

# Non-invasive assessment of porcine oocyte quality by supravital staining of cumulus–oocyte complexes with lissamine green B

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Date submitted: 18.03.2015. Date revised: 26.05.2015. Date accepted: 05.06.2015

## Summary

We evaluated the usefulness of lissamine green B (LB) staining of cumulus–oocyte complexes (COC) as a non-invasive method of predicting maturational and developmental competence of slaughterhouse-derived porcine oocytes cultured *in vitro*. Cumulus cells of freshly aspirated COCs were evaluated either morphologically on the basis of thickness of cumulus cell layers, or stained with LB, which penetrates only non-viable cells. The extent of cumulus cell staining was taken as an inverse indicator of membrane integrity. The two methods of COC grading were then examined as predictors of nuclear maturation and development after parthenogenetic activation. In both cases LB staining proved a more reliable indicator than morphological assessment ( $P < 0.05$ ). The relationship between LB staining and cumulus cell apoptosis was also examined. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for DNA fragmentation revealed that oocytes within COCs graded as low quality by either LB staining or visual morphology showed significantly greater DNA fragmentation ( $P < 0.05$ ) than higher grades, and that LB and visual grading were of similar predictive value. Expression of the stress response gene *TP53* showed significantly higher expression in COCs graded as low quality by LB staining. However expression of the apoptosis-associated genes *BAK* and *CASP3* was not significantly different between high or low grade COCs, suggesting that mRNA expression of *BAK* and *CASP3* is not a reliable method of detecting apoptosis in porcine COCs. Evaluation of cumulus cell membrane integrity by lissamine green B staining thus provides a useful new tool to gain information about the maturational and developmental competence of porcine oocytes.

Keywords: Cumulus cells, IVM, Lissamine green B, Membrane integrity, Pig, TP53, TUNEL

## Introduction

Genome editing directly in mammalian embryos (Meyer *et al.*, 2010; Flisikowska *et al.*, 2013) is a powerful new technique that promises to radically streamline the genetic modification of large animal species, such as pigs, and so provide exciting new resources for biomedical research. However such an approach requires a reliable supply of development-

ally competent porcine embryos. The first successful birth of a piglet derived by *in vitro* fertilization (IVF) of porcine oocytes flushed from the reproductive tract was reported almost 3 decades ago (Cheng *et al.*, 1986), and there have since been numerous reports of live births in pigs by IVF (Gruppen, 2014), but obtaining *in vivo* matured oocytes is costly and time consuming (Nakai *et al.*, 2003). Moreover, unlike cattle, non-surgical ovum pick up is not practical in pigs due to their anatomy. Developing *in vitro* production (IVP) of pig embryos including *in vitro* maturation (IVM) has however proved problematic, and the systems available are still unacceptably inefficient, especially compared with other livestock species such as cattle (Gruppen, 2014). *In vitro* matured porcine oocytes have problems at several stages in the process. They have reduced ability to be fertilized *in vitro*,

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to block polyspermy, to undergo normal pronuclear formation upon sperm penetration (Abeydeera & Day, 1997; Abeydeera, 2002) and culture conditions are suboptimal for supporting embryo development beyond the initial cleavage divisions (Grupe, 2014).

Pig ovaries are usually obtained from slaughterhouses, which is convenient, but variations between animals and their handling can significantly affect the quality of the tissue arriving in the laboratory. For example, porcine oocytes are highly susceptible to temperature stress, and transient temperature shocks can occur *in vivo* during slaughter (Tong *et al.*, 2004), or ovaries be exposed to low temperature after removal (Yuge, 2003). These stresses result in poor maturation and subsequent embryo development and are often outside the control of the experimenter. A simple, reliable means of identifying cumulus–oocyte complexes (COC) with high maturational competence before IVM culture would reduce an important source of variability and aid the improvement and standardisation of subsequent IVM conditions.

As with many mammalian species, porcine COCs are usually graded by visual assessment of morphological features such as the thickness and compactness of the cumulus investment, ooplasm homogeneity (Akshey *et al.*, 2011; Garg *et al.*, 2012) and the size of follicles (Hyttel *et al.*, 1997; Hendriksen *et al.*, 2000) or oocytes (Coticchio *et al.*, 2004). Based on light microscopy, mammalian COCs surrounded by several layers of cumulus cells and with evenly granulated ooplasm have higher developmental competence *in vitro* than oocytes with irregularly granulated ooplasm and fewer cumulus layers (Wang *et al.*, 1997; Sirard *et al.*, 2006; Miyano & Manabe, 2007). Many other different selection criteria have also been reported (Galeati *et al.*, 1991; Gandhi *et al.*, 2001; Qian *et al.*, 2003).

