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## Ovarian responses to undernutrition in pregnant ewes, USA

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### Abstract

In most mammals oogonia proliferate by mitosis and begin meiotic development during fetal life. Previous studies indicated that there is a delay in the progression to the first stage of meiotic arrest in germ cells of female fetuses of undernourished ewes. We report that underfeeding (50% NRC requirement beginning on Day 28 of pregnancy) provokes an increase in oxidative base lesions within DNA of mid-gestational (Day 78) fetal oogonia; this condition was associated with up-regulation of the tumor suppressor/cell-cycle arrest modulator p53, antiapoptotic factor Bcl-2, and base-excision repair polymerase  $\beta$ . Fetal ovarian weights and germ cell concentrations were not altered by nutrient deprivation. Ovaries of ewes on control diets (100% NRC) contained more tertiary follicles than their restricted counterparts; however, peripheral venous estradiol-17 $\beta$  was not different between groups. There was no effect of treatment on p53 accumulation in maternal oocytes. Luteal structure-function was not perturbed by undernutrition. No fetal losses were attributed to the dietary restriction. It is proposed that DNA of interphase fetal oogonia is vulnerable to oxidative insults perpetrated by a nutritional stress to the dam, and that multiple/integrated adaptive molecular response mechanisms of cell-cycle inhibition (providing the time required for base repairs) and survival hence sustain the genomic integrity and population stability of the germline.

### Background

That maternal undernutrition can affect fetal ovarian development has been documented [1]. Experiments using the sheep as a paradigm indicate that meiotic maturation of germ cells is delayed by feed restriction [2,3]; putative molecular mechanisms which mediate this effect are unknown. We hypothesized that fetal oogonia distressed as a result of nutrient deprivation express the tumor suppressor protein p53. Cells respond to p53 by cycle arrest at the G1/S or G2/M genomic checkpoints. Outcome is dictated by the intensity of the affliction. The apoptotic pathway is invoked in cells subjected to a severe trauma that causes ir-

reparable damages. In the presence of sublethal disturbances to DNA, p53 affords the time necessary for enzymatic repairs and proof-reading. Thus, p53, the so-called "guardian of the genome," serves to maintain genetic fidelity [4–7].

There is a lack of fundamental information on potential influences of undernutrition on the functional morphology of ovaries of pregnant animals. The corpus luteum provides steroid hormonal support essential for the establishment and maintenance of early pregnancy [8,9].

Follicles undergo cycles of antral development and atresia throughout gestation [10–12].

The primary objective of this investigation was to compare p53 responses of germ cells in fetal ovaries of ewes receiving adequate or restricted diets. Alterations in p53 were related to accretions of apoptotic/internucleosomal DNA cleavage sites [13], the oxidative DNA stress marker 8-oxoguanine [14], the survival protein Bcl-2 [15], and base-excision repair polymerase  $\beta$  [16]. Corpora lutea were evaluated for progesterone and steroidogenic cell and vascular compositions. Tertiary follicles were quantified and maternal oocytes were assessed for p53.

### Materials and Methods

This project was conducted with the approval of the University of Wyoming Animal Care and Use Committee. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

#### Animals and diets

Multiparous western-range ewes were synchronized to estrus (= Day 0) with prostaglandin  $F_2\alpha$  (dinoprost tromethamine i.m.; Pharmacia & Upjohn, Kalamazoo, MI) and bred to fertile rams. Diets consisted of a pelleted beet pulp (79.68% total digestible nutrients, 93.48% dry matter, 9.99% crude protein) supplemented with a mineral-vitamin mix (51.43% sodium triphosphate, 47.62% potassium chloride, 0.39% zinc oxide, 0.06% cobalt acetate; 8,000,000 IU vitamin A, 800,000 IU vitamin  $D_3$ , 400,000 IU vitamin E per pound). Animals which did not return to estrus ( $N = 13$ ) were weighed on Day 20 and dietary intakes calculated on a dry matter basis for total digestible nutrients recommended for early gestation (NRC). Feeding in individual pens commenced on Day 21. Ewes were assigned on Day 28 to a control (100% NRC;  $n = 7$ ) or nutrient-restricted group (50% NRC;  $n = 6$ ). Diets were adjusted for weight gain/loss (to maintain a constant level of energy) at seven-day intervals until slaughter (Day  $78 \pm 0.9$ ). Pregnancies were confirmed by ultrasonography on Day 45. Four-of-seven control and 5-of-6 restricted ewes were pregnant with female fetuses; twin females were present in two control animals and in one restricted animal.

