UV irradiation of pig metaphase chromosomes: maturation-promoting factor degradation, nuclear cytology and cell cycle progression

C. L. V. Leal^{1,2}, C. Lee¹ and R. M. Moor^{1*}

¹Protein Function Laboratory, The Babraham Institute, Babraham, Cambridge, CB2 4AT, UK; and ²Department of Animal Reproduction, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, CEP 05580-900, Brazil

Experiments were designed to test two hypotheses. The first was that irradiation of pig metaphase chromosomes would block the normal sequence of cytological and molecular events associated with activation; the second postulated that damaged DNA would prevent eggs from progressing through the first mitotic cleavage cycle. The experimental protocol involved selectively irradiating the metaphase II plate of pig oocytes with highly focused 254 nm ultraviolet (UV) light, followed by activation using standard electroactivation procedures. The following assessments were made of different groups of eggs: (i) nuclear membrane reassembly; (ii) chromosomal cytology; (iii) changes in maturation-promoting factor kinase (MPF kinase) activity at 1 h intervals after activation; and (iv) mitotic progression of eggs containing damaged chromosomal fragments. UV irradiation neither prevented the reassembly of nuclear membranes required for pronuclear formation nor interfered with the normal pattern of MPF kinase degradation after egg activation. UV irradiation did induce a wide range of chromatin defects, including condensation and dispersal of DNA fragments which, in turn, resulted in the formation of micronuclei in the treated eggs and embryos. The presence of damaged DNA retarded, but did not inhibit, progression through the first mitotic cycle. No evidence was obtained that the subsequent mitotic cycle was adversely affected by the presence of UV-damaged DNA. Overall, these results indicate that early cleavage divisions in pig eggs are not blocked by the presence of damaged, hypercondensed chromatin. In this respect, pig eggs are similar to *Xenopus* eggs, but are different from bovine eggs. On the basis of these findings it is suggested that focused UV irradiation offers a simple and rapid technique for the non-invasive enucleation of pig oocytes provided that the residual hypercondensed chromatin does not affect later developmental stages.

Introduction

Ultraviolet (UV) irradiation of the nucleus of oocytes provides a simple means of non-invasive enucleation for future cloning programmes provided that the presence of damaged maternal DNA in the resulting cytoplasts does not interfere with subsequent cell cycle progression. In testing this proposition in bovine oocytes, Bradshaw *et al.* (1995) demonstrated that accurately focused short wavelength UV light effectively destroyed chromosomal function without causing cytoplasmic damage as occurs after irradiation of the entire cell. However, after irradiation of the second metaphase plate in bovine oocytes, maturation-promoting factor (MPF) kinase degradation and pronuclear formation were compromised. The experiments in the present study are based on preliminary observations indicating that, in

contrast to bovine oocytes, pronuclear formation in pig oocytes is not compromised after UV irradiation. The objective of the present study was to extend these preliminary observations by determining the effect of short wavelength UV light on chromatin organization and cell cycle progression in pig oocytes. In addition to determining the UV sensitivity of pig oocytes, the experiments were also designed to contribute to the long-term aim of improving enucleation procedures in mammalian eggs.

After escape from the extended G2-phase block, mammalian oocytes progress to metaphase II, in which the meiotic cycle is subjected to a second period of arrest. Paradoxically, the presence of high concentrations of active cyclin-dependent kinase (referred to in oocytes as MPF kinase) is required both for escape from the first meiotic cycle block at the germinal vesicle (GV) stage and also for induction of the second period of arrest at metaphase II. Exit from metaphase II arrest and entry into the first mitotic cycle is initiated by a marked decrease in MPF kinase activity

*Correspondence.

Received 11 August 1998.

induced by sperm penetration, which in turn increases intracellular calcium and induces ubiquitin-mediated cyclin degradation (Glutzer *et al.*, 1991). In a study of bovine oocytes, Bradshaw *et al.* (1995) showed that early events in the metaphase II to anaphase II transition are interrupted by irradiation. Although sperm penetration and decondensation occurred normally, the subsequent degradation of cyclin and the decrease in MPF kinase did not occur in bovine oocytes subjected to UV irradiation. The resulting inhibition of meiosis is paralleled in somatic cells in which UV irradiation also induces cell cycle arrest (Devary *et al.*, 1992; Dhanwada *et al.*, 1995). However, detailed studies on somatic cells revealed that irradiation-induced arrest occurs at a number of different points in the mitotic cell cycle (Terada *et al.*, 1995). In general, these forms of mitotic cycle inhibition are closely associated with mechanisms of DNA repair (Carrier *et al.*, 1994). The specific points of arrest are invariably correlated with well established mitotic checkpoints, the function of which is to delay or prevent either DNA replication or chromatid segregation in cells containing damaged DNA (see Hartwell and Weinert, 1989; Murray, 1993). It is during the period of enforced delay that the repair mechanisms within the nucleus operate to correct the DNA lesions or, failing that, to redirect the cell into an apoptotic pathway (Enoch and Norbury, 1995). There is evidence that the checkpoint control mechanisms in oocytes, and hence the response of these cells to DNA damage, may differ from those in somatic cells (Fulka *et al.*, 1994). These differences probably reflect the unique nature of the meiotic cycle (see Bradshaw *et al.*, 1995) and indicate that the response to UV-induced DNA damage may likewise be of a specialized nature in oocytes.

