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ORIGINAL ARTICLE

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Running Head: DNA REPAIR GENES IN PORCINE OOCYTES

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ABSTRACT

Boar sperm freeze-dried with trehalose showed a protective effect against sperm DNA fragmentation. However, normal fertilization and embryonic development were not improved. Damaged sperm may activate maternal DNA repair genes when injected to oocytes. Therefore, we investigated the expression profile of some DNA repair genes in porcine oocytes after ICSI. Firstly, the expression levels of MGMT, UDG, XPC, MSH2, XRCC6, and RAD51 genes that concerned with the different types of DNA repair were examined in *in vitro* mature (IVM) oocytes injected with ejaculated sperm, or freeze-dried sperm with or without trehalose. Quantitative RT-PCR revealed that expression of six DNA repair genes in the oocytes at 4 h after injection did not differ among four groups. Next, we investigated the gene expression levels of these genes at different stages of maturation. The relative expression levels of UDG and XPC were significantly up-regulated in mature oocytes compared with earlier stages. Furthermore, there was an increased tendency in relative expression of MSH2 and RAD51. These results suggested two possible mechanisms that mRNA(s) of DNA repair genes are either accumulated during IVM to be ready for fertilization or increased expression levels of DNA repair genes in oocytes caused by suboptimal IVM conditions.

Key words*: DNA repair genes, freeze-dried sperm, gene expression, ICSI, pig.*

INTRODUCTION

It is reported that newly fertilized embryos of some species possess the ability to sense, respond to, and repair at least some types of DNA damage, particularly in the incoming sperm DNA (Zheng *et al*. 2005). DNA repair is an essential process for maintenance of genomic integrity in the preimplanation embryos to correct the damage existed in the gametes. The damage may be either inherent or arose during DNA replication and is also

caused by genotoxic agents (Zheng *et al*. 2005). The DNA damage needs to be repaired before the first round of DNA replication of zygote to minimize the mutation load of the developing embryos (Zheng *et al*. 2005). There are several DNA repair pathways in mammalian cells: direct reversal of damage, nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and double strand break repair (DSBR) (Jaroudi *et al*. 2009). Direct reversal of damage is the simplest form of DNA repair and also the most energy efficient method; it does not require a reference template as the other single strand repair mechanisms. O-6-methylguanine-DNA methyltransferase (known as MGMT) a specific DNA repair enzyme can remove the alkyl group from the O^6-position of the guanine, thereby preventing its mutagenic and carcinogenic effects (Zuo *et al.* 2004), belonging to direct reversal pathway. There are a number of regulatory elements in the MGMT promoter region, and a number of stimuli may increase MGMT expression, such as irradiation, glucocorticoid exposure, and cAMP (Liu *et al*. 2012).

Expression profile of maternal DNA repair genes correlates the ability of the oocytes to recognize and repair DNA damage at certain stages (oocyte/blastocyst). For instance, Jaroudi *et al*. (2009) demonstrated that the mRNA level for most repair genes was higher in oocytes compared with blastocysts in human and this is to ensure sufficient availability of template until embryonic genome activation, and that the DNA repair transcripts accumulated in the human oocyte play an important role in chromatin remodeling and maintain chromatin integrity during fertilization. Furthermore, when the DNA damage caused during fertilization is recognized as irreparable, embryos are excluded by cell cycle arrests or activation of apoptotic pathways (Jaroudi *et al*. 2009). To our knowledge, there are only a few works about the expression profiling of DNA repair transcripts in human oocytes and early embryos due to the rare availability of the materials and the ethical considerations (Li *et al*. 2006). Usage of non-human primates, laboratory or

74 domestic animals in research is more feasible and has a great importance on providing
75 novel knowledge on this field.

76 It is considered that DNA repair ability of oocytes correlates to the amount of
77 maternal repair mRNA in the cytoplasm which accumulated during the growth phase of
78 oocytes and follicles, and required for completion of the meiotic cell cycle (Zheng *et al*.
79 2005). Moreover, *in vitro* culture of oocytes and embryos may lead to dysregulation of
80 many genes (Zheng *et al*. 2005; Jones *et al*. 2008; Salhab *et al*. 2013), resulting in low
81 cellular viability and long-term embryo viability by the impaired competence for the repair
82 of the DNA damage. Recent studies suggested the differential expression of several repair
83 genes between *in vivo* and *in vitro* matured (IVM) oocytes in cattle (Thelie *et al*. 2007), in
84 human (Jones *et al*. 2008) and in non-human primates (Zheng *et al*. 2005). However, the
85 expression of DNA repair-related genes after fertilization has not been examined in porcine
86 oocytes.

