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Antioxidants reversibly inhibit the spontaneous resumption of meiosis

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Takami, M., S. L. Preston, V. A. Toyloy, and Harold R. Behrman. Antioxidants reversibly inhibit the spontaneous resumption of meiosis. *Am. J. Physiol.* 276 (*Endocrinol. Metab.* 39): E684–E688, 1999.—We previously showed that the cell-permeant antioxidant 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) inhibited germinal vesicle breakdown (GVBD) in oocyte-cumulus complexes (OCC) of the rat. The objective of the present studies was to assess other antioxidants and whether such inhibition was reversible. Spontaneous GVBD in OCC incubated for 2 h was significantly inhibited ($P < 0.005$) by nordihydroguaiaretic acid (NDGA; GVBD = 19.4%), BHA (GVBD = 25.7%), octyl gallate (OG; GVBD = 52.2%), ethoxyquin (EQ; GVBD = 58.8%), 2,6-di-*tert*-butyl-hydroxymethyl phenol (TBHMP; GVBD = 59%), butylated hydroxytoluene (BHT; GVBD = 59.5%), and *tert*-butyl hydroperoxide (TBHP; GVBD = 60.0%). Other antioxidants that produced lower but significant ($P < 0.05$) inhibition of oocyte maturation included propyl gallate (PG; GVBD = 70.3%), 2,4,5-trihydroxybutrophenone (THBP; GVBD = 71.4%), and lauryl gallate (LG; GVBD = 71.4%). Antioxidants that had no effect on oocyte maturation at the same concentration (100 μM) included ascorbic acid, vitamin E, and Trolox. Inhibition of GVBD was evident for up to 8 h of incubation of OCC and denuded oocytes (DO) with BHA or NDGA and was reversed by washing. NDGA was less potent than BHA for inhibition of GVBD in DO, unlike that seen with OCC. Oocyte maturation was induced by incubation of follicles for 3 h with human chorionic gonadotropin (hCG), and this response was inhibited by BHA or NDGA. These findings support the conclusion that cell-permeant antioxidants inhibit spontaneous resumption of meiosis, which may implicate a role of oxygen radicals in oocyte maturation.

oxygen radicals; follicle; oocyte maturation

OOCYTES from preovulatory follicles spontaneously resume meiosis when removed from their follicular environment (16). The physiological stimulus for the resumption of meiosis that occurs before follicular rupture is the gonadotropin surge associated with ovulation (1, 19). The nature of the intrafollicular inhibitor of oocyte maturation is not known.

Ascorbic acid is the preeminent water-soluble antioxidant (5). Ascorbic acid deficiency results in infertility that in the female is associated with ovarian atrophy, follicular atresia, and the premature resumption of meiosis (3, 11). Moreover, in cultured rat follicles, ascorbic acid and other antioxidants inhibit apoptosis (17). Recently, we showed that isolation of oocyte-cumulus complexes (OCC) results in the almost immediate depletion of ascorbic acid in oocytes, followed by the onset of spontaneous oocyte maturation, and that gonadotropin depletes ascorbic acid in preovulatory follicles before the reinitiation of meiosis (9). These same studies showed that, whereas extracellular ascorbic acid did not inhibit spontaneous germinal vesicle breakdown (GVBD), the direct intraoocyte injection of ascorbic acid delayed oocyte maturation (9). These findings led to the hypothesis that antioxidants may serve a role in the inhibition of meiosis and that the resumption of meiosis may be induced by an increase in reactive oxygen species.

The objective of the present studies was to determine whether the meiosis-inhibiting activity of 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) was unique to this structure or whether other antioxidants share this activity, and to examine whether the inhibition of oocyte maturation by antioxidants was reversible.

MATERIALS AND METHODS

Hormones, drugs, and reagents. BHA and nordihydroguaiaretic acid (NDGA) were purchased from Sigma Chemical (St. Louis, MO). Octyl gallate (OG), ethoxyquin (EQ), 2,6-di-*tert*-butyl-hydroxymethyl phenol (TBHMP), *tert*-butylhydroquinone (TBHQ), propyl gallate (PG), 2,4,5-trihydroxybutrophenone (THBP), and lauryl gallate (LG) were purchased from Supelco (Bellefonte, PA). 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Sigma Chemical and dissolved in 50% ethanol-water. Stock solutions (100 mM) of BHA, NDGA, OG, EQ, TBHMP, butylated hydroxytoluene (BHT), TBHQ, PG, THBP, and LG were prepared in 95% ethanol. The final concentration of ethanol was <0.05%, and parallel controls were run to assess the effect of ethanol.

