


Effects of maternal liver abnormality on *in vitro* maturation of bovine oocytes

Shiori Ashibe , Yui Kobayashi, Shusuke Toishikawa* and Yoshikazu Nagao 

University Farm, Faculty of Agriculture, Utsunomiya University, Utsunomiya, Tochigi 321-4415, Japan

Research Article

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Corresponding author:

Yoshikazu Nagao;
Email: ynagao@cc.utsunomiya-u.ac.jp

*Current address: Sendai ART Clinic, Sendai, Miyagi 983-0864, Japan

Summary

In cattle, maternal metabolic health has been suggested to influence oocyte and embryo quality. Here, we examined whether maternal liver abnormalities affected *in vitro* oocyte maturation by screening meiotic maturation, spindle morphology, actin filaments, and lysosomes. In oocytes from the abnormal liver group, the maturation rate (80.2%) was significantly lower compared to a control group with healthy livers (90.8%; $P < 0.05$). Mean spindle area in oocytes of the abnormal group ($50.4 \pm 3.4 \mu\text{m}^2$) was significantly larger than in the control ($40.8 \pm 1.6 \mu\text{m}^2$; $P < 0.05$). Likewise, mean spindle width in the abnormal group ($8.8 \pm 0.3 \mu\text{m}$) was significantly larger than in the control group ($7.8 \pm 0.2 \mu\text{m}$; $P < 0.05$). The proportion of cells with correctly aligned chromosomes in the abnormal group (48.0%) was significantly lower than in the control (78.3%; $P < 0.05$). The number of cortical actin filaments in mature oocytes of the abnormal group (299.3 ± 3.7) was significantly lower than in the control (314.7 ± 3.2 ; $P < 0.05$). The number of lysosomes in mature oocytes of the abnormal group (1363.6 ± 39.0) was significantly higher than in the control (1123.4 ± 26.3 ; $P < 0.05$). In conclusion, our findings indicate that the quality of *in vitro* matured oocytes is lower in cattle with liver abnormalities than in healthy cattle.

Introduction

Advances in *in vitro* embryo production (IVP) have enabled this technology to be applied to livestock production. However, in cattle, only about 30% of oocytes used in IVP develop to the blastocyst stage (Lonergan and Fair, 2016; Hansen, 2020). It is known that a range of factors can influence embryo development *in vitro*. For example, the use of supplements such as growth factors and antioxidants during *in vitro* maturation (IVM) can increase the rate of success in IVP (de Matos and Furnus, 2000; Wasielek and Bogacki, 2007; Wang *et al.*, 2014). In recent years, maternal metabolic health has also been implicated in oocyte and embryo quality, the so-called “Origins of Health and Disease (DOHaD)” hypothesis (Fleming *et al.*, 2015; Leroy *et al.*, 2015; Sauer, 2015). In the abattoir where we regularly collect bovine ovaries, the livers of approximately 81.6% of dairy cattle are discarded because of abnormalities such as hepatitis and liver degeneration (Table 1). High energy demand for milk production following parturition causes a negative energy balance that can trigger metabolic disorders and liver damage (Valour *et al.*, 2013). In most instances, this liver damage is subclinical and is not the principal reason for slaughter (Lucy, 2001; González *et al.*, 2011). The liver is an important organ in insulin-mediated metabolic regulation. In addition to its important role in systemic, glucose, and lipid homeostasis, it is also the primary site of synthesis of factors such as plasma proteins and insulin-like growth factors I and II (IGF-I and IGF-II) and their binding proteins, thereby affecting systemic metabolism and growth (Postic *et al.*, 2004). IGF-I is involved in many processes during follicular development, oocyte maturation (Demeestere *et al.*, 2004), and subsequent embryonic development in many animals including bovine (Palma *et al.*, 1997) and porcine species (Xia *et al.*, 1994), mice (O’Neill, 1997), and humans (Lighten *et al.*, 1998). Therefore, liver disease may have a negative impact on reproduction. We previously reported that developmental potential is lower in oocytes derived from cattle with abnormal livers compared to those from cattle with healthy livers (Sarentonglaga *et al.*, 2013; Sarentonglaga *et al.*, 2021). Additionally, γ -glutamyl transpeptidase (γ -GTP) levels in follicular fluid (FF) are higher in cattle with an abnormal liver (Sarentonglaga *et al.*, 2013; Sarentonglaga *et al.*, 2021). To date, however, the influence of an abnormal liver on *in vitro* oocyte maturation in cattle has not been investigated.

