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Minireview

Trace Elements in Ovaries: Measurement and Physiology[1]

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ABSTRACT

Traditionally, research in the field of trace element biology and human and animal health has largely depended on epidemiological methods to demonstrate involvement in biological processes. These studies were typically followed by trace element supplementation trials or attempts at identification of the biochemical pathways involved. With the discovery of biological molecules that contain the trace elements, such as matrix metalloproteinases containing zinc (Zn), cytochrome P450 enzymes containing iron (Fe), and selenoproteins containing selenium (Se), much of the current research focuses on these molecules, and, hence, only indirectly on trace elements themselves. This review focuses largely on two synchrotron-based x-ray techniques: X-ray absorption spectroscopy and x-ray fluorescence imaging that can be used to identify the in situ speciation and distribution of trace elements in tissues, using our recent studies of bovine ovaries, where the distribution of Fe, Se, Zn, and bromine were determined. It also discusses the value of other techniques, such as inductively coupled plasma mass spectrometry, used to garner information about the concentrations and elemental state of the trace elements. These applications to measure trace elemental distributions in bovine ovaries at high resolutions provide new insights into possible roles for trace elements in the ovary.

bromine, iron, ovary, selenium, synchrotron x-ray fluorescence, trace elements, zinc

INTRODUCTION

When living organisms migrated to the land from water during the Paleozoic period, they had to depend on the soil for their source of biological trace elements [1]. A total of 26 of the 90 naturally occurring elements are known to be essential for animal life, including copper (Cu), iron (Fe), selenium (Se), zinc (Zn), cobalt, fluorine, iodine, manganese, molybdenum, nickel, silicon, and vanadium, with some disagreement over arsenic, chromium, and tin being essential trace elements [2–4]. Evidence for essentiality of a particular trace element is established when symptoms are induced by dietary deficiency, then subsequently reversed after a diet that is replete in this specific trace element is provided [5]. Essentiality is then generally acknowledged when it has been demonstrated by more than one independent investigator and in more than one animal species [6]. Evolutionary selection of these elements remains a mystery; the property that the suite of elements discussed in this review has in common is that they commonly occur and function in living organisms at low concentrations. Interest in the potential role of trace elements in human and animal physiology began over a century ago with the discovery that a number of compounds in living organisms contained metals not previously considered to be of biological significance [6]. When studying the relationship of trace elements in human health, it becomes increasingly evident that optimal levels of elements in every organ, tissue, and cell of the human body may be key to maintaining a healthy existence [7]. Trace elements are present in every biological process, from the production of hormones and energy, digestion, nerve transmission, and muscle contraction to the regulation of pH, metabolism, and cholesterol and blood sugar levels [7]. Stress and exposure to environmental pollution alter our requirements for these minerals, and scientists are becoming increasingly aware that trace element deficiencies can affect both physical and mental health.

In addition to identifying trace elements, elucidating the roles they might be playing at a biochemical level, and considering the optimal dietary intake, bioavailability, or amount of utilizable element relative to the amount present in the daily diet, are important considerations [8]. Trace element bioavailability is influenced by a complex matrix of interacting variables, including the chemical form of the element found in food, the nature of the food ingested, the composition of the total diet, and the health and nutritional well being of the individual [3, 9]. Dietary deficiencies of trace elements have been reported to alter various aspects of reproductive physiology [10]; however, to date, the majority of these studies have focused on their roles in male reproductive function.

Traditionally, research in the field of trace element biology and human health has largely depended on epidemiological methods to demonstrate involvement in biological processes. These studies were typically followed by trace element supplementation trials or attempts at identification of the biochemical pathways involved. With the discovery of

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TABLE 1. Newer technical approaches to study trace elements in tissues.

Analytical platform	Mass of tissue	Tissue preparation	In situ quantification	Range of elements[a]	Accuracy[b]	Relative cost[c]
S-XRF	1 mg–1 g (wet weight)	No preconditioning of tissue	Yes, morphology intact. Single-cell level	Multielement, simultaneous	High	Low
XRF	1–7 g (dry weight)	Decomposition to powder or emulsion	No	Multielement (Mg to U), difficult with lighter elements	Medium	Medium
XAS	0.02–0.5 g (dry weight)	Unnecessary	Possible, although bulk analysis more common	Multielement (Mg to U), nonsimultaneous	Low, generally used to provide chemical rather than elemental (quantitative) information	Medium
ICP-MS	0.2 mg (dry weight)	Decomposition to liquid phase	No	Multielement, simultaneous	Medium to high	Low

[a] Range of elements is generally considered to be from Mg to U for XAS and XRF, and lighter elements are possible with S-XRF and ICP-MS.
[b] Accuracy: nearness of result to the true result, is dependent on sample preparation, homogeneity of tissue, and interfering matrix effects (see text).
[c] Relative cost of analysis takes into account preparation of sample, start-up costs, etc. S-XRF and ICP-MS require expensive equipment that is normally shared between institutions, allowing for assay costs to be relatively low (shared) compared with platforms using less-expensive equipment, but operated within individual institutions.

biological molecules that contain the trace elements, such as matrix metalloproteinases containing Zn, superoxide dismutase containing Mn or Cu, cytochrome P450 enzymes containing Fe, or selenoproteins containing Se, much of the current research focuses on these proteins and, hence, only indirectly on trace elements themselves. While useful, these approaches overlook key roles of many trace elements in biological processes, including reproduction. In this review, we cover some of the newer techniques for studying trace elements and, in particular, their application to the study of the ovary.

NEWER TECHNICAL APPROACHES TO STUDY TRACE ELEMENTS

The objectives of newer approaches to study trace elements in cells and tissues are first to identify which trace elements are present, to quantify levels of trace elements, and to determine the location of the trace elements in tissues and cells. According to the findings of the study of these parameters, assumptions can be made with regard to tissue- and cell-specific function of trace elements. In a paper designed to evaluate laboratory methods for trace element determination, Bolann et al. [11] summarized that the analysis of trace elements in biological fluids and tissues serves multiple purposes. These include determining the concentration and distribution of essential trace elements in normal and disease conditions, detection and allocation of potentially toxic metals, diagnosis of trace element deficiency states, and trace element-related diseases [11]. According to their findings, the four most commonly used techniques for trace element analysis in human biological material are: 1) flame atomic absorption spectrometry, 2) graphite furnace atomic absorption spectrometry, 3) inductively coupled plasma atomic emission spectrometry, and 4) inductively coupled plasma mass spectrometry (ICP-MS), with ICP-MS being preferable for simultaneous screening of multiple elements [11].

In another study designed to ascertain the most effective means of analyzing the elemental composition of a geological sample (samples of sediment from the bottom of a lake), Phedorin et al. [12] compared synchrotron x-ray fluorescence (S-XRF), traditional XRF, ICP-MS, atomic absorption spectrometry, and instrumental neutron activation analysis, and concluded that S-XRF was superior from the point of view of its speed, ease of application, cost, nondestructive nature, and sensitivity [12]. These factors, coupled with the simultaneous determination of multiple elements of geochemical or biochemical interest, make synchrotron-based XRF a highly sought-after technique.

