Capacity of Trolox to improve the development and quality of metabolically compromised bovine oocytes and embryos in vitro during different windows of development

Capacity of Trolox to improve the development and quality of metabolically compromised bovine oocytes and embryos *in vitro* during different windows of development

J. De Bie[A], *A. Smits*[B], *W. F. A. Marei*[B,C] *and J. L. M. R. Leroy*[B,D]

[A]Centre for the Evaluation of Vaccination, Vaccine and Infectious Disease Institute, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium.

[B]Gamete Research Centre, Laboratory for Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium.

[C]Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.

[D]Corresponding author. Email: jo.leroy@uantwerpen.be

Trials to improve oocyte developmental competence under metabolic stress by using antioxidants may start before or after oocyte maturation. In the present conceptual study, we aimed to identify the most efficient timing of antioxidant application in relation to a metabolic insult using a bovine *in vitro* embryo production model. Pathophysiological concentrations of palmitic acid (PA) were used to induce metabolic stress during oocyte maturation or embryo development. Trolox (TR; antioxidant) treatment prior to, during or after the PA insult was tested to evaluate the protective, neutralising and rescuing capacity of TR respectively. Changes in embryo developmental competence, mitochondrial activity, reactive oxygen species (ROS) concentrations, blastocyst cell allocation and apoptosis and cell stress-related gene expression were monitored. The improvement in developmental capacity was most obvious when oocytes were preloaded with TR before the PA insult. This protective effect could be explained by the observed combination of increased mitochondrial activity with reduced ROS production. This resulted in blastocysts with normal cell counts and apoptosis, as well as increased Nuclear factor erythroid 2-related factor 2 (*NRF2)* expression (a marker for redox regulatory processes) and normalised the expression of the mitochondrial transcription factor A (*TFAM)*, a marker of mitochondrial biogenesis. These results indicate that 'pretreatment' of oocytes with antioxidants produces embryos that seem to be more resilient to a metabolic stress insult.

Keywords: fatty acid, fertility, IVM, oxidative stress.

Suboptimal nutrition and metabolic health can increase oxidative stress in oocytes and embryos, thereby affecting fertility. The strategic use of antioxidants protects oocytes against metabolic stress insults during embryo development. These findings highlight the importance of the timing of antioxidant treatment with the aim of protecting and safeguarding oocyte and embryo quality, and improving fertility.

Introduction

Oocyte and embryo development include a series of finely regulated events in a complex and dynamic microenvironment. First, the oocyte acquires full developmental competence in the ovarian follicle following important ultrastructural and molecular changes known as cytoplasmic and nuclear maturation (Conti and Franciosi 2018). Subsequently, after fertilisation, the preimplantation embryo develops in the oviduct and undergoes its first cleavages, embryo genome activation and DNA methylation. These windows of development are sensitive to any perturbations in their microenvironment, which may compromise oocyte and embryo quality and further development.

The microenvironment of the oocyte (i.e. follicular fluid (FF)) and preimplantation embryo (i.e. oviductal fluid) can be affected by nutrition and metabolic health (Leroy *et al*. 2004; De Bie *et al*. 2016; Jordaens *et al*. 2017). Results from *in vivo* studies using a high-fat diet-induced obesity mouse model describe the consequences of an altered metabolic microenvironment throughout preimplantation development from oocyte maturation to embryo development. For example, high-fat diet-induced obesity in mice has been associated with increased oxidative stress (OS) in oocytes and zygotes (Igosheva *et al*. 2010; Han *et al*. 2017; Wang *et al*. 2018), as well as altered mitochondrial function and reduced glutathione concentrations in the oocyte, leading to embryos with a reduced capacity to successfully develop (Igosheva *et al*. 2010).

Many *in vitro* studies have focused on oocyte maturation as a sensitive window and investigated the effects of metabolic disorders at this stage on oocyte developmental competence (Jorritsma *et al*. 2004; Aardema *et al*. 2011; Leroy *et al*. 2015). Valckx *et al*. (2015) showed that the addition of FF from obese women, containing high concentrations of free fatty acids (FFA), during bovine oocyte IVM impaired embryo development compared with oocytes matured with FF from non-obese individuals. In particular, increased palmitic acid (PA) in female FF was associated with reduced embryo cleavage rates (O'Gorman *et al*. 2013), reduced embryo quality and low pregnancy rates following intracytoplasmic sperm injection (Mirabi *et al*. 2017). Other *in vitro* models showed that direct exposure of bovine oocytes to elevated FFA, particularly PA, resulted in increased reactive oxygen species (ROS) production, OS, an increase in mitochondrial inner membrane potential (MMP) in oocytes and cumulus cells, reduced cleavage and blastocyst rates, increased blastomere apoptosis and altered blastocyst metabolism (Van Hoeck *et al*. 2011; Sutton-McDowall *et al*. 2016; De Bie *et al*. 2017; Marei *et al*. 2017*b*, 2019*b*). More in-depth molecular analyses of FFA- and PA-exposed bovine oocytes also point towards activation of several OS-related pathways (Van Hoeck *et al*. 2013; Marei *et al*. 2019*b*).

Preimplantation embryo development is also an important window that is sensitive to perturbations in the microenvironment. Exposure of bovine and murine embryos to FFA during *in vitro* culture hampered development and embryo quality (Jungheim *et al*. 2011; Van Hoeck *et al*. 2012). In line with this, hyperlipidaemic culture conditions (by adding serum) jeopardised embryo developmental

competence and resulted in inferior blastocyst quality, as evidenced by altered mRNA expression profiles related to metabolism, apoptosis and OS (Nonogaki *et al*. 1994; Rizos *et al*. 2003; Leroy *et al*. 2010; Marei *et al*. 2017*a*).

Because OS related pathways are clearly implicated in the observed reduced oocyte and embryo quality in mothers suffering from metabolic disorders, antioxidant (AO) supplementation is a promising strategy to maintain or improve oocyte and embryo quality in metabolically compromised women. Animal studies showed that AO supplementation is reflected in the composition of the FF (Dobbelaar *et al*. 2010; De Bie *et al*. 2016), and human studies indicate that AO supplementation before ovum pick-up may protect the oocyte, most probably via direct effects (Luddi *et al*. 2016). These observations suggest that AOs supplied before oocyte maturation may protect the oocyte via the FF from metabolic insults later in development.

Furthermore, other studies reported neutralising effects of AO treatment applied simultaneously with the metabolic insult. Melatonin supplementation to high-fat diet-induced obese mice markedly reduced OS in oocytes (Han *et al*. 2017). Others revealed that the addition of an AO, such as Trolox, to embryos exposed to FFA *in vitro* attenuated the lipotoxic and inhibitory effects observed on embryo development (Nonogaki *et al*. 1994; Rooke *et al*. 2012).

Very recently, the first study investigating the reparative or rescuing effects of AO treatment during embryo culture of metabolically compromised oocytes was published (Marei *et al*. 2019*a*). In that study, Marei *et al*. (2019*a*) revealed that the addition of MitoQ (an AO specifically targeting mitochondria) to embryo culture reduced OS in bovine embryos derived from metabolically compromised oocytes *in vitro*, leading to higher blastocyst rates and lower blastomere apoptosis. Interestingly, we also demonstrated that AOs such as selenium and transferrin (combined with insulin) during bovine *in vitro* embryo culture could improve the development of metabolically compromised oocytes, but could not repair the quality of the embryo, as evidenced by the high rates of apoptosis in blastocysts (Smits *et al*. 2016; De Bie *et al*. 2017).

From the above, it is evident that AO supplementation during different windows of development may improve oocyte and embryo development under metabolic stress conditions. Until now, it is not known whether the AO treatment is best implemented in a protective, reparative or neutralising manner. In the present study we investigated this concept with the aim of determining the most effective timing of AO treatment in relation to the moment of the metabolic insult using a bovine *in vitro* embryo production model. To this end, pathophysiologically relevant concentrations of palmitic acid (PA, 150 to 230 µM) were used to induce metabolic stress during oocyte maturation or early embryo development (Marei *et al*. 2019*a*, 2019*b*). Based on Rooke *et al*. (2012), Trolox (TR), a water-soluble vitamin E analogue and AO, was used as the AO treatment. Trolox treatment prior to, during or after the PA insult was tested to evaluate the protective, neutralising or rescuing capacity of

TR respectively on embryo developmental competence and embryo ROS and mitochondrial activity. The experimental conditions that successfully improved embryo development were further evaluated for their effects on embryo quality by assessing blastocyst cell counts, apoptosis and OS-related pathways in blastocysts at the transcriptome level.

Materials and methods

The bovine ovaries used for this study were derived from a licenced abattoir and therefore no ethics approval was required. Chemicals used to prepare embryo culture media were purchased from Life Technologies (Thermo Fisher Scientific). Other chemicals were purchased from Sigma-Aldrich, unless otherwise stated. All compounds were of analytical grade.

Experimental design and treatments

An earlier described and validated model was used to mimic maternal metabolic disorders *in vitro* during bovine oocyte maturation and embryo culture (Van Hoeck *et al*. 2012; Desmet *et al*. 2016; Marei *et al*. 2019*b*). Cumulus–oocyte complexes (COCs) and embryos were exposed to pathophysiologically relevant PA concentrations as measured in the FF (150 µM PA, added during IVM; Marei *et al*. 2019*b*) and in serum and oviductal fluid (230 µM PA, added during *in vitro* culture (IVC); Jordaens *et al*. 2017) respectively. These pathophysiological PA concentrations have been detected in obese women (Valckx *et al*. 2014), as well as in dairy cows during negative energy balance (Leroy *et al*. 2004). Trolox was chosen as the AO in this study because it is a water-soluble AO, which makes it applicable for *in vitro* use in aqueous culture conditions, it is a vitamin E analogue with strong AO capacity (Hamad *et al*. 2010) and it has proven efficacy in previous studies (Forrest *et al*. 1994; Rooke *et al*. 2012; Messier *et al*. 2013). Based on the study of Rooke *et al*. (2012), TR was used at a concentration of 100 µM during oocyte IVM or embryo culture.

Stock solutions of 150 and 230 mM PA and 100 mM TR were prepared in 100% ethanol as the solvent (VWR). These stock solutions were diluted in oocyte maturation or embryo culture medium to obtain the final concentrations of PA (150 and 230 µM) and TR (100 µM) used in the study. Fatty acid-free bovine serum albumin (BSA) was used as a carrier for PA (Salway 1999). Preliminary experiments (data not shown) and previous data from Marei *et al*. (2017*b*) showed that the addition of ethanol up to 0.2% v/v during IVM or IVC does not affect the developmental competence or quality of bovine oocytes and embryos compared with ethanol-free controls.

Four different experiments were performed with different conditions during IVM and IVC. The treatment groups are named according to their IVM and IVC conditions. In each experiment, a solvent control (SC) was included (i.e. 0.2% ethanol during IVM and 0.2% ethanol during IVC (SC-SC)) for comparison.

In Experiment 1 (neutralising approach during IVM), oocytes were exposed to elevated PA concentrations with or without TR during IVM, after which zygotes were cultured under solvent control conditions during IVC (PA-SC and PATR-SC respectively).