Animals. Follicle development was induced in immature (25- to 27-day-old) female rats (Sprague-Dawley strain; Taconic Farms, Germantown, NY) by subcutaneous injection of 10 IU pregnant mare serum gonadotropin (PMSG; Gestyl; Organon Pharmaceuticals, West Orange, NJ). Animals were housed and cared for in the fully accredited facilities operated by the Animal Resource Center (Yale University School of Medicine, New Haven, CT). All treatments and procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and a protocol approved by the Yale University Animal Care Committee.

Isolation and incubation of OCC. The animals were euthanized 44–48 h after PMSG treatment. The ovaries were removed and placed in Earle's minimal essential medium (MEM 2360; GIBCO, Grand Island, NY) containing bovine serum albumin (BSA; 1 mg/ml), glutamine (0.29 mg/ml), and IBMX (100 μM). After the fat was trimmed from the ovaries, preovulatory follicles were bluntly dissected from the ovaries under a stereomicroscope. OCC from large preovulatory follicles were expelled by puncturing antral follicles with a

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stainless steel needle. Isolated OCC were pooled, washed three times in fresh medium without IBMX, and allotted to treatment groups. The isolation procedure from the time of puncture of the follicle to the allocation to treatment groups took ~30–45 min.

OCC were incubated for 2 h under a humidified atmosphere of 5% CO_2-95% air at 37°C in 1 ml of medium (MEM 2360) containing BSA (1 mg/ml), glutamine (0.29 mg/ml), and the antioxidant to be tested in Costar multiwell plates (Falcon 3047, Becton-Dickinson, Lincoln Park, NJ). Longer-term incubation of OCC was treated identically, either in the absence or the presence of BHA (100 μM) or NDGA (50–100 μM).

After incubation, OCC were scored for oocyte maturation by utilizing a squash technique that enables visualization of the oocyte with its surrounding investment of cumulus cells, as described earlier (15). Briefly, the OCC were placed on slides pretreated with Sigmacote (Sigma Chemical) that were rinsed and dried. Spots of silicone lubricant were mixed with a small amount of Sephadex LH 20 placed on the edges of the slides over which a coverslip was placed. Oocytes that retained a germinal vesicle and/or nucleolus were considered to show inhibition of maturation. Oocyte maturation was expressed as a percentage of GVBD.

Isolation and incubation of follicles. After PMSG treatment (see *Animals*), the largest follicles were dissected from each ovary and incubated in sterile 25-ml Erlenmeyer flasks containing 2.5 ml of medium (MEM 2360) with BSA (1 mg/ml), glutamine (0.29 mg/ml), and the agent to be tested. Follicles were incubated in a shaking water bath at 37°C and 95% oxygen-5% CO_2. After 15 min of preincubation with diluent or antioxidant, human chorionic gonadotropin (hCG; 20 IU/ml; Pregnyl; Organon, Boxtel, The Netherlands) was added to the medium, and follicles were incubated for 3 h. After incubation, follicles were placed in medium (MEM 2360) with BSA (1 mg/ml), glutamine (0.29 mg/ml), and IBMX (100 μM) to isolate the OCC, and oocyte maturation was scored as just described in *Isolation and incubation of OCC*.

Reversibility of inhibition of oocyte maturation by antioxidants. OCC were preincubated for 30–120 min in 1 ml of medium (MEM 2360) with BSA (1 mg/ml), glutamine (0.29 mg/ml), and either BHA or NDGA (100 μM) in Costar multiwell plates. After preincubation, OCC were washed three times with medium and then incubated without antioxidant for a total interval of 4 h (preincubation + postincubation). Other groups were incubated with BHA or NDGA (100 μM) continuously for 4 h. GVBD was scored in OCC as we have described.

Isolation and incubation of denuded oocytes. OCC were denuded of their cumulus corona investments by exposure to hypotonic media (1:1 ratio of 0.7% sodium citrate in distilled water-MEM 2360 containing 200 μM IBMX) and repeated pipetting through a narrow-bore glass pipette. Denuding was carried out immediately after isolation of the OCC.

