Influence of visible light and room temperature on cell proliferation in preimplantation rabbit embryos*

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Summary. During in-vitro culture rabbit early cleavage stages (Day 1 *p.c.*) and compacted morulae (Day 3 *p.c.*) were exposed to visible light or to room temperature (23°C) for various lengths of time (0.5–24 h). The light source used resembled closely routine laboratory lighting. Controls were cultured simultaneously for 24 h under standard conditions (37°C, darkness). Development was assessed by incorporation of tritiated thymidine as an indicator of cell proliferation.

In comparison to non-exposed controls cell proliferation of Day-1 embryos was more impaired by light than by room temperature whereas in Day-3 embryos thymidine incorporation was more reduced following exposure to room temperature than to light. No statistically significant decrease in thymidine incorporation was detectable up to 1 h (light) and 8 h (room temperature) in Day-1 embryos. Morulae tolerated room temperature and visible light for up to 3 h and 8 h, respectively. Split-dose exposure (e.g. 4 × 1 h) to visible light or room temperature revealed no statistically significant differences compared with one long en-bloc exposure (e.g. 1 × 4 h). These results demonstrate a stage-dependent susceptibility of preimplantation embryos to physical environmental factors. The major risk, indicated by the shortest tolerance times, was provoked by visible light to early cleavage stages.

Keywords: light; room temperature; preimplantation embryos; cell proliferation; rabbit

Introduction

Although in-vitro culture of preimplantation embryos is a well established technique, surprisingly little information is available concerning the influence of physical factors on embryos. During recovery and handling oocytes and embryos are almost inevitably exposed to visible light and reduced temperatures compared to physiological conditions *in vivo*.

Visible light is reported to provoke a delay in cleavage of rabbit embryos (Daniel, 1964) and to disturb the completion of meiosis in fertilized hamster oocytes (Hirao & Yanagimachi, 1978a). In contrast to these scanty data concerning light exposure, there has been more investigation of the viability of oocytes and embryos stored below 37°C. Several events in early development have been shown to be temperature dependent, e.g. oocyte maturation and viability (Lenz *et al.*, 1983; Katska & Smorag, 1985; Moor & Crosby, 1985; Syms *et al.*, 1985; Barros *et al.*, 1986; Morstin & Katska, 1986), post-fusion events (Hirao & Yanagimachi, 1978b) and cell proliferation and viability of cleavage stages (Chang, 1947, 1948a, b, c; Hafez, 1963, 1969; Whittingham & Wales, 1969) and blastocysts (Chang, 1950). Most of these investigations, however, focussed on storage and cryo-preservation of oocytes and embryos (for review see Wilmut, 1986). Routine working conditions in

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basic research as well as in human in-vitro fertilization/embryo transfer programmes involve temperatures which are far from those used for long-time preservation.

The purpose of the present study was to assess systematically the potential risk of short-term exposures to room temperature or to visible light for preimplantation embryos. Rabbit embryos of different developmental stages were chosen to disclose possible stage-specific reactions towards these physical factors.

Materials and Methods

Embryos and in-vitro culture. The superovulation procedure, embryo recovery and in-vitro culture were performed as previously described by Fischer (1987) and Fischer & Meuser-Odenkirchen (1988). Briefly, preimplantation embryos were collected from FSH-primed rabbits of mixed breed 26 or 72 h after mating (*p.c.*) and injection of hCG. Two-cell embryos and compacted morulae were recovered by flushing the oviducts with BSM II (Maurer, 1978) containing 0.1% bovine serum albumin (BSA). After washing 5 times, embryos from all donors were pooled and allocated randomly to the various experimental groups. Embryos were cultured in BSM II containing 1.5% BSA in a humidified atmosphere of 5% CO₂ in air in water-jacketed incubators at 37°C. Experiments with early cleavage stages and morulae were run separately.

