Membrane and intracellular effects of ultraviolet irradiation with Hoechst 33342 on bovine secondary oocytes matured *in vitro*

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Ultraviolet (UV) irradiation in combination with the bisbenzimidazole stain Hoechst 33342 has been used to visualize the chromosomes before removal of oocytes to be used as cytoplasmic hosts for embryo nuclear transplantation. Short term effects of UV irradiation on the cytoplasmic viability of bovine oocytes matured *in vitro* were assessed by performing membrane and intracellular studies at 2 and 20 h after exposure to UV irradiation for 0, 30 or 60 s. At the membrane level, loss of integrity was shown by increased lysis and increased retention of the fluorescein diacetate dye in oocytes exposed to 60 s of UV irradiation and uptake of methionine was higher in both irradiated groups. At the intracellular level, methionine incorporation into protein was 5.8 times higher in controls than in oocytes exposed to UV irradiation for 60 s and there was a marked difference in the pattern of protein synthesis. Some changes in protein synthesis were also found in oocytes after 30 s exposure. Moreover, high levels of fluorescence with the dye rhodamine 123 at 20 h after exposure indicated large increases in mitochondrial membrane potential in both groups of UV-irradiated oocytes. Together, these findings indicate that exposure to UV irradiation for periods as short as 60 s causes alterations to both membrane and intracellular components of bovine oocytes matured *in vitro*. It is concluded that care must be taken when using this methodology to visualize or destroy metaphase chromosomes during enucleation in the embryo cloning protocol.

Introduction

Nuclear transplantation, and its application to the production of genetically identical mammals, has the potential to become an important procedure for studying nuclear–cytoplasmic interactions in embryos and to improve the rate of genetic gain in animal production (Bondioli *et al.*, 1990; Robl and Stice, 1990). Lack of knowledge in some of the many steps involved in the procedure have led to a low overall efficiency of approximately 1–6% in producing offspring (Yang, 1991). One of these steps involves the removal of the genetic material contained within the recipient oocyte (enucleation). Total chromosomal removal is necessary to (1) eliminate any genetic contribution of the host cytoplasm and thus assure maximal similarity between cloned animals and (2) avoid ploidy abnormalities with its deleterious consequences to normal development. Owing to the presence of dense cytoplasmic granules in the oocytes of most domestic species, metaphase chromosomes cannot be readily visualized, a factor which causes about 30% errors in enucleation (Prather *et al.*, 1987; Prather *et al.*, 1989; Smith and Wilmut, 1989).

Ultraviolet irradiation (UV) and DNA-specific binding stains have been used to visualize oocyte and embryonic nuclei, and to assess enucleation efficiency after micromanipulation (Critser and First, 1986; Prather *et al.*, 1989; Smith and Wilmut, 1989).

Studies in mice have shown that the pronuclei and the cytoplasm have different sensitivities to Hoechst staining and UV irradiation (Tsunoda *et al.*, 1988). Whereas developmental potential of pronuclei was completely blocked after 20–30 s exposure to UV, developmental arrest due to cytoplasmic effects could be detected only with exposures of more than 30 s. Moreover, a recent report in cattle has shown that exposure to UV irradiation for 10 s has no effect on embryo viability and production of live calves (Westhusin *et al.*, 1992).

Destruction without the removal of oocyte metaphase chromosomes has been attempted in rabbits using exposure to UV irradiation for 3 min (Yang *et al.*, 1990). Culture of the reconstituted embryos *in vitro* showed a decrease in the viability when using irradiated oocytes, suggesting deleterious cytoplasmic effects of the longer exposures. Studies on the effects of UV irradiation in *Xenopus* eggs, where functional enucleation is a normal practice, showed that exposure of eggs for periods of between 15 and 50 s was without effect (Gurdon, 1960). However, 30% abnormal development was obtained when using a 30–80 s exposure suggesting that there is some cytoplasmic effect. Further studies are required to improve the current techniques for enucleating oocytes for use in nuclear transplantation. The following study was undertaken to verify the effects of UV irradiation on membrane and intracellular components of *in vitro* matured bovine oocytes stained with Hoechst 33342.