Differences in response to UV irradiation in oocytes is important not only from a cell cycle perspective but also for more practical reasons. The elimination of the chromosome complement of the oocytes is essential for nuclear transplantation. Although physical methods of enucleation are widely used at present, the results of Procháska and Fiser (1995) indicate that this approach adversely affects the ability of the resulting cytoplasm to reprogramme the transplanted nucleus appropriately. Non-invasive enucleation systems have been suggested as a means of overcoming this problem, including the use of chemical agents (Fulka and Moor, 1993) and centrifugation (Tatham *et al.*, 1995). UV irradiation has been suggested both as a method for the full functional enucleation of oocytes (Yang *et al.*, 1990) and for the production of gynogenic oocytes by inactivating sperm DNA (Guo *et al.*, 1993). Although focused UV irradiation effectively inactivates the DNA in bovine oocytes with minimal damage to the cytoplasm, its usefulness in that species is limited because of the concomitant arrest of cell cycle progression (Bradshaw *et al.*, 1995).

The present study was conducted with three specific objectives in mind. The first was to determine whether focused UV irradiation inactivates DNA in pig oocytes without causing general damage to other cellular components. The second was to investigate the ability of irradiated pig oocytes to respond to activation stimuli by inhibiting MPF kinase activity, completing meiosis and supporting pronuclear formation. The third was to

determine the ability of eggs to undergo mitosis and cleavage after irradiation of the metaphase II plate. The pig oocyte was studied not only because of its commercial importance, but also because it provides the closest convenient model to the human oocyte.

Materials and Methods

Collection and culture of oocytes

Pig ovaries were obtained from a local abattoir and transported to the laboratory in a thermocontainer at 24°C. Oocytes were collected by aspiration of antral follicles (2–5 mm diameter) using a 16-gauge needle and syringe. Oocytes were cultured according to the method of Staigmiller and Moor (1984). Briefly, oocytes were washed twice in dissection medium (Hepes-buffered TCM 199; Sigma, St Louis, MO) and transferred to 35 mm Petri dishes containing 2 ml maturation medium (bicarbonate-buffered TCM 199, Sigma) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco-BRL, Burlington, Ontario), 0.3 iu hFSH ml⁻¹ and 0.3 hLH iu ml⁻¹ (Pergonal, Serono, Rome). Two follicle shells prepared from 3–5 mm non-atretic follicles were added to each dish at the initiation of culture, which was thereafter carried out at 38.5°C in 5% CO₂ in humidified air (Mattioli *et al.*, 1988). All oocytes were matured for 44 h and then the cumulus cells were removed. Oocytes that had extruded the first polar body were allocated randomly to either control or UV-irradiated groups. Oocytes allocated to the treated groups were irradiated at 44 h; control and irradiated groups were cultured for a further 8 h (to 52 h) before they were subjected to electrical activation. The culture of oocytes after electrical activation varied from 8 to 72 h (see Tables 1 and 2). Thereafter, eggs and embryos were prepared for morphological or chemical analysis.

UV irradiation

The irradiation protocols were as described Bradshaw *et al.* (1995). Briefly, denuded oocytes were incubated in Hepes-buffered TCM-199 with 10% (v/v) FCS, supplemented with 1 µg bis-benzimide Hoechst stain 33342 ml⁻¹ (Calbiochem, La Jolla, CA) for 5 min at 38.5°C and were then washed four times in TCM-199. Groups of five to ten oocytes were placed in a drop of TCM-199 on a quartz coverslip held in a perspex chamber. A tight focus of the UV light source was obtained by using a ×32 quartz objective (Ultrafluor, Zeiss Inc., Thornwood, NY) placed in a modified inverted epifluorescence microscope (TMD Diaphot, Nikon, London). This was equipped with a 100 W mercury lamp (HBO 100 W/2, Zeiss); all optical elements in the light path were constructed from quartz. A shutter held the following filters in parallel: (i) a total UV light cut-off and (ii) a UV-A filter (330–380 nm excitation). The third position in the shutter was left open for irradiation at 254 nm (UV-C). Individual oocytes were examined using the UV-A filter together with a 420 nm emission filter (UV-2A, Nikon) to identify the position of the Hoechst-stained chromosomes. The use of an adjustable

Table 1. Number of control and ultraviolet-irradiated pig oocytes showing pronuclear development and chromatin defects 8 h after activation

Treatment	Pronuclear formation				Chromatin defects	
	Number of oocytes	Total number	Abnormal pronuclei	Two pronuclei	Non-activated oocytes	Activated oocytes
Activated controls	45	29 ^a	3 ^a	3 ^a	2 ^a	1 ^a
Irradiated and activated oocytes	43	29 ^a	18 ^b	2 ^a	12 ^b	11 ^b

^{ab}Within columns, different letters indicate that means are significantly different ($P < 0.05$).

