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Ascorbic acid and CoQ_{10} ameliorate the reproductive ability of superoxide dismutase 1-deficient female mice†

Naoki Ishii[1], Takujiro Homma[1], Jaeyong Lee[1], Hikaru Mitsuhashi[2], Ken-ichi Yamada[3,4], Naoko Kimura[5], Yorihiro Yamamoto[2], and Junichi Fujii[1,*]

[1]Department of Biochemistry and Molecular Biology, Graduate School of Medical Science, Yamagata University, Yamagata, Japan [2]School of Bionics, Tokyo University of Technology, Hachioji, Japan [3]Department of Bio-functional Science, Faculty of Pharmacological Science, Kyushu University, Fukuoka, Japan [4]JST, PRESTO, Kawaguchi, Japan and [5]Laboratory of Animal Reproduction, Faculty of Agricultural Sciences, Yamagata University, Tsuruoka, Japan

***Correspondence:** Department of Biochemistry and Molecular Biology, Graduate School of Medical Science, Yamagata University, 2-2-2 Iidanishi, Yamagata 990-9585, Japan. Tel: +81-23-628-5227; Fax: +81-23-628-5230; E-mail: jfujii@med.id.yamagata-u.ac.jp

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Abstract

Superoxide dismutase 1 suppresses oxidative stress within cells by decreasing the levels of superoxide anions. A dysfunction of the ovary and/or an aberrant production of sex hormones are suspected causes for infertility in superoxide dismutase 1-knockout mice. We report on attempts to rescue the infertility in female knockout mice by providing two antioxidants, ascorbic acid and/or coenzyme Q_{10}, as supplements in the drinking water of the knockout mice after weaning and on an investigation of their reproductive ability. On the first parturition, 80% of the untreated knockout mice produced smaller litter sizes compared with wild-type mice (average 2.8 vs 7.3 pups/mouse), and supplementing with these antioxidants failed to improve these litter sizes. However, in the second parturition of the knockout mice, the parturition rate was increased from 18% to 44–75% as the result of the administration of antioxidants. While plasma levels of progesterone at 7.5 days of pregnancy were essentially the same between the wild-type and knockout mice and were not changed by the supplementation of these antioxidants, sizes of corpus luteum cells, which were smaller in the knockout mouse ovaries after the first parturition, were significantly ameliorated in the knockout mouse with the administration of the antioxidants. Moreover, the impaired vasculogenesis in uterus/placenta was also improved by ascorbic acid supplementation. We thus conclude that ascorbic acid and/or coenzyme Q_{10} are involved in maintaining ovarian and uterus/placenta homeostasis against insults that are augmented during pregnancy and that their use might have positive effects in terms of improving female fertility.

Summary sentence

The results suggest that antioxidants support fertilizing ability against oxidative stress during parturition.

Key words: oxidative stress, fertility, ovary

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Introduction

Infertility increases in proportion to maternal aging and various types of stress are serious problems, especially for women over the age of 40. While multiple physiological processes and molecules are involved in female fertility, only few connections have been identified as its cause [1]. Reactive oxygen species (ROS) are produced not only under pathological conditions, such as inflammation, but also during normal physiological processes, such as steroidogenesis and ovulation [2], and represent a potential cause for the dysfunction of ovaries [3] and male germ cells [4]. The incidence of deletion in mitochondrial DNA in oocytes increases with age in women [5], which may be associated with low energy production and triggering the release of ROS from the mitochondrial respiratory chain. Repeated stimulation by hormone treatment causes multiple rearrangements in mitochondrial DNA molecules and the oxidative modification of lipids and proteins and may be associated with ovary damage in assisted reproductive technology [6, 7].

A variety of protective systems, including antioxidative enzymes and low molecular weight antioxidative compounds, are produced to suppress ROS levels and protect ovaries from oxidative damage [8]. Decreases in antioxidative systems or a concomitant increase in oxidative insult are associated with female infertility. Superoxide dismutase (SOD) plays a central role in antioxidative systems because it scavenges primary ROS, superoxide anions that are produced by a one-electron donation to molecular oxygen, and results in preventing oxidative stress at the beginning of the radical chain reaction [9]. SOD1-null sperm have fertilizing ability but show a rapid decline in their motility in vitro [10]. Sperm competition trials have also shown a low fertilization success [11].

In the meantime, SOD1 disruption is associated with severe female infertility in mice [12, 13]. Based on the results showing that the levels of serum follicle stimulating hormone and luteinizing hormone (LH) are decreased in SOD1-null mice, Matzuk et al. [13] have proposed that ovary dysfunction is secondary to decreased gonadotropin levels and/or a decreased responsiveness of the ovaries to physiological concentrations of gonadotropins. No significant difference, however, is observed in the number of embryos in uteri at an early embryonic stage, but lethality becomes elevated at advanced embryonic stage in the uteri of the SOD1-null mice [12]. Impaired luteal formation and decreased progesterone production have been proposed as causes for the embryonic lethality in the SOD1-null mice [14]. We also reported that embryos derived from oocytes of SOD1-null mice show developmental arrest at the two-cell stage under conventional culture conditions [15]. This developmental defect in SOD1-deficient mouse embryos appears to be associated with oxygen toxicity because, under hypoxic culture conditions (1% atmospheric oxygen with 5% CO_2), this two-cell arrest is overcome and normal development to the blastocyst stage is observed. However, SOD1-null mice are born with Mendelian characteristics from heterozygous female mice, and it is known that SOD1-null embryos that had been transplanted to wild-type (WT) female mice develop normally [14, 15], which suggests that low oxygen concentrations in the female reproductive tract might be involved in the normal development of the SOD1-null embryos and that the infertility is mainly caused by a maternal abnormality.

Nutritional components or supplements with antioxidant functions have been examined in attempts to rescue aging-dependent infertility [16]. Contrary to difficulties in demonstrating the effectiveness of these compounds in human beings [17], animal models, especially genetically modified mice, can be useful. An antioxidant *N*-acetyl-L-cysteine actually delays oocyte aging in mice [18], and melatonin improves the extent of fertilization of aged mouse oocytes [19]. Although these reports provide convincing data in favor of the effectiveness of antioxidants against oocyte aging, direct evidence for the involvement of antioxidants against oxidative stress-mediated female infertility is missing.

In this study, we examined the use of ascorbic acid (AsA) as an antioxidant because substantial decreases in the plasma levels of AsA have been reported in the mice lacking SOD1 and/or SOD3 [20]. We also employed coenzyme Q_{10} (CoQ_{10}), either singly or in combination with AsA, because it is a promising nutrient with antioxidant activity [21] and actually rescues age-related declines in oocyte quality by improving the mitochondrial function in mice [22]. Herein, we report of a partial but significant improvement in the reproductive ability of SOD1-null mice by providing these nutritional antioxidant factors as supplements.

Materials and methods

Experimental animals

All mice were bred in the Animal Center, Institute for Promotion of Medical Science Research, Yamagata University. The SOD1-knockout (KO) mice used in this study were originally established by Matzuk et al. [13] and backcrossed with C57BL/6 N mice more than 10 times as reported previously [23]. We bred WT mice pairs and, separately, homozygous SOD1-KO male × heterozygous SOD1-KO female mouse pairs in our institution. Genotypic analyses of the mice were performed using polymerase chain reaction (PCR) with specific primers, and WT and homozygous SOD1-KO mice were used in this study. The animal room was maintained under specific pathogen-free conditions at a constant temperature of 20–22 °C with a 12-h alternating light–dark cycle. Animal experiments were performed in accordance with the Declaration of Helsinki under protocols approved by the Animal Research Committee at Yamagata University.

In vitro fertilization

In vitro fertilization (IVF) was performed as previously reported [15]. Cumulus cell–oocyte complexes were collected from WT and KO female mice at 8 weeks of age, which had been superovulated by the intraperitoneal administration of 7.5 IU of pregnant mare serum gonadotropin (PMSG; ASKA Pharmaceutical, Tokyo, Japan), followed by 7.5 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical) 48 h later. Human tubal fluid medium (HTF; Zenith Biotech, Warrington, UK) [24] supplemented with 4 mg/mL of bovine serum albumin (BS) was used for the IVF, and potassium simplex optimization medium (KSOM; Zenith Biotech) [25] supplemented with 1 mg/mL of BSA was used for cultivating the zygotes. For IVF, sperm from WT male mice at 8 weeks old were collected by squeezing the cauda epididymis and then placing it into 500 ul (micro litter) HTF in a 1.5-mL microtube. The tubes were then preincubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 1 h, and only swim-up sperm were collected and adjusted to final concentrations of 5.0×10^5 sperm/mL. The cumulus cell–oocyte complex was inseminated with sperm for 5 h. After insemination, the embryos were transferred to new KSOM media with or without AsA (100 μM) and cultivated for 4 days. Each culture was maintained in 50-μL droplets of medium overlaid with mineral oil in 35-mm-

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diameter plastic culture dishes. Incubations were conducted at 37 °C, 5% CO_2, and 95% air.

