Development 109, 501–507 (1990)
Printed in Great Britain © The Company of Biologists Limited 1990

Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed *in vitro* or *in vivo*

MOHAMMED H. NASR-ESFAHANI[1], JOHN R. AITKEN[2] and MARTIN H. JOHNSON[1]

[1]*Department of Anatomy, Downing Street, Cambridge CB2 3DY, UK*
[2]*MRC Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, UK*

Summary

We describe a fluorimetric method for measuring the level of H_2O_2 in individual mouse oocytes and early embryos. Levels of H_2O_2 are low but detectable in unfertilized oocytes recovered freshly from the female reproductive tract. The levels in early cleaving embryos (1-cell to 8-cell stages) immediately after recovery from the female tract seem to be slightly higher the later the stage examined. However, when embryos are cultured *in vitro* from the 1-cell or early 2-cell stage, H_2O_2 levels rise when the embryos reach the mid-2-cell stage and remain elevated until they enter the early 4-cell stage. No equivalent elevation of H_2O_2 is seen during the transition from 1-cell to 2-cell or from 4-cell to 8-cell stages. Embryos that are able to develop successfully *in vitro*, as well as those that show a developmental block at the 2-cell stage on culture *in vitro*, both show this rise in H_2O_2 levels after *in vitro* culture. The relationship between the rise in H_2O_2 and the '2-cell block' to development is discussed.

Key words: hydrogen peroxide, 2-cell block, oocyte, embryo, oxygen radicals, reactive oxygen species.

Introduction

The culture of preimplantation mammalian embryos from fertilization to the blastocyst stage has not been achieved for most species. In all species studied, evidence has been found for arrest of development at a specific cell stage, e.g. mouse 2-cell (Whittingham, 1974; Goddard and Pratt, 1983), human 4- to 8-cell (Braude *et al.* 1988), hamster 2-cell (Carney and Bavister, 1987; Bavister, 1988), sheep and goat 8- to 16-cell (Sakkas *et al.* 1989), cow 4- to 8-cell (Camous *et al.* 1984) and pig 4-cell (Davis, 1985). For many of these species, the stage of developmental arrest coincides with certain other developmental transitions, including the activation of transcription by the embryonic genome (Bolton *et al.* 1984; Sakkas *et al.* 1988; Braude *et al.* 1988), the selective inactivation or destruction of much of the preexisting maternal mRNA (Bolton *et al.* 1984; Paynton *et al.* 1988), and a transition from extended to shorter cell cycles (Smith and Johnson, 1986; Crosby *et al.* 1988).

The phenomenon of developmental arrest has been studied most extensively in the mouse, in which 1-cell embryos from most outbred and inbred strains do not develop to blastocysts when cultured in a chemically defined medium, but arrest at the 2-cell stage, a phenomenon referred to as the '2-cell block' (Goddard and Pratt, 1983). In contrast, embryos from certain inbred strain crosses can develop into normal blastocysts in the same culture medium. Studies involving reciprocal crosses between different strains of mice suggest that the genotype of the oocyte alone determines whether the embryos block at the 2-cell stage (Goddard and Pratt, 1983; Loutradis *et al.* 1987). The 2-cell block can be overcome by the transfer of a small amount of cytoplasm from a non-blocking strain in the G, or G_2 phase of the second cell cycle to the embryos of a blocking strain. It has been proposed that a specific factor(s) might be absent in blocking strains as the result of a deficiency in the *in vitro* environment (Muggleton-Harris *et al.* 1982; Pratt and Muggleton-Harris, 1988; Muggleton-Harris and Brown, 1988), but no specific agent has been identified. Although the mechanism(s) underlying the 2-cell block in mice is not yet established, it is clear from the studies of cell lines *in vitro* that oxidative species including hydrogen peroxide and free radicals (ie superoxide anions and hydroxy radicals) can be involved in damaging cells. These reactive oxygen species have also been implicated in the damage seen in certain pathological conditions associated with the arrest of cell division and loss of cell function (Halliwell and Gutteridge, 1989; Halliwell, 1987; Aitken and Clarkson, 1987, 1988; Aitken *et al.* 1989). It is possible therefore that such reactive oxygen species might also be involved in the generation of the 2-cell block. In order to examine this hypothesis, we have developed a technique for assessing the level of H_2O_2 in individual oocytes and cleavage stage embryos.

To quantify H_2O_2 in individual oocytes and embryos, we have modified an assay for H_2O_2 in aqueous solution

(Keston and Brandt, 1965; Brandt and Keston, 1965) for use by photocytometry. 2′,7′- dichlorodihydrofluorescein diacetate (DCHFDA) and 5(and 6)carboxy-2,7, dichlorofluorescein diacetate (CDCFDA) are related compounds which, because of their non-ionized state, are membrane permeant and therefore are able to diffuse readily into cells. Within the cell, the acetate groups are hydrolyzed by intracellular esterase activity forming 2′,7′-dichlorodihydrofluorescein (DCHF) and 5(and 6)carboxy-2,7,dichlorofluorescein (CDCF) which are polar and thus trapped within the cell. CDCF is fluorescent at intracellular pH and provides a measure of the uptake and hydrolysis of the dyes. However, DCHF fluoresces only after oxidation by H_2O_2 to yield 2′,7′-dichlorofluorescein (DCF). The level of DCF produced within the cells appears to be related linearly to that of H_2O_2 present (Bass *et al.* 1983, 1986; Cathcart *et al.* 1983) and thus its fluorescent emission provides a measure of H_2O_2 levels. We have applied this technique to embryos from two strains of mice: MF1 (a blocking strain) and F1 (a non-blocking strain) over the period during which the 2-cell block develops. Freshly recovered oocytes and embryos have been compared with those cultured *in vitro* for varying times.

