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Meiosis progression and donor age affect expression profile of DNA repair genes in bovine oocytes

S. Bilotto[2,3], *R. Boni*[1], *G.L. Russo*[3] *and M.B. Lioi*[2]
Department of Sciences, University of Basilicata, Potenza; and Institute of Food Sciences, National Research Council, Avellino, Italy

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Summary

Several genetic and physiological factors increase the risk of DNA damage in mammalian oocytes. Two critical events are: (i) meiosis progression, from maturation to fertilization, due to extensive chromatin remodelling during genome decondensation; and (ii) aging, which is associated with a progressive oxidative stress. In this work, we studied the transcriptional patterns of three genes, *RAD51*, *APEX-1* and *MLH1*, involved in DNA repair mechanisms. The analyses were performed by real-time quantitative PCR (RT-qPCR) in immature and *in vitro* matured oocytes collected from 17 ± 3-month-old heifers and 94 ± 20-month-old cows. Batches of 30–50 oocytes for each group (three replicates) were collected from ovarian follicles of slaughtered animals. The oocytes were freed from cumulus cells at the time of follicle removal, or after *in vitro* maturation (IVM) carried out in M199 supplemented with 10% fetal calf serum, 10 IU luteinising hormone (LH) /ml, 0.1 IU follicle-stimulating hormone (FSH) /ml and 1 μg 17β-oestradiol/ml. Total RNA was extracted by Trizol method. The expression of bovine *GAPDH* gene was used as the internal standard, while primers for bovine *RAD51*, *APEX-1* and *MLH1* genes were designed from DNA sequences retrieved from GenBank. Results obtained indicate a clear up-regulation of *RAD51*, *APEX-1* and *MLH1* genes after IVM, ranging between two- and four-fold compared with germinal vesicle (GV) oocytes. However, only *RAD51* showed a significant transcript increase between the immature oocytes collected from young or old individuals. This finding highlights *RAD51* as a candidate gene marker for discriminating bovine immature oocytes in relation to the donor age.

Keywords: *APEX-1*, Bovine, DNA repair genes, IVM, *MLH1*, Oocyte, *RAD5*

Introduction

Reproductive activity is theoretically possible from puberty to senescence; however, senescence declines this activity in all animals studied up to its complete interruption (Harman, 2006). Cattle show a large variability in the age to conceive and being able to bring to term a pregnancy. Unfortunately, the low heritability ($h_2 = 0.09$) for traditional fertility parameters makes the discrimination of more fertile genotypes difficult; consequently, selection for fertility is frustrated (Darwash *et al.*, 1997). Erickson *et al.* (1976) analysed reproductive performance of aged Hereford cows and established that 15 years old was the age at which over 50% of the herd became infertile. This threshold may decrease under stressful conditions, as in the case of high-producing dairy cows.

Cattle have been proposed as a suitable model for studying reproductive aging in humans as follicular wave emergence, follicle selection, ovulation, and age-associated endocrinal changes are similar in the two species (Baerwald *et al.*, 2003; Malhi *et al.*, 2005; Malhi *et al.*, 2006). Although age may affect many aspects of female reproduction, oocytes have been assumed as a major focus in reproductive aging studies. Malhi *et al.* (2007) analysed the superovulatory efficiency in old versus young cows and observed the comparative recovery of fewer embryos and a greater proportion of unfertilized oocytes in old cows.

[1]All correspondence to: Raffaele Boni. University of Basilicata, Via dell'Ateneo Lucano, 10–85100–Potenza, Italy. Tel: +39 0971 205017. Fax: +39 0971 205099. e-mail: raffaele.boni@unibas.it
[2]Department of Sciences, University of Basilicata, 85100 Potenza, Italy.
[3]Institute of Food Sciences, National Research Council, 83100 Avellino, Italy.