Weekley et al. [13] assert that XRF imaging and x-ray absorption spectroscopy (XAS) are ideal techniques for investigating the chemical speciation and distribution of elements heavier than silicon in biological systems, with minimal sample preparation required [13]. XRF imaging is now a well-established, nondestructive analytical method applicable across a large variety of fields, including materials science, environmental science, geology, life science, and archeological science [14]. The discussion below describes the newer approaches to studying trace elements, detailing the basic principle behind the approach, including the advantages and limitations of each platform. Table 1 compares the platforms with regard to sample preparation and salient features.

S-XRF Imaging

The principle of XRF involves using x-rays of sufficient energy to expel tightly held inner electrons of an element

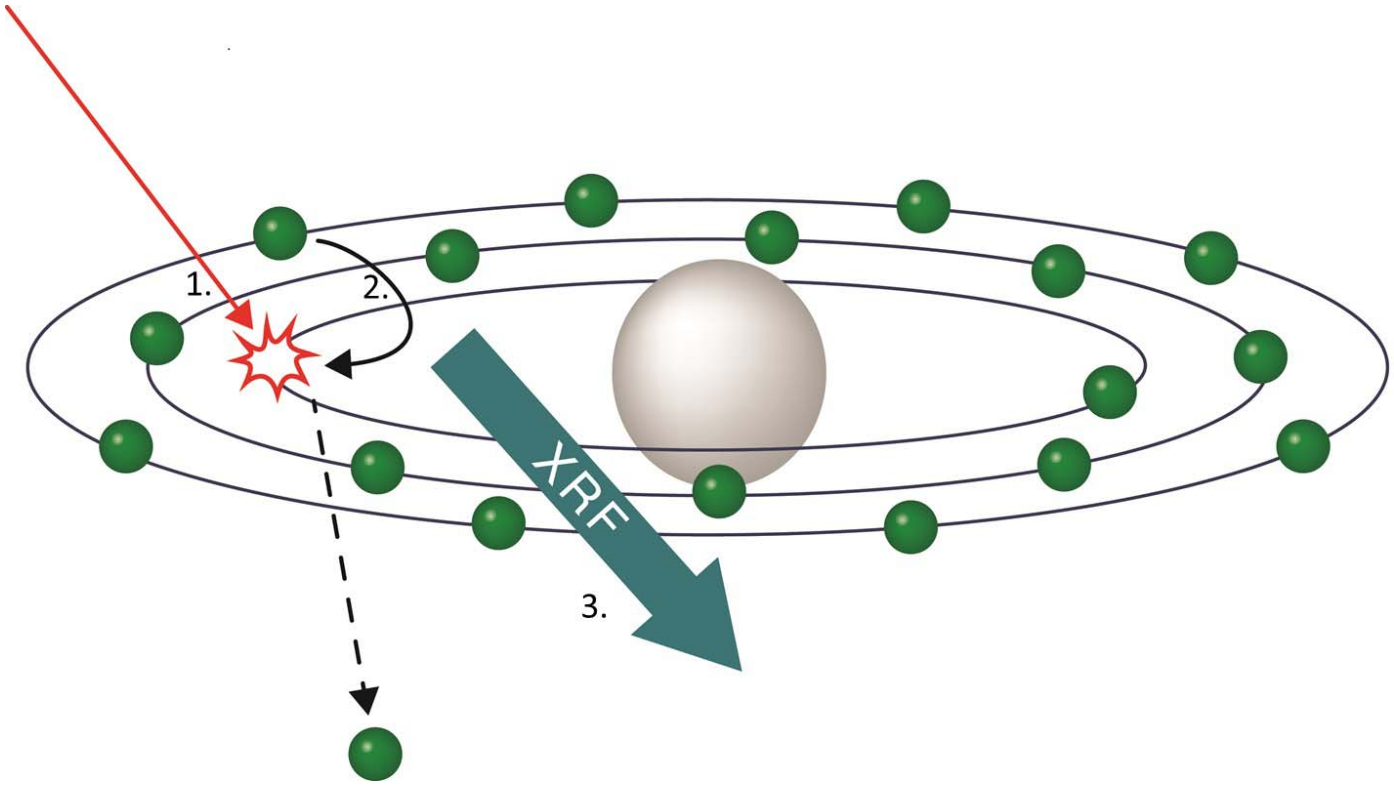

FIG. 1. Schematic diagram demonstrating the physical principles associated with S-XRF. 1) X-rays that strike an atom may knock electrons out of it, leaving behind an ionized version of the atom (i.e., one missing electron), and an unoccupied electron energy level. 2) An electron of a higher energy will then drop down to fill the lower-energy, unoccupied level, releasing an x-ray photon as a means to conserve energy. 3) The emission of the x-ray photon is the source of the fluorescence. The electron energy levels are very specific to each element, so that the energy of the S-XRF produced precisely describes the elements present in the biological sample.

within a sample. This makes the electronic structure of the element unstable, and an electron from a higher orbit replaces the inner electron, at the same time releasing energy in the form of a fluorescent photon. The fluorescent photon has a characteristic energy equal to the difference in energy between the outer and inner orbital [15]. As the electron energy levels are specific for each element, the fluorescence produced by the x-ray precisely identifies the elements within the sample (see Fig. 1).

A synchrotron is a circular particle accelerator that uses both magnetic and electric fields to accelerate charged particles. During this process, bunches of electrons are accelerated to about the speed of light within an orbital storage ring, and give off synchrotron radiation, which can then be harnessed for the x-ray source for XRF (see Fig. 2). S-XRF imaging is a powerful technique that harnesses the finely focused x-ray beam that is several orders higher in intensity than radiation from traditional x-ray sources, and focuses this onto a small spot on a biological sample (e.g., an ovarian section). Digital images of microscopic tissue samples are built, pixel by pixel, by scanning the sample through the beam, and mathematical deconvolution analysis of the fluorescence spectrum reveals the elemental composition. Quantitative elemental distribution maps of the sample can be assembled [16] to reveal the spatial organization of trace elements within the sample with high spatial resolution and sensitivity (to ppm or better) for a large number of elements (Fig. 2) [17]. The S-XRF process is undertaken without destroying the tissue under study [18].

Applications of S-XRF Imaging to Biological Systems

To date, there is a plethora of studies, ranging from elemental tissue profiles and identifying toxic metals in human teeth [19–21], to determining levels of calcium and phosphorous in the bones in osteoporosis research [22, 23], and comparing chemical composition in liver biopsies from untreated cirrhosis patients with healthy controls [19, 24]. However, only a limited number of XRF imaging studies have been carried out involving male or female reproductive organs.

Quantitative analysis of Zn and Ca in human prostate cancer and normal tissues by S-XRF imaging was undertaken by Ide-Ektessabi et al. [25] in order to investigate differences in the distribution and concentrations of Zn in these tissues. Ortega et al. [26] mapped the distribution of chromium in the reproductive organs of male mice after exposure to $CrCl_3$ to elucidate the specific cellular regions where it had accumulated. Two more recent papers presented S-XRF imaging of the ovaries to investigate the bioaccumulation of dietary Zn in *Daphnia magna*, a common ecotoxicological model [27, 28]. In both cases, the researchers set out to investigate the hypothesis that Zn selectively accumulates in female reproductive tissues and adversely effects reproduction. S-XRF has also been used in recent years to analyze the trace elemental composition of human breast tissue, and has revealed statistically significant elevation of a multitude of elements in neoplastic tissue compared to normal samples [29–33].