Materials and methods

MF1 female mice (3–4 weeks; Central Biological Services, Cambridge, UK) and F1 female mice (C57BL/10ScSn/Ola female×CBA/Ca/Ola male, bred in the laboratory) were superovulated by intraperitoneal injection of 5 or 10 i.u. of pregnant mare's serum gonadotrophin (PMS; Intervet) and human chorionic gonadotrophin (hCG; Intervet) 48 h apart. To obtain embryos, females were paired individually over night with HC-CFLP males (Interfauna) and inspected for vaginal plugs the next day as an indication of successful mating.

Unfertilized oocytes were recovered from unmated females at about 13 h post-hCG. 2-, 4- and 8-cell embryos were recovered at 36–48, 50–54 and 69–70 h post-hCG respectively. Oocytes and embryos were released from the oviduct into warmed H6+BSA (a Hepes buffered variant of T6), and then cultured in drops of T6+BSA (Howlett *et al.* 1987) under paraffin oil (Martindale), in Falcon tissue culture dishes, in 5 % CO_2 in air. All manipulations were carried out at 37 °C on heated stages, pads or in incubators. Microinjection of 2-cell embryos was carried out as described in McConnell *et al.* (1990).

A stock solution of H_2O_2 (30 % weight/volume) was obtained from BDH plc (Poole; England). Bovine liver catalase was obtained in lyophilized form from Calbiochem (Cambridge Bioscience, UK).

Stock solutions of DCHFDA (Kodak, Eastman Kodak Company, Rochester, NY, USA.) and CDCFDA (Molecular Probes Inc, Pitchford Ave, Eugene, USA) were prepared in acetone at 1×10^{-3} M. The stock solutions were diluted in H6+PVP to the required concentration. The solutions of CDCFDA were prepared freshly before use, since CDCFDA becomes denatured on exposure to air. DCHFDA stock solutions were also prepared just before the start of each experiment and were kept in the dark and used over a maximum period of 48 h. Oocytes and embryos were washed thoroughly in H6+PVP after their removal from H6 or T6+BSA and then loaded with the dyes in cavity blocks for a specified time (generally 15 min). The oocytes or embryos were then washed in H6+BSA to remove traces of the dyes and were placed in specially designed small chambers containing H6+BSA and covered by a coverslip (Maro *et al.* 1984). The fluorescence emissions of the oocytes and embryos were measured immediately by photocytometry, using a perspex carrying slide for viewing with a long working distance×32 objective on a Leitz Ortholux II microscope with stabilized HBO100 mercury vapor lamp and filter set L1 for FITC. For quantification of fluorescence, the photomultiplier housing of a Leitz MPV-L was fitted to the Ortholux III phototube (McConnell *et al.* 1990). The phototube contained a variable measuring diaphragm that could be adjusted to surround the periphery of an individual oocyte or embryo, thus excluding background. A 6.25 % transmission neutral density filter (Leitz N16) was placed in the path of the exciting light, to minimise any potential damage to the cells. Fluorescent emission was deflected to the amplifier/discriminator (Model 1140B, SSR Instruments Co, USA) of a quantum photometer (Model 1140A, SSR Instruments) that had been zeroed against background and set to read in a counts s^{-1} mode *via* a deflection meter. The individual oocyte or embryo was positioned within the adjusted diaphragm and exposed to the excitation wavelength for a period of less than 10 s and the fluorescent emission recorded (counts s^{-1} on the 1 M scale to a maximum reading of 10). This set up, involving short exposure to the exciting light was designed to avoid damage to the oocytes or embryos, which was only detected with greater than a 40 s exposure to the exciting light or removal of the 6.25 % transmission neutral density filter. Under these latter conditions, a rapid rise in fluorescent emission, reflecting conversion of DCHT to DCF, was detected, probably as a secondary consequence of lipid peroxidation. For each data point in each experiment, the fluorescent emissions of 10 to 20 oocytes or embryos were measured and their mean values were expressed as the 'mean reading'.

Results

A technique for measuring H_2O_2 in individual mouse embryos

Experiments were designed to observe whether we could measure a fluorescence signal from DCF or CDCF in single embryos. Within 1 h of their removal from the oviducts, freshly recovered oocytes and embryos were incubated for 15 min in the dyes, washed and their fluorescent emission measured immediately. Fig. 1 shows that the level of CDCF fluorescence remains constant from the unfertilized oocyte to the 8-cell stage. This result suggests that the uptake and de-esterification of the dyes does not vary with developmental stage. In contrast, the fluorescence from DCF is much lower and appears to increase slightly between the unfertilized oocyte and 8-cell stages, the biggest change appearing during the transition from the 1- to 2-cell stage, but even then does not approach the level of fluorescence generated by incubation in equimolar concentrations of CDCFDA. Since any fluorescence deriving from incubation in DCHFDA requires an oxidative step, these results suggest (a) that such H_2O_2 as is produced is insufficient to oxidize all the DCHF generated by esterase activity, (b) that the level of H_2O_2 may increase slightly with the more advanced developmental stages, and (c) that the increase ob-