Hyperovulation and oocyte counting

Vaginal cytology was performed for determining the day of ovulation. Mice were then euthanized on the morning of estrus, and oocytes harvested from the ampulla of the oviducts were counted. Hormone-stimulated ovulation was induced in mice, as described previously [15]. Briefly, SOD1-KO and C57BL/6 background genetic control (WT) female mice at 8 weeks of age were injected intraperitoneally with 5 IU of PMSG once. At 48 h after the PMSG administration, 5 IU of hCG was administered intraperitoneally once. At 14 h after hCG administration, oocytes in the ampullary sites of both fallopian tubes were collected and counted. For repeated superovulation, PMSG/hCG injections were repeated 1–4 times to corresponding mouse groups (n = 9–13, each group) at intervals of 7 days. The oocytes were then isolated from the ampulla of mice after receiving the final hCG injection and counted.

Supplementation of CoQ_{10} and/or ascorbic acid

AsA was purchased from Wako Pure Chemical (Osaka, Japan). Reduced form of CoQ_{10} (Kaneka QH) was kindly provided by Kaneka Corp. (Tokyo, Japan). After weaning at 4 weeks of age, AsA (1.5 g/L) and/or CoQ_{10} (1 g/L) that were dissolved in the drinking water were supplemented ad lib to the KO mice. The AsA concentrations used in this study have been reported to be sufficient and safe to the mice that have a defect in AsA synthesis due to the genetic ablation of Akr1a in the AsA producing pathway [26]. Based on the preliminary data on the volume of water intake, the daily intake of CoQ_{10} was calculated to be about 600 mg/kg/day, which is an effective dose for rats and dogs [27] and a dose similar to that has been administered to human patients (500 mg/kg/day) [28].

Measurement of ascorbic acid in plasma

15-(Naphthalen-1-ylamino)-7-aza-3,11-dioxadispiro [5.1.5^8.3^6]hexadecan-7-oxyl (Naph-DiPy), a fluorescent probe, was synthesized [29] and used to measure the AsA. Fresh blood plasma was used for the AsA assay. In a typical run, in the presence of ethylenediaminetetraacetic acid (EDTA), a blood sample was collected from the tail vein of the mice. After centrifugation at 900× g for 5 min at room temperature (RT), the blood plasma was incubated with Naph-DiPy for 30 min at RT. The AsA concentration was calculated by measuring the fluorescence at an excitation wavelength of 310 nm and an emission wavelength of 430 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Yokohama, Japan). The plasma AsA concentrations were calculated from a standard curve constructed using an authentic standard sample of AsA.

Measurement of total CoQ_9 and CoQ_{10} in plasma and tissues

Plasma levels of CoQ_9 and CoQ_{10} were determined by a previously reported method [27] with minor modifications. In a typical run, plasma treated with 2-propanol was analyzed by high-performance liquid chromatography (HPLC) equipped with a guard column (Type Supelguard LCABZ, 5 Am, 33_4.6 mm i.d., Supelco, Osaka, Japan), an analytical column (Type Supelcosil LC-8, 5 Am, 250_4.6 mm i.d., Supelco), a reduction column (Type RC-10-1, Irica, Kyoto, Japan), a UV detector (210 nm), and an amperometric electrochemical detector (ECD; Model A985, Irica). The ECD oxidation potential was +600 mV (vs Ag/AgCl) on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/2-propanol (9/1, v/v) delivered at a rate of 0.8 mL/min. The detection limits of CoQ_9 and CoQ_{10} are 10 and 2 fmol, respectively. The plasma concentrations of CoQ_9 and CoQ_{10} were calculated from standard curves constructed using authentic CoQ_{10}.

Measurement of plasma progesterone

Blood was collected from the tail vein of the mice at 7.5 days of pregnancy in the presence of an excess of EDTA. After centrifugation at 800× g for 5 min, plasma progesterone (P4) concentrations were measured using a Progesterone ELISA kit (ADI-900-011, Enzo Life Sciences, New York, USA) according to the manufacturer's instructions.

Histological analyses of ovary and placenta

Ovaries and placenta of mice after the first parturition or placenta at 17.5 days of pregnancy were dissected and fixed in 15% buffered formalin. After embedding in paraffin, 7-μm-thick sections of the ovaries were prepared and then stained with hematoxylin and eosin (H&E). Photographs of the sections were obtained using a BZ-X700 microscope (KEYENCE, Osaka, Japan), and area occupied by a corpus luteum cell in ovaries was measured by Image J (NIH, Bethesda, MD). We randomly chose two corpus luteum regions (0.2 mm × 0.3 mm) in one longitudinal section of the ovary (middle part) from each genotypic mice (n = 4), which mostly covered all corpus luteum regions in each section and measured the area for more than 300 cells in each genotypic mouse.

Immunohistochemical analysis of placenta

Rehydrated placenta sections from mice at pregnancy day 17.5 were subjected to immunohistochemical (IHC) analysis. Briefly, the tissue sections were incubated with a 3% hydrogen peroxide solution to eliminate intrinsic peroxidase activity. The target retrieval procedure involved immersing the tissue sections in a citrate-based buffer solution (pH 6.0) followed by heating in an autoclave. After rinsing, the sections were incubated for 60 min with 3% BSA in phosphate-buffered saline (PBS) at RT, followed by incubation with a rabbit polyclonal antibody against CD31 (Genetex, Tokyo, Japan, GTX130274, dilution 1:200) overnight in a humidified chamber at 4 °C. After rinsing, the sections were sequentially treated with a peroxidase-labeled goat anti-rabbit IgG polymer for 60 min. After a final rinse, specific immunolabeling was visualized by placing a chromogen, 3,3′-diaminobenzine (K1391, Dako, Tokyo, Japan) on the tissue sections for a few minutes. Slides were counterstained with hematoxylin. The sections were then dehydrated, mounted, and examined via light microscopy, as described above. Dark signals on the stained sections, corresponding to the CD31 protein, were extracted from pictures and quantified.

Protein preparation

Ovaries were homogenized in five volumes of RIPA buffer, which contains 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% Nonidet P-40; 0.5% deoxycholate; and 0.1% SDS, supplemented with a protease inhibitor cocktail (Sigma-Aldrich Corporation, Tokyo, Japan). After centrifugation at 15 000 rpm, the protein concentration in the supernatant was determined using a BCA protein assay reagent (Thermo Fisher Scientific).

Western blot analysis

The following antibodies were used: SOD1 [23], SOD2 [23], Catalase (Calbiochem, 219010), steroidogenic acute regulatory

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Table 1. Rescue of developmentally arrested KO embryos by AsA.

Oocyte genotype and treatments	Number of oocytes cultured	Number of embryos (%)		
		Two-cell stage ≤ at day 1	Four-cell stage ≤ at day 2	Blastocyst at day 4
WT	130	112 (86.2)[a,c]	105 (80.8)[a,c]	101 (77.7)[a,c]
KO	148	129 (87.2) [a,c]	11 (7.4)[b]	0 (0)[b]
KO + AsA	161	144 (89.4)[a]	129 (80.1)[a,c]	97 (60.3)[c]

Embryos developed from KO or WT mouse oocytes inseminated with WT sperm were cultured under conventional conditions. After insemination in HTF media, the embryos were transferred to new KSOM media with or without AsA (100 μM) and cultivated for 4 days. Female mice were bred under conventional conditions without AsA supplementation and used for oocyte collection. Numbers of female mice used for oocyte collection were 8 WT mice and 13 KO mice. To avoid the impact of individual difference, oocytes from individual female KO mouse were divided into two groups: one with and the other without AsA supplementation. So that the total number of KO female mice used for IVF was the same. We performed one-way ANOVA followed by Tukey test. Different superscript letters within the table indicate a statistical difference ($P < 0.05$).

protein (StAR) (Santa Cruz, Santa Cruz, USA, sc-25806), $P450_{Scc}$ (Chemicon, AB1244), 4-hydroxynonenal (HNE; Nikken Seil, Tokyo, Japan, MHN-100), or β-actin (Santa Cruz, sc-69879). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies (Santa Cruz Biotechnology) were used as the secondary antibodies. After washing, immune reactive bands were detected by measuring the chemiluminescence using an Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (EMD Millipore, Temecula, CA) on an image analyzer (ImageQuant LAS500; GE Healthcare Life Sciences, Buckinghamshire).

Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using one-way or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple groups or by Dunnett multiple comparison using the GraphPad Prism 6 software program. A *P*-value of less than 0.05 was considered to be significant.