In summary, the use of S-XRF in elucidating the distribution and quantification of both essential and toxic trace elements in tissues provides new insights into diseases and provides ever-increasing applications to biological systems

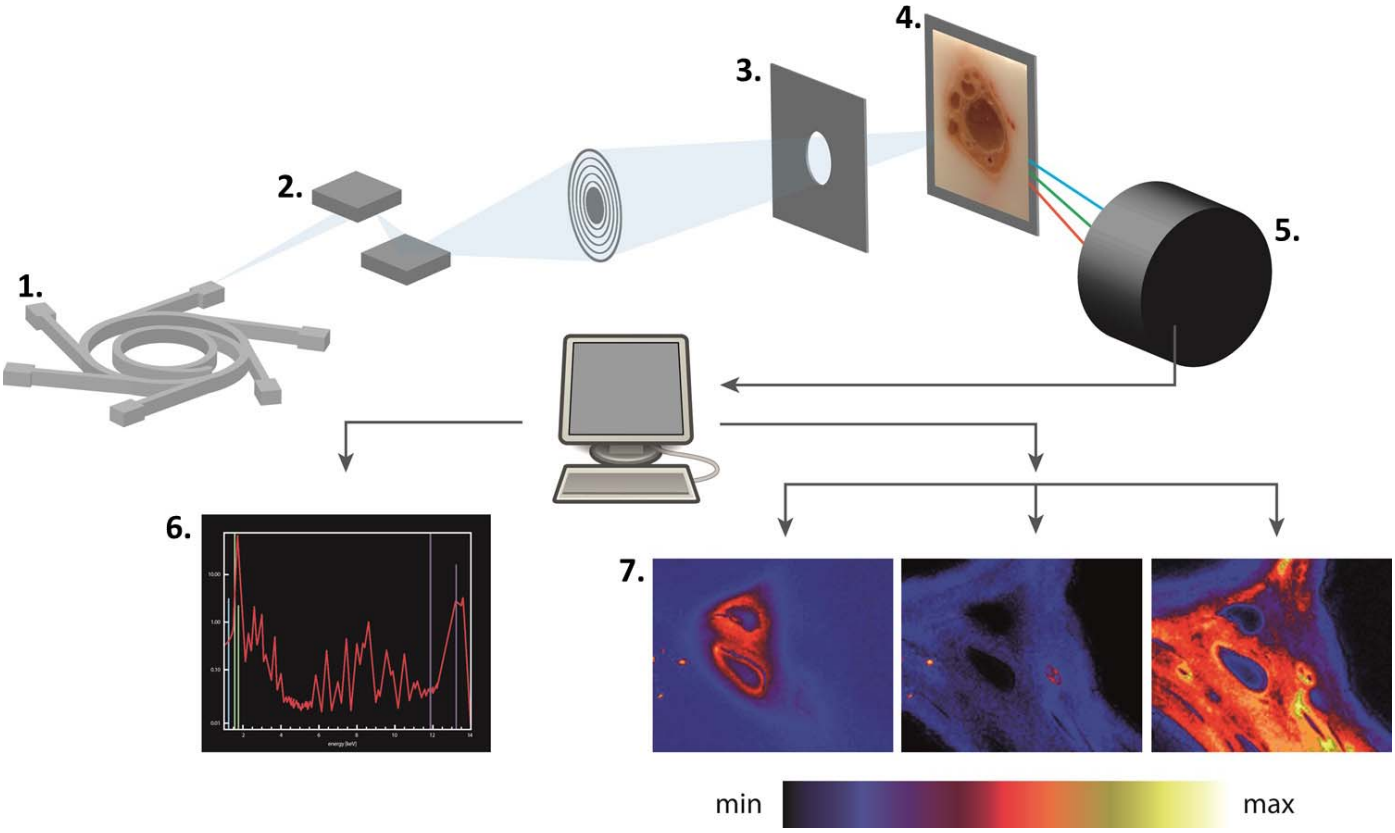

FIG. 2. Schematic representation of S-XRF. The sequence of steps includes: 1) X-rays produced by highly energetic electrons accelerated in the synchrotron storage ring; 2) x-ray optics are then used to energy filter; and 3) focus synchrotron sourced x-rays onto a small spot (several nanometers to micrometers in diameter) on the sample; 4) the sample is then raster scanned through the beam; 5) an energy-dispersive fluorescence detector is used to produce a full-fluorescence spectrum; and 6) peaks correspond to specific elements. By comparing these spectra to reference standards of known concentration, quantitative in situ maps of multiple elements (7) can be created simultaneously.

[34]. However, despite there being about 90 synchrotrons worldwide, only a handful have the capability of XRF imaging, and even fewer have the advanced capability to collect large amounts of data quickly, as was recently undertaken in the ovary [35, 36].

X-Ray Absorption Spectroscopy

The principle of XAS is similar to XRF, where synchrotron-sourced x-rays are used to ionize core electrons, creating an absorption edge with an energy that is characteristic to each element. XAS determines the local geometric and/or electronic structure of trace elements in matter [37]. More specifically, information can be gleaned on bond lengths, coordination numbers, local coordination geometry, and the oxidation state of atoms for a wide range of solid or liquid samples. Traditionally, the XAS spectrum is divided into two regions: the low-energy region, which covers photon energy up to about 50 eV above the absorption edge, often referred to as x-ray absorption near-edge structure (XANES); and the higher-energy region, from 50 to 1000 eV above the absorption edge, which is called the extended x-ray absorption fine structure (EXAFS) [38, 39]. Although the two regions have the same physical origin, this distinction is convenient for their interpretation [40]. XANES is highly sensitive to the spectral oxidation state and coordination chemistry of the absorbing atom, while the EXAFS is used to determine the distances, coordination number, and identity of the neighboring atoms. Thus, the information obtained using XAS is biologically useful in ascertaining which biological molecules the elements are associated and, hence, their functions.

Inductively Coupled Plasma-Mass Spectrometry

The principle of ICP-MS involves a high-temperature inductively coupled plasma source, which converts the atoms of the elements within a sample into gaseous ions. These ions are then separated and detected by mass spectroscopy due to their mass-to-charge ratio. The multielemental capability of ICP-MS for the analysis of biological samples makes this an ideal supplementary technique for quantifying the trace elements identified by XRF imaging. ICP-MS is capable of detecting metals and several nonmetals at concentrations as low as one part per trillion [41].

ROLE OF TRACE ELEMENTS IN FEMALE REPRODUCTIVE FUNCTION

Over the last four decades, countless studies have been conducted on the role of dietary trace elements on reproductive function; however, a review of the literature quickly highlights how heavily the research has been weighted toward male compared to female reproductive function. Studies with a great deal of evidence on the impact of micronutrients, including trace elements, on female fertility are rare [42]. Although there are many human and animal studies focusing on the dietary intake of trace elements during pregnancy, and the resultant effects on pregnancy outcome, very few studies take into account the preconception period, or focus on general

TABLE 2. Trace elements and proposed effect on ovarian function.