Therefore, they suggested that there was a reduced developmental competence of oocytes in old cows; although, there was no difference in embryo quality and in the proportion of calves born. Yamamoto *et al.* (2010) found that oocytes from old cows had a lower fertilizing ability and showed a faster meiosis progression and a rapid disappearance of gap junctions between oocytes and cumulus cells compared with those derived from young cows. This finding suggested that the premature meiosis progression in old cow oocytes was partly due to impaired cumulus–oocyte complex communication.

DNA damage has been demonstrated as one of the main consequences of aging (Bertram & Hass, 2008; Schumacher *et al.*, 2008; Maynard *et al.*, 2009). Genome integrity is an essential condition for assuring cell functions and division. In order to maintain this prerequisite, several DNA repair strategies have been established in cells (Bessho *et al.*, 1993; Wood *et al.*, 2001). Different types of damage, i.e. wrong nucleotide insertion during DNA replication, chemical modifications of nitrogenous bases, spontaneous hydrolysis of N-glycoside binding, pyrimidine alteration due to reactive oxygen species (ROS), ultraviolet (UV) and ionic radiations, chemotherapy drugs, genotoxic substances, oxidation or alkylation base, occur daily within the DNA of each individual (Wood *et al.*, 2001). These alterations are counteracted by DNA repair systems, such as nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and double-stranded break (DSB) repair in DNA. Under genotoxic alert, these systems interfere with the cell cycle and, in some cases, activate specific checkpoints in order to repair the damage, or induce apoptosis when cytoprotection cannot be achieved (Jaroudi & SenGupta, 2007). Cell lifespan depends on the efficacy of DNA repair and aging has been proposed as a consequence of declining DNA repair activity (Best, 2009). In fact, the silencing of genes involved in DNA repair causes an accelerated aging and an early manifestation of aging-associated diseases in mouse (de Boer *et al.*, 2002; Espejel *et al.*, 2004). DNA repair is supposed to be one of the crucial activities established during gamete growth and maturation, fertilization and embryo development (El-Mouatassim *et al.*, 2007; Ménézo *et al.*, 2010). The role played by genotoxic stress has been deeply studied in somatic and differentiated cell types; however, little information is available on the activity of DNA repair genes during gametogenesis and embryo growth. In rhesus monkey, the expression of *APEX-1*, a component of BER genes, did not show significant variations in either immature (germinal vesicle stage, GV) or mature (metaphase II stage, MII) oocytes (Zheng *et al.*, 2005). *MLH1*, which is a component of the MMR gene family, is highly expressed in growing and maturing oocytes, but its expression decreases during early embryo development (Zheng *et al.*, 2005). Genes related to NER seem to be weakly expressed in oocytes (Zheng *et al.*, 2005; Jaroudi & SenGupta, 2007). In monkey, some DNA repair genes have been found to be up-regulated in oocytes in respect to early embryos (Zheng *et al.*, 2005). This observation may represent an adaptive strategy that allows oocytes to remain arrested for long time at first meiotic division to guarantee genome integrity (Jaroudi & SenGupta, 2007). *RAD51*, involved in the DSB repair mechanism (Hsieh, 2001; Kelley & Parsons, 2001), is an essential gene for embryo vitality and is up-regulated during embryo development (reviewed in Ozturk & Demir, 2011). Several studies on mammalian germ cells have established a close relationship among DNA repair failure, embryo mortality, infertility and aging (Hart & Setlow, 1974; Grube & Bürkle, 1992; Cortopassi & Wang, 1996; Hsieh, 2001). However, to date, studies on the involvement of DNA repair systems in mammalian gametes are scarce and most of these are limited to monkeys and mice.

In this context, we hypothesized that defects in DNA repair gene expression could represent key factors affecting the donor age-related oocyte quality and fertility in cattle. Here, we present the transcriptional profile of three genes, *APEX-1*, *RAD51*, and *MLH1*, belonging to BER, DSB and MMR pathways, respectively. We compared their expression in bovine immature and in *in vitro* matured oocytes obtained from sexually mature heifers or old cows.