The quality of the oocytes after *in vitro* maturation (IVM) is an important factor that determines subsequent developmental competence (Lonergan and Fair, 2016). Oocyte maturation includes both nuclear and cytoplasmic modifications. During oocyte maturation, dynamic morphological changes are observed, such as cumulus expansion, formation of a spindle, and chromosome alignment on the spindle (Duan and Sun, 2019). At meiosis, the morphology of the spindle and the rate of chromosome alignment can be adversely affected by temperature (Tamura *et al.*, 2013), reactive oxygen species (Sasaki *et al.*, 2019), and chemicals

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Table 1. Number (%) of animals with liver disease among cattle sent for slaughter

No. cattle inspected	No. liver diseases				
	Total	Hepatitis	Degeneration	Telangiectasis	Fatty liver
4091	3338 (81.6)*	2235 (67.0) [#]	544 (16.3) [#]	544 (16.3) [#]	15 (0.4) [#]

Data from an annual report of the Meat Inspection Office, Yamanashi Prefecture, 2020. *Percentage of cattle inspected. [#]Percentage of the total number of cattle with liver disease.

(Machtinger *et al.*, 2013). Abnormalities in spindle morphology and chromosome alignment at meiosis are associated with impaired embryonic development after fertilization. It has also been reported that the cytoskeleton and organelles play an important role in oocyte maturation (Reader *et al.*, 2017). Spindle migration and positioning in the oocyte cortex are precisely controlled by actin filaments and are essential for polar body release; these critical functions ensure the asymmetric cytoplasmic division of the oocyte during meiotic maturation (Sun and Kim, 2013; Almonacid *et al.*, 2014). Lysosomes also have an important role in oocyte meiosis, particularly in protein hydrolysis and signalling transduction (Perera and Zoncu, 2016). Impairment of lysosomal storage and lysosome degradation are associated with several diseases and affect oocyte meiosis (Darios and Stevanin, 2020).

The present study was initiated to examine the possible influence of maternal liver abnormality in cattle on *in vitro* oocyte maturation. Various aspects of maturing oocytes were screened, namely, meiotic maturation, spindle morphology, actin filaments, and lysosomes.

Materials and methods

Ovary collection and classification

Donor cow selection and diagnosis of liver abnormality were performed as described in our previous reports (Sarentonglaga *et al.*, 2013; Sarentonglaga *et al.*, 2021). In brief, ovaries were obtained from Holstein dairy cows from a slaughter house; any ovaries with abnormalities, such as follicular cysts and atrophy, were not included in the experiments. The livers of the cows were assessed by an experienced veterinarian and two groups of cows were established: a control group with normal livers; and a group with structural abnormality of the liver, such as fatty liver, hepatitis, or liver degeneration. The ovaries from the two groups were maintained at 15–20°C and transported to the laboratory in saline solution supplemented with 0.1% antibiotics and antimycotics (AB; Invitrogen, Carlsbad, CA, USA); the transport time was approximately 1 h from the slaughter house to the laboratory.

Oocyte collection and *in vitro* maturation (IVM)

Oocyte collection and IVM were carried out as previously described (Nagao *et al.*, 1994). In short, cumulus oocyte-complexes (COCs) were aspirated from 2–6 mm diameter follicles using a 20-gauge needle attached to a 5 ml syringe. Oocytes with three or more layers of compact cumulus cells and evenly granulated cytoplasm were selected as good quality and used for experiments. A pool of COCs were collected from cattle with healthy livers and low concentration of γ -GTP that were lower than 50 IU/L in the FF (Control group, mean: 29.7 \pm 1.5 IU/L) and a pool of COCs were collected from cattle with liver disorders and high concentration of γ -GTP that were over 50 IU/L in the FF (Abnormal group, mean: 103.6 \pm 13.8 IU/L) (Sarentonglaga *et al.*, 2013). Selected oocytes were washed, placed in 50 μ l drops of modified TCM-199 (m-