Element	Bromine	Iron	Selenium	Zinc
Source	Soils, anthropogenic release	Food/supplements	Food/supplements	Food/supplements
Proposed mechanism/ function	Halogen replacement BDBP & PBBs, collagen formation	Cell growth/function, LH & FSH secretion	↓ROS/oxidative stress	↓ROS/oxidative stress
Deficiency	↓ Tissue integrity[56]	Infertility [102], anovulation [193], hypoxia (?)	Unexplained infertility [194], POF (?)	↓ Oocyte maturation anovulation [195]
Toxicity	Transgenerational infertility [78]	Infertility [103, 104] iron-induced oxidative stress (?)	n/a	n/a

BDBP, brominated disinfection by-product; n/a: information not available; POF, premature ovarian failure; (?), putative mechanism.

reproductive health when it comes to ovarian function, including hormone synthesis and follicular development.

In the late 1990s, Xu et al. hypothesized that a disorder in the metabolism of trace elements may be one of the important factors causing unexplained infertility [43]. Recent studies, which are reviewed elsewhere [44], have reported that changes in the levels of the trace elements, Fe, Zn, and Ca, play important roles in female infertility, but there is still debate about the relationship between Fe and unexplained infertility [44]. A better understanding of the role of trace elements in the underlying mechanisms in infertility, as well as more rigorous studies clarifying the effectiveness of nutritional factors, are needed to improve diagnosis and treatment [45]. Despite the paucity of studies, it is clear that there is a strong relationship between nutritional status of trace elements prior to conception and successful conception and healthy progression of pregnancy [46].

In order to gain a better understanding of the role of trace elements in the ovary, we recently undertook quantitative XRF imaging of bovine ovaries and conducted ICP-MS and XAS analyses [35, 36, 47, 48]. In particular, we obtained data on bromine (Br), Fe, Se, and Zn. These trace elements and their potential roles in the ovary form the basis of this review. However, some ovaries showed evidence of other trace elements, including Cu, cobalt, chromium, manganese, nickel, and titanium, but these were not in particularly high concentrations, nor were they found in a significant portion of samples (<10%), and so these elements were not examined further. In the following sections, we discuss what is known about Br, Fe, Se, and Zn in general and in reproduction, and, in particular, in the ovary. Table 2 summarizes the proposed effects of these trace elements on female fertility and ovarian function. Table 2 also includes proposed mechanisms associated with deficiency, and where possible toxicity of these trace elements is associated with ovarian function.

BROMINE

Br is one of the most abundant and ubiquitous trace elements in the biosphere, but, until recently, had not been conclusively shown to perform any essential function in plants, micro-organisms, or animals [2]. Up until the mid-1980s, only weak evidence existed to support the view that Br was essential, with one of the key findings being that bromide ($Br^-_{[aq]}$) could substitute for part of the chloride (Cl^-) requirement for chickens [49]. In 1990, one additional study reported that, when compared to goats fed a 20-mg Br/kg diet, goats fed a 0.8-mg Br/kg diet exhibited depressed growth and fertility, increased abortions, and reduced life expectancy [50]. In addition, microscopic examination of tissues and organs of Br-deficient goats showed abnormalities in tissue sections of the thyroid, heart, lungs, pancreas, and ovaries [51]. In 1998, it was suggested that, although these additional studies gave more credibility to the concept of Br essentiality, the findings were still regarded as too limited [52]. The evidence provided little insight into a possible biochemical role for this element, beyond saying that it may serve as an electrolyte [52]. This led to the general assumption that the biological behavior of Br was similar to chlorine (Cl) in that the administration of it results in some displacement of body Cl^-, and vice versa [53]. This assumption has since been found to be invalid for the thyroid gland. In studies on the interaction of Br with I in the rat thyroid, under the conditions of enhanced Br intake, Pavelka et al. found that, contrary to other tissues, $Br^-_{(aq)}$ replaced iodide (I^-) as opposed to Cl^- [54, 55]. As previously mentioned, a recent study demonstrated that Br is required as a cofactor for peroxidasin-catalyzed formation of sulfilimine crosslinks in *Drosophila* [56]. This process is involved in cross-linking the noncollagenous domains of collagens type IV, which are the collagen components of basal laminas found in many tissues. Br dietary deficiency led to physiologic dysfunction in *Drosophila,* while repletion of this trace element reversed the dysfunction, a key criterion for justifying essentiality [56].

Biological Activity of Brominated Species

Potential biological roles of brominated species have been under scrutiny for the past 13 yr, with several authors making important contributions to this area of knowledge. Hawkins et al. conducted an experiment to show that hypochlorous acid (HOCl) and hypobromous acid (HOBr) reacted with different selectivity with cellular targets, and that the resultant radical formation may result in cell lysis [57]. The reactivity of HOBr with biological molecules was not well characterized, but amino acids and proteins appear to be major targets, with the species reacting with fatty acid side chains and lipid-soluble antioxidants to a much greater extent than HOCl [58–60]. This species has also been reported to induce red blood cell lysis approximately 10 times more rapidly than HOCl [57]. In a later study to ascertain the rate constants for reactions of HOBr with protein components, most residues reacted 30- to 100-fold faster with this species than with the Cl^--containing acid [58]. More recently, the contribution of HOBr to optimal and efficient microbial killing has been recognized as essential [61]. Marcinkiewicz et al. [62] investigated the role of another brominating oxidant, taurine bromamine, and found that this species exerted strong bactericidal effects on *Escherichia coli*. Maines et al. [63] investigated the cytotoxic effects of activated Br on human fetal osteoblasts in vitro and showed that sodium

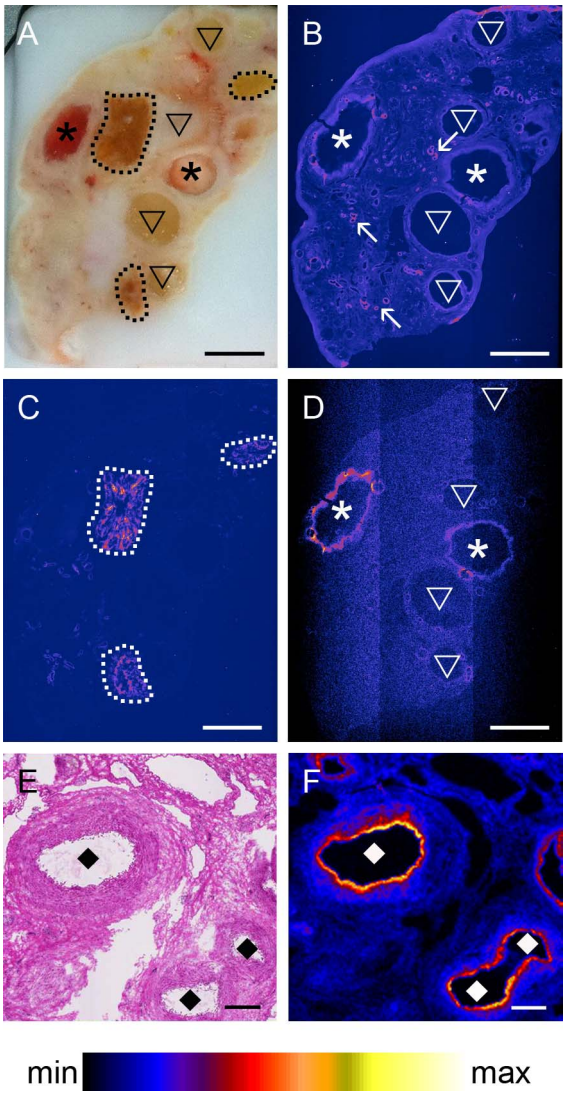

FIG. 3. Distribution of Zn, Fe, Se, and Br in bovine ovaries, as imaged by XRF. Comparison with the adjacent fresh frozen tissue (**A**) indicates that regions of high Zn (**B**, pink) intensity correspond to arterioles and capillaries; high Fe (**C**, pink) correspond to corpora lutea; and high Se (**D**, pink) is localized to the granulosa cell layers of the two largest healthy follicles. **E**) H&E-stained section of a bovine ovary containing arterioles alongside Br elemental distribution map (**F**) generated by XRF imaging. ▽, atretic follicles; *, healthy antral follicles; ----, corpus luteum or regressed follicles; ↑, capillaries; ♦, arterioles. Scale bars = 4 mm (**A**–**D**) and 200 μm (**E** and **F**). Reprinted from Ceko et al. Metallomics 2015; 7:71–82 and Ceko et al. Metallomics, 2015; 7:756–765 [48] and reproduced by permission from The Royal Society of Chemistry.

