Maternal supply of omega-3 polysaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse

Sarah L. Wakefield,1 Michelle Lane,1 Samantha J. Schulz,1 Michelle L. Hebart,2 Jeremy G. Thompson,1 and Megan Mitchell1

1Discipline of Obstetrics and Gynaecology, School of Paediatrics and Reproductive Health, and 2Discipline of Agricultural and Animal Science, School of Agriculture and Wine, The University of Adelaide, Adelaide, Australia

Submitted 28 June 2007; accepted in final form 4 December 2007

Wakefield SL, Lane M, Schulz SJ, Hebart ML, Thompson JG, Mitchell M. Maternal supply of omega-3 polysaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. Am J Physiol Endocrinol Metab 294: E425–E434, 2008. First published December 11, 2007; doi:10.1152/ajpendo.00409.2007.—Despite the well-known benefits of omega-3 (n-3) polysaturated fatty acid (PUFA) supplementation on human health, relatively little is known about the effect of n-3 PUFA intake on fertility. More specifically, the aim of this study was to determine how oocyte and preimplantation embryo development might be influenced by n-3 PUFA supply and to understand the possible mechanisms underlying these effects. Adult female mice were fed a control diet or a diet relatively high in the long-chain n-3 PUFAs for 4 wk, and ovulated oocytes or zygotes were collected after gonadotropin stimulation. Oocytes were examined for mitochondrial parameters (active mitochondrial distribution, mitochondrial calcium and membrane potential) and oxidative stress, and embryo developmental ability was assessed at the blastocyst stage following in vitro fertilization (IVF) or culture of in vivo-derived zygotes. This study demonstrated that exposure of the oocyte during maturation in the ovary to an environment high in n-3 PUFA resulted in altered mitochondrial distribution and calcium levels and increased production of reactive oxygen species. Despite normal fertilization and development in vitro following IVF, the exposure of oocytes to an environment high in n-3 PUFA during in vivo fertilization adversely affected the morphological appearance of the embryo and decreased developmental ability to the blastocyst stage. This study suggests that high maternal dietary n-3 PUFA exposure periconception reduces normal embryo development in the mouse and is associated with perturbed mitochondrial metabolism, raising questions regarding supplementation with n-3 PUFAs during this period of time.

oxidative stress; mitochondria

A large body of evidence exists to suggest that nutrients supplied to the prospective parents can influence various aspects of fertility, from events periconception and throughout the duration of the pregnancy, culminating in the birth of a healthy offspring (16, 24). Fat is an important dietary component, since both obesity and undernutrition have detrimental effects on reproductive functions, resulting in adverse outcomes for mother and baby (12). Polyunsaturated fatty acids (PUFAs) can be classified as omega-3 (n-3), omega-6 (n-6), or omega-9 (n-9) and are essential in the diet, with the longer chain n-3 PUFAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) readily available from marine sources. n-3 PUFAs are incorporated into many cells and recently have been shown to have significant and far-reaching health benefits in a wide range of physiological systems (for reviews, see Refs. 7, 37, 39). Despite these findings, the average Western diet has a higher ratio of n-6 to n-3 PUFAs (10:1) than is currently recommended (1).

The relative amount of PUFAs in the diet, compared with saturated and monounsaturated fatty acids, also can affect fertility, and there is a growing body of evidence, particularly in animal models, of their role in female fertility (for reviews, see Refs. 1, 27, 28). Previous studies have shown that supplementation increased gestation length and differences in onset of labor (4), altered fatty acid profiles of ovarian follicle components, and increased follicle number and size in cattle (35, 47), as well as increased ovulation rate in mice (42). In contrast to these beneficial effects, PUFAs, including linoleic acid, perturbed embryo development after in vitro culture (31). However, relatively less is known about the effect of longer chain marine n-3 PUFA intake on oocyte and preimplantation embryo development and the possible mechanisms underlying any potential effects.

Although the various mechanisms behind the human health benefits of n-3 PUFA supplementation are complex and remain largely elusive, they include altered eicosanoid production, via regulation of the cyclooxygenase-2 (COX-2) enzyme responsible for the conversion of PUFAs to prostaglandins or via alterations in the substrate availability for COX enzymes, thus changing the chemical composition of prostaglandins synthesized. Eicosanoid-independent mechanisms, such as modulation of intracellular signaling pathways, transcription factor activity, and altered gene expression, also are involved (11, 14, 37), as well as the modulation of oxidative stress and mitochondrial function (9). In addition, recent evidence suggests that oocyte fatty acids are utilized during oocyte maturation and that they are incorporated into the oocyte cytoplasm during this process (18, 23), perhaps for a role in oocyte development. Whether PUFAs are utilized as an energy source or for other cellular functions in the oocyte, changes in the n-3 PUFA levels in the diet may alter the fatty acid composition of the oocyte and its surrounding environment, potentially having an impact on oocyte maturation and developmental competence, as measured by its ability to fertilize and form a viable embryo.

