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# Effect of ambient light exposure of media and embryos on development and quality of porcine parthenogenetically activated embryos

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## Summary

Light exposure is a common stress factor during *in vitro* handling of oocytes and embryos that originates from both microscope and ambient light. In the current study, the effect of two types of ambient light (daylight and laboratory light) on porcine parthenogenetically activated (PA) embryos was tested in two experiments: (1) ambient light on medium subsequently used for embryo *in vitro* development; and (2) ambient light exposure on activated oocytes before *in vitro* development. The results from Experiment 1 showed that exposure of culture medium to both types of ambient light decreased the percentage of blastocysts that showed good morphology, only after 24 h exposure. The results from Experiment 2 revealed a reduction in both blastocyst formation and quality when activated oocytes were exposed to both types of ambient light. This effect was seen after only 1 h exposure and increased with time. In conclusion, exposure to ambient light can be harmful to embryo development, both when medium is exposed for a long period of time and, to a greater extent, when the embryo itself is exposed for >1 h. In practice, it is therefore recommended to protect both culture medium and porcine embryos against ambient light during *in vitro* handling in the laboratory.

Keywords: Apoptosis, Medium stability, Parthenote embryos, Pig

## Introduction

Oocytes and embryos can develop under a wide range of *in vitro* conditions, but their viability can decrease during manipulation and culture (Blockeel *et al.*, 2009; Heo *et al.*, 2010). One factor in this decrease is exposure to visible light during *in vitro* handling, as this step can compromise embryo quality and thus implantation potential (Nakayama *et al.*, 1994; Takahashi *et al.*, 1999; Takenaka *et al.*, 2007; Korhonen *et al.*, 2009). Damage to oocytes and zygotes caused by light can be seen at various different levels, such as DNA damage (Takahashi *et al.*, 1999), mitochondria degeneration (Gil *et al.*, 2012) and formation of reactive oxygen species in the cytoplasm (Oh *et al.*, 2007). Such damage can lead to apoptosis (Kulms & Schwarz 2002; Oh *et al.*, 2007; Takenaka *et al.*, 2007) of the embryonic cells and will reduce subsequent implantation potential and post-gestation development (Hnida *et al.*, 2004; Sela *et al.*, 2012).

Oocytes and embryos are exposed to three different sources of visible light during routine *in vitro* manipulations: the first source is by direct exposure to light from the microscope; the other two sources are types of ambient light, which come either from ceiling lamps or from daylight directly through the window. Ottosen *et al.* (2007) reported that 95% of the total light to which embryos were exposed during *in vitro* fertilization was from microscopes, such that the contribution from ambient light was not significant, therefore the use of dark laboratories was not justified (Ottosen *et al.*, 2007). Microscope light has been shown to decrease development rates and impair quality of human embryos only if the embryos were being observed frequently (once per day; Zhang *et al.*, 2010). Other studies have also shown that exposure to extra ambient light can affect embryonic development and quality, but in these cases the levels of sensitivity of the oocytes and embryos were fairly different between species. After extra visible light

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exposure, no significant decrease was observed in embryonic quality in mice (Takenaka *et al.*, 2007), or in *in vivo* development in rabbit (Bedford & Dobrenis, 1989); bovine embryo morphology was similar when oocytes were handled using unfiltered compared with green-filtered visible light, but the mRNA level of heat shock protein70 (Hsp70) in embryonic cells was decreased (Korhonen *et al.*, 2009). In contrast, hamster zygotes were severely affected by visible light from common fluorescent ceiling lights (Nakayama *et al.*, 1994; Takenaka *et al.*, 2007). The tolerance levels of porcine oocytes and embryos to visible light has not been reported.

Light exposure may also affect the stability of the medium used for embryo *in vitro* development, although the quality of mouse embryos exposed to light was seen to improve after addition of antioxidants to the medium (Moshkdanian *et al.*, 2011). However, no direct experimental data has shown the outcome on embryonic development of exposure of the culture medium to light.

We deduce therefore that ambient light exposure may still be a possible concern in routine procedures with embryos and culture medium, because these will be exposed to light at several steps even with the most optimized and careful *in vitro* handling. Illustration of such eventual effects would be optimized by choice of a species that is known to be particularly sensitive, such as the pig (Liu *et al.*, 2003), and in which no such systematic studies have been made. In the present study, the effects of two types of ambient light (daylight and laboratory light) on porcine parthenogenetically activated (PA) embryos were tested in two experiments: (1) ambient light on medium used subsequently for embryo *in vitro* development; and (2) ambient light exposure on activated oocytes before *in vitro* development.