#### Processing of samples

Maternal blood samples were collected by jugular venipuncture on the day of slaughter, placed into heparinized tubes, and mixed by inverting. Plasma was harvested from cells after centrifugation and stored at  $-20\text{ C}$ .

Parameters recorded after slaughter included: weights of ewes, fetuses, maternal and fetal ovaries, and isolated corpora lutea; fetal sex; corpora lutea per ewe; and numbers of follicles  $\geq 3$  mm diameter visible at the surface of mater-

nal ovaries. A small portion of luteal tissue was excised from each gland and frozen in liquid nitrogen.

Fetal ovaries, corpora lutea, and maternal interstitial/follicular (residual) tissues were fixed by immersion in 10% buffered formalin, washed in phosphate-buffered saline (PBS), dehydrated in a graded series of ethanol, cleared in xylene, infiltrated with and embedded in paraffin wax, and sectioned at  $6\ \mu\text{m}$  thickness. Sections were floated on deionized water, transferred onto microscope slides treated with subbing solution (0.025% chromium potassium sulfate, 0.25% gelatin), air-dried, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H & E) or processed for fluorescence microscopy (Olympus BH-2, Tokyo, Japan).

#### General morphology and cellular composition of fetal ovaries

Fetal ovarian sections stained with H & E were examined for surface epithelium and arrangements of pregranulosa and germ cells. Germ cells were counted (two fields from three different mid-ovarian sections per fetus at  $\times 400$  magnification) and relative manifestations of apoptosis (nuclear pyknosis and cytoplasmic condensation) were noted.

#### p53, 8-oxoguanine, Bcl-2, and polymerase $\beta$ immunohistochemistry

Purified antibodies to p53 (mouse monoclonal KAM-CC002) and Bcl-2 (rabbit polyclonal AAP-070) were obtained from StressGen Biotechnologies (Victoria BC, Canada). Mouse monoclonal anti-8-oxoguanine (4355-MC-100) was purchased from Trevigen (Gaithersburg, MD). Antipeptide polymerase  $\beta$  antibodies were affinity-purified from rabbit serum [17].

Sections of fetal and residual maternal ovarian tissues were incubated for 30 min with 10% normal goat serum and for 1 h with primary antibodies ( $1\ \mu\text{g}/\text{ml}$ ), washed in two changes of PBS, incubated for 30 min with secondary goat antirabbit (F0382) or antimouse (F0257) immunoglobulin G-fluorescein isothiocyanate (FITC) (1:40), and washed in two changes of PBS. Serum and antibodies were diluted in freshly-prepared PBS containing 0.5% bovine serum albumin. Negative controls were carried out without primary antibodies and with primary antibodies preabsorbed (100-fold molar excess, 2 h,  $25\text{ C}$ ) with an 8-oxoguanine oligonucleotide (3850-100-01; Trevigen), human recombinant p53 (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 peptide (amino acids 41–54; StressGen), or recombinant polymerase  $\beta$  (Trevigen).

#### DNA fragmentation analysis of germ cells

End-labeling of fragmented DNA was used to monitor progressive (nuclear) apoptosis [18,19]. Briefly, 3'-OH

**Table 1: Dietary impact on maternal and fetal weights**

	Control	Restricted
Ewes (kg, Day 28)	94 ± 5	92 ± 5
Ewes (kg, Day 78)*	102 ± 5	86 ± 5
Lambs (g)**	326 ± 20	221 ± 8

\*  $P < 0.05$ , \*\*  $P < 0.01$ .

ends of DNA were linked with digoxigenin-11-d uridine triphosphate by terminal deoxynucleotidyl transferase (TdT) catalysis. Incorporated nucleotide heteropolymers were localized with antidigoxigenin Fab-FITC (ApopTag Kit S7110; Intergen Co., Purchase, NY). Conjugate or TdT were omitted in negative control reactions.