Results

Ascorbic acid rescues developmental arrest of knockout embryos in vitro

We previously reported that the development of embryos from the oocytes of KO mice is arrested at two-cell embryonic stage under conventional culture conditions and is rescued by cultivation under 1% oxygen but not by supplementation with a reducing agent, 2-mercaptoethanol [15]. In the preliminary experiment, we have examined the effects of AsA at 50 and 125 μM and found improvement in the developmental efficiency, which showed about 30% embryos developed to blastocyst by 125 μM but none by 50 μM AsA in mice. We thought that 125 μM might be a little high because the plasma level is ∼70 μM and hence used 100 μM for this experiment (unpublished observation). While two-cell embryos that were derived from KO oocytes fertilized with WT sperm rarely develop to the four-cell stage, supplementation of 100 μM AsA markedly stimulated the development of the embryos (Table 1).

Low fertility in young knockout mice

Because the KO mice are slightly smaller than the WT mice [23], we suspected that sexual maturation might be delayed in the female KO mice. To confirm this, we examined the fertility of female mice ($n = 9$–10 each) at 4–6 weeks of age. After cohabitation of the female mice with a fully matured WT male mouse in an individual cage for 1 week, fecundity was assessed by counting the number of litters in the following 3 weeks. Regarding the WT mice, half of the female mice were already fertile at 4 weeks and all were fertile at 5 weeks of age (Figure 1A). On the contrary, no parturition was observed in the KO mice at 4 weeks. Thereafter, the parturition rate increased but litter sizes were remained less than those for the WT mice. A follow-up survey of the pregnant female mice that finally gave birth indicated that the weight gain of KO mice was suppressed compared with WT mice during pregnancy and the body weights of the KO mice were low at the time of delivery (Figure 1B). To minimize the influence of the delay in sexual maturation and to examine the effects of SOD1 ablation on female reproductive ability more specifically, we started mating the female mice at 8 weeks of age in the following experiments.

No difference in the number of ovulated oocytes by repeated hormone treatment was observed

The experiment on natural ovulation did not show a significant difference in the numbers of ovulated oocytes among three groups of mice (Figure 2A). To evaluate the status of ovaries, we examined the effects of hyperstimulation on ovulation from the WT and KO female mice at 8 weeks of age by repeated hormone treatments (up to four times). The drinking water for a group of the KO mice was supplemented with AsA (1.5 g/L) after weaning at 4 weeks of age. As results, numbers of ovulated oocytes increased compared with natural ovulation, but no significant differences were observed between the WT and KO mice regarding the numbers of ovulated oocytes at any of the repeated cycles of hormone treatment. Supplementation with AsA also has no effect on the number of ovulated oocytes in the KO mice.

Supplementation of ascorbic acid and/or CoQ_{10} increases the number of female KO mice capable of the second parturition

We then conducted experiments to determine whether the administration of antioxidants might have beneficial functions in maintaining the fertility of the KO mice. Supplementation of the KO mice with either a hydrophilic antioxidant AsA (1.5 g/L) and/or a lipophilic antioxidant CoQ_{10} (1 g/L) started at 4 weeks of age, after which the mice were cohabited with sexually matured male mice at 8 weeks of age for another 8 weeks. After the first delivery, the newborn mice were removed immediately, and the mating was continued. At the first parturition, 80% of the untreated KO mice produced relatively small litter sizes (average 2.8 pups/mouse) compared with the WT mice (average 7.3 pups/mouse), and no substantial changes were observed as the result of the supplementation of these antioxidants (Figure 3A, $P > 0.05$ in any combination). Regarding the second parturition, however, while only 18% of the KO mice without supplementation produced offspring, 44–75% of the KO mice that had the supplementation of AsA and/or CoQ_{10} produced offspring again (Figure 3B; $P = 0.0089$, WT vs KO; $P = 0.012$, KO vs KO + QA;

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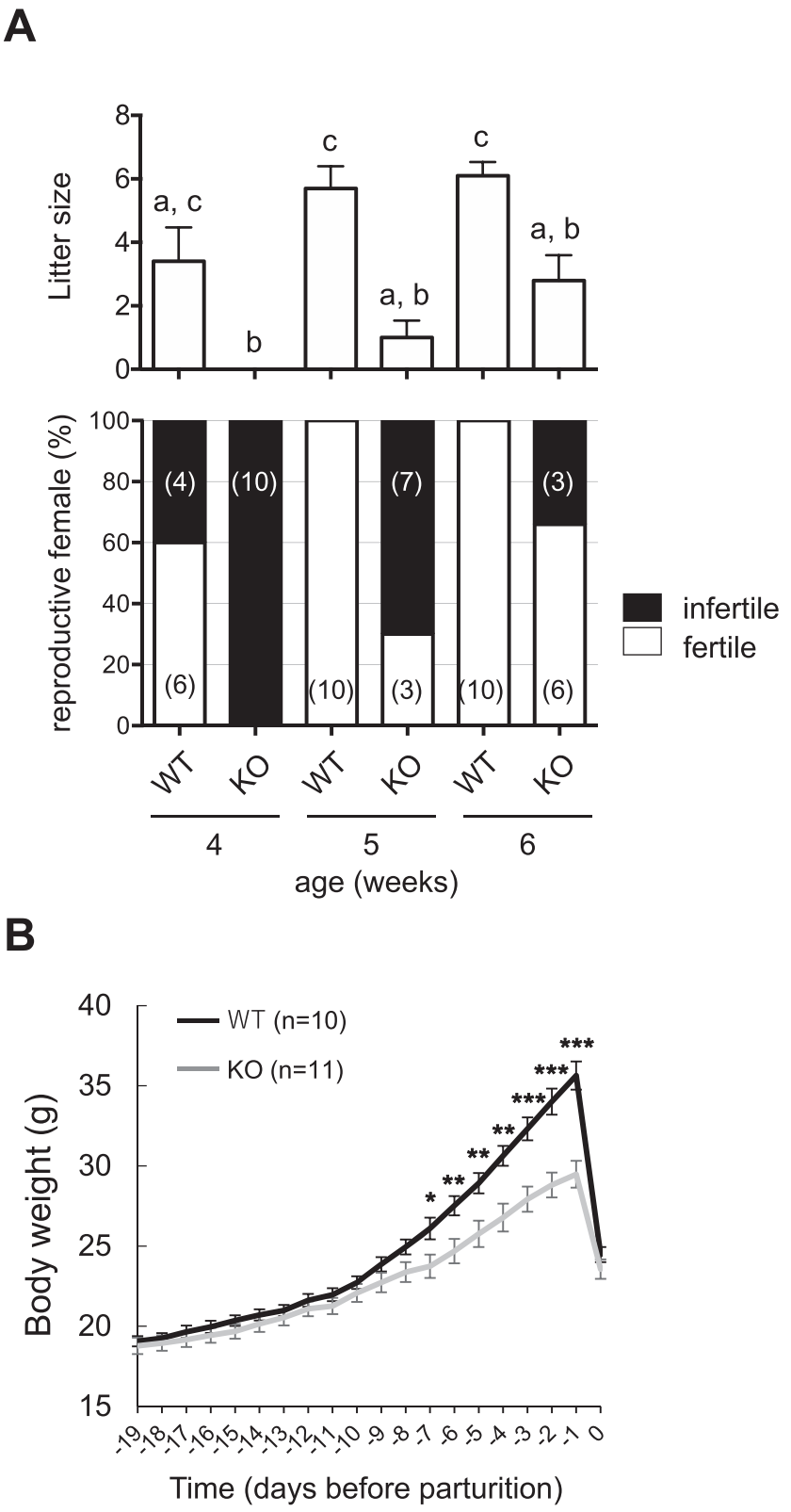

Figure 1. Changes in reproductive ability during maturation of the female mice. (A) The mean ± SEM of numbers of neonates born from each group are shown in the top panel. The bottom graph indicates the percentage of female mice that could give parturition with actual numbers in parentheses ($n = 9$–10). We performed two-way ANOVA followed by Tukey test. Different letters above the columns indicate a statistical difference ($P < 0.05$). (B) Changes in the body weight of the KO and WT female mice. Day 0 indicates the delivery date. We performed two-way ANOVA followed by Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Mean ± SEM ($n = 10$–11).