Although dietary n-3 PUFA intake has the potential to affect reproductive processes, including ovulation and oocyte development, little is known with respect to how PUFAs may modulate follicular development, oocyte maturation, and sub-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpendo.org 0193-1849/08 $8.00 Copyright © 2008 the American Physiological Society E425
sequent embryo development. A physiological system that may have parallels to oocyte and embryo development is human spermatozoa, where PUFAs stimulate the generation of reactive oxygen species (ROS) in vitro (3). Hong et al. (22) also have shown that n-3 PUFAs increase apoptosis in colonicocytes when coincubated with butyrate, because of the increase in oxidative stress. Furthermore, EPA increases oxidative stress via changes in lipid peroxidation in Walker 256 rat tumor cells, in addition to altering the mitochondria via a decrease in mitochondrial membrane potential (9). This is of particular interest, since changes in mitochondrial membrane potential have been measured in oocytes as a consequence of metabolic inhibitors (44) and have been correlated with developmental arrest in mouse two-cell embryos (2), increased fragmentation (2), and the rate of embryo development in the human (45). Finally, studies implicating PUFAs with an increase in mitochondrial synthesis and oxidation (19) and improved mitochondrial properties and function in aged cardiac cells (32) lend further support for a possible role of n-3 PUFAs in regulating oxidative stress and mitochondrial function.

Numerous studies have correlated mitochondrial function (45), organization (6), and oxidative stress (38) with early embryo development. This study examined the influence of maternal supplementation with n-3 PUFAs on oocyte and early embryo development and endeavored to ascertain whether altered mitochondrial function and oxidative stress are potential mechanisms underlying the developmental effects.

**MATERIALS AND METHODS**

**Experimental animals and diets.** The use and care of all animals used in this study were approved by the Animal Ethics Committee, The University of Adelaide. Five-week-old female C57BL/6 mice were assigned to one of two diets for a 3- to 5-wk duration: (1) a diet high in n-3 PUFAs, particularly DHA and EPA (SF06-001; Specialty Feeds, Perth, Western Australia), or (2) a control diet low in n-3 PUFAs (SF06-002; Specialty Feeds). The fatty acid concentration for each diet was determined by an external commercial service (Glennforrest Stock Feeds, Glennforrest, Western Australia) (Table 1), and diets were matched for total fat (7%), protein, and energy levels. Animals were group housed, with each cage forming a replicate for each of the experiments outlined below. Two to three females per diet were used for each replicate, unless stated otherwise.

**Body composition and fatty acid analysis.** Weekly measurements of individual body weight and group feed intake were recorded (3 replicate experiments, weekly measurements for 4 wk).

**Table 1. Fatty acid composition of experimental diets**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>High n-3 PUFA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total n-3 PUFA</td>
<td>19.09</td>
<td>0.58</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>3.67</td>
<td>7.86</td>
</tr>
<tr>
<td>Total saturates</td>
<td>38.05</td>
<td>38.54</td>
</tr>
<tr>
<td>Total monounsaturates</td>
<td>39.17</td>
<td>53.03</td>
</tr>
<tr>
<td>EPA (20:5 n-3)</td>
<td>11.40</td>
<td>0.00</td>
</tr>
<tr>
<td>DHA (22:6 n-3)</td>
<td>6.93</td>
<td>0.00</td>
</tr>
<tr>
<td>LA (18:2 n-6)</td>
<td>1.97</td>
<td>7.86</td>
</tr>
<tr>
<td>AA (20:4 n-6)</td>
<td>0.00</td>
<td>1.21</td>
</tr>
<tr>
<td>LNA (18.3 n-3)</td>
<td>0.76</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Data are expressed as percentages of total fatty acids. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; LNA, α-linolenic acid; LA, linoleic acid. Total n-3 polyunsaturated fatty acid (PUFA) includes values for EPA, DHA, and LNA, and total n-6 PUFA includes values for LA and AA.

Female mice were euthanized by cervical dislocation, and then peritoneal fat and retroperitoneal fat were dissected and weighed, with values expressed as a percentage of final body weight. Blood samples were obtained by suborbital bleed just before death (n = 6 samples per diet), and ovaries were dissected (n = 3 samples per diet) for the determination of fatty acid concentration by gas chromatography (Nutrition and Functional Food Science, The University of Adelaide). Plasma free fatty acid, glucose, cholesterol, and triglyceride concentrations were determined using enzymatic calorimetry with a COBAS Mira automated sample system and the assay kits as described previously (13).

**Handling and embryo culture media.** G-MOPS handling medium (26) was used for oocyte and zygote collection. The commercial sequential G-series media G1 V.3 plus and G2 V.3 plus, containing 10% human serum albumin (HSA; Vitrolife, Gothenburg, Sweden), were used for embryo culture.

**Oocyte and zygote collection.** Ovulated cumulus-oocyte complexes (COCs) or fertilized zygotes were collected from adult C57BL/6 female mice fed a diet high in n-3 PUFA or a control diet. Mice were superovulated with 5 IU of pregnant mare serum gonadotropin (Folligon; Intervet Australia, Bendigo, Victoria, Australia), followed 48 h later by 5 IU of human chorionic gonadotropin (hCG; Pregnyl; Organon, Oss, The Netherlands), administered intraperitoneally.