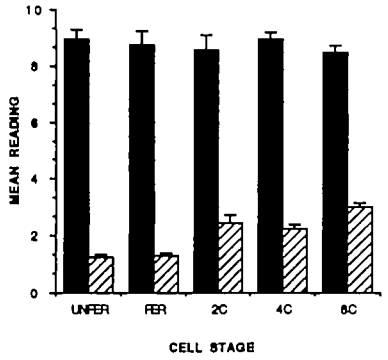

Fig. 1. Unfertilized MF1 oocytes and embryos were recovered; within one hour of recovery, they were incubated for 15 min in DCHFDA or CDCFDA at 1×10^{-5} M and the fluorescent emissions of DCF (hatched bar) and CDCF (solid bars) were measured immediately. S.D.s are shown. Unfer=unfertilized oocyte; Fer=fertilized oocyte; 2C, 4C and 8C=2-, 4-, 8-cell stages.

served is not due simply to an increased rate of uptake and/or hydrolysis of the dyes.

Kinetic studies of (i) the rate of dye uptake and conversion and (ii) the rate of decay of fluorescent signal after a loading period followed by transfer to washing medium were carried out on unfertilized oocytes, fertilized oocytes and 2-cell embryos of both MF1 and F1 strains of mice. Fig. 2 (panels A–C) show typical plots obtained from kinetic studies of the three different cell stages with embryos incubated in CDCFDA or DCHFDA. The 'increase in reading' from the fluorescent derivatives of CDCFDA and DCHFDA remains approximately the same at each of the three different stages. However, the rise in signal after incubation in DCHFDA is much slower than that seen from oocytes and embryos incubated in one tenth the concentration of CDCFDA because of the requirement for oxidation of the DCFH. In contrast, the decay of signals for both dyes is less after the transition from the 1- to 2-cell stage, a result that probably reflects reduced leakage of the dyes from the cells. This result might suggest that the apparent slight rise in DCF levels between the 1- and 2-cell stages described earlier (Fig. 1) could be due simply to a decrease in decay rate at the 2-cell stage. However, there are two reasons why this cannot be the case. First, examination of the fluorescent signal resulting from incubation in CDCFDA reveals similar kinetic decay patterns to those seen after incubation in DCHTDA, but no increase in fluorescence from CDCF occurs during the transition from the 1- to 2-cell stage. Second, all experimental fluorescence readings are taken after a 15 min incubation in dye and within 5 min of removal from the dye, in which time the decay of signal does not differ between stages. Thus, the increased fluorescence emission from DCF at the 2-cell stage cannot be explained simply on the basis of greater retention of the fluorochrome, but must also involve greater production of DCF as a result of oxidation by H_2O_2 (see also below).

