Damage of embryo development caused by peroxidized mineral oil and its association with albumin in culture

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Objectives: To examine the effect of free radicals from peroxidized oil and the role of albumin on the passage of radicals.
Design: Prospective study.
Setting: Clinical IVF laboratory and university department.
Patient(s): Blood samples were donated by laboratory staff.
Intervention(s): Examination of the effects of mineral oil samples with various peroxide value (POV) on culture of erythrocytes and on the passage of a lipophilic tracer, DiI, into the zona pellucida.
Main Outcome Measure(s): Time required for hemolysis of red blood cells by peroxidized oil, staining of zona pellucida from human oocytes and embryos by lipophilic tracer, and POV analysis of mineral oil samples in relation to various storage conditions.
Result(s): The time for hemolysis was related to the POV levels of oil samples covering the culture medium. Albumin in the medium facilitated hemolysis and the passage of DiI into the zona. Peroxidized oil (POV >0.02 meq/kg) blocked the entry of DiI into the zona.
Conclusion(s): The presence of albumin in the medium was associated with the entry into the human zona of agents present in peroxidized mineral oil. This process and variable oil peroxidation could be deleterious to embryos in culture. (Fertil Steril® 2009;91:1745–9. ©2009 by American Society for Reproductive Medicine.)

Key Words: Peroxidation, mineral oil, free radicals, albumin, human zona pellucida

We previously reported that peroxidation of mineral oil used in droplet culture had adverse effects on fertilization and embryo development (1). Because significant peroxide elevation was detected in unopened mineral oil samples, we proposed that a precise evaluation of mineral oil deterioration with time after manufacture is required before the oil is used by IVF laboratories or distributed by the supplier (1).

In the present study, we further investigated the effect of free radicals derived from oil peroxidation and examined the possible role of albumin in the passage of free radicals to oocytes or embryos via the zona pellucida. Because albumin has lipophilic and hydrophilic binding sites, washing an oil with a medium containing albumin may eliminate toxic lipophilic and hydrophilic contaminations from mineral oil. Also, mineral oil washing with albumin medium may avoid absorption of fatty acids present in the culture medium by the mineral oil. Some suppliers provide mineral oil that has been washed with culture medium containing albumin. However, it has been reported that paraffin oil washed with medium that contained albumin became toxic to mouse embryos upon its exposure to sunlight for 4 h (2).

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Mineral oil is produced from crude oil. If the purification process of the mineral oil is not closely controlled, contamination with multicyclic aromatic hydrocarbons, unsaturated hydrocarbons, and aromatic hydrocarbons may occur. When these unsaturated hydrocarbons are present in mineral oil, it becomes susceptible to peroxidation. It is likely that the embryo culture problem that we previously encountered with oil (1) could also occur with a variety of other mineral oil samples being supplied by various companies. Therefore, in the present study, the peroxide value (POV) of mineral oil samples supplied by nine companies was examined upon delivery and after periods of storage. For most mineral oil samples, the suggested expiry period is 1–2 years after production. However, as we previously reported, the POV of mineral oil that had been stored for 6 months was 0.12 meq/kg (1), which can adversely affect blastocyst development. Therefore, the expiry date should be monitored, and quality control of mineral oil used for embryo culture is required.