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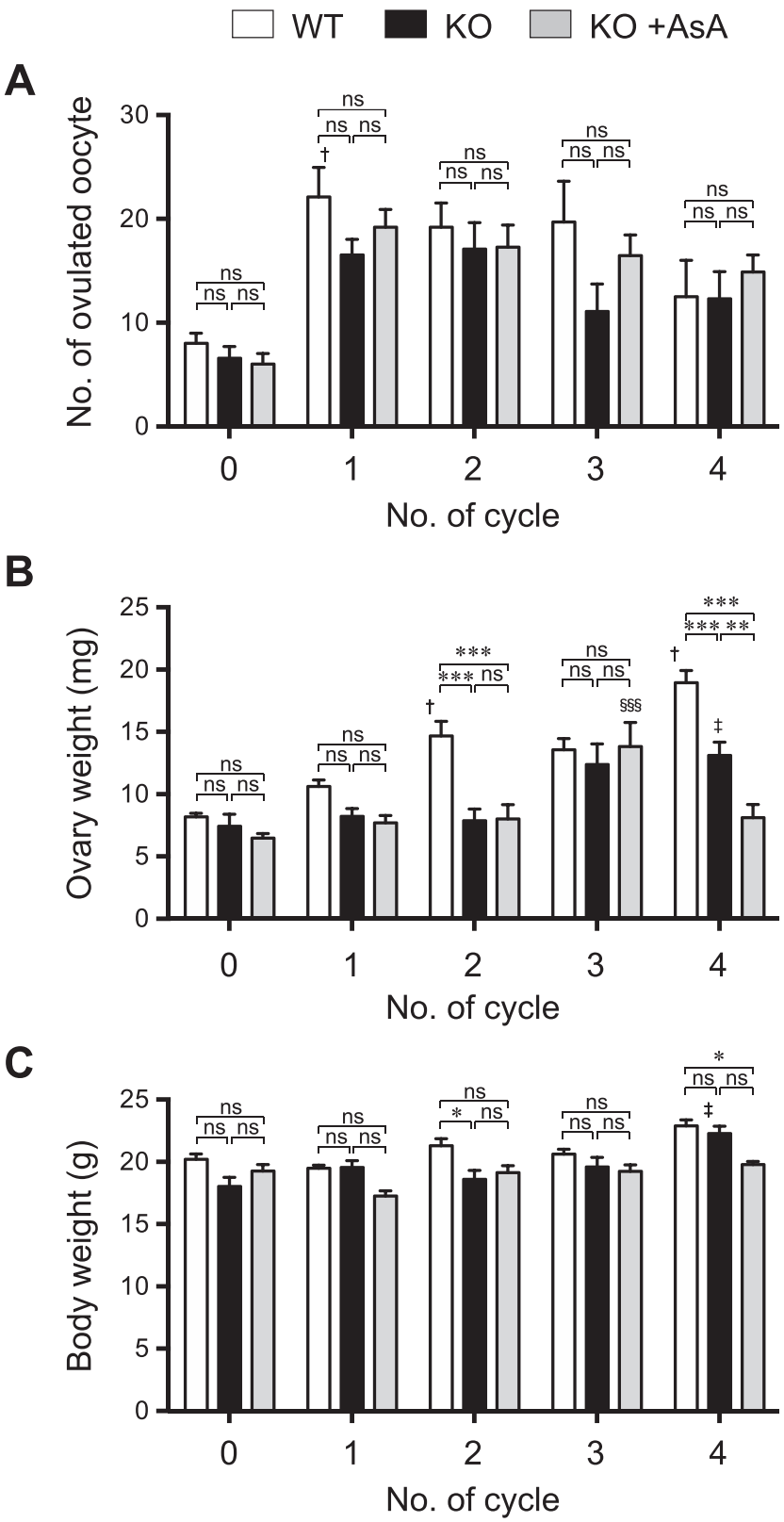

Figure 2. Numbers of ovulated oocytes by hyperstimulation with hormone treatment. (A) Numbers of ovulated oocytes, (B) ovary weight, and (C) body weight of the mice at natural ovulation or at the end of the indicated hormonal treatment were shown. Data are the mean ± SEM ($n = 5$–8 for mice with natural cycle; $n = 9$–13 for mice with hormonal treatment). We performed two-way ANOVA followed by Tukey test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant. †, vs WT control (0 cycle); ‡, vs KO control (0 cycle); §, vs KO + AsA control (0 cycle).

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$P > 0.05$ in other combination). Thus, these antioxidants appeared to have a beneficial role in maintaining the reproductive ability of the female KO mice, although there were no significant differences in the weights of the ovaries (Figure 3C), uterus (Figure 3D), and body (Figure 3E) of the mice after the first parturition.

An aberrant progesterone (P4) balance has been reported to be associated with infertility in KO mice [14]. To gain insights into the roles of the supplements in ovary function, we measured the levels of progesterone in plasma collected from mice at 7.5 days of pregnancy. Because the incidence of abortion was high in the KO mice as reported [12], plasma samples from the mice that finally completed the first parturition were subjected to an enzyme-linked immunosorbent assay (ELISA) assay. As a result, however, no difference was observed in progesterone production among the mouse groups (Figure 4A), indicating that the KO mice enabling the first parturition were in normal hormonal balance. We measured plasma AsA and the tissue levels of CoQ in the mice at 20 weeks of age. Although the mouse is able to biosynthesize AsA from glucose, AsA levels were lower in both the control and CoQ groups of the KO mice (Figure 4B). Plasma AsA concentrations were recovered in the mice with the drinking water containing AsA (1.5 g/L). We also measured the levels of CoQ_9, which is the dominant form produced in mice, and CoQ_{10}, which is largely derived from the supplement, in the plasma, ovary, liver, and kidney (Figure 4C–F and Supplementary Figure S1). CoQ_{10} levels were high in the plasma, ovary, and liver but not in the kidney of the mice that were administered CoQ_{10}. AsA had no effect on the CoQ levels in these tissues.

Small corpus luteum cells in ovary in postpartum KO mice and partial amelioration by ascorbic acid and/or CoQ_{10}

To gain insights into the protection of ovarian function by antioxidants, we performed histological analyses of ovaries from the five experimental groups of mice immediately after the first parturition. Ovarian sections from the KO mice showed that the corpus luteum cells in ovaries of the KO mice were smaller in size compared with those from the WT mice (Figure 5). Supplementation with AsA and/or CoQ_{10} in the KO mice increased the cellular size significantly, implying that these antioxidants are beneficial in maintaining cellular homeostasis. We then examined the levels of major antioxidative enzymes: SOD2 and catalase as well as SOD1, and two essential steroidogenic proteins: the steroidogenesis acute regulatory protein (StAR), which moves cholesterol into mitochondria, and cholesterol side-chain cleavage enzyme P450scc, which is encoded by the cytochrome P450 11A1 (CYP11A1) gene, in ovaries at time after the first parturition (Figure 6). As results, no significant difference was observed in the levels of these proteins among the groups of the mice. We also examined the possible involvement of oxidative stress in the female infertility by measuring the degree of protein modification caused by HNE, a reactive lipid peroxidation product [30]. There appeared to be large individual difference in HNE-modified proteins in the ovaries, and no significant changes were observed (Supplementary Figure S2). Thus, improvement in the reproductive ability in the KO mice by the supplementation was not supported by the oxidative stress marker.

Histological examination of placental vasculogenesis

Because the resorption rate is high in KO mice [12], we hypothesized that a defect in placental function was involved in the infertility. To examine this possibility, we performed histological analyses of placenta tissue in mice after they were mated with WT male mice. Placenta was dissected from the female mice at pregnancy day 17.5 and subjected to H&E staining (Figure 7A). Although no extensive damage was observed in the KO placenta, the histological appearance suggested that the vasculature in the labyrinth of the KO placenta was less developed compared with the WT mice. Supplementation with AsA improved the vascularization to some extent. Moreover, immunostaining of the placental sections with an antibody against CD31, a maker protein for vascular endothelial cells [31], confirmed these trends (Figure 7B).

Discussion

This study was conducted to determine whether supplementation with antioxidants, AsA and/or CoQ_{10}, rescues the infertility in the KO female mice and to reveal the potential target of oxidative stress caused by a SOD1 deficiency. Supplementation with these nutritional antioxidants resulted in a partial rescue, as evidenced by an increased rate of offspring production at the second parturition (Figure 3B). The improvement in the fertility rate of the female KO mice appeared to be, at least partly, due to the protection of corpus luteum cells (Figure 5) and uterus/placenta function (Figure 7) from injury during pregnancy and delivery.

There are a few inconsistencies between our data and previous reports [12–14]. The initial study on the fertility of the female KO mice implies an insufficient response to gonadotropins as the main cause for severe infertility [13]. Other investigators have independently established KO mice and reported high embryonic lethality before 10 days of pregnancy [12]. Aberrant luteal formation and decreased progesterone production have been reported to be a potential cause afterward [14]. We found that infertility was very severe in the KO female mice at younger ages but improved parallel with their growth (Figure 1), which suggests that the sexual maturation of the KO female was delayed. The litter sizes of the KO mice at 8 weeks of age were still less than a half those of the WT mice. While we backcrossed the KO mice more than 10 times with C57BL/6 N mice [23], a mixed background of the 129SvJ and C57BL/6 was used by Ho et al. [12], but in the paper by Matzuk et al. [13], this was not a subject that was raised. Hence, we considered that differences in the backgrounds of the mouse strains used for studies might also partly explain these inconsistent results. Moreover, because superoxide exerts epigenetic effects [32], it is also possible that superoxide stimulated epigenetic changes in the years of the breeding period of the KO mice, leading to a phenotypic difference in these mice.

