



Published in final edited form as:

Reproduction. 2010 May ; 139(5): 871–881. doi:10.1530/REP-09-0390.

Profiling of SOD isoenzymes in compartments of the developing bovine antral follicle

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Abstract

The antral follicle constitutes a complex and regulated ovarian microenvironment that influences oocyte quality. Oxidative stress is a cellular state that may play a role during folliculogenesis and oogenesis, although direct supporting evidence is currently lacking. We thus evaluated the expression of the three isoforms (SOD1, 2, 3) of the enzymatic antioxidant superoxide dismutase in all of the cellular (granulosa cells, cumulus cells, oocytes) and extracellular (follicular fluid) compartments of the follicle. Comparisons were performed in bovine ovaries across progressive stages of antral follicular development. Follicular fluid possessed increased amounts of SOD1, 2, 3 in small when compared to large antral follicles; concomitantly, total SOD activity was highest in follicular fluids from smaller diameter follicles. SOD1, 2, and 3 proteins were expressed in granulosa cells without any fluctuations with follicle sizes. All three SOD isoforms were present but distributed differently in oocytes from small, medium, or large antral follicles. Cumulus cells expressed high levels of SOD3, some SOD2, but no detectable SOD1. Our studies provide a temporal and spatial expression profile of the three SOD isoforms in the different compartments of the developing bovine antral follicles. These results lay the ground for future investigations into the potential regulation and roles of antioxidants during folliculogenesis and oogenesis.

INTRODUCTION

Good quality oocytes are a prerequisite to a multitude of clinical and agricultural applications. There is thus a critical need to identify and understand the cellular and molecular factors that influence oocyte and follicle health during antral folliculogenesis. The antral follicle constitutes the ovarian microenvironment within which the oocyte undergoes key developmental transitions, including preparatory events and eventually maturation at ovulation. It is while the antral follicle develops, grows, and differentiates that the oocyte acquires its meiotic and developmental competencies (Mermillod *et al.* 1999; Hendriksen *et al.* 2000; Sirard *et al.* 2006). The oocytes that are used for *in vitro* maturation (IVM) and later embryo production originate from antral follicles. These oocytes are of known heterogeneous quality, and developmental competence improves with advanced stages of antral folliculogenesis (Pavlok *et al.* 1992; Lonergan *et al.* 1994; Blondin & Sirard 1995; Hagemann *et al.* 1999; Lequarre *et al.* 2005). Much may thus be learnt from identifying the cellular and biochemical differences between follicles of varying sizes.

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Several compartments ensure the normal functioning and development of antral follicles: three cellular ones (the oocyte, cumulus, and granulosa cells) and an acellular one (the follicular fluid). Much attention has focused on a variety of gene groups that modulate the antral follicle microenvironment, including endocrine and paracrine factors (Knight & Glistler 2006; Webb & Campbell 2007). High steroidogenic and metabolic demands characterize the developing follicle (Boland *et al.* 1993; Fortune *et al.* 2001; Harris & Picton 2007), and as a result oxidative stress may ensue. Oxidative stress (OS) refers to an imbalance between the concentrations of pro- and anti-oxidants, thereby resulting in compromised cellular functions; sources of pro-oxidants are extensive, including their generation as byproducts of cellular respiration and steroid metabolism (Halliwell 1991). Previous findings support the relevance of OS to female reproduction, including endometriosis, polycystic ovarian syndrome, age-related fertility decline, early embryogenesis, and pregnancy (Tarin 1996; Guerin *et al.* 2001; Sabuncu *et al.* 2001; Agarwal *et al.* 2008; Ruder *et al.* 2008). However, there is a conspicuous lack of understanding when it comes to OS and normal ovarian function, especially with respect to folliculogenesis and oogenesis. Most studies in the ovary have focused on OS during ovulation, luteogenesis, luteal function, and luteolysis (Bertout *et al.* 2004; Sugino 2005); and follicular analyses of pro- and anti-oxidants have predominantly focused on human follicular fluid samples (Combelles *et al.* 2009).

The present study examines the enzymatic antioxidant superoxide dismutases (SOD) that convert the pro-oxidant superoxide into hydrogen peroxide. Three isoforms of SOD exist with varying structures, regulation, localizations, and functions (Johnson & Giulivi 2005). Cu, Zn-SOD (SOD1) is typically cytosolic and Mn-SOD (SOD2) mitochondrial, while extracellular SOD (SOD3) scavenges superoxide radicals in extracellular fluids and spaces (Fattman *et al.* 2003; Nozik-Grayck *et al.* 2005). Previous studies in the ovary have focused on SOD1 and SOD2, or total SOD enzymatic activity with neither a concomitant analysis of all three isoforms nor an evaluation of their cellular distributions within the developing antral follicle. Total SOD enzyme activity is present in bovine oocytes and cumulus cells obtained from 2–5 mm follicles (Cetica *et al.* 2001), with no consideration of the SOD isoforms and potential changes during later stages of antral folliculogenesis. Lonergan *et al.* (2003) documented the downregulation of *SOD1* and *SOD2* mRNAs in *in vivo* matured bovine oocytes when compared to *in vitro* matured and immature oocytes from 2–6 mm follicles. However, no comparison was made in oocytes derived from antral follicles larger than 6 mm, and analysis was limited to gene transcripts for unfertilized oocytes (Lequarre *et al.* 2001; Lonergan *et al.* 2003). In the rat, transcripts for the three SOD isoforms were measured in whole ovaries without an evaluation of individual follicles (Tilly & Tilly 1995). Bovine granulosa cells from dominant follicles showed changes in *SOD1* and *SOD2* mRNAs between days 4 and 8 of the first follicular wave with differences in SOD activity albeit no protein regulation (Valdez *et al.* 2005). In porcine (Peterson & Stevenson 1992) and goat (Behl & Pandey 2002) granulosa cells from follicles of increasing sizes, catalase activity augmented, thereby indicating the dynamic regulation of an antioxidant enzyme during antral folliculogenesis. In a pig model, follicular fluid SOD activity decreased with follicle growth (Basini *et al.* 2008) and in human, increased follicular fluid SOD activity was correlated with low fertilization rates (Sabatini *et al.* 1999). Lastly, mice with gene deletions for either SOD1 or its copper chaperone display reduced female fertility and notably abnormal follicular development (Ho *et al.* 1998; Matzuk *et al.* 1998; Wong *et al.* 2000). Taken together, previous studies point towards the need for a careful analysis of SOD proteins in the dynamic microenvironment of the developing antral follicle.