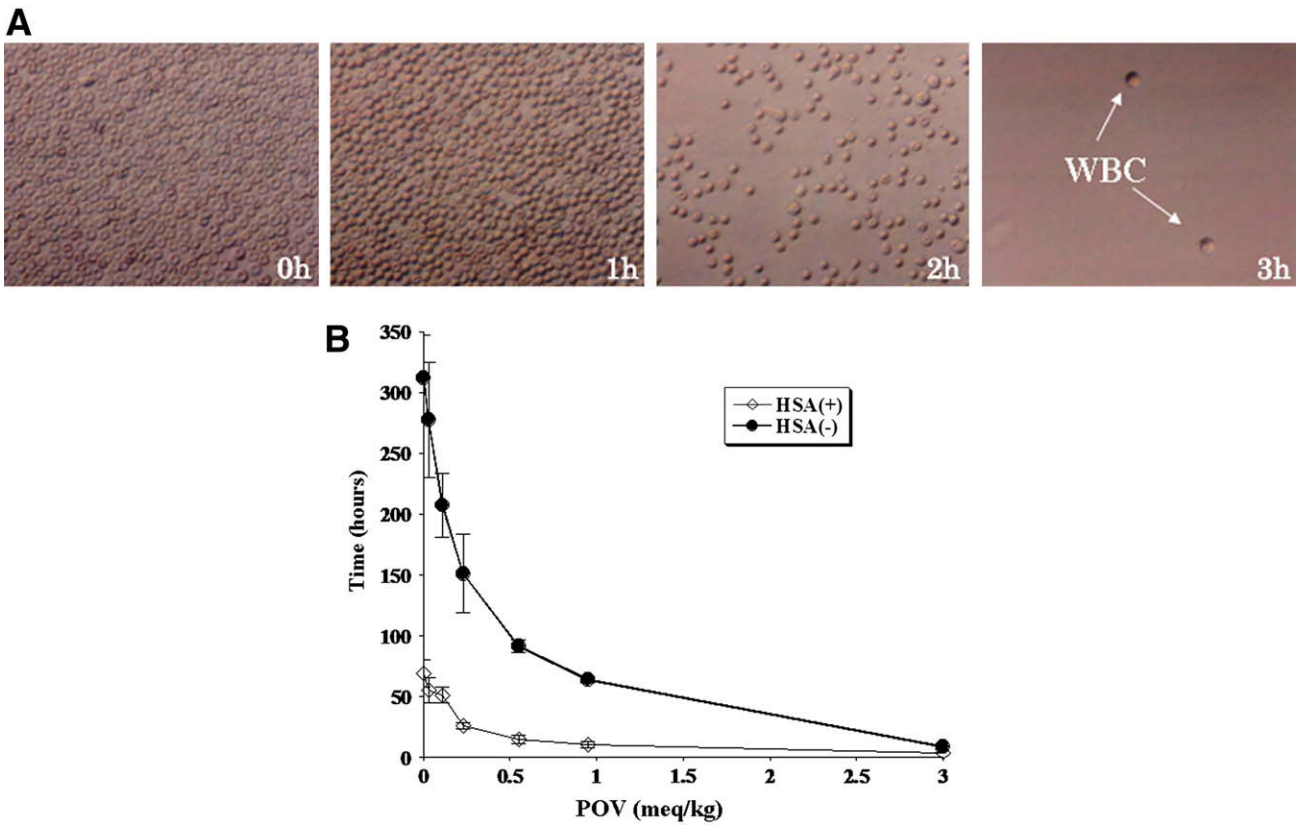
MATERIALS AND METHODS

Culture of Erythrocytes at Different POV Levels With or Without HSA

Blood samples were donated by laboratory staff and were collected into test tubes containing EDTA. Erythrocyte numbers were determined using the Sysmex (Kobe, Japan) K-800 automated cell counter. They were then centrifuged at 400 *g*

FIGURE 1

Incubation of erythrocytes under oil samples containing different peroxide value (POV) levels in the presence or absence of human serum albumin (HSA in the medium. (**A**) When culture medium (human tubal fluid [HTF]) was covered by peroxidized mineral oil (POV 3.0 meq/kg), erythrocytes started to become spherical in 1 h and hemolysis began to occur. Hemolysis progressed after 2 h and completed in 3 h. However, white blood cells (WBC) remained intact. (**B**) The time for hemolysis depended on the oil POV levels. The presence of 10 mg/mL HSA in the HTF culture medium facilitated hemolysis at each POV level. The values given in Figure 1B are the times for 100% hemolysis.