## Materials and methods

All chemicals were purchased from Sigma–Aldrich Corp. (St. Louis, Missouri, USA) unless otherwise indicated.

### Production of porcine PA embryos

Cumulus–oocyte complexes (COCs) were aspirated from 2–6 mm follicles in slaughterhouse-derived sow ovaries and matured as described earlier by Li *et al.* (2013). Briefly, COCs with at least two layers of compact cumulus cells were selected and cultured for 42–44 h in 4-well dishes (Nunc, Roskilde, Denmark). Groups of 50–60 COCs were matured in 400 μl bicarbonate-buffered TCM-199 supplemented with 10% (v/v) cattle serum (CS; Danish Veterinary Institute, Frederiksberg, Denmark), 10% (v/v) sow follicular fluid, 10 IU/ml pregnant mare serum gonadotrophin and 5 IU/ml human chorionic gonadotrophin (Suigonan Vet, Boxmeer, Holland), covered with 400 μl mineral oil and incubated at 38.5°C in 5% $CO_2$ in air with 100% humidity.

After maturation, cumulus cells were removed from COCs by pipetting for 1 min in hyaluronidase (1 mg/ml); the oocytes with even texture and smooth membranes were collected for parthenogenetic activation. Briefly, oocytes were equilibrated for 10–15 s in drops of activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.01% polyvinyl alcohol). Under a 0.12 kV/cm alternating current, oocytes were aligned to the wire of a fusion chamber (Microslide 0.5-mm fusion chamber, model 450; BTX, San Diego, California, USA) and a single direct current pulse (1.26 kV/cm, 80 μs) was applied. Groups of 100 oocytes were washed twice in drops of TCM-199 supplemented with 10% CS (v/v), and then incubated in 400 μl culture medium [porcine zygote medium 3 (PZM-3) supplemented with 4 mg/ml bovine serum albumin, 5 μg/ml cytochalasin B and 10 μg/ml cycloheximide] at 38.5°C covered with 400 μl mineral oil in an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ with 100% humidity for 4 h before the putative PA embryos were distributed for the experiments (see below). Time of activation was defined as day 0.

### Routine light and experimental light exposure

After maturation, oocyte and embryo handling was routinely performed in a room with the windows curtained and with light from standard fluorescent ceiling lights (220 V, 40 W each). Oocytes and embryos were only handled outside the incubator during the period of activation and for most of this time they were under the microscope, in the light from its incandescent lamp (6 V, 20 W). The total time for denudation and activation was about 30 min.

Experimental light exposure was achieved using two main types of light: daylight and laboratory light; both the culture medium and embryos in the medium were exposed. For this procedure, the dishes were placed in a plastic foil bag (as described by Vajta *et al.*, 1997) filled with the appropriate gas mixture (5% $CO_2$, 5% $O_2$ and 90% $N_2$, passed through water) during light exposure and placed on a heating plate (38.5°C). It should be noted that the time inside these foil bags is considered to be *in vitro* culture time, equivalent to being inside the incubator (Vajta *et al.*, 1997). The experimental groups were exposed to light for 1 or 4 h; these two periods reflected the time necessary for the processes of intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT), respectively; the time required for oocyte preparation, about

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20 min/200 oocytes for both denudation and morphology evaluation, was excluded. Furthermore, to illustrate the severe damage caused by light exposure on both medium and embryos, an extra time point of 24 h was also included as a separate group.

For daylight, the foil bag was placed approximately 4 cm from a window without curtains in a room with the ceiling lights off, and with the foil bag placed behind a shelter to avoid direct sunlight. Experiments were conducted in the summer in Denmark when there was about 16 h daylight and 8 h darkness out of the 24 h exposure time for the daylight group.

For laboratory light, the foil bag was placed under a warm white lamp (12 V, 40 W) at a distance of 40 cm in a room with windows curtained and the ceiling lights off.