For the collection of ovulated COCs, mice were killed 14 h post-hCG injection and COCs were collected from the oviduct into G-MOPS handling medium. Before the measurement of oocyte parameters, surrounding cumulus cells were removed using 0.5 mg/ml hyaluronidase (Sigma Chemical, St. Louis, MO).

For the collection of in vivo fertilized zygotes (single-cell embryos), C57BL/6 female mice were housed overnight with a proven adult CBA/C57BL6 F1 male immediately following hCG injection. Presumed zygotes were collected into G-MOPS handling medium 22 h post-hCG injection, and the remaining cumulus cells were removed using 0.5 mg/ml hyaluronidase before culture.

**Measurement of mitochondrial parameters in ovulated oocytes.** Mitochondrion-specific fluorescent probes and confocal microscopy were used to analyze mitochondrial distribution, mitochondrial calcium levels, and membrane potential in ovulated oocytes recovered from females fed a diet high in n-3 PUFA or the control diet (n ≥ 28 oocytes per diet per mitochondrial measurement within 2 replicate experiments). The distribution of active mitochondria and levels of mitochondrial calcium were determined in ovulated oocytes by dual staining with MitoTracker green FM and rhod-2 AM (Molecular Probes, Eugene, OR). Ovulated oocytes were coincubated immediately after collection with a final concentration of 100 nM MitoTracker green FM and 5 μM rhod-2 AM, in G-MOPS, for 15 min at 37°C in the dark. Oocytes were then washed and imaged immediately using a confocal microscope (Nikon C1 confocal scanning head, Nikon TE2000E).

Mitochondrial membrane potential was determined by staining ovulated oocytes with the mitochondrial stain JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carboxyanine iodide; Molecular Probes) at a concentration of 1.5 mM for 15 min at 37°C in the dark. Oocytes were then imaged immediately using a confocal microscope.

All images of fluorescently labeled oocytes were analyzed using the Adobe Photoshop Pro software package (version 13: Adobe Systems). A grease spacer was used between the slide and coverslip to ensure there was no compression of the oocyte. One optical section was examined for each oocyte, in the plane where both the polar body and metaphase plate were able to be visualized. The regional distribution of mitochondrial properties was examined, because mitochondria migrate within the developing oocyte, and this is thought to be related to successful preimplantation development. The mean red and green staining intensities were determined in four different regions (regions 1–4) within three areas of the oocyte (areas 1–3), as depicted
in Fig. 1. The average pixel intensity was then determined for red and green within each of the three areas. Ratios of pixel intensity were then calculated to compare active mitochondria distribution and mitochondrial calcium levels, as well as the ratio of mitochondrial calcium to active mitochondria in each area of the oocyte. Membrane potential was determined similarly by expressing the average red reading (high membrane potential) as a ratio of the average green reading (low membrane potential) in each area of the oocyte.

**ROS levels in ovulated oocytes.** Ovulated oocytes were collected as described previously in two replicate experiments (n = 17 oocytes per diet within 2 replicate experiments). The level of ROS was determined using a 2′,7′-dichlorodihydrofluorescein diacetate (DCHDFA; Sigma Chemical) for 20 min and measuring the fluorescence of CDCF using fluorescence spectroscopy. Oocytes were stained for 30 min in 1 μM DCDHF DA (Sigma Chemical Co), and the level of DHF was measured using fluorescence spectroscopy. The relative fluorescence for each oocyte (DHF) was then determined comparatively to the average of the esterase control oocytes (CDCF) for the corresponding treatment and expressed as mean fluorescence units.

**Colocalization of ROS and mitochondria in oocytes.** Ovulated oocytes (n = 5 per diet) were coincubated in 17 μM Redox Sensor red (Molecular Probes) and 100 nM MitoTracker green FM (Molecular Probes) for 15 min in the dark at 37°C. The areas of oxidative stress in relation to regions of active mitochondria were localized and imaged as for the previously described mitochondrial stains.

**RNA extraction, reverse transcription, and real-time PCR.** Ovulated COCs collected 14 h post-hCG, with cumulus cells intact, were pooled in groups of 10 COCs within diet (n = 5 groups of COCs per diet within 2 replicate experiments), and total RNA was extracted using an RNeasy microkit (Qiagen, Doncaster, Victoria, Australia) per the manufacturer’s instructions. Reverse transcription was carried out using SuperScript III (Invitrogen, Eugene, OR) and stored at −20°C before PCR. Real-time PCR was performed using a Rotor Gene 6000 PCR machine (Corbett Life Science, Sydney, New South Wales, Australia) to determine the expression levels of superoxide dismutase 2 (SOD2), catalase, and COX-2 in whole COCs. The reaction was performed in 20-μl volumes by using a master mix containing 10 μl SYBR green mix (Applied Biosystems, Foster City, CA), 5 pM of each forward and reverse primer (Table 2), and the equivalent of 0.064 COCs per reaction. For the housekeeping gene, cDNA from the equivalent of 0.016 COCs per reaction was analyzed. The thermal cycling program was as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Rotor Gene 6000 analysis software was used to generate a cycle threshold (Ct) for each sample, and the expression level for each of the samples was determined relative to a standard curve of COC cDNA, corrected for 18S rRNA levels in each sample. Each sample was run in triplicate for each gene, and data for the group supplemented with high n-3 PUFA were expressed as the degree of change in gene expression relative to the expression levels in COCs from animals fed the control diet.