Because KO mice are born with Mendelian characteristics from heterozygous female mice, it can be rationally hypothesized that a maternal deficit rather than embryonic one causes the infertility of the KO mice. It has been reported that ovarian AsA concentrations decline to 50% after LH treatment and that this level is sustained for 8 h [33], suggesting that AsA is consumed in the metabolic process that is stimulated by LH. When female mice are fed a diet supplemented with a mixture of AsA and vitamin E, the percentage of aneuploidy and diploidy is less in the supplement-fed females compared with those with the control diet [34]. Thus, AsA appears to improve reproductive function via the amelioration of multiple processes.

The ovulation process itself is comparable to an inflammation-like reaction [35] and produces ROS during normal reproductive cycles [2]. The levels of superoxide and SOD1 change inversely during the reproductive cycle in rats [36], suggesting that SOD1

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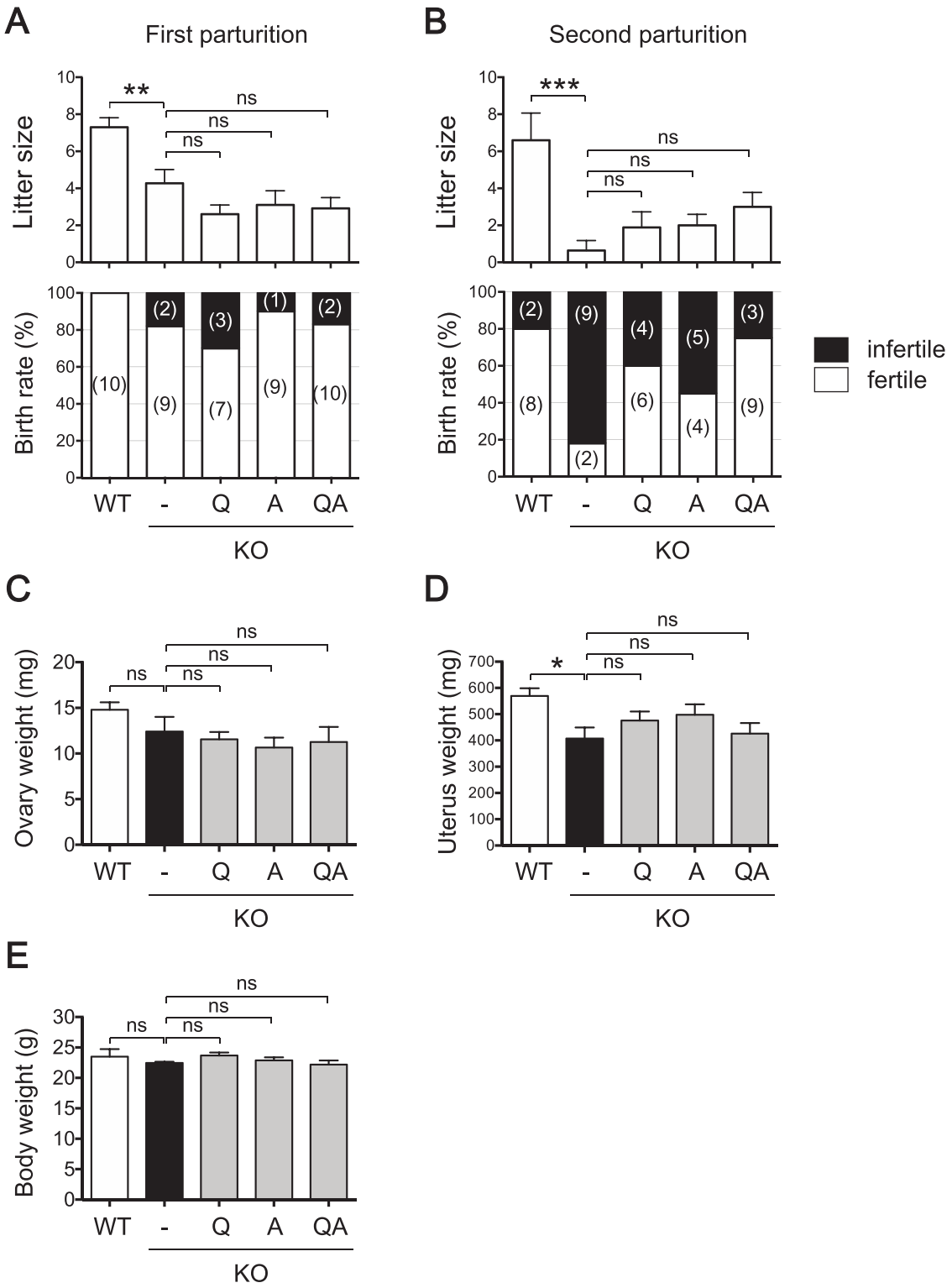

Figure 3. Effects of supplementation of AsA and/or CoQ_{10} on the reproductive ability of the KO mice. The lower and upper graphs, respectively, indicate the percentage of female mice and their litter sizes that could result in (A) the first parturition and (B) the second parturition. Empty column and numbers in parentheses in the lower graphs indicate the actual number of female mice used in this experiment. Another set of mouse groups were administered with the antioxidants and cohabitated with male mice in the same way as above. After the first parturition, the weights of the (C) ovary, (D) uterus, and (E) body of the mice were measured. All data are the mean ± SEM ($n = 4$–6). We performed Pearson χ^2 test (A and B) or one-way ANOVA followed by Dunnett multiple comparison to KO control (without supplements) (C—E). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant. Q, KO mice administered CoQ_{10}; A, KO mice administered AsA; QA, KO mice administered CoQ_{10} and AsA.

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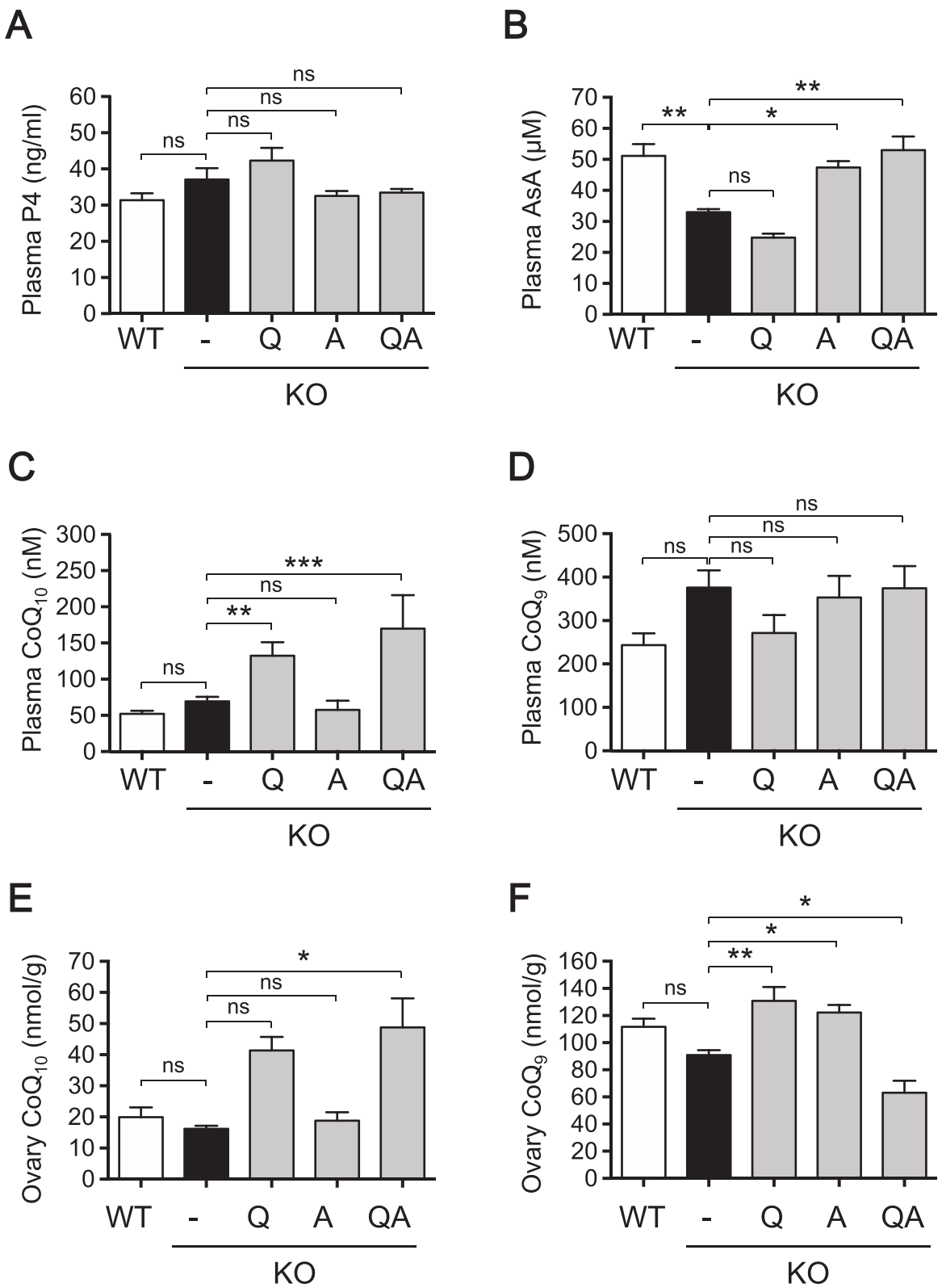

Figure 4. Levels of AsA, progesterone (P4), and CoQs. (A) Blood plasma were collected from 7.5-day pregnant mice and subjected to progesterone assay by ELISA ($n = 4$–6). (B) After weaning, the KO mice were continued to be administered AsA (1.5 g/L) and/or CoQ_{10} (1 g/L) and sacrificed at 8 weeks of age ($n = 3$–6). Plasma levels of AsA were measured using the fluorescent probe for AsA. Levels of CoQ_{10} (C, E) and CoQ_9 (D, F) in plasma (C, D) and ovary (E, F) in the mice were measured. All data are the mean ± SEM. We performed one-way ANOVA followed by Dunnett multiple comparison to KO control (without supplements). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant. Q, KO mice administered with CoQ_{10}; A, KO mice administered with AsA; QA, KO mice administered with CoQ_{10} and AsA.

