) which may correspond with spindle functions. Nevertheless, at this point it remains unclear if the actual spindle length affects its functionality and the ability of oocytes to develop to normal embryos. The

480 ability of metaphase spindle in oocytes to proceed chromosome segregation during
481 fertilization might be a key factor defining developmental competence. Defects of the
482 meiotic spindle can potentially result in aneuploidy due to mistakes in assortment of
483 sister chromatids during the extrusion of the second polar body (Wang & Sun 2006) or
484 digynic triploidy by the complete failure of polar body extrusion during fertilization
485 (Rosenbusch 2008), both causing impaired competence of resultant embryos. The
486 contribution of such chromosome abnormalities in oocytes to reduced developmental
487 competence of IVF embryos remains unknown and requires further research.
488

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496

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634

635 **FIGURE LEGENDS**

636

637 **Figure 1** Matured bovine oocytes after simultaneous staining of chromosomes (blue),
638 microtubules (green) and microfilaments (red).
639 Treatment groups: A) FGT–/IVM (conventional OPU followed by IVM, note the lack of
640 actin microfilament network from oocyte center); B) FGT+/IVM (FGT–OPU followed
641 by IVM); C) FGT+/in vivo (*in vivo* maturation). A'-C') meiotic spindles of
642 corresponding oocytes. Arrows denote pole to pole length of spindles. Scale bars
643 represent 100 μm in micrographs A-C and 10 μm in micrographs A'-C'.

644

645 **Figure 2** Distribution of mitochondria in OPU-derived MII stage oocytes stained by
646 MitoTracker Red CMXRos. Distribution types: A) peripheral; B) semi-peripheral; C)
647 diffused; D) percentages of distribution types among treatment groups. Scale bars
648 represent 100 μm. Total numbers of oocytes (n) examined in each group are given in
649 parentheses. 3 biological replications were performed. Percentage data are presented as
650 mean ± SEM. Significant difference was not detected among the treatment groups at $P <$
651 0.05 (One way ANOVA).

652

Table 1. Nuclear status in OPU-derived oocytes after maturation *in vitro* and *in vivo*

FGT	Maturation	No of donor cows	Total oocytes	Nuclear status of oocytes				
				GV	MI	AI/TI	MII	Deg
–	*In vitro*	12	60	0 (0 ± 0)	8 (12.9 ± 1.8)	1 (1.3 ± 1.3)	49 (82.1 ± 3.0)	2 (3.5 ± 3.5)
+	*In vitro*	7	50	0 (0 ± 0)	5 (9.2 ± 9.2)	5 (9.7 ± 3.4)	39 (79.1 ± 14.5)	1 (1.8 ± 1.8)
+	*In vivo*	4	44	0 (0 ± 0)	7 (18.1 ± 7.0)	7 (17.6 ± 6.8)	30 (64.2 ± 12.1)	0 (0 ± 0)

3 biological replications were performed. Percentage data are presented as mean ± SEM. Significant difference was not detected among the treatment groups at $P < 0.05$ (One way ANOVA).