Materials and methods

If not otherwise stated, all chemicals were purchased from Sigma-Aldrich (Milano, Italy).

Oocyte source

Ovaries from slaughtered 17 ± 3-month-old Holstein Friesian heifers or from 94 ± 20-month-old Holstein Friesian cows were collected separately from the abattoir and transported in a thermal bag at 27–30°C to the laboratory within 3–4 h from collection. The laboratory temperature was 30°C. Immature oocytes were collected from 2–8 mm follicles by an 18-gauge needle under controlled pressure (50–70 mm Hg). Cumulus–oocyte complexes (COCs) with suitable morphological characteristics (Boni *et al.*, 2002) were isolated from the follicular fluid and washed three times with TCM-199 supplemented with 0.05% (w/v) polyvinyl alcohol and 10 mM HEPES. The COCs were then divided randomly into homogeneous groups for *in vitro* maturation (IVM) or immediately prepared for treatment for RNA extraction in Trizol (Life

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Table 1 qPCR primer sequences for bovine *APEX-1*, *MLH1* and *RAD51* genes

Gene	Primer sequence 5′→3′	Amplicon size (bp)	Accession number
RAD51	F-CGGAAGCAGTATGTGTCT R-CTGCCTCCAAAACACTTCAC	144	NW_003104278
APEX-1	F-CTCTGTTGCCTGCATTGTGT R-GCTCTGTGACAACCTCTTCCA	101	NW_001492801
MLH1	F-GAAATATCATTGCTCACGCCT R-CAGATGGAGTGGGAGAT	147	NW_003104540
*GAPDH**	F-CCAACGTGTCTGTTGTGGATCTGA R-GAGCTTGACAAAGTGGTCGTTGAG	226	NW_003103940

*Vallèe *et al.*, 2006.

Technologies, Monza, Italy). In the latter case, oocytes were freed from the cumulus cells by vortexing for 4 min. The resultant intact oocytes without cumulus traces were counted under a stereomicroscope and transferred into tubes with the lowest quantity of washing medium. Trizol was added to the tubes (1 μl Trizol/oocyte) and samples immediately stored at –80°C until analysis.

In vitro maturation

The COCs destined for IVM were transferred into 4-well maturing plates (Nuclon, Nunc, Roskilde, DK) containing a maturation medium (MM) that consisted of TCM-199 supplemented with 10% fetal bovine serum (FBS), 10 IU/ml luteinising hormone (LH), 0.1 IU/ml follicle-stimulating hormone (FSH), 1 μg/ml of 17β-estradiol (approximately 30 μl MM/COC). The plates were then transferred to an incubator at 39.0°C in 5% CO_2 humidified air for 24 h. At the end of the incubation period, COCs were placed in 15-ml tubes that contained 1 ml of washing medium and vortexed, as described above. The IVM efficiency was assessed in retrieved oocytes by evaluating the presence of a polar body. The MII oocytes were counted and transferred in Trizol for RNA extraction.