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Materials and Methods

Production of secondary oocytes

Bovine ovaries were collected at a local abattoir and transported to the laboratory in phosphate-buffered saline (PBS; pH 7.4), supplemented with 100 iu penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Pen/Strep; Gibco, Grand Island, NY) in an insulated container. At the laboratory, ovaries were washed twice in PBS and the contents of non-haemorrhagic follicles with a diameter between 1 and 5 mm were aspirated using a 20 gauge needle. Cumulus-oocyte complexes with complete cumulus layers and homogeneous cytoplasm were selected and washed twice in Hepes-buffered tissue culture medium 199 (TCM 199, Gibco) and placed into bicarbonate-buffered TCM 199 (Gibco) supplemented with 10% heat-treated oestrous cow serum (ECS) for maturation. Groups of approximately 100 cumulus-oocyte complexes supplemented with granulosa cells were placed in 1 ml wells and cultured for 26–28 h at 38.5°C in a humidified atmosphere of 5% CO₂ in air. At the end of the maturation period, expanded cumulus-oocyte complexes were placed in Ca²⁺- and Mg²⁺-free Ham's medium (Gibco) supplemented with 300 iu bovine testis hyaluronidase ml⁻¹ (Sigma, St Louis, MO) and vigorously shaken for 3 min to remove the cumulus cells. Denuded oocytes with a homogeneous cytoplasm were selected for further use in experiments.

Staining, ultraviolet irradiation and activation protocols

Unless specified, oocytes to be exposed to UV were incubated in bicarbonate-buffered TCM199 with 5 µg Hoechst stain 33342 ml⁻¹ (bisbenzimidazole trihydrochloride; Sigma) for 30 min at 38.5°C. After staining, the oocytes were washed in Hepes-buffered TCM199 and placed in a glass chamber for fluorescence and irradiation studies. Oocytes were randomly distributed into three groups and exposed to 55 nW of UV irradiation for 0 s (control group), 30 s or 60 s. The UV irradiation dose was measured using a photodetector (model 818-UV; Newport Corp., CA) with an optical power meter (model 840; Newport Corp.). Irradiation was performed on individual oocytes with a ×40 objective (DIC LWD; Nikon, Tokyo) on an inverted microscope equipped with Epi-fluorescence (TMD-Diaphot; Nikon), a 50 W mercury lamp (Philips, Germany) and an ultraviolet filter block (330–380 nm excitation and 420 nm emission, UV-2A; Nikon). After UV irradiation, oocytes were removed from the glass chamber and placed for a 10 min equilibration period in a low electrolyte solution of 0.3 mol mannitol l⁻¹, 0.1 mmol MgSO₄ l⁻¹ and 0.05 mmol CaCl₂ l⁻¹ in water (Willadsen, 1986). Oocytes were placed in an electrofusion chamber containing activation medium and positioned between two parallel platinum electrodes 200 µm apart. Activation was produced by a 20 V square direct current pulse (1.0 kV cm⁻¹) with 70 µs duration (Electro Cell Manipulator 200; BTX, CA). After the activation stimulus, oocytes were removed from the electrofusion chamber, washed three times in TCM 199 and incubated until further analysis. Control and UV irradiated oocytes were examined either after 2 h to determine the most immediate effects or after 20 h to determine the progression of these effects. A small group of oocytes was examined before the activation stimulus to determine the effects of this procedure on the different aspects of oocyte viability.