bromide was more cytotoxic than either sodium hypochlorite or activated sodium hypobromite. Thus, it appears that there is a range of chemical modifications induced by $Br^-_{(aq)}$-based species, many of which are still in the process of being fully elucidated.

Br Byproducts and Reproduction

In nature, Br is found mostly as Br^-, bound to metals in the form of inorganic salts [64]. The main natural source of Br^-(aq) is in sea water, where the average concentration is several orders of magnitude larger than in freshwater systems [65]; however, it is also found naturally in soils [66] and a range of plant- and animal-based foods [65]. Major contributors to the anthropogenic release of Br into the environment include mining, industrial emissions, disinfectants, flame retardants, and the use of fertilizers and pesticides in agriculture [65]. These processes can significantly increase the concentration of Br in the environment. With such prevalent exposure to Br, coming from natural and anthropogenic sources, it is not surprising that numerous studies have been conducted on the impact of chronic Br exposure on mammalian systems. Throughout the 1980s, several authors confirmed that large doses of Br^- could reduce fertility, impact the rate of survival of offspring, and result in depressed production of thyroxine, because excess $Br^-_{(aq)}$ competes with I^- in the synthesis of this hormone [67–69]. Flury et al. [65] suggested that chronic Br toxicity affects mainly the endocrine and reproductive systems of mammals, and Pavelka [64] further suggested that high $Br^-_{(aq)}$ levels can influence iodine metabolism by either decreasing the iodine accumulation in the thyroid and skin and/or by increasing the excretion of iodine by the kidneys. More recent studies have focused less on $Br^-_{(aq)}$ and more on the potential toxicology of brominated disinfection by-products. Several authors focusing on dibromoacetic acid have concluded that chronic exposure to even low levels of this chemical (1 mg/kg body weight/day) adversely affects reproductive function in male rabbits, and delays pubertal development and compromises sperm quality in rats [70–72].

In humans, most of the research investigating the effects of Br is centered around the possible toxic effects of brominated compounds and residues in food and water supplies [73, 74]. As a member of the halogen family, the propensity for substitution with other halogens (chlorine and iodine in particular) and the advent of newer technologies that identify and quantify the bioaccumulation of Br in tissue, more research is examining the toxic effects of Br exposure on pregnancy outcome. However, to date, there are inconsistent associations with spontaneous abortion, preterm birth, small-for-gestational-age babies, and birth defects [73]. In a study spanning 3 decades, Small et al. [75] investigated the effects of exposure of brominated flame retardants and polybrominated biphenyls (PBBs), a class of halogenated, brominated flame retardants, during pregnancy on the fertility and reproductive outcomes of offspring more than 20 yr after exposure. Of 194 women that were exposed to PBBs while in utero, there was a decrease in fertility and an increased risk of spontaneous abortions with increasing risk associated with greater exposure to PBBs [75]. The finding of an increase in spontaneous abortions suggests that the in utero exposure on reproductive outcomes is likely due to direct exposure to the fetal ovaries at the time when early follicular development is initiated. A hypothesis to account for the findings includes the effect that PBBs have on estrogen activity and progesterone production in in vitro models and animal studies [75]. Concurrent exposure to PBBs in pregnancy has led to spontaneous abortions in cattle [76, 77], but not in humans [75].

To our knowledge, our group was the first to identify and document the presence and nature of distribution of Br in the mammalian ovary [35]. Qualitative observations of the XANES region of the spectra coupled with principal component analyses of all XAS data led us to conclude that the predominant form of Br was aqueous Br^-. Using S-XRF, Br was identified in high concentrations in all sections from 45 bovine ovaries and was widely distributed throughout the ovarian tissue, but heavily concentrated in the walls of arterioles (Fig. 3F). The only known biological function of Br is for cross-linking of type IV collagen [56], which is predominantly a basal lamina component. Cross-linking of subunits would presumably increase the tensile strength of

basal laminas. The basal laminas of subendothelial cells of arterioles, venules, and capillaries and the basal laminas of smooth muscle cells of arterioles in bovine ovaries contain type IV collagens α1 and α2 [78]. Cross-linking of both type IV collagens α1 and α2 has been observed previously in bovine follicles [78]. That Br would be heavily concentrated in the walls of arterioles would suggest that its function is to facilitate substantial cross-linking of type IV collagens in the subendothelial basal laminas and that of the smooth muscles of arterial walls to withstand the high intra-arteriole blood pressures.

In our study [48], we observed tenfold higher concentrations of Br in bovine serum versus normal human serum concentrations, which raises some important questions regarding the dietary and environmental exposure to Br for the cattle. Due to previously determined toxic nature of Br, the origin and source of Br requires further investigation to determine whether it is from anthropogenic or natural sources.

IRON

Fe has the longest and best-described history among all the trace elements; however, it was not until 1932 that the importance of Fe was finally confirmed with the evidence that inorganic Fe was needed for hemoglobin synthesis [79]. It is a key element in the metabolism of almost all living organisms, as it is involved in central cellular processes, including DNA synthesis, respiration, and oxygen transport. The biological functioning of Fe depends upon its ability to readily accept or donate electrons, interconverting between Fe^{3+} and Fe^{2+} forms, as it does in enzymes, such as cytochromes [80]. In the human body, Fe is an essential component of hundreds of proteins and enzymes [7], where it mainly exists in complex forms bound to heme protein (hemoglobin or myoglobin), as heme compounds, as heme enzymes, or as nonheme compounds (flavin-Fe enzymes, transferrin, and ferritin) [81]. In hemoglobin, Fe is in the Fe^{2+} form, whereas Fe transported on transferrin in the blood, or stored intracellularly in ferritin, is in the Fe^{3+} form [80]. An understanding is emerging on how these proteins, both individually and in unison, maintain cellular and whole-body homeostasis of this crucial trace element [82]. Fe hemostasis is a complex process, involving many proteins that respond not only to Fe load (intake and storage), but also to stimuli including inflammation, hypoxia, and anemia [83].