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for 5 min, and the sedimented erythrocytes were diluted to 50×10^4/mL with a physiologic salt solution. One μL of erythrocyte solution was added to 200 μL human tubal fluid (HTF; In Vitro Care, Japan) medium containing 10 mg/mL human serum albumin (HSA; In Vitro Care) in a culture dish (Falcon 3001) and incubated at 37°C under 5% CO_2 in air. The test culture dishes were pre-equilibrated in an incubator for 12 h before use. Drops of equilibrated medium were covered with mineral oil samples containing seven different POV levels (0.00, 0.03, 0.11, 0.23, 0.55, 0.95, 3.0 meq/kg). The time required for complete hemolysis was measured in each sample. Also, the experiments were performed in the presence and absence of albumin in the test medium. Each procedure was repeated 3 times.

Transfer Into the Zona of DiI Used as a Model for Free Radicals

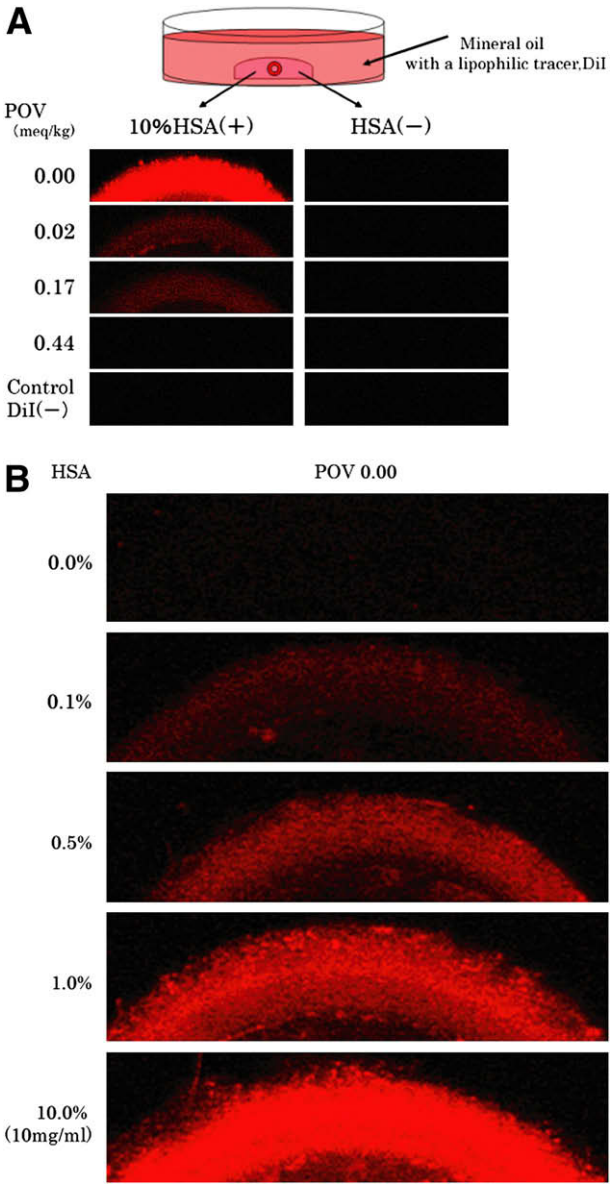
A lipophilic tracer, DiI (1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate) was dissolved in mineral oil that contained various POV levels (0.00, 0.03, 0.17, 0.44, 1.0 meq/kg). Droplets of 200 μL HTF (In Vitro Care) medium containing 10 mg/mL HSA (In Vitro Care) were covered by each of the above mineral oil samples and incubated for 12 h at 37°C under 5% CO_2 in air. Four to five unfertilized human oocytes or embryos that had arrested were placed in droplets with each POV level and cultured for 12 h. The oocytes or embryos were then washed three times with HEPES-buffered HTF medium containing 10 mg/mL HSA. The transfer of tracer into the zona pellucida was observed by means of a confocal laser microscope.