Before incubation, denuded oocytes (DO) were washed three times with fresh medium and incubated in 100 μl of medium in a 4-well glass slide that was placed on moistened filter paper within a covered Petri dish to guard against evaporation. Medium (MEM 2360) was supplemented with BSA (1 mg/ml), glutamine (0.29 mg/ml), sodium pyruvate (1 mM), and BHA or NDGA (100 μM). After 30–360 min of incubation, DO were visualized under Nomarski optics, and DO that retained a germinal vesicle and/or nucleolus were considered to show inhibition of maturation.

Statistical analysis. The effect of treatments on oocyte maturation was evaluated by χ^2 analysis. Each experiment was independently repeated at least three times.

RESULTS

The effect of various antioxidants on oocyte maturation is shown in Fig. 1. Spontaneous GVBD in control OCC that were incubated for 2 h was 89.7% (n = 68). The most effective antioxidants that produced significant ($P < 0.005$) inhibition of oocyte maturation were NDGA (19.4% GVBD; n = 36), BHA (25.7% GVBD; n = 35), OG (52.2% GVBD; n = 46), EQ (58.8% GVBD; n = 34), TBHMP (59.0% GVBD; n = 39), BHT (59.5% GVBD; n = 37), and TBHP (60.0% GVBD; n = 45). Other antioxidants that produced minimal, but significant ($P < 0.05$) inhibition of oocyte maturation included PG (70.3% GVBD; n = 37), THBP (71.4% GVBD; n = 42), and LG (71.4% GVBD; n = 35). Antioxidants that had no effect on oocyte maturation at a concentration of 100 μM were ascorbic acid, vitamin E, and Trolox (data not shown).

Dose-response studies were carried out with NDGA and BHA, the two most potent inhibitors of oocyte maturation. At a concentration of 30 μM, neither BHA nor NDGA inhibited oocyte maturation in OCC, whereas at 50 μM, both antioxidants inhibited oocyte maturation (Fig. 2). The more potent of the two antioxidants was NDGA, because near-maximal inhibition was evident at 50 μM, in contrast to BHA, where a concentration of 100 μM still did not show inhibition of oocyte maturation equivalent to that of NDGA. Cytotoxicity was visually assessed by the granular appearance of the ooplasm, shrinkage of the ooplasm, or oocyte fragmentation. No cytotoxic effect of either antioxidant on the oocytes was evident over the 2-h time course of these studies.

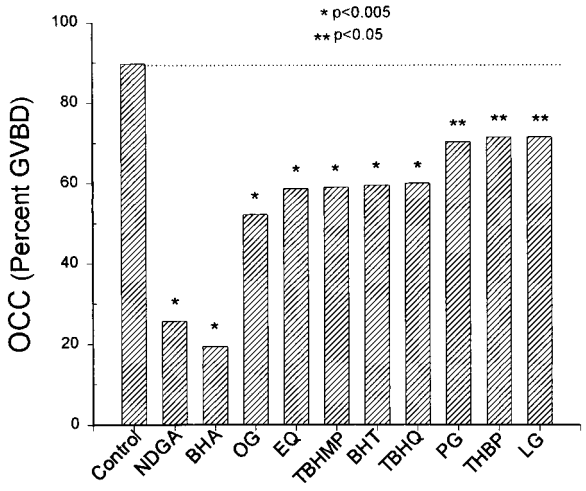

Fig. 1. Inhibition of oocyte maturation by antioxidants in oocyte-cumulus complexes (OCC). OCC were incubated with 100 μM of antioxidant for 2 h, and the percentage of germinal vesicle breakdown (GVBD) was scored as described in MATERIALS AND METHODS. The number of individual oocytes examined for each antioxidant treatment [nordihydroguaiaretic acid (NDGA); 2(3)-*tert*-butyl-4-hydroxyanisole (BHA); octyl gallate (OG); ethoxyquin (EQ); 2,6-di-*tert*-butyl-hydroxymethyl phenol (TBHMP); butylated hydroxytoluene (BHT); *tert*-butyl hydroquinone (TBHQ); propyl gallate (PG); 2,4,5-trihydroxybutrophenone (THBP); and lauryl gallate (LG)] ranged from 34 to 42 and was 68 for the control group. Results are means of ≥3 independent experiments.