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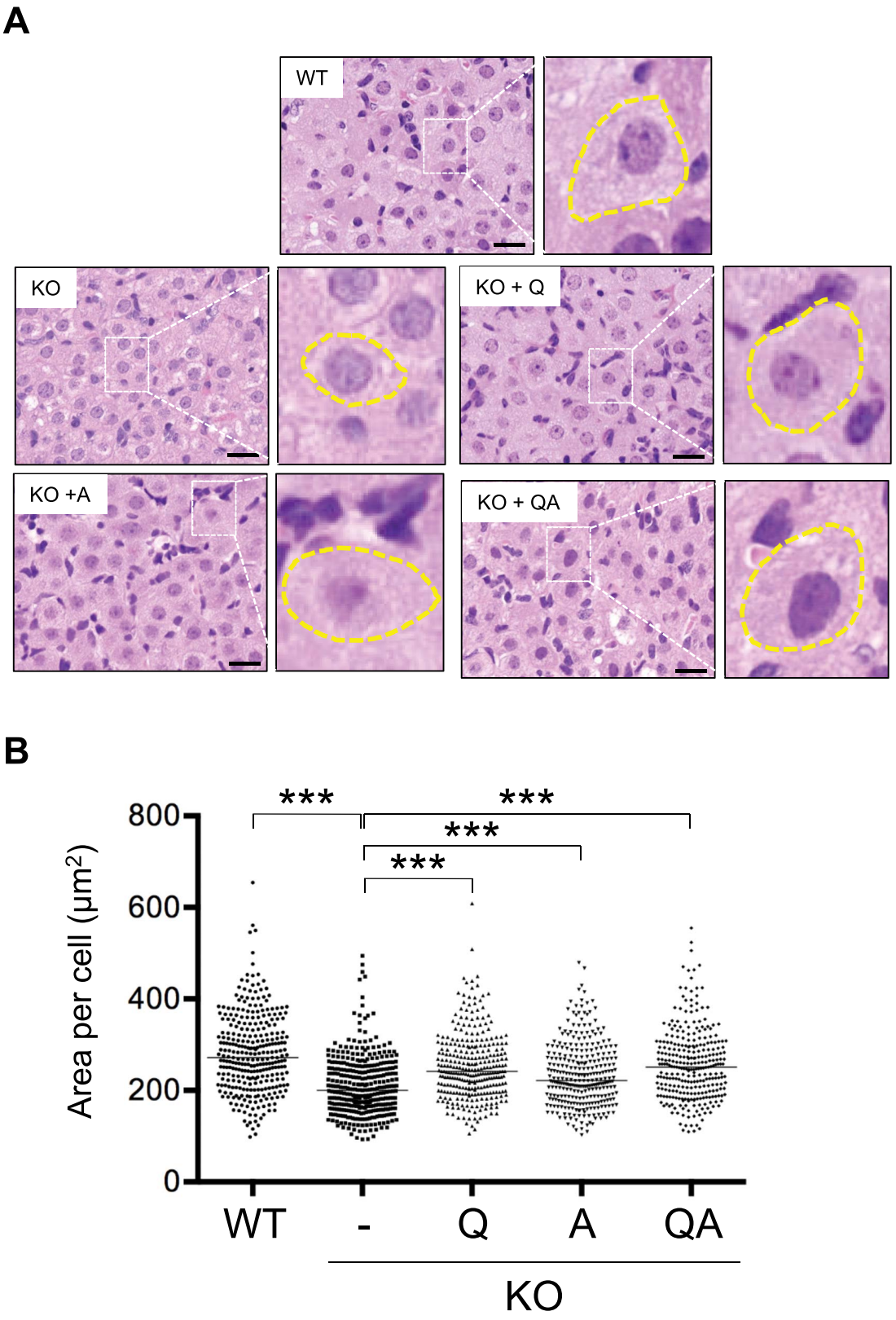

Figure 5. Atrophic changes observed after the first delivery in corpus luteum cells in ovary from KO mice and rescue by supplementation with AsA and/or CoQ_{10}. One ovary of each of the mice was fixed in formalin, and 7-μm sections were prepared and stained with H&E. (A) Representative images of ovaries in each group of the mice were shown. Bar, 20 μm. (B) Area of more than 300 cells, exemplified as a circled cell with a dotted yellow line, in each genotypic mice was measured by Image J. Mice numbers were four for each genotype. Beeswarm plots of the data together with the median line are shown. We have used the mean cell area for each of the four mice per group and performed one-way ANOVA followed by Dunnett multiple comparison to KO control (without supplements). $^{***}P < 0.001$; Q, KO mice administered with CoQ_{10}; A, KO mice administered with AsA; QA, KO mice administered with CoQ_{10} and AsA.

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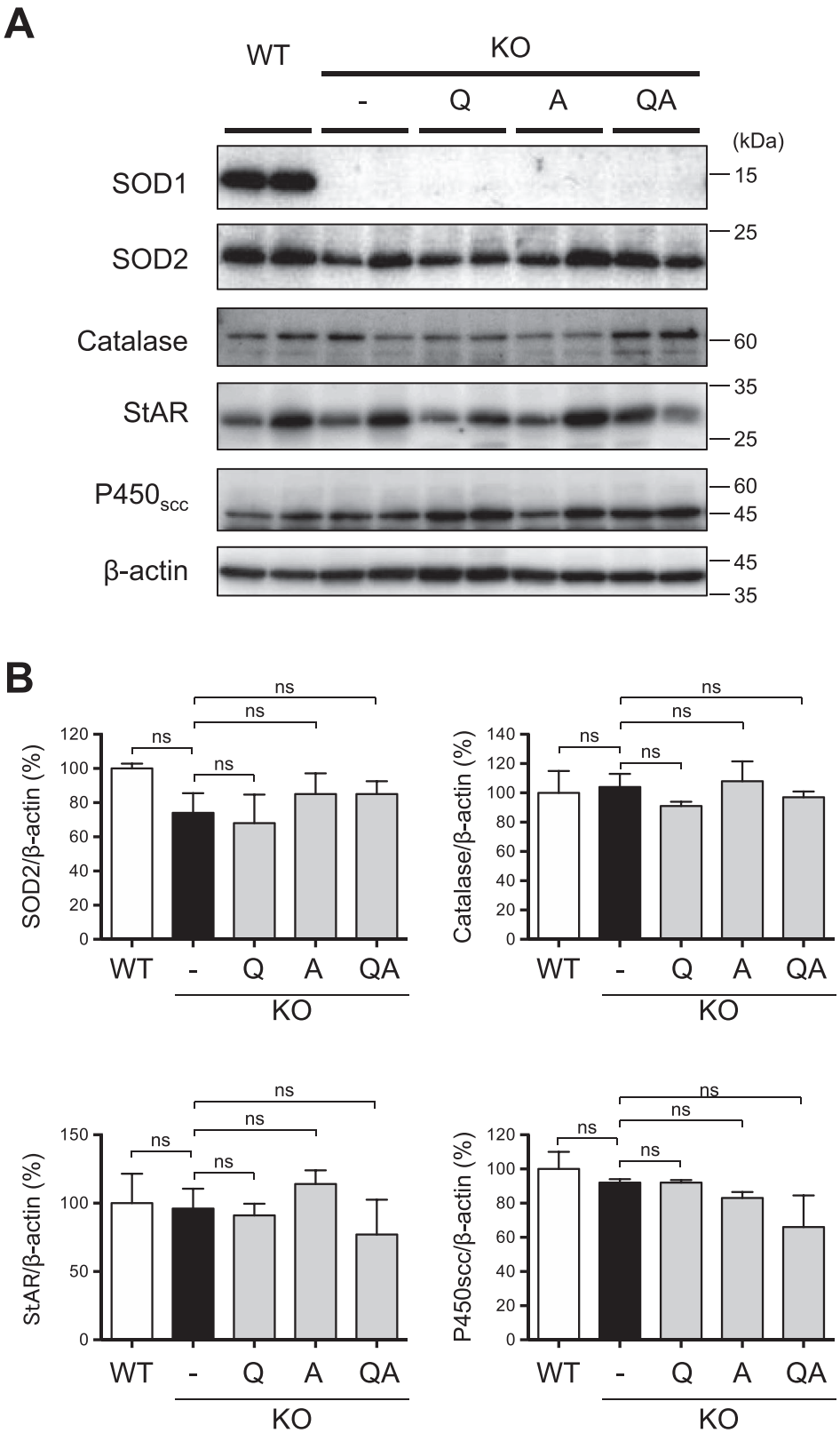