The influence of albumin on the transfer of tracer into the zona was examined at various HSA concentrations (0, 0.1, 0.5, 1.0, 10 mg/mL) in the HTF medium. Each of these experiments was repeated three times.

Informed consent for this study was obtained from couples being treated at the Nagai Clinic. All patients undergoing

FIGURE 2

The transfer of a tracer, DiI, for free radicals into the zona pellucida. (**A**) When albumin (HSA) was absent in the culture medium, DiI was not observed in the zona pellucida. In the presence of albumin, DiI transfer was seen when the peroxide value (POV) was undetectable. However, DiI entry into the zona was blocked when the POV level was more than 0.02 meq/kg. (**B**) HSA concentration–dependent DiI transfer into the zona was seen when the POV was undetectable.



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controlled ovarian hyperstimulation treatment consented to the use of their unfertilized oocytes and arrested embryos for the studies described in this report.

Analysis of Mineral Oil Samples From Nine Suppliers

Mineral oil samples were obtained from nine suppliers and stored for 12 months either at room temperature or at 4°C in the dark. The POV levels of nine mineral oil samples obtained from various suppliers were measured at the time of delivery and after 4 and 12 months of storage. The method used for measuring peroxide levels was described in our previous report (1). Briefly, POV was measured using a POV meter (Iijima Denshi, Aichi, Japan). Peroxide value is the value of oil oxidation (mainly hydroperoxide) associated with molecular oxygen. POV = (S × N × 1000)/W, where POV = meq hydroperoxide/kg oil, S = volume of sodium thiosulfate for titration (mL), N = normality of sodium thiosulfate (0.01 N), and W = weight of sample (g). A total of 0.5 mL of 0.01 mol/L sodium thiosulfate solution (5 μmol) is required to titrate 5 g of 1 POV oil.

RESULTS

Incubation of Erythrocytes Under Different POV Oil Samples in Droplets With or Without HSA

To evaluate the toxic effects of free radicals derived from oil peroxidation, erythrocytes in culture medium containing 10 mg/mL HSA were covered with mineral oil samples with different POV values. When mineral oil with a POV of 3.0 meq/kg was used, the erythrocytes started to become spherical in 1 h and then hemolysis occurred. Hemolysis of all erythrocytes was completed in 210–240 min. The time required to attain complete hemolysis was dependent on the POV level of the covering oil. Moreover, the presence of 10 mg/mL HSA in the HTFculture medium facilitated hemolysis at each POV level (Fig. 1).

Transfer of "Free Radicals" Into the Zona Pellucida

Preliminary studies indicated that albumin may be involved in the transfer of free radicals into cells. To visualize the influence of albumin on the transfer of a lipid-soluble tracer, we added a fluorescent marker, DiI, to the mineral oil and performed experiments in the presence and absence of albumin in the culture droplets. When albumin was absent in the culture medium, DiI transfer was not observed. On the other hand, in the presence of albumin, DiI transfer into the zona pellucida was observed when the POV level was undetectable in the oil. However, DiI transfer into the zona was blocked when the oil POV level was more than 0.02 meq/kg (Fig. 2A). Furthermore, HSA concentration–dependent transfer of DiI into the zona was seen when the oil's POV was undetectable (Fig. 2B).

Analysis of Mineral Oil Samples From Nine Suppliers

The analysis showed that three of the nine mineral oil samples had elevated POV levels (0.002, 0.005, and 0.020 meq/kg) at the time of delivery (Table 1). After 4 months of storage, POV levels increased further in these three mineral oil samples after storage both at room temperature and at 4°C. The mineral oil sample provided by another supplier, which

TABLE 1

Peroxide value (POV) levels in nine mineral oil samples at delivery from suppliers and at 4 months and 12 months after the delivery when the oil samples were stored at room temperature and at 4°C.