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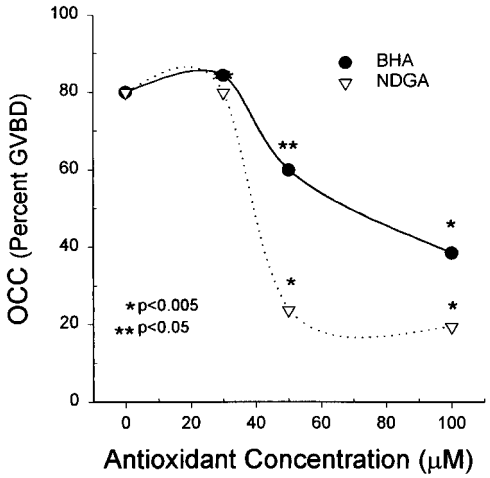

Fig. 2. Dose-response effect of BHA and NDGA on inhibition of oocyte maturation in OCC. OCC were incubated with the indicated concentration of BHA and NDGA for 2 h, and percent GVBD was scored as described in MATERIALS AND METHODS. The number of individual oocytes examined for each antioxidant treatment ranged from 32 to 39 and was 74 for the control group. Results are means of ≥3 independent experiments.

The time course for inhibition of oocyte maturation by BHA and NDGA in OCC is shown in Fig. 3. Although significant inhibition of oocyte maturation was evident for up to 8 h of incubation of OCC with BHA (100 μM) or NDGA (50 and 100 μM), a gradual reduction in inhibition occurred to the extent that at 10 and 24 h no inhibition was evident. A twofold increase in the concentration of NDGA did not appear to further reduce the rate of oocyte maturation (note the similarity in slope of lines). This finding may indicate that depletion of antioxidant may not be the cause for the loss of inhibition of oocyte maturation. This conclusion was verified in other studies in which freshly prepared BHA or NDGA (100 μM) was added 4 h after incubation with the same antioxidants, and the rate of oocyte maturation 2, 4, and 6 h later was compared with that of a single addition of antioxidant at the beginning of the incubation. These studies showed no significant difference in the inhibition of GVBD by the antioxidants between a single addition of antioxidant at the beginning of the incubation compared with addition of freshly prepared antioxidant (data not shown). In other studies, OCC were isolated in the presence of NDGA or BHA (100 μM) and incubated for up to 24 h with the same concentration of antioxidants. Although GVBD was characteristically inhibited after 4 and 6 h with both antioxidants, no inhibition was seen after 24 h (data not shown). Whereas no oocyte toxicity was evident with BHA at 100 μM with long-term incubation, 100 μM NDGA was cytotoxic in contrast to a concentration of 50 μM. Cytotoxicity was visible by granulation of the oocyte and shrinkage of the ooplasm.

To further assess whether inhibition of oocyte maturation by BHA and NDGA was reversible, OCC were preincubated with antioxidant, washed, and reincubated to determine whether oocyte maturation occurred. Preincubation of OCC with 100 μM BHA or NDGA for 30, 60, 90, or 120 min, followed by washing, showed levels of oocyte maturation similar to the level of control OCC (Fig. 4). However, continuous incubation of OCC for the entire 4-h period with BHA or NDGA caused a marked and significant inhibition of oocyte maturation.

The time course for inhibition of oocyte maturation by antioxidants in DO is shown in Fig. 5. In these studies, DO were incubated with 100 μM BHA or

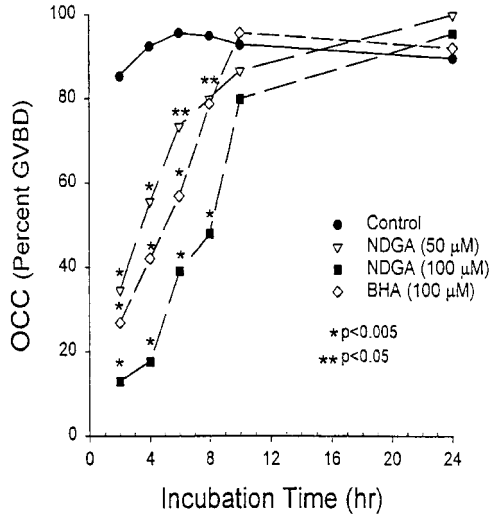

Fig. 3. Duration of inhibition of oocyte maturation by BHA and NDGA in OCC. OCC were incubated with 50 and 100 μM BHA and NDGA for the indicated intervals, and percent GVBD was scored as described in MATERIALS AND METHODS. The numbers of individual oocytes examined for each antioxidant, treatment, and time interval ranged from 24 to 51 and from 40 to 46 for the control group. Each time interval and antioxidant concentration represent individual treatment groups for each experiment. Results are means of ≥3 independent experiments.