**Zygote production and embryo culture.** Embryo development was compared for 1) zygotes produced in vivo or 2) zygotes produced in vitro following in vitro fertilization (IVF). Embryos from individual animals were cultured separately in groups of up to 15 in 20 μl of G1 medium overlayed with mineral oil (Sigma Chemical). On day 2 of culture, all two-cell embryos were transferred into new warmed and equilibrated 20-μl drops of G1 medium, and on day 3, embryos were transferred into 20-μl drops of G2 medium for the remainder of culture. All embryo culture was carried out at 37°C in 6% CO2, 5% O2, and 89% N2. Embryo development was assessed at the same time daily after the commencement of culture (days 2, 3, 4, and 5, respectively).

In vivo zygotes were retrieved from the oviducts 22 h post-hCG injection as described previously, and ovulation rate was determined for each female (n = 7 animals per diet within 3 replicate experiments). Zygotes were graded as grade 1, normal morphology and even cytoplasm; or grade 2, unusual morphology and an uneven cytoplasm. Presumed grade 1 zygotes were cultured separately for each animal to the blastocyst stage, to determine developmental potential.

Zygotes were produced in vitro following collection of mature oocytes 14 h post-hCG (n = 11 animals per diet within 4 replicate experiments). Mature sperm was collected from the caudal region of the epididymis and vas deferens of male CBA/C57BL6 F1 mice and capacitated in Quinns Advantage fertilization medium (SAGE; Cooper Surgical, Trumball, CT) with the addition of 6% HSA (Vitrolife). After capacitation, oocytes and sperm were coincubated in G1 medium for 4 h at 37°C, at 6% CO2, 5% O2, and 89% N2, with a final sperm concentration of 35,000 ± 5,000 sperm/ml. Presumed zygotes were washed in G1 medium following incubation, and any remaining cumulus cells were removed manually using a glass pipette. Zygotes were then graded using the following scoring system: grade 1, normal morphology and even cytoplasm; grade 2, slightly unusual morphology or uneven cytoplasm; or grade 3, unusual morphology and uneven cytoplasm. Grade 1 zygotes were then transferred to 20-μl culture drops of G1 medium, cultured for 96 h, and assessed as described above.

**Statistics.** Quantitative data are means ± SE, with the statistical analysis performed using a general linear model in SAS version 9.1 (SAS Institute, Cary, NC). Analysis examined the fixed effect of dietary treatment (n-3 PUFA vs. control) and experimental replicate (cage effect). Embryo development data were weighted in relation to the starting number of oocytes, grade 1 COCs, or grade 1 zygotes (zygote number, in vitro two-cell number, and in vivo two-cell number, respectively) or the number of two-cell embryos per animal (day 4 and day 5 embryo development) to account for female-to-female variation. To analyze ovulation rate and embryo development, only animals producing multiple zygotes were included, and animals were distributed randomly to each diet.
that did not respond to stimulation were excluded. For analysis of oocyte and embryo development following IVF, only animals producing multiple grade 1 COCs were assumed to have responded to stimulation, and all other animals were excluded from analysis. To analyze differences in mitochondrial staining, potential hierarchical variability resulting from maternal effects on the oocyte were taken into account by fitting a mixed model with fixed effects of diet and replicate and mother identification as a random effect. For gene expression and ROS analysis, oocytes were pooled within each diet; therefore, no random effect was included in the analysis. A univariate general linear model with the fixed effect of diet was used to analyze all other data using SPSS version 13 (SPSS, Chicago, IL).

RESULTS

Body and organ weights. Dietary supplementation with n-3 PUFAs did not alter feed intake, final body weight, weight gain, or the weight of retroperitoneal and peritoneal fat deposits (data not shown). There was also no difference in plasma metabolites, triglycerides, total cholesterol, glucose, and free fatty acids (data not shown).

Ovary and plasma fatty acid profiles. To determine the level of incorporation of dietary fatty acids into animal tissue after 3–5 wk of dietary supplementation, fatty acid profiles were determined for plasma and ovarian tissue (Table 3). Animals receiving the n-3 PUFA diet had significantly enriched plasma EPA and DHA levels of 0.8 and 5.4% of total fatty acids, respectively, compared with 0.1 and 3.6% of total fatty acids for the control diet (P < 0.001). Ovary samples were also significantly enriched for EPA and DHA, with 0.6 and 8.7% for the n-3 PUFA diet, respectively, compared with 0.1 and 4.5% for the control diet samples (P < 0.05). The ratio of n-3 to n-6 PUFA was significantly greater in both ovary and plasma samples for animals fed a diet high in n-3 PUFA (P < 0.001).