Exposure. In total, experimental procedures and in-vitro culture lasted for 24 h. Embryos were exposed to routine laboratory light or to room temperature. The light source used was a neon tube (Philips, TL 8W/33), kept inside the incubator, with emission in the 320–740 nm range (see Fig. 1). The neon tube was installed 12 cm above the culture dish lid. Light intensity at that distance was 1600 lx. The purpose of this arrangement was to imitate strictly the routine working conditions in our sterile laminar flow hood. Temperature was controlled and a heating effect due to the neon tube was excluded. Unexposed controls were cultured simultaneously in a second incubator for 24 h in darkness under standardized conditions as described above. To investigate the influence of room temperature, heating of the incubator was switched off several days before the start of experiments, and the temperature within the incubator decreased to the environmental level (23°C). During experiments the temperature changed $\pm 1^\circ\text{C}$.

Fig. 1. Spectrum of the neon tube showing emission in the 320–740 nm range.

Embryos were subjected to both physical factors for various lengths of time (see Table 1). After exposure, culture dishes were removed from the first into the second incubator where they were cultured for the remaining time in the same way as controls, for example for 20 h after 4 h exposure. In split-dose experiments exposure times were distributed homogeneously over the 24-h experimental period, e.g. 4×1 h exposure = 1 h exposure and 5 h standard culture conditions alternating. After the 24 h experimental period embryos were classified morphologically and labelled with [³H]thymidine.

Thymidine incorporation. Cell proliferation of embryos was evaluated by incorporation of tritiated thymidine as described in detail by Fischer (1987). Day-1 embryos were incubated in 25 μCi methyl-[³H]thymidine/ml (sp. act. 25 Ci/mmol or 925 GBq/mmol; Amersham Buchler, Braunschweig, F.R.G.) for 6 h. Day-3 embryos were labelled with 5 μCi methyl-[³H]thymidine/ml (sp. act. 5 Ci/mmol or 185 GBq/mmol; Amersham Buchler) for 4 h. Incubation periods and specific activities were chosen to obtain valid incorporation data on the one hand and to avoid radiotoxic effects due to prolonged exposure to isotopes on the other (for further details see Fischer, 1987).

Preparation of embryos for liquid scintillation counting involved washing for 5 times in cold phosphate-buffered saline (PBS; Dulbecco & Vogt, 1954), followed by precipitation with trichloroacetic acid and addition of a tissue homogenizer (Lumasolve; Lumac, Schaesberg, The Netherlands) as well as neutralization with glacial acetic acid. Liquid scintillation counting was carried out in an LKB RackBeta 1219 (tritium efficiency of 58%; scintillation fluid:

Table 1. Experimental protocols

Physical factor	Exposure time (h)	Embryo stage (days <i>post coitum</i>)
Visible light	0.5, 1, 2, 3, 4, 8, 12, 24	1
	2, 4, 8, 12, 24	3
	4 × 0.5	1
	4 × 1	1
Room temperature	2, 4, 5, 6, 7, 8, 12, 24	1
	2, 3, 4, 8, 12, 24	3
	4 × 2	3
	3 × 4	3

Lipoluma, Lumac). Most embryos were processed individually. Results are expressed as mean percentages $\pm$ s.e.m. of control incorporation values.

Data were derived from at least 3 replicates. Split-dose treatments were performed in two series of experiments.

Statistical analyses. Reduction in cell proliferation was analysed by a pair-wise comparison of thymidine incorporation in exposed and control embryos using the non-parametric Mann-Whitney test. Due to variations in incorporation data between individual experiments, each replicate was analysed independently. The threshold value was defined by first evidence of damage, i.e. by statistically significant decreased thymidine incorporation.

Results

Exposure to visible light

The first statistically significant impairment of thymidine incorporation was found in Day-1 embryos (Fig. 2a) at 1 h exposure ($P < 0.05$) and in morulae (Fig. 2b) at 8 h ($P < 0.05$). At the longest exposure time investigated (24 h) 2-cell stages incorporated $<20\%$ that for unexposed controls, whereas in compacted morulae incorporation levels still amounted to $\sim 60\%$ at that time. In Day-1 embryos exposure to visible light as split-dose treatment or as one long en-bloc exposure (Fig. 3a) did not lead to a different response ($P > 0.05$).