87 In our previous study (Men *et al*. 2013), we have shown that sperm freeze-dried in
88 the basic medium containing 15 mmol/L trehalose showed less DNA damage compared
89 with control group without trehalose treatment. However, normal fertilization and
90 subsequent embryonic development were not different between both two groups. It is still
91 unknown that whether the increased DNA damage of sperm in the control group leads to
92 increase the expression of DNA repair genes in oocytes after injection of a spermatozoon
93 from this group. The objective of this study was to estimate the expression levels of DNA
94 repair-related genes in porcine oocytes after fertilization by intra-cytoplasmic of sperm
95 injection (ICSI) before early cleavage stage. In addition, the expression profile of these
96 genes was also detected in different stages of oocyte maturation.

97 Based on previous reports (Harrouk *et al*. 2000; Wood *et al*. 2001; Zheng *et al*.
98 2005; Jaroudi & SenGupta 2007), we focused on the expression of six DNA repair genes

related to repair ability of different kinds of DNA damage: MGMT (for direct reversal), UDG (for NER), XPC (for BER), MSH2 (for MMR), XRCC6 (for DSBR by homologous recombination) and RAD51 (for DSBR by non-homologous end-joining) (Tables 1 and 2).

Many reports showed that DNA repair in oocyte occurs in the first few hours after fertilization (in rats, Harrouk *et al*. 2000; in mice, Derijck *et al*. 2006), or prior to S-phase (pronuclear stage) (in humans, Aitken & Koppers 2011). Therefore, in this study, mRNAs were extracted from oocytes at 4 h after sperm injection (Experiment 1) and at four time points of oocyte maturation (Experiment 2) and were subjected to quantitative RT-PCR to examine the expression of these genes.

MATERIALS AND METHODS

Chemicals and media

All chemicals were obtained from Sigma-Aldrich (St. Louis, MA, USA), unless otherwise stated. The freeze-drying medium contained 10 mmol/L Tris-HCl buffer supplemented with 50 mmol/L ethylene glycol tetraacetic acid (EGTA; 346-01312; Dojindo Laboratories, Kumamoto, Japan) and is referred to as basic freeze-drying medium. This medium was then supplemented with different concentrations of trehalose (T0167; 0 (referred as control) and 15.0 mmol/L) and also NaCl (50.0 and 40.0 mmol/L, respectively). The osmolality and pH of the final solutions were adjusted to 265 to 270 mOsm/kg and 8.0 to 8.5, respectively, and then they were filtered and stored at 4^{o}C. The *in vitro* maturation medium for oocytes was modified North Carolina State University (NCSU)-37 solution containing 10% (v/v) porcine follicular fluid, 0.6 mmol/L cysteine, 50 mmol/L β-mercaptoethanol, 1 mmol/L dibutyl cAMP, 10 IU/mL eCG (Serotropin; ASKA Pharmaceutical Co. Ltd., Tokyo, Japan), and 10 IU/mL hCG (Puberogen 500 units, Novartis Animal Health, Tokyo, Japan) (Kikuchi *et al*. 2002). Medium used for *in vitro* culture (IVC) of sperm-injected oocytes was

modified NCSU-37 supplemented with 0.17 mmol/L sodium pyruvate, 2.73 mmol/L sodium lactate, 4 mg/mL bovine serum albumin (BSA) and 50 mmol/L β-mercaptoethanol (IVC-PyrLac) (Kikuchi *et al*. 2002).

Sperm collection and freeze-drying

Protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences, Tsukuba, Japan. Sperm collection and freeze-drying were conducted as described previously (Men *et al*. 2013). In brief, ejaculated semen was collected from a Landrace boar, which is used for reproductive program at National institute of Livestock and Grassland Science, Tsukuba Japan, and transferred to the laboratory within 30 min. After determination of the sperm concentration, the semen was centrifuged for 10 min at 900 × *g* at 30ºC and the seminal plasma was removed. The pellet was re-suspended in freeze-drying medium containing different concentrations of trehalose pre-warmed at 30ºC, and the supernatant was removed after centrifugation. The final pellet was then re-suspended in freeze-drying media containing 0 or 15 mmol/L trehalose at a final concentration of 4×10^8 cells/ mL. One milliliter of sperm suspension was placed into an individual glass vial (15-mL glass vial, Maruemu Corporation, Tokyo, Japan), then the vials were covered with aluminum foil and placed in a refrigerator at –80ºC for at least 4 h. The aluminum foil was replaced by a rubber cap (Maruemu) with small gaps between the cap and the vial, and then they were placed in a freeze-drying system (FTS systems DuraDry μP, SP Scientific, Warminster, PA, USA). The freeze-drying program was as follows: specimens were dried primarily for 19 h at 0.13 hPa and secondarily for 3 h at 0.13 hPa. During the process of primary drying, the shelf temperature was controlled at –30ºC and then increased to 30ºC during the last 1 h 20 minutes (0.75ºC/min). After filling with N_2 gas, the vials were sealed with rubber caps and