#### Fluorescence measurements of immunostained oogonal oocytes

Images of cells sectioned through the nucleus were captured ( $\times 400$  magnification) by computer-interfaced digital photography (1.2 million pixel resolution; Pixera, Los Gatos, CA) and assessed for luminance intensities (continuous inverted gray-scale = 0 [black]-255 [white]; Optimas Software, Bothell, WA). Measures were made on twenty germ cells (within a respective section) per fetus for each analytical procedure: p53, apoptotic DNA fragmentation, 8-oxoguanine, Bcl-2, and polymerase  $\beta$ . Ten oocytes per ewe (primary-antral follicles;  $n = 4-6$  ovarian sections) were evaluated for p53 immunoreactions.

#### Radioimmunoassays

Maternal plasma were assayed for progesterone [20] and estradiol-17 $\beta$  [21] and luteal tissues (wet basis) for progesterone [22] using validated procedures. All samples were analyzed in the same assays; intra-assay coefficients of variation were  $< 10\%$ .

#### Luteal morphometry

Percentage areas occupied by large steroidogenic cells, small cells, and blood vessels (luminal space) were determined (Optimas) within images of H & E-stained tissues ( $\times 400$  magnification; two fields within each of three different sections per gland). Small luteal cells (12–22  $\mu\text{m}$  diameter) were defined as spindle-shaped with dark-staining cytoplasm. Large luteal cells ( $> 30 \mu\text{m}$  diameter) were distinguished as polyhedral with light-staining cytoplasm [23].

#### Statistics

Assignments of animals to treatments and selections of fields/cells for microscopic examination were made at random. Subsample values were averaged. Fetal twin and luteal data were averaged within-ewe. Treatment (control

vs. restricted) mean comparisons were made by Student's t-test. Contrasts were considered significantly different at  $P < 0.05$ . Data are presented as means  $\pm$  standard errors.