The cumulus layer is undoubtedly vital for oocyte development, for example it plays an important role in the distribution of cortical granules, and thus the ability to undergo sperm penetration (Cheng *et al.*, 2013). However visual observation provides only limited information about its functional competence. Alternative methods of analysing cumulus cells are available, including evaluation of telomere length (Lee *et al.*, 2001), cumulus cell apoptosis (Corn *et al.*, 2005; Assou *et al.*, 2010) and gene expression profiling using microarray analysis (Ruppert-Lingham *et al.*, 2006), but are too time consuming and expensive for routine laboratory use. Propidium iodide staining has also been used as a non-invasive method of determining cumulus cell integrity (Uchikura *et al.*, 2011), but this requires fluorescence microscopy, which may not be available for some researchers and may be potentially mutagenic.

Here we report the use of lissamine green B (LB) dye to visually assess the quality of COCs used for *in vitro* culture. LB is used in ophthalmology to evaluate ocular membrane integrity (Hamrah *et al.*, 2011), but to our knowledge has never been used previously in reproductive technology. LB is a non-toxic synthetic organic acid dye that can be used as a supravital stain and, unlike propidium iodide, is directly visible, making staining rapid and simple. LB offers an efficient, objective, non-invasive and directly visible means of selecting good quality COCs and oocytes.

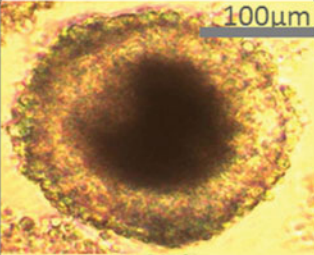
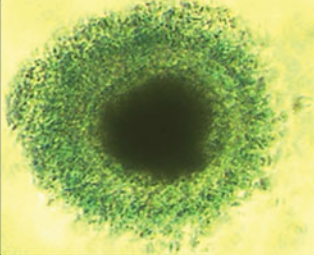
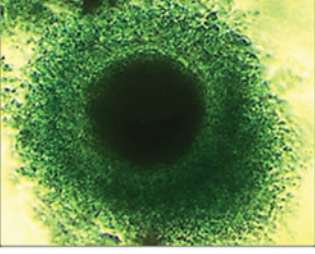
## Materials and methods

### Chemicals, cell culture media and supplements

Chemicals, reagents and culture media were purchased from Sigma Chemical Co., St. Louis, MO, USA unless otherwise indicated. Media were prepared fresh and sterilised by 0.2 µm membrane filtration.

### Collection of oocytes

Slaughterhouse ovaries were brought to the laboratory within 1 h of collection in a temperature-controlled box maintained at 39°C. Ovaries were thoroughly washed with pre-warmed (39°C) Dulbecco's phosphate-buffered saline (DPBS) solution containing 0.1% polyvinyl alcohol and penicillin, streptomycin solution. Follicles with a diameter of 3–8 mm were aspirated using an 18-gauge needle and a 5 ml syringe. Aspiration medium consisted of HEPES-buffered TCM199 (M 2520) with 26 mM NaHCO<sub>3</sub> (S 5761), 1 mM L-glutamine (G8540), 0.5 mg/ml polyvinyl alcohol (P 8136), 50 µg/ml gentamicin (G1264) and 20 U/ml heparin (H3149). Oocytes (COCs) were retrieved in the same medium without heparin using a stereomicroscope. Oocytes with even, dark cytoplasm and more than one compact layer of cumulus cells were selected. In total, 767 oocytes (COCs) were obtained from ~70 ovaries. These were divided randomly into two groups, one to study nuclear maturation and one to study parthenogenetic activation. Each one was further divided randomly into two sub-groups, one was graded by the morphology of their surrounding cumulus investments: grade A, at least three layers; grade B, less than three layers; or grade C, only corona radiata. The other sub-group was LB stained and scored according to a system previously used for propidium iodide staining (Ruppert-Lingham *et al.*, 2006; Uchikura *et al.*, 2011), with grade 1 having <25% cumulus cells stained; grade 2, 25–50% cumulus cells

Oocyte quality	LB staining under light microscopy (40)	Extent of cumulus damage
Grade 1		<25% of cumulus cells were stained by lissamine green B
Grade 2		25–50% of cumulus cells were stained by lissamine green B
Grade 3		>50% of cumulus cells were stained by lissamine green B

**Figure 1** Classification of COCs by lissamine green B staining.

stained; and grade 3, >50% cumulus cells stained, as shown in Fig. 1.