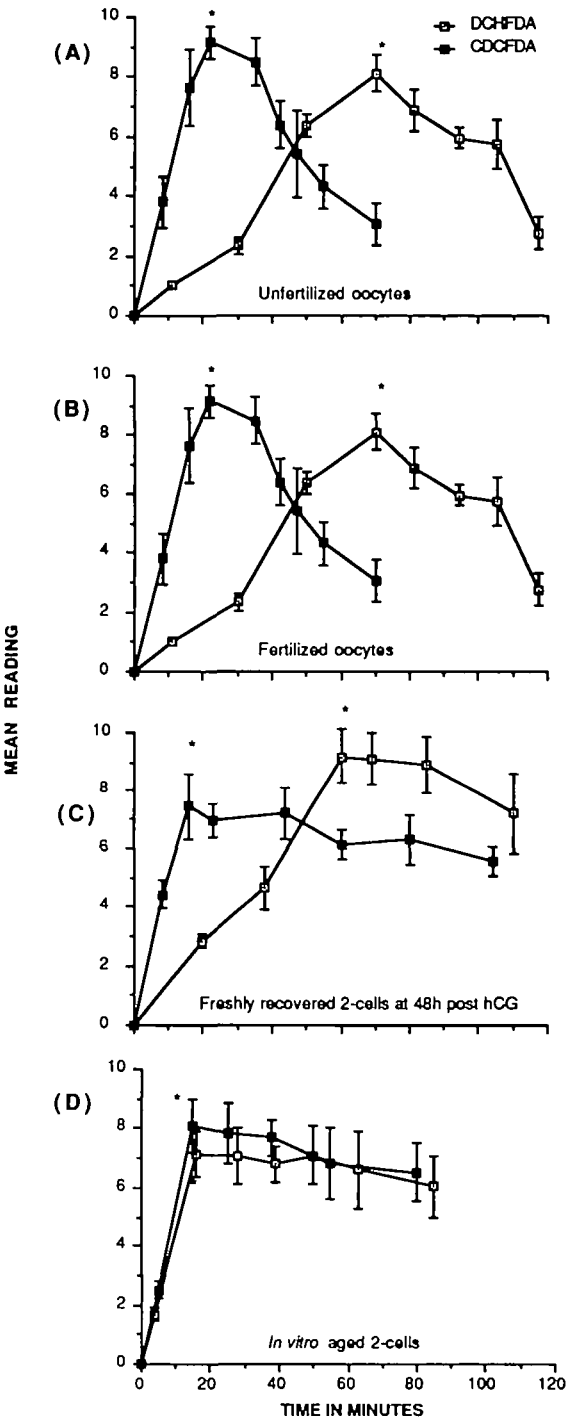

Fig. 2. MF1 (A) unfertilized oocytes or (B) fertilized oocytes or (C) fresh 2-cell embryos or (D) *in vitro* aged 2-cell (cultured from 30–48 h post hCG) embryos were incubated with CDCFDA (5×10^{-6} M) or DCHTDA (1×10^{-5} M) for a period, the end of which is indicated by the astrisk (*). Then the embryos were washed in and transferred to H6+BSA and the amount of CDCF or DCF present was measured at different time points. Each point on the graph presents the mean reading for 10 embryos (±S.D.).

Table 1. *Effects of exogenous hydrogen peroxide or lipid peroxidation by UV irradiation on DCF and CDCF emission in fertilized MF1 oocytes*

Treatment	Mean reading (in units) after incubation in* DCHFDA (0.00001 M)	CDCFDA (0.00001 M)
Control	2.5 (0.4)	8.3 (1.2)
UV Irradiation	9.0 (0.7)	8.3 (1.2)
Hydrogen peroxide:		
0.0003 %	2.1 (0.6)	8.8 (0.8)
0.003 %	9.2 (1.2)	9.4 (0.7)

* Values are mean for readings from 10 fertilized oocytes (plus S.D.).

In order to establish further that our method was providing a quantitative measure of H_2O_2 levels in mouse embryos, we tried to enhance DCF formation from DCFH either by the addition of exogenous H_2O_2 or by Lipid-peroxidation using high levels of UV radiation which increase intracellular peroxide levels. The results in Table 1 show that the DCF formation is increased if an extracellular threshold level of H_2O_2 greater than 0.0003 % by volume is achieved or if lipid peroxidation is induced. Moreover, the signal from DCF under such conditions approximates to that of CDCF at the same concentration. Addition of catalase blocked the increased DCF formation induced by exogenous H_2O_2 but not that intrinsic to the cell or induced by UV radiation (Table 2). In contrast, endogenous DCF formation was reduced significantly (paired student *t*-test; $P<0.02$) if catalase was injected into one blastomere prior to incubation in DCHFDA (Table 2).

Table 2. *Fertilized MF1 oocytes were incubated in DCHFDA under various conditions and the emission from DCF recorded*

Treatment	Mean reading (in units) after incubation in DCHFDA at 0.00001 M for 15 minutes
Fresh fertilized oocytes:	
Control	1.4 (0.2)
Hydrogen peroxide (0.003 %)	10.0*
Hydrogen peroxide+catalase	1.0 (0.1)
UV Irradiation	10.0*
UV Irradiation+catalase	10.0*
2-cells:	
Fresh 2-cells	2.4 (0.3)
2-cells cultured from 28–42 h post hCG**	9.2 (0.6)
2-cells cultured from 33–49 h post hCG:	
Microinjected with water	6.2 (0.7)
Microinjected with 0.5 mg/ml catalase	4.5 (0.4)

Values are mean for the reading from 10 eggs (plus S.D.).
* The readings were off scale in excess of 10.
** Cultured in catalase for one hour before incubation in DCHFDA.

Comparison of H_2O_2 production in vitro *and* in vivo

Since DCHFDA appears to provide a valid method for the quantitative analysis of H_2O_2 production in individual mouse embryos at different times over the early cell cycles, we compared the fluorescent emission of embryos developed *in vitro* with those developed *in vivo* in two strains of mice, only one of which (MF1) shows the 2-cell block. Figs 3 and 4 show (a) that, as suggested earlier, there may be a slight rise in DCF formation when freshly recovered 2-cell embryos are compared with those freshly recovered at 1-cell stage; and (b) that in both MF1 (Fig. 3) and F1 (Fig. 4) embryos the fluorescence emission from DCF increases markedly during G_2 of the second cell cycle when embryos are cultured *in vitro* but not when they have

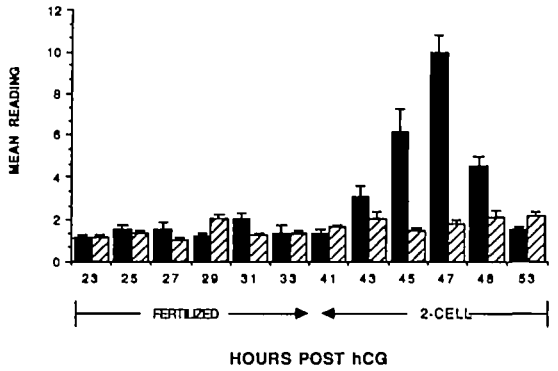

Fig. 3. Freshly recovered MF1 embryos (hatched bars) and MF1 embryos cultured *in vitro* from 23 h post-hCG (solid bars) were incubated for 15 min in DCHFDA (1×10^{-5} M) at various times post hCG. Each bar represents the mean reading of DCF emission for 10 embryos plus S.D. Between 23 and 33 h post hCG all the readings were from 1-cell fertilized oocytes. Thereafter all the readings were from 2-cell embryos. Note that in non-blocking strains, embryos start dividing to 4-cells at shortly after 48 h post hCG (see Fig. 4) but do not do so in blocking strains such as MF1.

