Peroxidation of mineral oil used in droplet culture is detrimental to fertilization and embryo development

Unexpected deterioration of human embryos led us to an investigation of possible toxic factors in the oil covering culture medium. Elevated levels of peroxidation in the oil by oxygen exposure or unfavorable storage were found to be the main causes of toxicity. (Fertil Steril® 2007;88:741–3. ©2007 by American Society for Reproductive Medicine.)

Mineral oil has been used for embryo culture since 1963 (1). The drop culture method, in which the embryo culture medium is covered by mineral oil, is now a standard method in many IVF laboratories. The oil cover prevents the culture medium from evaporation and maintains the appropriate pH and osmotic pressure. For these reasons microdrops are also used in intracytoplasmic sperm injection and insemination procedures. On the other hand, the use of mineral oil can damage oocytes and embryos because of toxic contamination or deterioration of oil quality (2).

Commercial suppliers often test mineral oil for microbial contamination and endotoxin. They also perform a mouse embryo culture test with a requirement of 70% to 80% blastocyst development for a satisfactory oil. However, damage to oocytes and embryos has been observed with use of some commercially available mineral oils without microbial contamination (2). It has been very difficult to identify the exact cause, and indeed in many of these cases the cause of damage has not yet been determined.

In July 2003, damage to cultured oocytes and embryos occurred when we used a new bottle of oil. We knew that there were no notable problems with the culture medium being used, the conditions of oil storage, the technical procedures, or other laboratory conditions. The damage to cultured oocytes and embryos occurred soon after opening a new bottle of oil that was stored for 2 weeks at room temperature in the dark. We strongly suspected that the embryo damage was related to the new mineral oil.

The oil bottle was opened inside a clean bench with use of strict aseptic conditions. The affected mineral oil (Reproline lot No. 45020301) was purchased from a Japanese distributor that distributed the product from a US supplier. According to the Certificate of Analysis from the supplier, the mineral oil had passed the mouse embryo culture test with more than 70% blastocyst development and had been sterilized by filtration.

To examine for possible causes of toxicity in the mineral oil, we ordered endotoxin and bacteriologic analyses at a clinical analysis center (Mitsubishi Kagaku Bio-Chemical Labs, Inc., Tokyo, Japan). In addition, infrared spectroscopic analysis, ultraviolet (UV) spectroscopic analysis, fluorescent spectroscopic analysis, high-performance liquid chromatography, and the acidic value were ordered at Nippon Kaiji Kentei Kyokai, Physical and Chemical Analysis Centre (Kanagawa, Japan). The tests showed that the endotoxin level was <10 ng/mL in all the samples of oil, and no bacteria or fungus was detected in any of the samples. Also, no significant problems were detected in any of the other analyses.

We then investigated the peroxide value (POV) of the mineral oil. The POV reflects on oil oxidation (mainly the presence of hydroperoxide) associated with molecular oxygen. This can be quantified by using the formula $POV = (S \times N \times 1000)/W$, where POV = 1 mEq hydroperoxide/kg oil, S = volume of sodium thiosulfate used for titration (milliliters), N = normality of sodium thiosulfate (0.01N), and W = weight of sample (grams). We used a highly sensitive method employing a POV meter (Iijima Denshi, Aichi, Japan) and performed electric titrations to obtain precise data. Fixed volumes of sodium thiosulfate (A) and potassium iodide were added, and reverse titration was performed to detect residual sodium thiosulfate. The volume of sodium thiosulfate consumed (B) was converted to the production of iodine by electrolysis according to Faraday's law: $\text{-CH}_2\text{-CH(OOH)-CH}_2\text{-} + 2\text{KI} \rightarrow \text{-CH}_2\text{-CH(OH)-CH}_2 + \text{I}_2 + \text{K}_2\text{O}$, $\text{I}_2 + 2\text{Na}_2\text{S}_2\text{O}_3 \rightarrow \text{Na}_2\text{S}_4\text{O}_6 + 2\text{NaI}$ $\text{-CH}_2\text{-CH(OOH)-CH}_2\text{-} + 2\text{KI} \rightarrow \text{-CH}_2\text{-CH(OH)-CH}_2 + \text{I}_2 + \text{K}_2\text{O}$, $2\text{I-} \rightarrow \text{I}_2 + 2\text{e-}$. From these reactions POV = A − B.

The POV of the affected oil was found to be 2.97 mEq/kg and that of unaffected oil was 0.00 mEq/kg. We therefore provisionally concluded that the cause of the oocyte and embryo deterioration observed at our clinic during microdrop cultures was related to peroxidation (POV elevation) of the mineral oil.