#### Optimization of lissamine green B staining

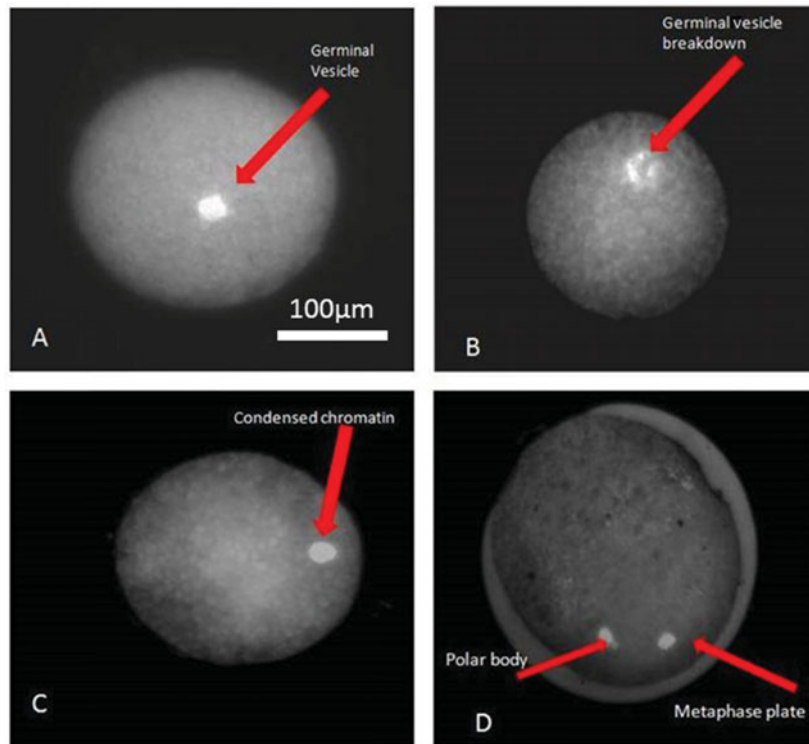
A stock solution of 1% LB (199583) in DPBS (pH = 7.0) was prepared and further diluted into TCM199 medium. All staining was performed at 39°C. Suitable staining conditions to detect cumulus cell damage were determined using COCs artificially damaged by treatment with 0.1% hyaluronidase (H3506) for 30 min at room temperature. Treated COCs were placed in groups of 25 in 500 µl droplets at four different LB concentrations (1, 0.5, 0.25 or 0.1%) under mineral oil for 2.5, 5 or 10 min. Each treatment was repeated three times. Following staining, COCs were washed in phosphate-buffered saline (PBS) to remove residual stain, observed by zoom stereomicroscope, and the number of stained COCs was recorded.

#### Determination of nuclear maturation after IVM culture

After quick assessment of cumulus membrane integrity, oocytes were washed twice in *in vitro* maturation

(IVM) medium and placed in groups of 40–60 in a final volume of 500 µl IVM medium in 4-well dishes (Nunc GmbH, Co. KG, Germany). Oocytes were cultured at 39°C under 5% CO<sub>2</sub> in air and maximum humidity for 46 h. The maturation medium consisted of TCM 199 (M 2154) with 1 mM L-glutamine, 1 mM sodium pyruvate (P3662), 0.1 mM 2-mercaptoethanol (M7522), 50 ng/ml epidermal growth factor (E4127), 50 µg/ml gentamicin and 0.1% (w/v) BSA (A3311). During the first 24 h of IVM, the medium was supplemented with 10 IU/ml PMSG and 5 IU/ml hCG (MSD Tiergesundheits, Germany) and 5 µl/ml ITS (recombinant human insulin, human transferrin, and sodium selenite, I3146) (Sjunnesson *et al.*, 2013).

After IVM culture, cumulus investments were removed from the oocytes by gentle pipetting. Oocytes were then fixed for 30 min in 0.5% glutaraldehyde (500 µl PBS + 10 µl 25% glutaraldehyde G-5882), washed in 500 µl PBS for 5 min then transferred to 500 µl Hoechst (B2261) stain solution (0.1%). The nuclear status was evaluated using an epifluorescence microscopy (ultraviolet (UV) light filter; Carl Zeiss) using standard measures like germinal vesicle breakdown, condensation of chromatin and appearance of the



**Figure 2** Progression of lissamine green B stained oocytes through different stages of maturation. (A) Immature oocyte. (B) Germinal vesicle breakdown. (C) Appearance of first polar body. (D) Mature oocyte with visible polar body and metaphase plate.

first polar body as confirmation of nuclear maturation (Fig. 2).