149 further fastened with aluminum caps (Maruemu). The freeze-dried (FD) samples were
150 transferred to a refrigerator and stored at 4°C under dark condition until usage.
151
152 **Oocyte collection and *in vitro* maturation (IVM)**
153 Oocyte collection and IVM were conducted as described (Kikuchi *et al*. 2002). In brief,
154 ovaries were obtained from prepubertal crossbred gilts (Landrace-Large White-Duroc
155 breeds) at a local slaughterhouse and transported to the laboratory at 35°C. Cumulus-oocyte
156 complexes (COCs) were collected from follicles 2 to 6 mm in diameter in Medium 199
157 (with Hanks' salts) supplemented with 10% (v/v) fetal bovine serum (Gibco, Life
158 Technologies Corporation, Grand Island, NY, USA), 20 mmol/L Hepes (Dojindo), 100
159 IU/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate. About 40 to 50 COCs
160 were cultured in 500 μL of maturation medium for 20 to 22 h in four-well dishes (Nunc,
161 Thermo Fisher Scientific, MA, USA). The COCs were subsequently cultured for 24 h in
162 maturation medium without dibutyl cAMP and hormones. IVM was carried out at 39°C
163 under conditions in which CO_2, O_2, and N_2 were adjusted to 5%, 5%, and 90%, respectively.
164 After IVM, the cumulus cells were removed from the oocytes by repeated pipetting in
165 Medium 199 supplemented with 150 IU/ mL hyaluronidase. Denuded oocytes with the first
166 polar body (PB) were harvested under a stereomicroscope and used as IVM oocytes.
167
168 **ICSI and oocyte stimulation**
169 FD sperm were re-hydrated with deionization distilled water. The sperm suspension was
170 centrifuged for 2 min at 600 × *g* and the sperm were washed with PBS(–) containing 5
171 mg/mL BSA, then re-suspended in the same buffer. The sperm suspension was sonicated
172 for about 5 to 10 sec to isolate a sperm head and kept at room temperature prior to ICSI,
173 which was carried out as previously described (Nakai *et al*. 2006) with some modifications

(Men *et al*. 2013). In brief, sperm were kept in IVC-PyrLac supplemented with 20 mmol/L Hepes and 4% (w/v) polyvinylpyrrolidone (MW 360,000) (IVC-PyrLac-Hepes-PVP). About 30 IVM oocytes were transferred to a 20-μL drop of Medium 199. A small volume (0.5 μL) of the sonicated sperm suspension was transferred to a 2-μL drop of IVC-PyrLac-Hepes-PVP. All drops were covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Inc., Kyoto, Japan). A single sperm head was aspirated into the injection pipette, and injected into the ooplasm using a piezo-actuated micromanipulator (PMAS-CT150; Prime Tech Ltd., Tsuchiura, Japan). ICSI was completed within 2 h after re-hydration of FD sperm, and sperm-injected oocytes were recovered in IVC-PyrLac for 1 h before electrical activation. The end of injection was considered as 0 h post-injection. As a control group, mature oocytes were injected with ejaculated sperm (fresh sperm group). One hour post-injection, the oocytes were transferred to an activation solution consisting of 0.28 mol/L d-mannitol, 0.05 mmol/L $CaCl_2$, 0.1 mmol/L$MgSO_4$, and 0.1 mg/mL BSA. Once the oocytes were sunk at the bottom of the drop, they were then stimulated with a direct current pulse of 1.5 kV/cm for 20 μs under the same condition for each group using a somatic hybridizer (SSH-10; Shimadzu, Kyoto, Japan), then washed three times and cultured in IVC PyrLac for 3 h before RNA extraction.

RNA extraction, purification, cDNA systhesis and quantitative RT- PCR (qRT-PCR)

Total RNAs of the pooled oocytes or putative zygotes were purified using an RNeasy Micro Kit (QIAGEN) according to manufacturer's instructions. Reverse Transcription was performed to synthesize cDNA using a Primescript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan) according to manufacturer's instructions. qRT-PCR was performed using a LightCycler® 480 SYBR Green I (Roche) according to standard

198 protocols. For each gene, the quantities of transcript were normalized to the reference
199 transcript and TUBULIN α1 was used to standardize the data.
200
201 **Decondensation status of sperm head**
202 For assessment of fertilization status, putative zygotes were fixed at 4 h post-injection (3 h
203 after electric activation) in fixative solution with a 1:3 mixture of acetic acid:ethanol (v/v)
204 under vaseline-supported coverslips for several days. They were stained with 1% (w/v)
205 orcein in 45% (v/v) acetic acid for several minutes, and then their fertilization status was
206 examined using a phase-contrast microscopy.
207
208 **Experimental design**
209 Experiment 1: mRNAs were extracted from IVM oocytes or those oocytes injected with a
210 fresh sperm or a FD sperm at 4 h after injection and were subjected to quantitative RT-PCR
211 to examine the expression of these genes. Sperm injected oocytes were also fixed at 4 h
212 after injection to examine decondensation status of sperm head and pronuclear formation.
213 Three trials were performed.
214 Experiment 2: mRNAs were extracted from oocytes at different stages of IVM and were
215 subjected to quantitative RT-PCR to examine the expression of these genes. Oocytes at four
216 maturational stages were examined; 1) germinal vehicle (GV, just after collection–0 h of
217 IVM), 2) late GV (GVL, 20 h of IVM), 3) metaphase I (M I, 33 h of IVM (according to
218 Maedomari *et al*. (2007)); oocytes with a first PB at 33 h of IVM by light microscope were
219 discarded) and 4) metaphase II (M II, 44 h of IVM; oocytes with the first PB). Some
220 oocytes in each group were fixed at respective stages to examine the nuclear status. Three
221 trials were carried out.
222