To examine the possible cause of mineral oil peroxidation, we next analyzed the time-dependent change in POV exposed to sunlight, or to UV light, or kept at 50°C or room temperature in the dark. Because the oil may have been stored without being protected from light, the time-dependent change in POV on exposure to sunlight (poured into a 50 cc Falcon conical tube [352070]) or UV light (the tube being placed 50 cm below the UV light, Hitachi

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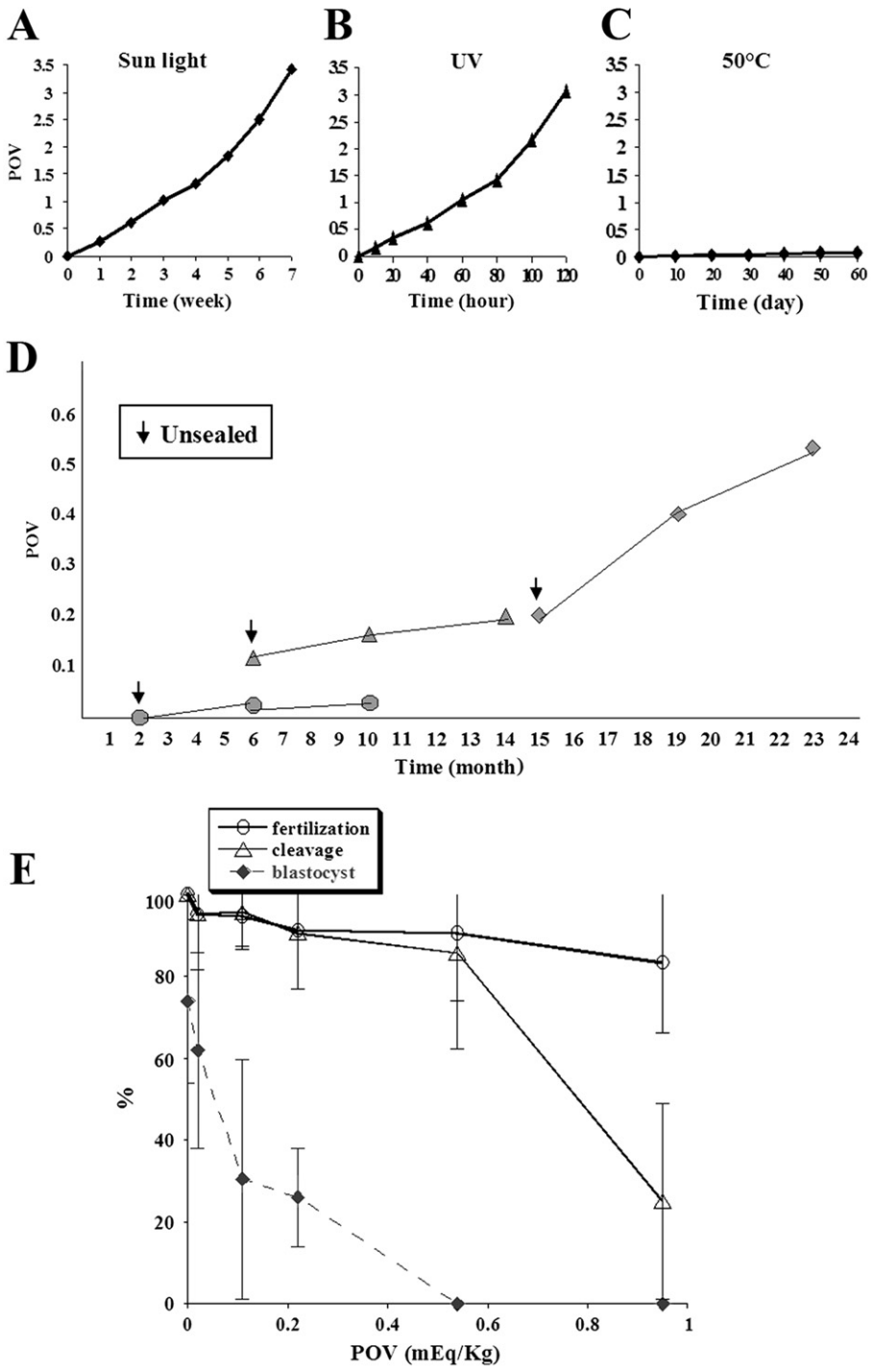
Reprint requests: Junko Otsuki, M.Sc., Nagai Clinic, 607-1 Kamihikona Misato, Saitama 341-0004, Japan (FAX: 81-48-959-1125; E-mail: midori@nagai-cl.com).

