The effect of micronutrients in oxidative stress, proliferation and apoptosis in placenta

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- All researchers who never give up

- All healthcare providers who are passionate about improving people's health

- All mothers who give life to their little creations

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Habibi N, Leemaqz SYL, Jankovic-Karasoulos T, Grieger JA, McCullough D, Wilson RL, St. John JC, Roberts CT, Bianco-Miotto T. Reduced buffy coat mitochondrial DNA content is associated with a greater risk of pregnancy complications. Target journal: International Journal of Molecular Sciences. (To be submitted)

Chapter 1

General Introduction

"The only way to do great work is to love what you do."
Steve Jobs

Thesis Introduction

A healthy pregnancy plays an essential role in fetal growth and development as well as future health. Due to the major biochemical, physiological and anatomical alterations that occur to the body during pregnancy, a woman's future health is impacted by her pregnancy outcome. Adverse pregnancy outcomes include preeclampsia, miscarriage, spontaneous preterm birth, gestational diabetes mellitus, intrauterine growth restriction and preterm premature rupture of the membranes, all of which are associated with an increased mortality and morbidity rate for both mother and offspring. Therefore, it is of high importance to investigate factors that contribute to adverse pregnancy outcomes.

The placenta, which is a multifunctional bridge between mother and fetus, has a dynamic system and its development during pregnancy is pivotal for pregnancy success. Therefore, factors that may impact placental structure and function can contribute to pregnancy outcome. Too much free radicals such as reactive oxygen species can cause oxidative stress when there is not enough antioxidants to neutralise them. Placental maldevelopment and oxidative stress contribute to adverse pregnancy outcomes. The mechanisms that underlie how oxidative stress may result in poor placental development require further investigation, particularly the role of maternal nutrition in oxidative stress and pregnancy outcome.

Mechanistically there is little evidence defining how maternal micronutrient intake can impact placental development and pregnancy outcome. However, poor maternal micronutrient status may be associated with increased oxidative stress and inflammation. Selenium and iodine are two essential micronutrients that are vital for a healthy pregnancy and childbirth. A deficiency of selenium or iodine can cause health issues such as impaired executive function, thyroid dysfunction, reduced fetal growth and insufficient neurodevelopment in offspring and is associated with a higher risk of pregnancy

complications. A plethora of research on the thyroid has demonstrated that selenium and iodine contribute to oxidative stress but their potential synergistic effect within the placenta and in relation to oxidative stress has not been investigated.

Copper is an essential micronutrient with a complex association with free radicals metabolism. Determination of how it may affect the placenta and its role in oxidative stress may provide additional knowledge to understand its association with pregnancy complications in population studies.

Fetal and maternal new tissue synthesis during pregnancy needs higher energy and nutrients. In humans, the mitochondria metabolises macronutrients to provide energy to the cell with the highest efficiency. Micronutrients are involved in mitochondrial electron transport chain and their deficiency may result in mitochondrial dysfunction in producing energy. Mitochondria contain DNA and electron respiratory chain. Mitochondrial electron respiratory chain is a source of electron leakage and oxidative stress. Changes to the electron respiratory chain and mitochondrial DNA copy number have been shown in some oxidative stress related diseases. The question that arises is how mitochondrial DNA content may change in relation to maternal micronutrient status and if pregnancy outcomes are associated with mitochondrial DNA content.

Telomere length is one of the markers used to predict later risk of non-communicable diseases and telomere length can be shortened by oxidative stress. Because maternal diet during pregnancy can impact offspring chronic disease incidence, a review that systematically summarises and discusses the literature assessing the association between maternal diet and offspring telomere length would reflect the available knowledge and future direction in this area.

Thesis Structure / Abstract

This thesis is comprised of seven chapters: a general thesis introduction (Chapter 1), one Published review of the literature (Chapter 2), one published research article (Chapter 3), one submitted research article (Chapters 4), one unpublished research chapter written in manuscript style (Chapters 5), a published systematic review (Chapter 6), and a final general discussion (Chapter 7).

Chapter 2 is a published comprehensive review of the impact of maternal selenium and iodine on placental and child health (Habibi *et al*., Nutrients 2020). A few studies showed that selenium could increase antioxidant enzyme activity in placental cell lines. Additionally, population studies revealed that selenium and iodine deficiency separately were associated with a greater risk of pregnancy complications. However, there are no studies that have investigated the potential synergistic effect of iodine and selenium combined on placental health. To address this gap in the literature, the role of these two essential micronutrients during pregnancy and in relation to oxidative stress has been comprehensively reviewed. This review supports the hypothesis that selenium and iodine could potentially have a synergistic role in protecting against oxidative stress in the placenta. We went on to test this hypothesis in Chapters 3 and 4.

Chapter 3 is a published original research study describing the effect of iodine and selenium on proliferation, viability, and oxidative stress in HTR-8/SVneo placental cells (Habibi *et al.*, Biological Trace Element Research 2020). Our study showed that oxidative stress reduces HTR-8/SVneo cells viability and increases lipid peroxidation, which is oxidative damage to the cell membrane. Interestingly, selenium and iodine supplementation separately or together could protect cells against oxidative stress. A supraphysiological concentration of selenium caused some toxic effects in cells that were not exposed to oxidative stress.

Interestingly, the same concentration in cells treated with oxidative stress was protective suggesting a higher demand of selenium in HTR-8/SVneo placental cells to combat oxidative stress. The combination of selenium and iodine provided a greater protection against oxidative stress to the cells compared to their individual supplementation suggesting a synergistic effect in HTR-8/SVneo placental cells.

Chapter 4 is original research describing how selenium and iodine can protect first trimester human placenta against oxidative stress and what the effect of copper is in the placenta (submitted to Human Reproduction on 19th August 2020). For the first time, using laser ablation inductively coupled plasma-mass spectrometry, we showed the selenium and copper distribution in the treated human first trimester placenta explants. We investigated the potential mechanism of the effect of iodine, selenium and copper on first trimester human placenta. Oxidative stress increased DNA damage and apoptosis and supplementation with iodine or selenium was protective resulting in reduced DNA damage and apoptosis in first trimester placenta explants. Supplementation with a combination of iodine and selenium provided a greater reduction in oxidative damage to DNA molecules suggesting a synergistic effect against oxidative stress in the placenta. A high concentration of copper increased DNA damage and apoptosis in the placenta in the absence of induced oxidative stress. However, this was not seen when placenta tissue explants were exposed to oxidative stress suggesting a higher consumption of copper during oxidative stress.

Chapter 5 is an unpublished original manuscript describing associations between buffy coat mitochondrial DNA content and maternal micronutrient status and pregnancy outcome (To be submitted to the International Journal of Molecular Sciences). Using 317 samples of participants of Screening for Pregnancy Endpoints (SCOPE) study in Adelaide we investigated the association between maternal mitochondrial DNA content at 15 ± 1 weeks' gestation with micronutrient status and pregnancy outcomes. We found that higher

inflammation, defined by increased C-reactive protein concentration, was associated with reduced mitochondrial DNA content. A lower mitochondrial DNA content at 15 ± 1 weeks' gestation was associated with a greater risk of pregnancy complications. There was no association between mitochondrial DNA content and micronutrient status. Mitochondrial DNA content may reflect risk of pregnancy complications but it is not a strong predictor and it was not associated with maternal micronutrient status.

Chapter 6 is a published systematic review on maternal diet and offspring telomere length (Habibi *et al.*, Nutrition Reviews 2020). Maternal nutrition may be an important determinant of offspring telomere length that is a biomarker of ageing and chronic diseases. Therefore, we systematically reviewed all available literature about the association of maternal nutrition and offspring telomere length. We found little evidence on such an essential topic. However, there were seven studies on this topic and collectively they showed that higher maternal circulating folate and 25(OH)D3 and dietary caffeine intake, were associated with longer offspring telomere length, whereas dietary intake of carbohydrate, folate, n-3 PUFA, vitamin C or sodium, was not. This systematic review highlighted the necessity for further research in this area.

Chapter 7 is the final chapter that presents a summary of my PhD work and general discussion of all findings of this project and suggests future research directions.

Chapter 2

A Review of the Potential Interaction of Selenium and Iodine

on Placental and Child Health

"The beginning of knowledge is the discovery of something we don't understand."
Albert Einstein

Statement of Authorship

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Contribution to the Paper	Contributed to designing the review, wrote the first draft and critical reviewing and revisions of the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports a comprehensive review I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10.07.2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Principal Author	Tina Bianco-Miotto		
Contribution to the Paper	Contributed to designing the review, drafting the manuscript and manuscript critical review and revision		
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Name of Principal Author	Jessica Grieger		
Contribution to the Paper	Contributed to designing the review and manuscript critical review and revision		
Signature		Date	5 August, 2020

Please cut and paste additional co-author panels here as required.

nutrients

MDPI

Review

A Review of the Potential Interaction of Selenium and Iodine on Placental and Child Health

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Abstract: A healthy pregnancy is important for the growth and development of a baby. An adverse pregnancy outcome is associated with increased chronic disease risk for the mother and offspring. An optimal diet both before and during pregnancy is essential to support the health of the mother and offspring. A key mediator of the effect of maternal nutrition factors on pregnancy outcomes is the placenta. Complicated pregnancies are characterized by increased oxidative stress in the placenta. Selenium and iodine are micronutrients that are involved in oxidative stress in placental cells. To date, there has been no comprehensive review investigating the potential synergistic effect of iodine and selenium in the placenta and how maternal deficiencies may be associated with increased oxidative stress and hence adverse pregnancy outcomes. We undertook a hypothesis-generating review on selenium and iodine, to look at how they may relate to pregnancy complications through oxidative stress. We propose how they may work together to impact pregnancy and placental health and explore how deficiencies in these micronutrients during pregnancy may impact the future health of offspring.