### Parthenogenetic activation of porcine oocytes

Oocytes were activated chemically to assess their developmental competence. After 46 h IVM culture, COCs were manually denuded by mouth pipetting, then activated by incubating in IVM medium containing 5  $\mu$ M Ca-ionophore A23187 (C7522) for 5 min at 39°C under 5% CO<sub>2</sub> in air. Oocytes were washed three times in IVM culture medium and incubated in 500  $\mu$ l droplets containing 2 mM 6-dimethylaminopurine (6-DMAP, D2629) and covered with mineral oil in an incubator at 39°C under 5% CO<sub>2</sub> in air for 4 h. After activation, oocytes were cultured in 500  $\mu$ l porcine zygote medium (PZM 5 IFP) in 4-well dishes (Nunc GmbH, Co. KG, Germany) overlaid with 500  $\mu$ l mineral oil (M8410). The dish was incubated undisturbed at 39°C in 5% CO<sub>2</sub> in air for 7 days. Embryo development was observed microscopically and cleavage assessed at 48 h post-activation.

### RNA isolation and reverse-transcription

Apoptotic gene expression was evaluated in groups of 50–60 COCs. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Europe) according to

the manufacturer's instructions. Sample purity was assessed using the  $A_{260}/A_{280\text{ nm}}$  ratio with expected values between 1.8 and 2.0, RNA samples were treated with TURBO DNA-free™ Kit (Life Technologies) to remove genomic DNA. Reverse-transcription reactions were performed with 0.5–1  $\mu$ g total RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Life Technologies) and random hexamer as a primer, according to the manufacturer's instructions. For each sample, negative controls lacking reverse transcriptase were prepared using the identical procedure.

### Quantitative real-time PCR

Gene expression was assessed by quantitative RT-PCR. Primers for *BAK*, *CASP3* and *TP53*, and a housekeeping gene *ACTB* (beta-actin) were designed using 'Primer3' software. The predicted fragment sizes are listed in Table 1. Real-time PCR amplification was conducted using an ABI 7300 real-time PCR System (Applied Biosystems). A KAPA SYBR FAST q PCR Kit (Kapa Biosystems) was used to provide real-time quantification of the amplified product. Three replicates of each reaction were measured and the mRNA level of each sample was normalised to that of

**Table 1** Primer sequences and annealing temperature

Genes	Primer sequences	Annealing temperature (°C)	Product size (bp)	Accession no.
$\beta$ -Actin	F-5'GTGGACATCAGGAAGGACCTCTA3'; R-5'ATGATCTTGATCTTCATGGTGCT3'	58	135	U07786
Bak	F-5'CAGCACCATGGGGCAGGTAG3'; R-5'AGGCTGGAGGCGATCTTGGT3'	58	150	AJ001204.1
Caspase 3	F-5'GCCGAGGCACAGAATTGGAC3'; R-5'GCGCTGCACAAAGTGACTGG3'	58	180	AB029345.1
TP53	F-5'CTTTGAGGTGCGTGTTTGTG3'; R-5'CGGATCTGGAGGGTGAATA3'	60	152	AF098067.1

beta-actin. Relative mRNA levels were determined by the  $\Delta\Delta$ Ct method (Livak & Schmittgen, 2001).

### Measurement of DNA fragmentation

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) procedure was used to fluorescein-dUTP label 3'-OH ends of DNA fragments generated by apoptosis, allowing detection of apoptotic cells by fluorescence microscopy. Fixed and permeabilized embryos were subjected to the TUNEL assay procedure using an *in situ* apoptosis detection kit (TaKaRa Bio Inc., Japan) according to the manufacturer's instructions. COCs were fixed for 30 min at room temperature in PBS (pH 7.4), permeabilized for 5 min on ice in permeabilization buffer, and then washed once in PBS. COCs were incubated with terminal transferase and labelled nucleotide solution in a humidified, sealed chamber in the dark at 37°C for 1 h. Slides were overlaid with a coverslip and the edges sealed with quick drying nail polish, then evaluated using an epifluorescence microscopy (green excitation filter, Carl Zeiss). Oocytes showing discrete pinpoint fluorescent green signals were judged to be positive for DNA fragmentation. The apoptosis score was expressed as a percentage of the total.

### Statistical analysis

Data were analysed using GraphPad Prism (GraphPad Software, Inc. USA) after arcsin transformation of percentage values. Differences between mean percentages were analysed by two-way analysis of variance (ANOVA). A value of  $P < 0.05$  was considered to be statistically significant.

## Results

### Lissamine B staining

A staining regimen of 0.50% LB for 5 min was found to be optimum for staining in terms of specificity and

easily detectable coloration. Under light microscopy, damaged cumulus cells were stained greenish blue, while healthy cumulus cells remained unstained. The ooplasm of denuded oocytes that incurred damage to the zona pellucida stained greenish blue.