Exposure to room temperature

Room temperature displayed a less harmful effect on Day-1 cleavage stages than did lighting. Thymidine incorporation was significantly decreased at 8 h exposure (Fig. 4a; $P < 0.05$). Compared with light exposure, variation in incorporation data was notably increased (Figs 2a, 4a). In Day-3 morulae impairment of thymidine incorporation was first detectable at 3 h exposure (Fig. 4b; $P < 0.05$). Symptomatic for the differences in susceptibility to room temperature exposure were the incorporation levels persisting after 24 h exposure: values ranged from 65% of controls for 2-cell embryos to 24% of controls for compacted morulae. Split-dose exposures of Day-3 embryos to room temperature did not result in different incorporation data compared with single dose exposures (Fig. 3b; $P > 0.05$).

Discussion

A stage-dependent susceptibility to visible light and room temperature is demonstrable by impairment of cell proliferation in preimplantation rabbit embryos. With cleavage progressing from the

Fig. 2. $[^3\text{H}]$ Thymidine incorporation (as % of non-exposed controls) in (a) Day-1 and (b) Day-3 rabbit embryos exposed to visible light. Values are mean $\pm$ s.e.m. for the no. of embryos indicated. For (a), controls (0 h) = 314 ± 27 d.p.m./embryo; for (b), controls (0 h) = 2157 ± 200 d.p.m./embryo. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with controls (0 h).

Fig. 3. $[^3\text{H}]$ Thymidine incorporation (as % of non-exposed controls) in (a) Day-1 rabbit embryos exposed to split doses of visible light (stippled columns) and (b) Day-3 rabbit embryos exposed to split doses of room temperature (stippled columns). Open columns are exposures in single blocks. Values are mean $\pm$ s.e.m. for the no. of embryos indicated. For (a), controls (0 h) = 511 ± 45 d.p.m./embryo; for (b), controls (0 h) = 2738 ± 274 d.p.m./embryo. N.S. = not significant, $P > 0.05$.

Fig. 4. $[^3\text{H}]$ Thymidine incorporation (as % of non-exposed controls) in (a) Day-1 rabbit embryos and (b) Day-3 rabbit embryos exposed to room temperature. Values are mean $\pm$ s.e.m. for the no. of embryos indicated. For (a), controls (0 h) = 368 ± 43 d.p.m./embryo; for (b), controls (0 h) = 2164 ± 190 d.p.m./embryo. * $P < 0.05$; ** $P < 0.01$ compared with controls (0 h).

2-cell to the morula stage, sensitivity to light exposure decreases while sensitivity to room temperature increases. Susceptibility can be demonstrated by threshold values of significantly decreased thymidine incorporation as well as by minimal incorporation levels persisting after elongated exposure. For both environmental factors studied the most severe effects were those of visible light on 2-cell stages. As early as after 1 h exposure a statistically significant impairment of cell proliferation was detectable.

Similar to the present findings stage-specific susceptibility to physical factors has been reported for X-irradiation (Alexandre, 1974) and u.v. irradiation (Eibs & Spielmann, 1977) in preimplantation mouse embryos. Stage-dependent susceptibility of rabbit embryos to temperatures below 37°C became manifest by differences in viability between cleavage stages and blastocysts after storage at 10°C (Chang, 1948a, b, 1950).

Irradiation experiments indicate that the deleterious effects of light are probably attributable to DNA lesions. Ultraviolet irradiation of preimplantation mouse embryos (Müller & Spindle, 1986) and mammalian cells in culture (Kato, 1977) led to a significant increase in sister-chromatid exchange, provoked the induction of pyrimidine dimers (Beukers & Berends, 1961), produced single-strand breaks in DNA (Elkind, 1971; Friedman *et al.*, 1975) and was followed by excision repair (Pedersen & Cleaver, 1975) and post-replication repair of DNA (Eibs & Spielmann, 1977; Müller & Spindle, 1986). Cell damage after irradiation depends on the duration of exposure,