TCM199) in culture dishes (Falcon351007, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), covered with mineral oil (M8410, Sigma-Aldrich), and cultured for 24 h at 39°C under 5% CO₂ in humidified air. The m-TCM199 consisted of HEPES-buffered medium 199 (No.12340, Invitrogen) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA; P8136, Sigma-Aldrich), 0.5 mM sodium pyruvate (Nacalai Tesque, Tokyo, Japan), 1% AB, 0.02 AU/ml FSH (Antrin, Kyoritsu Seiyaku, Tokyo, Japan) and 1 μ g/ml oestradiol-17 β (E2758, Sigma-Aldrich). Following maturation, oocytes were individually placed on microscope slides and fixed with 3:1 ethanol: acetic acid; the cells were stained with 1% orcein. Oocytes undergoing germinal vesicle breakdown and maturing to metaphase II (MII) were selected for detailed analysis.

Collection and analysis of follicular fluid (FF)

After aspiration of COCs, residual FF from each cow was centrifuged at 1000 \times g at 4°C for 10 min. The concentration of γ -glutamyl transpeptidase (γ -GTP) in each FF sample was measured using the SPOTCHEMTM II assay (Glutamyl Transpeptidase Kit, ARKRAY, Kyoto, Japan) (Sarentonglaga *et al.*, 2013).

Assessment of spindle morphology and chromosome alignment

MII oocytes were washed in PBS + PVA (1 mg/ml) at 37°C and fixed in 2% paraformaldehyde containing 0.1% Triton X-100 for 30 min. Fixed oocytes were then washed in PBS + PVA. For spindle and chromatin staining, oocytes were first blocked in PBS supplemented with 4% bovine serum albumin (BSA) for 30 min at room temperature and then incubated overnight at 37°C in mouse monoclonal anti- α -tubulin (diluted 1:500 in PBS + 1.5% BSA, Invitrogen, USA). After two washes in PBS + 1.5% BSA (15 min each), oocytes were incubated in Alexa Fluor 488-labelled goat anti-mouse secondary antibody (diluted 1:100, abcam) at 37°C for 1 h. Oocytes were then mounted on a glass slide with 4',6-diamidino-2-phenylidole (DAPI; VECTASHIELD, VECTORLABS, USA) and analyzed using a FV10i FLUOVIEW (OLYMPUS, Japan). Spindles were analyzed as previously described (Ueno *et al.*, 2005) with regard to area, width, and length using the scale tool on the FL10-ASW3.1 (OLYMPUS, Japan) (Figure 2A). The meiotic stages of the oocytes were determined from the organization of the microfilaments, microtubules, and chromatin according to previously described criteria (Campen *et al.*, 2018). Spindles with two defined and focused poles were classified as bipolar (Figure 3A). Spindles with two poles but with structural abnormalities (e.g. splayed or disorganized microtubule fibres, broad or unfocused poles, protrusions of the spindle) were classified as abnormal bipolar. Spindles that had no apparent organization, that were monopolar or tripolar were considered undeterminable. Chromosome alignment was determined in all MII oocytes regardless of spindle morphology. Chromosomes that were located

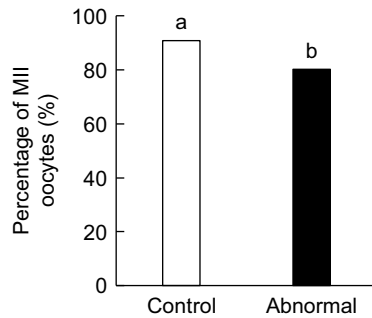


Figure 1. Effect of liver abnormality on maturation rate of the bovine oocytes. Different letters indicate significant differences ($P < 0.05$). Data were obtained from 9 cows from the control group and 7 cows from the abnormal group. Number of oocytes cultured: control group, 109; abnormal group, 96.

at the equatorial metaphase plate were classified as aligned. Where one to six chromosomes were slightly displaced from the metaphase plate, the chromosomes were classified as mostly aligned. Where more than six chromosomes were displaced from the metaphase plate, the chromosomes were considered undeterminable.