Keywords: iodine; micronutrients; selenium; pregnancy; placenta; oxidative stress

1. Introduction

Pregnancy is characterized by a pro-inflammatory, hyperlipidemic and hyperinsulinemic state [1]. A normal healthy pregnancy is important for the growth and development of the baby and for the lifelong health of the mother and offspring [2]. To provide a suitable condition for the developing fetus, the maternal body undergoes major biochemical, physiological and anatomic adaptations [3], which typically revert back to the condition they were in prior to pregnancy [4].

Human pregnancy is also associated with an increase in oxidative stress markers, due to increased placental mitochondrial activity and the production of reactive oxygen species, which have pronounced effects on placental function [5]. The placenta is the interface between the mother and baby. It mediates nutrient and waste exchange to support a healthy pregnancy. Factors such as poor maternal diet or obesity impact placental health and function, such as proliferation, invasion and apoptosis of trophoblast cells [6,7]. This can have a profound effect on fetal growth and development, and pregnancy success [6,7]. As such, a balance of antioxidant and oxidant status is key to a healthy placenta and hence a successful pregnancy outcome for mother and baby.

There is a clear role for an optimal diet both before and during pregnancy to support the health of the mother and offspring [8–12]. Higher dietary scores for a high-protein/fruit pattern before pregnancy, was associated with decreased likelihood of preterm birth (adjusted odds ratio; 95% confidence interval, OR: 0.31; 95% CI: 0.13, 0.72), whereas the reverse direction was apparent for the high-fat/sugar/takeaway pattern (adjusted OR: 1.54; 95% CI: 1.10, 2.15) [13]. Increasing dietary quality,

calculated by the Alternative Healthy Eating Index 10 during pregnancy, was associated with a decrease in the likelihood of delivering a small-for-gestational-age (SGA) baby [14]. Supplementation with vitamins and minerals may also have a positive effect on infant birthweight [15]. Some micronutrients, such as selenium and iodine, are essential micronutrients because the body cannot produce them and the mother needs to obtain them from her diet or from supplemental intake. Deficiencies in iodine can lead to thyroid hormone deficiency, and therefore improper neurodevelopment and mental retardation in the fetus [16]. Selenium deficiency is associated with pregnancy-induced hypertensive disorders [17], miscarriage [18], preterm birth [19], and gestational diabetes [20,21].

Importantly, the key mediator of the effect of maternal nutrition factors on pregnancy outcomes is the placenta. However, such pregnancy outcomes do not just end after pregnancy, but can have lifelong consequences for a mother and her offspring. This is depicted by the developmental origins of health and disease (DOHaD) hypothesis, which emphasizes the importance of fetal life exposure to maternal factors, such as diet, in the development of chronic diseases later in life [22]. Yet, while maternal nutrition and the placenta are important factors regarding pregnancy success, there is a paucity of information examining multiple nutrients in combination, and the potential synergistic effect they may have in pregnancy.

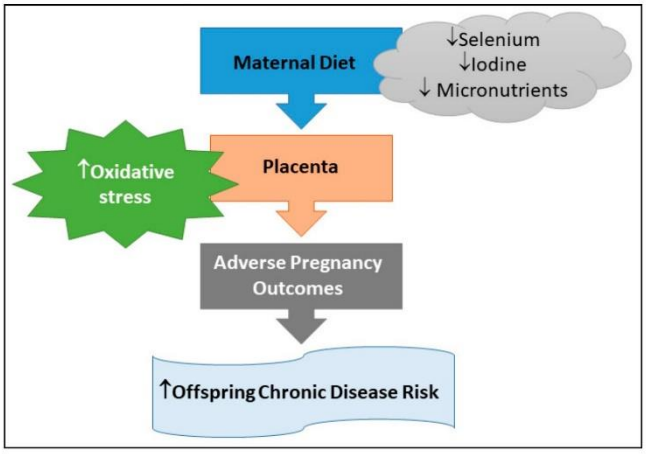

Figure 1. Deficiencies (↓) in selenium and iodine result in placental oxidative stress which may contribute to adverse pregnancy outcomes and hence increased (↑) risk of chronic disease in offspring.

Adverse pregnancy outcomes are associated with increased oxidative stress in the placenta [23] and since selenium and iodine are involved in oxidative stress in placental cells [24], it is important to investigate the role of both micronutrients. To the best of our knowledge, no study has reviewed current evidence regarding the potential synergistic effect of iodine and selenium in the placenta. Our knowledge from other organs, such as the thyroid, highlights the importance of these two micronutrients and their combined impact on oxidative stress [25]. We identified studies that investigated the association between maternal selenium and iodine status with pregnancy complications using the following keywords: "maternal blood selenium concentration AND pregnancy complication" and "maternal urinary iodine concentration AND pregnancy complication" in PubMed. The last search was performed in July 2020. In addition, any relevant studies identified from the reference list of selected studies were also included. The exclusion criteria were: (1) review papers, (2) unavailable full text, (3) non-English language, (4) animal studies, and (5) taking any medication during pregnancy. Pregnancy outcomes were defined as healthy if there was no complication or complicated, if there was

one of the following pregnancy complications: preeclampsia (PE), pregnancy-induced hypertension, miscarriage, gestational diabetes mellitus (GDM), preterm birth (PTB), intrauterine growth restriction (IUGR), or premature rupture of membrane (PROM). In this review we outline how selenium and iodine may relate to pregnancy complications through oxidative stress, and we propose how they may work together to impact pregnancy and placental health (Figure 1). We also explore how deficiencies in these two micronutrients during pregnancy may impact on the future health of the offspring, which has not been previously reviewed as a combination.

2. Oxidative Stress and the Importance of Antioxidants

Oxidative stress occurs when free radicals are generated to a level higher than the physiological level and the antioxidant system cannot neutralize them [26,27]. Free radicals, namely reactive oxygen species (ROS), are unstable molecules that have unpaired electrons and can donate or accept an electron to be stabilized [28]. As a result, the new molecule is unstable and formation of free radicals continues. This chain reaction can cause oxidative damage to nucleic acids, lipids, carbohydrates and proteins, which results in the decaying of tissues and the disruption of homeostasis [27]. The production of ROS is a continuous phenomenon in cells and is essential for cell-signaling, however, excessive ROS levels are detrimental to cells [27,29].

To stop the accumulation and damage of ROS, free radicals need to be neutralized by pairing unpaired electrons. This is how the antioxidant system can protect the cell against oxidative damage [30]. Antioxidants typically work in three ways, including the prevention of ROS formation, the interception of a ROS chain reaction and the repairing of damaged molecules [31,32]. The antioxidant defense system is made up of enzymes and non-enzyme components. Glutathione peroxidase (GPx), catalase, superoxide dismutase and thioredoxin reductase (TRx) are some of the enzymes [33] but they need cofactors such as micronutrients to work properly. For example, superoxide dismutase requires manganese or copper and zinc, while GPx and TRx need selenium to function properly. Therefore, micronutrient deficiencies can reduce the ability of the antioxidant system to protect cells against free radicals [34]. Vitamin C, vitamin E and beta-carotene are other micronutrients involved in the antioxidant defense system [35,36].

Oxidative Stress and Pregnancy Complications

Pregnancy complications are adverse outcomes of pregnancy such as preeclampsia (PE), gestational diabetes mellitus (GDM), intrauterine growth restriction (IUGR), preterm birth (PTB), and premature rupture of membrane (PROM). They can increase morbidity and mortality rates for both the mother and her offspring [37–40].

Preeclampsia is a pregnancy-specific, multisystem disorder, presenting as hypertension with new onset of one or more of proteinuria or renal, liver, neurological or hematological complications, or uteroplacental dysfunction after 20 weeks' gestation [41]. Patients with a history of PE have around a 2-fold increased risk for vascular diseases such as hypertension and ischemic heart disease [42], stroke [42] and chronic kidney disease [43], at 10–15 years post-partum. Infants of mothers who had PE are also at an increased risk of small-for-gestational-age [44] and perinatal mortality [45]. Children exposed to preeclamptic pregnancies have increased hospitalizations due to infectious and parasitic, nutritional and metabolic diseases of the respiratory system, blood, and blood-forming organs at ages 1–13, 16, 18–21 and 24 years compared to unexposed children [46]. Moreover, women who experience PE in their first pregnancy have a higher risk of myocardial infarction and cardiovascular death compared to non-PE mothers [47]. Currently, delivery of the fetus and placenta is the only treatment [41,42,45,48,49].

The pathogenesis of PE involves improper placental development as a result of dysfunctional proliferation, migration, and invasion of placenta-derived extravillous cytotrophoblast cells into the uterine vasculature, along with maternal endothelial and vascular dysfunction [48]. This leads to placental hypoxia and subsequent reperfusion, resulting in oxidative stress and inflammation [50,51].

Similar to PE, oxidative stress also contributes to PTB, IUGR and PROM. IUGR refers to a fetus that is smaller than expected for their gestational age. One of the most common causes of IUGR is uteroplacental insufficiency in which the placenta is unable to provide the developing fetus with sufficient nutrients and oxygen. The growth-restricted offspring have a 2–6 fold increased risk of developing chronic diseases such as type 2 diabetes mellitus, coronary heart disease and chronic kidney disease later in life [52].