Amino acid uptake and incorporation of oocytes

Oocytes were washed in modified Tyrode's medium, supplemented with 0.6% BSA (Fraction V; Sigma), lactate, pyruvate and gentamicin (TALP, as described by Parrish *et al.* (1986)). After washing, oocytes were incubated for 2 h in 50 µl drops of TALP containing L-[³⁵S]methionine (1 mCi ml⁻¹, >800 Ci mmol⁻¹; Amersham, Canada). After incubation, they were washed free of radiolabelled precursor by three transfers through TALP and a final wash in PBS to remove protein present in the medium. Washed individual oocytes were disrupted by adding 50 µl SDS dissociation buffer (62.5 mmol Tris-HCl l⁻¹, pH 6.8, containing 2% w/v SDS, 10% glycerol and 5% v/v 2-mercaptoethanol) followed by heating at 95°C for 15 min. Uptake of L-[³⁵S]methionine by individual oocytes was assayed by counting 4 µl of SDS-dissociated extracts in Aquasol scintillation cocktail (New England Nuclear, Boston). Incorporation of acid precipitable protein was measured in individual oocytes by transferring 4 µl of the same SDS-dissociated extracts onto Whatman GF/C filters. Dried filters were washed with 20 ml 10% trichloroacetic acid and 10 ml absolute ethanol, dried, and counted in the scintillation cocktail.

One-dimensional SDS-PAGE and densitometric analysis

Individual oocytes obtained 20 h after the activation stimulus were used in this experiment. Radiolabelled proteins were separated and resolved by SDS-PAGE carried out on an electrophoresis cell (Mini-Protean II; BioRad, Richmond, CA) attached to a power supply (PS2500 DC; Hoefer Scientific Inst., San Francisco) according to the method of Laemmli (1970). The remaining SDS-dissociated individual oocyte extracts were loaded onto 12% slab polyacrylamide gels that were 0.75 mm thick. After electrophoresis, the gels were stained and treated with an autoradiography enhancer (En³Hance; New England Nuclear, Boston), dried and then exposed to pre-flashed X-ray film at -70°C for 3 weeks. Densitometric analysis was performed using a laser densitometer (UltraScan XL; Pharmacia, Sweden) and computer analysis (GelScanXL Software Package; Pharmacia).

Rhodamine 123 staining and fluorescence quantification

The purified laser dye rhodamine 123 (R123; Sigma) was dissolved in distilled water at a concentration of 1 mg ml⁻¹ and subsequently diluted to 10 µg ml⁻¹ in TCM199 for incubation (Johnson *et al.*, 1980). Oocytes were incubated for 30 min at 38.5°C after which they were washed several times in TCM199 without R123 and examined by epifluorescent illumination using a green filter block (510–560 nm excitation and 610 nm emission; G-2A; Nikon). Quantitative measurement of the emitted fluorescence from individual oocytes was obtained using a microphotometry system (System P1; Nikon) attached to the microscope.

Fluorescein diacetate (FDA) staining and fluorescence quantification

A stock solution of 5 mg FDA ml⁻¹ (Sigma) in acetone was prepared and stored at -20°C. Just before use, a stock solution

Fig. 1. Bovine secondary oocytes matured *in vitro* after cumulus removal and staining with the bisbenzimidazole dye Hoechst 33342. (a) and (b) show a single oocyte with polar body located close to the metaphase chromosomes; (c) and (d) show another oocyte with polar body positioned away from the metaphase chromosomes before and during excitation with UV light (365 nm main wavelength), respectively. Arrows indicate first polar bodies; arrowheads indicate metaphase chromosomes; the dotted line separates the oocyte into two hemispheres according to the position of the metaphase chromosomes. Bar = 20 μm .

of $0.5 \mu\text{l ml}^{-1}$ in PBS was prepared giving a final concentration of $2.5 \mu\text{g FDA ml}^{-1}$ (Mohr and Trounson, 1980). Oocytes were incubated in this solution for 1 min at room temperature after which they were washed several times in PBS. After incubation at room temperature for 10 min, the oocytes were examined by epifluorescent illumination using a blue filter block (450–490 nm excitation and 520 nm emission; B-2A: Nikon). Quantitative measurements of the emitted fluorescence from individual oocytes were obtained using the same microphotometric system described for R123.

Statistical analysis

Data collected from individual oocytes grouped into three to five replications were assembled for statistical analysis and analysed using the General Linear Model procedure. Individual means were compared using Duncan's multiple range test (SAS Inc., Cary, NC). Data on the proportion of oocytes lysed after UV irradiation were analysed by χ^2 test.