In Experiment 2 (neutralising approach during IVC), oocytes were matured under SC conditions during IVM, then exposed to elevated PA concentrations with or without TR during IVC (SC-PA and SC-PATR respectively).

In Experiment 3 (reparative approach), oocytes were exposed to elevated PA concentrations during IVM, after which zygotes were cultured under solvent control (PA-SC) or TR (PA-TR) conditions during IVC.

In Experiment 4 (protective approach), oocytes were matured under SC or TR conditions during IVM, then exposed to elevated PA concentrations during IVC (SC-PA and TR-PA respectively).

Different outcome parameters were evaluated in these experiments. Developmental competence (Day (D) 2 cleavage and D7 and D8 blastocyst rates) and mitochondrial activity and intracellular ROS in D2 cleaved embryos were assessed in all experiments. In the experiments with an effective TR enhancement of development competence, additional parameters were assessed to evaluate blastocyst quality, namely blastocyst cell numbers of the trophectoderm (TE) and inner cell mass (ICM), apoptotic cell indices and blastocyst gene expression related to OS, mitochondrial function, lipid metabolism and apoptosis. The number of oocytes or embryos evaluated and the number of replicates performed for each outcome parameter in Experiments 1–4 are indicated below (see Outcome Parameter Assessment).

In vitro *maturation*

IVM of bovine oocytes was performed as described previously (Van Hoeck *et al*. 2011). Briefly, immature Grade I COCs were retrieved from abattoir-derived ovaries and selected for serum-free IVM. COCs were matured for 24 h in groups of 45 ± 10 COCs (mean ± s.e.m.) in a volume of 500 µL per well in a humidified atmosphere containing 5% CO_2 at 38.5°C.

The serum-free maturation medium used in the present study consisted of TCM199 (Life Technologies) containing 50 µg mL^{-1} gentamycin (Life Technologies), 0.1 mM cysteamine, 0.75% v/w fatty acid-free BSA and 20 ng mL^{-1} epidermal growth factor, and was supplemented with or without solvent, PA and/or TR according to the experimental design.

In vitro *embryo production*

Mature COCs were fertilised for 22 h using frozen semen of a bull with proven *in vitro* fertility, as described previously (Leroy *et al*. 2005). Presumptive zygotes were transferred to HEPES-buffered Tyrode's balanced salt solution (HEPES-Tyrode's albumin–lactate–pyruvate (TALP)) and vortexed for 3.5 min to remove cumulus cells. Finally, zygotes were cultured in groups of 25 ± 4 (mean ±

s.e.m.) in 75 µL culture medium. Zygotes were incubated in a 96-well dish (in an atmosphere of 90% N_2, 5% CO_2 and 5% O_2, at 38.5°C and maximum humidity) until D2 or D8 postinsemination (p.i.) depending on the outcome parameter being assessed.

The serum-free culture medium used in the present study consisted of modified synthetic oviductal fluid (mSOF) with 2% w/v fatty acid-free BSA supplemented with or without solvent, PA and/or TR.

Outcome parameter assessment

Embryo developmental competence

Total cleavage rates and the proportion of embryos with ≥4 cells per total cleaved embryo were recorded at 48 h p.i. On D7 and D8 p.i., blastocyst rates were evaluated and are presented as the number of blastocysts per total number of oocytes and per total cleaved embryos (801 COCs in Experiment 1, 1294 COCs in Experiment 2, 861 COCs in Experiment 3 and 760 COCs in Experiment 4; six replicates each). The proportion of embryos in each developmental stage (i.e. the number of young, normal, expanded and hatching or hatched blastocysts (YB, NB, EB and HB respectively) as a proportion of the total number of blastocysts) on D8 p.i. in each treatment group in each experiment was also calculated.

Mitochondrial activity and intracellular ROS in D2 cleaved embryos

As in previous research performed in our laboratory (Marei *et al*. 2019*b*) and to be able to build on existing research, MMP and ROS were determined in D2 cleaved embryos (≥2-cell stage, 48 h p.i.). A combined fluorescence staining technique with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1; Invitrogen) and CellROX Deep Red Reagent (ThermoFisher) was used, as described previously (Komatsu *et al*. 2014; Marei *et al*. 2019*b*) to estimate MMP and ROS. Briefly, cleaved embryos were selected and transferred to HEPES-Tyrode's albumin–lactate–pyruvate (TALP). Cleaved embryos were then immediately stained for 30 min in wash-TALP containing 5 µg mL^{-1} JC1 and 2.5 µg mL^{-1} CellROX Deep Red (from 1000× stock solutions in dimethylsulfoxide) under 5% CO_2 at 38.5°C. The embryos were then washed and kept in wash-TALP droplets under oil in a glass-bottomed 35-mm dish. Stained embryos were immediately examined under a Leica SP8 confocal microscope enclosed in a humid chamber (37°C) equipped with white laser source (Leica WLL) at excitation and emission wavelengths of 488 and 525 nm respectively (to detect JC-1 monomers or less active mitochondria; green), 561 and 590 nm respectively (to detect JC1-aggregates or active mitochondria; yellow) and 644 and 665 nm respectively (to detect CellROX or OS; red). One optical section was examined for each embryo at the level where the different embryo cells were visible. The grey scale intensity in each channel was measured using Leica Application Suite X software. MMP was calculated as the ratio of the grey scale intensity at 590 nm to that at 590 + 525 nm (i.e. J-aggregates/(J-monomers + aggregates) or active mitochondria as a percentage total of mitochondrial mass). ROS were estimated based on the

grey scale intensity at 665 nm, with results expressed as absolute ROS and as the ratio of ROS to total of active mitochondria. In all, 142 embryos in Experiment 1, 111 embryos in Experiment 2, 99 embryos in Experiment 3 and 101 embryos in Experiment 4 were evaluated across three replicates.

Blastocyst cell numbers of the TE and ICM and apoptotic cell indices

At D8, fixed EB and HB were stained immunohistochemically with anti-CDX2 (TE) and anti-cleaved caspase-3 (apoptotic cells) antibodies and 4′,6′-diamidino-2-phenylindole (DAPI; total cells), as described previously (De Bie *et al.* 2017). The number of ICM, TE and apoptotic cells was counted using ImageJ software 1.41 (National Institutes of Health). The ICM/TE cell ratio and the apoptotic cell index (i.e. number of apoptotic cells as a percentage of total cells) were calculated. Data are expressed as the overall mean ± s.e.m. or classified based on the blastocyst stage (35 EB and 19 HB in Experiment 4; three replicates).

Blastocyst RNA extraction, reverse transcription and quantification of gene expression by quantitative polymerase chain reaction

D8 blastocysts were washed and transferred to a 1.5-mL vial in minimal volume of phosphate-buffered saline (PBS) and immediately snap frozen in liquid nitrogen in pools of ≥10 blastocysts (including all blastocysts produced per treatment per replicate). Samples were stored at –80°C (seven YB, 11 NB, 18 EB and eight HB in Experiment 4; three replicates).

Total RNA was extracted from blastocysts using the PicoPure RNA isolation kit (Thermo Fisher Scientific) following the manufacturer's instructions. Extracted RNA was treated with DNase (Qiagen). The concentration and purity of the isolated RNA samples were determined using a BioAnalyser (Agilent). Total RNA (50 ng) from each sample was reverse transcribed using a Sensiscript RT kit (Qiagen). A reverse transcription negative control sample (no reverse transcriptase) was included.

Gene transcripts were quantified by real-time quantitative polymerase chain reaction (qPCR) using SYBR Green (SsoAdvanced Universal SYBR Green Supermix; Bio-Rad). Quantification was normalised using the geometric mean of two housekeeping genes, namely H2A histone family, member Z (*H2AFZ*) and tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*; Robert *et al.* 2002; Goossens *et al.* 2005), calculated using geNorm software (https://genorm.cmgg.be). The comparative quantification cycle (Cq) method (i.e. $2^{-\Delta\Delta Cq}$) was used to quantify the relative expression of each gene, as described by Livak and Schmittgen (2001). Fold changes in the expression of all studied genes were calculated using the control as a reference (= 1 fold-change) and are expressed as the mean ± s.e.m.

The abundance of transcripts of genes involved in redox regulation (i.e. catalase (*CAT*), glutathione peroxidase 1 (*GPX1*), superoxide dismutase 1 and 2 (*SOD1*, *SOD2*), peroxiredoxin 1 and 3 (*PRDX1*, *PRDX3*) and nuclear factor, erythroid 2-like 2 (*NFE2L2*)), mitochondrial function (i.e. transcription

factor A, mitochondrial (*TFAM*) and heat shock protein family D member 1 (*HSPD1*)), lipid metabolism (i.e. peroxisome proliferator-activated receptor gamma (*PPARγ*)) and apoptosis (i.e. BCL2 associated X (*BAX*)) was analysed. NCBI reference sequences of *Bos taurus* were used for primer design using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were intron flanking. Primer details are provided in Table 1.

Statistical analyses

All statistical procedures were performed in IBM SPSS Statistics 25 for Windows. Categorical data of developmental competence were compared among the three treatment groups using a binary logistic regression model. Numerical data of cumulus cell expansion scores, MMP, OS, embryo cell counts, apoptotic cell indices and relative transcript abundance (ΔCq) were compared among all treatments using a linear mixed-model analysis of variance (ANOVA). Prior to ANOVA, data were analysed for normal distribution and homogeneity of variances by performing the Kolmogorov–Smirnov and Levene's test respectively. Log transformation was applied (MMP, OS, embryo cell counts, apoptotic cell indices and ΔCq) to correct for inhomogeneity of variances when necessary. If Levene's test failed after log transformation, a non-parametric Kruskal–Wallis followed by a Mann–Whitney post hoc test was performed for each comparison. A Bonferroni post hoc test was performed to correct for multiple comparisons. Replicate (random factor), treatment (fixed factor) and the interaction of both factors were taken into account. When the interaction term was not significant, it was omitted from the final model. Statistical significance was set at two-tailed $P < 0.05$. Differences with $P < 0.1$ and >0.05 are described as 'tendencies'.

Results

To facilitate interpretation, the term 'exposure' is used if the effect of PA is tested and the term 'treatment' is used if the effects of TR effects were tested in combination with PA, regardless of whether TR could actually 'treat' any effect.

Effects of TR treatment on embryo developmental competence (Experiments 1–4)

Neutralising approach (Experiments 1 and 2)

Results for Experiments 1 and 2 are presented in Tables 2 and 3. Exposure of oocytes or embryos to PA (PA-SC or SC-PA) tended to reduce or significantly reduced cleavage rates respectively compared with controls (SC-SC). Embryo development into 4-cell embryos and D7 and D8 blastocyst rates among total oocytes were significantly reduced in the PA-SC and SC-PA treatments compared with controls. TR treatment simultaneously with the PA insult during IVM or IVC (PATR-SC or SC-PATR) could not alleviate the reduction in cleavage rates and blastocyst development induced by PA exposure (PA-SC or SC-PA). The proportion of different embryo developmental stages at D8 was not significantly different among treatments (Fig. 1).