The same placental dysfunction seen in PE is also seen in IUGR with deficient spiral artery remodeling resulting in malperfusion [53]. The malperfusion results in oxidative stress within the placenta and overwhelms the antioxidant system. In addition, the enzymes which act as antioxidants require micronutrients to work and maternal diets deficient in these micronutrients impact on the placenta's ability to combat oxidative stress [53].

3. Micronutrients and Pregnancy

To compensate for the higher demand of growing maternal tissues and fetus and also for hemodilution that occurs in pregnancy, a higher dietary intake of many micronutrients and trace elements are recommended [4,54]. In developed countries, despite the availability of suitable nutritional foods, many pregnant women have an imbalanced diet that puts them at risk of an inadequate intake of micronutrients like folate, vitamin D, vitamin B12, iron and iodine [55,56]. There is considerable evidence that shows that deficiency of micronutrients such as iodine, selenium, zinc, vitamin E, folate and iron, adversely impacts maternal and fetal health, and pregnancy outcome [19,57–69]. One of the reasons for this association may be the bio-functionality of vitamins and minerals in pregnancy, besides their classical roles in health and disease in the general population [56]. Although maternal micronutrient intake through the entire gestational period can affect fetal growth and development, the peri-implantation stage and placental development are critical windows for programming a healthy birth and future life [70,71].

3.1. Selenium

Selenium is an essential trace element that plays a pivotal role in the antioxidant defense system, cell cycle, and immune function, because of its contribution to selenoproteins [72]. Selenoproteins such as glutathione peroxidases (I, II, III, IV, and VI); thioredoxin reductases (I, II, and III); and selenoprotein H, P, and W, have antioxidant properties and are involved in managing ROS production. Selonoproteins also possess a vast range of functions such as protein folding, signaling, lipid biosynthesis, cell cycle, and calcium regulation [73–79].

Selenium is found in a broad range of foods including brazil nuts, wholegrain foods and cereals, fish, beef, eggs, and some fortified ready-to-eat breakfast cereals. The recommended dietary intake of selenium has been calculated by the amount of selenium needed to maximize the synthesis of glutathione peroxidase (GPx) [80]. During pregnancy, it is 60 micrograms per day in European countries [81] and 65 micrograms per day in Australia and New Zealand [82]. The requirement for selenium is higher during pregnancy, up to 4 micrograms per day, due to fetal requirements [83].

Globally, up to one in seven people have a low selenium dietary intake [84]. Selenium levels can be influenced by multiple factors including drinking water, soil, plant and animal tissue content of selenium [84], as well as different intakes in different regions. For example, the average intake of selenium in Eastern Europe is lower than that of Western Europe [85]. In addition, in New Zealand [85] and most areas of China [86], selenium deficiency is evident. In the Middle East, selenium intake is dependent on socio-economic status [85].

While studies across different countries report various levels of selenium during pregnancy (Table 1), using the data reviewed by Mariath et al. (2011) [87], Perkins and Vanderlelie (2016) proposed that selenium levels below 45 micrograms per litre can be dangerous and can be associated with adverse birth outcomes. They further concluded that selenium levels above 95 micrograms per litre can be considered as seleno-sufficient because the majority of selenoproteins can be maximally

expressed at this level [57]. In addition to the measurement of maternal selenium levels, markers of functional effects of selenium may provide additional information. For instance, assessment of selenoproteins such as GPX3 and SEPP1 are suitable to investigate the functional effects of selenium such as antioxidant activity, nutritional selenium deficiency, and evaluating responses of deficient individuals to selenium supplementation [88]. However, more studies would be needed to determine whether these selenoproteins provide additional information for interpreting clinical outcomes in addition to, or instead of, maternal selenium levels. The time point of selenium assessment can also affect the interpretation of the data, since pregnant women that were selenium sufficient in the first trimester have been shown to become selenium deficient later in pregnancy [89].

Table 1. Selenium levels in women of reproductive age.

References (Ref.)	Country Year	Sample Size (Women)	Time of Sampling	Sample Type	Selenium Concentration (μg/L)
[90]	Indonesia 2019	25 pregnant	Delivery	Serum	76.42 ± 16.30
[91]	Turkey 2019	30 pregnant	Delivery	Blood, urine, amniotic fluid	Median (min-max): Maternal blood: 78.98 (72.36–84.14) Maternal urine: 23.44 (19.66–26.69) Amniotic fluid: 26.00 (22.56–29.88)
[92]	Australia 2018	558 pregnant	15 ± 1 weeks' gestation	Plasma	Mean ± SD: 71.93 ± 11.05
[93]	Sudan 2014	31 pregnant	Not reported	Serum	Median (25–75th quartile): 204 (68–541)
[94]	Iran 2011	40 pregnant	34–39 weeks' gestation	Blood	Mean ± SD: 58.51 ± 11.85
[95]	UK 2008	27 pregnant 22 non-pregnant	Delivery	Blood	Mean ± SD: Pregnant: 58.4 ± 14.9 Non-pregnant: 69.8 ± 11.7
[20]	Hungary 2008	20 pregnant 24 non-pregnant	24–28 weeks' gestation	Serum	Mean ± SD: Pregnant: 40.5 ± 8.03 Non-pregnant: 77.4 ± 14.82
[96]	Turkey 2005	28 pregnant 25 non-pregnant	28–39 weeks' gestation	Serum	Mean ± SD: Pregnant: 87.50 ± 10.96 Non-pregnant: 109.0 ± 6.34
[97]	Kuwait 2004	15 pregnant	Delivery	Blood	Mean ± SEM: Maternal vein: 102.3 ± 3.1 Umbilical artery: 85.4 ± 4.2 Umbilical vein: 82.6 ± 4.1
[98]	USA 2004	22 pregnant	12 and 34 weeks' gestation	Plasma	Mean ± SD: 12 weeks' gestation: 126.0 ± 15.0 34 weeks' gestation: 111.0 ± 12.0
[99]	Poland 2001	36 pregnant 28 non-pregnant 36 pregnant 28 non-pregnant	Before or within 12 hours after uterine curettage	Whole blood and plasma	Mean ± SD: Whole blood: Pregnant: 74.1 ± 11.6 Non-pregnant: 90.5 ± 11.2 Plasma: Pregnant: 54.6 ± 11.1 Non-pregnant: 66.1 ± 13.1

SD, Standard deviation; SEM, standard error of the mean; μg/L, micrograms per litre.

3.2. Selenium and Pregnancy Complications

Selenium is necessary for human reproduction [100]. We have recently shown that in 1060 Australian pregnant women, those who had lower selenium concentrations (<0.95 micromol per litre) took a longer time to conceive (1.19 (1.01–1.40)), an equivalent of around 0.6 months, and they were at greater risk of infertility (1.46 (1.06–2.03)) [101]. Deficiency in selenium, measured by lower concentrations either in plasma, toenail, red blood cells or hair, has also been associated with a higher risk of pregnancy complications such as pregnancy-induced hypertension, PTB, impaired glucose tolerance and GDM [19–21,67,95–97,99,102,103] (Table 2). Comparatively, selenium supplementation was associated with a lower incidence of PE and PROM [104–106]. Importantly, inflammatory indicators such as high sensitivity C-reactive protein were higher in selenium-deficient pregnant women and antioxidant defense indicators namely GPx, catalase, thiobarbituric acid reactive substances (TBARS), superoxide dismutase, and glutathione S-transferase were altered [20,95,99,107,108] (Table 2).

This supports that selenium deficiency may contribute to higher oxidative stress levels, inflammation, and subsequent pregnancy complications (Table 2).

3.3. Iodine

Iodine is a vital trace element required for thyroid hormone synthesis and plays a fundamental role in fetal brain development [109,110]. Maternal iodine deficiency during pregnancy is associated with insufficient neurodevelopment, and defective intellectual skills such as poorer school performance and language delay as well as attention-deficit/hyperactivity disorder in offspring later in life [111,112]. While iodine deficiency is described as the most common cause of a child's central nervous system maldevelopment [113], mandatory iodine fortification programs such as salt iodization have helped address this common health issue among women of child-bearing age globally [114–117]. In Australia, although this preventive program seems to be successful among the general population, 16–44 year old pregnant women's median urinary iodine concentrations (MUIC: 116 micrograms per litre) still indicate insufficient iodine intake [118]. In New Zealand, MUIC of 68 micrograms per litre indicates a mild iodine deficiency in 18–44 year old women post-mandatory iodine fortification [118]. A similar situation has been reported in other countries with iodine fortification such as Austria, Croatia, Egypt, and Iran, all reporting that pregnant women's iodine status was insufficient [119]. Pregnancy iodine status should be at an optimal level to avoid the potentially harmful consequences of iodine deficiency [120].

Iodine intake during pregnancy should cover the needs of both the mother and her developing fetus. Thus, it is recommended that dietary intake increases from 150 micrograms perday for a non-pregnant woman to 220 micrograms per day during pregnancy [82]. The World Health Organization recommends a daily iodine supplement of 250 micrograms per day during pregnancy, or an annual dose of iodized oil supplement of 400 milligrams per year [121]. While maternal dietary intake of iodine can impact placental iodine content, and therefore control the effect of iodine on fetal thyroid gland activity [122], placental iodine accumulation plays a significant role in iodine availability to the fetus [123].

In thyroid hormone production, I- oxidation occurs to form iodine (I_2). This reaction uses H_2O_2 and thyroid peroxidase and inhibits H_2O_2 accumulation or its decomposition to a hydroxyl radical [25,124]. A normal level of thyroid hormone exerts a negative feedback on thyroid-stimulating hormone (TSH). Iodine deficiency is associated with several health issues including a reduction in thyroid hormone production [125], which results in the absence of the negative feedback on TSH. Therefore, a cascade of signals and reactions, including increasing TSH secretion, occurs [126]. TSH stimulates H_2O_2 generation for I^- oxidation but in severe iodine deficiency this process does not occur, resulting in thyroid hormone insufficiency [125,126]. Therefore, TSH continues to increase H_2O_2 generation, which can be higher than the antioxidant capacity of GPx; H_2O_2 will accumulate and more ROS is produced, resulting in oxidative stress and apoptosis [126].