Statistical analysis

Data were expressed as mean ± SEM. The percentage data were arcsine-transformed (Snedecor & Cochran 1989) then subjected to one way-ANOVA using R packages 3.0.1 (R Core Team 2013). As the difference is found in groups by ANOVA, further analysis is conducted by Tukey's posthoc test using the R packages. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Expression levels of DNA repair genes in IVM- oocytes injected with sperm

Damaged DNA in sperm should be repaired by oocytes before pronuclear formation. According to Nakai *et al*. (2006), the rate of pronuclear formation sharply increased at 4 h after electric stimulation, therefore, we analyzed the expression of DNA repair genes at the time point of 3 h after stimulation. There were no differences in expression level of 6 investigated genes in oocytes at 4 h post-injection (Fig. 1).

Decondensation status of sperm head at 4 h post-injection (3 h after electric activation)

DNA damage is believed to be detected during decondensation of the sperm head and induces activation of essential DNA repair pathways. Therefore, the decondensation status of FD sperm head and the pronuclear formation rate also were examined at 4 h post-injection (Fig. 2A and 2B). As shown in Table 3, relatively high percentages of decondensed sperm head were observed in all three groups (62 to 71%) and there were no differences among groups. Similarly, the rate of zygotes with two PBs and two pronuclei (PNs) was also not different and ranged from 8 to 20% among groups. Newly formed PNs

at 4 h post-injection were smaller, separately located and the female or male pronucleus was easily distinguishable (Fig. 2C).

Results of nuclear status of oocytes at different stages of IVM

The data obtained from fixation and staining of oocytes (Table 4) showed that all COCs were at the GV stage just after collection and at the GVL stage 20 h after IVM. And then 5.3% of oocytes show the first polar body at 33 h of IVM and 61.8% of oocytes show the first PB at 44 h of IVM by light microscope. At 33 h of IVM, oocytes showing the first PB were removed by light microscope and remaining oocytes were fixed and stained. The data reveal that 95.7% of these remaining oocytes were at the M I stage and 4.3% were at the GVL stage (Table 4).

Expression levels of DNA repair genes in oocytes at different maturational stages

The expression level of DNA repair genes in oocytes at different maturational stages were summarized in Figure 3. Of the six genes investigated, expression levels of UDG and XPC were significantly up-regulated in M II oocytes compared with earlier stages but did not differ between GV and GVL stage. There was an increased tendency in relative expression of MSH2 and RAD51 over time during IVM process although no differences in MGMT and XRCC6.

DISCUSSION

It is generally accepted that there are mechanisms in a cell to maintain genome integrity including DNA damage detection, repair, cell cycle arrest and apoptosis. Such mechanisms coordinate together to protect the fetus from potential DNA damage originating either in

parental gametes or in the embryo's somatic cells (reviewed by Jaroudi & SenGupta 2007). Moreover, the cell cycle of embryonic cells is much shorter than that of adult cells. The integrity of its genome is thus easy to be influenced. In other word, DNA repair at the early stages is of great significance for the later development. During the early embryonic development, three main transitions occur in preimplantation development, and each transition is reflected by changes in gene expression patterns (Zheng *et al*. 2005). The first transition is the maternal to zygotic transition. The second transition occurs during compaction at the 8-cell stage (Fleming *et al*. 2001) and the last one occurs during blastocyst formation at the 32- to 64-cell stage (Zheng *et al*. 2005). In the first transition, maternal factors – mRNA and proteins- that are stored in the cytoplasm of the oocyte during oogenesis and necessary for oocyte maturation, homeostasis and the first stages of embryogenesis but become unnecessary or potential deleterious as the embryo develops are destroyed and replaced by novel transcripts which are specific to the zygotes or embryos (so-called zygotic or embryonic genome activation (ZGA or EGA), respectively) (Zheng *et al*. 2005). It is well-documented that genome activation is an essential event in order to synthesize new protein preparing for the first cell division and subsequent events as well. The phenomenon of *in vitro* cultured mouse 1-cell embryos arrested at the 2-cell stage (2-cell block) was found to be related to the delay of ZGA (Qiu *et al*. 2003). The initiation of EGA varies between species. In mice, event of genome activation begins during the 1-cell stage and becomes evident by the 2-cell stage with a transcriptional and translational burst (Schultz 2002). In porcine embryos, EGA occurs during the 4-cell stage, promoting a dramatic reprogramming of gene expression accompanied by the generation of novel transcripts that are not expressed in the oocytes (Jarrell *et al*. 1991; Hyttel *et al*. 2000). A recent study uncovered a series of successive waves of embryonic transcriptional initiation that occur as early as the 2-cell stage in human preimplantation embryos (Vassena *et al*.