The vulnerability of growing antral follicles to OS remains to be tested. This is relevant given the changes in oxygen availability and metabolic activity that follicles and oocytes are subjected to whether under *in vivo* or *in vitro* conditions (Fischer *et al.* 1992; Sutton *et al.*

2003; Harris & Picton 2007). We predict that antral follicles are properly and dynamically equipped to withstand OS; more specifically, granulosa cells, follicular fluid, and the cumulus-oocyte-complex would provide protection by tightly and coordinately regulating the expression of the various SOD isoforms. Towards this goal, the total SOD enzymatic activity was measured in follicular fluid and the protein expression of SOD isoforms were analyzed in granulosa cells, follicular fluid, and cumulus-oocyte-complexes from bovine antral follicles containing oocytes of different developmental competencies.

MATERIALS AND METHODS

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

Collection, categorization, and processing of bovine antral follicles, follicular fluid, granulosa cells, and cumulus-oocyte-complexes

Bovine ovaries were obtained from Champlain Beef slaughterhouse (Whitehall, NY) and transported to the laboratory in 0.9% NaCl, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B at 20°C. All samples were collected and processed within 2–4 hours of slaughter. As previously described, the routine assessment of corpus luteum morphology was used to determine the stage of the estrous cycle (Ireland *et al.*, 1980). Antral follicles were dissected and stored in a Minimum Essential Medium with Hanks Balanced Salt Solution (MEM-HBSS), 25 mM Hepes, 1 mM sodium pyruvate, 2 mM L-glutamine, 4 µg/ml bovine serum albumin (BSA), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml heparin). Obviously atretic follicles were avoided based on published morphological characteristics (Kruip & Dieleman 1982). Dissected follicles were grouped according to the following size categories: small (2–5 mm), medium (6–8 mm), and large (>8 mm). These size ranges were selected based on established key transitions in antral folliculogenesis and accompanying changes in the acquisition of oocyte developmental competence (Lonergan *et al.* 1994; Lequarre *et al.* 2005).

Follicular fluid was collected from each follicle by aspiration with an insulin syringe. Due to the low volume of removed follicular fluid from small follicles, samples were pooled (usually between 5 and 10 follicles) within a given ovary. No sample pooling was needed for the medium and large size categories. Samples were kept on ice until centrifugation for 4.5 minutes at 5400 g. The supernatant was evaluated for the presence of any residual cells under the microscope and then stored at –80°C. For follicular fluids to be analyzed by immunoblotting, protease inhibitor cocktail was added to each sample at a 1:100 dilution.

Granulosa cells were manually detached from the follicular wall with an inoculation loop and pooled within a size category. Granulosa cells were washed (by natural sedimentation, 2×10 minutes), and allowed to dissociate in MEM-HBSS without calcium and magnesium (MEM-HBSS-Ca²⁺-Mg²⁺) supplemented with 25 mM Hepes and 4 mg/ml BSA. The cell pellet was exposed for 5 seconds to a 100 µl of double-distilled water (for the lysis of erythrocytes) followed by immediate neutralization with 10 ml of supplemented MEM-HBSS-Ca²⁺-Mg²⁺ medium. Cells were then centrifuged at 800 g for a minute, and the resuspended pellet layered over a 50% Percoll gradient in supplemented MEM-HBSS-Ca²⁺-Mg²⁺. After a 10 minute spin, any remaining erythrocytes were separated from granulosa cells, which were retrieved from the Percoll-MEM-HBSS interface. The purified pellet of granulosa cells was centrifuged and washed in 200 µl of cold PBS at 800 g, 4°C for 3 minutes. The pellet was resuspended in 500 µl cold lysis buffer consisting of 2X RIPA buffer (0.2% SDS, 2% NP-40, 1% Na-deoxycholate, and 1X PBS), 100 µM PMSF, 100 µM Na-vanadate, 1X protease inhibitor cocktail, and 1X PBS. The suspension was passed 10 times through an insulin syringe and placed on a rotating platform for 30 minutes at 4°C.

After a 16000 g, 4°C, 10 minute spin, the supernatant or whole cell lysate was frozen and stored at -80°C until use for immunoblotting.

Cumulus-oocyte-complexes (COCs) were released from sized follicles after bisection of the follicle with a scalpel. COC quality was scored based on morphological features of the oocyte and cumulus layers (Blondin & Sirard 1995). COCs were stripped manually of their outer cumulus layers with few cumulus cells kept attached to the zona pellucida. All partially denuded oocytes were fixed in freshly prepared 2% paraformaldehyde (PFA) in PBS, pH 7.4 for 15 minutes at room temperature (RT) in darkness with shaking. Following fixation, cells were extracted in 0.2% Triton-X100 in PBS for 30 minutes at RT with shaking. Samples were stored in a blocking WASH solution (0.2% Na-azide, 0.2% nonfat powdered milk, 2% normal donkey serum, 1% BSA, and 0.1 M glycine in PBS) at 4°C until labelling by immunocytochemistry.

Immunoblotting of follicular fluid and granulosa cell lysates

Each SOD isoform was detected and semi-quantified in follicular fluid and granulosa cells lysates using SDS-PAGE and immunoblotting. An identical amount of protein (10 µg for granulosa cell lysate and 30 µg for follicular fluid) was loaded per well, based on total proteins quantified with a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Protein lysates were prepared with a 6X protein dye (0.5 M Tris pH 6.8, 29% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 5 minutes. Samples were separated using a 15% acrylamide gel and proteins were transferred to PVDF membrane.