Oocyte morphology and mitochondrial properties. There was no significant effect of diet on the number of in vivo matured oocytes retrieved or their morphology (data not shown). Oocyte mitochondrial properties were examined using the mitochondrial-specific dyes rhod-2 AM and MitoTracker. The distribution of active mitochondria throughout the oocyte was altered due to maternal diet such that there were more active mitochondria in the intermediate region (area 2) of the oocyte compared with the outer region (area 1) (area 2/area 1 ratio), but no difference in the ratio between areas 3 and 4. Areas 2 and 3, when females were fed the diet high in n-3 PUFA (Fig. 2A). In contrast, diet had no significant effect on mitochondrial calcium levels throughout the different areas of the oocyte (Fig. 2B).

Whilst there was no change in the distribution of mitochondrial calcium, the altered distribution of active mitochondria resulted in a significant increase in the ratio of mitochondrial calcium to active mitochondria in this outer region of oocytes from mothers fed high n-3 PUFA (P = 0.008; Fig. 2C), but there was no difference in the ratio of calcium per active mitochondria in either area 2 or 3. This indicates that active mitochondria within oocytes mothers fed high n-3 PUFA contained a higher portion of calcium in the outer region of the oocytes (area 1) compared with oocytes from animals fed the control diet.

The mitochondrial membrane potential of oocytes was determined by staining with JC-1, where at high membrane potential the dye is converted to j-aggregates, which fluoresce red, rather than the monomer, which exists at low membrane

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer Sequence</th>
<th>Molecular Weight</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD2</td>
<td>CCTGCTCTATAACGAGCAGATT</td>
<td>6630</td>
<td>59</td>
</tr>
<tr>
<td>Catalase</td>
<td>AATGCGACACGAGGAACTT</td>
<td>5791</td>
<td>83</td>
</tr>
<tr>
<td>COX-2</td>
<td>AGATCGACAGAAATACCAAAACAG</td>
<td>6980</td>
<td>103</td>
</tr>
<tr>
<td>18S</td>
<td>AGAAGGCGGATCAGCATCCCA</td>
<td>6373</td>
<td>91</td>
</tr>
</tbody>
</table>

SOD2, superoxide dismutase 2; COX-2, cyclooxygenase-2; 18S, housekeeping ribosomal gene.

Table 2. Details of primers used for analysis of gene expression in mature cumulus oocyte complexes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Molecular Weight</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD2</td>
<td>CCTGCTCTATAACGAGCAGATT</td>
<td>6630</td>
<td>59</td>
</tr>
<tr>
<td>Catalase</td>
<td>AATGCGACACGAGGAACTT</td>
<td>5791</td>
<td>83</td>
</tr>
</tbody>
</table>

Table 3. Fatty acid composition of plasma and ovary from females fed a high n-3 PUFA or control diet

Data are means ± SE, expressed as percentages of total fatty acids; n = 6 plasma samples per diet, n = 3 ovary samples per diet. *P < 0.001; †P < 0.001; ‡P < 0.05; §P < 0.05, different superscripts within plasma and ovary measurements indicate differences between diets.
potential and fluoresces green. Despite the perturbed distribution of active mitochondria and mitochondrial calcium due to dietary n-3 PUFA consumption, the mitochondrial membrane potential did not differ in any area of the oocyte (Fig. 2D).

Oxidative stress in the oocyte. The level of oxidative stress within an ovulated oocyte, as measured by DCDHF DA oxidation and expressed as a mean fluorescence reading, significantly increased due to high maternal dietary n-3 PUFA consumption (6.0 vs. 3.1 fluorescence units, respectively, for high n-3 PUFA vs. control diet, \( P < 0.001 \); Fig. 3).

Because mitochondria are a major source of ROS production, the regions within the oocyte producing high levels of ROS were colocalized with the active mitochondria by coinoculating oocytes with Redox Sensor red and MitoTracker green. The red staining of the Redox Sensor red was found to colocalize to the green staining of active mitochondria in the majority of oocytes visually assessed using confocal microscopy (Fig. 4).

Gene expression in the oocyte-cumulus complex. The expression levels of genes that are crucial for scavenging ROS (SOD2 and catalase) and a gene required for the production of prostaglandins (COX-2), were examined in COCs retrieved from animals fed a diet high in n-3 PUFA or the control diet, using real-time PCR. There was no significant difference in expression of any of these genes due to maternal dietary n-3 PUFA supply, expressed relative to the levels measured for the control diet (Fig. 5).

In vivo zygote morphology. A diet high in n-3 PUFA tended to increase the total number of zygotes collected per female (\( P = 0.08 \); Table 4), which was attributable to a significant increase in the number of poor-quality, grade 2 zygotes, compared with the control diet (6.7 and 2.6 grade 2 zygotes retrieved, respectively, \( P = 0.03 \); Table 4).