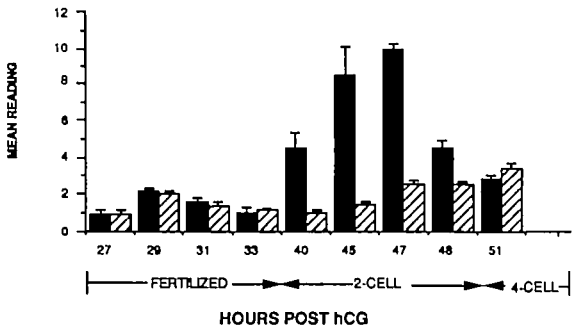

Fig. 4. Freshly recovered F1 embryos (hatched bars) and F1 embryos cultured *in vitro* (solid bars) were incubated for 15 min in DCHFDA (1×10^{-5} M) at various times post hCG. Each bar represents the mean reading of DCF emission for 10 embryos plus S.D. Between 27 and 33 h post hCG all reading were from 1-cell fertilized oocytes. From 40 to 48 h post hCG, all reading were from 2-cell embryos, thereafter only 4-cell embryos were read.

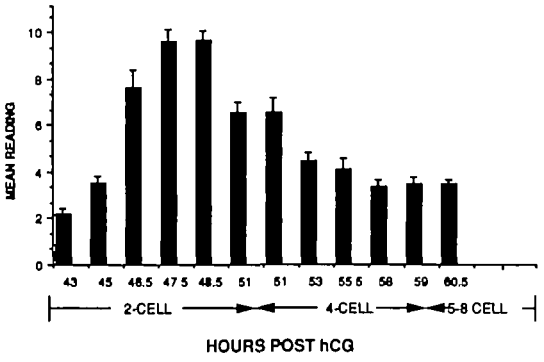

Fig. 5. F1 embryos were recovered at 43 h post hCG and cultured *in vitro* for periods up to 60.5 h post hCG. Samples of 10 embryos were taken at the times indicated, and incubated in DCHFDA at 1×10^{-5} M for 15 min. The mean values of DCF emission plus S.D. are shown. The reading are from 2-cell, 4-cell or 5–8 cell embryos for each bar as indicated beneath.

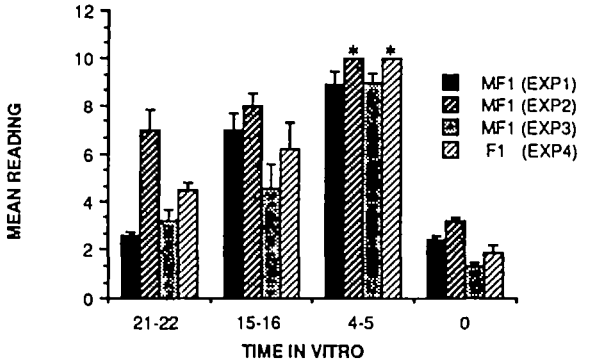

Fig. 7. MF1 and F1 embryos were recovered at various times post hCG, were cultured *in vitro* for 21–22 h, 15–16 h, 4–5 h or 0 h, and were then incubated for 15 min in DCHFDA (1×10^{-5} M) at 45–47 h post hCG (which=time 0 h). Each bar represents the mean reading for 10–15 embryos. S.D. shown except in two bars (*) for which all readings were off scale in excess of 10.

developed *in vivo*.The DCF fluorescence from *in vitro* cultured embryos declines during late G_2/M (or its equivalent in developmental time in blocking embryos), and is restored to background levels on entry into the 4-cell stage. When embryos were incubated *in vitro* for a longer period through the third mitotic division into the 8-cell stage, no equivalent marked rise in DCF formation occurred (Fig. 5 shows the data for F1 embryos but the same result has been obtained with MF1 embryos – data not shown). The higher DCF levels after *in vitro* culture are due to an increase in the rate of its formation from DCHF oxidation (compare panels C and D in Fig. 2). In contrast to the results with DCHFDA, fluorescence from embryos incubated in CDCFDA is similar throughout whether they have developed *in vitro* or *in vivo*, indicating that differences in uptake, de-esterification and decay of the dyes cannot be responsible for the change in DCF fluorescence described above (Fig. 6 and compare panels C and D in Fig. 2).

To determine whether the absolute time spent *in vitro* influences the pattern of DCF fluorescence emission, embryos were cultured for different periods *in vitro* but

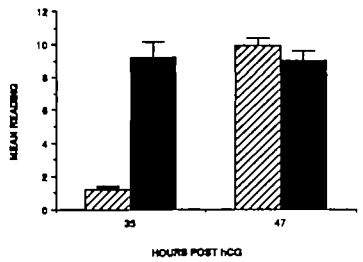

Fig. 6. MF1 embryos were recovered and used immediately at 35 h post hCG or cultured until 47 h post hCG before use. Both sets of embryos were incubated for 15 min in CDCFDA or DCHFDA at 1×10^{-5} M. Each bar represents the mean reading of CDCF (solid bars) or DCF (hatched bars) emission for 10 embryos plus S.D.

were assayed at the same time e.g. 45–47 h post-hCG (Fig. 7). The results from this experiment show that, regardless of the time spent *in vitro*, the H_2O_2 levels are elevated by 45–47 h, corresponding to the late G_2 period of the second cell cycle. Paradoxically, in the experiments recorded here some of those embryos that had spent the longest time *in vitro* (21–22 h) showed the least marked rise in H_2O_2 production. This may reflect the fact that their development had been slowed and they were reaching the G_2 period of the second cell cycle slightly later than the other groups. In confirmation of this, we have shown in subsequent experiments that the H_2O_2 levels in these embryos rose to maximal 2–3 h later than those in the other two groups.