Results

After removing the cumulus cells from the cumulus–oocyte complexes, a total of 507 *in vitro* matured bovine oocytes were

Fig. 2. Intensity of emission from the fluorescein diacetate (FDA) dye retained in oocytes exposed to UV irradiation for periods of 0 (□), 30 (▨) or 60 s (■) and measured at 0, 2 and 20 h after exposure. Values with different letters are significantly different ($P < 0.05$).

considered suitable, selected and randomly allocated for use in these experiments. The position of the first polar body within the perivitelline space and its relationship to the metaphase chromosomes was recorded in most UV irradiated oocytes (Fig. 1). Proper positioning was possible by rotating the oocyte to place both polar body and metaphase chromosomes on the same focal plane. From a total of 220 oocytes examined, 158

Fig. 3. Incorporation (▨) and uptake (■) of [³⁵S]methionine by control non-exposed oocytes and oocytes exposed to 30 s of UV after incubation with different concentrations of H-33342 (range from 0 to 100 µg ml⁻¹). Values with different letters are significantly different ($P < 0.05$).

(72%) had the polar body located in the same hemisphere, whereas 62 oocytes (28%) had polar bodies located in the hemisphere opposite to the metaphase chromosomes.

Oocytes were considered to have undergone lysis either when no plasma membrane was observed or when the plasma membrane appeared rough and fragmented under differential interference contrast optics. Loss of membrane integrity by lysis was observed in 16 out of a total of 149 oocytes (11%) in the control group, eight out of 125 oocytes (7%) exposed to 30 s, and 45 out of 145 oocytes (31%) exposed to 60 s of UV irradiation. The proportion of lysis was significantly higher ($P < 0.01$) in the 60 s group than in both 30 s and untreated control groups which did not differ from each other ($P > 0.05$). The presence of lysed oocytes in the control group indicated that either the mannitol solution or the electric stimulus or both were causing some loss in membrane integrity.

Accumulation of fluorescein diacetate (FDA) in oocytes increased significantly with time and after prolonged exposure to UV irradiation. A total of 210 non-lysed oocytes were analysed for FDA in three replicates with an average of 30 oocytes per experimental group (Fig. 2). Although slightly increased, the amounts of FDA in oocytes exposed for 30 s were not significantly different from that in controls at 2 h after irradiation ($P > 0.05$). However, the amount of FDA found in oocytes exposed for 60 s was significantly higher than that in controls at this time ($P < 0.05$). A large increase in FDA accumulation was observed at 20 h in control and UV irradiated groups. At this time, the control and 30 s groups did not differ ($P > 0.05$), but oocytes exposed to 60 s of UV irradiation had significantly more FDA accumulated in the cytoplasm ($P < 0.05$).

Uptake and incorporation of radiolabelled methionine were analysed at 20 h after activation in a total of 24 oocytes used in protein synthesis analysis. Counting of [³⁵S]methionine in the SDS-dissociated extracts from individual oocytes indicated an average uptake during the 2 h incubation of 35.7 ± 1.8 fmol by the non-exposed controls, 52.7 ± 7.0 fmol by the group exposed to UV irradiation for 30 s and 51.8 ± 7.5 fmol of methionine by the oocytes exposed to 60 s of UV irradiation. Both UV irradiated groups had significantly higher uptake than controls ($P < 0.05$). Incorporation of [³⁵S]methionine into