Reparative approach (Experiment 3)

Results for Experiment 3 are presented in Table 4. Exposure of oocytes to PA (PA-SC) significantly hampered oocyte and embryo developmental competence compared with controls (SC-SC). TR treatment during IVC could not significantly increase D7 and D8 blastocyst rates of PA-exposed oocytes (PA-TR) compared with PA-SC blastocysts. The proportion of blastocysts that developed into YB by D8 was significantly higher in the PA-TR than PA-SC group (Fig. 1).

Protective approach (Experiment 4)

Results for Experiment 4 are presented in Table 5. Similar to Experiment 2, exposure of embryos to PA during culture (SC-PA) significantly hampered oocyte and embryo developmental competence compared with controls (SC-SC). Compared with PA-exposed embryos (SC-PA), the addition of TR during IVM before the PA insult (TR-PA) tended to increase cleavage rates and D8 blastocysts as a proportion of total number of cleaved zygotes. Moreover, treatment of oocytes with TR before the PA insult during embryo culture (TR-PA) significantly increased the proportion of embryos that developed into 4-cell embryos and significantly increased D8 blastocyst rates as a proportion of total oocytes to levels significantly higher than the SC-PA treatment, but significantly lower than the SC-SC treatment. The proportion of different embryo stages at D8 was not significantly different among treatments (Fig. 1).

Effects of TR treatment on cleaved D2 embryo mitochondrial activity and ROS (Experiments 1–4)

Effects on embryo mitochondrial activity (Experiments 1–4)

PA exposure (PA-SC and SC-PA) significantly increased MMP in cleaved embryos at D2 compared with controls (SC-SC) (Fig. *2*). Regardless of the moment of PA exposure, TR treatment during IVM (TR-PA and PATR-SC) increased MMP even more compared with SC-PA and PA-SC. TR treatment during IVC did not alter the increased MMP levels in D2 embryos from PA-exposed oocytes and embryos compared with SC-PA and PA-SC.

Effects on embryo ROS (Experiments 1–4)

D2 embryo ROS was not altered when oocytes were exposed to PA during IVM (PA-SC), but embryo ROS tended to increase when embryos were exposed to PA during IVC (SC-PA vs SC-SC; $P < 0.1$) (Fig. *3*). TR treatment of these SC-PA embryos during IVM (TR-PA) and IVC (SC-PATR) significantly reversed embryo ROS to levels similar to those in the controls (SC-SC).

Effects on embryo ROS/active mitochondria (Experiments 1–4)

When ROS levels were expressed as a proportion of active mitochondria, TR treatment before the PA insult (TR-PA) generated D2 embryos that had significantly lower ROS/active mitochondria ratios than SC-PA treated embryos (Fig. *4)*. No significant differences in ROS/active mitochondria ratios were found in the other approaches.

Effects of TR treatment on blastocyst cell number of the TE and ICM and apoptotic cell indices (protective approach, Experiment 4)

Because preloading of oocytes with TR during IVM enhanced the developmental competence of metabolically stressed embryos in Experiment 4, further blastocyst quality parameters were evaluated. The results of these evaluations are presented in Table 6. No significant differences in blastocyst quality were detected when embryos were exposed to PA (SC-PA), or when TR was added during oocyte maturation (TR-PA). However, the TE/ICM ratio in SC-PA-derived EBs tended to be lower compared with controls, an effect that was alleviated when TR was added during oocyte maturation (TR-PA). In addition, TR-PA EBs tended to have a lower apoptotic cell index than SC-PA EBs.

Effects of TR treatment on blastocyst gene expression of selected genes (protective approach, Experiment 4)

Exposure of embryos to PA (SC-PA) significantly increased the abundance of *TFAM* mRNA in D8 blastocysts compared with controls (SC-SC), which could be decreased by TR treatment during oocyte maturation before the PA insult (TR-PA). Although SC-PA did not significantly alter the mRNA transcript abundance of the other selected genes, TR treatment during oocyte maturation before the PA insult (TR-PA) significantly increased *NRF2* gene (Nuclear factor erythroid 2-related factor 2) expression compared with control (SC-SC; Fig. 5).

Discussion

We showed that PA exposure of bovine COCs and embryos reduced cleavage rates, increased mitochondrial activity in cleaved D2 embryos, lowered the proportion of good-quality ≥4-cell stage embryos and reduced blastocyst rates. Moreover, PA-exposed embryos tended to have increased ROS at the ≥4-cell stage and showed significantly increased expression of genes implicated in mitochondrial biogenesis. We hypothesised that the efficacy of AO treatment of metabolically compromised oocytes and embryos in terms of improving embryo developmental competence and quality depends on the timing of the treatment. We found that the protective approach, in which oocytes were preloaded with TR during maturation before PA exposure after fertilisation, significantly improved the development of ≥4-cell embryos and D8 blastocysts, reduced ROS in cleaved D2 embryos and resulted in blastocysts of a similar quality (based on cell counts and gene expression patterns) to untreated PA-exposed embryos.

Neutralising approach: TR treatment simultaneous with the metabolic stress insult during IVM or IVC cannot improve embryo developmental competence (Experiments 1 and 2)

Similarly to previous reports, our data demonstrated that embryo development was significantly hampered by PA exposure during IVM (Marei *et al.* 2019*a*, 2019*b*) or IVC (Jungheim *et al.* 2011). Many *in vitro* studies have investigated the efficacy of AO supplementation in improving oocyte and embryo developmental capacity. Mostly, AOs could not improve developmental competence in the absence of a stress insult. For example, Dalvit *et al.* (2005) and Rooke *et al.* (2012) showed that the

addition of vitamin E or TR during routine IVM or IVC respectively could not improve blastocyst rates. These results are in line with our findings, whereby TR during IVM or IVC did not affect developmental competence (data not shown). However, when oocytes or embryos were exposed to a stress insult, such as lipoproteins in the study of Rooke *et al*. (2012), the induced stress could be alleviated by the addition of an AO (TR) and blastocyst rates increased again. In the study of Asadi *et al*. (2012), increased oocyte apoptosis in nicotine-exposed mice could be overcome by *in vivo* vitamin E administration. Overall, it has been reported that AOs may enhance the *in vitro* development of embryos by neutralising ROS and protecting the embryos against oxidative damage (Natarajan *et al*. 2010). In an attempt to neutralise (i.e. TR treatment at the same time as the PA insult) the PA-induced stress at the oocyte or embryo level, we showed that if cellular ROS tended to increase, TR treatment was able to neutralise this increase in PA-exposed embryos (SC-PATR, Experiment 2; Fig. 3). Nevertheless, this was not associated with improved development.

Preserving the intracellular redox environment is crucial for normal cellular functions. Persistent excess to fatty acids may lead to increased fatty acid metabolism and energy production, mitochondrial dysregulation and excess production of ROS and/or fatty acid accumulation of triacylglycerols in non-adipose tissues, both of which lead to cellular dysfunction (Cortassa *et al*. 2017). In the present study, embryos resulting from TR-treated PA-exposed oocytes (PATR-SC, Experiment 1) showed further increased mitochondrial activity (Fig. 2), but normal ROS levels (Fig. 3) compared with their non-treated PA-exposed counterparts (PA-SC). As such, embryos may be better at using their intracellular PA for energy production without exhibiting cellular stress if they are simultaneously treated with AOs during oocyte maturation. Nevertheless, this does not give them a selective growth advantage, as seen in the reduced developmental rates in the present study. More research to further understand these observations is definitely needed.

Reparative approach: metabolic stress insult at the oocyte level can only be partially alleviated by TR treatment later in development (Experiment 3)

In IVF clinics, IVC media are frequently supplemented with growth- and quality-improving factors to improve the IVF success rates with oocytes of different quality. Among these factors, ascorbic and folic acid and vitamin E are often added because they seem to reduce oxidative damage in mouse embryos and improve developmental rates (Gruber and Klein 2011; Koyama *et al*. 2012). Marei *et al*. (2019*a*) showed that supplementation of embryos derived from PA-exposed oocytes with MitoQ (a mitochondrial targeted AO) during IVC alleviated increased embryo ROS, prevented mitochondrial uncoupling and completely rescued blastocyst development and quality.

In the present study we showed that metabolically compromised oocytes could only be partially rescued by AO treatment with TR during IVC (PA-TR, Experiment 3; Table 4). TR treatment during IVC slightly improved development to levels similar to control oocytes (SC-SC), but not different from metabolically compromised oocytes (PA-SC). A significantly increased proportion of YBs was

detected in TR-treated embryos originating from PA-exposed oocytes (PA-TR) compared with PA-SC (Experiment 3; Fig. 1), indicating catch-up growth of embryos that were lagging behind in development.

TR governs a key protective mechanism against emerging lipid peroxides, which are specific ROS present not only in mitochondria, but also in lipid membranes throughout the cell. TR, as a water-soluble analogue of vitamin E, has the main advantage over lipid-soluble AOs of being incorporated both in the water and lipid compartments of cells to scavenge and neutralise peroxides throughout the cell (Hamad *et al*. 2010). TR treatment during IVC (PA-TR) allowed increased embryo mitochondrial activity (Experiment 3; Fig. 2) without a concomitant increase in ROS (Experiment 3; Fig. 3) compared with controls. This may indicate increased fatty acid oxidation of the high PA depots present in the embryo without leading to OS and subsequent cell damage. However, this is also true for PA-SC embryos, in which an increased mitochondrial activity, but no increase in embryo ROS, was observed, indicating that TR may partially rescue embryo development via a different mechanism than relieving OS.

Protective approach: 'pretreatment' of oocytes with TR significantly improves embryo development under metabolic stress conditions (Experiment 4)

The only approach in which TR significantly improved the development of metabolically compromised oocytes or embryos was when oocytes were preloaded with TR during maturation, after which subsequent embryos were exposed to PA (Experiment 4; Table 6). Importantly, although developmental competence was significantly improved in the protective approach, blastocyst rates did not reach control levels.

To the best of our knowledge, the present study is the first showing that the 'pretreatment' of oocytes with AOs can generate embryos that are more resilient to a metabolic stress insult. When embryos were exposed to PA during culture, the cleaved embryos at D2 p.i. exhibited a tendency for increased ROS (Experiments 2 and 4; Fig. 3). Interestingly, TR treatment significantly reduced embryo ROS to levels similar to those in the controls, regardless of the timing of the treatment. As such, it can be assumed that the antioxidative potential of TR could both neutralise PA-induced ROS (Experiment 2; Fig. 3) and protect against increased ROS (Experiment 4; Fig. 3). However, protecting oocytes from a ROS insult later in development was the only approach that improved embryo development (Experiment 4; Table 6). In addition to a reduction in embryo ROS, 'pretreatment' of oocytes before the PA insult further increased mitochondrial activity to levels higher than in SC-PA embryos (Experiment 4; Fig. 2). Interestingly, when ROS were expressed as a proportion of active mitochondria (Fig. 4), we found that TR 'pretreatment' before the PA insult generated D2 embryos that had significantly lower ROS/active mitochondria ratios. Because no differences in the ROS/active mitochondria ratios were observed in the other approaches (neutralising and reparative), we may hypothesise that the combination of relatively low ROS levels with highly active mitochondria may

be a mechanism implicated in the protective effect of TR before the PA insult on embryo developmental competence. Preloading the oocyte with sufficient AO capacity may render the early embryo more capable of coping with the increased generation of ROS in the active mitochondria as a consequence of the oxidation of excess PA during embryo development. As such, fatty acid oxidation during embryo development can continue without increases in ROS, which may partially safeguard further embryo development. However, in addition to OS, other pathways are involved in lipotoxic effects on oocytes and embryos that could not be abrogated by preloading oocytes with AOs, such as altered metabolism, endoplasmic reticulum stress, mitochondrial dysfunction and the increased production of deleterious complex lipids (Palomer *et al*. 2018). This may explain why no complete recovery of developmental capacity was observed.