### Nuclear maturation

Of the 372 COCs in the nuclear maturation group, 191 were graded by morphology, with 63 assessed as grade A (32.99%), 67 grade B (35.07%) and 61 grade C (31.93%); 181 were graded by LB staining as 59 grade 1 (32.59%), 65 grade 2 (35.91%), and 57 grade 3 (31.49%). Nuclear maturation of each oocyte was scored by the attainment of metaphase II, as judged by the presence of condensed chromosomes in an equatorial position and extrusion of the first polar body (Fig. 2). Nuclear maturation data (Table 2) showed no significant difference between morphological grade A ( $79.9 \pm 3.82\%$ ), grade B ( $86.48 \pm 3.07\%$ ), or grade C ( $82.00 \pm 3.00\%$ ) oocytes. The nuclear maturation rates of LB graded oocytes were: grade 1 ( $84.70 \pm 3.36\%$ ), grade 2 ( $83.61 \pm 3.44\%$ ) and grade 3 ( $62.26 \pm 3.69\%$ ). The difference between grade 3 and grades 1 and 2 was significant ( $P < 0.05$ ).

### Developmental competence of oocytes

The parthenogenetic activation group comprised 395 oocytes. 204 were graded by morphology as 67 grade A (32.84%), 71 grade B (34.80%) and 66 grade C (32.35%); 191 were graded by LB staining as 64 grade 1 (33.50%), 65 grade 2 (34.03%), and 62 grade 3 (32.46%). Development was scored as the number of embryos consisting of two to eight evenly sized blastomeres 48 h after parthenogenetic activation. There was no significant difference between morphological grade A ( $84.55 \pm 4.45\%$ ), grade B ( $85.06 \pm 4.49\%$ ), or grade C ( $81.60 \pm 4.64\%$ ) oocytes. The rates of parthenogenetic activation in LB graded oocytes were: grade 1 ( $87.71 \pm 2.46\%$ ), grade 2 ( $82.72 \pm 3.39\%$ ) and grade 3 ( $62.88 \pm 3.85\%$ ). Again this shows a statistically significant

**Table 2** Effects of cumulus morphological categories on maturational and developmental ability of porcine oocytes

Item	Nuclear maturation and parthenogenetic activation					
	Grades based on cumulus investment			Grades based on membrane integrity		
	Grade A	Grade B	Grade C	Grade 1	Grade 2	Grade 3
Nuclear maturation (%)	79.9 ± 3.8 <sup>a</sup>	86.48 ± 3.07 <sup>a</sup>	82.00 ± 3.00 <sup>a</sup>	84.70 ± 3.36 <sup>a</sup>	83.61 ± 3.44 <sup>a</sup>	62.26 ± 3.69 <sup>b</sup>
Activation (%)	84.55 ± 4.4 <sup>a</sup>	85.06 ± 4.49 <sup>a</sup>	81.60 ± 4.64 <sup>a</sup>	87.71 ± 2.46 <sup>a</sup>	82.72 ± 3.39 <sup>a</sup>	62.88 ± 3.85 <sup>a</sup>

<sup>a,b</sup>Values with different superscripts differ significantly ( $P < 0.05$ ). Values are means ± standard deviation (SD).

**Table 3** Rate of DNA fragmentations as detected by TUNEL assay in different category of porcine oocytes

Item	DNA fragmentation assay					
	Grades based on cumulus investment			Grades based on membrane integrity		
	Grade A	Grade B	Grade C	Grade 1	Grade 2	Grade 3
Apoptosis (%)	20.97 ± 3.52 <sup>a</sup>	22.69 ± 4.05 <sup>a</sup>	38.42 ± 4.03 <sup>b</sup>	21.66 ± 4.08 <sup>a</sup>	21.82 ± 5.85 <sup>a</sup>	37.08 ± 2.76 <sup>b</sup>

<sup>a,b</sup>Values with different superscripts differ significantly ( $P < 0.05$ ). Values of DNA fragmentation are means ± standard deviation (SD).

difference between grade 3 and grades 1 and 2 ( $P < 0.05$ ) (Table 2).

### TUNEL assay for oocytes and cumulus cells

In total, 176 COCs were graded by either morphology or LB staining. For each grade a TUNEL score was determined as the percentage of observed COCs showing signs of DNA fragmentation, as indicated by a fluorescent signal (Table 3 and Fig. 3). Throughout, fluorescent signals were observed only in the ooplasm and not in the cumulus cells. There was no significant difference between morphological grade A (20.97 ± 3.52%) and grade B (22.69 ± 4.05%) oocytes, but grade C oocytes showed significantly higher (38.42 ± 4.03%) incidence of DNA fragmentation ( $P < 0.05$ ). The TUNEL score in LB graded oocytes was similar: grade 1 (21.66 ± 4.08%), grade 2 (21.82 ± 5.85%), with grade 3 significantly higher (37.08 ± 2.76%) ( $P < 0.05$ ). There was no significant difference in TUNEL score between morphological grade C and LB grade 3 COCs.