Embryo development (from in vivo zygotes). The development potential of grade 1 zygotes was assessed on days 2, 4, and 5 of in vitro culture. Consumption of a diet high in n-3 PUFA significantly reduced the ability of zygotes produced in vivo to cleave and form a two-cell embryo in culture on day 2, compared with zygotes from a female fed the control diet (61% of grade 1 zygotes cleaved vs. 91% cleaved, \( P = 0.04 \); Table 4). Thus there were an equal number of two-cell embryos in total for each dietary treatment on day 2 of culture (Table 4).

Dietary n-3 PUFA did not effect the mean number of cleaved embryos that reached the compact morula stage of development on day 4 of culture (\( P = 0.6 \); Fig. 6A), and although the animals fed high n-3 PUFA tended to produce
fewer blastocyst-stage embryos (2.6 vs. 4.6 embryos per animal in high n-3 PUFA vs. control diet, respectively), this also did not differ significantly ($P = 0.23$; Fig. 6A). However, significantly more embryos were arrested at the two-cell stage or various cleavage stages thereafter when cultured from embryos treated with high n-3 PUFA compared with the control diet; that is 3.3 vs. 0.7 delayed embryos per animal, respectively (Fig. 6A, $P = 0.04$).

A similar but nonsignificant pattern was observed on day 5 of culture for the diet high in n-3 PUFA, with a decrease in the number of blastocyst-stage embryos per animal (4.6 vs. 6.5 for high n-3 PUFA vs. control diet, respectively, $P = 0.35$) and more embryos with delayed development (4.1 vs. 1.6 embryos per animal for the high n-3 PUFA vs. control diet, respectively, $P = 0.12$; Fig. 6B). Thus dietary supplementation with n-3 PUFA altered the on-time developmental potential of in vivo zygotes examined on day 4 of culture.

**IVF and embryo development.** Mature oocytes were fertilized in vitro to determine whether dietary supplementation with n-3 PUFA altered the ability of the oocyte to fertilize in vitro. Dietary supplementation with n-3 PUFAs did not significantly alter the number of COCs retrieved, and it did not alter the morphological appearance of the COCs or subsequent zygotes after fertilization, as determined by a visual morphological scale (data not shown).

Furthermore, supplementation did not alter the ability of the in vitro-fertilized zygotes to cleave and form two-cell embryos on day 2 of culture, and it did not alter the mean number of in vitro-fertilized two-cell embryos that developed to either the morula or blastocyst stage of development on day 4 of culture (Table 5). There was no effect of diet on the mean number of embryos reaching the blastocyst stage of development on day 5 of culture, and in contrast to the embryo development from in vivo-derived zygotes, there was no difference in the number

---

Fig. 4. Confocal images of oocytes from animals fed a diet high in n-3 PUFA (a–f) and oocytes from animals fed a control diet (g–i) showing areas of redox (Redox Sensor red; left) and active mitochondria (MitoTracker green; middle) and their colocalization (right).
of embryos showing delayed development on either day 4 or day 5 of culture (Table 5).

DISCUSSION

As is well described by the Fetal Origins hypothesis (5), the environment to which a conceptus is exposed in utero can have an impact on fetal development and adult health. The realization that environmental conditions during the window of time of oocyte and preimplantation embryo development can also perturb subsequent development has only relatively recently been explored (17, 41, 46). Given the growing interest in diet and lifestyle factors around the time of conception, this study examined the impact of periconception consumption of an \( n \)-3 PUFA-enriched diet on oocyte and embryo development and hypothesized that the underlying mechanisms responsible for altered development were impaired mitochondrial metabolism.

The utilization of embryo culture in this study, following either in vitro or in vivo zygote production, permitted the investigation of potential developmental effects of \( n \)-3 PUFA exposure before conception, to be contrasted with extended exposure during fertilization and pronuclear formation in vivo. Diet did not influence the number of oocytes collected before fertilization, their morphological appearance, or their development in culture following in vitro fertilization in this study, which is in contrast to findings of one other study in ewes, where a diet high in \( n \)-3 PUFA improved the morphology of oocytes recovered in vivo (48). In cows, supplementation with the shorter chained PUFAs contained in sunflower and linseed oils had little effect on in vitro maturation, subsequent oocyte quality (morphology), fertilization, or embryo development (8). However, we have shown that exposure to the reproductive tract for a further period of time (22 h post-hCG) increased the

![Fig. 5. Expression of superoxide dismutase 2 (SOD2), catalase, and cyclooxygenase-2 (COX-2) in cumulus oocytes complexes (COCs) from animals fed a diet high in \( n \)-3 PUFA, relative to the levels measured for the control diet. Data are means ± SE, expressed as fold change from the control-fed group and have been normalized to 18S rRNA; \( n \) = 5 groups of 10 COCs per diet within 2 replicate experiments.]