Discussion

Hydrogen peroxide has been proposed as a second messenger in various cell stimulation and regulation systems (May and deHaen, 1979; Oberley *et al.* 1981; Skoglund *et al.* 1988; Laloraya *et al.* 1988, 1989), but may also, if the regulation of its levels is uncontrolled, contribute to cell damage by excessive peroxidation of lipids and proteins. In this paper, we describe a method for quantifying the production of reactive oxygen species in individual cells or embryos, thereby allowing us to examine the changes in these species over the early period of development.

The results presented here suggest that the amount of fluorescence emission from individual mouse oocytes and embryos is related to the rate of oxidation of DCHF by H_2O_2 produced within the oocytes and embryos. Thus, the fluorescence emission of DCHF but not that of CDCF can be enhanced by exogenous H_2O_2 or *via* lipid-peroxidation induced by irradiation with UV light. The signal from DCHF oxidation under such conditions approximates to that of CDCF. These results with single cells and embryos resemble those obtained fluorimetrically by Bass *et al.* (1986) using suspensions of polymorphonuclear neutrophilic leukocytes (PMNL).

These workers showed that the rate of DCF formation was related linearly to that of H_2O_2 production, that the increase in the rate of DCF formation after activation of PMNL could be mimicked by addition of extracellular H_2O_2 or an H_2O_2 generating system (e.g. xanthine-oxidase+acetaldehyde), and that the increase seen with exogenous stimulation was inhibited by catalase but not by superoxide dismutase (SOD). We also were able to inhibit the oxidising effect of exogenous H_2O_2 with catalase, and the H_2O_2 generated within the cell was reduced by intracellular injection of catalase. Thus, it seems reasonable to conclude that the changing levels of DCF formation reflect changes in H_2O_2 levels in oocytes and embryos.

Our preliminary results suggested that there was a gradual increase in H_2O_2 levels as embryos proceeded from the unfertilized stage toward the 8-cell stage. However, a more detailed analysis of H_2O_2 levels in embryos freshly recovered from mice over the first three cell cycles *(in vivo* embryos) suggested that this increase may not be as marked as at first evident and could reflect, at least in part, the consequences of exposure of embryos to conditions *in vitro* during recovery and handling. Thus, when analysis of *in vivo*-derived embryos is conducted rapidly after their recovery (as shown in Figs 3, 4), the rise in H_2O_2 levels on transition to the 2- and 4-cell stages is much reduced. In contrast, when embryos spend a period *in vitro* before being incubated with fluorochrome, a marked rise in the fluorescent signal occurs, reflecting increased conversion of DCHF to DCF by oxidation. However, this increase in oxidative conversion *in vitro* is restricted to the G_2/M phase of the second cell cycle, but is not evident before this time, nor after entry to the 4-cell stage is completed, nor during passage through the 4-cell stage and into the 8-cell stage. This temporal restriction on DCF formation is not a consequence of the total time spent *in vitro*, but reflects an interaction between *in vitro* conditions and the stage of the cell cycle. The possibility that this increase was due simply to a greater retention of DCF was excluded by comparing the kinetics of handling of the two dyes by the embryos.

This increase in H_2O_2 production *in vitro*, compared with *in vivo* derived embryos, takes place during the atypically long G_2 period of the second cell cycle, shortly after the time at which embryonic gene activation has occurred. This period coincides with that of the 2-cell block, suggesting a possible relationship between the block and the potential rise in damaging free radicals generated from the H_2O_2. Indeed, it has been proposed that activated oxygen species may arrest normal cell division (Oberley *et al,* 1981). However, when F1 embryos, which did not exhibit a 2-cell block *in vitro*, were compared with MF1 embryos, which did, both showed a rise in peroxide production in the latter half of the second developmental cell cycle. Thus, the absence of reactive oxygen species as a source of damaging free radicals cannot provide the simple explanation for the development of non-blocking strain embryos *in vitro*. However, it is possible that the MF1 embryos, unlike the F1 embryos, are less able to scavenge the reactive oxygen species induced by *in vitro* culture. The inability of MF1 embryos to develop normally *in vitro* might be due to a deficiency of cytoplasmic protective enzyme activity, such as is provided by superoxide dismutase, catalase or the glutathione peroxide/reductase couple.