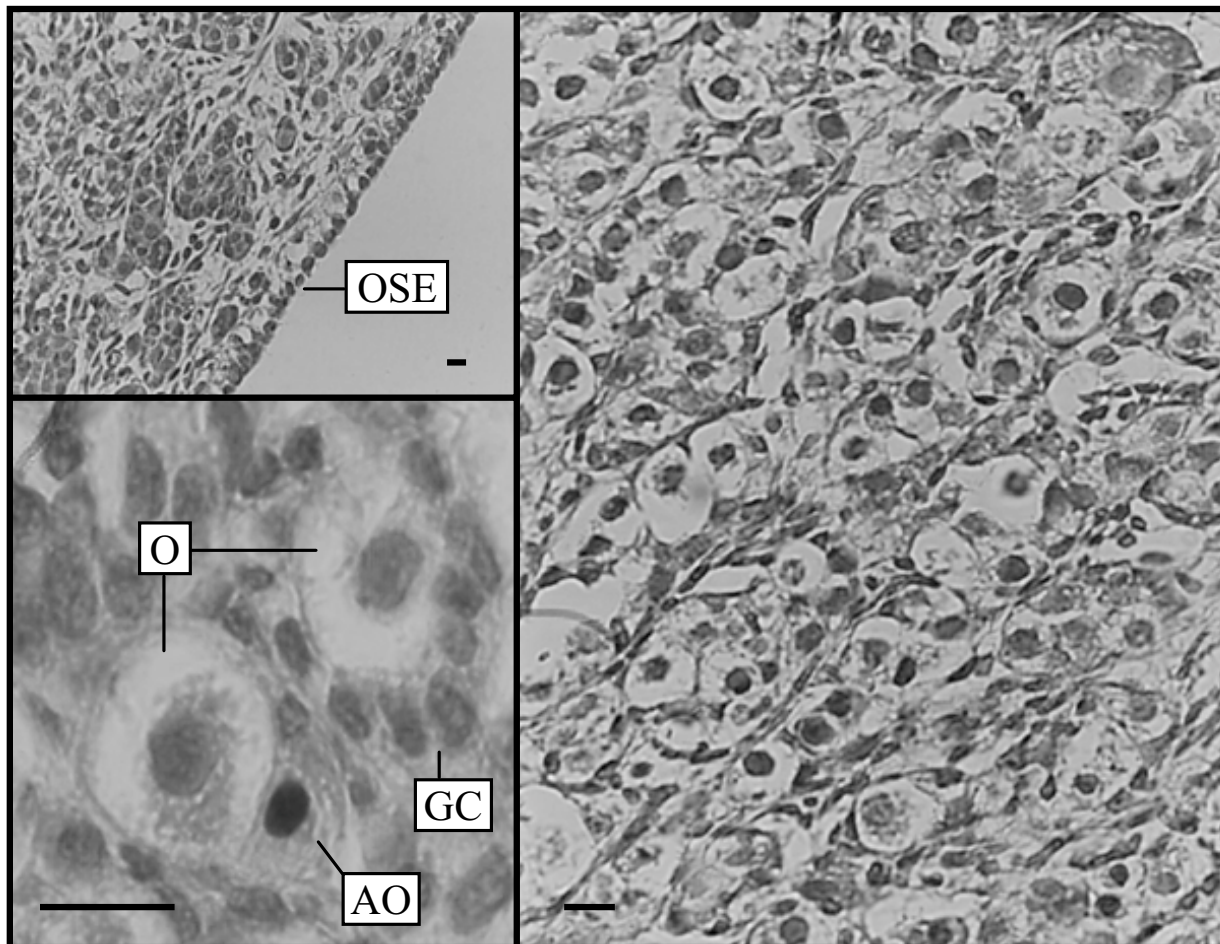
#### Results

Ewes in the restricted-diet group weighed less at slaughter than controls. Fetal weights also were reduced by nutritional deprivation (Table 1).

There were no salient differences observed in the histoarchitecture of ovaries recovered from fetal lambs of control and restricted ewes. Surface epithelia of ovaries consisted of cuboidal cells; a definitive basal lamina (which eventually supports and separates surface cells from the ovarian cortical interstitium) was not readily discernible. Germ cells were typically spherical in shape, contained a conspicuous centrally-located nucleus, were larger than other cell-types, and were organized into clusters or cords sometimes associated with progenitor granulosa cells. Evidence of apoptotic oogonal degeneration was apparent (approximately 10% of cells) within ovaries of both nutritional groups (Figure 1).

There were no significant influences of nutrition on weights of fetal ovaries or tissue concentrations of germ cells (Table 2). Increases above controls in immunoreactive 8-oxoguanine, p53, Bcl-2, and polymerase  $\beta$  were detected in germ cells of fetal ovaries from diet-limited ewes. No group differences in rates of apoptosis as indicated by immunoreactive DNA fragments were detected. Levels of p53 in maternal oocytes were not altered overall (irrespective of follicular classification) by undernutrition (Figure 2); and therefore, we did not monitor the inductive damage (8-oxoguanine) or response survival (Bcl-2)/repair (polymerase  $\beta$ ) markers.

Additional data relative to maternal ovaries and jugular plasma sex steroid hormone determinations are compiled in Table 3. Weights of maternal ovaries and (dissected) corpora lutea were not influenced by nutritional status. Numbers of corpora lutea and fetuses diagnosed by ultrasound matched the number of fetuses retrieved at slaughter (i.e., no embryonic/fetal losses occurred during gestation or due to treatments). Ovaries of control ewes