It is important to consider the duration of exposure during IVM (24 h) and IVC (7 days) to PA and/or TR. From this study, there is evidence that PA exposure during IVC has a more detrimental effect on embryo developmental competence than exposure during IVM. Accordingly, we would expect a more pronounced beneficial effect of TR treatment during the 7-day IVC window compared with the 24-h IVM window. However, the opposite was true in the present study. Comparison of oocyte maturation and embryo culture *in vivo* and *in vitro* showed that the intrinsic quality of the oocyte appears to be the main factor determining blastocyst yields (Rizos *et al*. 2002). From this perspective, we showed that 'preloading' the oocyte with AO provides them with a selective growth advantage. This can be explained by the facts that: (1) AO can prevent DNA fragmentation in the cumulus cells surrounding the oocyte; and (2) oocytes are capable of accumulating more AO when cumulus cells are present (Tao *et al*. 2004). As such, good cumulus cell quality nourishes and supports the developing oocyte and is instrumental for oocyte maturation and the acquisition of developmental competence. Moreover, the AO accumulated in the oocyte may protect the embryo against a metabolic stress insult later in development.

Blastocyst quality (Experiment 4)

Mechanistic research revealed that metabolic stress during oocyte maturation or embryo culture resulted in higher rates of apoptosis in the blastocyst and in altered expression patterns in oocytes, cumulus cells and blastocysts of genes in pathways mainly related to metabolism, mitochondrial dysfunction, endoplasmic reticulum stress and OS (Van Hoeck *et al*. 2011; Marei *et al*. 2019*b*). In the study of Rooke *et al*. (2012), TR treatment of lipoprotein-exposed bovine embryos (cf. neutralising approach, Experiment 2) could neutralise the induced stress at the embryo level. In contrast, we previously demonstrated that AOs such as selenium and transferrin (combined with insulin) during IVC of bovine embryos improved the development of metabolically compromised oocytes, but could not repair the quality of the embryo (Smits *et al*. 2016; De Bie *et al*. 2017). In the protective approach (Experiment 4), blastocyst *NRF2* and *TFAM* expression patterns were altered due to PA and/or TR. NRF2 is a transcription factor that regulates the expression of AO proteins and is thus important in the

protection against OS. Marei *et al*. (2019*b*) previously showed that NRF2-mediated OS responses are implicated in PA-exposed oocytes. An NRF2-mediated OS response has recently been shown to mediate bovine embryo survival under OS conditions (Amin *et al*. 2014), as shown in the present study. Glutathione reductase transcription was increased in mouse models with enhanced Nrf2 activation (Wu *et al*. 2011). This can be related to the significantly reduced ROS observed in D2 embryos when metabolically compromised embryos were pretreated with TR during IVM (protective approach). Similar to the findings of Van Hoeck *et al*. (2013) and Marei *et al*. (2019*a*), *TFAM*, an important gene for mitochondrial biogenesis, was significantly upregulated in PA-exposed blastocysts. This altered gene expression, indicative of mitochondrial dysfunction, normalised again when TR treatment was applied before the PA insult (TR-PA). The same neutralising effect on *TFAM* transcript abundance was seen when metabolically compromised oocytes were treated with MitoQ (Marei *et al*. 2019*a*).

In addition, the protective approach (Experiment 4) could reverse the small difference in blastocyst cell allocation observed in PA-exposed embryos and tended to reduce blastocyst apoptosis. These observations point towards improved blastocyst quality with the protective approach, indicating that the 'pretreatment' of oocytes with TR produces embryos that may be more resilient to a metabolic stress insult.

Conclusions

The results in this conceptual study emphasise the difference in the efficacy of AO treatment in relation to a metabolic stress insult during oocyte maturation or embryo culture. We showed that the enhancement of the developmental capacity of metabolically compromised bovine oocytes and embryos by TR treatment was most obvious when oocytes were pretreated with, and thus protected by, TR before the PA insult. Although developmental competence was significantly improved in the protective approach, blastocyst rates did not reach control levels. We showed that the combination of relatively low ROS levels with highly active mitochondria may be a mechanism implicated in the protective effect of TR against a metabolic insult later in development. The subsequent PA-exposed embryos from these TR-'pretreated' oocytes were of good quality, based on normalised cell allocation, normalised expression of genes related to mitochondrial function and increased expression of genes involved in redox regulation. The results from this conceptual *in vitro* study indicate that the 'pretreatment' of oocytes with TR produces embryos that may be more resilient to a metabolic stress insult.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This project was funded by the Special Research Fund, Small Project, University of Antwerp). The authors acknowledge Els Merckx, Silke Andries, Lies Jordaens, Kerlijne Moorkens and Inne Xhonneux for support and assistance with the *in vitro* production of bovine embryos. The authors also thank the abattoirs in Heist-Op-Den-Berg (Mortelmans, Belgium) and Hoogstraten (Swaegers, Belgium) for providing the ovaries.

References

<jrn>Aardema, H., Vos, P. L., Lolicato, F., Roelen, B. A., Knijn, H. M., Vaandrager, A. B., Helms, J. B., and Gadella, B. M. (2011). Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol. Reprod.* **85**, 62–69. doi:10.1095/biolreprod.110.088815</jrn>

<jrn>Amin, A., Gad, A., Salilew-Wondim, D., Prastowo, S., Held, E., Hoelker, M., Rings, F., Tholen, E., Neuhoff, C., Looft, C., Schellander, K., and Tesfaye, D. (2014). Bovine embryo survival under oxidative-stress conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway. *Mol. Reprod. Dev.* **81**, 497–513. doi:10.1002/mrd.22316</jrn>

<jrn>Asadi, E., Jahanshahi, M., and Golalipour, M. J. (2012). Effect of vitamin E on oocytes apoptosis in nicotine-treated mice. *Iran. J. Basic Med. Sci.* **15**, 880–884.</jrn>

<jrn>Conti, M., and Franciosi, F. (2018). Acquisition of oocyte competence to develop as an embryo: integrated nuclear and cytoplasmic events. *Hum. Reprod. Update* **24**, 245–266. doi:10.1093/humupd/dmx040</jrn>

<jrn>Cortassa, S., Sollott, S. J., and Aon, M. A. (2017). Mitochondrial respiration and ROS emission during β-oxidation in the heart: an experimental-computational study. *PLOS Comput. Biol.* **13**, e1005588. doi:10.1371/journal.pcbi.1005588</jrn>

<jrn>Dalvit, G., Llanes, S. P., Descalzo, A., Insani, M., Beconi, M., and Cetica, P. (2005). Effect of alpha-tocopherol and ascorbic acid on bovine oocyte *in vitro* maturation. *Reprod. Domest. Anim.* **40**, 93–97. doi:10.1111/j.1439-0531.2004.00522.x</jrn>

<jrn>De Bie, J., Langbeen, A., Verlaet, A. A. J., Florizoone, F., Immig, I., Hermans, N., Fransen, E., Bols, P. E. J., and Leroy, J. L. M. R. (2016). The effect of a negative energy balance status on β-carotene availability in serum and follicular fluid of nonlactating dairy cows. *J. Dairy Sci.* **99**, 5808–5819. doi:10.3168/jds.2016-10870</jrn>

<jrn>De Bie, J., Marei, W. F., Maillo, V., Jordaens, L., Gutierrez-Adan, A., Bols, P. E., and Leroy, J. L. (2017). Differential effects of high and low glucose concentrations during lipolysis-like conditions on bovine *in vitro* oocyte quality, metabolism and subsequent embryo development. *Reprod. Fertil. Dev.* **29**, 2284–2300. doi:10.1071/RD16474</jrn>

<jrn>Desmet, K. L. J., Van Hoeck, V., Gagné, D., Fournier, E., Thakur, A., O'Doherty, A. M., Walsh, C. P., Sirard, M. A., Bols, P. E. J., and Leroy, J. L. M. R. (2016). Exposure of bovine oocytes and embryos to elevated non-esterified fatty acid concentrations: integration of epigenetic and transcriptomic signatures in resultant blastocysts. *BMC Genomics* **17**, 1004. doi:10.1186/s12864-016-3366-y</jrn>

<jrn>Dobbelaar, P., Bouwstra, R. J., Goselink, R. M., Jorritsma, R., van den Borne, J. J., and Jansen, E. H. (2010). Effects of vitamin E supplementation on and the association of body condition score with changes in peroxidative biomarkers and antioxidants around calving in dairy heifers. *J. Dairy Sci.* **93**, 3103–3113. doi:10.3168/jds.2009-2677</jrn>

<jrn>Forrest, V. J., Kang, Y.-H., McClain, D. E., Robinson, D. H., and Ramakrishnan, N. (1994). Oxidative stress-induced apoptosis prevented by trolox. *Free Radic. Biol. Med.* **16**, 675–684. doi:10.1016/0891-5849(94)90182-1</jrn>

<jrn>Goossens, K., Van Poucke, M., Van Soom, A., Vandesompele, J., Van Zeveren, A., and Peelman, L. J. (2005). Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. *BMC Dev. Biol.* **5**, 27. doi:10.1186/1471-213X-5-27</jrn>

<jrn>Gruber, I., and Klein, M. (2011). Embryo culture media for human IVF: which possibilities exist? *J. Turk. Ger. Gynecol. Assoc.* **12**, 110–117. doi:10.5152/jtgga.2011.25</jrn>

<jrn>Hamad, I., Arda, N., Pekmez, M., Karaer, S., and Temizkan, G. (2010). Intracellular scavenging activity of Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) in the fission yeast, Schizosaccharomyces pombe. *J. Nat. Sci. Biol. Med.* **1**, 16–21. doi:10.4103/0976-9668.71667</jrn>

<jrn>Han, L., Wang, H., Li, L., Li, X., Ge, J., Reiter, R. J., and Wang, Q. (2017). Melatonin protects against maternal obesity-associated oxidative stress and meiotic defects in oocytes via the SIRT3–SOD2-dependent pathway. *J. Pineal Res.* **63**, e12431. doi:10.1111/jpi.12431</jrn>

<jrn>Igosheva, N., Abramov, A. Y., Poston, L., Eckert, J. J., Fleming, T. P., Duchen, M. R., and McConnell, J. (2010). Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PLoS One* **5**, e10074. doi:10.1371/journal.pone.0010074</jrn>