### Experimental design

Experiment 1 (light on medium): The culture medium (PZM-3), in standard 5 ml tubes, was placed in a foil bag at room temperature and exposed to either daylight or laboratory light for 1, 4 or 24 h, before being placed inside an incubator until use. As a control, the tubes that contained the medium were placed in the incubator until use without prior exposure to light. Within 2 h of oocyte activation, dishes were prepared with either treated or control medium. The putative PA embryos were divided randomly into seven groups, and groups of 15–20 embryos were cultured in 400 μl PZM-3 covered with 400 μl mineral oil and placed in the incubator at 38.5°C in an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ with 100% humidity until day 6.

Experiment 2 (light on embryos): The putative PA embryos were transferred into dishes that contained freshly prepared PZM-3 and placed in a foil bag, which was then placed on a heating plate and exposed to either daylight or laboratory light for 1, 4 or 24 h. As the control, putative PA embryos were transferred to two dishes, one of which was placed in a foil bag; both dishes were then placed in an incubator until day 6. After light exposure, the embryos were divided randomly into eight groups, and groups of 15–20 embryos were cultured in 400 μl PZM-3 covered with 400 μl mineral oil in an incubator at 38.5°C in an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ with 100% humidity until day 6.

The number of available oocytes was too limited to use for all the groups in Experiment 2, so the experiments were performed unpaired for each replicate, but the non-foiled control was included in each replicate together with one or two of the experimental groups. The foiled control was performed in three replicates, and was only used to investigate the effect of the foil bag on embryonic development.

### Evaluation of the embryonic development by morphology and apoptosis

On day 6, all embryos that had formed a blastocoel cavity (i.e. were blastocysts) were evaluated morphologically under a stereomicroscope and scored into the following four grades as described previously by Li *et al.* (2013): (1) excellent: spherical, regular border, symmetrical with cells of uniform size, even distribution, colour and texture; (2) good: few small blastomeres, irregular shape; (3) fair: vesiculation, few blastomeres; and (4) poor: numerous extruded blastomeres, varying sizes of cells, numerous vesicles.

After the morphological evaluation, the total cell number and the number of apoptotic cells were assessed for all the blastocysts from three replicates using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (*in situ* Cell Death Detection Kit, Cat No. 11684795910, Roche, Germany) as described previously by Li *et al.* (2013). Briefly, all blastocysts were washed three times with 0.1% polyvinylpyrrolidone and fixed in 4% paraformaldehyde for 1 h at room temperature, then permeabilized by incubation in 0.5% Triton X-100 for 1 h. Fixed embryos were incubated in TUNEL reaction medium for 1 h at 38.5°C in complete darkness. After the reaction was stopped, the embryos were washed and transferred into 1 μg/ml Hoechst 33342 for 15 min at room temperature in the dark. The embryos were washed three times and mounted on slides with fluorescent mounting medium (Cat. S3023, DAKO, USA). Stained embryos were examined and images taken under a fluorescence microscopy (360 ± 20 nm excitation, ebq 100 Filter, Leica, Germany).

### Statistical analysis

For each experiment, the data that included percentages of blastocyst, live cell number, total cell number and apoptosis ratio were checked for normality, and all values were fitted approximately to a normal distribution. Therefore, all analyses were made by one-way analysis of variance (ANOVA), and Fisher's least significant difference (LSD) test in R version 2.14.2. A probability of $P < 0.05$ was considered to be statistically significant.

## Results

### Experiment 1: Development of porcine PA embryos cultured in medium previously exposed to light

In total, 584 activated oocytes were cultured, and the results are shown in Table 1. There was no significant difference in percentage of total blastocysts or grades 1 and 2 blastocysts compared with activated oocytes.

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**Table 1** Effect of light exposure of *in vitro* culture medium on porcine *in vitro* embryo development