Table 2. Rate of pronuclear formation and cleavage in electroactivated control and UV-irradiated pig oocytes cultured for 24, 48 or 72 h before examination

Treatment	Time from activation to fixation (h)	Total oocytes	Number of activated oocytes	One-cell (%)	Two-cell (%)	Three- to four-cell (%)
Control	24	21	17 ^a	10 (59) ^a	7 (41) ^a	0 ^a
Irradiated	24	46	29 ^a	29 (100) ^b	0 ^b	0 ^a
Control	48	46	21 ^a	14 (67) ^a	3 (14) ^a	4 (19) ^a
Irradiated	48	20	16 ^a	9 (56) ^a	3 (19) ^a	4 (25) ^a
Control	72	24	12 ^a	6 (50) ^a	3 (25) ^a	3 (25) ^a
Irradiated	72	46	25 ^a	14 (56) ^a	4 (16) ^a	7 (28) ^a

^{ab}Within columns, different superscripts indicate that means are significantly different ($P < 0.05$).

Comparisons were made between control and irradiated oocytes for each period of culture (24, 48 and 72 h).

diaphragm enabled the field of exposure to be restricted to the metaphase plate. Once tight focus on the metaphase chromosomes was achieved, irradiation was performed for 0.5–1.0 s through the UV-C shutter. After irradiation, oocytes were cultured for 4 h (to 48 h) before activation. It was not possible to measure accurately the precise dose of UV at the chromosomes because of the tight focus of the beam (see Bradshaw *et al.*, 1995 for discussion on UV dose monitoring). However, the use of a UVX radiometer (UVP Ltd, Cambridge) equipped with a 254 nm short wavelength sensor and careful standardization of all parameters (for example lamp, filters, quartz objectives and coverslips) enabled the conditions described by Bradshaw *et al.* (1995) to be replicated accurately in all the experiments. It is necessary to make a clear distinction between short wavelength UV light (254 nm) and the longer wavelength UV components of the spectrum, the effects of which on DNA are much more attenuated.

Electrical activation

Oocytes were equilibrated for 5 min in pulse medium (0.28 mmol inositol l⁻¹, 0.1 mmol CaCl₂ l⁻¹, 0.1 mmol MgSO₄ l⁻¹, 10 mmol histidine l⁻¹, pH 7.0) and were then submitted to three DC pulses of 1.0 kV cm⁻¹ field strength for 50 µs at 5 min intervals (Liu and Moor, 1995) using a BTX Electro Cell Manipulator 200 (BTX Inc., San Diego, CA). Oocytes were incubated for a further 8 h to 72 h before they were harvested for morphological or biochemical studies.

Morphological analysis

In the first experiment (see Table 1), both UV-irradiated and control oocytes were activated at 48 h and then cultured for a further 8 h before fixation to analyse pronuclear formation and chromatin cytology. In the second experiment (Table 2) irradiated and control oocytes were cultured for 24, 48 and 72 h after activation to assess rate of cleavage.

Oocytes were fixed for 1 h in a fixative solution of PBS (Oxoid), 4% (w/v) paraformaldehyde and 3 µl Triton-X100 ml⁻¹, and washed three times in PPB (PBS with 1 mg polyvinyl-alcohol (PVA) ml⁻¹ and 1% (w/v) BSA). Oocytes were treated with goat serum for 45 min and incubated overnight in anti-lamin A and C antibody (MRC Laboratory of Molecular Biology, Cambridge) at a 1:10 dilution in a humidified chamber at 4°C. Primary antibody oocytes were washed three times for 15 min in PPB and incubated for 45 min in 2.5% (v/v) fluorescein isothiocyanate (FITC)-conjugated goat anti-IgG. Oocytes were then washed three times in PPB, stained with propidium iodide (200 µg ml⁻¹) for 15 min, washed three times in PPB, and mounted using antifade Vectashield mountant (Miyano *et al.*, 1996). The slides were analysed using a laser scanning confocal microscope (MRC 600, Bio-Rad, Hemel Hempstead).