<jrn>Jordaens, L., Van Hoeck, V., De Bie, J., Berth, M., Marei, W. F. A., Desmet, K. L. J., Bols, P. E. J., and Leroy, J. (2017). Non-esterified fatty acids in early luteal bovine oviduct fluid mirror plasma concentrations: an *ex vivo* approach. *Reprod. Biol.* **17**, 281–284. doi:10.1016/j.repbio.2017.05.009</jrn>

<jrn>Jorritsma, R., Cesar, M. L., Hermans, J. T., Kruitwagen, C. L., Vos, P. L., and Kruip, T. A. (2004). Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes *in vitro*. *Anim. Reprod. Sci.* **81**, 225–235. doi:10.1016/j.anireprosci.2003.10.005</jrn>

<jrn>Jungheim, E. S., Louden, E. D., Chi, M. M., Frolova, A. I., Riley, J. K., and Moley, K. H. (2011). Preimplantation exposure of mouse embryos to palmitic acid results in fetal growth restriction followed by catch-up growth in the offspring. *Biol. Reprod.* **85**, 678–683. doi:10.1095/biolreprod.111.092148</jrn>

<jrn>Komatsu, K., Iwase, A., Mawatari, M., Wang, J., Yamashita, M., and Kikkawa, F. (2014). Mitochondrial membrane potential in 2-cell stage embryos correlates with the success of preimplantation development. *Reproduction* **147**, 627–638. doi:10.1530/REP-13-0288</jrn>

<jrn>Koyama, H., Ikeda, S., Sugimoto, M., and Kume, S. (2012). Effects of folic acid on the development and oxidative stress of mouse embryos exposed to heat stress. *Reprod. Domest. Anim.* **47**, 921–927. doi:10.1111/j.1439-0531.2012.01992.x</jrn>

<jrn>Leroy, J. L. M. R., Vanholder, T., Delanghe, J. R., Opsomer, G., Van Soom, A., Bols, P. E., Dewulf, J., and de Kruif, A. (2004). Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* **62**, 1131–1143. doi:10.1016/j.theriogenology.2003.12.017</jrn>

<jrn>Leroy, J. L. M. R., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A., Genicot, G., and Van Soom, A. (2005). Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes *in vitro*. *Reproduction* **130**, 485–495. doi:10.1530/rep.1.00735</jrn>

<jrn>Leroy, J. L. M. R., Van Hoeck, V., Clemente, M., Rizos, D., Gutierrez-Adan, A., Van Soom, A., Uytterhoeven, M., and Bols, P. E. (2010). The effect of nutritionally induced hyperlipidaemia on *in vitro* bovine embryo quality. *Hum. Reprod.* **25**, 768–778. doi:10.1093/humrep/dep420</jrn>

<jrn>Leroy, J. L. M. R., Valckx, S. D., Jordaens, L., De Bie, J., Desmet, K. L., Van Hoeck, V., Britt, J. H., Marei, W. F., and Bols, P. E. (2015). Nutrition and maternal metabolic health in relation to oocyte and embryo quality: critical views on what we learned from the dairy cow model. *Reprod. Fertil. Dev.* **27**, 693–703. doi:10.1071/RD14363</jrn>

<jrn>Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408. doi:10.1006/meth.2001.1262</jrn>

<jrn>Luddi, A., Capaldo, A., Focarelli, R., Gori, M., Morgante, G., Piomboni, P., and De Leo, V. (2016). Antioxidants reduce oxidative stress in follicular fluid of aged women undergoing IVF. *Reprod. Biol. Endocrinol.* **14**, 57. doi:10.1186/s12958-016-0184-7</jrn>

<jrn>Marei, W. F., Alvarez, M. A., Van Hoeck, V., Gutierrez-Adan, A., Bols, P. E., and Leroy, J. L. (2017*a*). Effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality depends on the type of major fatty acid in the diet. *Reprod. Fertil. Dev.* **29**, 1856–1867. doi:10.1071/RD16297</jrn>

<jrn>Marei, W. F., De Bie, J., Mohey-Elsaeed, O., Wydooghe, E., Bols, P. E. J., and Leroy, J. (2017*b*). Alpha-linolenic acid protects the developmental capacity of bovine cumulus-oocyte complexes matured under lipotoxic conditions *in vitro*. *Biol. Reprod.* **96**, 1181–1196. doi:10.1093/biolre/iox046</jrn>

<jrn>Marei, W. F., Van Den Bosch, L., Pintelon, I., Mohey, O., Bols, P. E. J., and Leroy, J. L. M. (2019*a*). Mitochondria-targeted therapy rescues development and quality of embryos derived from metabolically compromised oocytes: a bovine *in vitro* model. *Hum. Reprod.* **34**, 1984–1998. doi:10.1093/humrep/dez161</jrn>

<jrn>Marei, W. F. A., Van Raemdonck, G., Baggerman, G., Bols, P. E. J., and Leroy, J. L. M. R. (2019*b*). Proteomic changes in oocytes after *in vitro* maturation in lipotoxic conditions are different from those in cumulus cells. *Sci. Rep.* **9**, 3673. doi:10.1038/s41598-019-40122-7</jrn>

<jrn>Messier, E. M., Bahmed, K., Tuder, R. M., Chu, H. W., Bowler, R. P., and Kosmider, B. (2013). Trolox contributes to Nrf2-mediated protection of human and murine primary alveolar type II cells from injury by cigarette smoke. *Cell Death Dis.* **4**, e573. doi:10.1038/cddis.2013.96</jrn>

<jrn>Mirabi, P., Chaichi, M. J., Esmaeilzadeh, S., Ali Jorsaraei, S. G., Bijani, A., Ehsani, M., and Hashemi Karooee, S. F. (2017). The role of fatty acids on ICSI outcomes: a prospective cohort study. *Lipids Health Dis.* **16**, 18. doi:10.1186/s12944-016-0396-z</jrn>

<jrn>Natarajan, R., Shankar, M. B., and Munuswamy, D. (2010). Effect of alpha-tocopherol supplementation on *in vitro* maturation of sheep oocytes and *in vitro* development of preimplantation sheep embryos to the blastocyst stage. *J. Assist. Reprod. Genet.* **27**, 483–490. doi:10.1007/s10815-010-9430-7</jrn>

<jrn>Nonogaki, T., Noda, Y., Goto, Y., Kishi, J., and Mori, T. (1994). Developmental blockage of mouse embryos caused by fatty acids. *J. Assist. Reprod. Genet.* **11**, 482–488. doi:10.1007/BF02215713</jrn>

<jrn>O'Gorman, A., Wallace, M., Cottell, E., Gibney, M. J., McAuliffe, F. M., Wingfield, M., and Brennan, L. (2013). Metabolic profiling of human follicular fluid identifies potential biomarkers of oocyte developmental competence. *Reproduction* **146**, 389–395. doi:10.1530/REP-13-0184</jrn>

<jrn>Palomer, X., Pizarro-Delgado, J., Barroso, E., and Vazquez-Carrera, M. (2018). Palmitic and oleic acid: the yin and yang of fatty acids in type 2 diabetes mellitus. *Trends Endocrinol. Metab.* **29**, 178–190. doi:10.1016/j.tem.2017.11.009</jrn>

<jrn>Rizos, D., Ward, F., Duffy, P., Boland, M. P., and Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol. Reprod. Dev.* **61**, 234–248. doi:10.1002/mrd.1153</jrn>

<jrn>Rizos, D., Gutiérrez-Adán, A., Pérez-Garnelo, S., De La Fuente, J., Boland, M. P., and Lonergan, P. (2003). Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biol. Reprod.* **68**, 236–243. doi:10.1095/biolreprod.102.007799</jrn>

<jrn>Robert, C., McGraw, S., Massicotte, L., Pravetoni, M., Gandolfi, F., and Sirard, M. A. (2002). Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. *Biol. Reprod.* **67**, 1465–1472. doi:10.1095/biolreprod.102.006320</jrn>

<jrn>Rooke, J. A., Watt, R. G., Ashworth, C. J., and McEvoy, T. G. (2012). Inclusion of bovine lipoproteins and the vitamin E analogue, Trolox, during *in vitro* culture of bovine embryos changes both embryo and fetal development. *Reprod. Fertil. Dev.* **24**, 309–316. doi:10.1071/RD11034</jrn>

<bok>Salway, J. (1999). 'Metabolism at a Glance.' (Blackwell Science: Oxford, UK.)</bok>

<conf>Smits, A., De Bie, J., Bols, P. E. J., Marei, W. F., and Leroy, J. L. M. R. (2016). Effect of embryo culture conditions on developmental potential of bovine oocytes matured under lipotoxic conditions. In 'Proceedings of the 18th International Congress on Animal Reproduction', Tours, France. (Eds XX.) pp. PW552. (Publisher name: City.)</conf>

<jrn>Sutton-McDowall, M. L., Wu, L. L. Y., Purdey, M., Abell, A. D., Goldys, E. M., Macmillan, K. L., Thompson, J. G., and Robker, R. L. (2016). Nonesterified fatty acid-induced endoplasmic reticulum stress in cattle cumulus oocyte complexes alters cell metabolism and developmental competence1. *Biol. Reprod.* **94**, 23. doi:10.1095/biolreprod.115.131862</jrn>

630 <jrn>Tao, Y., Zhou, B., Xia, G., Wang, F., Wu, Z., and Fu, M. (2004). Exposure to L-ascorbic acid or alpha-
631 tocopherol facilitates the development of porcine denuded oocytes from metaphase I to metaphase II and
632 prevents cumulus cells from fragmentation. *Reprod. Domest. Anim.* **39**, 52–57. doi:10.1046/j.1439-
633 0531.2003.00478.x</jrn>

634 <jrn>Valckx, S. D., Arias-Alvarez, M., De Pauw, I., Fievez, V., Vlaeminck, B., Fransen, E., Bols, P. E., and
635 Leroy, J. L. (2014). Fatty acid composition of the follicular fluid of normal weight, overweight and obese
636 women undergoing assisted reproductive treatment: a descriptive cross-sectional study. *Reprod. Biol.*
637 *Endocrinol.* **12**, 13. doi:10.1186/1477-7827-12-13</jrn>

638 <jrn>Valckx, S. D., De Bie, J., Michiels, E. D., Goovaerts, I. G., Punjabi, U., Ramos-Ibeas, P., Gutierrez-Adan,
639 A., Bols, P. E., and Leroy, J. L. (2015). The effect of human follicular fluid on bovine oocyte developmental
640 competence and embryo quality. *Reprod. Biomed. Online* **30**, 203–207.
641 doi:10.1016/j.rbmo.2014.10.008</jrn>

642 <jrn>Van Hoeck, V., Sturmey, R. G., Bermejo-Alvarez, P., Rizos, D., Gutierrez-Adan, A., Leese, H. J., Bols, P.
643 E., and Leroy, J. L. (2011). Elevated non-esterified fatty acid concentrations during bovine oocyte maturation
644 compromise early embryo physiology. *PLoS One* **6**, e23183. doi:10.1371/journal.pone.0023183</jrn>