![Fig. 6. Development potential of in vivo-fertilized zygotes from animals fed a diet high in \( n \)-3 PUFA or a control diet on day 4 (A) and day 5 (B) of embryo culture. Data are expressed as the mean number of embryos per animal (±SE); \( n \) = 7 animals per diet within 3 replicate experiments. a,b \( P < 0.05 \), different letters indicate differences within development stages.]

Table 4. Effect of diet on mean number of in vivo-fertilized zygotes recovered and their morphological grading and subsequent embryo cleavage

<table>
<thead>
<tr>
<th>Zygotes</th>
<th>Control</th>
<th>High ( n )-3 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of zygotes</td>
<td>9.0±2.70</td>
<td>16.6±2.70</td>
</tr>
<tr>
<td>Grade 1</td>
<td>8.3±2.54</td>
<td>12.7±1.96</td>
</tr>
<tr>
<td>Grade 2</td>
<td>2.6±1.28*</td>
<td>6.7±0.99†</td>
</tr>
<tr>
<td>Number of 2-cell embryos on day 2</td>
<td>7.6±1.45</td>
<td>7.6±1.23</td>
</tr>
<tr>
<td>Percentage of 2-cell embryos on day 2</td>
<td>91.3±11.19†</td>
<td>60.9±9.43*</td>
</tr>
</tbody>
</table>

Morphological descriptions: grade 1, good; grade 2, poor. Data are means ± SE, expressed as the mean number of embryos per animal; \( n \) = 7 animals per diet within 3 replicate experiments. *\( P < 0.05 \); †\( P < 0.05 \), different superscripts indicate differences within parameters.

Table 5. Effect of diet on developmental competence of in vitro-fertilized oocytes derived from animals fed a high \( n \)-3 PUFA or control diet on day 2, 4, and 5 of subsequent embryo culture

<table>
<thead>
<tr>
<th>Embryo Development</th>
<th>Control</th>
<th>High ( n )-3 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of 2-cell embryos</td>
<td>4.9±0.88</td>
<td>5.3±0.91</td>
</tr>
<tr>
<td>Percentage of 2-cell embryos</td>
<td>70.1±5.93</td>
<td>70.0±6.14</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compact morula</td>
<td>4.0±0.69</td>
<td>4.4±0.72</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>0.4±0.22</td>
<td>0.6±0.23</td>
</tr>
<tr>
<td>Delayed</td>
<td>0.6±0.20</td>
<td>0.4±0.21</td>
</tr>
<tr>
<td>Day 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastocyst</td>
<td>3.4±0.65</td>
<td>3.7±0.68</td>
</tr>
<tr>
<td>Delayed</td>
<td>1.8±0.71</td>
<td>1.9±0.75</td>
</tr>
</tbody>
</table>

Data are means ± SE, expressed as the mean number of embryos per animal; \( n \) = 11 animals per diet within 4 replicate experiments.
fed a diet high in n-3 PUFA. Of the morphologically normal in vivo-derived zygotes that were subsequently cultured from both diets, a greater proportion from the high n-3 PUFA treatment failed to cleave and form two-cell embryos and had delayed development. Similarly, Nonogaki et al. (31) inhibited embryo development following in vitro exposure of zygotes to the PUFAs linoleic and α-linolenic acid, and this was overcome with the addition of antioxidants, suggesting the increased oxidative stress from the PUFAs may be at least partially responsible for the reduced development.

To examine the possible mechanisms underpinning this intriguing observation, that prolonged exposure to an environment high in n-3 PUFA during fertilization impairs embryo development, we examined mitochondrial function and the production of ROS in the oocyte. Mitochondria are vital for energy metabolism, including ATP production, and cellular homeostasis in the oocyte and early embryo (reviewed in Refs. 10, 43). Supplementation of the maternal periconception diet with n-3 PUFA caused a significant shift in the distribution of active mitochondria in the oocyte away from the outer region of the oocyte, where the polar body and metaphase plate reside. Because the incorporation of sperm and decondensation of the sperm head are energy dependent, it is proposed that this shift in distribution has an impact on energy production in this outer region and hence reduces fertilization. Mitochondrial calcium levels per active mitochondria in this outer region of the oocyte were also greater, and whereas calcium signals are transmitted directly to the mitochondria during sperm-triggered oscillations at fertilization (15), in other physiological settings increased intracellular calcium results from injury or stress (21, 33), suggesting that calcium levels are a marker of cellular health. In embryos, increased intracellular free calcium was associated with reduced development of hamster preimplantation embryos (25), and intracellular calcium levels are linked with mitochondrial calcium levels as mitochondria attempt to regulate the intracellular calcium. Thus it is proposed that there is an unknown threshold level for mitochondrial calcium, a balance between the beneficial and detrimental cellular effects in the oocyte. Although the precise functional significance of perturbed mitochondrial calcium is unclear, the increase described in this study indicates altered calcium homeostasis in the mature oocyte as a consequence of n-3 PUFA supplementation.