**Figure 1**

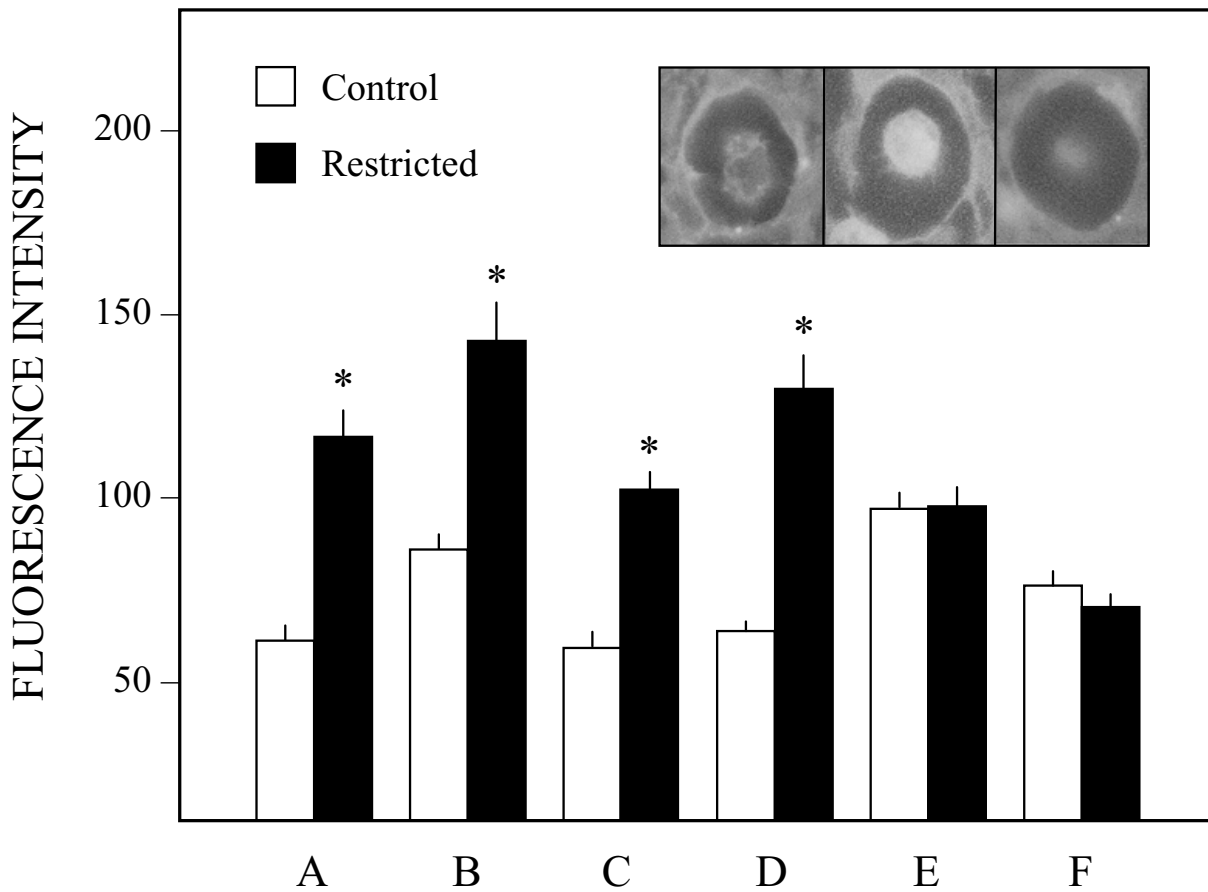
Light microscopic morphology of fetal ovaries. A low-power view of the ovarian surface epithelium (OSE) and cortex are shown in the upper left panel. High-power views of healthy oogonia (O), an apoptotic oogonium (AO), and pregranulosa cells (GC) are illustrated in the lower left panel. A mid-power view of oogonial clusters/cords is depicted in the right panel. Scale bars = 5  $\mu$ m.

contained more follicles 3 mm or greater in diameter than animals receiving restricted diets; albeit, there was no significant difference in jugular venous concentrations of estradiol-17 $\beta$ . Likewise, there was no differential effect of treatments on peripheral progesterone. Luteal progesterone and tissue areas occupied by blood vessels and large and small steroidogenic cells were similar between groups.