Iodide is the ionic state of iodine, occurring when iodine forms a salt with another element, such as potassium. Iodide may have an ancestral antioxidant function in various iodide-concentrating cells not only similar to thyroid cells where iodine consumes H_2O_2, but also because iodide can reduce the lipid peroxidation rate by reacting with double bonds of the cell membrane polyunsaturated fatty acids and make iodolipids that will be less reactive to ROS [127].

Table 2. Association between maternal selenium levels or selenoproteins and pregnancy complications.

Ref.	Country Year	Sample Size (Pregnant Women)	Time of Sampling	Sample Type and Assays	Outcomes
[128]	Norway 2020	2638 (2558 term, 80 PTB)	17–18 weeks	Blood Se	No association between blood Se and PTB risk with adjustment for iodine intake (population had moderate iodine deficiency)
[90]	Indonesia 2019	51 (25 term, 26 PTB)	Delivery	Serum, placental and cord blood Se	Lower Se in the placenta and cord blood from PTB compared to term deliveries ($p < 0.05$)
[91]	Turkey 2019	50 (30 term, 20 PTB)	Delivery	Blood, urine and amniotic fluid Se	Lower blood, urine and amniotic fluid Se in PTB compared to term ($p < 0.05$)
[92]	Australia 2018	1065 (480 healthy, 585 complicated)	15 ± 1 weeks	Plasma Se, C-reactive protein	No difference between complicated and healthy pregnancies. No association between Se and C-reactive protein.
[129]	Iran 2017	60 at risk for IUGR (30 Se-supplementation, 30 placebo); RCT	Week 17 and end of intervention (week 27)	Plasma total antioxidant capacity, glutathione Serum C-reactive protein	Higher total antioxidant capacity and glutathione and lower C-reactive protein after 100 µg/d Se supplementation
[130]	South Africa 2017	66 (23 healthy, 43 PE)	Delivery	Serum and hair Se	Lower Se concentration in PE, no difference in hair Se between healthy and PE
[131]	Bangladesh 2015	74 PE (52 mild, 22 severe 118 normotensive)	≥20 weeks	Serum Se	Lower Se concentration in mild and severe PE, lower Se in severe PE compared to mild PE
[132]	Iran 2015	65 with GDM (32 Se-supplementation 33 placebo)	Week 24 and end of intervention (week 28)	Plasma MDA and glutathione	Higher glutathione and lower MDA after 200 µg/d Se-supplementation
[133]	Australia 2015	716 (472 healthy, 244 PE)	15 ± 1 weeks	Plasma Se	No difference in plasma Se between healthy and PE
[104]	UK 2015	230 primiparous 60 µg/d selenium yeast or placebo	(1) 12 and 35 weeks (2) 16 weeks	(1) Whole-blood Se (2) Toenail Se	After Se supplementation, low toenail Se was associated with decreased OR for PE/PIH (OR 0.30, 95% CI 0.09, 1.00, $p = 0.049$)
[93]	Sudan 2014	62 (31 healthy,31 GDM)	Not reported	Serum Se	No difference in Se level between healthy and GDM pregnancies
[106]	UK 2014	229 primiparous (115 Se-treated, 114 placebo) 60 µg/d selenium yeast	(1) 12 and 35 weeks (2) 35 weeks	(1) Whole-blood Se (2) selenoprotein P	Higher Se and selenoprotein P in Se-treated group at 35 weeks. Reduced PE/PIH odds in all Se-treated participants (OR 0.350, 95% CI 0.126, 0.974; $p = 0.044$)
[134]	Iran 2013	76 (38 healthy, 38 PE)	24 weeks–2 days after delivery	Plasma Se	Lower Se in women with PE compared to healthy pregnancies
[135]	UK 2013	50 (27 healthy, 23 PE)	Delivery	Serum Se, placental deiodinase mRNA and enzyme activity	Lower Se in women with PE, correlation between placental deiodinase III mRNA expression and its enzyme activity only in PE
[136]	Indonesia 2013	71 (46 healthy, 25 miscarriage)	8–20 weeks	Serum Se, GPx	Lower Se in women with miscarriage, no difference in GPx activity
[94]	Iran 2011	80 (40 healthy, 40 PE)	34–39 weeks	Plasma Se	Lower Se in women with PE

Table 2. *Cont.*

Ref.	Country Year	Sample Size (Pregnant Women)	Time of Sampling	Sample Type and Assays	Outcomes
[19]	Netherlands 2011	1129 (60 PTB, 21 PROM, 13 PE)	12 weeks	Serum Se	Higher risk for PTB with lower Se (OR 2.18, 95% CI 1.25–3.77)
[105]	Iran 2010	166 primigravid (83 Se supplement, 83 placebo) 60 µg/d selenium yeast	1st trimester and delivery	Serum Se	Increased Se ($p < 0.01$) and lower incidence of PROM ($p < 0.001$) in 100 µg/d of selenium yeast-supplemented group
[95]	UK 2008	74 (27 healthy, 25 PE, 22 non-pregnant age-matched)	Delivery	Serum, plasma, umbilical venous Se. Plasma and umbilical venous TBARS. Plasma and placental GPx.	Decreasing trend of plasma Se from non-pregnant to normal pregnant and PE; Lower serum Se and plasma GPx in PE compared to healthy pregnancies; higher level of maternal and umbilical venous TBARS in PE group; Lower placental GPx in PE
[20]	Hungary 2008	61 (20 healthy, 17 GDM, 24 healthy non-pregnant)	24–28 weeks	Serum Se, high sensitivity C-reactive protein	Negative correlation between serum Se and high-sensitive C-reactive protein
[21]	Turkey 2008	180 (101 healthy, 30 GDM, 49 glucose intolerance)	24–28 weeks	Serum Se	Lower Se in GDM and glucose intolerance compared to healthy pregnancies
[137]	Kuwait 2007	20 obese (10 GDM, 10 control)	Delivery	Blood Se, GPx, SOD and total antioxidant enzyme activity	Higher SOD activity in maternal vein, umbilical artery and umbilical vein blood of control obese women
[96]	Turkey 2005	85 (32 PE, 28 healthy pregnant, 25 non-pregnant)	28–39 weeks	Serum and placenta MDA, SOD and catalase in erythrocytes. Placental GSH and GPx. Serum Se	Lower serum Se, erythrocyte SOD activity, and placental GPx and higher erythrocyte catalase activity in PE; negative correlation between placental MDA level and serum Se in PE
[67]	Italy 2005	504 (210 gestational hyperglycemic, 294 normoglycemic)	24–28 weeks	Serum Se Dietary intake of Se	Lower dietary intake of Se in gestational hyperglycemic women; lower serum Se in women with impaired glucose tolerance; negative association between Se (OR 0.92, 95% CI = 0.87 to 0.95, $p < 0.0001$) and gestational hyperglycemia
[97]	Kuwait 2004	30 (15 healthy, 15 GDM)	Delivery	Blood Se from maternal vein, umbilical artery and umbilical vein	Lower maternal vein Se in GDM compared to healthy pregnancies

Table 2. *Cont.*

Ref.	Country Year	Sample Size (Pregnant Women)	Time of Sampling	Sample Type and Assays	Outcomes
[108]	Turkey2003	36 (16 healthy, 9 PIH and PE, 3 IDDP; 3 GDM, 3 OP, 2 PAP)	3rd trimester and immediately after delivery	GST, GPx and catalase activity and TBARS in maternal erythrocyte, plasma, and umbilical cord blood	Higher erythrocyte GPx activity and increased plasma TBARS in PIH and IDDP; higher cord blood GST activity (2–3 fold) in PE and PIH and IDDP compared to maternal activity before delivery; lower cord blood GPx activity compared to before delivery in PE and PIH; lower cord blood GPx (in PE and PIH, IDDP) and catalase activity (in PE and PIH) compared to maternal values; higher plasma TBARS in PE and PIH and IDDP in the antenatal period; lower cord blood erythrocytic TBARS in PE and PIH compared to maternal value
[102]	UK 2003	106 (53 healthy, 53 PE)	Not reported	Toenail Se	Lower Se in PE; more severe PE (delivery <32 weeks') with lower Se within the PE group; higher PE risk in the bottom tertile of Se (OR 4.4, 95% CI 1.6–14.9)
[103]	China 2001	251 (98 IGT, 46 GDM, 90 healthy, 17 healthy non-pregnant)	20 and 42 weeks	Serum Se	Lower Se at 33–42 weeks' than at 20–33 weeks' in all pregnant women; lower Se in IGT and GDM; lower Se in healthy pregnancies compared to healthy non-pregnant

CI, Confidence Interval; GDM, Gestational diabetes mellitus; GPx, Glutathione Peroxidase; GSH, Glutathione; GST, Glutathione S-transferase; Hct, Haematocrit; IDDP, Insulin-Dependent Diabetes mellitus in Pregnancy; IGT, Impaired glucose tolerance; IUGR, Intrauterine growth restriction; MDA, Malondialdehyde; µg/d, micrograms per day; OP, Oligohydramniotic Pregnancy; OR, Odds Ratio; PAP, Pregnancy with Abruption Placentae; PE, Preeclampsia; PIH, Pregnancy-induced hypertension; PROM, Premature rupture of membrane; PTB, Preterm birth; RBC, Red blood cell; RCT; Randomized controlled trial; Se, selenium; SOD, superoxide dismutase; TBARS, Thiobarbituric acid reactive substances.