Equal loading of protein and effective transfer was verified by incubating membrane in Ponceau-S and visualized using Kodak 440 Gel Imager (Norwalk, CT). Membranes were blocked in 1X TBST (0.066M Tris, 0.054M NaCl, 0.1% Tween 20) with 5% powdered nonfat milk for one hour, and exposed for 2 hours at RT to working dilutions of primary antibody prepared in 1× TBST/5% nonfat milk. Working dilutions of primary rabbit antibodies were used as follows for follicular fluid and granulosa cell samples, respectively: anti-SOD1, 0.2 µg/ml and 0.1 µg/ml (Assay Designs, Ann Arbor, MI), SOD2, 0.5 µg/ml and 0.1 µg/ml (Millipore/Upstate Biotechnology, Billerica, MA), and SOD3, 1 µg/ml and 0.1 µg/ml (Millipore/Upstate Biotechnology, Billerica, MA). Controls were run for each antibody and blot using a non-specific rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a working dilution identical to the one used for the test primary antibody, as well as a secondary only application. For granulosa cell samples, blots were also probed for β-actin (0.02 µg/ml) as a loading control. For follicular fluid samples, blots were also probed for mitochondrial and cytoplasmic markers to ensure the lack of contamination from cells or cell lysis: OxPhos Complex V inhibitor protein (mouse IgG, 0.2 µg/ml; Invitrogen, Carlsbad, CA), β-tubulin (mouse IgG, 0.01 µg/ml), and β-actin (0.02 µg/ml). After primary antibody exposure, three washes in 1X TBST were conducted with agitation for 10 minutes. A one-hour secondary exposure was then performed with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:2000; Amersham/GE Healthcare, Piscataway, NJ). Membranes were subsequently washed as described before and then incubated 1 minute with ECL Western blotting detection solution (Pierce, Rockford, IL). Exposures were conducted to reach a linear range without signal saturation. Developed exposures were later quantified by digitizing blots and counting pixels from a set sized box over each signal band (Adobe Photoshop, San Jose, CA). For each blot, pixel data from follicular fluid or granulosa cells samples from small and medium follicles were arbitrarily normalized to pixel counts of samples from large follicle.

SOD enzyme activity assay in follicular fluid

Follicular fluid was evaluated for total superoxide dismutase activity using an assay kit from Dojindo Molecular Technology (Kurnamoto, Japan). The assay uses the activity of xanthine oxidase (XO), which produces the superoxide anion in the presence of oxygen. The water-soluble tetrazolium salt (WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) reacts with the superoxide anion reducing it; WST-1 now forms a water-soluble formazan dye measurable with spectrophotometry. SOD competes with WST-1 for the superoxide anion produced by XO, and the percent inhibition of XO activity is a quantification of SOD activity. Standards spanning the range of 0.1 to 10 U/ml were prepared as serial dilutions from a 200 U/ml stock of bovine erythrocytes SOD. Initial testing showed the need to dilute follicular fluid samples from medium/large and small follicles 1:10 and 1:50, respectively. Reactions were initiated by adding 20 μ l of the XO enzyme working solution to the WST reaction mixture. After incubating at 37°C for 20 minutes, samples were read at 450 nm with a Synergy HT spectrophotometric plate reader (BioTek, Winooski, VT). All standards, blanks, and samples were run in triplicates, and final SOD activity measurements are reported as U per ml of follicular fluid. Intra- and inter-assay coefficients of variation averaged 8.4% and 10.2%, respectively, and linearity of the assay for a range of erythrocyte SOD concentrations during the first 20 min of incubation is shown in Supplementary Figure 1.

Immunocytochemistry of oocytes and cumulus cells

Following fixation and blocking (see above), oocytes and cumulus cells were processed for immunocytochemistry with the aforementioned SOD antibodies. Working solutions of primary antibodies were prepared in WASH at the following optimized dilutions: SOD1, 2 μ g/ml; SOD2, 5 μ g/ml; SOD3, 5 μ g/ml. All incubations were undertaken in microwells of a 60-well plate, and routine labelling controls included a group of oocytes incubated with non-immune rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a same concentration as primary test antibodies as well as a secondary only group. After overnight incubation with the primary antibody (4°C, shaking) and three washes (with WASH, 10 minutes each at 37°C shaking), cells were incubated in a mixture of secondary Alexa Fluor 488 anti-rabbit (1:800 dilution of a 2 mg/ml stock; Invitrogen, Carlsbad, CA) and Texas-red phalloidin (1:20 dilution; Invitrogen, Carlsbad, CA) in WASH for 90 minutes at 37°C shaking. After a 10 minute wash, cells were exposed to DAPI (1 μ g/ml) for 30 minutes at 37°C shaking. Following two final washes, oocytes and cumulus cells were mounted onto a glass slide in 50% glycerol:50% sodium azide-PBS and a coverslip added with minimal compression. Labelled samples were visualized and scored by conventional fluorescence microscopy with a Zeiss AxioScope 2 *plus* (Thornwood, NY). Imaging was undertaken by confocal laser microscopy with a Zeiss LSM 510 META. Fluorescent signals were captured after excitation with 405 nm, 488 nm, and 543 nm laser lines. Qualitative comparisons of labelling patterns and/or intensities in oocytes originating from small, medium, or large antral follicles were made only for oocytes that were collected, fixed, labelled, and imaged on a same day using identical conditions and acquisition parameters. Optical sections of 0.5 μ m thickness were acquired and a total of 10 of them projected into a single image.

Statistical analysis

Numbers of samples and experimental replicates are provided below with each analysis. SOD activity in follicular fluid and signal intensity data obtained from immunoblotting experiments in granulosa cells or follicular fluids from small, medium, or large follicles were compared using a nonparametric Kruskal-Wallis test followed by Mann-Whitney tests for two independent samples (Statistics Package for Social Sciences 16.0, Chicago, IL). Statistical significance was accepted for p-values \leq 0.05.

RESULTS

Presence and variation in the amounts of SOD isoforms in follicular fluids from progressive stages of antral development

Immunoblotting detected the presence of all three SOD isoforms in bovine follicular fluids with a single and specific SOD1 band at 21 kDa (Figure 1A), SOD2 at 24 kDa (Fig. 1C), and SOD3 at 45 kDa (Fig. 1E) when compared to negative controls (for both control IgG and secondary only; data not shown). Quantification of the signal bands revealed significantly higher levels of each SOD type in follicular fluids from small when compared to large antral follicles (Fig. 1B, D, F). SOD1 and SOD3 expressions were more elevated in small than medium and large antral follicles (Fig. 1B, F) with SOD2 and SOD3 higher in medium than large follicles (Fig. 1F). These expression patterns were obtained from the following numbers of total blots and samples: 13 and 42 for SOD1; 9 and 27 for SOD2; and 11 and 33 for SOD3. No mitochondrial (OxPhos complex V inhibitor) or cytoplasmic (β -tubulin and β -actin) proteins were detected in follicular fluid samples (Fig. 1G).