297 2011), in contrast to the previously accepted time point of embryonic genome activation at
298 the 4- to 8-cell stage (Braude *et al*. 1988). EGA occurs in bovine embryos by the 8- to 16-
299 cell stage (Memili & First 2000). In the present study, we attempted to investigate the
300 expression pattern of DNA repair genes in porcine zygotes before genome activation.
301 Previously, we showed that sperm freeze-dried in the presence of trehalose showed less
302 DNA fragmentation than that in the absence of trehalose. However, the rates of oocytes
303 with two PBs, two PNs (referred as normal fertilization), and blastocyst formation were not
304 different between two groups (Men *et al*. 2013). Therefore, we speculated that DNA
305 damage in FD sperm might be repaired through pre- and postreplication repair mechanisms
306 in oocytes (Genesca *et al*. 1992). This repair capacity depends mainly on the extent of
307 sperm DNA fragmentation and the cytoplasmic quality of the oocyte. Single strand breaks
308 (SSBs) could be quickly repaired by oocytes after fertilization, but double strand breaks
309 (DSBs) could be responsible for chromosome aberrations and loss of genetic materials, thus
310 the repair of DSBs in oocytes is more difficult than that of SSBs (Enciso *et al*. 2009).
311 The objective of this study initially was to compare the induction of DNA repair
312 genes in newly fertilized oocytes by ICSI of sperm freeze-dried in the presence or absence
313 of trehalose because of observed different DNA integrity of two these groups after freeze-
314 drying. Six candidate genes participating in the repair of various types of DNA damage
315 were selected and their expressions were analyzed at a given time after fertilization. Three
316 genes (MGMT, UDG, and XPC) are candidate genes for repair of SSBs; one gene for
317 mismatch repair (MSH2) and two remaining genes (XRCC6 and RAD51) are for repair of
318 DSBs. As a result, there were no differences in relative gene expression level of six genes
319 in sperm injected oocytes at 4 h post-injection and mature oocytes. This means the
320 expression level of these genes might have already been abundant in the oocytes matured at
321 the M II stage. Also probably, at 4 h post-injection, these genes have not been induced

significantly or the DNA fragmentation of fresh sperm and two types of FD sperm was not sufficient to induce the differential expression. Harrouk *et al*. (2000) indicated that fertilization with sperm exposed to a DNA damaging agent alters the expression of DNA repair genes as early as the 1-cell stage in the rat preimplantation embryo. However, the expression of DNA repair genes in 1-cell embryos is limited since 1-cell embryos are completely dependent on maternal proteins for DNA repair. The zygotes may be able to regulate its repair efficiency only after the first cell division (Harrouk *et al*. 2000).

In the genes investigated in this study, MGMT works as DNA methyltransferase with the function of direct reversal alkylation at the O^6 position of guanine, and it has an important role to avoid the lethal cross-linking resulting in enhanced resistance to alkylating agents (Pegg *et al*. 1995). The expression level of MGMT gene was not different in all groups. UDG gene was expressed abundantly in oocytes and zygotes and revealed a significantly higher level in the M II oocytes compared with the oocytes at earlier stages. Similarly, expression level of XPC gene in the M II oocytes was significantly higher than those in oocytes at the earlier stages. For MSH2 gene, the expression of this gene had an increased tendency in the M II stage oocytes relative to oocytes at the GV, GVL and M I stages. Zheng *et al*. (2005) showed that the MSH2 gene was expressed throughout development from oocytes at the GV stage to hatched blastocyst, with a transient increase in expression in embryos at the 8-cell and morula stages in non-human primate, and our data were in agreement with this report. RAD51 is involved in the homologous recombination pathway of DSBR, and is essential for embryo viability (Zheng *et al*. 2005; Jaroudi & SenGupta 2007). In the rhesus monkey, this gene was expressed in abundance in oocytes, but its expression decreased during oocyte maturation and then increased again at the 8-cell stage (Zheng *et al*. 2005). Unlike observed patterns in the rhesus monkey, our data in pigs revealed that the expression of RAD51 tends to up-regulate during IVM.

According to results of Jaroudi *et al*. (2009), RAD51 and MSH2 were expressed at high levels in both human oocytes and blastocysts, on the other hand, XRCC6 had medium to high expression levels in the M II oocytes and blastocysts.