radiation intensity and on the wavelengths emitted. Daniel (1964) observed an inhibition of cleavage in Day-1 rabbit embryos after 12 h exposure to visible light. In the present study the 2-cell stage embryos cleaved at least once after 12 h and even after 24 h exposure to light. The different results might be explained by the higher light intensity (2600 lx) used by Daniel (1964). Emission values ranged from 320 to 740 nm in our study (see Fig. 1). Damage provoked by wavelengths below 300 nm should be negligible because the plastic of the culture dishes absorbs more than 99% of these ultraviolet wavelengths (Rauth, 1970; Bradley & Sharkey, 1977). Emissions in the range of 300–500 nm seem to be more important, as shown by the use of appropriate filters for the protection of early rabbit embryos (Daniel, 1964) and in the damage of human D 98/AH₂ cells (Wang, 1975) and hamster oocytes (Hirao & Yanagimachi, 1978a). The near-ultraviolet range (300–400 nm) was particularly suspected of causing cell death in different mammalian cells in culture (Wang *et al.*, 1974). There are indications that a small but significant portion of light in the spectrum of > 500 nm penetrates transabdominally into the uterine lumen of mammals (Jacques *et al.*, 1987). Jacques *et al.* (1987) suggest that light may induce direct and/or indirect beneficial effects on fetal development, e.g. concerning circadian rhythms.

Photochemical reactions between light and components of the culture medium have been reported to cause lethal effects on various mammalian cells (Stoien & Wang, 1974; Wang, 1975; Pereira *et al.*, 1976). To exclude such influences we pre-irradiated for 24 h medium which was to be used for cultures. The incorporation data of embryos cultured in this medium were not different from those of controls (data not shown), indicating that the deleterious effects of light were not due to phototoxic reactions within the culture medium. Light therefore seems to damage blastomeres directly.

Each physical factor investigated in the present study most probably affects blastomeres at different cellular targets. In a related study we found ultrastructural evidence that light provoked mainly cell degeneration and cell death while room temperature seemed to alter cytoskeleton organization and intracellular transport of organelles (Hegele-Hartung *et al.*, 1988). The dynamic equilibrium between free tubulin and polymerized microtubules (Pickering & Johnson, 1987) and molecular mechanisms of membrane fluidity (Breisblatt & Ohki, 1975; Jacobson & Papahadjopoulos, 1975) also seem to be influenced by temperature. Cooling leads to a depression of rabbit embryonic metabolism during storage at 10°C (Anderson & Foote, 1974, 1975b). After rewarming, energy metabolism as well as nucleic acid and protein synthesis were not significantly decreased compared with controls cultured continuously at 37°C. However, the number of embryos developing into blastocysts was clearly reduced after cooling (Anderson & Foote, 1975a). In agreement with these findings we observed no blastocyst formation in Day-3 rabbit embryos after exposure to room temperature for 24 h, while all light-exposed morulae did cavitate. Possible mechanisms involved may be temperature-sensitive trophoblast transport systems which are essential for accumulation of fluid, a prerequisite for blastocyst formation and expansion. Measurements of transtrophoblast potential difference (Cross & Brinster, 1970) and reconstitution of blastocysts after mechanical injury (Daniel, 1963) indicated an optimum temperature in the 34–37°C range.

The mode of light or room temperature exposure did not display a significant effect on thymidine incorporation. Short split-dose exposures of Day-1 embryos to visible light (e.g. 4 × 1 h) and of Day-3 embryos to room temperature (e.g. 4 × 2 h) revealed no statistically significant differences in incorporation values compared with embryos subjected to a single long exposure (4 h and 8 h, respectively). Obviously, the periods between the exposure times did not allow significant recovery or, alternatively, the induced damage was irreversible. Relevant for laboratory conditions is the combined action of both stressors. Early results indicate that a simultaneous exposure towards light and room temperature does amplify the adverse effects (B. Fischer, A. Schumacher, C. Hegele-Hartung & H. M. Beier, unpublished observation).

The amount of damage to and viability of embryos depends on the number of cells surviving after exposure. The reports of Seidel (1952, 1960) and Tarkowski & Wróblewska (1967) clearly

indicate the ability of single blastomeres to regulate and to secure further development. Embryo transfer experiments with exposed embryos will therefore have to be performed. The inevitable exposure to visible light and room temperature of human embryos in in-vitro fertilization/embryo transfer programmes could contribute to the surprisingly low rate of pregnancies after embryo replacement.

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