645 <conf>Van Hoeck, V., De Bie, J., Andries, S., Merckx, E., Bols, P. E. J., and Leroy, J. L. M. R. (2012).
646 Elevated concentrations of saturated NEFA during bovine in vitro embryo culture compromise pre-
647 implantation embryo development. In 'Proceedings of the 28th Scientific Meeting AETE', 11-13 September
648 2012, St-Malo, France. (AETE.) pp. 224.</conf>

649 <jrn>Van Hoeck, V., Leroy, J. L. M. R., Arias-Alvarez, M., Rizos, D., Gutierrez-Adan, A., Schnorbusch, K.,
650 Bols, P. E. J., Leese, H. J., and Sturmey, R. G. (2013). Oocyte developmental failure in response to elevated
651 non-esterified fatty acid concentrations: mechanistic insights. *Reproduction* **145**, 33–44. doi:10.1530/REP-
652 12-0174</jrn>

653 <jrn>Wang, H., Cheng, Q., Li, X., Hu, F., Han, L., Zhang, H., Li, L., Ge, J., Ying, X., Guo, X., and Wang, Q.
654 (2018). Loss of TIGAR induces oxidative stress and meiotic defects in oocytes from obese mice. *Mol. Cell.*
655 *Proteomics* **17**, 1354–1364. doi:10.1074/mcp.RA118.000620</jrn>

656 <jrn>Wu, K. C., Cui, J. Y., and Klaassen, C. D. (2011). Beneficial role of Nrf2 in regulating NADPH generation
657 and consumption. *Toxicol. Sci.* **123**, 590–600. doi:10.1093/toxsci/kfr183</jrn>

Figure captions

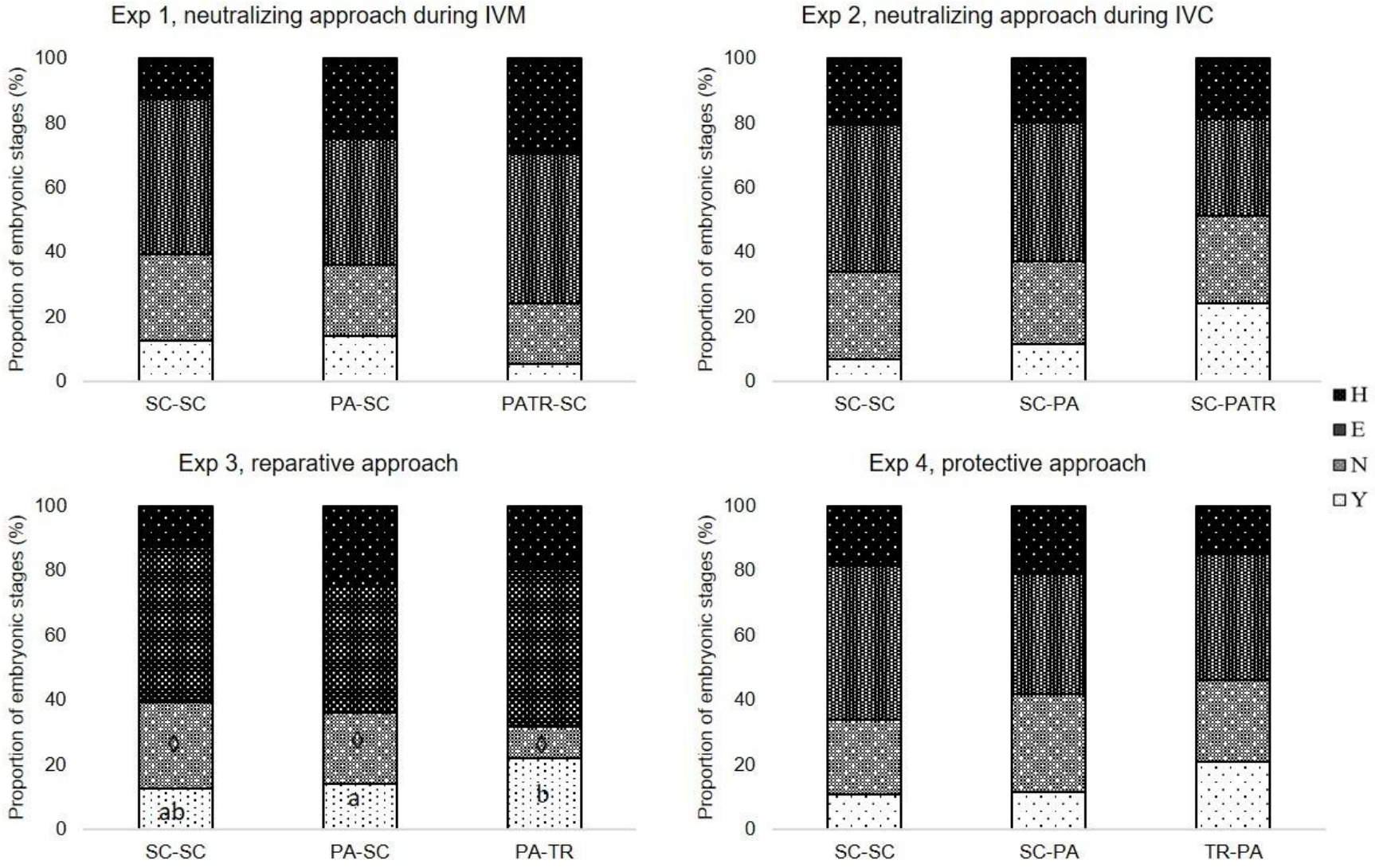
Fig. 1. Proportion of embryo stages (young (Y), normal (N), expanded (E) and hatching or hatched (H) blastocysts) at Day 8 after insemination in each treatment group and in each experiment used for gene expression analysis. Within each experiment, different letters within a blastocyst stage indicate significant differences among groups. Diamonds (◊) indicate that statistical analysis could not be performed due to a significant Treatment × Repeat interaction (i.e. the effect of the treatment was dependent on the experimental repeat).

Fig. 2. Mean (±s.e.m.) embryo mitochondrial activity as a proxy of mitochondrial membrane potential (mean grey intensity of J-aggregates/(J-monomers + aggregates) or 590/(590 + 525) nm or active mitochondria/total mitochondrial content) in each treatment group and each experiment (n = 142, 111, 99 and 101 embryos in Experiments 1, 2, 3 and 4 respectively; three repeats for each). Mitochondrial activity was evaluated 48 h after insemination in ≥2-cell embryos. Different letters above columns indicate significant differences ($P < 0.05$).

Fig. 3. Mean (±s.e.m.) embryo ROS (mean grey intensity of CellROX or 630 nm) in each treatment group and each experiment (n = 142, 111, 99 and 101 embryos in Experiments 1, 2, 3 and 4 respectively; three repeats for each). Embryo ROS levels were evaluated 48 h after insemination in ≥2-cell embryos. Different letters above columns indicate significant differences. $^{\S}P < 0.1$ between these groups.

Fig. 4. Mean (±s.e.m.) embryo ROS as a proportion of active mitochondria (mean grey intensity of CellROX/J-aggregates or 630/590 nm) in each treatment group and each experiment (n = 142, 111, 99 and 101 embryos in Experiments 1, 2, 3 and 4 respectively; three repeats for each). Embryonic ROS and mitochondrial activity were evaluated 48 h after insemination in ≥ 2-cell embryos. Different letters above columns indicate significant differences. $^{\S}P < 0.1$ between these groups.

Fig. 5. Gene expression patterns in Day 8 blastocysts in Experiment 4 (protective approach). Data are the mean ± s.e.m. fold change in gene expression relative to the control treatment (SC-SC; fold change = 1). $^{*}P <$ 0.05 compared with control (SC-SC).