### Expression of stress and apoptosis-related genes in lissamine B graded porcine COCs.

Differential expression of the stress-associated gene *TP53* and two genes related to apoptosis, *BAK* (BCL2-antagonist/killer) and *CASP3* (caspase 3), was analysed by quantitative real-time RT-PCR in three pools of 50–60 COCs representing LB-stained grades 1, 2 and 3 (Fig. 4). Three replicates were conducted for each experiment. Figure 4 shows that *BAK* and *CASP3* expression was similar in all grades of LB-stained COCs, but *TP53* expression was significantly

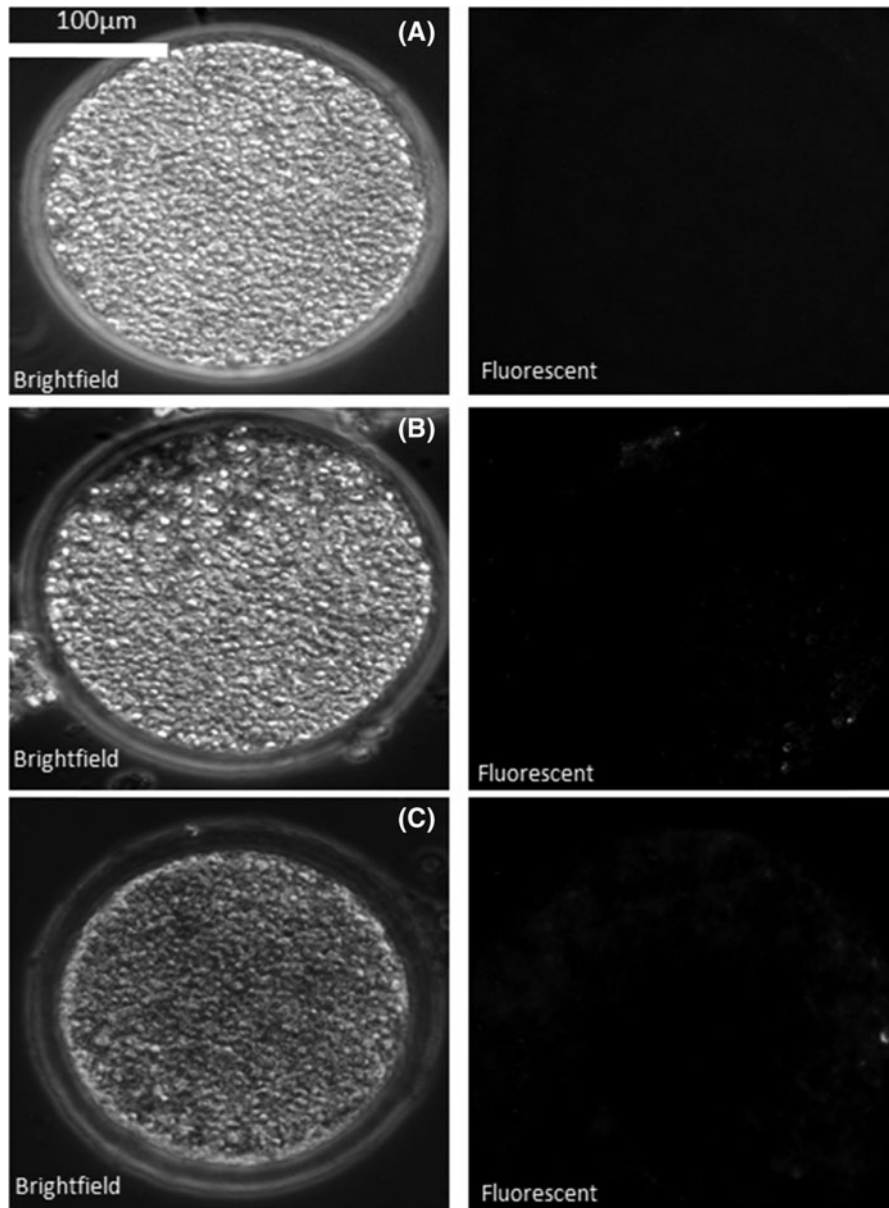
greater ( $P < 0.05$ ), in grade 3 COCs than grades 1 and 2.

### Discussion

We evaluated the usefulness of lissamine green B (LB) staining for non-invasive prediction of maturational competence of porcine oocytes. LB preferentially stains membrane-damaged or devitalised cells (Kim & Foulks, 1999). Studies using LB to stain rabbit and human corneal epithelial cells *in vitro* show that it does not stain healthy, proliferating cells and has a minimal effect on cell viability (Kim, 2000). Evidence to date indicates no carcinogenic or cytotoxic properties (Brantom *et al.*, 1987; Clode, 1987; Moorhouse *et al.*, 1987).