In the present study, it could not be neglected that relative expression value of UDG gene in the M II oocytes in Experiment 1 was almost two-fold lower than those in Experiment 2. M II oocytes used in Experiment 2 were denuded at 44 h of IVM and immediately subjected to RNA extraction. The M II oocytes used in Experiment 1 were also denuded at 44 h of IVM but subjected to RNA extraction at about 2 h later when ICSI was completed. As mentioned before, this gene showed highly expression in the M II oocytes in Experiment 2 relative to other genes at 44 h of IVM. It may be possible that lower expression value of the M II oocytes in Experiment 1 was attributed to age-related degeneration of some DNA repair mRNAs in oocytes or the expression of this gene reached peak at 44 h then decreased. This explanation is clearly supported by the fact that the relative expression of this gene in other groups of Experiment 1 also did not achieve the similar level of Experiment 2 irrelevant to treatments.

There are two possible explanations for the maturational stage-dependent changes of most of DNA repair genes in IVM oocytes observed in Experiment 2. One possibility is that during IVM, oocytes have accumulated mRNAs of DNA repair genes being ready for fertilization. The other possibility is that suboptimal IVM system stressed oocytes and it might induce up-regulation of these DNA repair genes. The later appears to be a more satisfactory explanation. Indeed, Jones *et al*. (2008) reported that several genes involved in many signaling pathways, such as response to stress, cell cycle, cell proliferation, cell division and cell death and so on, were up-regulated in *in vitro* matured oocytes compared with *in vivo* matured oocytes, and this up-regulation may attribute to dysregulation occurring during IVM. Cumulus cells play an essential role in whole process of oocyte

372 growth and maturation; therefore, their gene expression profiles according to IVM
373 condition have been also investigated in detail (Tesfaye *et al*. 2009; Ouandaogo *et al*. 2012).
374 In bovine cumulus cells, isolated from *in vitro* matured COCs, genes involved in response
375 to stress were up-regulated and genes related to cumulus expansion and oocyte maturation
376 were down-regulated compared with cumulus cells isolated from *in vivo* matured COCs
377 (Tesfaye *et al*. 2009). Similarly, expression of genes involved in DNA replication,
378 recombination and repair in human cumulus cells isolated from *in vivo* and *in vitro* matured
379 COCs at different nuclear maturation stages were up-regulated in cumulus cells after IVM
380 (Ouandaogo *et al*. 2012).

381 It should be noted that there are so many DNA repair genes and enzymes in the
382 oocytes and zygotes in addition to those examined in the present study. Expression level of
383 these genes may depend on unknown cellular signals related to normal development or
384 delayed development in the oocytes injected with sperm that have various types or levels of
385 DNA damage. The expression of those genes depends on embryo development stage and/or
386 influenced by many other unknown factors.

387 In conclusions, the present study revealed that expression of DNA repair genes in
388 fertilized oocytes at 4 h after ICSI using fresh sperm, FD sperm in the presence or absence
389 of trehalose was not different. Likewise, no difference was observed in the expression of
390 DNA repair genes between the sperm injected groups and the M II oocyte group without
391 sperm injection. On the other hand, during IVM, the expression of XPC was significantly
392 increased in the M II oocytes compared with earlier stages. The expression of UDG was
393 significantly increased from the GV through GVL, M I and M II stages. Further
394 experiments are needed to confirm whether increased expression levels of DNA repair
395 genes in oocytes are caused by suboptimal IVM conditions or their accumulation. If
396 increased level of DNA repair genes is confirmed to be caused by IVM conditions, the

efficient improvement of IVM system can be achieved based on expression profile of DNA repair genes.

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Table 1. Repairable DNA damage type, proper functions, accession number and primer sequence of six investigated genes

Genes symbol	Full name	DNA damage type	Proper function	References
MGMT	O-6-methylguanine-DNA methyltransferase	Direct reversal: alkylated guanine	Removes alkylating lesions at the O6 of guanine residues	Wood *et al.* 2001; Jaroudi *et al.* 2009
UDG	Uracil -DNA glycosylase	Single strand break (Base)	DNA glycostylase, recognize and remove uracil opposite A	Harrouk *et al.* 2000; Wood *et al.* 2001; Zheng *et al.* 2005; Jaroudi *et al.* 2009
XPC	Xeroderma pigmentosum, complementation group C	Single strand break (Nucleotide)	DNA binding	Harrouk *et al.* 2000; Wood *et al.* 2001; Zheng *et al.* 2005;
MSH2	mutS homolog 2, colon cancer	Mispairing	Mismatch and loop recognition	Harrouk *et al.* 2000; Wood *et al.* 2001; Zheng *et al.* 2005; Jaroudi *et al.* 2009
XRCC6	X-ray repair cross-complementing 6	Double strand break	Non-homologous end joining	Jaroudi *et al.* 2009
RAD51	Sus scrofa RAD51 homolog (S.cerevisiae)	Double strand break	Homologous recombination	Wood *et al.* 2001; Zheng *et al.* 2005; Jaroudi *et al.* 2009
α TUB	Alpha tubulin	–	Reference gene	