Although low Fe intake and its poor bioavailability are responsible for most anemia in industrialized countries, this accounts for only about half of the causes of anemia in developing countries [84]. In these regions, infectious and inflammatory diseases, such as malaria, blood loss caused by parasitic infections, and nutrient deficiencies, including those for vitamins A, B_2, B_9, and B_{12}, are also important causes [85]. The role of Fe deficiency in detrimentally influencing pregnancy outcomes is well established, and governments and researchers have focused a significant amount of attention on this aspect [86–91]. There has been little research, however, into the relationship between Fe deficiency and unexplained infertility [92].

Metabolism of Fe

The fraction of Fe absorbed relative to the amount ingested is typically quoted in the range of 5%–35%, depending on the combination of foods ingested and the oxidation state of Fe [81]. Fe absorption predominantly takes place in the duodenum and upper jejunum [93], and occurs by the enterocytes by divalent metal transporter 1, a member of the solute carrier group of membrane transport proteins. From here, it is transferred across the duodenal mucosa into the blood, where it is transported by transferrin to the cells or the bone marrow for red blood cell production [94, 95]. Fe uptake, storage, and export by cells are tightly regulated by Fe regulatory proteins (IRPs) [96]. IRPs are cytosolic *trans* regulators that bind to specific RNA Fe-responsive elements that are present within the mRNA encoding transferrin receptors and ferritin, thus controlling the intracellular flux of Fe [97].

Dietary Fe occurs in both heme and nonheme forms [98]. Primary sources of heme Fe are hemoglobin and myoglobin from consumption of red meat, poultry, and fish, whereas nonheme Fe is obtained from cereals, pulses, legumes, fruits, and vegetables [99]. The difference in bioavailability of the two Fe forms (15%–35% and 2%–20% for heme and non-heme, respectively), in addition to the fact that dietary factors have little effect on absorption of heme Fe, make red meats an excellent nutrient source of this trace element [98]. Major inhibitors of Fe absorption are phytic acid, polyphenols, calcium, and peptides from partially digested proteins [98]. Enhancers include ascorbic acid and muscle tissue, which may reduce Fe^{3+} to Fe^{2+} and bind it in soluble complexes, which are available for absorption [98].

Fe and Reproduction

As previously mentioned, the effect on pregnancy of Fe deficiency in the mother and, more specifically, its negative impact on fetal growth and development, has been the focus of many studies [100]. Pregnancy increases the Fe requirement to nearly 6 mg/day by the second and third trimesters, due to the high growth rates of the placenta and the fetus, and the expansion of the maternal red blood cell mass [101]. Few studies have been published on the effect of Fe on female fertility; however, the preliminary consensus is that a greater intake of Fe, either due to increased dietary intake or supplementation, increases fertility levels in women, with the comment that the utilization by the ovary of this element is the likely reason for this observation [42]. In a large study involving 18 555 women, Chavarro et al. [102] evaluated whether Fe supplement use or greater intake of total heme and nonheme Fe is associated with lower risk of ovulatory infertility. Women who consumed Fe in supplements had a significantly lower risk of ovulatory infertility than women who did not use Fe. More specifically, total nonheme Fe intake, consumed as multivitamins and Fe supplements, was inversely associated with the risk of ovulatory infertility, whereas heme Fe intake was unrelated to ovulatory infertility in multivariable adjusted analyses [102]. A recently published study to ascertain the effects of severe Fe deficiency on fertility in female rats found that there was a significantly lower conception rate and a disruption of estrus in an Fe-deficient group relative to the controls [92]. These results suggest that the importance of Fe in female reproductive function begins well before pregnancy.

There are few studies investigating the effect of Fe overload (toxicity) and fertility, and even less with regard to female fertility. However, there is a link between Fe overload and female infertility [103, 104]. Excess Fe leads to reduced production of the hormones LH and FSH from the anterior pituitary, suggesting impaired oocyte maturation and low ovarian reserve [103]. In patients with beta thalassemia, multiple blood transfusions and increased gastrointestinal Fe absorption leads to Fe overload in the body [105] and infertility [103], likewise in patients with hemochromatosis, a genetic condition that leads to increased serum Fe levels and also subfertility in women [104].

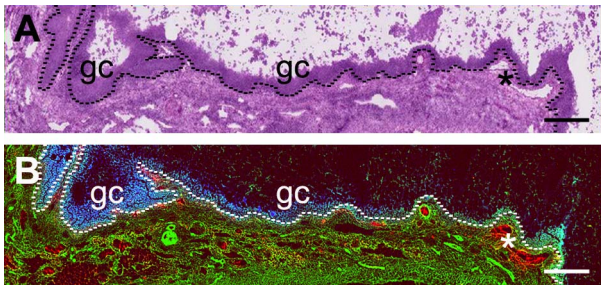

FIG. 4. Localization of Zn, Fe, and Se in a large bovine antral follicle. (**a**) H&E-stained serial section of a 15-mm-diameter healthy follicle. (**b**) The corresponding RGB image was generated from XRF elemental distribution maps and depicts the distribution of Zn (green), Fe (red), and Se (blue). *, vasculature; - - - - -, the separation between granulosa layer and the thecal interna; gc, granulosa cells. Scale bar = 500 μm. Reprinted from Ceko et al. Metallomics 2015; 7:71–82 [47] and reproduced by permission from The Royal Society of Chemistry.

Using S-XRF, we were able to determine the precise location and levels of Fe in bovine ovarian sections [35, 36]. Bioaccumulation of Fe in the ovary is elevated in corpora lutea: low at early stages of development and high in mature corpora lutea. This would be consistent with Fe as a major component of cytochrome P450 cholesterol side-chain cleavage, which is elevated in mature bovine corpora lutea [106], and with the Fe in hemoglobin in the highly vascularized corpora lutea [76]. Fe in capillaries of follicles, likely due to hemoglobin, can be seen in Figure 4. Regressing corpora lutea and regressed follicles (Fig. 3C) are also high in Fe, which probably represents hemosiderin derived from the dead cells that contained the steroidogenic cytochrome P450 enzymes. Fe levels are also elevated in the walls of arterioles, and these levels are five times that of the levels within the lumen of the arterioles [36]. At least one other study has reported high Fe concentrations in blood vessels and in smooth muscle cells imaged by XRF [77]. They hypothesized that the elevated levels are owing to the presence of Fe in smooth muscle myoglobin. In addition to a clear higher density of Fe in the compartments discussed above, Fe is crucial for many basic processes in all ovarian cells, including respiration, DNA synthesis, antioxidant activities, and steroidogenesis, which is representative in the more dispersive distribution shown in Figure 4.

SELENIUM

The trace element Se was discovered in 1817 by the Swedish physician and chemist Berzelius; however, its essentiality in mammals was not discovered until the 1950s [107]. Conclusive evidence for the essentiality of Se in humans came with publication of the results of large-scale trials in China that showed the protective effect of Se supplementation on children and young adults suffering from Keshan disease, a cardiomyopathy endemic in regions with low soil Se levels [108]. Whole-body Se is about 15 mg, as estimated by direct tissue analysis and radioisotope techniques, with the tissue concentrations of this essential trace element being highest in the kidney and liver [109]. The most important biologically active compounds contain selenocysteine, where Se is substituted for sulfur in cysteine. The insertion of selenocysteine to form a selenoprotein is specified by the UGA codon in mRNA under specific conditions [110, 111].