Our data indicate that LB staining of COCs immediately after collection is superior to morphological grading as a predictor of oocyte viability, nuclear maturation after IVM culture, and successful parthenogenetic activation and development. Parthenogenesis was used as a convenient and valid functional assay of developmental competence (Rougier & Werb, 2001; McElroy *et al.*, 2010).

While higher rates of nuclear maturation, fertilization and development after IVP have been reported in bovine oocytes graded on the basis of the thickness of the cumulus cell layers, our data show that this cannot automatically be extended to porcine oocytes. Our findings clearly reaffirm the importance of a cumulus layer for oocyte maturation and development (Davachi *et al.*, 2012; Prates *et al.*, 2014), but suggest



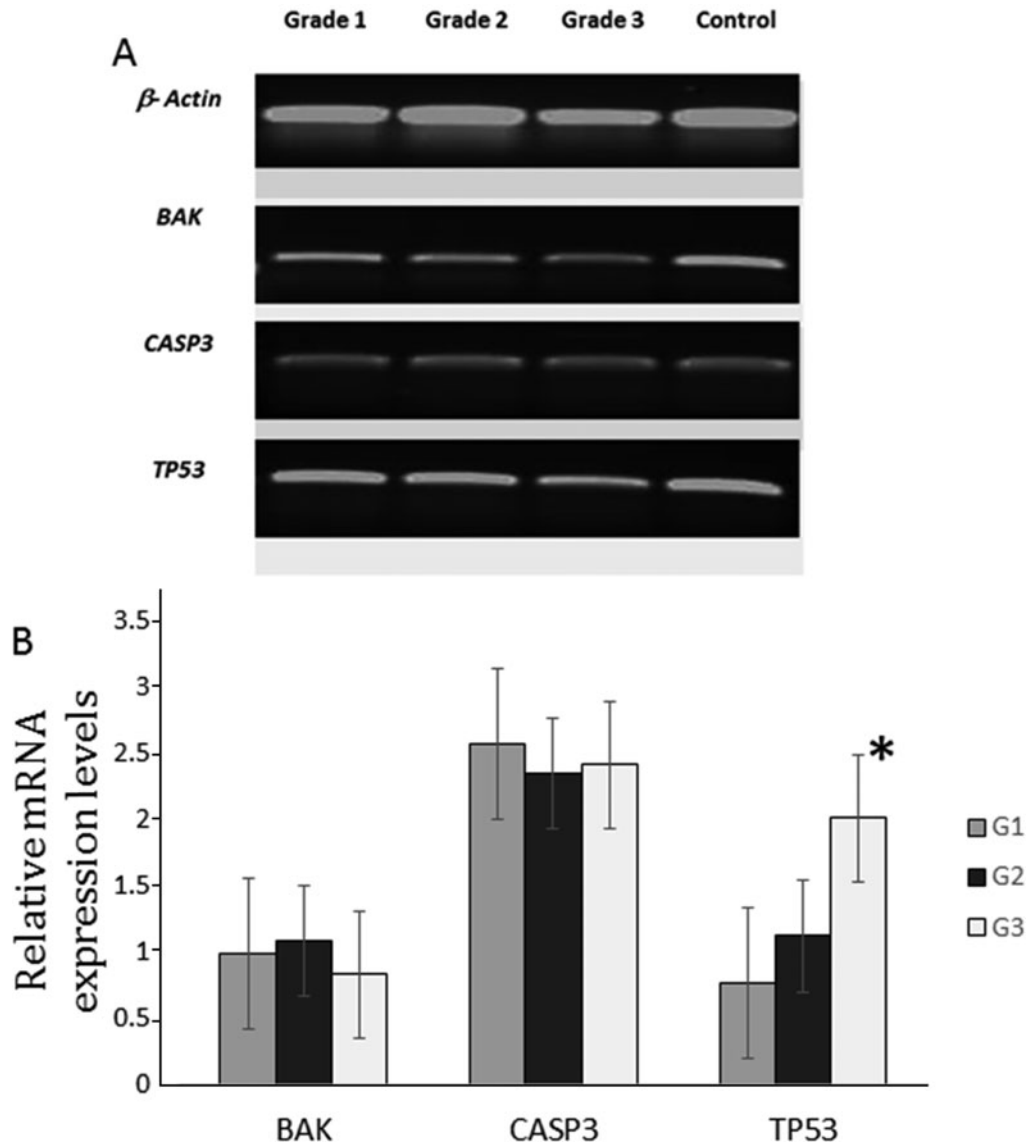
**Figure 3** DNA fragmentation detection by TUNEL assay. (A) Porcine oocyte with no detectable DNA fragmentation. (B) Porcine oocyte with moderate DNA fragmentation. (C) Porcine oocyte with extensive DNA fragmentation.

that measures of cumulus cell integrity are more useful than morphological parameters alone.