Figure 6. Levels of proteins involved in antioxidation or steroidogenesis. Ovary samples, which were obtained from the female mice described in Figure 5 and stored at −80 °C, were subjected to protein analyses. Three or four out of four ovaries in each group were derived from the same mice used in Figure 5. (A) Proteins were subjected to immunoblot analyses using antibodies against SOD1, SOD2, Catalase, StAR, $P450_{scc}$, and β-actin. (B) The panels depict the quantification of the intensity of proteins and normalized to the corresponding β-actin band. Data are the mean ± SEM ($n = 4$). We performed one-way ANOVA followed by Dunnett multiple comparison to KO control (without supplements). ns, not significant. Q, KO mice administered with CoQ_{10}; A, KO mice administered with AsA; QA, KO mice administered with CoQ_{10} and AsA.

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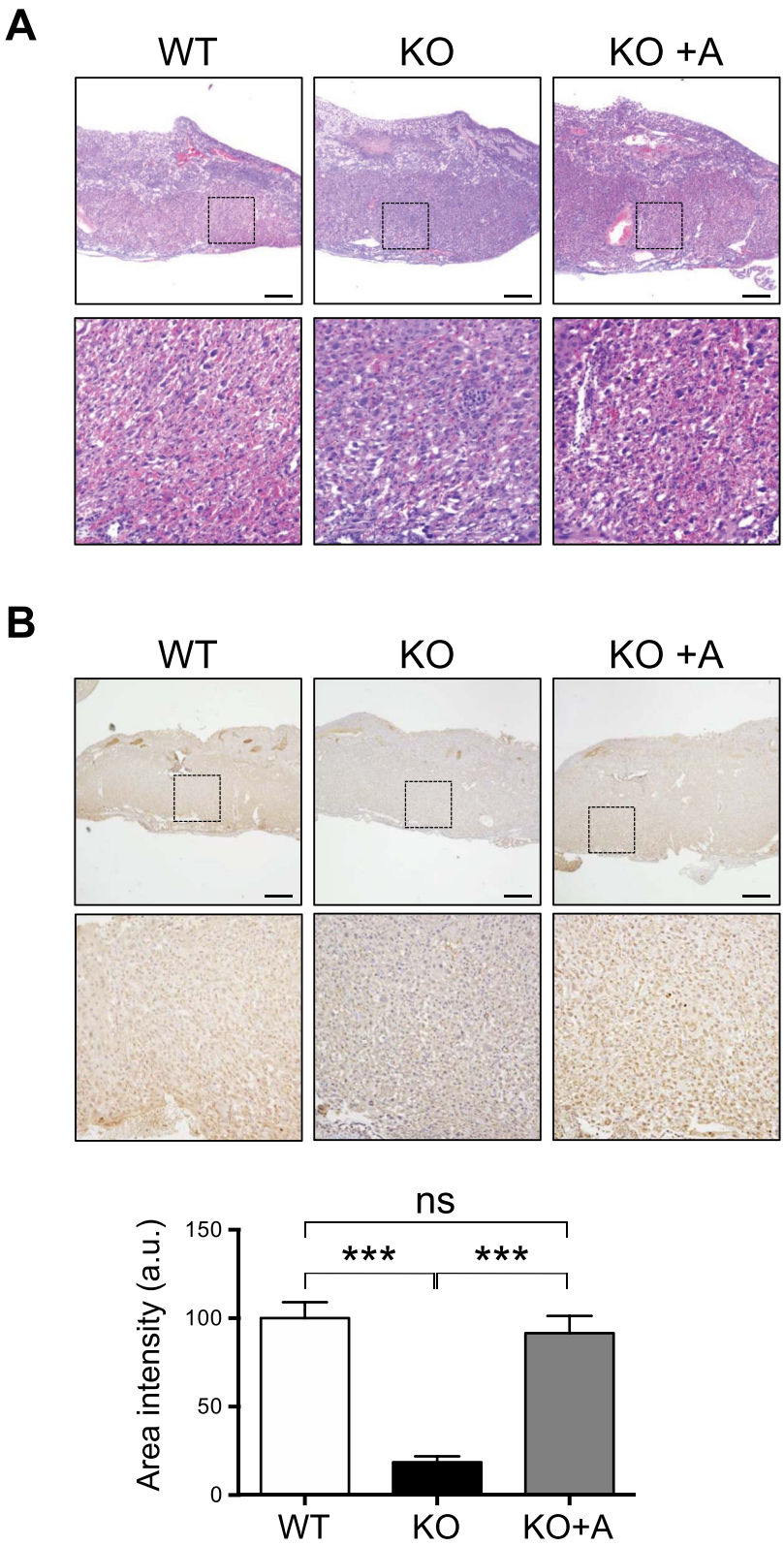

Figure 7. Impaired vasculogenesis of KO placenta and partial amelioration by AsA. (A) The 7-μm sections were prepared and stained with H&E. (B) IHC staining of the sections was carried out using an antibody against CD31 as the primary antibody. Representative images of placenta tissue from each group of the mice are shown. Bars, 500 μm. Quantification of the CD31-derived signal was performed on four sections from two mice in each experimental group. We performed one-way ANOVA followed by Tukey multiple comparison. ***$P < 0.001$; ns, not significant.

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has a beneficial function in suppressing the production of excessive levels of ROS. Because we were interested in determining whether progesterone concentrations affected the litter size of the KO mice, we measured the hormone levels in plasma only from the mice at 7.5 days of pregnancy that finally gave the first parturition. Given the observations reported by Noda et al. [14], KO mice with lower progesterone production might be associated with the high rate of embryo resorption. Consistent with this notion, impaired placental development, notably poor labyrinth formation, was observed in the KO mice (Figure 7). Thus, AsA supplementation did not affect the litter size, but it might contribute to the maintenance of ovary and uterus functions and enable the second pregnancy.

In turn, in a report that examined 483 pregnant women, low plasma CoQ_{10} levels were found to be associated with the incidence of spontaneous abortion [37]. Supplementation with CoQ_{10} improved the reproductive ability of the KO mice at the second parturition, similar to the case of supplementation with AsA (Figure 3B). The ovary is the organ that effectively takes up CoQ_{10} (Figure 4E), consistent with a report by Bentinger et al. [38], although some organs such as the kidney are not, a conclusion that was confirmed in this study (Supplementary Figure S1). CoQ_{10} improves mitochondrial function and rescues age-related declines in oocyte quality in mice [22]. Similarly, CoQ_{10} has been demonstrated to be effective in treating ischemia-reperfusion injuries triggered by ovarian torsion in rats [39]. CoQ_{10} levels in human follicular fluid are correlated with oocyte maturation and embryo grade during in vitro maturation [40]. The findings reported herein imply that the combined administration of CoQ_{10} with AsA may also improve clinical pregnancy rates. A possible explanation for this is that oxidative damage by elevated ROS causes infertility in KO mice. Consistently, HNE-modified proteins appeared to be elevated slightly in the KO mice and this elevation tended to be suppressed by AsA (Supplementary Figure S2). To the contrary, however, CoQ_{10} did not suppress the formation of HNE-modified proteins but rather caused an increase. Thus, at this moment, the causal connection between SOD1 ablation and impairments in the ovary and uterus/placenta remain unclear. A possible explanation would be the disruption of the signaling function of superoxide [41] rather than robust oxidative modification. In this scenario, supplemented AsA and/or CoQ_{10} might eliminate excessive superoxide, leading to the normalization of the signaling pathway.

In conclusion, we employed two nutritional components with antioxidant functions, AsA and CoQ_{10}, and found them to be of benefit in improving the reproductive ability of KO mice. The administration of either supplement alone was effective in improving ovarian and uterus/placental function and that their combined administration was even more effective, despite the absence of synergism. These antioxidants appear to improve ovarian function by protecting against damage caused during pregnancy and parturition. Thus, these exogenous antioxidants may have protective roles against ROS-triggered infertility in KO mice and may also exert beneficial function in cases of infertile women.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Acknowledgments

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Author Contributions

NI performed the majority of the experiments and JL performed some experiments. TH and NK provided technical support for NI. HM and YY measured the CoQ levels in the samples. KY provided the fluorescent probe for detecting AsA. JF conducted the study, performed some experiments, and wrote the paper.