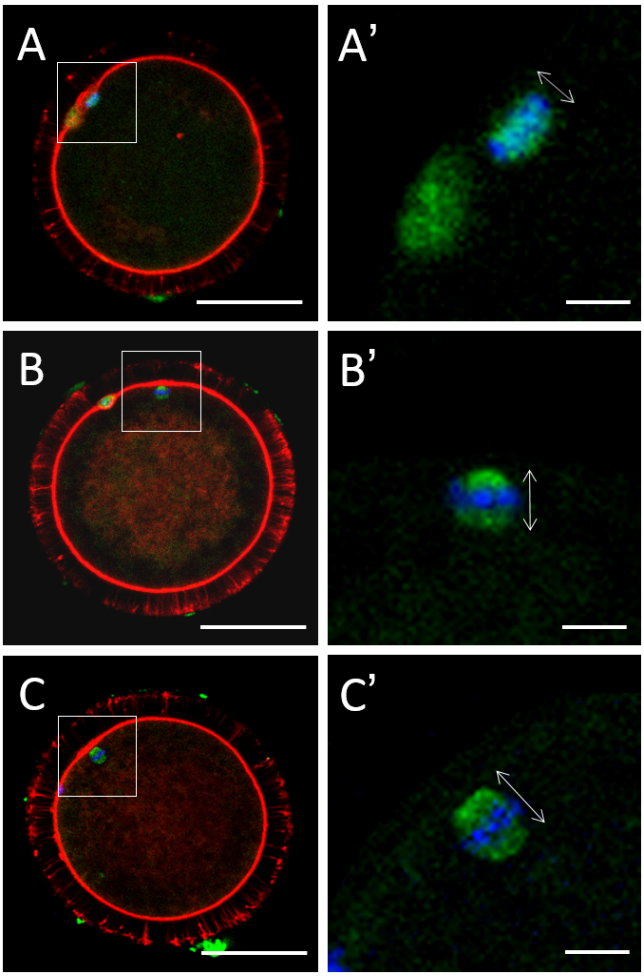
Abbreviations: OPU＝Ovum Pick-Up, FGT=Follicle Growth Treatment, GV = Germinal Vesicle, MI= Metaphase-I, AI=Anaphase-I, TI=Telophase-I, MII=Metaphase-II, Deg= Degenerated

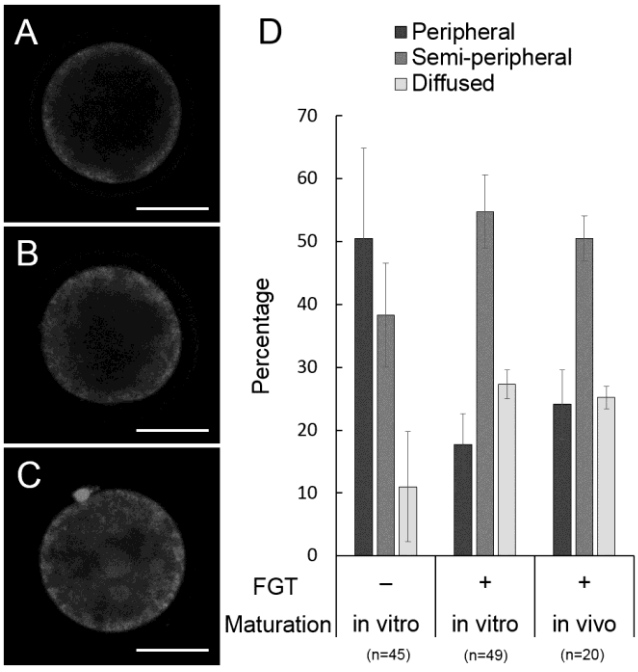
Table 2. Characteristics of cytoskeleton in OPU-derived MII stage oocytes

FGT	Maturation	No of donor cows	Total oocytes	No. (%) of oocytes with abnormalities observed in			Spindles from lateral view	
				Spindle	Cortical filaments	Inner filaments	Total measured	Length (μm)
–	*In vitro*	12	49	2 (3.9±2.0)	2 (4.4±4.4)	22 (44.2±15.9)	11	8.28±0.16a
+	*In vitro*	7	39	1 (3.7±3.7)	0	3 (11.1±11.1)	12	12.88±0.85b
+	*In vivo*	4	30	0	1 (2.7±2.7)	2 (5.5±5.5)	8	12.45±1.3b

3 biological replications were performed. Data are presented as mean ± SEM. Values with different superscripts in the same column are significantly different at $P < 0.05$ (One way ANOVA).

Abbreviations: OPU＝Ovum Pick-Up, FGT=Follicle Growth Treatment, MII=Metaphase-II


A
A'
B
B'
C
C'


A
B
C
D
Peripheral
Semi-peripheral
Diffused
Percentage
70
60
50
40
30
20
10
0
FGT
Maturation
–
+
+
in vitro
in vitro
in vivo
(n=45)
(n=49)
(n=20)