Histone H1 kinase assay

H1 kinase activity was assayed according to the method of Christmann *et al.* (1994), with minor modifications. Groups of three oocytes were placed in Eppendorf tubes containing

Fig. 1. Confocal photomicrographs of nuclear architecture in control pig oocytes and in oocytes subjected at 44 h after the initiation of maturation to focused 254 nm ultraviolet irradiation. In the first experiment, oocytes were returned to culture for 4 h after (a) irradiation or (b) sham treatment before fixation and staining with anti-tubulin antibody (green) and propidium iodide (red fluorescence). The hypercondensed metaphase chromatin in the irradiated oocyte (a) is in contrast to the normal metaphase II plate in the controls (b). Pronucleus configuration (c) and pronuclear chromatin organization (e) in oocytes irradiated at 44 h, activated by electrostimulation at 48 h, and fixed at 56 h; corresponding pronuclei in sham irradiated oocytes are shown in (d) and (f), respectively. The aberrant pronuclear structure and chromatin organization in irradiated eggs (c,e) is in contrast to the normal pronuclei containing decondensed chromatin observed in sham irradiated eggs (d,f). Pronuclear membranes were stained with fluorescein isothiocyanate (FITC)-labelled anti-lamin A and C (green), while chromatin was stained with propidium iodide (red). Scale bar represents 5 μm .

3 μl collection buffer (6.4 mmol EDTA 1^{-1} , 10 mmol NaF 1^{-1} and 100 mmol Na_3VO_4 1^{-1} in PBS) and stored at -70°C until used for the kinase assay. Samples were collected at 0 h (just before activation), 30 min, 1 h, 2 h, 3 h, 4 h and 8 h after activation. Non-irradiated but activated control oocytes were

collected at the same intervals as the irradiated group. After thawing, 3 μl homogenization buffer (HB) (45 mmol β -glycerophosphate 1^{-1} , 12 mmol *p*-nitrophenylphosphate 1^{-1} , 20 mmol 3-(*N*-morpholino)-propanesulfonic acid 1^{-1} , pH 7.2, 12 mmol MgCl_2 1^{-1} , 12 mmol ethyleneglycol-*bis* (b-amino-

ethylether) *N,N,N',N'*-tetra-acetic acid 1^{-1} , 0.1 mmol ethylenediamine tetra-acetic acid 1^{-1} , 0.8 mmol dithiothreitol (DTT) 1^{-1}) was added to the sample. After incubation at 37°C for 15 min, 4 μ l kinase buffer (KB) was added (45 mmol b-glycerophosphate 1^{-1} , 12 mmol *p*-nitrophenylphosphate 1^{-1} , 20 mmol 3-(*N*-morpholino)-propanesulfonic acid 1^{-1} , pH 7.2, 12 mmol $MgCl_2$ 1^{-1} , 12 mmol ethyleneglycol-*bis* (b-aminoethylether) *N,N,N',N'*-tetra-acetic acid 1^{-1} , 0.1 mmol ethylenediamine tetra-acetic acid 1^{-1} , 0.8 mmol DTT 1^{-1} , 1 mg histone H1 (type III-S from calf thymus) ml^{-1} , 2.2 μ mol protein kinase inhibitor peptide (TTYADFIASGRTGRRNAIHD 1^{-1}) and 1.8 MBq ml [γ - ^{32}P] ATP 1^{-1} (166 TBq mmol 1^{-1}). The reaction was allowed to proceed for 30 min at 37°C and was then terminated by the addition of 10 μ l 2 $\times$ SDS sample buffer (Laemmli, 1970) to a final volume of 20 μ l. The sample was boiled for 5 min at 90°C and then loaded on to a 10% (w/v) polyacrylamide gel for one-dimensional SDS electrophoresis for the separation of the radiolabelled histone H1. After electrophoresis, the gel was dried and the kinase activity was quantitated by direct analysis of radioactivity of the gels using a GS-525 Molecular Imager System (Bio-Rad, Hemel Hempstead). The activity was measured for each sample and an average measure from the experiments was determined. Oocytes at 48 h were arbitrarily designated to represent 100% activity (this stage showed the highest activity); the activities of oocytes at the other stages are presented as a proportion of this activity.

Statistical analysis

The statistical significance of the rate of pronuclear formation, abnormal pronuclear formation, chromatin defects and cleavage was evaluated by the chi-squared test. For H1 kinase activity, analysis of variance was performed using a randomized design with a scheme of parcels subdivided in time, considering the groups as parcels (controls and UV-irradiated oocytes) and time after activation as subparcels (0, 0.5, 1, 2, 3, 4 and 8 h). The data, in percentage, were corrected by the arcsine transformation of the square root of the percentage, and means were compared by Tukey's test. Analysis was carried out using the GLM (general linear model) procedure of the Statistical Analysis System (SAS) software, with a significance level of 5%.