In humans, the nutritional functions of Se are achieved by its incorporation into 25 selenoproteins (including the glutathione peroxidases [GPXs], thioredoxin reductases and deiodinases) that have selenocysteine at their active center [110]. These selenoproteins play an important role in many biological functions, such as antioxidant defense, formation of thyroid hormones, DNA synthesis, fertility, and reproduction [112]. By working synergistically with vitamin E, GPXs play an important role in the body, catalyzing the conversion of peroxides to nontoxic alcohols and thus protecting cells from membrane damage and oxidative stress [113, 114]. The concentrations of Se in whole blood and in plasma and/or serum are related to dietary intake, with 50%– 60% of the total plasma Se being present as selenoprotein P [108, 109], about 30% being present as GPX3, and the remainder incorporated into albumin as selenomethionine [115]. Low serum Se levels have been linked to higher cancer risk based on evidence from observational studies conducted over a 40-yr period (see reviews [116–119]. Thus, adequate Se intake may have an anticancer effect, due to its contribution to antioxidant function or to immune system function, but the precise mechanism is uncertain. Weekley and Harris [117] highlight that epidemiological studies have reported an inverse relationship between Se status and the incidence of various diseases, but subsequent studies of Se supplementation and disease prevention have presented mixed results, with concerns about this trace element's toxicity [117].

Bioavailability and Absorption of Se

The Se content of food is dependent upon the Se content of the soil in which the plants are grown or the animals are raised. Se is found in highest amounts in organ meats, such as kidney and liver, while some seafood contains nearly as much [120]. That said, plant foods, such as grains, legumes, and cruciferous vegetables, are a major dietary sources of Se in most countries, where it enters the food chain predominantly as selenomethionine [109, 121]. Se, which is initially taken up from the soil and concentrated by plants, is absorbed in the small intestine and incorporated into proteins by complex mechanisms that remain to be fully elucidated [122]. Preintestinal absorption of Se is negligible, so the absorption operates mainly in the duodenum and caecum [112]. Similar to Fe, the mechanisms of intestinal absorption are different depending on the chemical form of the element. A recent study designed to investigate the bioavailability of four Se species using an in vitro model of the intestinal barrier determined that the efficiency of Se absorption decreased in the following order: selenomethionine, methylselenocysteine, selenate (SeO_4^{2-}), and selenite (SeO_3^{2-}) [123]. An earlier report presented by the U.S. Institute of Medicine, however, summarized that Se from the inorganic salts, SeO_4^{2-} and SeO_3^{2-}, is more rapidly incorporated into GPX and other selenoproteins than Se from organic sources containing selenomethioine. SeO_3^{2-} is absorbed by simple diffusion, and its subsequent reduction is a well-characterized metabolic pathway in animals [124]. The proportion of ingested Se excreted is dependent upon dietary intake, with high consumption resulting in high urinary and fecal excretion and vice versa [125, 126]. In cases of dietary Se deprivation, the synthesis of some selenoproteins (e.g., GPX4) is prioritized over that of others [111]. Conversely, GPX1 is one of the most highly sensitive selenoproteins to changes in Se status, with levels of mRNA and protein dramatically reduced under low Se conditions [127].

Se and Reproduction

Se has long been recognized in animal husbandry as being essential for successful reproduction [2]. Of particular importance to reproduction and pregnancy are the GPXs,

which play a crucial role in reducing hydrogen peroxide (H_2O_2) and lipid peroxides to harmless products, thereby reducing cellular damage by reactive oxygen species (ROS) [128]. The emerging role of Se in maintaining healthy reproductive function has been extensively studied in males, but data pertaining to Se status and fertility in females are sparse, with the majority of female-based studies having a tendency to focus on the role of this element in pregnancy [129–133].

Over 30 yr ago, Behne et al. conducted several studies in male rats and observed that, during insufficient Se intake, the supply of this trace element to the testes was prioritized over other tissues. This suite of studies led the authors to postulate the involvement of Se in the biosynthesis of testosterone [134, 135], and it is now commonly known that testicular tissue contains high concentrations of Se, predominantly as GPX4, and that this element is essential for the formation and normal development of spermatozoa [136, 137]. Some evidence suggests that increasing Se dietary intake increases antioxidant GPX activity, thereby increasing male fertility [138]. In a study of subfertile Norwegian men, the Se concentration of seminal plasma correlated positively with the concentration of spermatozoa [139]. Scott et al. [140] supplemented subfertile men with 100 μg of Se per day for 3 mo and, in addition to their sperm motility significantly increasing, 11% of men receiving the active supplement fathered a child compared with none in the placebo group. It should be noted, however, that a similar study carried out over a similar length of time, but with double the daily dose of Se, showed no beneficial effect on sperm motility in subfertile Polish men [141]. Closer observation of the form of Se administered in each of these studies indicates that they differed, with the first being selenomethionine versus SeO_3^{2-} in the latter Polish study. This highlights that it is not necessarily the quantity of the trace element ingested that matters with regard to exerting a beneficial biological effect, but more so its bioavailability and subsequent absorption by the body.

With regard to Se status and fertility in females, Paszkowski et al. [142] completed a study of 135 follicular fluid samples collected from patients during transvaginal oocyte retrieval, and found that patients with unexplained infertility had significantly decreased follicular Se concentrations compared with those with other known causes of infertility [142]. A case-controlled study from Turkey found lower Se concentrations in the serum and follicular fluid of women undergoing in vitro fertilization treatment compared with age-matched, nonpregnant control women [143], and a recent study, also from Turkey, found that plasma Se levels were significantly lower in women with polycystic ovary syndrome relative to the control group, indicating that this element may play a role in the pathogenesis of polycystic ovary syndrome related to hyperandrogenism [144]. Finally, women presenting with unexplained infertility or premature ovarian failure were found to have significantly increased serum levels of the ovarian autoantibody protein, Se-binding protein-1 [145]. Ultimately, Se plays a significant role in living organisms, and owing to its contribution to the formation of selenoproteins combined with its antioxidant action, a low Se status in the body induces a low resistance to free radical damage [112].

Our investigations into the bioaccumulation of Se in ovarian tissue using S-XRF demonstrated that Se was consistently localized to the granulosa cell layer of large, healthy follicles (Fig. 4) [36]. In addition, the increase in Se in these follicles was likely due to the increased expression of selenoprotein *GPX1*, which was significantly upregulated compared to small healthy or atretic follicles. Western blot analyses also confirmed a strong association between GPX1 and large, healthy follicles, suggesting a role for this antioxidant in follicle dominance, protecting the dominant follicle from increasing levels of ROS [47]. Furthermore, an additional study using human cumulus cells derived from cumulus-oocyte complexes collected for both in vitro fertilization and intracytoplasmic sperm injection were assessed using RT-PCR for *GPX1* expression before fertilization [47]. These cumulus cells retrieved from cumulus-oocyte complexes prior to embryo transfers that resulted in a pregnancy ($n = 12$) had significantly higher expression of *GPX1* compared with those that did not result in a pregnancy ($n = 18$) [47]. Notwithstanding the fact that much further research is needed, these observations do suggest a strong role for selenoprotein GPX1 in determining follicle growth, maturation, and dominance in both the cow and in women, and lead to further specific dietary recommendations for Se and the role oxidative stress may play in infertility.