RNA extraction, reverse transcription and real-time PCR quantification

Total RNA extraction from at least three independent pools (30–50) of oocytes was performed using the Trizol reagent. An amount of 1–4 μg glycogen was added as carrier to facilitate RNA precipitation. RNA was resuspended in an appropriate volume of RNase-free water. Each aliquot of extracted RNA was subjected to treatment with RNase-free DNase using 1 U/μl (BD-Clontech, Milan, Italy) in order to eliminate any possible contamination of residual genomic DNA. cDNA synthesis was performed by using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies) with random hexamers, following the manufacturer's protocol in a final volume of 20 μl and carried out on five oocytes equivalent RNA. Real-time qPCR analysis was performed on randomized cDNA equivalent to 1.33 oocyte using the SYBR Green PCR Master Mix (Applied Biosystems, Monza, Italy) in a final volume of 25 μl according to the manufacturer's protocol in a 7000 Real-Time PCR System (Applied Biosystems). Quantification was in triplicate and samples for each gene were analysed on the same run in order to minimize inter-experiment variation. The expression levels of candidate genes were normalised using the internal standard glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as reported previously (Vallée *et al.*, 2006). *GAPDH* was stable in both immature and *in vitro* maturated oocytes (data not shown; Somfai *et al.*, 2011). The gene sequences used in the experiments have been retrieved from GenBank. Primers were designed using the Plexor™ Primer Design system (http://www.promega.com) and sequences reported in Table 1. The amplification protocol included: denaturation (95°C for 10 min), 40 amplification cycles (15 s at 95°C, 1 min at 60°C). The specificity of each amplification reaction was verified by melting curve analysis. The ΔΔCt method was used to calculate the expression level of each gene (Pfaffl, 2001) and PCR efficiency was 83–95% (calculated from the slope of a standard curve based on the formula: efficiency = –1 + 10(–1/slope)).

Statistical analysis

Values are presented as means ± standard deviation (SD). Differences in gene expression between the age and meiotic stage groups were analysed by analysis of variance (ANOVA) (Systat 11.0 release). Pairwise comparison of the means was made with Fisher's least significant difference (LSD) test. COVARIANCE procedure was applied in order to analyse the age and the meiotic stage effects within each gene transcript singly, as well as to compare the relative transcript abundance between the analysed genes independently of age and meiotic stage effects. A P-value < 0.05 indicated significance.

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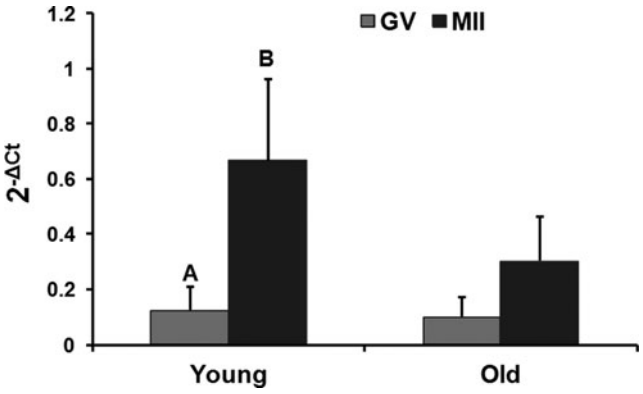

Figure 1 Relative expression profiles of *APEX-1* gene in immature (GV) and *in vitro* matured (MII) bovine oocytes in relation to the donor age. The mRNA abundance of the candidate gene was normalised against the internal standard (*GAPDH*). Relative abundance values are expressed as $2^{-\Delta Ct}$ and show the mean value ± standard deviation (SD). A,B indicates $P < 0.01$.

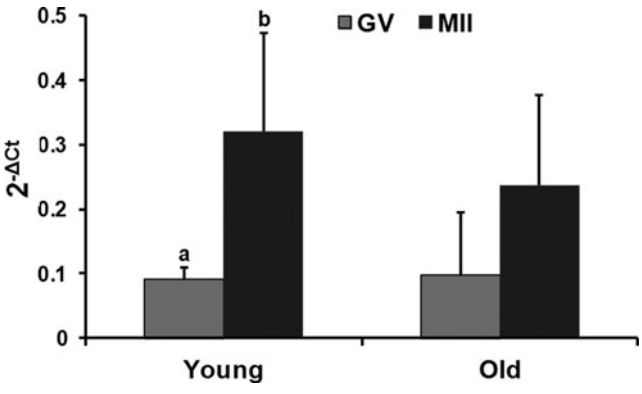

Figure 2 Relative expression profiles of *MLH1* gene in immature (GV) and *in vitro* matured (MII) bovine oocytes in relation to the donor age. The mRNA abundance of the candidate gene was normalised against the internal standard (*GAPDH*). Relative abundance values are expressed as $2^{-\Delta Ct}$ and show the mean value ± standard deviation (SD). a,b indicates $P < 0.02$.