Assessment of cortical actin microfilaments

MII oocytes were washed in PBS + PVA (1 mg/ml) at 37°C and fixed in 2% paraformaldehyde containing 0.1% Triton X-100 for 30 min at 4°C. The fixed oocytes were then washed in PBS + PVA and then incubated in 1 µg/ml Phalloidin (diluted 1:80 in PBS + PVA, Sigma, USA) at 37°C for 1 h. After two washes in PBS + PVA, oocytes were mounted on a glass slide DAPI and examined using an FV10i FLUOVIEW. Cortical actin filaments were evaluated as previously described (Feitosa *et al.*, 2020). Briefly, digital images of oocytes were analyzed using FL10-ASW3.1. The circular draw function was used to quantify the total actin pixel intensity (TAPI) of each oocyte and the medullar actin pixel intensity (MAPI; 70% of TAPI in the centre of the oocyte). TAPI and MAPI were used to calculate cortical actin pixel intensity (CAPI) where $CAPI = [(TAPI - 0.7 \times MAPI)/0.3]$. Cortical actin pixel intensity was normalized by the ratio cortex:medullar pixel intensity (Figure 4A).

Assessment of lysosomes

MII oocytes were incubated in modified synthetic oviduct fluid supplemented with 0.1% (w/v) PVA (SOF-PVA) and 1 µM Lyso Tracker (Invitrogen, USA) for 30 min at 37°C. The oocytes were then washed twice in PBS + PVA, transferred to a glass-bottomed dish (Matsunami Glass, Osaka, Japan) and immediately viewed under a laser confocal fluorescence microscope (FV10i FLUOVIEW). Digital images were analyzed using FL10-ASW3.1 to determine the intensity of fluorescence and provide a measure of lysosome levels in each oocyte.

Statistical analysis

Differences in maturation rates, spindle morphology, and chromosome alignment were compared between the two groups of cattle using X^2 tests. Spindle size, cortical actin microfilament and lysosome data were analyzed using analyses of variance with F-tests and *t*-tests. In all experiments, values were considered to be significantly different when $P < 0.05$.

Results

The rates of oocyte maturation in the two groups of cattle are shown in Figure 1. A significantly lower rate of maturation was found in the abnormal group compared to the control group (80.2% vs 90.8%, respectively; $P < 0.05$).

The mean area of the spindle in oocytes from the abnormal group was larger than in the control group ($50.4 \pm 3.4 \mu\text{m}^2$ vs $40.8 \pm 1.6 \mu\text{m}^2$, respectively; $P < 0.05$; Figure 2B). Furthermore, the mean width of the spindle in oocytes from the abnormal group was larger than in the control group ($8.8 \pm 0.3 \mu\text{m}$ vs $7.8 \pm 0.2 \mu\text{m}$, respectively; $P < 0.05$). However, oocytes from the two groups showed no significant differences in spindle length ($5.7 \pm 0.2 \mu\text{m}$ in the abnormal group vs $6.1 \pm 0.3 \mu\text{m}$ in the normal group, respectively).

There were no significant differences in the frequencies of oocytes with normal bipolar spindles or abnormal bipolar spindles in the two cattle groups (Figure 3B). However, oocytes from the abnormal liver group had had a lower rate of aligned chromosomes than in the control group (40.8% vs 78.3%, respectively; $P < 0.05$).

The levels of cortical actin filaments in oocytes of the two groups were assessed using mean fluorescence intensity (MFI; Figure 4C). Oocytes from the abnormal group showed a significantly lower MFI than the control group (299.3 ± 3.7 vs 314.7 ± 3.2 , respectively; $P < 0.05$). Oocytes from the abnormal group had a higher lysosomal MFI than the control group (1363.6 ± 39.0 vs 1123.4 ± 26.3 ; $P < 0.05$; Figure 5B).