Table 2. Repairable DNA damage type, proper functions, accession number and primer sequence of six investigated genes

Gene symbol	Genebank accession no.	Primers	Product Size (bp)
MGMT Direct Reversal	XM_003483574	F/acttgcaggtccagaggaga R/tgcagcagcttccataacac	168
UDG Base excision repair	XM_003132925	F/cagctccgtcaagaagatcc R/gctgaggtgcttcttccaac	175
XPC Nucleotide excision repair	AF041032	F/atccgacgaagattctgagc R/tcttcttgcctcctttacgc	179
MSH2 Mismatch repair	NM_001195357	F/tggtcccaatatgggaggta R/catttcagccatgaatgtgg	184
XRCC6 Non-homologous end-joining	NM_001190185 XR_045703	F/aacggaaggtgccctttact R/cttttagccattgcctcagc	223
RAD51 Homologous recombination	NM_001123181	F/attctgaccgaggcagctaa R/atgggaagctggcatgttac	224
α TUB* Cell cycle	NM_001044544	F/tggaccacaagtttgacctgatg R/gtcctcacgggcctcagaaa	101

*indicate endogenous reference gene

Table 3. Decondensation status of sperm head at 4 h post-injection (3 h after electric activation)

Treatment	No of examined oocytes	Oocyte with decondensed sperm head (%)	Oocyte with 2PB+2PN (%)
Fresh	108	62.49 ± 9.97	12.80 ± 5.29
Tre 0 mmol/L	123	71.53 ± 10.39	8.86 ± 5.21
Tre 15 mmol/L	130	67.23 ± 6.61	20.44 ± 6.10

Data were presented as mean ± SEM of 5 replicates. PB; polar bodies, PN; pronucleus(ei), Fresh; ejaculated fresh sperm

Tre 0 mmol/L; sperm freeze-dried in basic freeze-drying medium without trehalose

Tre 15 mmol/L; sperm freeze-dried in basic freeze-drying medium supplemented with 15 mmol/L trehalose

Table 4. Nuclear status of oocytes assessed by staining at different maturational stages

Time of IVM (h)	No of oocyte examined	Maturational stages			
		No (%) GV	No (%) GVL	No (%) M I	No (%) M II
0	120	120 (100 ± 0.0)	–	–	–
20	122	–	122 (100 ± 0.0)	–	–
33	116	–	5 (4.3± 0.3)	111 (95.7 ± 0.3)	#
44	309	–	–	118 (38.2 ± 0.9)	191 (61.8 ± 0.9)

GV; germinal vesicle, GVL; late germinal vesicle, M I; metaphase I and M II; metaphase II stages. #: at 33 h of IVM, M II oocytes were removed by observation (5.3%) before staining

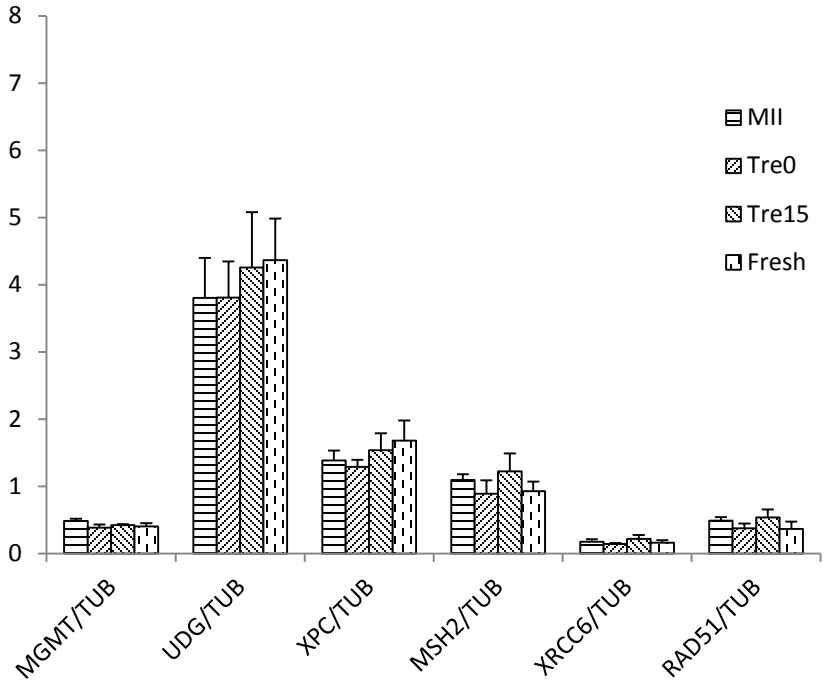

Figure 1 Relative gene expression of six DNA repair genes in oocytes at 4 h after ICSI. (Data were obtained from 3 biological samples with duplicate)