| Daylight (h) | Laboratory light (h) | Activated oocytes (replicates) | Total blastocysts/ Activated oocytes (%)* | Grades 1 and 2 blastocysts/Activated oocytes (%)** | Grades 1 and 2 blastocyst/Total blastocysts (%)** |
|---|---|---|---|---|---|
| – | – | 114 (6) | 69.2 ± 4.2 (79) | 55.9 ± 3.7 (64) | 80.8 ± 3.0 (64)$^{a}$ |
| 1 | – | 99 (6) | 68.0 ± 2.0 (67) | 45.9 ± 4.1 (45) | 67.1 ± 5.0 (45)$^{a,b}$ |
| 4 | – | 99 (6) | 70.2 ± 5.1 (69) | 46.7 ± 7.8 (46) | 64.1 ± 8.1 (46)$^{a,b}$ |
| 24 | – | 98 (6) | 69.0 ± 4.1 (68) | 42.4 ± 6.9 (42) | 60.1 ± 7.9 (42)$^{b}$ |
| – | 1 | 98 (6) | 71.9 ± 3.3 (70) | 50.6 ± 4.4 (49) | 70.0 ± 4.1 (49)$^{a,b}$ |
| – | 4 | 78 (5) | 77.8 ± 3.7 (61) | 50.0 ± 6.8 (39) | 63.8 ± 7.5 (39)$^{a,b}$ |
| – | 24 | 98 (6) | 72.1 ± 6.4 (71) | 45.4 ± 6.7 (45) | 61.1 ± 4.9 (45)$^{b}$ |

$^{a,b}$Different superscripts in the same column indicate significant difference ($P < 0.05$).
*Mean of replicates ± standard error of the mean (SEM) (number of total blastocysts).
**Mean of replicates ± SEM (number of grades 1 and 2 blastocysts).

**Table 2** Effect of light exposure of the porcine parthenogenetically activated embryos on their subsequent *in vitro* development

| Daylight (h) | Laboratory light (h) | Activated oocytes (replicates) | Total blastocysts/ Activated oocytes (%)* | Grades 1 and 2 blastocysts/Activated oocytes (%)** | Grades 1 and 2 blastocyst/Total blastocysts (%)** |
|---|---|---|---|---|---|
| – | – | 245 (11) | 73.2 ± 2.9 (180)$^{a}$ | 57.3 ± 2.4 (141)$^{a}$ | 78.5 ± 1.9 (141)$^{a}$ |
| – (Foiled) | – | 82 (3) | 74.45.7(60)$^{a}$ | 59.8 ± 5.4 (48)$^{a}$ | 80.2 ± 1.1 (48)$^{a}$ |
| 1 | – | 200 (10) | 69.2 ± 3.4 (139)$^{a}$ | 42.9 ± 2.6 (87)$^{b}$ | 62.2 ± 3.2 (87)$^{b}$ |
| 4 | – | 144 (7) | 67.6 ± 5.5 (98)$^{a}$ | 37.0 ± 4.8 (54)$^{b}$ | 53.4 ± .4.4 (54)$^{b}$ |
| 24 | – | 118 (6) | 18.2 ± 7.1 (20)$^{c}$ | 5.5 ± 2.8 (6)$^{d}$ | 17.5 ± 9.7 (6)$^{d}$ |
| – | 1 | 209 (11) | 67.4 ± 7.2 (141)$^{a}$ | 44.8 ± 5.7 (93)$^{b}$ | 60.4 ± 7.1 (93)$^{b}$ |
| – | 4 | 213 (11) | 69.9 ± 4.4 (152)$^{a}$ | 41.1 ± 3.4 (90)$^{b}$ | 58.3 ± 2.3 (90)$^{b}$ |
| – | 24 | 174 (8) | 46.4 ± 4.9 (82)$^{b}$ | 18.8 ± 3.9 (33)$^{c}$ | 36.5 ± 6.2 (33)$^{c}$ |

$^{a,b,c,d}$Different superscripts in the same column indicate significant difference ($P < 0.05$).
*Mean of replicates ± standard error of the mean (SEM) (number of total blastocysts).
**Mean of replicates ± SEM (number of grades 1 and 2 blastocysts).

However, the percentage of grades 1 and 2 blastocysts to total blastocysts was higher in the control groups compared with the groups in which medium was exposed for 24 h, either via daylight or laboratory light.

### Experiment 2: Development and quality of porcine PA embryos after direct light exposure

In total, 1385 activated oocytes were cultured, and the results are shown in Table 2. All blastocyst rates were similar in the two control groups (without and with foil). A significant decrease in the percentage of grades 1 and 2 blastocysts was found after only 1 h exposure, for both types of light. Furthermore, the percentage of total blastocysts was significantly lower than in the other groups after 24 h exposure to either daylight or laboratory light.

In total, 306 blastocysts were stained on day 6, and the results are shown in Table 3. The numbers of live cells and total cell decreased with increasing lengths of light exposure and reached significant differences after 24 h, using either daylight or laboratory light. No regular pattern was found in the apoptotic cells or in the ratio of apoptotic cell number to total cell number.