3.4. Iodine and Pregnancy Complications

There is limited but supporting evidence that iodine contributes to the antioxidant system. In a small sample of 74 women, there was decreased total antioxidant status and superoxide dismutase activity in women with mild iodine deficiency in the 2nd and 3rd trimesters of pregnancy compared to pregnant women with optimal iodine levels [138]. Iodine sufficient pregnant women, as indicated by more than 150 micrograms per litre urinary iodine concentration, had higher superoxide dismutase enzyme activity compared to iodine-deficient pregnant women [66]. In vitro studies support this and have shown that iodine supplementation reduces ROS production in a dose-dependent manner [139]. While to date there is no study about the potential antioxidant effect of iodine in the placenta, studies have shown a role for iodine in the antioxidant system of other organs such as thyroid, breast, stomach and eye [25,140–143]. We have recently shown in a placental cell line that treatment with iodine resulted in a lower lipid peroxidation compared to control upon induction of oxidative stress [24], further supporting a potential role of iodine as an antioxidant in the placenta.

Importantly, several studies have shown that iodine-deficient pregnant women are at an increased risk of pregnancy complications such as maternal high blood pressure, PE, IUGR and PTB [64–66,138,144,145] (Table 3). Iodine assessment methods can significantly impact the iodine measurement [146]. Iodine intake can be assessed indirectly by thyroid hormone level, urine spot or 24 h urinary samples [146]. Although spot urinary samples in population studies with more than 500 people is a more feasible approach and provides a good estimate of iodine status, it is not a valid measure for individual iodine status; 24 h urine analysis is a more appropriate assessment among individuals or in studies with smaller sample sizes [146]. Adjusting for urinary creatinine to avoid the influence of fluid intake also improves the reliability of the measurement of iodine status [146,147]. However, regardless of the assays used to measure iodine, it is clear that iodine deficiency is associated with pregnancy complications (Table 3). Because complications in pregnancy are exacerbated by increased oxidative stress, the role of iodine as an antioxidant may positively impact pregnant complications.

3.5. Potential Synergistic Effects of Selenium and Iodine

To date, most studies have investigated the impact of micronutrients on oxidative stress in the placenta, separately. Unfortunately, this is unlikely to provide more information on potential interactions or synergistic effects than if they were assessed in combination. In particular, selenium and iodine are essential micronutrients that may affect oxidative stress synergistically. Deiodinases are selenocysteine-containing enzymes that can regulate thyroid hormone bioavailability by removing iodide from different positions on the tyrosine ring. There are various deiodinases in different tissues (deiodinase I, II, III), however type III is dominant in the placenta. It inactivates the T3 (triiodothyronine) and T4 (thyroxine) hormones by removing an inorganic iodine from their inner ring and converting them to T2 (diiodothyronine) and reverse T3 (rT3), respectively [148,149]. Deiodinase type II can produce T3 from T4, and increase the bioavailability of T3 [150]. Thus, the activity of both deiodinase II and deiodinase III leads to the release of iodine in the placenta [151].

Selenoprotein expression in endocrine tissues is precisely controlled to be maintained, even in Se deficiency, and deiodinases seem to be higher than GPxs hierarchically in some tissues [152]. It is also unclear whether there are similar protective mechanisms for deiodinase in the placenta because of the lack of systematic investigation of the placental deiodinases and other selenoproteins. Only one study has reported that placental deiodinase III mRNA expression and its enzyme activity were correlated in PE but not in normotensive pregnant women [135]. In this study, maternal selenium levels were significantly lower in PE compared to normotensive women. This suggests that in normotensive pregnant women where selenium level is optimal, translation of deiodinase III is conserved, while in PE women with low selenium levels, deiodinase III enzyme regulation is altered; therefore iodine metabolism may be affected [135]. However, further studies are required and in larger sample sizes. This will help identify whether and how fetal adaptations occur, in order to maximize iodide uptake.

Table 3. Iodine status and pregnancy complications.

Ref.	Country Year	Sample Size (Pregnant Women)	Time of Sampling	Sample Type and Assays	Outcome
[153]	UK 2018	3182 (3140 with child alive at 1st year, 42 pregnancy/infant loss)	Not reported	Urinary iodine-to-creatinine ratio(spot urine)	No association between iodine status and pregnancy complications or infant loss
[69]	China 2018	1569 euthyroid and primapara	1st trimester	Urinary iodine concentration (spot urine)	Mild iodine deficiency (urinary iodine 100–150 μg/L) was an independent risk factor for GDM (OR 1.669, 95% CI 1.114–2.501, $p < 0.05$). More than adequate and excessive iodine (urinary iodine ≥250 μg/L) was an independent risk factor for macrosomia (OR = 2.116, 95% CI 1.218–3.676, $p < 0.05$)
[154]	China 2018	2347	1st, 2nd, 3rd trimester	Urinary iodine concentration (spot urine)	Lower incidences of PE in pregnant women with UIC 150–249 μg/L compared to the reference group of UIC < 50 μg/L (OR = 0.12, 95% CI 0.01–0.87, $p < 0.05$)
[66]	Mexico 2017	57 (37 normotensive, 20 HPD)	3rd trimester	Urinary iodine concentration (spot urine), SOD, CAT, TBARS	Significant association between iodine deficiency and hypertensive disease of pregnancy (HPD); lower level of urinary iodine, SOD and CAT and higher level of TBARS in HPD compared to normotensive
[64]	Thailand 2016	390	Each trimester	Urinary iodine concentration (spot urine)	Higher PTB risk (OR 2.69, 95% CI 1.38–5.24, $p = 0.004$) and low birthweight (OR 2.66, 95% CI 1.40–5.05, $p = 0.003$) among urinary iodine < 150 μg/L
[155]	Argentina 2012	77	Not reported	Urinary iodine concentration (morning and evening urine samples, placental weight, placental index	Higher risk of lower placental weight in iodine deficiency (urinary iodine < 150 μg/L) (OR 3, 95% CI 1.06–8.5)
[156]	Turkey 2010	58 (40 severe PE, 18 healthy)	Not reported	Urinary iodine concentration (spot urine), thyroid hormone levels (T3, T4, TSH, fT3, fT4), blood magnesium	Positive correlation between urinary iodine and blood magnesium level in PE; higher T3 and fT3 levels in PE
[144]	Turkey 2009	40 (24 severe PE, 16 healthy)	Not reported for blood	Serum protein-bound iodine	Lower level of serum protein-bound iodine in maternal blood in PE; higher serum protein-bound iodine level in umbilical cord blood of infants in severe PE

Table 3. *Cont.*

Ref.	Country Year	Sample Size (Pregnant Women)	Time of Sampling	Sample Type and Assays	Outcome
[65]	Turkey 2007	35 (20 severe PE, 15 healthy)	Not reported for blood	Placental tissue iodine content, blood magnesium	Lower placental iodine in PE; positive correlation between placental iodine and blood magnesium level in PE
[145]	Senegal 2000	882 (462 pregnant, 420 non-pregnant)	Not reported	Urinary iodine concentration (spot urine), rate of miscarriage and stillbirth	Higher risk of miscarriage and stillbirth in iodine deficiency; highest rate in severe iodine deficiency (urinary iodine ≤ 20 µg/L) (OR 3.64, 95% CI 2.92–4.55)
[157]	China 1997	>60,000 Iodine supplementation to water	Not reported	Neonatal and infant mortality rate after iodine supplementation	Large reduction in both neonatal and infant mortality with iodine supplementation of water among all population in three severely iodine-deficient townships

CAT, Catalase; CI, Confidence Interval; fT3, Free triiodothyronine; fT4, Free thyroxine; GDM, Gestational diabetes mellitus; IUGR, Intra uterine growth restriction; µg/L, micrograms per litre; OR, Odds Ratio; PE, Preeclampsia; PIH, Pregnancy-induced hypertension; PTB, Preterm birth; SOD, superoxide dismutase; T3, triiodothyronine; T4, thyroxine; TBARS, Thiobarbituric acid reactive substances; TSH, thyroid-stimulating hormone; UIC, Urinary iodine concentration.

4. Impact of Maternal Selenium and Iodine, via Oxidative Stress, on Child Health

Maternal deficiencies in selenium or iodine may result in oxidative stress in the placenta, which may impact on future offspring health through developmental origins of health and disease [158–161]. Numerous studies support that an adverse in utero environment contributes to future chronic disease risk in adult offspring (reviewed in [162]) and that this may be mediated by the placenta [159,163].

It is known that during a healthy pregnancy there is a large amount of oxidative stress, especially since the placenta initially develops in a hypoxic environment with maternal blood flow established at approximately 10 weeks of gestation [164]. During this time, reactive oxygen species are produced and the antioxidant system combats this, however, if there are deficiencies in micronutrients involved in this antioxidant system, then it is likely that oxidative stress will occur which will lead to damaged placenta and potentially pregnancy complications. These pregnancy complications are associated with future chronic disease risk in the offspring. Several animal studies have shown that the use of maternal antioxidant supplementation can prevent placental oxidative stress and the associated programming of cardiovascular disease risk to the offspring (reviewed in [165]). Human randomized controlled trials with antioxidants have not shown improvements in pregnancy complications and some have in fact been associated with an increased risk (reviewed in [166]). However, what needs to be considered is the micronutrient status of the mother as deficiencies in elements such as selenium and iodine, which are required for antioxidant enzymes, may diminish the effectiveness of simply adding antioxidant supplements to the maternal diet.