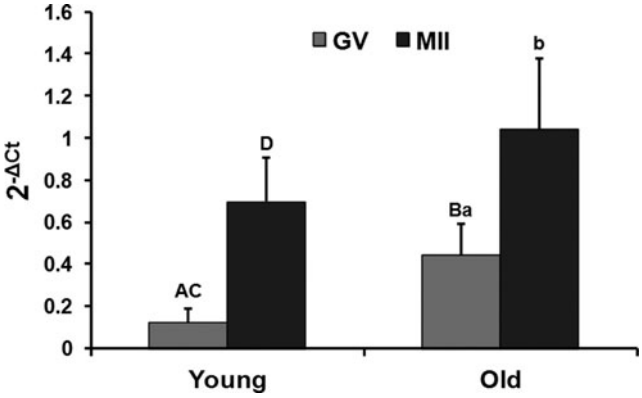

Figure 3 Relative expression profiles of *RAD51* gene in immature (GV) and *in vitro* matured (MII) bovine oocytes in relation to the donor age. The mRNA abundance of the candidate gene was normalised against the internal standard (*GAPDH*). Relative abundance values are expressed as $2^{-\Delta Ct}$ and show the mean value ± standard deviation (SD). A,B indicates $P < 0.01$ between GV from the young and old groups; a,b indicates $P < 0.05$ between GV and MII from the old group. C,D indicates $P < 0.01$ between GV and MII from the young group.

Results

This study analysed data of combined batches, including three replicates of 30–50 bovine oocytes for a cumulative number of 230 at the immature (92 and 138 from young and old individuals, respectively) and 217 (87 and 130 from young and old individuals, respectively) at the *in vitro* matured stage. We did not find any significant difference in maturation rate between young and old oocytes on the basis of polar body evaluation (80 ± 7% versus 76 ± 6% in young and old oocytes, respectively). The three transcripts analysed were differently expressed in bovine oocytes, independently from meiotic stage and donor age. The relative amount measured indicated the following order of abundance: *RAD51* > *APEX-1* > *MLH1* ($P < 0.01$). However, the amount of *APEX-1* and *MLH1* transcripts did not significantly differ between them. At the end of IVM, all transcripts were up-regulated in comparison with the immature stage (0.117 ± 0.05 vs 0.505 ± 0.05; $P < 0.01$, data not shown). The relative abundance of each single transcript increased after IVM from two-fold (*MLH1*), up to four-fold (*APEX-1*) compared with the immature stage. In contrast in the meiotic stage, the effect of donor age did not produce homogeneous changes of gene transcript patterns, hence when the analysis was carried out on overall data, no significant differences were found between the two age classes (0.267 ± 0.047 versus 0.355 ± 0.058; $P > 0.05$, data not shown).

APEX-1 transcript patterns are shown in Fig. 1 in relation to the age and meiotic stage. An up-regulation of gene transcripts following *in vitro* maturation was clearly found in oocytes from young animals ($P < 0.01$), but it was not statistically significant ($P = 0.066$) in oocytes from old animals. Similarly, *MLH1* transcripts showed a significant increase ($P < 0.02$) after *in vitro* maturation in oocytes from young donors, but not in old animals (Fig. 2). In the case of *RAD51*, oocytes from both young and old donors showed a significant increase of transcript abundance (Fig. 3). In addition, in immature oocytes a significant ($P < 0.01$) difference between the two age classes was found with an up-regulation of this gene in the old donor cows.

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Discussion

Data obtained in the present study showed a clear up-regulation of all three DNA repair genes evaluated, i.e. *RAD51*, *APEX-1* and *MLH1*, in bovine oocytes after IVM. Among these genes, only the expression of *RAD51* significantly increased in immature oocytes collected from old individuals compared with young ones. This finding suggests that *RAD51* may represent a helpful candidate in discriminating bovine immature oocytes in relation to the donor age.