Variation in total SOD enzyme activity in follicular fluids originating from small, medium, and large antral follicles

The analysis of total SOD activity in follicular fluid showed levels that fluctuated with developmental stages of antral folliculogenesis (Figure 2). SOD activities were significantly higher in follicular fluids from small antral follicles (51.1 ± 4.5 U/ml, $n=88$; mean \pm SD) than in those from medium antral follicles (29.9 ± 2.3 U/ml, $n=61$); similarly, follicular fluids from medium antral follicles possessed elevated levels of SOD activity when compared with large follicles (14.8 ± 1.1 U/ml, $n=99$; Figure 2). SOD activity levels were compared when expressed as units per ml, providing identical results and statistical significance as when units per mg of total protein were used. In line with this, total protein levels did not vary among follicular fluids from small (83.0 ± 17.7 mg/ml, $n=100$; mean \pm SD), medium (84.3 ± 17.7 mg/ml, $n=65$), and large (88.5 ± 17.3 mg/ml, $n=113$) antral follicles ($p>0.05$). Over a span of 29 collections, a total of 116 ovaries from 72 animals were used for SOD activity assays yielding 88, 61, and 99 follicular fluid samples from small, medium, and large antral follicles, respectively.

SOD activity was also evaluated based upon estrous stage of the animal together with follicle size from which the follicular fluid was obtained (Supplementary Figure 2). Stages of the estrous cycle were estimated based on the morphology of the corpus luteum with stage I corresponding to days 1–4, II to days 5–10, III to days 11–17, and IV to days 18–20 (Ireland et al., 1980). Statistical analysis revealed no significant difference between estrous stages in follicular fluids derived from small, medium, or large follicles.

Expression of SOD isoforms in granulosa cells from small, medium, and large antral follicles

Granulosa cells express at the protein level all three types of SOD isoforms whether they originate from small, medium, or large antral follicles (Fig. 3A, C, E). Specificity of the single protein bands obtained with granulosa cell lysates were confirmed with routine negative controls (control rabbit IgG and secondary only; data not shown). Testing for SOD3 showed a second faint band at about 47 kDa, which also showed no sign of developmental regulation. Furthermore, the relative amounts of SOD1, 2, and 3 protein expression were comparable in cell lysates from small, medium, or large antral follicles (Fig. 3B, D, F; 12 collected sets of small, medium, and large samples or a total of 36 cell lysates for each SOD isoform). Equal protein loading was confirmed for each blot with β -actin expression (Fig. 3A, C, E).

Expression and localization of SOD isoforms in oocytes and cumulus cells during antral folliculogenesis

An immunocytochemical approach demonstrated the spatially and temporally dynamic expression of each SOD isoform in intact germinal vesicle stage (prophase-I) oocytes as well as surrounding cumulus cells (Figures 4 and 5). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and phalloidin counterlabels permitted evaluation of chromatin stages, nuclear status, and cell contours for the two somatic and germ cell compartments of the COC. While morphology was evaluated for each COC, no differences in any of the SOD labelling patterns were apparent across COC grades.

At all stages of antral folliculogenesis (> 2 mm in diameters), oocytes display intracellular SOD1 proteins in a fine, dense, and punctate pattern throughout the volume of the oocyte (Fig. 4A, a; n=84, 42, and 61 oocytes from small, medium, and large antral follicle, respectively). This is in contrast to probing with secondary alone (n=46) or control rabbit IgG (n=42), which both yielded only background non-specific labelling (Fig. 5F, f). Interestingly, SOD1 positive foci were also present within the nucleoplasm of all oocytes, with some oocytes further displaying an accumulation of SOD1 within the nuclear confines (Fig. 4A, arrow; with an incidence of 79/84 or 94% of oocytes from small follicles when compared to 18/42 or 43% and 0/61 or 0% from medium and large antral follicles, respectively). The zona pellucida remained devoid of SOD1 across all size categories of antral follicles (Fig. 4A, a). Cumulus cells of the corona radiata also failed to demonstrate any reactivity to SOD1 antibodies whether they originated from small, medium, or large antral follicles (Fig. 4A, asterisk).

Oocytes and cumulus cells expressed SOD2 proteins at all of the analyzed stages of antral folliculogenesis (Fig. 4B; n=67, 48, and 52 oocytes from small, medium, and large antral follicle, respectively). SOD2 levels were elevated in comparison to control oocytes (Fig. 5F), with a strong and dense labelling pattern through the ooplasm with the exception of the nucleoplasm (Fig. 4B, b). Each cumulus cell showed a few specific foci that were positive for SOD2 in their cytoplasm (Fig. 4B, asterisk).

SOD3 was present not only in the oocyte cortex (Fig. 5A–B; n=81, 38, and 57 oocytes from small, medium, and large antral follicle, respectively) but also in cumulus cells (Fig. 5A–B, D–E). It was distributed in the cytoplasm of cumulus cells as well as in the extracellular spaces adjoining cells; furthermore, SOD3 labelling was characteristically strong in the oocyte and cumulus cell layer of COCs from large (Fig. 5B, E) when compared to small antral follicles (Fig. 5A, D). The zona pellucida was itself devoid of SOD3 (Fig. 5C, c, double-ended arrow: width of the zona pellucida), but the entire lengths of transzonal projections (TZPs) were positive for SOD3 as demonstrated by co-localization with actin microfilaments (Fig. 5C, c, arrow). TZPs all co-localized with SOD3 whether they originated from small, medium, or large antral follicles. The oocyte nucleoplasm always contained SOD3 (Fig. 5A–B); however, only in small oocytes was more SOD3 present in the nucleoplasm than in the surrounding ooplasm (Fig. 5A, a). SOD3 was also associated with the chromatin (Fig. 5A, arrow).

DISCUSSION

Our detailed spatial and temporal analysis of the three SOD isoforms revealed the expression of SOD1, 2, and 3 in the granulosa cells, follicular fluid, and cumulus-oocyte-complex of the developing antral follicle. In granulosa cells, there was no change in SOD expression as antral follicles increased in size, while follicular fluid showed highest SOD activity and SOD proteins in small when compared to medium and large follicles. COCs

exhibited a compartmentalized and varying distribution of SOD1, 2, and 3 protein expression.

In light of the differential and prevalent expression of SOD isoenzymes in all compartments of the antral follicle, it may be speculated that antioxidants, and by inference pro-oxidants, play a role in follicle development. Reactive oxygen species (ROS) are not always detrimental to the cell as they can carry out functional roles at certain concentrations, notably during embryonic development (Covarrubias *et al.* 2008). In large diameter antral follicles, ROS may need to be above a certain threshold and keeping SODs at reduced concentrations within the follicular fluid milieu may provide the appropriate balance of superoxide anion and hydrogen peroxide for normal cell function. Of relevance is the documented need for free radicals at ovulation (Miyazaki *et al.* 1991). Given the follicular changes in oxygen availability (Fischer *et al.* 1992) and metabolic requirements (Harris & Picton 2007), it is likely that mechanisms exist to manage oxidative stress in antral follicles, and altered SOD levels may be one manifestation of this need.