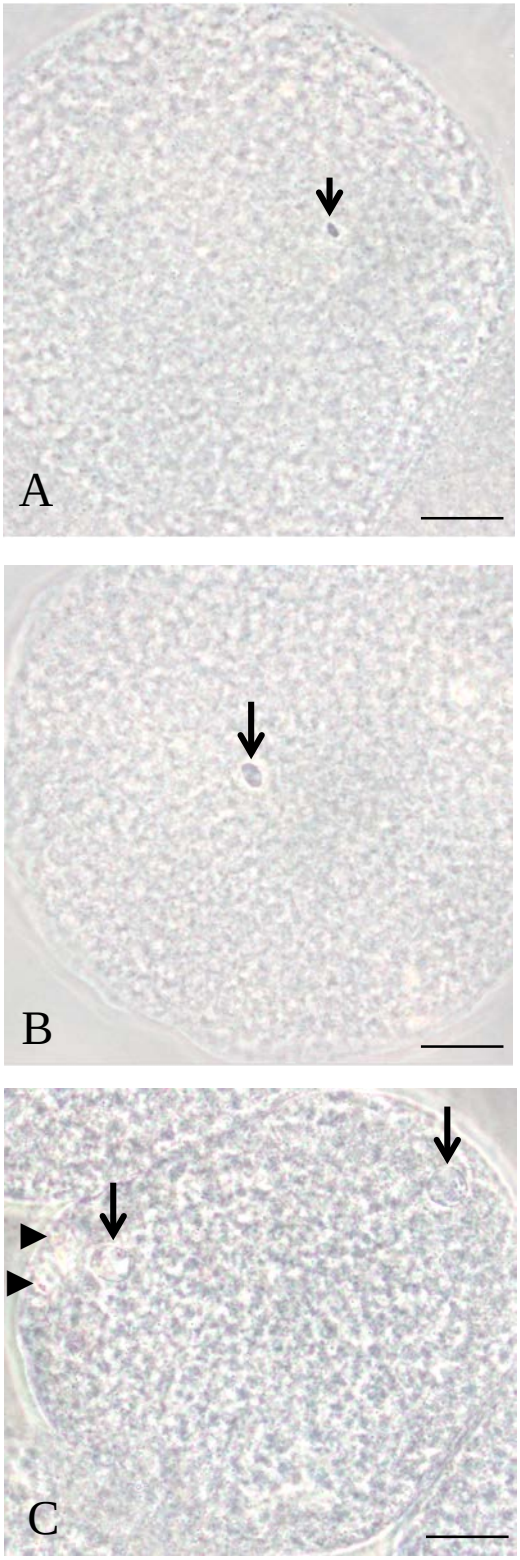

Fig. 2. Decondensation status and PN formation of sperm at 4 h after ICSI. Intact sperm head and decondensed sperm head (arrows in A and B, respectively). Newly formed female (left) and male (right) pronuclei (arrows in C). The first (upper) and second (lower) polar bodies (arrowheads in C). Scale bar = 20 μm.

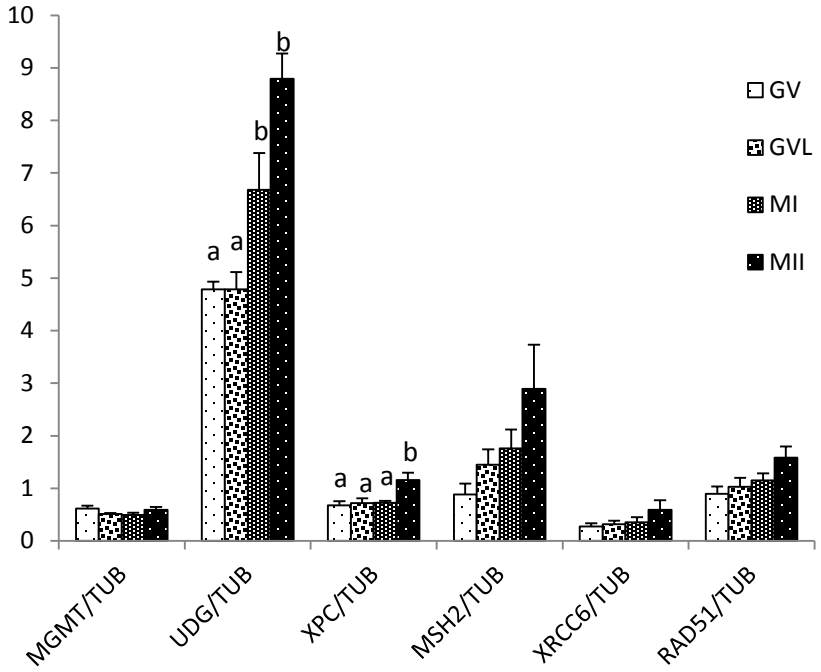

Figure 3 Relative gene expression of six DNA repair genes in oocytes at different maturational stages. (Data were obtained from 3 biological samples with duplicate;
[a, b] different superscripts in each gene show significant difference, $P < 0.05$)