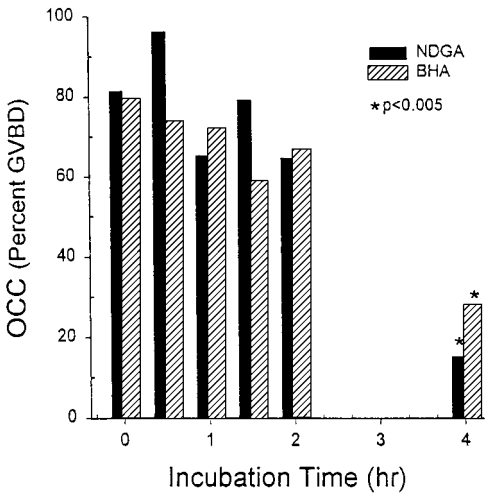

Fig. 4. Reversibility of antioxidant inhibition of oocyte maturation in OCC. OCC were preincubated for 0.5, 1.5, or 2 h with 100 μM BHA or NDGA, washed three times, and then incubated without antioxidant for an interval such that the total incubation period was 4 h for all groups. Percent GVBD was scored as described in MATERIALS AND METHODS. The numbers of individual oocytes examined for each antioxidant treatment interval ranged from 31 to 64 and from 70 to 75 for the control (0 h) groups. Results are means of ≥3 independent experiments.

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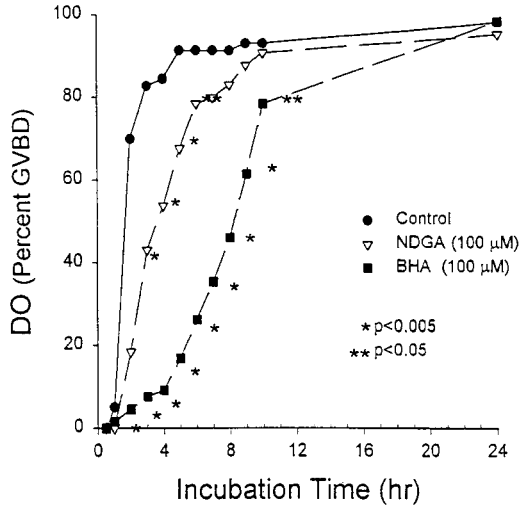

Fig. 5. Time course for inhibition of oocyte maturation in denuded oocytes (DO). DO were incubated with 100 μM BHA or NDGA, and GVBD was scored after the indicated intervals, as described in MATERIALS AND METHODS. The number of individual oocytes examined for each antioxidant treatment was 65 and was 58 for the control group. Results are means of ≥3 separate experiments.

NDGA, and GVBD was scored at various time intervals up to 24 h of incubation. Although both NDGA and BHA produced long-lasting inhibition of oocyte maturation, BHA was more potent, and inhibition was longer lasting than that seen with NDGA. In addition, the duration of inhibition by BHA and NDGA slowly diminished and with a time course similar to that seen with OCC. Little evidence of cytotoxicity was seen for BHA, whereas NDGA produced significant granulation and shrinkage of the ooplasm at 4 h of incubation that became progressively greater with longer periods of incubation.

Figure 6 shows that whereas spontaneous maturation was minimal in follicle-enclosed oocytes (21% GVBD; $n = 88$), maturation was induced by incubation

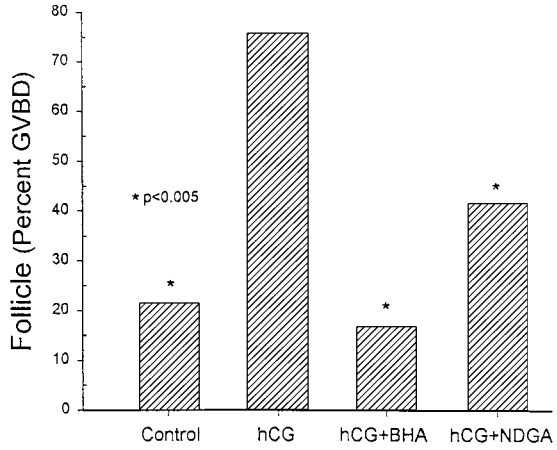

Fig. 6. Inhibition of gonadotropin-induced oocyte maturation in isolated intact follicles by BHA and NDGA. Follicle-enclosed oocytes were incubated with 300 μM BHA or NDGA for 3 h, and percent GVBD was scored as described in MATERIALS AND METHODS. Numbers of follicle-derived oocytes examined for control, human chorionic gonadotropin (hCG), hCG+BHA, and hCG+NDGA were 100, 28, 23, and 25, respectively, in 3 independent experiments.

of follicles for 3 h with hCG (75.4% GVBD; $n = 111$). Both BHA and NDGA at a concentration of 300 μM significantly blocked ($P < 0.005$) hCG-induced maturation of follicle-enclosed oocytes (16.3% GVBD; $n = 31$ and 41.1% GVBD; $n = 34$, respectively). However, no significant effect of these antioxidants was seen at 100 μM, a dose that was highly effective in OCC or DO.