Discussion

We previously reported that γ -GTP concentration in FF from cattle with abnormal livers was higher than in cattle with healthy livers. Moreover, the developmental potential of oocytes from cattle with abnormal livers was lower than for oocytes derived from healthy cattle (Sarentonglaga *et al.*, 2013; Sarentonglaga *et al.*, 2021). These results indicated that liver abnormalities may impair IVM and reduce the rate of production of blastocysts. Thus, in the present study, we focused on maturation of oocytes from dairy cattle with a liver disorder, which was confirmed by the elevated γ -GTP level in FFs (Supplementary 1). Various factors influence oocyte quality and thereby affect the success of IVP (Lonergan and Fair, 2016). For example, delayed progression of nuclear maturation in oocytes derived from cattle with abnormal livers has been reported (Iwata *et al.*, 2010). Tanaka *et al.* (2014) also reported similar results to (Iwata *et al.*, 2010); however, nuclear maturation rates did not differ significantly after 21 h IVM. In this study, we used dairy cattle with liver disease and high γ -GTP levels in FFs and found that maturation rates were significantly lower compared to cows with healthy livers. The liver plays an important role as the primary site of IGF-I synthesis by stimulation of growth hormone (Matsumoto *et al.*, 2018). IGF-I is involved in many metabolic pathways during follicular development, oocyte maturation, and embryonic development (Xia *et al.*, 1994; O'Neill, 1997; Palma *et al.*, 1997; Lighten *et al.*, 1998; Demeestere *et al.*, 2004). Supplementation of IGF-I during IVM promotes steroid synthesis in granulosa cells (Mani *et al.*, 2010) and decreases the rate of apoptosis in oocytes (Wasielak and Bogacki, 2007). Furthermore, it has been shown that γ -GTP levels in the blood are negatively correlated with IGF-I levels in cows with liver disorders (Matsumoto *et al.*, 2018). These findings suggest that a high concentration of γ -GTP in FF causes a reduction in the levels of IGF-I and consequently may decrease maturation rates.

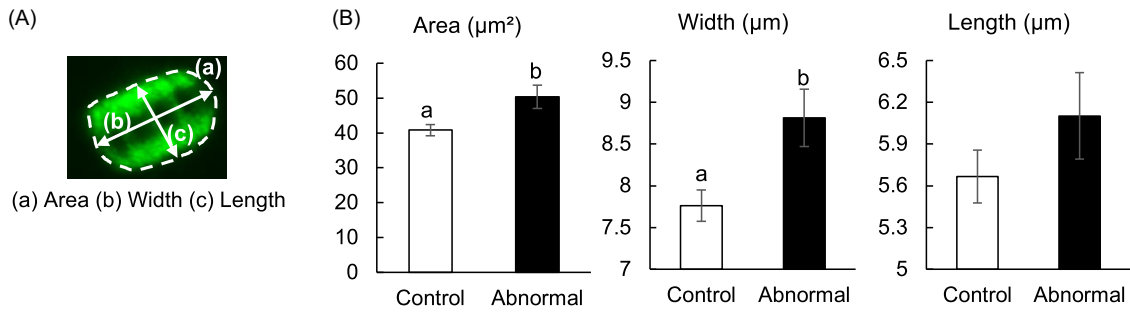


Figure 2. (A) Immunofluorescence images of spindles in metaphase II oocytes. The dashed line shows (a) the area measured; the arrows indicate (b) width and (c) length of the spindle. (B) Comparison of spindle morphology in oocytes from the control and abnormal liver groups. Different letters indicate significant differences ($P < 0.05$). Error bars represent the standard error of the mean. Data were obtained from 5 cows from the control group and 4 cows from the abnormal group. Measurements were obtained from 27 oocytes from the control group and 13 oocytes from the abnormal group.