682
683 **Figure 1**

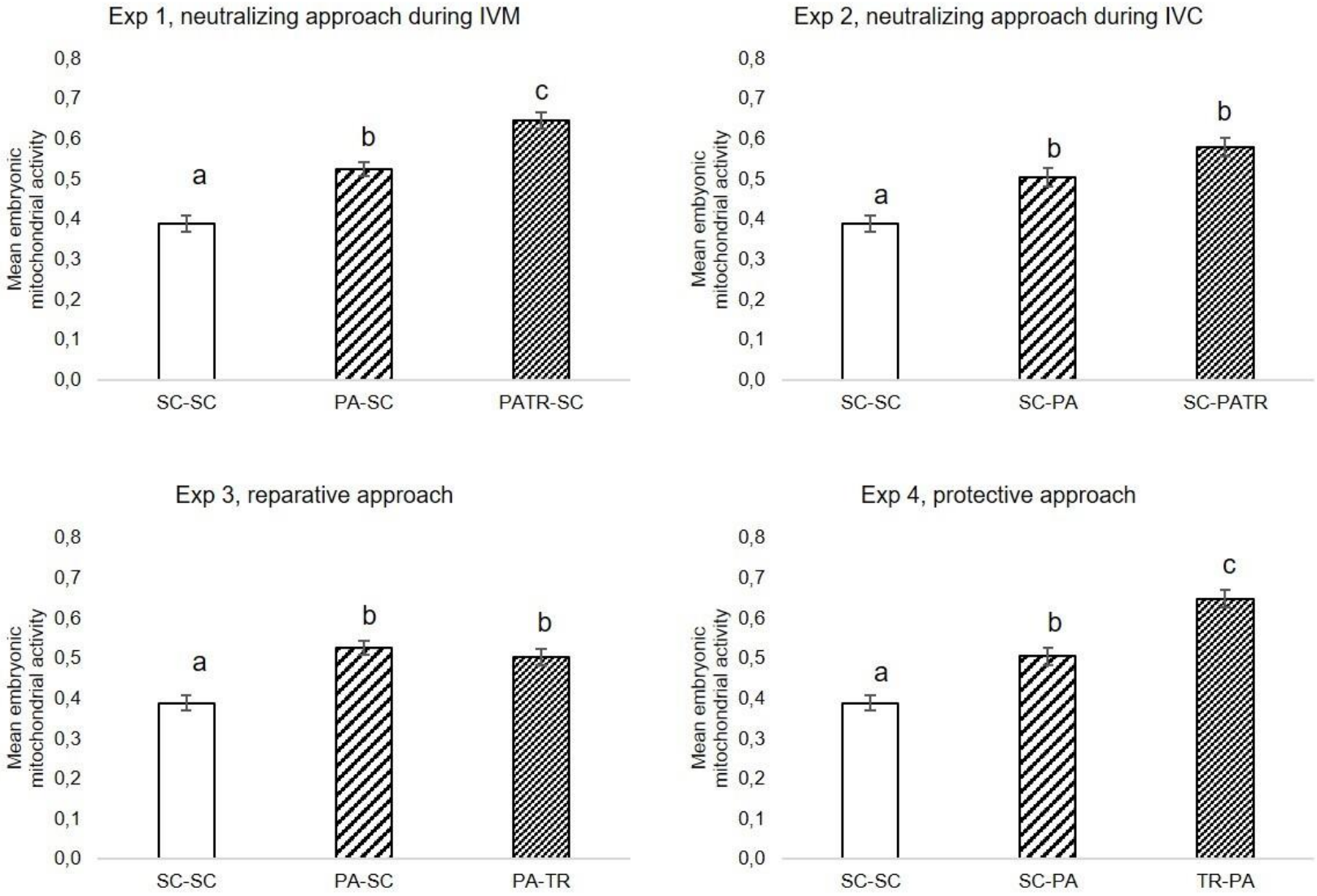
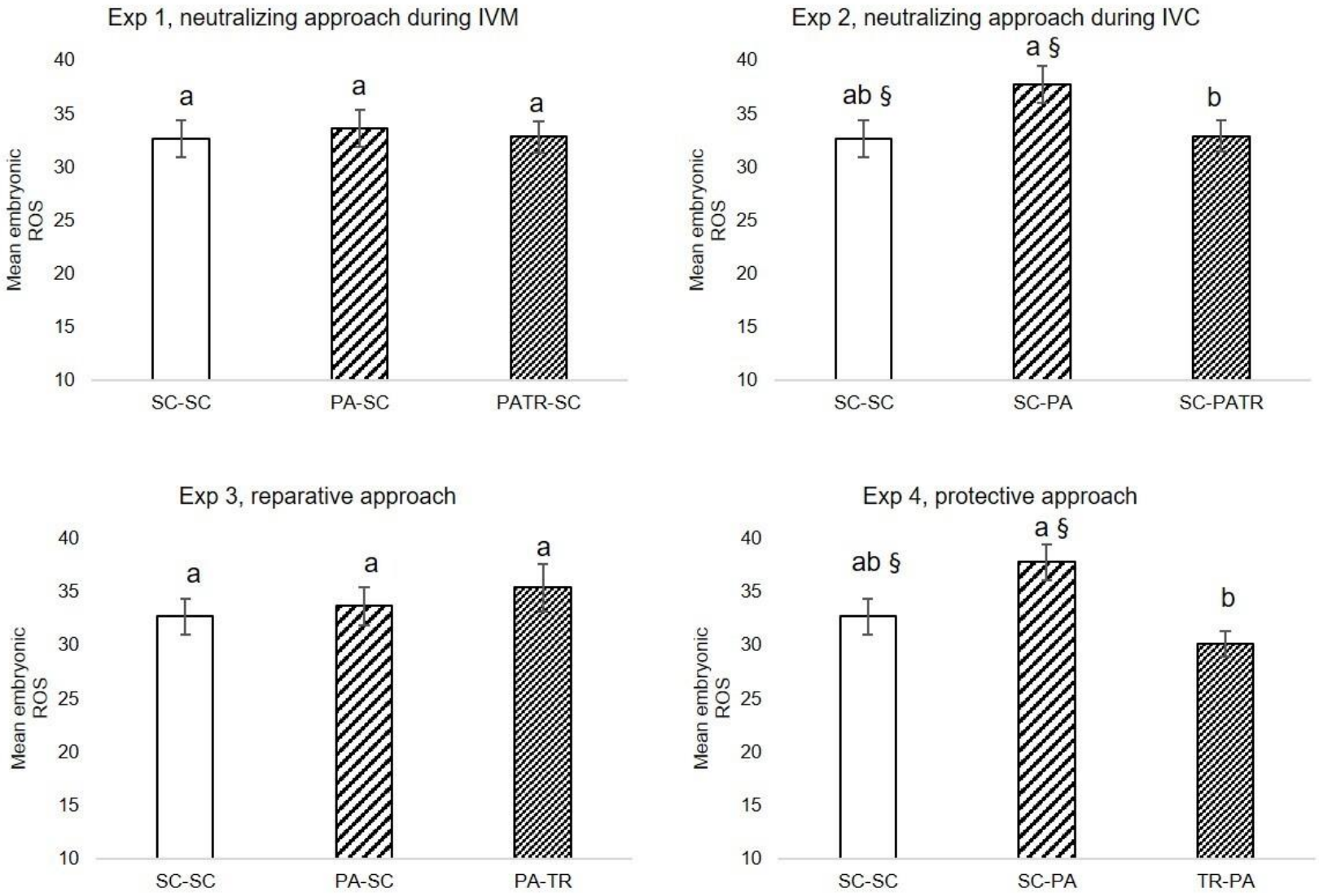

Exp 1, neutralizing approach during IVM
Mean embryonic mitochondrial activity
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
a
b
c
SC-SC
PA-SC
PATR-SC
Exp 2, neutralizing approach during IVC
Mean embyonic mitochondrial activity
a
b
b
SC-SC
SC-PA
SC-PATR
Exp 3, reparative approach
Mean embryonic mitochondrial activity
a
b
b
SC-SC
PA-SC
PA-TR
Exp 4, protective approach
Mean embryonic mitochondrial activity
a
b
c
SC-SC
SC-PA
TR-PA

Figure 2



686
687 **Figure 3**

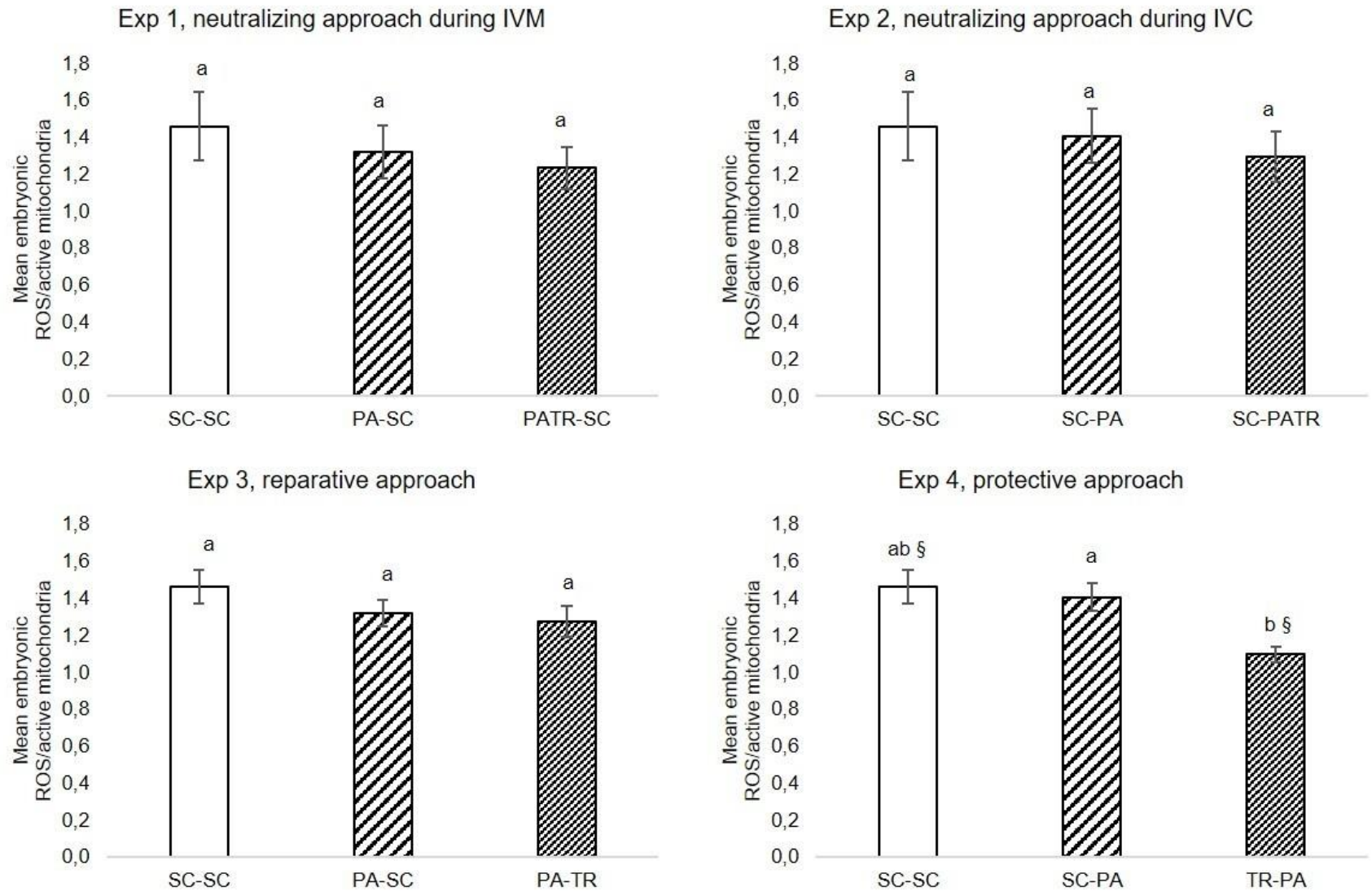
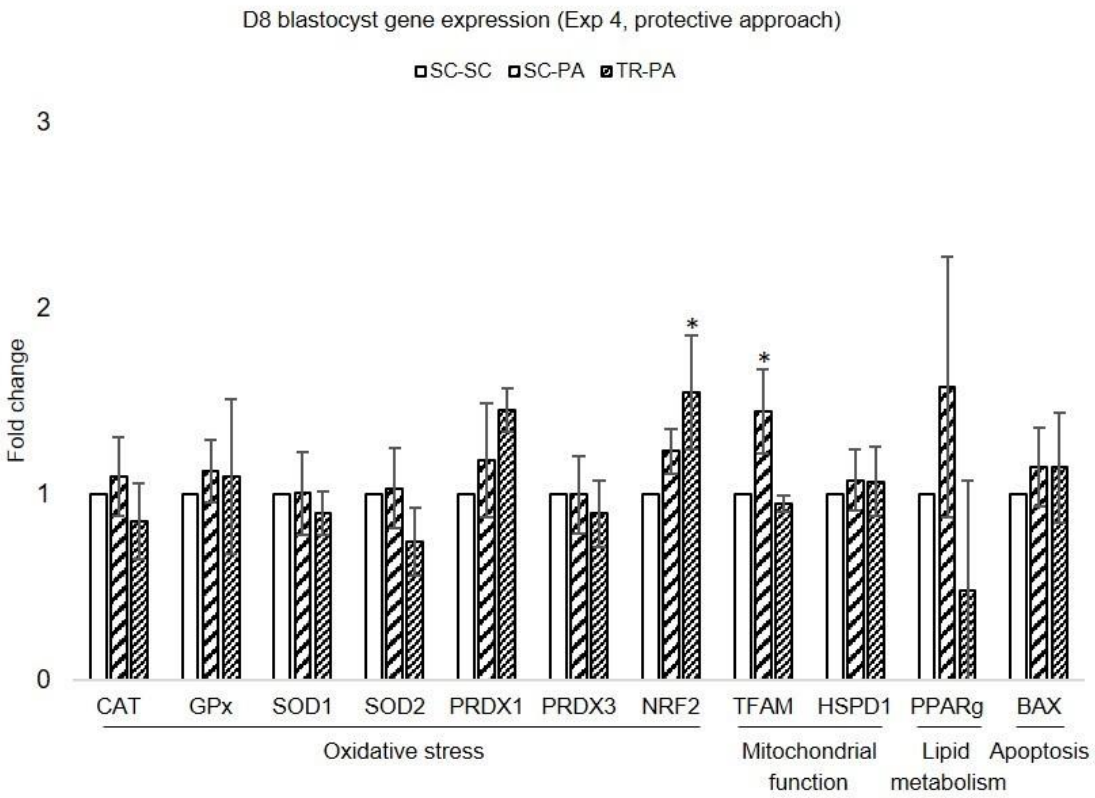