During oocyte maturation, differences in transcriptional activity result in either over-expression, under-expression or stable expression of genes that concur to establish the maternal cytoplasm inherence (Memili *et al.*, 1998; Tomek *et al.*, 2002). The complex genetic remodelling that occurs during oocyte maturation, in which homologous chromosome pairing, synapse and recombination take place, requires a perfectly active and functional DNA repair machinery. This factor may explain why all three transcripts (and possibly others) quantified in the present study increased their expression significantly from GV to MII. Conversely, following a dated, but still current, theory the adaptive advantage of meiosis consists in facilitating recombinational repair of DNA damage that is otherwise difficult to repair, and occurs especially as the result of oxidative stress (Bernstein *et al.*, 1985). In mouse, a massive destruction of transcripts has been described during oocyte maturation. Of the estimated 85 pg of mRNA present at the GV stage, polyadenylated mRNA declines during oocyte maturation to 50 pg; about half undergoes deadenylation, while the remaining part is subject to degradation (Paynton *et al.*, 1988). Su *et al.* (2007) using the Affymetrix Mouse Genome 430 v2.0 GeneChip observed that this degradation is a selective process that involves transcripts associated with meiotic arrest at the GV stage and during oocyte maturation. An up-regulation of some genes that are likely to be involved in DNA replication, amino acid metabolism and signaling molecules has been described during oocyte maturation, with 1682 genes under-expressed and 1936 over-expressed out of 12,164 analysed in *in vivo* matured oocytes (Cui *et al.*, 2007).

In bovine, Mamo *et al.* (2011) using the Affymetrix GeneChip Bovine Genome Array, analysed global mRNA expression in immature and *in vitro* matured oocytes. They found that approximately 25% of the 8489 analysed transcripts significantly changed their abundance from immature to *in vitro* matured stage. In particular, 72% of them were under-expressed and 28% were over-expressed throughout IVM. Microarray analysis was further supported by qPCR, which showed in all but one of the 25 analysed genes a similar expression pattern to the microarray data. Furthermore, treatment with α-amanitin significantly decreased transcript abundance for all the genes analysed (Leal *et al.*, 2012). The same authors compared the transcriptional activity of 35 selected genes related to cell cycle regulation and oocyte competence in *in vitro* matured bovine oocytes after temporary inhibition of meiosis using the cyclin-dependent kinase inhibitor butyrolactone I. Also in this study, after IVM, most of the examined transcripts (54%) were down-regulated, whereas 20% of them were up-regulated in control and inhibited oocytes (Leal *et al.*, 2012). More exhaustive information can be obtained when the transcriptional analysis is completed by protein expression. Recently, we analysed *NFKBIA* (NFκB inhibitor α) gene and its product, IκBα, during the oocyte-to-embryo transition period in bovine. Interestingly, during meiosis progression, we found a decrease in maternal *NFKBIA* mRNA and a parallel increase of IκBα, indicating a different regulation of the two processes (Paciolla *et al.*, 2011).

DNA repair genes should play a crucial role in the fertilized egg due to the extensive chromatin remodelling that occurs at the time of genome decondensation. This event may result in the modified expression of several members of the DNA repair machinery. Rad51 protein is involved in the homologous repair of DNA DSBs, playing a relevant role in the cell fate. Perez *et al.* (2007) found that apoptosis in MII oocytes from inbred mouse strains is affected by genetic background, as demonstrated by numerous DNA DSBs in freshly isolated oocytes. Microinjection of Rad51 protein reduced DNA damage, suppressed apoptosis and improved embryonic development (Kujjo *et al.*, 2010). *RAD51* was also related to the halting cell death programme induced by ionizing radiation in bovine oocytes (Kujjo *et al.*, 2012). In particular, these authors found that either inhibition of Rad51 or increase in caspase-3 activity, before irradiation, caused an increased cytoplasmic fragmentation and DNA damage in mouse and bovine oocytes. Conversely, both types of cell damage markedly decreased when recombinant human Rad51 was microinjected in oocytes. Bovine oocytes were more resistant than mouse oocytes to these environmental stimuli and showed a faster protection rate following Rad51 treatment. In the present study, *RAD51* expression increased significantly both in *in vitro* matured oocytes and in aged donor oocytes. At the present time, it is not easy to formulate hypotheses on why only *RAD51* expression increased in aged donors. The larger abundance of the *RAD51* transcript may reflect a biological strategy towards conditions of high sensitivity to DNA damage. Future studies will be devoted to clarify and extend the primary observation reported in the present work. Our initial attempts to investigate this issue have been focused on the possibility that