Beyond physiological changes during follicular growth are selective pressures imposed on antral follicles since one will become dominant and others subordinate and destined for atresia. Follicular atresia involves apoptosis, a program of cell death during which ROS and antioxidants play a role (Ott *et al.* 2007; Covarrubias *et al.* 2008). This study only considered follicle size as a potential factor influencing antioxidant expression, but future work should test the involvement of oxidative stress during follicle selection and atresia. In the rat, exogenous SOD inhibits apoptosis in cultured rat follicles (Tilly & Tilly 1995) while in cows, no correlations were found between SOD1/SOD2 protein expression and granulosa cell apoptosis in the dominant follicle of the first follicular wave (Valdez *et al.* 2005). Nonetheless, *SOD1/SOD2* mRNAs and SOD activity rose when nonviable granulosa cells also increased (Valdez *et al.* 2005). Lastly, it is conceivable that external factors may influence the oxidative stress status and in turn expression of SODs in the follicle. For our study, ovaries from adult (< 3 years old), cycling, non-pregnant animals were used without consideration of nutritional status and general or reproductive health; notably, infertility diagnosis appeared to influence SOD expression in human cumulus cells (Matos *et al.* 2009) and dietary supplements may impact ovarian function (Forges *et al.* 2007). Taken together, our work documents the dynamic expression of SOD isoforms during antral folliculogenesis and future studies will need to examine factors that may underlie any differences between animals and/or follicles.

Intra-follicular variations in antioxidants also prove relevant since not all follicular compartments exhibited similar changes in SODs. Indeed, SOD1, 2, 3 proteins and activity were elevated in follicular fluid from small follicles while SOD3 protein appeared most abundant in oocytes and cumulus cells from large relative to small follicles. Future work should thus examine follicular variances in not only the potential reliance of oocytes on exogenous sources of antioxidants but also the amount of oxidative stress that follicles are subject to. Our preliminary findings also support the need to quantify SOD protein and activity within single oocytes and cumulus cells of different follicular origins. Regardless, the bovine COC showed compartmentalization with respect to each SOD isoform and its cell types and regions. SOD2 is the predominant SOD expressed in the ooplasm while SOD3 is the main SOD type in the layer of cumulus cells and the only one located in TZPs. The localization of SOD3 in both cumulus cells and oocytes may reflect its intracellular processing, whether prior to exocytosis to impart oxidative protection (Tan *et al.* 2006) or upon endocytosis (Chu *et al.* 2006) as demonstrated in other cell systems. SOD1 showed an enhanced nuclear accumulation in oocytes from the majority of small and half of medium antral follicles. Previous reports in liver and brain cells documented the presence of SOD1 in the nucleoplasm (Slot *et al.* 1986; Moreno *et al.* 1997) but the functional significance of the

observed change in localization during oogenesis is unknown. Similarly, SOD3 was present in the oocyte nucleus and a previous study in cultured somatic cells proposed a protective role for the regulated translocation of SOD3 to the nucleus (Ookawara *et al.* 2002). It may thus be relevant to test the potential role of SOD1 and SOD3 in the protection of genomic DNA and/or transcriptional regulation of redox-sensitive genes.

The distinct expression of each SOD isoform within the COC can be further rationalized: SOD1 neutralizes superoxide anions in the cytoplasm of cells, and in this case the oocyte is likely an abundant producer of ROS as it is metabolically active during the acquisition of developmental competence (Sutton *et al.* 2003); mammalian oocytes possess an abundance of mitochondria, the organelle where SOD2 is preferentially localized (Johnson & Giulivi 2005); and SOD3 acts in the extracellular matrix (Nozik-Grayck *et al.* 2005), a dynamically regulated and essential component of the developing COC. In addition, oocytes, or COCs as a whole developmental unit, may stockpile the various SOD isoforms for the upcoming events of oocyte maturation, fertilization, and early embryonic development. These processes occur precisely when the oocyte is faced with augmented oxidative challenges with the proposed burst of ROS at ovulation (Fujii *et al.* 2005) and the increase in oxygen tension within the oviduct (Fischer *et al.* 1992; Fischer & Bavister 1993). The hypothesis of antioxidant stockpiling by the oocyte was previously proposed based on the analysis of transcripts (Guerin *et al.* 2001); our study now define the intrinsic sources of SOD proteins that oocytes are equipped with prior to meiotic resumption. Also worthy of future investigation is the possibility that differences in the total SOD neutralizing capacity of COC reflect different vulnerability of COCs to oxidative stress, thereby influencing their subsequent developmental competence. In this vein, cumulus cells provide protection against ROS-induced apoptosis in porcine COCs during IVM (Tatemoto *et al.* 2000) and in the bovine during *in vitro* fertilization (Fatehi *et al.* 2005). Most recently, an analysis of human cumulus cells showed a positive relationship between total SOD activity and cycle success as defined by a live birth (Matos *et al.* 2009). Cell localization studies of the type undertaken here are insightful, notably in the case of SOD since the superoxide anion is largely produced in mitochondria and doesn't diffuse readily through the plasma membrane, thereby mostly reacting within the cell where it is produced. The prevalence of SODs in all regions of the COC is thus not surprising, although the expression patterns of each isoform may point towards cell-type specific reliance on different protective mechanisms against superoxide. One must also consider the possibilities that the metabolite of SOD (hydrogen peroxide) plays a role in either the oocyte or cumulus cells, that SOD influences nitric oxide bioavailability (Nozik-Grayck *et al.* 2005), or that SOD possesses another, yet unknown, function.