Exp 1, neutralizing approach during IVM
Mean embryonic ROS/active mitochondria
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
a
a
SC-SC
PA-SC
PATR-SC
Exp 2, neutralizing approach during IVC
Mean embryonic ROS/active mitochondria
a
a
a
SC-SC
SC-PA
SC-PATR
Exp 3, reparative approach
Mean embryonic ROS/active mitochondria
a
a
a
SC-SC
PA-SC
PA-TR
Exp 4, protective approach
Mean embryonic ROS/active mitochondria
ab §
a
b §
SC-SC
SC-PA
TR-PA

Figure 4



690
691 **Figure 5**

692 **Table 1. Primers used for real-time quantitative polymerase chain reaction**

Gene symbol	Gene name	Accession no.	Primer sequence (5′–3′)		Product length (bp)
			Forward	Reverse	
H2AFZ	H2A histone family, member Z	NM_174809.2	CGGAATTCGAAATGGCTGGC	TCTTTCGATGCATTTCCTGCC	238
YWHAZ	Tryptophan 5-monooxygenase activation protein zeta	BM446307.1	GCATCCCACAGACTATTTCC	GCATCCCACAGACTATTTCC	120
CAT	Catalase	NM_001035386.2	CTATCCTGACACTCACCGCC	GAAAGTCCGCACCTGAGTGA	268
GPX1	Glutathione peroxidase 1	NM_174076.3	AACGTAGCATCGCTCTGAGG	TCTCCTCGTTCTTGGCGTTT	145
SOD1	Superoxide dismutase 1, cytoplasmic (formerly Cu/Zn-SOD)	NM_174615.2	GGTGTTGCCATCGTGGATATT	CAGCGTTGCCAGTCTTTGT	145
SOD2	Superoxide dismutase 2, mitochondrial (formerly Mn-SOD)	NM_201527.2	TGCAAGGAACAACAGGTCTTATC	CTCAGTGTAAGGCTGACGGTT	181
PRDX1	Peroxiredoxin 1, cytoplasmic	NM_174431.1	AGCCTAGCTGACTACAAAGGAA	GTGTTGATCCATGCCAGGTG	182
PRDX3	Peroxiredoxin 3, mitochondrial	NM_174432.2	ACCGAGGAAGAATGGTGGTTT	CTCAGATGCTTGATGACTCCGT	159
NFE2L2	Nuclear factor, erythroid 2-like 2	NM_001011678.2	GTCCCAGCAGGACATGGATTTG	ATTCGCCGGTCTCTTCATCTAGT	212
TFAM	Transcription factor A, mitochondrial	NM_001034016.2	GCCAAGCTATGGAGGGAACT	AGCTTTACCTGTGATGTGCCA	293
HSPD1	Heat shock protein family D member 1	NM_001166608.1	CTACTGTACTGGCACGCTCT	CAATCTCTTCGGGGGTTGTC	159
PPARγ	Peroxisome proliferator-activated receptor gamma	NM_181024.2	GCCGAGAAGGAGAAGCTGTTA	CAAACGGTGATTTGTCTGTCGT	181
BAX	BCL2 associated X	NM_173894.1	AGCAGATCATGAAGACAGGG	TCAGACACTCGCTCAGCTTC	141

693

Table 2. Embryo developmental competence in Experiment 1 (neutralising approach during IVM; *n* = 801, six repeats)

Cleavage parameters were determined 48 h after insemination and blastocysts were scored on Days 7 and D8 after insemination. Within rows, different superscripts indicate significant differences among treatment groups ($P < 0.05$). $^{§}P < 0.1$ between these groups. SC-SC, solvent control (0.2% ethanol) during IVM and IVC; PA-SC, 150 µM PA during IVM and SC during IVC; PATR-SC, 150 µM PA and 100 µM Trolox during IVM and SC during IVC

	SC-SC	PA-SC	PATR-SC
Total no. oocytes used	259	278	264
Day 2			
No. cleaved embryos (% total oocytes)	191 (76.15)^{a§}	183 (66.57)^{ab§}	172 (65.20)^{b}
No. ≥4-cells (% total oocytes)	120 (48.20)^{a§}	94 (33.53)^{b}	98 (37.88)^{ab§}
Day 7			
No. blastocysts (% total oocytes)	45 (18.47)^{a}	24 (8.85)^{b}	27 (10.19)^{b}
No. blastocysts (% cleaved embryos)	45 (24.13)^{a}	24 (12.57)^{b}	27 (15.39)^{ab}
Day 8			
No. blastocysts (% total oocytes)	76 (31.27)^{a}	51 (18.73)^{b}	51 (19.45)^{b}
No. blastocysts (% cleaved embryos)	76 (40.27)^{a§}	51 (26.58)^{b}	51 (29.28)^{ab§}

Table 3. Embryo developmental competence in Experiment 2 (neutralising approach during IVC; *n* = 1294, six repeats)

Within rows, different superscripts indicate significant differences among treatment groups ($P < 0.05$). SC-SC, solvent control (0.2% ethanol) during IVM and IVC; SC-PA, SC during IVM and 230 µM PA during IVC; SC-PATR, SC during IVM and 230 µM PA and 100 µM Trolox during IVC

	SC-SC	SC-PA	SC-PATR
Total no. oocytes used	295	498	501
Day 2			
No. cleaved embryos (% total oocytes)	241 (80.70)^{a}	318 (64.07)^{b}	329 (66.10)^{b}
No. ≥4-cells (% total oocytes)	151 (53.68)^{a}	182 (36.95)^{b}	179 (37.05)^{b}
Day 7			
No. blastocysts (% total oocytes)	53 (21.19)^{a}	46 (10.04)^{b}	44 (9.57)^{b}
No. blastocysts (% cleaved embryos)	53 (26.29)^{a}	46 (15.79)^{b}	44 (14.51)^{b}
Day 8			
No. blastocysts (% total oocytes)	74 (25.72)^{a}	77 (14.67)^{b}	87 (18.28)^{b}
No. blastocysts (% cleaved embryos)	74 (32.05)	77 (23.20)	87 (27.35)

Table 4. Embryo developmental competence in Experiment 3 (reparative approach; *n* = 861, six repeats)

Within rows, different superscripts indicate significant differences among treatment groups ($P < 0.05$). $^{\S}P < 0.1$ between these groups. SC-SC, solvent control (0.2% ethanol) during IVM and IVC; PA-SC, 150 µM PA during IVM and SC during IVC; PA-TR, 150 µM PA during IVM and 100 µM Trolox during IVC

	SC-SC	PA-SC	PA-TR
Total no. oocytes used	289	289	283
Day 2			
No. cleaved embryos (% total oocytes)	212 (73.65)§	187 (64.88)§	195 (69.17)
No. ≥4-cells (% total oocytes)	143 (49.37)^{a}	102 (34.70)^{b}	116 (40.85)^{ab}
Day 7			
No. blastocysts (% total oocytes)	48 (16.47)^{a}	28 (9.71)^{b}	31 (11.06)^{ab}
No. blastocysts (% cleaved embryos)	48 (22.62)	28 (14.14)	31 (15.72)
Day 8			
No. blastocysts (% total oocytes)	83 (28.60)^{a§}	53 (18.27)^{b}	59 (21.02)^{ab§}
No. blastocysts (% cleaved embryos)	83 (38.58)§	53 (26.82)§	59 (30.17)

Table 5. Embryo developmental competence in Experiment 4 (protective approach; *n* = 760, six repeats)

Within rows, different superscripts indicate significant differences among treatment groups ($P < 0.05$). $^{\S}P < 0.1$ between these groups. SC-SC, solvent control (0.2% ethanol) during IVM and IVC; SC-PA, SC during IVM and 230 µM PA during IVC; TR-PA, 100 µM Trolox during IVM and 230 µM PA during IVC

	SC-SC	SC-PA	TR-PA
Total no. oocytes used	245	254	261
Day 2			
No. cleaved embryos (% total oocytes)	190 (79.18)^{a}	149 (58.75)^{b§}	177 (68.07)^{b§}
No. ≥4-cells (% total oocytes)	124 (52.13)^{a}	59 (23.27)^{c}	92 (35.30)^{b}
Day 7			
No. blastocysts (% total oocytes)	42 (18.12)^{a}	10 (3.71)^{b}	19 (7.40)^{b}
No. blastocysts (% cleaved embryos)	42 (22.40)^{a}	10 (5.99)^{b}	19 (10.70)^{b}
Day 8			
No. blastocysts (% total oocytes)	74 (31.63)^{a}	23 (8.78)^{c}	44 (16.60)^{b}
No. blastocysts (% cleaved embryos)	74 (39.23)^{a}	23 (13.98)^{b§}	44 (23.70)^{b§}

Table 6. Quality of Day 8 expanded and hatched blastocysts (EB and HB respectively) in Experiment 4 (protective approach; *n* = 54, three repeats)

Data are presented as the mean ± s.e.m., with the number of blastocysts evaluated in parentheses. The total number of cells per blastocyst and the ratio of trophectoderm cells (TE) to inner cell mass (ICM) cells are presented. The apoptotic cell index is the number of apoptotic cells as a percentage of total cells. Within rows, values with the same signs tend to differ ($P < 0.01$). SC-SC, solvent control (0.2% ethanol) during IVM and IVC; SC-PA, SC during IVM and 230 μM PA during IVC; TR-PA, 100 μM

		SC-SC	SC-PA	TR-PA
All	Overall total cell count	162.1 ± 7.8 (21)	179.2 ± 10.9[§] (14)	149.3 ± 7.2[§] (19)
	Overall TE/ICM ratio[A]	1.8 ± 0.1 (21)	1.6 ± 0.3 (7)	2.1 ± 0.1 (4)
	Overall apoptotic cell index (%)	3.6 ± 0.9 (21)	3.7 ± 0.6 (14)	2.4 ± 0.5 (19)
EB	Total cell count	147.1 ± 7.6 (14)	145.7 ± 3.5 (7)	135.2 ± 5.5 (14)
	TE/ICM ratio[A]	1.8 ± 0.1[§] (14)	1.1 ± 0.3[§‡] (3)	2.1 ± 0.1[‡] (4)
	Apoptotic cell index (%)	3.1 ± 0.6 (14)	4.4 ± 1.0[§] (7)	1.9 ± 0.7[§] (14)
HB	Total cell count	192.3 ± 11.4 (7)	212.7 ± 11.3 (7)	188.6 ± 9.2 (5)
	TE/ICM ratio[A]	1.7 ± 0.1 (7)	2.0 ± 0.4 (4)	– (0)
	Apoptotic cell index (%)	4.7 ± 2.5 (7)	2.9 ± 0.5 (7)	3.6 ± 0.6 (5)

[A]In some blastocysts, TE and ICM cells could not be counted and so the number of blastocysts evaluated may differ from the total number.