**Discussion**

Results of this study indicate that in underfed ewes p53, Bcl-2, and polymerase  $\beta$  are up-regulated in fetal oogonia

containing elevated contents of 8-oxoguanine. Oxoguanine has become the benchmark for oxidative DNA modifications; it is arguably the most important mutagenic lesion in DNA (mispairing with adenine during chromosomal replications causes GC  $\rightarrow$  TA transversions) [24]. It is suggested that untoward oxidative stresses to DNA prompt cell-cycle delay, antiapoptotic, and repair responses. Indeed, p53 controls the low-level damage-dependent premeiotic checkpoint which facilitates DNA repair during spermatogenesis [25]. Gametogenesis is inherently error-prone [26] and subject to mutations [27].



**Figure 2**

Fluorescence intensity scores of oogonia/oocytes: A, fetal 8-oxoguanine; B, fetal p53; C, fetal Bcl-2; D, fetal polymerase  $\beta$ ; E, fetal apoptosis (DNA fragmentation); F, maternal p53 (fetal control, n = 4; fetal restricted, n = 5; maternal control, n = 7; maternal restricted, n = 6). Asterisks indicate pairwise increases ( $P < 0.01$ ). Values for negative controls (not subtracted from the data shown) were  $< 45$ . Representative photomicrographs of p53-immunostained oogonia of control (left panel) and restricted (center panel) fetuses are shown in the inset (note the contrast in magnitudes of nuclear fluorescence); a negative preabsorption control cell (restricted fetus) is shown in the right panel.

**Table 2: Substance of fetal ovaries**

	Control	Restricted
Ovarian (combined) weight (mg)	54.3 $\pm$ 6.2	41.6 $\pm$ 8.0
Germ cells (# per field, $\times$ 400)	27.3 $\pm$ 1.5	29.4 $\pm$ 1.0

**Table 3: Maternal ovarian/follicular/luteal and steroidogenic indices**

	Control	Restricted
Ewes (number)	7	6
Ovarian (combined) weight (g)	5.4 ± 0.7	5.7 ± 1.0
Corpus luteum weight (g)	0.93 ± 0.1	0.84 ± 0.1
Corpora lutea (number per ewe)	1.9 ± 0.3	1.7 ± 0.3
Tertiary follicles (number per ewe)*	7.2 ± 0.4	3.0 ± 0.6
Jugular estradiol-17β (pg/ml)	3.0 ± 0.5	3.9 ± 0.8
Jugular progesterone (ng/ml)	5.8 ± 0.4	7.0 ± 1.0
Luteal progesterone (ng/mg)	19.7 ± 1.8	22.1 ± 5.5
Luteal vascular space (%)	9.5 ± 0.3	9.2 ± 0.4
Large luteal cells (%)	38.9 ± 0.4	37.9 ± 1.1
Small luteal cells (%)	19.5 ± 0.5	19.1 ± 0.4

\*  $P < 0.01$ .

Nevertheless, it is imperative to avoid genotoxic complications that could become integrated into the germline.

Bcl-2 belongs to a family of cellular proteins which arbitrate decisions in life-or-death situations. In the presence of Bcl-2, irregularities within the DNA sequence are corrected before the p53 suicidal program is executed. Bcl-2 impedes the subcellular trafficking of p53, inhibits downstream adapters necessary for stimulation of the apoptotic caspases, and can act as an antioxidant [15,28].

Polymerase β is a penultimate mediator of mammalian DNA base-excision repair. The base-excision cascade is characteristically limited to the repair of small lesions in DNA (e.g., single nucleotide modifications). Short-patch reconstruction is initiated by a proof-reading glycosylase that hydrolyzes the N-glycosylic bond linking an improper base to deoxyribose. The abasic sugar-phosphate backbone is then cleaved by an apurinic/apyrimidinic endonuclease or lyase. Polymerase β fills the nucleotide gap created in DNA with the deoxyribonucleoside triphosphate complementary to the template. Finally, the nick is sealed by a DNA ligase [16]. The base-excision pathway is a principal contributor to the amendment of 8-oxoguanine corruptions in DNA [29].