Interestingly, despite perturbed mitochondrial calcium and distribution, inner mitochondrial membrane potential, which is thought to be correlated to embryo development and fragmentation (2, 45), was unaffected by diet. In vitro studies of colonocytes demonstrated that the n-3 PUFA DHA increased membrane potential in a dose-dependent manner (30), but dietary supplementation with this PUFA reduced membrane potential in rat colonocytes (22). Thus it appears difficult to predict changes in membrane potential as a consequence of fatty acid exposure.

PUFA exposure has been implicated with increased oxidative stress in spermatozoa (3) and in colonocytes (22, 36). ROS are generated by lipid peroxidation, which alters mitochondrial metabolism and increases ROS, or perturbed mitochondrial function, which directly increases the level of ROS (for review, see Ref. 20). ROS are important signaling molecules in the cell, and although originally regarded as cytotoxic, these molecules are considered important in many cell pathways (20, 40). Furthermore, triglycerides are an important energy source for oocytes during maturation (18, 23), and a change in the fatty acid profiles of the ovary potentially alters the energy source of the developing oocyte, which could in turn have an impact on mitochondrial metabolism and ROS generation, a link that remains to be examined. Previously, culture of mouse embryos with both n-3 and n-6 PUFAs increased lipid peroxidation and decreased embryo development, which was attenuated by the addition of antioxidants (31). In this study, the level of ROS in mature oocytes from supplemented animals increased more than twofold relative to those from control-fed animals. The areas of oxidative stress measured in the oocyte were predominantly colocalized with areas of active mitochondria, suggesting that oxidative metabolism in the mitochondria is the source of ROS. Furthermore, expression of the antioxidant genes SOD2, which catalyzes the conversion of the superoxide anion to hydrogen peroxide, and catalase, which is involved in the reduction of hydrogen peroxide to water, were unchanged in the mature COC as a result of supplementation. Together, these results suggest that the increase in production of ROS in the oocyte following n-3 PUFA supplementation results from perturbed mitochondrial homeostasis, not from deregulated control of ROS conversion into less harmful molecules by antioxidant proteins.

Although the mechanism for this increased ROS, as well as the precise effects of altered mitochondrial calcium levels, remains to be fully elucidated, we have clearly shown that both ROS production and mitochondrial calcium are altered in the mature oocyte due to maternal high n-3 PUFAs, a complex relationship also described in aged mouse oocytes (38). It is proposed that exposure to this environment for a longer period of time, during fertilization, results in perturbed sperm-triggered calcium oscillations and energy homeostasis, subsequently reducing fertilization and early embryo development. The study of Takahashi et al. (38), in which a brief and moderate exposure of oocytes to hydrogen peroxide perturbed the sperm-triggered calcium oscillations and resulted in decreased fertilization and early embryo development, lends support for this theory. The partial reversibility of increased intracellular calcium due to hydrogen peroxide in Takahashi’s study further suggests that removal of oocytes from an environment of oxidative stress reverses perturbed calcium homeostasis. These findings are consistent with our study showing that removal of the oocyte from this high n-3 PUFA environment before fertilization (in vitro) partially or fully reverses the perturbations observed in embryos produced in vivo.

An alternative hypothesis is that the high n-3 PUFA environment has an impact on the spermatozoa directly. The enrichment of plasma and ovary tissue with n-3 PUFAs following supplementation is suggestive of probable alterations in the entire reproductive tract, potentially increasing the levels of oxidative stress to which both the sperm and egg is exposed. Spermatozoa contain very high levels of PUFA, including arachidonic acid and DHA, and are therefore susceptible to oxidative damage via lipid peroxidation, thus decreasing sperm motility (3). Therefore, it is possible that the altered early embryo development following in vivo fertilization could involve perturbed sperm function resulting from oxidative stress experienced in the reproductive tract. Whether the re-
duced fertilization and development following in vivo fertilization are related solely to the developing oocyte or occur because of the interaction with sperm in the oviductal environment remains to be explored further.

Finally, it would be remiss not to discuss the role of prostaglandins and PUFA s in the context of this study, given the numerous studies examining these eicosanoids and ovarian function (28, 34, 35). COC expression of COX-2, the enzyme responsible for the conversion of long-chain PUFA s to proinflammatory prostaglandins, was unchanged by dietary treatments, suggesting that any prostaglandin-mediated effects of supplementation directly on the oocyte are unlikely, but they cannot be ruled out entirely.

In conclusion, this study examined oocyte and early embryo development following maternal supplementation with a diet high in the n-3 PUFA s DHA and EPA. Although fertilization and development in vitro were normal following IVF, the exposure of oocytes to a high n-3 PUFA environment during in vivo fertilization adversely affected zygote morphology and delayed embryo development. Finally, supplementation altered mitochondrial properties and increased the levels of ROS in oocytes, suggestive of a role for mitochondrial function in mediating the effects of maternal n-3 PUFA supplementation on impaired embryo development.

ACKNOWLEDGMENTS
We thank Dr. Karen Kind for technical advice and Prof. David Armstrong for fruitful discussions.

GRANTS
This work was supported by a National Health and Medical Research Council (NHMRC) of Australia Program Grant and a NHMRC Project Grant.

REFERENCES