In the context of these results, it is of interest that the development of preimplantation mouse embryos *in vitro* is sensitive to oxygen concentration, raising the possibility that oxygen toxicity might lead to developmental arrest *via* formation of oxygen radicals (Whitten, 1971; Quinn and Harlow, 1978; Pabon *et al.* 1989). Nonphysiological oxygen concentrations can lead to free radical generation *via* interaction with a number of cellular or media components, including hypoxanthine (Loutradis *et al.* 1987), catecholamines (Misra and Fridovich, 1972), thiols (Baccanari, 1978) and flavin (Ballou *et al.* 1969). An adverse effect of hypoxanthine on mouse embryo development was reported recently (Loutradis et *al.* 1987). Additionally, high oxygen tensions *in vitro* can influence the balance between the synthesis of glycogen (from exogenous glucose) and its degradation, probably *via* accumulation of ATP (Ozias and Stern, 1973; Quinn and Wales, 1973; Barbehenn *et al.* 1974; Edirisinghe *et al.* 1984; Spielmann *et al.* 1984). These disturbances in glucose metabolism could lead to the formation of free radicals by generating NADPH *via* the pentose phosphate shunt, indeed glucose has been reported as deleterious for development through the 2-cell stage, its omission from the media improving development (Flood and Wiebold, 1988; Chatot *et al.* 1989). Finally, it may also be relevant that embryos blocked at the 2-cell stage show abnormalities in the organization of their newly synthesised membrane lipids and in their mitochondria (Muggleton-Harris and Brown, 1988; Pratt and George, 1989), both important targets for damage by free radicals.

We thank our colleagues in the Embryo and Gamete Research Group for their support, in particular Martin George and Brendan Doe. The work was supported by grants from Medical Research Council to MHJ and Dr PR Braude and the Cancer Research Campaign to MHJ and Dr J. McConnell.

References

Aitken, R. J. and Clarkson, J. S. (1987). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fert.* **81**, 459–469.

Aitken, R. J. and Clarkson, J. S. (1988). Significance of reactive oxygen species and antioxidant in defining the efficacy of sperm preparation techniques. *J. Andrology* **9**, 367–376.

Aitken, R. J., Clarkson, J. S. and Fishel, S. (1989). Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol. Reprod.* **41**, 183–194.

Baccanari, D. P. (1978). Coupled oxidation of NADPH with thiols at neutral pH. *Arch. Biochem. Biophys.* **191**, 351–357.

Ballou, D., Palmer, G. and Massey, V. (1969). Direct demonstration of superoxide anion production during the oxidation of reduced flavin and its catalytic decomposition by erythrocuprein. *Biochem. Biophys. Res. Commun.* **36**, 898–904.

Barbehenn, E. K., Wales, R. G. and Lowry, O. H. (1974). The explanation for the blockade of glycolysis in early mouse embryos. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1056–60.

Bass, D. A., Prace, W. J., Dechatelet, L. R., Szejda, P., Seeds, M. C. and Thomas, M. (1983). Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J. Immunology* **130**, 1910–1917.

Bass, D. A., Olbrantz, P., Szejda, P., Seed, M. C. and McCall, C. E. (1986). Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. *J. Immunology* **136**, 860–866.

Bavister, B. D. (1988). Role of oviductal secretions in embryonic growth in vivo and vitro. *Theriogenology* **29**, 143–154.

Bolton, V. N., Oades, P. J. and Johnson, M. H. (1984). The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. *J. Embryol. exp. Morph.* **79**, 139–163.

Brandt, R. and Keston, A. S. (1965). Synthesis of diacetyldichlorofluorescin: a stable reagent for fluorometric analysis. *Anal. Biochem.* **11**, 6–9.

Braude, P., Bolton, V. and Moore, S. (1988). Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332**, 459–456.

Camous, S., Heyman, Y., Meziou, W. and Menezo, Y. (1984). Cleavage beyond the clock stage and after transfer of early bovine embryos cultured with trophoblastic vesicles. *J. Reprod. Fert.* **72**, 479–485.

Carney, E. W. and Bavister, B. D. (1987). Stimulatory and inhibitory effects of amino acids on the development of hamster eight-cell embryos in vitro. *J. in vitro Fert. & Embryo Transfer* **4**, 162–167.

Cathcart, R., Schwiers, E. and Ames, B. N. (1983). Detection of picomole levels of hydroperoxides using a fluorescein assay. *Anal. Biochem.* **134**, 111–116.

Chatot, C. L., Ziomek, B. D., Lewis, J. L. and Torres, I. (1989). An improved culture medium supports development of random-berd 1-cell mouse embryos in vitro. *J. Reprod. Fert.* **86**, 679–688.

Crosby, I. M., Gandolfi, F. and Moor, R. M. (1988). Control of protein synthesis during early cleavage of sheep embryos. *J. Reprod. Fert.* **82**, 769–775.

Davis, D. L. (1985). Culture and storage of pig embryos. *J. Reprod. Fert. Suppl.* **33**, 115–124.

Edirisinghe, W. R., Wales, R. G. and Pike, I. L. (1984). Degradation of biochemical pools labelled with [^{14}C]glucose during culture of 8-cell and morula-early blastocyst stage mouse embryos *in vitro* and *in vivo*. *J. Reprod. Fert.* **72**, 59–65.

Flood, M. R. and Wiebold, J. L. (1988). Glucose metabolism by preimplantation pig embryos. *J. Reprod. Fert.* **84**, 7–12.

Goddard, M. J. and Pratt, H. P. M. (1983). Control of events during early cleavage of the mouse embryo: an analysis of the "2-cell block". *J. Embryol. exp. Morph.* **73**, 111–133.

Halliwell, B. and Gutteridge, J. M. C. (1989). *Free Radicals in Biology and Medicine*. 2nd edition. Oxford University Press.