	Washing with culture medium including HSA	Recommended storage temp.	Shelf life (months)	POV (meq/kg)				
				Upon delivery	4 months storage		12 months storage	
					Room temp.	4°C	Room temp.	4°C
Company A	No	Room temp.	24	0.000	0.000	0.000	0.064	0.000
Company B	No	Room temp.	12	0.000	0.000	0.000	0.029	0.000
Company C	No	Cold and dark	12	0.000	0.000	0.000	0.006	0.000
Company D	No	Room temp.	24	0.000	0.000	0.000	0.021	0.000
Company E	No	2~8°C	10	0.000	0.000	0.000	0.021	0.000
Company F	Yes	2~8°C	3	0.000	0.012	0.000	0.052	0.000
Company G	No	Room temp.	60	0.002	0.003	0.003	0.038	0.003
Company H	Yes	2~5°C	6	0.005	0.016	0.012	0.079	0.017
Company I	Yes	Room temp.	12	0.020	0.051	0.034	0.099	0.037

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had been washed with culture medium containing albumin, also showed an elevated POV level after 4 months of storage at room temperature. After 12 months of storage, all mineral oil samples stored at room temperature showed elevated POV levels. Additional POV elevation after 4 months of storage at 4°C was not detected except in the three mineral oil samples that had elevated POV levels at delivery.

DISCUSSION

In this study we examined the effects of free radicals that enter the culture medium from an external covering of mineral oil, using erythrocytes as an experimental model. When hemolysis was used as an end point of toxicity, we found that the presence of albumin in the culture medium facilitated erythrocyte damage. It has been proposed that cysteine-34 in the albumin molecule has free sulfhydryl (SH) groups that capture radicals (3), and this may promote their transfer into the plasma membrane or into the interior of the cells to damage erythrocytes.

A model that proposed an explanation for the results obtained in the experiments on the transfer of free radicals into the zona pellucida is shown in Figure 3. When the POV level in the oil is more than 0.02 with the presence of albumin in culture medium, transfer of free radicals into zona pellucida is facilitated. This is likely to be damaging to embryo development. Therefore, when the POV in mineral oil is more than 0.02 we conclude that it should not be used for embryo culture in the presence of albumin or, possibly, other protein supplements.

The analysis of nine mineral oil samples obtained from different suppliers, showed that three had elevated POV levels. It is likely that all three would be unfavourable for embryo culture, particulary in the presence of HSA in the medium. Three of the analyzed oil samples were washed with culture medium, including albumin, and two of these showed the elevated POV levels.