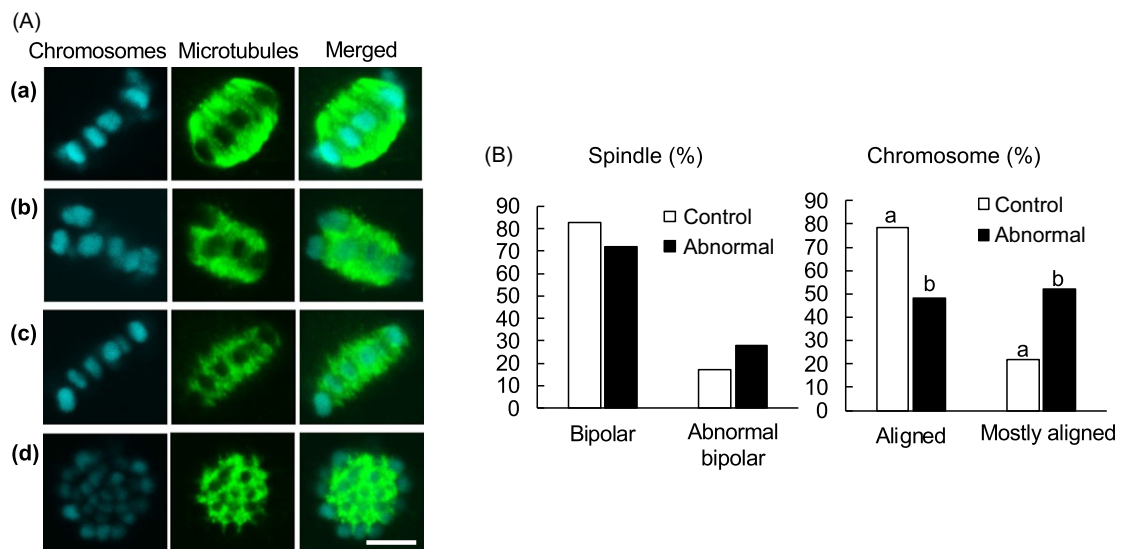


Figure 3. (A) Representative images of spindle and chromosome classifications in metaphase II oocytes. Chromosomes are shown in blue (left panels), microtubules are shown in green (centre panels), and merged images are shown in the right panels. (a) Bipolar spindle with aligned chromosomes. (b) Bipolar spindle with unfocused poles and a single misaligned chromosome. (c) Flattened bipolar spindle with extremely broad poles and aligned chromosomes. (d) Non-bipolar spindle with dispersed chromosomes. Bar (for all figures) = 5 µm. (B) Effect of liver abnormality on the frequencies of different spindle and chromosome types. Different letters indicate significant differences ($P < 0.05$). Data were obtained from 5 cows. We analyzed 46 oocytes from the control group and 25 oocytes from the abnormal group.

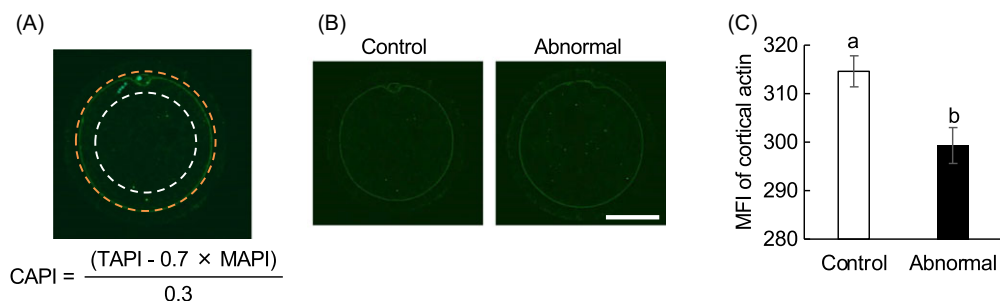


Figure 4. (A) Measurement of cortical actin filament levels in metaphase II oocytes. TAPI = Total actin pixel intensity (orange dashed line), MAPI = Medullar actin pixel intensity (white dashed line), CAPI = Cortical actin pixel intensity. (B) Representative images of cortical actin filament staining of oocytes. Bar = 50µm. (C) Comparison of cortical actin filament levels in oocytes of the control and abnormal liver groups. Different letters indicate significant differences ($P < 0.05$). Mean fluorescence intensity (MFI) indicates cortical actin levels. Error bars indicate the standard error of the mean. Data were obtained from 3 cows. Measurements were made in 41 oocytes from the control group and 19 oocytes from the abnormal group.

We found that oocytes from the abnormal group had a significantly larger mean spindle area. Additionally, mean width of the spindle was significantly larger in oocytes of the abnormal group. Ueno *et al.* (2005) reported that maturation conditions

influenced the morphogenesis of MII spindles in porcine oocytes. Machtinger *et al.* (2013) further reported that exposure to 200 ng/ml of Bisphenol-A increased the width of spindles in human oocytes; bisphenol-A treatment also significantly increased the

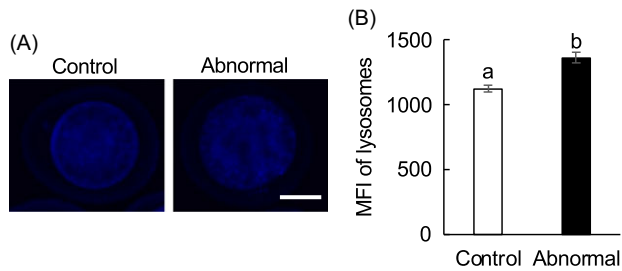


Figure 5. (A) Representative images of lysosome staining of oocytes. Bar = 50 μ m. (B) Mean fluorescence intensity (MFI) of lysosomes in metaphase II oocytes from control and abnormal liver groups. Different letters indicate significant differences ($P < 0.05$). Error bars show the standard error of the mean. Data were obtained from 4 cows. Measurements were made in 52 oocytes from the control group and 49 oocytes from the abnormal group.