5. Conclusions

This is the first comprehensive review examining the potential synergistic effects of selenium and iodine (Figure 2). In addition, we have discussed whether the association between maternal selenium and iodine status as measured in biological specimens is associated with pregnancy complications due to their roles in oxidative stress. In future, studies that assess maternal dietary intake of selenium and iodine should also be examined. Iodothyronine deiodinases are selenoenzymes involved in thyroid hormone metabolism. The incorporation of selenium into deiodinases causes it to play an essential role in the metabolism of thyroid hormones and in the release of iodide. In addition, iodine deficiencies result in greater production of H_2O_2, which requires the selenoenzyme GPx to remove the excess H_2O_2. Thus, selenium and iodine may have some combined effects that should be investigated. Maternal diet is essential for the health of the placenta and baby, and deficiencies in micronutrients impact placental health potentially via oxidative stress pathways. This in turn not only increases the risk of an adverse pregnancy outcome, but is associated with poor future health outcomes for mother and offspring. Supplementation with antioxidants is not necessarily the key if the underlying selenium and iodine levels are low, as antioxidants require these and other micronutrients for optimal activity. Therefore, to address adverse pregnancy outcomes and the impact they have on future offspring health, a better understanding of the role of each micronutrient, alone and in combination, in placental development and hence pregnancy success is needed.

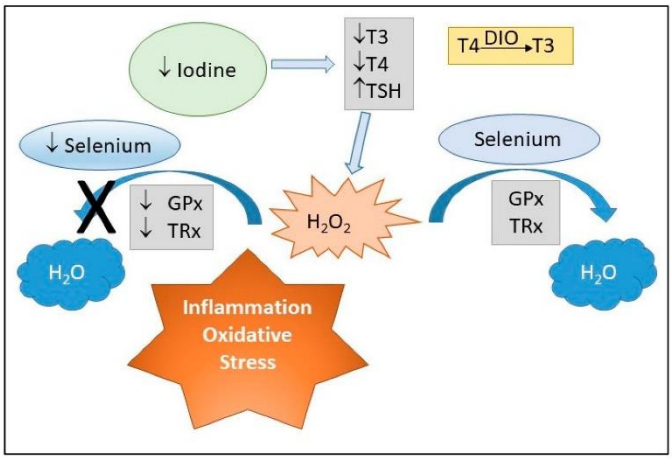

Figure 2. Deficiencies (↓) in iodine result in decreased (↓) T3 (triiodothyronine) and T4 (thyroxine) levels but increased (↑) TSH (thyroid-stimulating hormone) which results in more H_2O_2 (hydrogen peroxide). Antioxidants such as GPx (glutathione peroxidase) and TRx (thioredoxin reductase) are selenoproteins and in the presence of adequate selenium can convert H_2O_2 to H_2O (water). However, if there is a selenium deficiency (↓), H_2O_2 accumulates and can result in increased oxidative stress and inflammation. Deiodinases (DIO) are selenoproteins and deiodinase II converts T4 to T3.

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Chapter 3

Effect of Iodine and Selenium on Proliferation, Viability, and Oxidative Stress in HTR-8/SVneo Placental Cells

"There's a way to do it better. Find it!"
Thomas A. Edison

Statement of Authorship

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Effect of Iodine and Selenium on Proliferation, Viability, and Oxidative Stress in HTR-8/SVneo Placental Cells

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Abstract

Adequate maternal micronutrition is vital for placental formation, fetal growth, and development. Oxidative stress adversely affects placental development and function and an association between deficient placental development, oxidative stress, and micronutrient deficiency has been observed. Selenium and iodine are two essential micronutrients with antioxidant properties. Epidemiological studies have shown that poor micronutrient status in pregnant women is associated with a higher incidence of pregnancy complications. The aim of this study was to determine how selenium, iodine, and their combination impact oxidative stress in placental trophoblast cells. HTR8/SVneo extravillous trophoblasts were supplemented with a concentration range of organic and inorganic selenium, potassium iodide, or their combination for 24 h. Oxidative stress was then induced by treating cells with menadione or H_2O_2 for 24 h. Cell viability and lipid peroxidation as the biomarker of oxidative stress were assessed at 48 h. Both menadione and H_2O_2 reduced cell viability and increased lipid peroxidation ($P < 0.05$). Greater cell viability was found in selenium-supplemented cells when compared with vehicle treated cells ($P < 0.05$). Selenium and iodine supplementation separately or together were associated with lower lipid peroxidation compared with vehicle control ($P < 0.05$). Supplementation with the combination of selenium and iodine resulted in a greater reduction in lipid peroxidation compared with selenium or iodine alone ($P < 0.05$). Oxidative stress negatively impacts trophoblast cell survival and cellular integrity. Selenium and iodine protect placental trophoblasts against oxidative stress. Further research is warranted to investigate the molecular mechanisms by which selenium and iodine act in the human placenta.

Keywords Oxidative stress · Selenium · Iodine · Placenta · Lipid peroxidation · Reactive oxygen species

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Introduction

Although the prevalence of pregnancy complications such as preeclampsia, preterm birth, and gestational diabetes mellitus varies substantially across the world, they are associated with increased morbidity and mortality for both mother and offspring [1–4]. Many factors have been associated with the etiology of pregnancy complications and oxidative stress is one of those for which there is a large amount of evidence [5]. For instance, oxidative stress can cause apoptosis, thus, increasing deportation of placental fragments, mainly derived from syncytiotrophoblasts [6]. This apoptosis stimulates the inflammatory response which can lead to adverse pregnancy outcomes if anti-inflammatory mediators cannot effectively counterbalance it [6]. Oxidative stress occurs when free radicals, including reactive oxygen species (ROS), are generated to a greater than physiological level and the antioxidant system cannot neutralize them [7, 8]. ROS are unstable molecules that attack lipids, proteins, and nucleic acids to pair their unpaired electron. This can start an oxidative damage cascade with the continued formation of free radicals, thus cell death and tissue damage occur [8, 9]. An endogenous complex antioxidant system containing enzymes and non-enzyme components combats the effect of high levels of ROS. Glutathione peroxidase, catalase, superoxide dismutase, and thioredoxin reductase are some of these enzymes [10], and they need cofactors such as micronutrients to work appropriately. For instance, glutathione peroxidase requires selenium. Micronutrient deficiencies can interrupt mitochondrial respiratory chain activity that later lead to high levels of electron leakage and thus increased ROS production and oxidative stress [11]. Importantly, micronutrient deficiencies reduce cell protection by the antioxidant system, which is then overwhelmed by free radicals [7, 8, 12].

Selenium and iodine are essential for health but around one in seven people may have low dietary selenium intake globally [13]. Some selenoproteins, such as glutathione peroxidases, thioredoxin reductases, selenoproteins H, P, and W, have antioxidant properties and are involved in controlling ROS production [14–20]. Antioxidant enzyme activity of maternal plasma glutathione peroxidase and concentration of selenium in maternal serum or plasma were lower in women with pregnancy complications, such as miscarriage, preeclampsia, gestational diabetes mellitus, and preterm birth [21–26]. In addition, selenium supplementation was related to lower incidence of preeclampsia and premature rupture of membranes [27–29]. Selenium deficiency, therefore, may contribute to higher oxidative stress and inflammation and may contribute to pregnancy complications.

Iodine deficiency during pregnancy can impair thyroid hormone synthesis in both mother and fetus, which in early life can cause physical and cognitive impairment to the infant and child. Therefore, for many years, mandatory iodine fortification programs, such as salt iodization or iodine supplementation of bread and cereal products, have been put in place in many countries [30–33]. However, follow-up surveys show that iodine deficiency has not yet been eliminated. For instance, Global Scorecard 2016, that included a survey of 65 countries, showed that in 37 countries, iodine status of pregnant women is still insufficient as defined by median urinary iodine concentration (MUIC) of less than 150 μg/L [34]. In Australia, although mandatory iodine fortification programs seem to be successful among the general population and in 5–8 year old children, 16- to 44-year-old pregnant women have a MUIC of 116 μg/L which still points to iodine insufficiency [35]. In New Zealand, mild iodine deficiency in 18- to 44-year-old women was indicated by MUIC of 68 μg/L post-mandatory iodine fortification [35]. Iodine-deficient pregnant women and their offspring may be at increased risk of pregnancy complications such as gestational hypertension, intrauterine growth restriction, and preterm birth [36–41].

Similar to selenium, iodine may have a role in the antioxidant system. However, it has not been as well studied as selenium with minimal research in the placenta [42]. A role for iodine in the antioxidant system in organs such as the thyroid, breast, stomach, and eye is suggested based on biochemistry and evolution-related knowledge [43–47]. The oxidation of iodide to iodine by H_2O_2 is the first step of thyroid hormones biosynthesis. As H_2O_2 is a major oxidant in the body, the more it is used in iodide oxidation, the less will be available to cause oxidative damage to other parts of the body [46]. H_2O_2 can oxidize proteins in the lens of the eye and cause cataracts [48]. Therefore, iodine may contribute to control the detrimental effects of H_2O_2 in the eyes and other tissues by its antioxidant properties. Muranov et al. [49] reported that potassium iodide treatment protected the eye lens of young white rats against cataract formation caused by selenite. Because both iodide and selenite are anions, a redox interaction between them is impossible. Thus, protective effects of iodide are likely related to its contribution to the antioxidant defense system [49]. Iodine can react with double bonds of cell membrane lipids and make iodolipids. Because iodolipids are less vulnerable to ROS, lipid peroxidation will be reduced suggesting antioxidant activity of iodine in cells [50].