Halliwell, B. (1987). Superoxide-dependent formation of hydroxy radicals in presence of iron chelates. *FEB letters* **92**, 321–326.

Howlett, S. K., Barton, S. C. and Surani, M. A. (1987). Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos. *Development* **101**, 915–923.

Keston, A. S. and Brandt, R. (1965). The fluorometric analysis of ultramicro quantities of hydrogen peroxide. *Anal. Biochem.* **11**, 1–5.

Laloraya, M., Kumar, G. P. and Laloraya, M. M. (1988). Changes in the levels of superoxide anion radical and superoxide dismutase during the estrous cycle of Rattus norvegicus and induction of superoxide dismutase in rat ovary by lutropin. *Biophys. Res. Commun.* **157**, 146–153.

Laloraya, M., Kumar, G. P. and Laloraya, M. M. (1989). Histochemical study of superoxide dismutase in the ovary of the rat during the oestrous cycle. *J. Reprod. Fert.* **86**, 583–587.

Loutradis, D., John, D. and Kiessling, A. A. (1987). Hypoxanthine causes a 2-cell block in random-bred mouse embryos. *Biol. Reprod.* **37**, 311–316.

Maro, B., Johnson, M. H., Pickering, S. J. and Flach, G. (1984). Changes in actin distribution during fertilization of the mouse egg. *J. Embryol. exp. Morph.* **81**, 211–237.

May, J. M. and deHaen, C. (1979). Insulin-stimulated intracellular hydrogen peroxide production in rat epididymal fat cells. *J. biol. Chem.* **224**, 6049–6055.

McConnell, J., Pickering, S. J., Johnson, M. H. and Bashford, J. (1990). A technique for quantifying the amount of macromolecule injected into cells of the early mouse embryo. *J. Reprod. Fert.* **88**, 375–381.

Misra, H. P. and Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. biol. Chem.* **247**, 2170–2178.

Muggleton-Harris, A. L. and Brown, J. J. G. (1988). Cytoplasmic factors influence mitochondrial reorganization and resumption of cleavage during culture of early mouse embryos. *Human Reprod.* **3**, 1020–1028.

Muggleton-Harris, A. L., Whittingham, D. G. and Wilson, L. (1982). Cytoplasmic control of preimplantation development *in vitro* in the mouse. *Nature* **299**, 460–462.

Oberley, L. W., Oberley, T. D. and Buettner, G. R. (1981). Cell division in normal and transformed cells: The possible role of superoxide and hydrogen peroxide. *Med. Hyp.* **7**, 21–42.

Ozias, C. B. and Stern, S. (1973). Glycogen levels of preimplantation mouse embryos developing *in vitro*. *Biol. Reprod.* **8**, 467–479.

Pabon, J. E., Findly, W. E. and Gibbons, M. D. (1989). The toxic effects of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. *Fertil. Steril.* **51**, 896–900.

Paynton, D. V., Rompel, R. and Bachvarova, R. (1988). Changes in state of adenylation and time course of degradation of maternal mRNA during oocyte maturation and early embryonic development in the mouse. *Devl Biol.* **129**, 304–314.

Pratt, H. P. M. and George, M. A. (1989). Organisation and assembly of the surface membrane during early cleavage of the mouse embryo. *Roux's Arch. devl Biol.* **198**, 170–178.

Pratt, H. P. M. and Muggleton-Harris, A. L. (1988). Cycling cytoplasmic factors that promote mitosis in the cultured 2-cell mouse embryos. *Development* **104**, 115–120.

Quinn, P. and Harlow, G. M. (1978). The effect of oxygen on the development of preimplantation mouse embryos in vitro. *J. exp. Zool.* **206**, 73–80.

Quinn, P. and Wales, R. G. (1973). The effect of culture *in vitro* on the levels of adenosine triphosphate in preimplantation mouse embryos. *J. Reprod. Fert.* **32**, 231–241.

Sakkas, D., Batt, P. A. and Cameron, A. W. N. (1989). Development of preimplantation goat (Capra hiracus) embryos *in vivo* and *in vitro*. *J. Reprod. Fert.* **87**, 359–356.

Skoglund, G., Contgreave, I., Rincon, J., Patarroyo, M. and Ingelman-Sundberg, M. (1988). H_2O_2 activates CD11/CD18-dependent cell adhesion. *Biochem. Biophy. Res. Comm.* **157**, 443–449.

Smith, R. K. W. and Johnson, M. H. (1986). Analysis of the third and fourth cell cycles of mouse early development. *J. Reprod. Fert.* **76**, 393–399.

Spielmann, H., Jacob-Mueller, U., Schultz, P. and Schimmel, A. (1984). Changes in the adenine ribonucleotide content during preimplantation development of mouse embryos *in vivo* and *in vitro*. *J. Reprod. Fert.* **71**, 467–473.

Whitten, W. K. (1971). Nutrient requirements for the culture of preimplantation embryos *in vitro*. *Adv. Biosci.* **6**, 129–141.

Whittingham, D. G. (1974). Fertilization, early development and storage of mammalian ova in vitro. In *The Early Development of Mammals* (ed. M. Balls and A. E. Wild), pp. 1–24. Cambridge University Press.

(*Accepted 2 April 1990*)