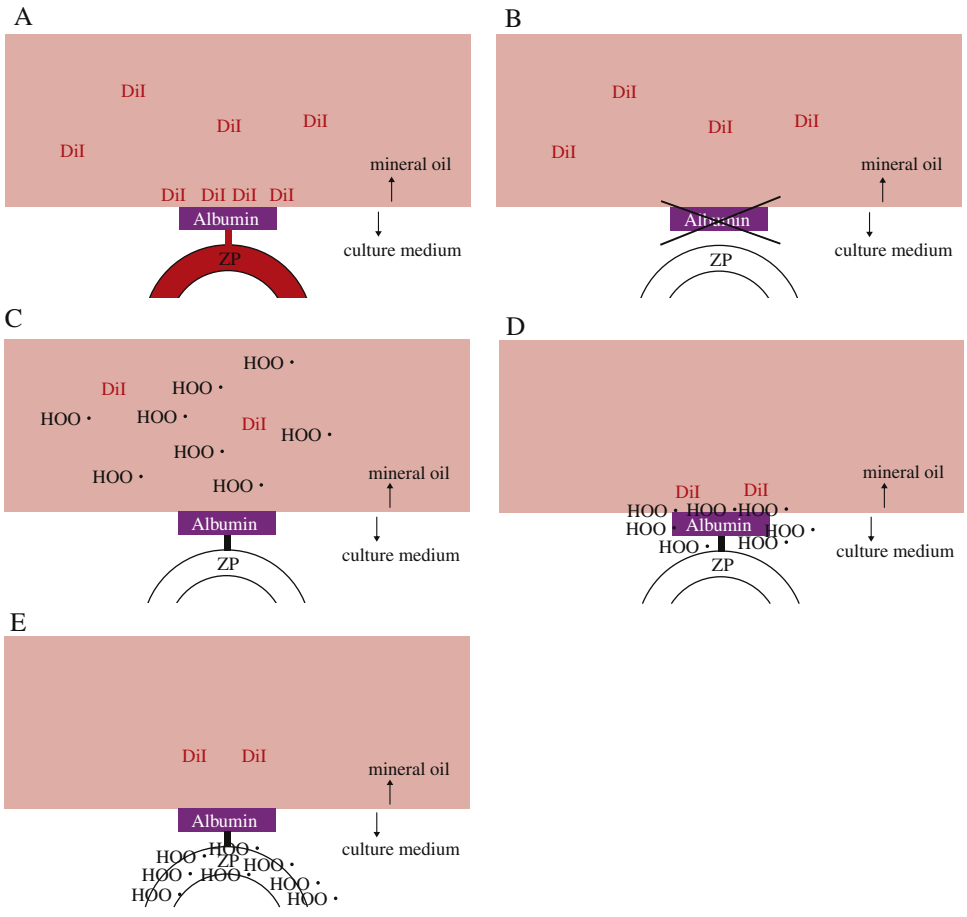
Because albumin has both lipophilic and hydrophilic binding sites, washing with a medium containing albumin may lower both toxic lipophilic and toxic hydrophilic contaminants in the mineral oil. However, it is possible that the oil becomes susceptible to oil peroxidation because of unsaturated fatty acids which may be released from the dissolved albumin.

When the nine different mineral oil samples were stored at room temperature, elevated POV levels were observed after 12 months. However, when the mineral oil samples were stored at 4°C, POV elevation was found only in the oil samples from suppliers G, H, and I, which already had elevated POV at delivery. It is suggested that the presence of free radicals may trigger chain reactions, causing further POV elevation. However, low levels, such as 0.002 meq/kg, may not be harmful during embryo culture. Nevertheless, the possibility of chain reactions of the free radicals should be considered.

The mineral oil sample of supplier F was washed with culture medium containing HSA. Its short shelf life may be related to the contact of the oil with HSA. The POV elevation occurred when the oil sample from supplier F was stored at

FIGURE 3

A proposed model of interactions between free radicals (or tracer DiI) in the oil, albumin in the culture medium, and the zona pellucida (ZP). When albumin is present, DiI enters the medium and attaches to albumin at its lipophilic binding site, facilitating the transfer of the tracer into the ZP. This is observed as the red staining in the ZP (**A**). When albumin is absent, DiI is not transfered into the ZP, which remains unstained (**B**). When free radicals are present in the mineral oil (**C**), the DiI and free radicals compete with each other for binding sites on the albumin (**D**). Therefore, DiI transfer into the zona pellucida is blocked by the presence of free radicals (**E**).



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room temperature but not at 4°C for 12 mouths. We recommend that mineral oil should be stored at 4°C, particularly those washed with albumin-containing culture medium. In the case of supplier I, the label on the bottle indicated that the mineral oil is to be stored at room temperature even though it had been washed with culture medium containing albumin. It is likely that this caused deterioration of the oil.

It should be noted that the oil manufacturers should recommend a shortened shelf life of the oil they manufacture. In addition, oil manufacturers should recommend storage of their oil samples at 4°C instead of at room temperature. We also propose that POV analysis is good practice, not only in assisted reproductive technology laboratories, but also for animal cell culture studies, especially in comparative investigations dealing with oxidative stress that require the use of mineral oil.

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