precipitable protein was substantially affected by a long exposure to UV irradiation. Counting of the radiolabelled precursor in the precipitable protein fraction of oocytes indicated an average incorporation during the 2 h incubation of 2.9 ± 0.2 fmol in the control group, 2.1 ± 0.4 fmol in the group exposed to UV irradiation for 30 s and 0.5 ± 0.1 fmol [³⁵S]methionine in the oocytes exposed to 60 s of UV irradiation. Incorporation in oocytes after 60 s exposure was significantly lower than in control and 30 s groups ($P < 0.05$). A subsequent experiment was performed to analyse the effect of a standard dose of UV (30 s) both on unstained oocytes and using different concentrations of H-33342 (Fig. 3). With the exception of the highest concentration (100 µg ml⁻¹), in which medium saturation and precipitation of stain were noticed, uptake of radiolabelled methionine decreased gradually and significantly by increasing the concentration of the stain. There was no difference between unexposed oocytes and oocytes exposed to UV in the absence of H-33342 or at concentrations up to 1 µg ml⁻¹. However, incorporation of radiolabelled methionine decreased substantially after exposure to UV regardless of whether the stain was present. The lowest incorporation was observed using H-33342 at a concentration of 10 µg ml⁻¹. These results indicate that although UV irradiation without the stain will not affect methionine uptake, this level of exposure will affect protein synthesis in oocytes. A synergistic effect of H-33342 was detected for uptake and incorporation at 10 µg ml⁻¹.

Qualitative analysis by one-dimensional SDS-PAGE of proteins that are synthesized at 20 h after activation showed marked differences between both irradiated groups and controls. Owing to the large variation in levels of incorporation between control and UV irradiated oocytes, direct visual analysis of autoradiograms provided limited information on the different patterns found in each group (Fig. 4a). Fine details of these changes were better visualized from densitometric scans of the same autoradiogram (Fig. 4b) and could be classified into two categories. First, protein bands present in the control group and in the group exposed to UV irradiation for 30 s that were not present in oocytes exposed to 60 s of irradiation (76, 100 and 120 kDa) and second, proteins not present in controls which appeared after irradiation. In this category were proteins present in both the 30 and 60 s exposure group (69, 82 and 104 kDa) and others, only observed in the 60 s group (42 and 124 kDa).

Fluorometric monitoring of mitochondrial membrane potential with the laser dye rhodamine 123 (R123) on individual oocytes was significantly higher and more variable after UV irradiation (Fig. 5). A total of 194 oocytes was analysed in three replicates. Although the 60 s group oocytes retained the amount of R123 fluorescence, the average readings for control and 30 s irradiated oocytes seemed to decrease slightly after 2 h ($P > 0.05$). However, at 20 h after irradiation, significantly more R123 fluorescence was observed in groups irradiated for both 30 and 60 s ($P < 0.05$).

Discussion

The present study shows that *in vitro* matured bovine oocytes stained with the bisbenzimidazole dye, Hoechst 33342, and

Fig. 4. Synthesis profile of [35 S]methionine-labelled polypeptides from control and UV irradiated (30 s and 60 s) *in vitro* matured oocytes incubated for 20 h after activation. (a) Autoradiograms obtained by one-dimensional SDS-PAGE; (b) densitometric reading of the polypeptide profiles obtained from the autoradiograms in (a). Arrows show the proteins that were absent in oocytes exposed to UV irradiation for 60 s. Arrowheads show protein bands present exclusively in UV-irradiated oocytes.

Fig. 5. Fluorometric monitoring of mitochondrial membrane potential with the laser dye rhodamine 123 (R123) in control (□), 30 (▨) and 60 (■) s UV irradiated individual oocytes at 0, 2 and 20 h after activation. Values with different letters are significantly different ($P < 0.05$).

exposed for different periods to UV irradiation are substantially affected. Effects were observed at the level of membrane integrity, such as lysis, FDA accumulation and methionine uptake, and also at the intracellular level, such as quantitative and qualitative changes in protein synthesis and mitochondrial activity. Although some of the effects were less severe in oocytes exposed to 30 s of UV irradiation, exposures for 60 s caused substantial changes to most aspects of membrane and intracellular viability analysed. These changes to the non-genomic components of the oocyte appear irreversible in most cases and are likely to be incompatible with normal development.