FIGURE 1

(**A**) Mineral oil exposed to sunlight caused a marked increase in POV that rose to 3.0 mEq/kg in 7 weeks. (**B**) Mineral oil exposed to UV light elevated its POV level to 3.0 mEq/kg in 120 hours. (**C**) Mineral oil kept in a constant-temperature oven, 50 °C, in the dark, resulted in a slight increase in POV to 0.04 in 20 days and to 0.10 in 50 days. (**D**) POV was measured in unopened mineral oils and then 4 and 8 months after opening. Although the POV value was 0.00 mEq/kg at 2 months after production, the oil without opening showed 0.12 mEq/kg and 0.21 mEq/kg at 6 and 15 months after production, respectively. (**E**) A significant decrease in fertilization (on day 0–1), cleavage (on day 1–2), and blastocyst development (on day 4–5) rates was observed when oils with elevated POV were used with droplet cultures.



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GL15; Tokyo, Japan) was investigated with use of a mineral oil (Reproline lot No. 4504305) that was known to have a POV of 0.0 mEq/kg. The POV markedly increased and reached 3.0 mEq/kg during 7 weeks of sunlight exposure (Fig. 1A) and during 120 hours of UV light exposure (Fig. 1B), respectively. To explore the possibility that the oil may have been stored at an elevated temperature, equivalent to being inside a car during summer, the same lot of mineral oil was stored in a constant-temperature oven at 50°C in the dark, and the time-dependent change in POV was analyzed. The POV of the oil became slightly increased to 0.04 in 20 days and to 0.10 in 50 days (Fig. 1C).

To determine the effect of prolonged exposure to atmospheric oxygen, we measured the POV of unopened mineral oils. Unopened bottles of mineral oil (Reproline lot Nos. 45030304, 45030301, 45020301) that had been stored by the distributor for 2, 6, and 15 months after production were obtained from the distributor. The POV of each mineral oil was measured just after initial opening and after 4 and 8 months of storage at room temperature in the dark. Although the POV value was 0.00 mEq/kg at 2 months after production, it rose to 0.12 mEq/kg and 0.21 mEq/kg at 6 and 15 months after production, respectively (Fig. 1D). Each mineral oil showed a slight time-dependent increase in POV. Differences in this may have been due to a difference in the purity of the mineral oils and/or conditions of oil storage at the same supplier and/or distributor.

To explore the influence of small increases in POV, ranging from 0.1 to 0.2 mEq/kg, which were observed before the expiration date, we studied fertilization and embryo development rates in a culture medium covered with mineral oils from several sources. In brief, 5-week-old ICR mice were superovulated with the injection of 7.5 IU hMG (Pergogreen; Serono, Tokyo, Japan), followed 48 hours later by 5 IU hCG (Profasi; Serono, Japan). Oocytes were retrieved 13 hours after hCG injection, washed, and randomly allocated to each POV treatment (POV: 0.00, 0.02, 0.12, 0.23, 0.54, 0.95 mEq/kg oil). This experiment was repeated three times. Significant POV-dependent decreases were seen in fertilization, cleavage, and blastocyst development rates (Fig. 1E). When the oil sample had a POV of 0.5 mEq/kg all of the embryos died by day 3 of culture, and when the POV was 1.0 mEq/kg all embryos died by day 2.

These results strongly suggest that the toxic effects of the mineral oil used in our laboratory were due to peroxidation during transport and/or storage by the supplier and/or distributor. Importantly, significant peroxide elevation was detected in unopened mineral oils that still had not reached the expiration date. The use of such oils was associated with significant decreases in fertilization, cleavage, and blastocyst development rates. It is likely that the embryo culture problem that we encountered could also occur with mineral oils being supplied by various companies. Thus, we propose that a precise indicator of mineral oil deterioration with time after manufacture be required by IVF laboratories and by the supplier and distributor.

All studies involving animals described herein were approved by and performed in strict accordance with the guidelines of Ochanomizu University Animal Care and Use Committee. The statistical package used for data analysis was SPSS version 12.0, SPSS Inc., Chicago, Illinois. The statistical analysis was performed with use of logistic regression analysis. Results were regarded as statistically significant if the P value was $<.05$.

Junko Otsuki, M.Sc.[a,b]
Yasushi Nagai, M.D.[b]
Kazuyoshi Chiba, Ph.D.[a]
[a] *Department of Biology, Ochanomizu University, Tokyo; and* [b] *Nagai Clinic, Saitama, Japan*

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