DISCUSSION

The present findings show that spontaneous resumption of meiosis in cumulus-enclosed oocytes was inhibited by a wide variety of phenolic antioxidants, all of which were lipophilic and therefore expected to readily penetrate cells. In contrast, ascorbic acid and vitamin E were inactive when added to media. Most potent of the antioxidants were NDGA and BHA. The inhibition of oocyte maturation by NDGA and BHA was long lasting, at least in OCC, but not permanent, and their inhibitory activity could also be reversed by washing of the OCC.

Antioxidants also inhibited oocyte maturation in DO, and this response was similar to that seen in OCC. However, BHA was a significantly more potent inhibitor of maturation in DO than NDGA, in contrast to that found in OCC. It should be noted that the action of the antioxidants in OCC and DO did not completely mimic the inhibitory environment within the follicle, in which spontaneous oocyte maturation does not occur for long intervals (18). Under conditions of culture used in these studies, we found that culture of control follicles for 24 h showed minimal oocyte maturation (GVBD = 14.3%; $n = 28$). A gradual and linear decrease in inhibition of oocyte maturation by antioxidants occurred in both OCC and DO.

The mechanism of action of antioxidants on inhibition of oocyte maturation is not known. One possibility is that reactive oxygen species induce the resumption of meiosis and that oxidant radical scavenging is the mechanism of antioxidant inhibition of oocyte maturation. However, other mechanisms of action of antioxidants cannot be ruled out, and such mechanisms may involve the maintenance of inhibitory levels of cAMP within the oocyte, an agent well known to prevent oocyte maturation (4, 6, 8, 13).

The differential inhibition of oocyte maturation by various antioxidants cannot readily be explained by their relative antioxidant activities in vitro. For example, PG surpasses BHA and BHT for scavenging superoxide and hydroxyl anions (10), yet BHA was more effective than PG on inhibition of oocyte maturation in the present studies. On the other hand, EQ surpassed OG, BHA, BHT, and PG for inhibition of lipid peroxidation in an in vitro system (10), but BHA was more effective than EQ on inhibition of oocyte maturation. The relative abilities of the various antioxidants to penetrate cells and enter appropriate cellular compartments would be expected to be more important characteristics that would be necessary for scavenging of intracellular oxidants, and this may explain the differences between potency of oxidant scavenging between cell-free and cellular systems. As we showed

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earlier, extracellular ascorbic acid has no effect on oocyte maturation unless injected within the oocyte (9). However, ascorbic acid is present almost entirely as the anionic species at physiological pH (12) and would therefore be excluded by cells. We previously described granulosa (2) and luteal (14) cell ascorbic acid transporters that are energy, sodium-dependent, and endocrine regulated. The kinetics of the ascorbic acid transporter are such that more than 2 h are necessary to saturate cell levels of ascorbic acid (2, 14) that are depleted by mere isolation of the OCC or oocyte (9). This interval is too long, we suggest, to accumulate a sufficient amount of ascorbic acid to block oocyte maturation, in which the early events that activate this process are probably very rapid. Notably the concentrations of the antioxidants that were effective in the present studies for inhibition of oocyte maturation are in the same range as those for effective oxidant scavenging in cell-free systems (10).

To our knowledge this is the first report of antioxidant inhibition of oocyte maturation by a wide variety of phenolic antioxidants, beyond our recent report of such activity by BHA (9). Earlier studies by others examined sulfhydryl compounds on oocyte survival and maturation (7, 20). These studies showed that dithiothreitol assisted oocyte survival in calcium-free media and extended inhibition of oocyte maturation by cAMP but did not inhibit oocyte maturation in the absence of cAMP.

The importance of the present studies resides in the potential use of antioxidants for studies of gamete function or increased survival after manipulation in vitro, in addition to implication of a role for oxidants in the reinitiation of meiosis. On the basis of morphological criteria, the antioxidants were not cytotoxic except at high levels, and the cells appeared less damaged when incubated with antioxidants than without, particularly over long time periods of incubation.

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