Most studies have investigated the effects of individual micronutrients on oxidative stress but do not provide any information on potential interactions. Selenium and iodine may work synergistically in different parts of the body including the placenta. Deiodinases (DIOs) are selenium-containing enzymes that can control thyroid hormone bioavailability by removing iodide from different positions on the hormone's tyrosine ring resulting in iodide release. Placental DIOs II and III can provide active thyroid hormone and release iodide [51]. Therefore, this study investigated whether selenium and iodine, separately and together, may be involved in placental cell protection against oxidative stress by assessing their impact on proliferation, viability, and lipid peroxidation.

Method and Materials

Cell Culture and Reagents

HTR8/SVneo cells were kindly provided by Professor Charles Graham, Queen's University, Kingston, Ontario. Cells were cultured in 75 ml flasks and maintained at 37 °C, 20% oxygen, and 5% CO_2 in Gibco 1X RPMI 1640 media (Life Technologies™) containing 10% v/v fetal bovine serum (FBS) (Sigma Aldrich). After reaching about 80% confluence, cells were trypsinized and 50,000 cells/well were seeded in each well of a 24-well plate in RPMI 1640 media with 0.5% FBS containing different concentrations of sodium selenite (0, 0.4, 2.8, and 5.5 μM NaSe), seleno-L-methionine (0, 0.4, 3.2, and 6.2 μM SeMet), potassium iodide (0, 0.1, 1, and 3 μM KI), NaSe + KI (0, 0.4 μM NaSe and 0.1 μM KI, 2.8 μM NaSe and 1 μM KI, 5.5 μM NaSe and 3 μM KI), or SeMet + KI (0, 0.4 μM SeMet and 0.1 μM KI, 3.2 μM SeMet and 1 μM KI, and 6.2 μM SeMet and 3 μM KI) for 24 h. Above-mentioned concentrations represent low, physiological, and super physiological selenium and iodine concentrations in maternal blood in pregnancy [52–55]. Solutions of sodium selenite (Sigma Aldrich), seleno-L-methionine (Sigma Aldrich) and potassium iodide (Sigma Aldrich) were made by dissolving in sterile water. All treatments were carried out in triplicate. Mass spectrometry analysis showed that RPMI 1640 media contained 63 nM selenium and less than 40 nM iodine.

Oxidative Stress

Menadione (Sigma Aldrich) made up in dimethyl sulfoxide (DMSO; Sigma Aldrich) and H_2O_2 (Vintessential Laboratories) diluted with water were used to induce oxidative stress. To find an appropriate dose of H_2O_2 and menadione for oxidative stress induction, HTR8/SVneo cells were cultured in a 24-well plate in RPMI 1640 media with 0.5% FBS for 24 h. Cells were then treated with different concentrations of menadione (0, 12, 15, 17, 20, 25 μM) and H_2O_2 (0, 25, 40, 50, 65 μM). After 24 h, cells were harvested to identify the concentration that reduced viability to 50–60%. This percentage was chosen to see if any protective effect would be achieved by supplementation.

To assess how selenium or iodine protect cells against oxidative stress, following 24 h of micronutrient supplementation (with NaSe, SeMet, KI, NaSe + KI, or SeMet + KI as shown above), an optimized dose of H_2O_2 (40 μM) or menadione (15 μM) was used for a further 24 h to induce oxidative stress in three independent experiments. For the control well, the appropriate volume of sterile water or 0.1% DMSO was added. Cells were then harvested or fixed for downstream analyses.

Assessment of Proliferation and Viability

HTR8/SVneo cells in each well were trypsinized and collected at the end of the 48 h treatment. Cells were counted with trypan blue dye and Countess II FL Automated Cell Counter (Thermo Fisher Scientific). Percentage of dead cells to total number of cells relative to the vehicle control was assessed to investigate the potential toxicity of different concentrations of micronutrients. Total number of cells per ml and ratio of live cells to total number of cells in vehicle control multiplied by 100 were calculated and compared between treatments to measure proliferation and viability.

Lipid Peroxidation

HTR8/SVneo cells were cultured on glass coverslips inside the 24-well culture plates with the same cell concentration and treatment time as above. Dead cells were removed and after a PBS wash the cells were fixed using 1:1 ice-cold acetone-methanol to evaluate lipid peroxidation as a biomarker of oxidative stress. HTR8/SVneo cells were then stained with primary polyclonal rabbit anti-4-hydroxynonenal antibody (4-HNE; Life Technologies™, dilution factor: 1:750) with an overnight incubation at 4 °C. Finally, nuclei and membrane lipid peroxidation were labeled using 4′, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) and secondary antibody Alexa Fluor 555 goat anti-rabbit IgG (H + L) (Life Technologies™), respectively. Cells of one well of the plate were used as a negative control incubated in diluent without primary antibody. To visualize 4-HNE and DAPI staining of HTR-8/SVneo cells, an Invitrogen™ EVOS ™ FL Auto II Imaging System (Life Technologies ™) was used. Twenty images were captured from randomly selected regions of each well per treatment at × 20 magnification and fluorescent intensity was quantified using Thermo Scientific HCS Studio: Cellomics Scan version 6.6.1.

Statistical Analysis

The numbers of dead, live, and total cells were modeled using Poisson (or negative binomial as appropriate) mixed effects models to account for repeated experiments as random effects to assess potential effect of supplementation on cell death, proliferation, and viability. Lipid peroxidation is expressed as a fold change (FC) relative to the control ±standard error. Linear regression model was used with log2-transformed 4-HNE intensity to estimate fold change compared to controls to test lipid peroxidation differences between micronutrient-treated and non-treated groups. This was followed by Tukey's post hoc test for multiple comparisons. For all analyses, P values < 0.05 were considered significant. All analyses were performed using R version 3.5.3 or later.

Results

Menadione and H_2O_2 Dose Optimization

A dose response experiment was performed with the following doses of menadione (0, 12, 15, 17, 20, 25 μM) and H_2O_2 (0, 25, 40, 50, 65 μM) to determine the optimal concentration of menadione and H_2O_2 for inducing oxidative stress in HTR8/SVneo cells. Cell viability assays showed that 15 μM menadione and 40 μM H_2O_2 decreased HTR8/SVneo cell viability to 50–60% (Fig. 1). These concentrations were used to induce oxidative stress in subsequent experiments.

Cell Viability Post Selenium Supplementation

Assessment of cells cultured in DMSO as the vehicle control for menadione showed that supplementation with 0.4 μM (0.873, 95% CI 0.768–0.993) and 2.8 μM NaSe (0.627, 95% CI 0.545–0.721), and 3.2 μM SeMet (0.719, 95% CI 0.624–0.828) significantly reduced the number of dead cells compared with control, while 5.5 μM NaSe increased dead cells (1.847, 95% CI 1.652–2.066) (Fig. 2a and b). Increased cell proliferation was seen after treatment with 0.4 μM (1.044, 95% CI 1.021–1.067) and 2.8 μM (1.088, 95% CI 1.064–1.112) NaSe, while 5.5 μM NaSe reduced cell proliferation (0.923, 95% CI 0.902–0.944). Similarly, supplementation with 3.2 μM SeMet increased cell proliferation (1.038, 95% CI 1.016–1.061) and the highest dose of SeMet (6.2 μM) caused a reduction in proliferation (0.977, 95% CI 0.956–0.998) (Fig. 2c and d). In terms of cell viability, supplementation with 0.4 μM and 2.8 μM NaSe and 3.2 μM SeMet resulted in a significant increase in comparison with the control group, whereas 5.5 μM NaSe and 6.2 μM SeMet were associated with a reduction in cell viability ($P < 0.05$) (Fig. 2e and f). In cells cultured in water as the vehicle of H_2O_2, 0.4 μM and 2.8 μM NaSe and 0.4 μM and 3.2 μM SeMet increased cell viability and 5.5 μM NaSe reduced it compared with the control group ($P < 0.05$) (Supplementary Fig. 1a and b).

Cell viability after induction of oxidative stress by menadione or H_2O_2 showed that all concentrations of both organic and inorganic selenium increased cell viability compared with the control. Interestingly, the highest concentrations of NaSe and SeMet that reduced cell viability in non-stressed cells were associated with increased cell viability in the presence of menadione or H_2O_2 (Fig. 3 and Supplementary Fig. 1c and d).

Lipid Peroxidation Post Selenium Supplementation

Lipid peroxidation of the cell membrane in selenium supplemented cells is shown in Fig. 4a–e. Lipid peroxidation decreased in selenium supplemented cells compared with control (Fig. 4f). Both 0.4 μM and 2.8 μM NaSe reduced lipid peroxidation significantly, with the most reduction at 2.8 μM NaSe (−0.8 ± 0.06 FC). All doses of SeMet reduced lipid peroxidation ($P < 0.05$) and the lowest level of lipid peroxidation was achieved at 3.2 μM SeMet (−0.6 ± 0.05 FC) (Fig. 4g). Membrane lipid peroxidation of cells treated with menadione is shown in Fig. 5a–e. All doses of both NaSe and SeMet significantly reduced lipid peroxidation compared with non-supplemented cells (Fig. 5f and g) after menadione treatment. The largest reduction in lipid peroxidation was seen at 2.8 μM NaSe (−1 ± 0.1 FC) and 3.2 μM SeMet (−0.7 ± 0.08 FC). Similar trends were seen when oxidative stress was induced using H_2O_2 (Supplementary Fig. 2).