As to the presence of SOD in follicular fluid, it may provide protection of the maturing pig oocytes against oxidative damage, in turn improving developmental competence (Tatemoto *et al.* 2004) while supplementation with exogenous SOD failed to influence bovine IVM (Ali *et al.* 2003). The stage is now set to examine the role of follicular fluid SOD isoforms on the prematuration of oocytes as the antral follicle grows. To our knowledge, this is the first report of SOD3 in follicular fluid, an expected association based on its known functions in body fluids (Nozik-Grayck *et al.* 2005). SOD2 is typically restricted to mitochondria although it is present in cerebrospinal fluid in bacterial meningitis (Hirose *et al.* 1995), one of its variant is secreted by a tumor cell line (Mancini *et al.* 2006), and one study detected SOD2 in human follicular fluid (Tamate *et al.* 1995). Importantly, we further demonstrated the lack of contaminants from cells or cell lysis within follicular fluid samples, and the functional significance of SOD2 in follicular fluid thus merits attention. Further studies should investigate potential releases of SOD2 and/or SOD1 in follicular fluid in responses to stress conditions. The presence of SOD1 in follicular fluid originates from the ability of SOD1 to be secreted in addition to its cytoplasmic functions (Mondola *et al.* 1996; Cimini *et*

al. 2002). SOD1 was also previously reported in human follicular fluid (Carbone *et al.* 2003). Given the size of SOD proteins and their expression in granulosa and cumulus cells, this study indicates that the likely source of follicular fluid SODs is from follicular cells. Furthermore, protein expression did not change in granulosa cells while follicular fluid SOD proteins and activity diminished with increased follicle diameter. There may thus be mechanisms to control the secretion of SOD proteins from follicular cells and/or to alter the turnover of SOD proteins once in follicular fluid. With SODs exhibiting comparable patterns at the protein and enzyme activity levels, post-translational control likely doesn't predominate in follicular fluid. However, post-translational regulation of SODs may operate in the cellular compartments of the follicle; of relevance are post-translational modifications of SOD isoforms (Johnson & Giulivi 2005; Nozik-Grayck *et al.* 2005). It must also be noted that data on SOD expression in granulosa cells were based on the pooling of cells from multiple follicles within a size category; thus, any intra-follicular wall or inter-follicular variances are not apparent.

During antral folliculogenesis, granulosa cells are steroidogenically active and SODs may play a role in controlling steroid production. SOD1 (indirectly via superoxide) inhibits progesterone production in rat luteal cells (Sugino *et al.* 1999) and in rat granulosa cells, SOD inhibited aromatase activity *in vitro* (LaPolt & Hong 1995). By analogy to the involvement of SOD and superoxide in corpus luteum steroidogenesis, SODs may thus provide a mechanism to regulate steroid output during follicular development. In turn SODs may be regulated by the steroid-rich environment of the antral follicle; notably, estradiol inhibited SOD1 and SOD2 mRNA expression in rat luteal cells (Sugino *et al.* 1998).

While this study lays the foundation for future work aimed at understanding the roles and regulation of SOD isoenzymes during antral folliculogenesis, consideration must also be given to (1) the dynamics of both membrane-impermeable superoxide (the free radical scavenged by SOD) and diffusible hydrogen peroxide (the ROS generated by SOD), and (2) the catalase and peroxidase enzymes that neutralize hydrogen peroxide. In the meantime, there are several potential applications of this novel knowledge on SOD expression during antral folliculogenesis. SODs may serve as biomarkers of follicle and in turn oocyte quality; indeed, it remains to be tested whether the concentrations and activity of SODs in follicular fluid can predict the developmental capacity of the oocyte originating from that follicle. Based on previous studies establishing the improved developmental competence of oocytes from increased size follicles, we hypothesize that lower SOD activity in follicular fluid corresponds to follicles containing oocytes of optimal quality. In such search for biomarkers, all follicular compartments should be examined since follicular fluid SOD activity was negatively correlated with oocyte fertilizability in human (Sabatini *et al.* 1999) but SOD activity was increased in human cumulus cells from successful cycles (Matos *et al.* 2009). Our findings also documented differences in SOD expression within the follicular microenvironment. Lastly, defining the physiologically relevant concentrations and proportions of each SOD isoform may contribute to the formulation of culture conditions for pre-maturation arrest or oocyte maturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dijana Poljak for her technical assistance with immunoblots. This work was supported by the National Institute of Child Health and Human Development (R15 HD05645-01), the Vermont Genetics Network through Grant Number P20 RR16462 from the INBRE program of the National Center for Research Resources (NCRR), a

component of the National Institutes of Health (NIH), and Middlebury College. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRN or NIH.

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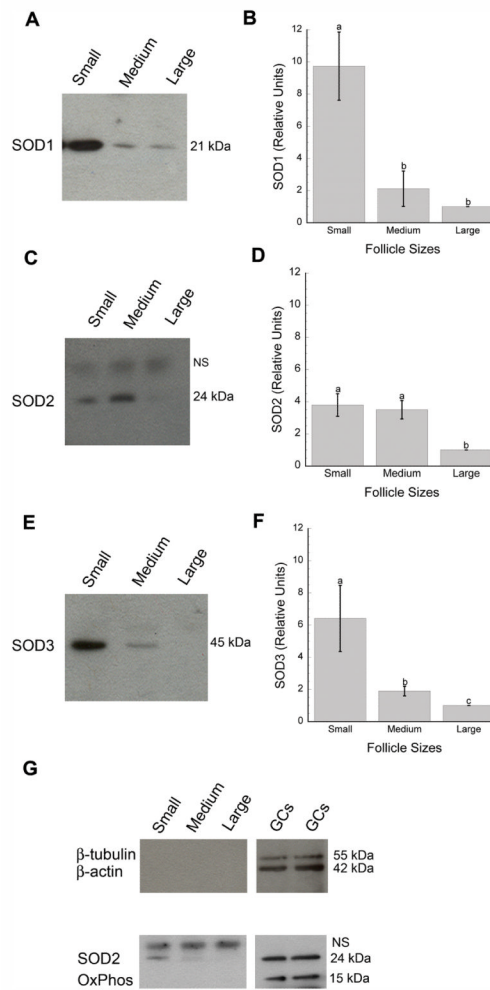


Figure 1.

Expression of SOD1 (A–B), SOD2 (C–D), and SOD3 (E–F) proteins in follicular fluids from small (2–5 mm), medium (6–8 mm), and large (>8 mm) antral follicles. Panels A, C, and E show representative immunoblots for SOD1 (21 kDa), SOD2 (24 kDa), and SOD3 (45 kDa). SOD protein concentrations were quantified and reported as arbitrary units relative to a set value of 1 for samples from large follicles (B, D, F: means \pm SD's). Each SOD isoform showed higher protein expression in follicular fluid derived from small when compared to large antral follicles (B, SOD1: (a,b), $p < 0.001$; D, SOD2: (a,b), $p = 0.001$; F, SOD3: (a,c), $p < 0.001$). SOD1 and SOD3 proteins were increased in follicular fluid from small when compared to medium antral follicles (B, F: (a,b), $p < 0.001$). Follicular fluid from large follicles also contained less SOD2 (D: (a,b), $p = 0.001$) and SOD3 (F: (b,c), $p = 0.002$) than fluid from medium follicles. Control blots (G) supported the lack of cytosolic and mitochondrial contaminations with the probing of follicular fluid for β -tubulin/ β -actin and OxPhos, respectively. The use of these markers was validated with granulosa cell lysates (GCs) as positive controls. (C, G): NS corresponds to a non-specific band at ~ 25 kDa based on rabbit IgG and secondary antibody alone negative controls.