Our investigation of the role of apoptosis in COC quality revealed some interesting findings. As expected, DNA fragmentation measured by TUNEL assay showed that LB grade 3 COCs were more damaged than higher grade COCs, consistent with their developmental properties and also in line with morphological grade C COCs. DNA fragmentation in immature oocytes (in particular, GV and MI stages) could be a consequence of stress during maturation of the ovarian follicle or hypoxia resulting from compromised microcirculation and correlate with

aneuploidy or other chromosomal abnormalities (Van Blerkom, 1996). It is notable that we found no evidence of DNA fragmentation in the cumulus layer in immature porcine COCs. This is consistent with a similar finding in cattle in which no TUNEL signal was obtained in cumulus cells of immature oocytes (Yuana *et al.*, 2005).

Gene expression analysis of three genes chosen for their well known roles in stress and apoptosis however revealed an interesting and unexpected distinction. The expression of *TP53*, a stress response gene induced by DNA damage, was significantly greater in LB-stained grade 3 COCs, consistent with



**Figure 4** (A) Reverse-transcription PCR of apoptosis-associated genes in lissamine B stained oocytes. Relative gene expression levels of lissamine B stained porcine oocytes. (B) The mRNA levels of BAK, Caspase 3 and TP53 were analysed in porcine oocytes. Results are displayed as fold changes in different grades of lissamine B stained oocytes relative to each other. Significant difference is marked with an asterisk (\* $P < 0.05$ ).

TUNEL assay findings and the poorer developmental parameters of this grade. Studies in humans and mice have revealed a clear relationship between *in vitro* culture related stress and TP53 expression in embryos (Chandrankanthan *et al.*, 2006). In mice, genetic inactivation of *Mdm2*, a negative regulator of p53, results in early embryonic lethality (Jones *et al.*, 1995) that can be reversed by deletion of *Trp53* (Luna *et al.*, 1995), demonstrating that the p53 response pathway plays a direct role in embryonic loss.

It was expected that BAK and CASP3 expression would also be greater in the lowest quality COCs, but this was not the case. BAK and CASP3 are members of two important regulatory families involved in

apoptosis. BAK is a pro-apoptotic member of the Bcl-2 family whose cytoplasmic elevation is sufficient to cause induction of oocyte apoptosis (Morita & Tilly, 1999). The lack of any clear correlation between the RNA level of these genes and COC grade, despite evident DNA damage, could be due to a variety of reasons. One is that there is abundant maternal CASP3 mRNA in the oocytes (Weil *et al.*, 1996), and this may mask any differential expression in the cumulus cells of different grades. Another is that regulation of caspase 3 activity is known to occur at the protein level, by modification of an inactive proenzyme. In which case direct immunocytological detection of the active form of caspase-3 may be a more reliable measure. It is



also known that caspase cleavage and DNA fragmentation do not take place simultaneously (Yuana *et al.*, 2005). However, our findings are also consistent with similar observations in bovine embryos treated with the apoptosis inducer staurosporine, which showed no difference in the expression of several apoptosis-related genes including *CASP3* and other Bcl family genes (Vandaele *et al.*, 2008).

Cumulus cells play a critical role in oocyte maturation and fertilization by releasing and mediating signals to oocytes (Vandaele *et al.*, 2008), but the relationship between cumulus cell apoptosis and the development potential of the oocyte is unclear. There are in fact contradictory reports in various species as to whether definitive apoptosis actually takes place in COCs (Tanghe *et al.*, 2002). For example, some studies have shown that human and bovine cumulus cells undergo apoptosis (Lee *et al.*, 2001; Mikkelsen *et al.*, 2001; Yuana *et al.*, 2005), While others report the absence of apoptosis in cumulus cells of rats (Zeuner *et al.*, 2003), pigs (Szoltys *et al.*, 2000), and cattle (Manabe *et al.*, 1996).

The simplicity and non-invasive nature of LB staining makes it straightforward to adopt as a quality control test for embryological labs. LB staining can give valuable information about the quality of oocytes in terms of maturational ability, developmental potential and extent of DNA damage. Further studies are clearly required to assess any long-term effects of LB on embryo survival and fetal health.

## Acknowledgements

We would like to thank Steffen Löbnitz for help collecting samples. This work was financially supported by grant 16EX1024B; 'Platform Advanced Preclinical Animal Models' from the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung) as part of the m<sup>4</sup> cluster initiative Personalized Medicine and Targeted Therapies. RD was supported by a scholarship from the German Academic Exchange Service (Deutscher Akademischer Austausch Dienst). The authors are members of COST Action BM1308 'Sharing Advances on Large Animal Models' (SALAAM).

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