Results

Effects of irradiation on metaphase II plate organization

In the first series of experiments, oocytes were cultured to metaphase II, subjected after polar body extrusion to focused 254 nm UV irradiation, returned to culture for 4 h and fixed for chromosomal analysis. The experiments were carried out on 80 oocytes, 41 of which were UV irradiated and the remainder were controls. Within 4 h, in 20 of 41 irradiated oocytes, the chromatin had undergone clumping and hypercondensation (Fig. 1a). In contrast, metaphase plate organization remained intact over the same period in all the control non-irradiated oocytes (Fig. 1b). After demonstrating that 254 nm UV irradiation induces rapid hypercondensation

and chromatin clumping in a similar manner to that in bovine oocytes (Bradshaw *et al.*, 1995), the second series of experiments was designed to test the hypothesis that UV irradiation at metaphase II would prevent, or significantly delay, the formation of female pronuclei in pig oocytes. In these experiments, irradiated and control oocytes were cultured for 8 h after electrical activation before the oocytes were fixed and stained with lamin antibody and propidium iodide to visualize nuclear membrane formation and chromatin organization, respectively. In addition, the pattern of MPF kinase degradation after activation of control and UV-irradiated oocytes was also assessed.

Effects of irradiation on pronuclear formation

Egg activation. The percentage of irradiated and non-irradiated control eggs showing pronuclear development and chromatin defects is presented (Table 1). Activation, as assessed by pronuclear formation, occurred in 29 of 45 (64.4%) of electrically stimulated control oocytes. A comparable percentage of pronucleate eggs (67.4%) was observed in oocytes irradiated at metaphase II and activated thereafter.

Pronuclear organization. Although pronuclei formed in almost 70% of irradiated eggs, they differed from the controls in a number of important respects. Firstly, a significantly higher proportion ($P < 0.05$) of pronuclei in irradiated oocytes showed structural abnormalities (18 of 29, 62%) compared with controls (3 of 29, 10%). These gross abnormalities were characterized by the formation of multiple pronuclei of different sizes (see Fig. 1c). In contrast, in controls, two pronuclei arising from failure of second polar body extrusion were observed in less than 10% of eggs (Fig. 1d). In addition to the effects on gross pronuclear structure outlined earlier, defects were also observed in the chromatin component within the pronuclei (Table 1). In the irradiated group of pronucleate eggs, almost 40% (11/29) displayed chromatin defects, which included hypercondensation, DNA strand breaks and chromosome dispersal (see Fig. 1c,e). In contrast, almost no chromatin abnormalities (Fig. 1f) were observed in the pronuclei of non-irradiated oocytes (1/29).

Non-activated oocytes. Hypercondensation and other chromosome defects were observed in virtually all (86%) of the irradiated oocytes that failed to respond to the activation stimulus (Table 1). These results are in marked contrast to the control oocytes that failed to activate, in which only 12% showed chromosomal defects.

MPF kinase activity

The effect on MPF kinase activity of activating irradiated or control oocytes was assayed using histone H1 kinase as substrate. In non-irradiated control oocytes, MPF kinase activity decreased sharply after activation; 60% of kinase

Fig 2. Relative changes in histone H1 kinase activity of (□) irradiated and (■) non-irradiated pig oocytes after activation. In each of three replicate experiments, the H1-kinase activity was measured in oocytes before activation (0 h) and at intervals after activation (1–8 h). Within each replicate, the measured activity was expressed as a percentage of that in the non-activated metaphase II group (0 h). The overall mean percentage change in 18 oocytes at each time point is shown.

activity was lost within the first 30 min after electrostimulation. The rate of histone H1 kinase inactivation in irradiated oocytes was similar to that in the controls; there was a rapid initial loss after electrostimulation of approximately 70% of peak metaphase II values in the first 30 min. Basal MPF kinase activity persisted for the ensuing 8 h in both groups of oocytes (Fig. 2).

As expected from the H1 kinase data presented above, B-type cyclin degradation and hyperphosphorylation of the p34^{cdc} catalytic subunit were observed after activation in both irradiated and control pig oocytes (data not shown).

Early mitotic progression

The final series of experiments tested the hypothesis that mitotic cleavage is blocked by the presence of damaged DNA. The results indicate that the effects of irradiation may be to retard rather than to block mitosis (Table 2). A total of 41% of control parthenogenetic eggs had undergone cleavage to the two-cell stage at 24 h after activation. Although there was some progression to the four-cell stage in control groups cultured for 48 or 72 h, the total number of cleaved eggs remained relatively constant at about 40%. In contrast, cleavage had not occurred in the irradiated oocytes at 24 h after activation. Irradiated groups examined at 48 or 72 h after activation were similar to controls: approximately 40% had undergone cleavage, indicating that the delay at 24 h was transient. The morphology of cleaved irradiated oocytes was interesting, as in addition to nucleated blastomeres, a number of micronuclei were also often observed in these eggs (Fig. 3).