frequencies of oocytes with spindle abnormalities and dispersed chromosomes (Campen *et al.*, 2018). Here, we found that frequency of oocytes with correctly aligned chromosomes was significantly lower in the abnormal group. In the mouse, a change in spindle integrity and chromosome alignment was found in oocytes exposed to reactive oxygen species (ROS) (Zhang *et al.*, 2006). Wang *et al.* (2017) reported that exogenous or endogenous sources of ROS could induce significant mitotic delays due to abnormal mitotic spindle assembly in HeLa cells. Moreover, the frequencies of misaligned chromosomes and multipolar spindles at metaphase are substantially increased in the presence of H_2O_2 (Wang *et al.*, 2017). In our previous study, we found that the levels of ROS in oocytes were significantly higher in the abnormal group (Sarentonglaga *et al.*, 2021). The present study might have occurred in oocytes from the abnormal group due to high levels of ROS, resulting in the occurrence of abnormal spindle morphology. Moreover, in our pre-experiment, the rates of development to the blastocyst stage in *in vitro* fertilization were significantly lower ($P < 0.05$) in the abnormal group (10.8%) than that in the control group (24.6%). Since abnormal spindle morphology may result in incorrect chromosome segregation and subsequent aneuploidy, spindle morphology is an important contributor to oocyte quality and subsequent embryonic developmental potential. Our findings support the view that liver abnormalities causing high γ -GTP concentration in FF have a negative influence on spindle architecture and chromosome organization during oocyte maturation.

Actin filaments that are located near the plasma membrane are important for progression of nuclear and cytoplasmic maturation in mammalian oocytes (Sun and Schatten, 2006). We found here that oocytes from the abnormal group had a significantly lower mean intensity of fluorescence; this indicates a lower level of cortical actin filaments in these oocytes. A previous study reported that a lower fluorescence intensity of cortical actin filaments in oocytes exposed to 2 or 4 h of heat shock (Ju and Tseng, 2004). In rabbits, it has been reported that hyperthermal stimulation during embryonic development destabilizes F-actin, resulting in failure of embryonic morphogenesis and apoptosis (Makarevich *et al.*, 2007). The present study indicates that healthy livers and low γ -GTP levels in the FF play critical roles in determining the F-actin structures in oocytes. Egerszegi *et al.* (2013) found that a higher frequency of spindle and actin filament damage was associated with a lower frequency of 2nd polar body formation in frozen-thawed oocytes. We found here that oocytes from cows with liver abnormalities and high γ -GTP concentrations in the FF had reduced actin filament formation, suggesting that actin

abnormalities could lead to abnormal spindle formation and blockage of meiosis in bovine oocytes. We also examined lysosome levels in oocytes using fluorescence intensities and found that oocytes of the abnormal group had significantly higher levels of lysosomes. Wang *et al.* (2021) reported that lysosome fluorescence intensity was higher in mouse oocytes after Fumonisin B1 treatment. Lysosomes have been suggested to sequester macromolecules from the endocytosis and autophagy pathways for degradation and recycling and to play important roles in regulating oocyte maturation and development (Miao *et al.*, 2019). Lysosomes can increase in number to fulfil different cellular demands, such as autophagy due to starvation and the distribution of lysosomes to daughter cells during cell division (Yang and Wang, 2021). In the present study, it is possible that oocytes from the abnormal liver group were starving and that the autophagy pathway increased the amount of nutrition needed for embryonic development. As described above, we also found oocytes from the abnormal liver group had altered lysosomal function, which might be related to autophagy pathways.

In conclusion, the present study showed that oocyte maturation potential was lower in dairy cattle with liver abnormalities than in healthy cattle and highlighted the possible influence of spindle morphology, actin filaments and lysosomes as factors that influence the success of *in vitro* maturation.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0967199424000352>

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Competing interests. None.

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