Unlike males, which continue to generate sperm cells by mitosis throughout their reproductive lives, mammalian females are generally born with their full complement of (meiotic) gametes [30]. The normal chronology of ovarian development follows a definitive pattern. Critical windows of activity in the sheep include: somatic ovarian tissue differentiation (from the gonadal ridges of the mesonephros) and growth (up to Day 50 of gestation); germ (from yolk sac endoderm) and pregranulosa (derived from the ovarian surface epithelium) cellular migration into and colonization of the ovaries (Days 30–65); clonal

expansion of oogonia (Days 35–75); onset of meiosis and arrest (by Day 80) at the dictyate stage of prophase (the first division of meiosis is not completed until ovulation); and ovigerous cord regression and primordial follicle emergence (Days 65–110) [1,31–33]. It seems apparent that germ cells are particularly vulnerable to a metabolic insult when in the phasic transition between mitosis and meiosis I.

Relatively innocuous base damages to DNA caused by oxidations are an inevitable by-product of physiological metabolism (e.g., leakage of radicals associated with the reduction of oxygen to water during mitochondrial respiration) [34]. The etiological basis for the formation of abnormally high levels of 8-oxoguanine adducts in oogonia of lambs of undernourished ewes is unknown. Altered placental vascular dynamics and shifts in fetal organ blood flow precipitated by nutrient deficiencies are of possible significance [35]. Ischemia-reperfusion is a well known stimulus of free radical formation [36]. Micronutrient imbalances or insufficiencies also can negatively impact the functional capacities of antioxidant vitamin (C, E) and enzymatic (superoxide dismutase, glutathione peroxidase, catalase) defense systems [37].

While cellular proliferation is a generic feature of normal germline development, so is programmed physiological death. In fact, the apoptotic demise (yielding double-stranded DNA breaks) of female germ cells throughout gestations of most vertebrates will encompass more than one-half of precursor populations [38,39]. Day 75 is the approximate time-point when a maximum number of oogonia is achieved in lambs; a nearly five-fold loss then occurs between Days 75 and 90 [40]. In some cases of nutritional deprivation cellular losses are accelerated [38] and reproductive performance of the female offspring during adulthood is attenuated [1]. Prenatal exposure to

famine (1944–45 Dutch cohort) did not affect subsequent fertility of women [41]. Numbers of oogonia/oocytes and weights of mid-gestational fetal ovaries of sheep were increased [2,42] or not influenced by under-nutrition [[3], present study]. It has been suggested that oogonia which have matured to oocytes are less susceptible to degeneration as pregnancy advances [2]. A prominent role for the aromatic hydrocarbon receptor (e.g., which is responsive to polycyclic ligands present in environmental toxicants) in promoting apoptosis during fetal oogenesis has recently been identified in mice [43,44]. Prospective endocrine/paracrine modifiers (which are altered by nutritional regimen) of the fates of fetal germ cells include glucocorticoids, thyroid hormones, insulin, growth hormone, insulin-like growth factors, and leptin [1,45].

The physiological relevance, if any, of the diminution in numbers of surface antral ovarian follicles in dams of the restricted nutrition group is unclear – this was not reflected by a perturbation in circulatory estradiol-17 $\beta$ . Structural and functional properties of ovine corpora lutea of pregnancy were not altered by plane of nutrition. Moreover, maternal nutrient restriction during the middle third of gestation in pigs had no effect on ovarian progesterone production or fetal survival [46]. Morphometric data for sheep corpora lutea are consistent with previous reports summarized by Sawyer [23]. It therefore appears that maternal ovaries are comparatively resilient to repercussions of undernutrition.

In conclusion, we surmise that oxidative base damages to the DNA of fetal oogonia of pregnant animals faced with a nutritional adversity are a potential threat to the genetic character and reproductive capacity of their progeny; the predicament can evidently be reconciled by response mechanisms of cell-cycle arrest, survival, and repair. Evolutionary pressures to resist or adapt to the stresses of caloric constraints have almost certainly served to assure the successes of mammalian reproduction (natural selection) for millions of years.

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