Discussion

The results of this study provide information on the effect of focused nuclear irradiation on meiotic progression in pig oocytes. Firstly, the results indicate that short wavelength (254 nm) UV light seriously damages the chromosome

complement. Secondly, destruction of the DNA by focused irradiation appears to occur with minimal damage to stored mRNA, mitochondrial DNA and cytoplasmic activity, as evidenced by subsequent normal synthesis of cell cycle molecules and mitotic progression. Thirdly, UV-damaged DNA in pig oocytes does not activate checkpoint-induced arrest in the transition between metaphase II and anaphase II. Activation induces MPF-kinase destruction, exit from metaphase II and pronuclear formation. Thus a comparison of histone H1 kinase activity in irradiated and control metaphase II oocytes before and after activation shows that the kinetics of inactivation of this enzyme are similar in both groups of oocytes, despite severely damaged chromatin characterized by the dispersal of hypercondensed DNA fragments in the cytoplasm. Finally, the presence of chromosome fragments delays, but does not block, progression of mitosis and cleavage.

Scattered fragments or clumps of DNA were frequently observed as membrane-bound micronuclei in irradiated pig eggs from the pronuclear to the four-cell stage. Similar chromatin clumping was reported in irradiated bovine oocytes (Bradshaw *et al.*, 1995). However, the presence of DNA fragments appears to be correlated with time in culture and would not therefore have been apparent in the study on bovine eggs, which was terminated at 16 h after egg activation. Although the formation of pyrimidine dimers between adjacent bases on the same DNA chain is the primary response to UV irradiation, strand breaks may occur during nucleotide excision repair. DNA repair in metaphase II oocytes is extremely limited (Masui and Pedersen, 1975), but increases substantially during mitosis. It is postulated that the appearance of DNA fragments reflects an increase in strand breaks during increased repair activity in early mitosis.

The use of lamin antibody as one of the probes has enabled comparison of the function of inner nuclear membrane proteins (Peter *et al.*, 1990) in irradiated and control oocytes. It is concluded that proteins associated with nuclear membrane formation are not adversely affected by irradiation and that after activation nuclear membranes are formed rapidly in both treated and control oocytes. It is also clear from the results of this study that the rate at which nuclear membranes form around chromatin is not affected by the state of the DNA; membranes form rapidly around normal, hypercondensed and fragmented chromatin after activation.

In both the present study and that of Kim *et al.* (1996a), a small number of control oocytes (10%) contained two pronuclei of equal size after activation. In contrast, a significantly increased number of irradiated oocytes contained multiple pronuclei of different size. There are at least two explanations that may account for this observation. Firstly, although the damage to the cytoplasm by focused irradiation appears to be very small, it is possible that there is localized damage to the spindle structure (see Kim *et al.*, 1996b for description of pig oocyte spindle). UV damage to microtubules was reported by Zamansky *et al.* (1991). However, the finding that MPF kinase degradation is unaffected by irradiation and occurs in a similar manner in control oocytes after activation (Kikuchi *et al.*, 1995) does not support this proposal. This is important since, in a variety of

Fig. 3. Confocal photomicrographs of cleavage of parthenogenic embryos activated by electrostimulation. (a,b) Control non-irradiated embryos; nuclei in each blastomere are uniform in size. (c,d) Embryos derived from oocytes irradiated 4 h before activation contain nuclei of different sizes at the two-cell and the three- to four-cell stages. Embryos were stained with fluorescein isothiocyanate (FITC)-labelled anti-lamin A and C to highlight nuclear membranes. Scale bar represents 5 μ m.

cells including eggs (Kubiak *et al.*, 1993), unimpeded cyclin proteolysis, and consequent MPF kinase degradation (as observed in this study) occurs only in the presence of an undamaged spindle. A second explanation for the formation of multiple pronuclei in treated eggs is that UV-induced chromosomal fragments become dislodged from the spindle during anaphase and subsequently provide the stimulus for nuclear membrane reassembly and pseudo-pronuclear formation.

The results of this study indicate that MPF kinase degradation after activation in pig oocytes is unaffected by irradiation. This finding is in marked contrast to the study of Bradshaw *et al.* (1995) in which bovine oocytes were irradiated at metaphase II using the same equipment and conditions used in the present study. Exposure of bovine oocytes to 254 nm UV irradiation completely blocked MPF kinase degradation after ethanol-induced activation and prevented female pronuclear formation in the majority of eggs. After fertilization of metaphase II-irradiated bovine eggs, female pronuclear formation was prevented while male pronuclear formation was only slightly reduced compared with controls (53% versus 72%). Since female pronuclear development occurred in irradiated pig oocytes,

it is predicted that male pronuclear development would also occur. The difference in both biochemical and cytological responses to focused UV irradiation in bovine and pig oocytes can be explained by differences in UV sensitivity between the two species. However, the two experiments also differed in that method of activation used (ethanol versus electrical) and this may have influenced development after activation in the two species.