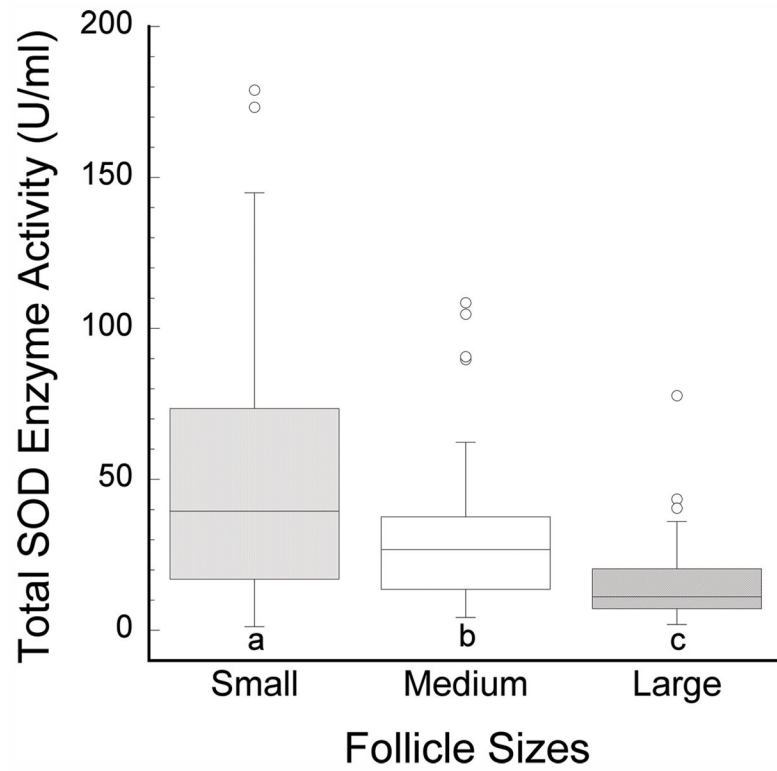


Figure 2. Total SOD enzyme activity (U/ml) in follicular fluid samples from small (2–5 mm), medium (6–8 mm), and large (> 8 mm) antral follicles. SOD activity is highest in follicular fluids from small antral follicles with greater concentrations in small than medium (a,b: $p < 0.05$), and in medium than in large (b,c: $p < 0.0005$) follicles.

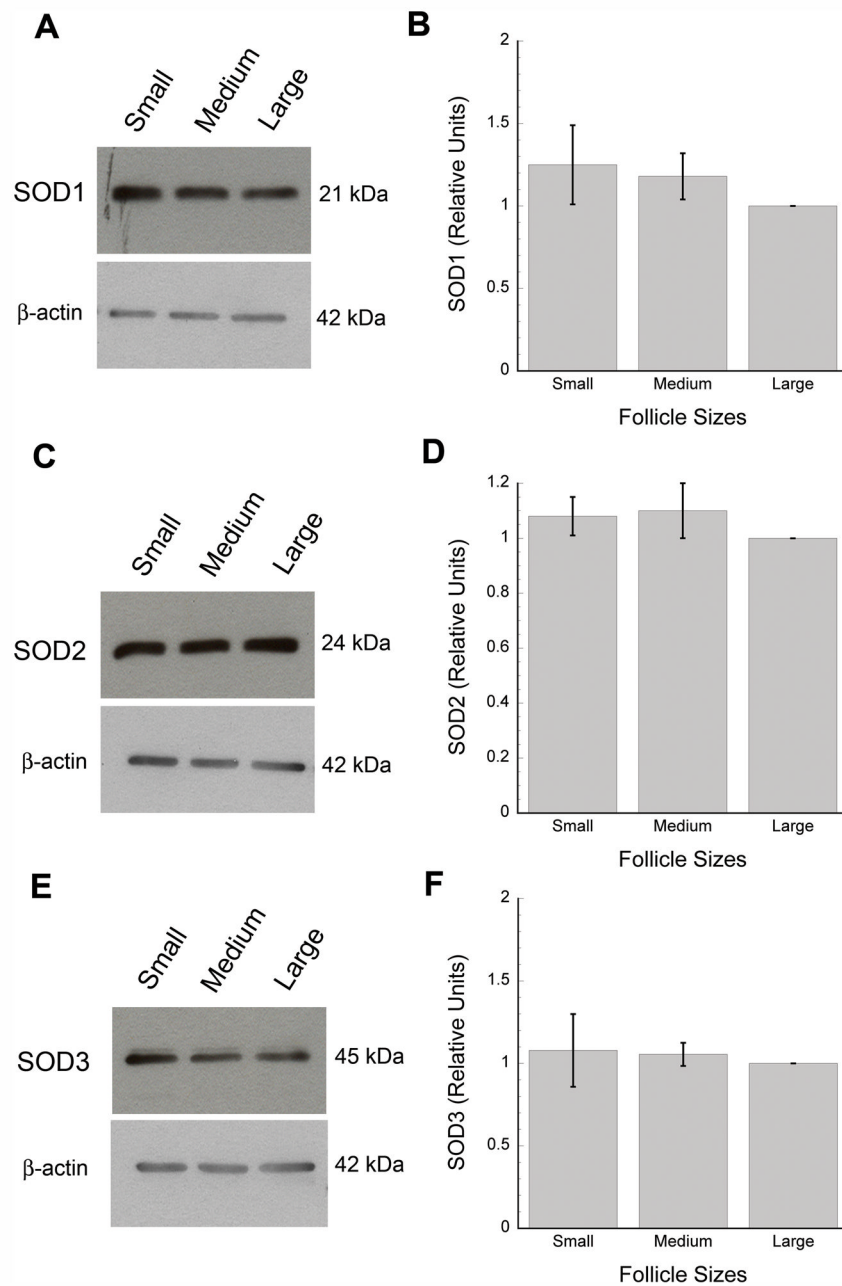


Figure 3. Expression of SOD1 (A–B), SOD2 (C–D), and SOD3 (E–F) proteins in pooled granulosa cell lysates from small (2–5 mm), medium (6–8 mm), and large (>8 mm) antral follicles. Panels A, C, and E show representative immunoblots for each SOD isoenzyme. SOD protein concentrations were quantified and reported as arbitrary units relative to a set value of 1 for samples from large follicles (in B, D, and F, Y-axis values are expressed as means \pm SD's). β -actin was used as a loading control for each SOD blot (A, C, E).