At the level of the plasma membrane, oocytes exposed to 60 s of UV irradiation undergo changes to all aspects analysed by 20 h after exposure. Most lysed oocytes possessed a rough and fragmented membrane by 2 h and cytoplasmic leakage into the perivitelline space was noticed by 20 h, suggesting a

gradual deterioration of the membrane integrity. Nevertheless, the ability of oocytes to accumulate FDA within the cell increased with time and with longer exposure to UV irradiation. It is interesting to note from these results that effects of UV irradiation on plasma membrane appear to be different from those found after freezing and thawing of cells in which there is an accelerated loss of FDA possibly due to an increase in membrane permeability (McGann *et al.*, 1988). According to Rotman and Papermaster (1966), as FDA is non-polar, it can readily pass into the oocyte where it is hydrolysed by esterases to yield polar fluorescein. Although polar fluorescein can cross the cell membrane, it does so less rapidly than the apolar FDA and, therefore, accumulates intracellularly. The effect of UV irradiation was to increase FDA accumulation in the non-lysed oocytes, which suggests either an increase in esterase activity or an alteration in the ratio between uptake and release of the apolar and polar compounds, respectively, by UV irradiation. The latter may have been due to a loss in the fluidity of the membrane due to lipid oxidation (Singh, 1989), leading to improper repair and closure of pores caused by the electric pulse. These effects may also have resulted in higher methionine uptake by irradiated oocytes.

Quantitative and qualitative changes to the intracellular protein synthetic activity were substantially affected by exposure to UV irradiation. Whereas uptake increased after UV irradiation, incorporation of radiolabelled methionine into protein in oocytes exposed for 60 s was reduced to 17% of that found in unexposed control oocytes. The patterns of proteins being synthesized were also markedly changed after irradiation. These changes are unlikely to be related exclusively to the repair of UV-induced damage, since not only did 'new' protein bands appear but also many stage-specific protein bands disappeared after prolonged irradiation, and suggest that changes to the endogenous developmental programme of protein synthesis

also occur. Although some of these changes may have been caused by post-translational modifications, changes at the pretranslational level of cytoplasmic mRNA stores may also have been involved as shown to occur in irradiated mouse and *Drosophila* embryos (Masui and Pederson, 1975; Kobayashi and Okada, 1989).

Rhodamine 123 readings were significantly higher and very variable in oocytes exposed to UV. The mechanism for selective uptake of this dye into mitochondria resides in the attraction of the rhodamine cationic molecule toward the relatively high electronegative potential that exists across the mitochondrial membrane (Waggoner, 1979). It, therefore, provides means to assess the total proton motive force within the mitochondrial mass that may reflect ATP requirements in cells (Johnson *et al.*, 1981). This positive correlation between rhodamine 123 fluorescence and the ATP requirement is supported by the strong mitochondrial staining of actively migrating or dividing cells and the reduced staining of confluent and progeria cells (Goldstein and Korczak, 1981). Although it is unclear why irradiated oocytes should produce high mitochondrial readings, increased energy requirement for the repair of membrane, or intracellular UV-induced damage or both are possibilities. However, UV irradiation has been shown to cause swelling and vesiculation of mitochondria, and fragmentation of germinal granules in *Xenopus* eggs (Ikenishi *et al.*, 1974). Effects on the metabolic activity of mitochondria would probably be detected only at the later stages of embryonic development when there is an exponential increase in aerobic respiration (Rieger, 1992).

Finally, these findings clearly demonstrate that Hoechst 33342 staining should not be used for UV exposure periods of more than 30 s. This level of exposure seems to be close to the threshold at which recovery from irradiation damage may be obtained. Cell viability should be assessed by other methods to determine the effects of UV irradiation at later stages of embryonic and fetal development. In *Drosophila* embryos, germ-line formation is blocked during embryogenesis in UV-irradiated oocytes and fertility can be restored by injections of non-irradiated polar cytoplasm (Okada *et al.*, 1974). This defect has been shown to be due to damage to the transcriptional ability of mitochondria and could be corrected by cytoplasmic injections of mitochondrial rRNA (Kobayashi and Okada, 1989). In mice, animals produced by nuclear transplantation carry mitochondrial DNA from both donor and recipient oocytes (Smith *et al.*, 1991) and this finding emphasizes the need for care when using UV-irradiation for enucleating oocytes in the embryo cloning procedure.

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