Although oocytes were able to form pronuclei and undergo cleavage to the four-cell stage, there was a delay in the first cell cycle of oocytes subjected to irradiation at metaphase II. The delay is probably due to the irradiation, since exposure to UV leads to unscheduled DNA synthesis in mouse oocytes (Masui and Pedersen, 1975) and to temporary arrest of the cell cycle in many somatic cells to allow repair of the damaged DNA (Hartwell and Weinert, 1989; Herzinger *et al.*, 1995). Although growing oocytes have the ability to repair DNA damage (Ashwood-Smith and Edwards, 1996), this function is reduced or absent during maturation (Masui and Pedersen, 1975). Even though focused UV irradiation causes severe damage to genomic DNA it is probable that most mitochondrial DNA and stored mRNA escapes damage. This conclusion is based on the fact that similar

numbers of irradiated and control pig eggs underwent cleavage to the four-cell stage in the present study. Furthermore, pig eggs are entirely dependent upon stored maternal mRNA for support during this period (Jarrell *et al.*, 1991). In the present study, cleavage of parthenotes did not proceed beyond the four-cell stage in both groups, probably due to both the activation procedure and the culture conditions, which can affect parthenogenetic activation rates (Hagen *et al.*, 1991; Funahashi *et al.*, 1994; Chian and Sirard, 1995). Until normal young are produced, the possibility that irradiation and the presence of damaged DNA has some adverse effects on cleavage at later stages of development cannot be ruled out. Indeed, Guo *et al.* (1993) reported that the introduction of damaged DNA into Pacific oyster oocytes led to lower rates of development. Similarly, in a study on rabbit eggs, Yang *et al.* (1990) observed that cytoplasts prepared by whole cell irradiation underwent cleavage at normal rates to the four-cell stage, but were retarded thereafter.

Full irradiation of somatic cells has a number of effects such as activation of transcription factors, Src tyrosine kinases, calcium signalling pathways, RNA-binding proteins and MAP kinase signalling pathways (Devary *et al.*, 1992; Schieven *et al.*, 1993; Carrier *et al.*, 1994; Dhanwada *et al.*, 1995), which can affect subsequent cell function in several ways. However, in an extensive series of pioneering experiments on nuclear transplantation in *Xenopus*, Gurdon (1986) used UV irradiation for cytoplasm enucleation. Despite the presence of damaged DNA, subsequent development of reconstructed eggs into fully viable young frogs was not compromised. The present results show that UV-irradiated pig oocytes degrade MPF kinase and form pronuclei in a manner that is indistinguishable from controls. However, it is clear that further experiments are required to determine whether pig cytoplasts produced from UV treatment are fully capable of supporting development to term.

C. L. V. Leal was supported by a scholarship granted by CNPq-Brazil, ref. number 200950/94-0, and FAPESP-Brazil, ref. number 93/4323-3. The authors thank Dianne Styles and Karen Waterton for editorial assistance. Anti-lamin A and C antibody was generously donated by Murray Stewart, MRC Laboratory of Molecular Biology, Cambridge, UK.