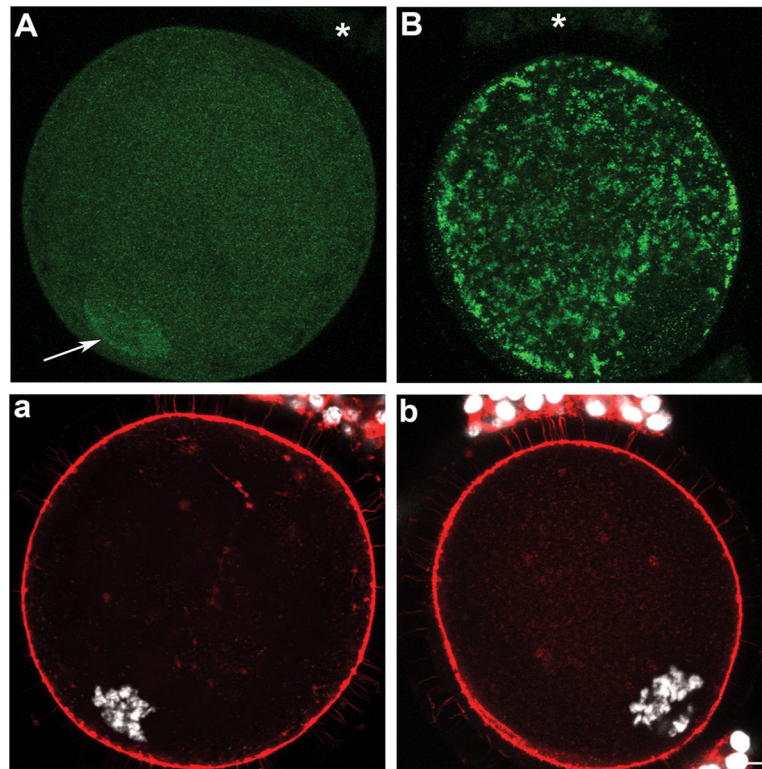


Figure 4.

Expression and localization of SOD1 (A) and SOD2 (B) in oocytes and cumulus cells upon isolation from sized antral follicles. Panels a and b show corresponding DNA (white) and microfilaments (red). (A) A fine punctuate SOD1 labelling was localized throughout the ooplasm and nucleoplasm in oocytes from small, medium, and large antral follicles with an enhanced nuclear accumulation indicated by the arrow. SOD1 was not detectable in cumulus cells (A, asterisk). (B) SOD2 was restricted to the ooplasm of oocytes from small, medium, and large follicles, with a detectable but much reduced labelling in cumulus cells (asterisk). (b) Scale bar for all panels: 10 μm .

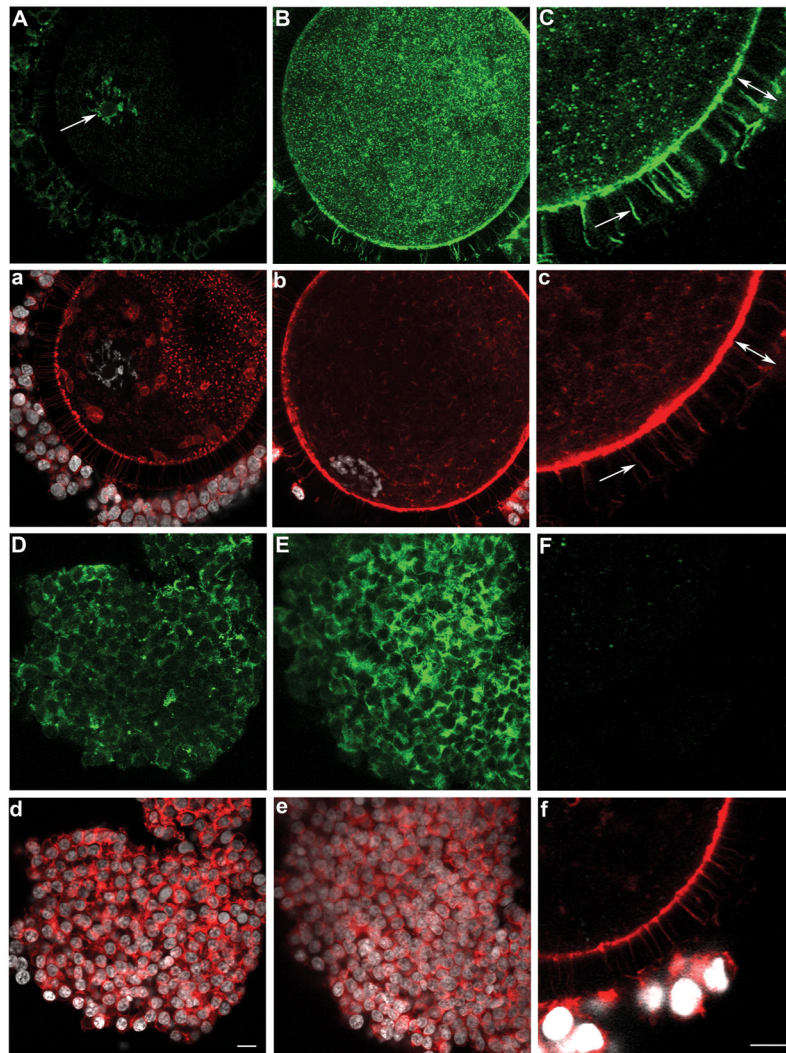


Figure 5. Expression and localization of SOD3 in oocytes (A–B), trans-zonal projections (C), and cumulus cells (D–E) upon isolation from sized antral follicles. Corresponding microfilament (red) and DNA (white) stains are shown (a–f), and all processing and imaging conditions were kept identical across panels. SOD3 was organized as small aggregates (reminiscent of a vesicular labelling) in the oocyte cortex of oocytes from small (A), medium (C), and large (B) antral follicles. SOD3 was also abundant in cumulus cells and trans-zonal projections (C, c; arrow) with the width of the zona pellucida delineated by a double-ended arrow (C, c). All oocytes had nuclear SOD3, but ones from small (A, a) when compared to large (B, b) follicles showed more SOD3 in the nucleoplasm than ooplasm; SOD3 also co-localized (A, arrow) with chromatin (a). The use of control rabbit IgG antibody (F, f) confirmed the specificity of SOD labelling. (d) Scale bar for all panels except C, c, F, and f for which a scale bar is provided in f: 10 μ m.