## Discussion

The present study showed that ambient light has a negative effect on the development and quality of porcine PA embryos, when the medium used for embryo culture was exposed for a long time period, and even more so when the embryos themselves were exposed for just 1 h to either daylight or laboratory light.

For microscopes used for embryo-related work, attention has focused on the light sources used in order to reduce or avoid the most harmful wavelengths and intensities. This procedure is also carried out in time-lapse systems that are being used more frequently in embryo research (Oh *et al.*, 2007). In addition, the effect of light present around the area where work on embryos is being carried out has not been studied very systematically, certainly not

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**Table 3** Effect of light exposure of porcine PA embryos on their quality

| Daylight (h) | Laboratory light (h) | Blastocysts (replicates) | Live cell number* | Total cell number* | Ratio of apoptosis/total* |
|---|---|---|---|---|---|
| – | – | 41 (3) | $54.8 \pm 2.7^{a}$ | $58.9 \pm 2.6^{a}$ | $7.4 \pm 1.1$ |
| 1 | – | 52 (3) | $51.3 \pm 2.4^{a,b}$ | $54.5 \pm 2.4^{a,b}$ | $7.3 \pm 1.9$ |
| 4 | – | 53 (3) | $46.5 \pm 2.4^{b}$ | $50.4 \pm 2.4^{b}$ | $8.8 \pm 1.1$ |
| 24 | – | NA | NA | NA | NA |
| – | 1 | 53 (3) | $51.8 \pm 2.5^{a,b}$ | $55.7 \pm 2.5^{a,b}$ | $8.3 \pm 1.0$ |
| – | 4 | 61 (3) | $47.5 \pm 2.6^{b}$ | $52.0 \pm 2.6^{a,b}$ | $10.8 \pm 1.4$ |
| – | 24 | 46 (3) | $38.7 \pm 2.5^{c}$ | $40.9 \pm 2.4^{c}$ | $8.9 \pm 1.5$ |

$^{a,b,c}$Different superscripts in the same column indicate significant difference ($P < 0.05$).
*Mean of each blastocyst ± standard error of the mean (SEM).
NA: not applicable.

in pigs. In practice, the action taken to protect medium and embryos against visible light rarely fulfills the standard recommendations for keeping medium and embryos outside the incubator for 'as short a time as possible', especially for reproductive technologies that require a long time to perform, such as ICSI and SCNT. The negative effect of visible light on embryonic development has been confirmed in different species (Nakayama *et al.*, 1994; Takenaka *et al.*, 2007; Korhonen *et al.*, 2009), however the boundaries are only vaguely known. It has been demonstrated that human embryos quickly reach their limit of tolerance to microscope light, based on development and quality, when there are frequent observations under the microscope (Zhang *et al.*, 2010). Ambient light has been documented to contribute less to effects of light exposure than does microscope light (5 versus 95%; Ottosen *et al.*, 2007). However, the current work shows that the development and quality of porcine PA embryos were decreased significantly when exposed to ambient light for >1 h. One possible reason is that porcine oocytes and embryos are comparatively more sensitive to different stresses, as documented for temperature (Pollard & Leibo 1994) and cryoprotectants (Fujihira *et al.*, 2004). This 1 h boundary will be easily crossed for techniques such as ICSI and SCNT, in which hours are spent on first enucleating the oocytes and then fusing electrically, when ambient light is present constantly.

Surprisingly, the current results illustrated that the stability of the medium was compromised by 24 h light exposure, as that level of exposure decreased the percentage of good morphology porcine PA embryos out of total blastocysts. This finding could be due to the presence of certain light-sensitive components in the culture medium: one example is riboflavin in serum, which after photosensitization can degrade tryptophan and result in slow growth of cell cultures (Zang *et al.*, 2011). Therefore, it is important to ensure that practical arrangements and working routines in reproduction laboratories avoid reaching the critical exposure time at which damaging medium can be formed, as the effect is likely to be cumulative rather than due to constant exposure for several hours.

In conclusion, the development of porcine PA embryos can be compromised by extra ambient light exposure both to culture medium and directly to embryos. This effect was seen in embryos after only 1 h of light exposure, therefore porcine PA embryos should be protected from light exposure as much as possible during *in vitro* manipulation.

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