References

- Ashwood-Smith MJ and Edwards RG (1996) DNA repair by oocytes *Molecular Human Reproduction* **2** 46–51
- Bradshaw J, Jung T, Fulka J, Jr and Moor RM (1995) UV irradiation of chromosomal DNA and its effect upon MPF and meiosis in mammalian oocytes *Molecular Reproduction Development* **41** 503–512
- Carrier F, Gagnon A, Hollander MC, Jeang KT and Fornace AJ, Jr (1994) Induction of RNA-binding proteins in mammalian cells by DNA-damaging agents *Proceedings National Academy of Sciences USA* **91** 1554–1558
- Chian RC and Sirard MA (1995) Effects of cumulus cells and follicle-stimulating hormone during *in vitro* maturation or parthenogenetic activation of bovine oocytes *Molecular Reproduction Development* **42** 425–431
- Christmann L, Jung T and Moor RM (1994) MPF components and meiotic competence in growing pig oocytes *Molecular Reproduction Development* **38** 85–90
- Devary Y, Gottlieb RA, Smeal T and Karin M (1992) The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases *Cell* **71** 1081–1091
- Dhanwada KR, Dickens M, Neads R, Davis R and Pelling JC (1995) Differential effects of UV-B and UV-C components of solar radiation on MAP kinase signal transduction pathways in epidermal keratinocytes *Oncogene* **11** 1947–1953
- Enoch T and Norbury C (1995) Cellular responses to DNA damage: cell cycle checkpoints, apoptosis and the roles of p53 and ATM *Trends in Biochemistry* **20** 426–430
- Fulka J, Jr and Moor RM (1993) Non-invasive chemical enucleation of mouse oocytes *Molecular Reproduction Development* **34** 427–430
- Fulka J, Jr, Bradshaw JP and Moor RM (1994) Meiotic cycle checkpoints in mammalian oocytes *Zygote* **2** 351–354
- Funahashi H, Cantley TC, Stumpf TT, Terlouw SL and Day BN (1994) *In vitro* development of *in vitro*-matured porcine oocytes following chemical activation or *in vitro* fertilization *Biology of Reproduction* **50** 1072–1077
- Glotzer M, Murray AW and Kirschner MW (1991) Cyclin is degraded by the ubiquitin pathway *Nature* **349** 132–138
- Guo X, Hershberger WK, Cooper K and Chew KK (1993) Artificial gynogenesis with ultraviolet light-irradiated sperm in the Pacific oyster, *Crassostrea gigas*. I. Induction and survival *Aquaculture* **113** 201–214
- Gurdon J (1986) Nuclear transplantation in eggs and oocytes *Journal of Cell Science Supplement* **4** 287–318
- Hagen DR, Prather RS and First NL (1991) Response of porcine oocytes to electrical and chemical activation during maturation *in vitro*. *Molecular Reproduction Development* **28** 70–73
- Hartwell LH and Weinert TA (1989) Checkpoints: controls that ensure the order of the cell cycle events *Science* **246** 629–634
- Herzinger T, Funk JO, Hillmer K, Eick D, Wolf DA and Kind P (1995) Ultraviolet B irradiation-induced G2 cell cycle arrest in human keratinocytes by inhibitory phosphorylation of the cdc2 cell cycle kinase *Oncogene* **11** 2151–2156
- Jarrell VL, Day BN and Prather RS (1991) The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, *Sus scrofa*: quantitative and qualitative aspects of protein synthesis *Biology of Reproduction* **44** 62–68
- Kikuchi K, Izaiki Y, Noguchi J, Furukawa T, Daen FP, Naito K and Toyoda Y (1995) Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged *in vitro*. *Journal of Reproduction and Fertility* **105** 325–330
- Kim NH, Moon SJ, Prather RS and Day BN (1996a) Cytoskeletal alteration in aged porcine oocytes and parthenogenesis *Molecular Reproduction Development* **43** 513–518
- Kim NH, Funahashi H, Prather RS, Schatten G and Day BN (1996b) Microtubule and microfilament dynamics in porcine oocytes during meiotic maturation *Molecular Reproduction Development* **43** 248–255
- Kubiak JZ, Weber M, Depennart H, Winston NJ and Maro B (1993) The metaphase-II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin-B in the presence of CSF *EMBO Journal* **12** 3773–3778
- Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of the bacteriophage T4 *Nature* **227** 680–685
- Liu L and Moor RM (1995) Optimisation of electrical activation of porcine oocyte matured *in vitro*. *Journal of Reproduction and Fertility Abstract Series* **15** Abstract 70
- Masui Y and Pedersen RA (1975) Ultraviolet light-induced unscheduled DNA synthesis in the mouse oocytes during meiotic maturation *Nature* **257** 705–706
- Mattioli M, Galeati G and Seren E (1988) Effect of follicle somatic cells during pig oocyte maturation on egg penetrability and male pronuclear formation *Gamete Research* **20** 177–183
- Miyano T, Moor RM, Wooding FBP and Shiroo M (1996) Localization and function of tyrosine phosphorylated protein in pig oocytes *Molecular Reproduction Development* **44** 408–416
- Murray AW (1993) Turning on mitosis *Current Biology* **3** 291–293
- Peter M, Nakagawa J, Doree M, Labbe JC and Nigg EA (1990) *In vitro* disassembly of the nuclear lamina and M-phase specific phosphorylation of lamins by cdc2 kinase *Cell* **61** 591–602
- Procházka R and Fiser PS (1995) Behaviour of blastomere nuclei fused to mouse oocytes is affected by oocyte enucleation and age *Reproduction Nutrition Development* **35** 695–701
- Schieven GL, Kirihaara JM, Gilliland LK, Uckun FM and Ledbetter JA (1993) Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signalling in lymphocytes *Molecular Biology of the Cell* **4** 523–530
- Staigmiller RB and Moor RM (1984) Effect of follicle cells on the maturation

- and developmental competence of ovine oocytes matured outside the follicle *Gamete Research* **9** 221–229
- Tatham BG, Dowsing AT and Trownson AO** (1995) Enucleation by centrifugation of *in vitro* matured bovine oocytes for use in nuclear transfer *Biology of Reproduction* **53** 1088–1094
- Terada Y, Tatsuka M, Jinno S and Okayama H** (1995) Requirements for tyrosine phosphorylation of Cdk4 in G1 arrest induced by ultraviolet irradiation *Nature* **376** 358–362
- Yang X, Zhang L, Kovács A, Tobback C and Foote RH** (1990) Potential of hypertonic medium treatment for embryo micromanipulation II. Assessment of nuclear transplantation methodology, isolation, subzona insertion, and electrofusion of blastomeres of intact or functionally enucleated oocytes in rabbits *Molecular Reproduction Development* **27** 118–129
- Zamansky GB, Perrino BA and Chou IN** (1991) Disruption of cytoplasmic microtubules by ultraviolet radiation *Experimental Cell Research* **195** 269–273