Conflict of interest

The authors have declared that they have no conflicts of interest.

References

1. Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. *Antioxid Redox Signal* 2008; **10**:1375–1403.
2. Jabbour HN, Sales KJ, Catalano RD, Norman JE. Inflammatory pathways in female reproductive health and disease. *Reproduction* 2009; **138**:903–919.
3. Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reprod Biol Endocrinol* 2005; **3**:43.
4. Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* 2011; **26**:1628–1640.
5. Keefe DL, Niven-Fairchild T, Powell S, Buradagunta S. Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertil Steril* 1995; **64**:577–583.
6. Chao HT, Lee SY, Lee HM, Liao TL, Wei YH, Kao SH. Repeated ovarian stimulations induce oxidative damage and mitochondrial DNA mutations in mouse ovaries. *Ann N Y Acad Sci* 2005; **1042**:148–156.
7. Miyamoto K, Sato EF, Kasahara E, Jikumaru M, Hiramoto K, Tabata H, Katsuragi M, Odo S, Utsumi K, Inoue M. Effect of oxidative stress during repeated ovulation on the structure and functions of the ovary, oocytes, and their mitochondria. *Free Radic Biol Med* 2010; **49**: 674–681.
8. Devine PJ, Perreault SD, Luderer U. Roles of reactive oxygen species and antioxidants in ovarian toxicity. *Biol Reprod* 2012; **86**:27.
9. Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 1995; **64**:97–112.
10. Tsunoda S, Kawano N, Miyado K, Kimura N, Fujii J. Impaired fertilizing ability of Sod1-deficient mouse sperm during in-vitro fertilization. *Biol Reprod* 2012; **87**:1–6.
11. Garratt M, Bathgate R, de Graaf SP, Brooks RC. Copper-zinc superoxide dismutase deficiency impairs sperm motility and in vivo fertility. *Reproduction* 2013; **146**:297–304.
12. Ho YS, Gargano M, Cao J, Bronson RT, Heimler I, Hutz RJ. Reduced fertility in female mice lacking copper-zinc superoxide dismutase. *J Biol Chem* 1998; **273**:7765–7769.
13. Matzuk MM, Dionne L, Guo Q, Kumar TR, Lebovitz RM. Ovarian function in superoxide dismutase 1 and 2 knockout mice. *Endocrinology* 1998; **139**:4008–4011.
14. Noda Y, Ota K, Shirasawa T, Shimizu T. Copper/zinc superoxide dismutase insufficiency impairs progesterone secretion and fertility in female mice. *Biol Reprod* 2012; **86**:1–8.
15. Kimura N, Tsunoda S, Iuchi Y, Abe H, Totsukawa K, Fujii J. Intrinsic oxidative stress causes either two-cell arrest or cell death depending on developmental stage of the embryo from SOD1-deficient mice. *Mol Hum Reprod* 2010; **16**:441–451.
16. Meldrum DR, Casper RF, Diez-Juan A, Simon C, Domar AD, Frydman R. Aging and the environment affect gamete and embryo potential: can we intervene? *Fertil Steril* 2016; **105**:548–559.

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17. Ruder EH, Hartman TJ, Blumberg J, Goldman MB. Oxidative stress and antioxidants: exposure and impact on female fertility. *Hum Reprod Update* 2008; **14**:345–357.
18. Liu J, Liu M, Ye X, Liu K, Huang J, Wang L, Ji G, Liu N, Tang X, Baltz JM, Keefe DL, Liu L. Delay in oocyte aging in mice by the antioxidant N-acetyl-L-cysteine (NAC). *Hum Reprod* 2012; **27**:1411–1420.
19. Dai X, Lu Y, Zhang M, Miao Y, Zhou C, Cui Z, Xiong B. Melatonin improves the fertilization ability of post-ovulatory aged mouse oocytes by stabilizing ovastacin and Juno to promote sperm binding and fusion. *Hum Reprod* 2017; **32**:598–606.
20. Sentman ML, Granström M, Jakobson H, Reaume A, Basu S, Marklund SL. Phenotypes of mice lacking extracellular superoxide dismutase and copper- and zinc-containing superoxide dismutase. *J Biol Chem* 2006; **281**:6904–6909.
21. Yamamoto Y. Coenzyme Q_{10} redox balance and a free radical scavenger drug. *Arch Biochem Biophys* 2016; **595**:132–135.
22. Ben-Meir A, Burstein E, Borrego-Alvarez A, Chong J, Wong E, Yavorska T, Naranian T, Chi M, Wang Y, Bentov Y, Alexis J, Meriano J et al. Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging. *Aging Cell* 2015; **14**:887–895.
23. Iuchi Y, Okada F, Onuma K, Onoda T, Asao H, Kobayashi M, Fujii J. Elevated oxidative stress in erythrocytes due to an SOD1 deficiency causes anemia and triggers autoantibody production. *Biochem J* 2007; **402**:219–227.
24. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril* 1985; **44**:493–498.
25. Erbach GT, Lawitts JA, Papaioannou VE, Biggers JD. Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol Reprod* 1994; **50**:1027–1033.
26. Takahashi M, Miyata S, Fujii J, Inai Y, Ueyama S, Araki M, Soga T, Fujinawa R, Nishitani C, Ariki S, Shimizu T, Abe T et al. In vivo role of aldehyde reductase. *Biochim Biophys Acta* 2012; **1820**:1787–1796.
27. Kitano M, Watanabe D, Oda S, Kubo H, Kishida H, Fujii K, Kitahara M, Hosoe K. Subchronic oral toxicity of ubiquinol in rats and dogs. *Int J Toxicol* 2008; **27**:189–215.
28. Sanoobar M, Eghtesadi S, Azimi A, Khalili M, Jazayeri S, Reza Gohari M. Coenzyme Q10 supplementation reduces oxidative stress and increases antioxidant enzyme activity in patients with relapsing-remitting multiple sclerosis. *Int J Neurosci* 2013; **123**:776–782.
29. Matsuoka Y, Yamato M, Yamasaki T, Mito F, Yamada K. Rapid and convenient detection of ascorbic acid using a fluorescent nitroxide switch. *Free Radic Biol Med* 2012; **53**:2112–2118.
30. Guéraud F, Atalay M, Bresgen N, Cipak A, Eckl PM, Huc L, Jouanin I, Siems W, Uchida K. Chemistry and biochemistry of lipid peroxidation products. *Free Radic Res* 2010; **44**:1098–1124.
31. Cheung K, Ma L, Wang G, Coe D, Ferro R, Falasca M, Buckley CD, Mauro C, Marelli-Berg FM. CD31 signals confer immune privilege to the vascular endothelium. *Proc Natl Acad Sci USA* 2015; **112**:E5815–E5824.
32. Afanas'ev I. Mechanisms of superoxide signaling in epigenetic processes: relation to aging and cancer. *Aging Dis* 2015; **6**:216–227.
33. Guarnaccia MM, Takami M, Jones EE, Preston SL, Behrman HR. Luteinizing hormone depletes ascorbic acid in preovulatory follicles. *Fertil Steril* 2000; **74**:959–963.
34. Tarin JJ, Vendrell FJ, Ten J, Cano A. Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. *Mol Hum Reprod* 1998; **4**:281–288.
35. Espey LL, Stein VI, Dumitrescu J. Survey of antiinflammatory agents and related drugs as inhibitors of ovulation in the rabbit. *Fertil Steril* 1982; **38**:238–247.
36. Laloraya M, Pradeep KG, Laloraya MM. 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. *Biochem Biophys Res Commun* 1988; **157**:146–153.
37. Noia G, Littarru GP, De Santis M, Oradei A, Mactromarino C, Trivellini C, Caruso A. Coenzyme Q_{10} in pregnancy. *Fetal Diagn Ther* 1996; **11**:264–270.
38. Bentinger M, Tekle M, Brismar K, Chojnacki T, Swiezewska E, Dallner G. Stimulation of coenzyme Q synthesis. *Biofactors* 2008; **32**:99–111.
39. Ozler A, Turgut A, Görük NY, Alabalik U, Basarali MK, Akdemir F. Evaluation of the protective effects of CoQ10 on ovarian I/R injury: an experimental study. *Gynecol Obstet Invest* 2013; **76**:100–106.
40. Turi A, Giannubilo SR, Brugè F, Principi F, Battistoni S, Santoni F, Tranquilli AL, Littarru G, Tiano L. Coenzyme Q10 content in follicular fluid and its relationship with oocyte fertilization and embryo grading. *Arch Gynecol Obstet* 2012; **285**:1173–1176.
41. Wang Y, Branicky R, Noë A, Hekimi S. Superoxide dismutases: dual roles in controlling ROS damage and regulating ROS signaling. *J Cell Biol* 2018; **217**:1915–1928.

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