an increased accumulation of ROS in aged oocytes could represent the molecular requirement for the increased synthesis of the *RAD51* transcript. However, preliminary observations obtained in our laboratory on oocytes treated with different concentrations of hydrogen peroxide seem to exclude this possibility (data not shown). Alternatively, a recent work suggests a novel role for human *RAD51* in mitochondria. The authors provided the first demonstration that Rad51 exists in human mitochondria and interacts physically with mitochondrial DNA (mtDNA), which suffers significant damage resulting from the production of ROS via the oxidative phosphorylation pathway (Sage *et al.*, 2010). Considering the importance of mitochondrial function in mammalian oocytes and embryos and their role in developmental competence (reviewed in Dumollard *et al.*, 2009; Van Blerkom, 2011), the hypothesis that high levels of *RAD51* transcript and protein are required to ensure the maintenance of the mitochondrial genome during aging cannot be excluded, a concept that deserves further study in the near future.

Apex/Ref-1 (apurinic/apyrimidic endonuclease) is an enzyme involved in the DNA BER pathway to remove abasic sites, the most common type of DNA decay. These gene transcripts increase greatly following IVM, but did not differ between oocytes of young and old individuals. In a previous comparative study, we analysed the expression patterns of *APEX-1* in human gametes and embryos and in sea squirt *Ciona intestinalis* (ascidian), an organism studied intensively in developmental biology and that has been proposed as a model to study meiotic regulation (Russo *et al.*, 1998, 2011). qPCR analysis clearly indicated that *APEX* transcripts were clearly detectable in oocytes and embryos until the larva stage, but not in spermatozoa, a finding that suggested the appearance of paternal contribution to DNA repair during development (El-Mouatassim *et al.*, 2007).

MLH1 belongs to the DNA MMR family that is involved in the process of meiotic recombination in germ cells. In particular, MMR gene mutations in mice result in meiotic disruption during prophase I (Edelmann *et al.*, 1996). MMR function is required for the formation and stabilization of crossovers in mammalian oocytes (Hunter & Borts, 1997) and, in the absence of a functional MMR system, the failure to maintain chiasmata results in a reduced ability to proceed normally through the first and second meiotic divisions, despite near-normal levels of meiotic resumption after dictate arrest (Kan *et al.*, 2008). The larger transcript abundance of this gene in MII oocytes, found in the current study, supports its role during meiosis progression in bovine oocytes. This finding, however, conflicts with data from Mamo *et al.* (2011) who described an opposite pattern of *MLH1* expression in bovine oocytes following IVM. There is no clear explanation for this discrepancy, which may be due to epigenetic effects associated with different IVM protocols used in the two studies.

Conclusions

The analysis of *RAD51*, *APEX-1* and *MLH1* gene expression reveals a clear up-regulation of these genes in bovine oocytes from the immature to the *in vitro* matured stage. Among these patterns, only *RAD51* expression differs significantly in relation to donor age with an up-regulation in immature oocytes of aged donor.

On the basis of the role played by these genes, it is likely that several additional sources of variability affecting oocyte quality (size and grade of atresia of the follicle, environment, etc.) may interfere with their expression in order to protect the oocytes' fate and allow fertility preservation.

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