ZINC

Zn is an essential element in the nutrition of human beings, other animal species, and plants, and is required in every cell [7]. Zn plays an important role in growth and development, the immune response, neurological function, and reproduction [146, 147]. Zn is needed for DNA and RNA synthesis and is required at every step of the cell cycle, including proliferation, differentiation, and apoptosis [147]. Zn plays an important role in the structure of proteins and cell membranes, and is an integral component of over 300 metalloenzymes, including carbonic anhydrase, alcohol dehydrogenase, thymidine kinase carboxypeptidase, glutamate dehydrogenase, lactate dehydrogenase, and alkaline phosphatase [5, 148]. Zn additionally participates in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids [3]. Furthermore, Zn finger proteins, formed when Zn atoms become tetrahedrally coordinated with histidine and cysteine [149], regulate the expression of many genes [150, 151], and Zn has been shown to have antiapoptotic and antioxidant properties [152–154]. Inadequate intake of this trace element in humans and other animal species results in immunodeficiency, increased numbers of infections, increased severity of infections, stunted growth, and delayed sexual maturation, as summarized by Qureshi et al. [7].

Bioavailability and Absorption of Zinc

Zn is found as organic complexes with protein in meats and as inorganic salts in plant foods [155, 156]; however, no absorption studies have been conducted in humans to determine whether these forms of Zn differ in their bioavailability [157]. Based on limited published data on the absorption of Zn from supplements ingested by humans, it appears that Zn gluconate, Zn citrate, and Zn sulfate are absorbed to a similar extent, and that Zn oxide is slightly less well absorbed [158, 159]. A recent study in rats, however, found all four of these species to be equally bioavailable [160]. Similar to the aforementioned trace elements (Cu, Fe, and Se), inadequate dietary intake of Zn can arise from low intake, poor bioavailability, or a combination of these dietary factors. Low dietary Zn intake is often associated with developing countries, owing to the prohibitive cost of Zn-rich foods (i.e., meat, poultry, and fish) or religious restrictions [161]. The total Zn content of one's diet is influenced, not only by the range of food items selected and their relative bioavailability, but also by the degree of refinement of any constituent cereals. In

addition, a diet rich in saturated or unsaturated fats tends to dilute the uptake of Zn [3].

Zn is absorbed in the small intestine by a carrier-mediated mechanism [162], with ongoing research over the last decade indicating that its transport across membranes is mediated by two subfamilies of mammalian Zn transporters, ZnT (Slc30) and Zip (Slc39) [163–166]. Following absorption through the enterocyte, dietary Zn is transported into the circulation and then to the tissues, where it is needed. Active transport of Zn into portal blood is mediated by metallothionein [167]. Zn is released from food as free ions during digestion and the liberated ions may then bind to endogenously secreted ligands before their transport into the enterocytes in the duodenum and jejunum [16, 168]. In contrast to Fe, the body regulates Zn homeostasis through gastrointestinal secretion and excretion of endogenous Zn, in addition to the absorption of exogenous Zn [169, 170]. Loss of Zn through the gastrointestinal tract accounts for approximately half of all Zn eliminated from the body, with pancreatic and biliary pathways being other modes of excretion. This is an important process in the regulation of Zn balance [171], and similar to Cu and Se, with higher Zn intake balanced by more secretion and vice versa [172].

As alluded to earlier, plant-based foods are the major source of dietary Zn in many low-income countries. Of the plant-based foods, cereals and legumes contain high levels of phytic acid and their magnesium, calcium, and potassium-associated salts, called phytates. While amino acids and peptides in chyme facilitate the absorption of Zn, the binding of Zn by phytate and dietary fibers forms insoluble complexes, rendering the Zn relatively unavailable [173, 174].

Zinc and Reproduction

Countless studies have focused on the role of Zn in male fertility, paying particular attention to its role in male sex hormone synthesis, as well as the resultant impact on sperm production and motility. Zn content is high in the adult testis, and the prostate has a higher concentration of Zn than any other organ of the body [1]. Zn deficiency first impairs angiotensin-converting enzyme activity, which leads to depletion of testosterone and inhibition of spermatogenesis. Defects in spermatozoa are frequently observed in Zn-deficient rats [175, 176]. In females, Zn also seems to be important in reproduction, but relatively few investigations have been performed [45]. Shaw et al. [177] found that Zn deficiency in female rabbits resulted in sexual disinterest by their male counterparts. Another study suggested that Zn deficiency in female rats led to abnormal estrous cycles [178], and the effects of Zn deficiency in two species of monkeys led the authors to conclude that normal reproduction was impaired in both the species through abnormal ovarian development [179]. Over 30 yr ago, Sato et al. [180] reported that even marginal Zn deficiency could affect oocyte maturation by doubling the number of degenerating oocytes and increasing chromosomal abnormalities in metaphase II oocytes. Despite the authors suggesting that preconception was a crucial time for women to ensure a sufficient intake of Zn, it would appear that attention in subsequent decades has shifted to Fe, folate, and, in more recent years, iodine supplementation [181–186]. In studies designed to ascertain the reproductive effects of feeding virgin female mice a Zn-deficient diet, Taneja and Kaur observed retardation of ovarian follicular growth with varying degrees of atresia, lack of preovulatory follicles, a reduced and shrunken corpus luteum, and a fragmented zona pellucida and vitelline membrane, indicating the cessation of oogenesis and ovulation [187, 188].

Kim et al. investigated the abundance of Zn throughout the ovarian cycle in mice using S-XRF [189]. Interestingly, during the period of meiotic maturation, between the LH surge and ovulation, there was a 50% increase in total Zn within the ovary [189]. Moreover, within the unfertilized egg, there is a polar distribution of Zn, which the authors suggest are Zn-enriched cortical granules that may play a role in the hardening of the zona pellucida required to block polyspermy at fertilization [189]. In a more recent study, Tian et al. [190] demonstrated that Zn deficiency in mice before fertilization led to reductions in placental and fetal development, highlighting the role of trace elements and the dependency that the embryo has on the integrity of the oocyte predecessor. Taken together, these studies demonstrate that Zn has a vital role in ovarian function, and cytoplasmic levels within the oocyte are crucial for early embryo and placental development [189, 190].

Similar to the alleged role of Se in reproduction, Zn is important for several antioxidant functions that provide protection of cells against oxidative and electrophilic stress caused by ROS. Many investigations have shown evidence for the role of ROS in the physiology and pathology of both male and female reproductive functions [191, 192], suggesting that the focus should shift to elucidating the biochemical roles these trace metals play in the process.

Our investigations using S-XRF into the accumulation of Zn in bovine ovarian tissue indicate that the highest Zn levels always corresponded to either the walls of arterioles or to capillaries (Figs. 3 and 4), and was associated with above-background Fe concentrations [36]. Although at lower levels than blood vessels, Zn was also elevated in the thecal and granulosa cell layers relative to the stromal tissue. Healthy follicles contain an average of three times more Zn than the regressed follicles. Using principal component analyses, a relationship was found to exist between levels of Br and Zn, showing that the healthy and early atretic follicles are different from atretic and regressed follicles, suggesting that the roles of these elements in healthy follicles may differ between growth and atresia [35].

FUTURE DIRECTIONS

The recent new capabilities to both localize and quantitate trace elements in tissues will allow us to identify where and when trace elements accumulate in reproductive tissues during their development and in disease states. Just as the recent studies in the ovary have demonstrated, this may lead to discoveries of new roles played by these elements in reproduction.

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