Cell Viability and Lipid Peroxidation Post Iodine Supplementation

No significant effect on cell death, proliferation, or viability was seen in HTR8SV/neo cells supplemented with potassium iodide and cultured in menadione or H_2O_2 and relevant

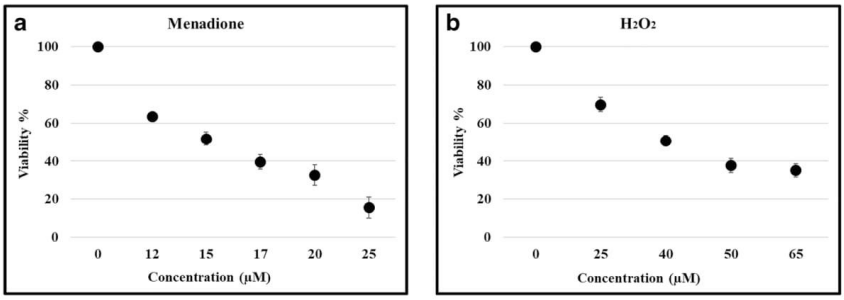

Fig. 1 Dose optimization for menadione and H_2O_2 in HTR8/SVneo cells. After culturing HTR8/SVneo cells in RPMI 1640 media containing 0.5% FBS for 24 h, they were treated with increasing concentrations of (**a**) menadione (12–25 μM) or (**b**) H_2O_2 (25–65 μM) for 24 h ($n = 3$). Data presented as percentage of cell viability relative to vehicle control. The optimal dose was chosen to achieve 50–60% cell viability

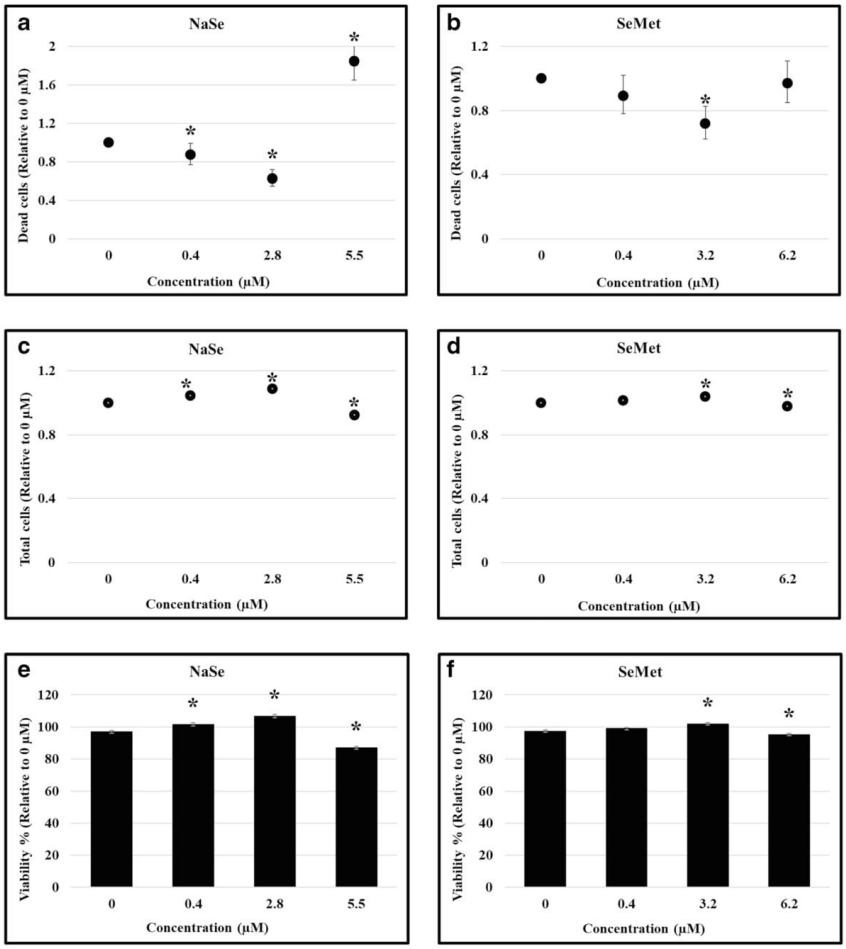

Fig. 2 Effect of selenium on dead cells, cell proliferation, and viability of HTR8/SVneo cells. HTR8/SVneo cells were cultured with 0, 0.4, 2.8, and 5.5 µM NaSe or 0, 0.4, 3.2, and 6.2 µM SeMet for 24 h followed by another 24 h in DMSO as the vehicle of menadione. After harvesting, dead cells, total cells (proliferation), and viability were assessed. Data are presented as estimated marginal mean ± 95% confidence interval. Statistical significance was assessed using Poisson (or negative binomial as appropriate) mixed effects models. *Denotes statistically different ($P < 0.05$) from control. **a** Dead cell post-NaSe supplementation ($n = 3$). **b** Dead cells post SeMet supplementation ($n = 3$). **c** Total cells (proliferation) post-NaSe supplementation ($n = 3$). **d** Total cells (proliferation) post-SeMet supplementation ($n = 3$). **e** Cell viability percentage post-NaSe supplementation ($n = 3$). **f** Cell viability percentage post-SeMet supplementation ($n = 3$)

controls (Fig. 6 and Supplementary Fig. 3). Potassium iodide at concentrations of 0.1, 1, and 3 µM reduced lipid peroxidation of cells in DMSO with the greatest reduction seen with 1 µM potassium iodide (− 0.7 ± 0.07 FC) and the least with 3 µM potassium iodide (− 0.3 ± 0.08 FC) (Fig. 7a). In menadione treated cells, the greatest reduction in lipid peroxidation was achieved with 1 µM potassium iodide (− 1.7 ± 0.23 FC) and cells supplemented with 3 µM potassium iodide (− 1.1 ± 0.18 FC) had a greater decrease in lipid peroxidation than those supplemented with 0.1 µM potassium iodide (− 0.6 ± 0.2 FC) (Fig. 7b). Similar trends were seen with H_2O_2 (Supplementary Fig. 4).

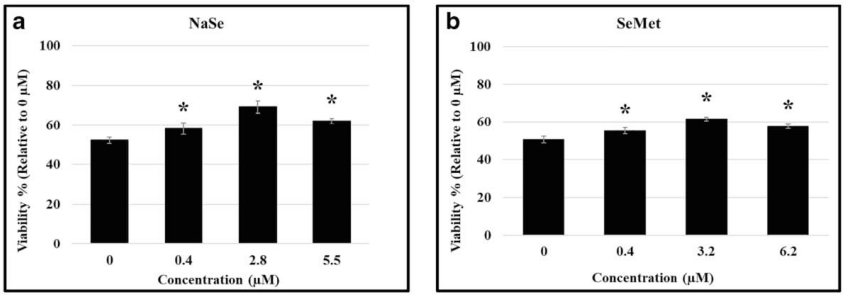

Fig. 3 Effect of selenium on HTR8/SVneo cell viability after oxidative stress induced by menadione. HTR8/SVneo cells were cultured with 0, 0.4, 2.8, and 5.5 μM NaSe or 0, 0.4, 3.2, and 6.2 μM SeMet for 24 h followed by another 24 h in 15 μM menadione. Data presented as estimated marginal mean ± 95% confidence interval. Statistical significance was assessed using Poisson (or negative binomial as appropriate) mixed effects models *Denotes statistically different ($P < 0.05$) from control. **a** Cell viability percentage post NaSe and menadione treatment ($n = 3$). **b** Cell viability percentage post SeMet and menadione treatment ($n = 3$)

Cell Viability with Combined Selenium and Iodine Supplementation

Combinations of selenium and iodine were evaluated for synergistic effects in low, physiological, and high concentrations of selenium and iodine on cell protection against oxidative stress. In the absence of menadione or H_2O_2, 0.4 μM NaSe and 0.1 μM KI and 2.8 μM NaSe and 1 μM KI reduced dead cells (0.833, 95% CI 0.725–0.958) and (0.673, 95% CI 0.582–0.779), respectively, while 5.5 μM NaSe and 3 μM KI increased dead cell ratio compared with control (1.634, 95% CI 1.446–1.846). Similarly, supplementation with the combination of SeMet and KI in low (0.4 μM SeMet and 0.1 μM KI) and physiological (3.2 μM SeMet and 1 μM KI) concentrations decreased dead cells with the greatest reduction caused by 3.2 μM SeMet and 1 μM KI (0.687, 95% CI 0.604–0.781) followed by (0.876, 95% CI 0.776–0.989) caused by 0.4 μM SeMet and 0.1 μM KI (Fig. 8a and b).

There was a significant increase in cell proliferation after supplementation with the combination of NaSe and KI in both low (0.4 μM NaSe and 0.1 μM KI) and 2.8 μM NaSe and 1 μM KI concentration while supplementation with high concentrations (5.5 μM NaSe and 3 μM KI) was associated with decreased cell proliferation (Fig. 8c). A similar trend was seen in cell proliferation post-supplementation with the combination of SeMet and KI at low and middle concentrations (Fig. 8d). Supplementation with a combination of all concentrations of NaSe or SeMet and KI was associated with a significant increase in cell viability compared with cells that were not supplemented with selenium and iodine (Fig. 8e and f and Supplementary Fig. 5) in the presence of oxidative stress induced by menadione or H_2O_2.

Further analyses showed that cells treated with menadione or H_2O_2, after supplementation with 5.5 μM NaSe and 3 μM KI, had a greater increase in cell viability compared with individual supplementation with 5.5 μM NaSe ($P < 0.05$). Viability of menadione treated cells was significantly greater post supplementation with 3.2 μM SeMet and 1 μM KI compared with supplementation with 3.2 μM SeMet alone ($P < 0.05$).

Lipid Peroxidation Post Combined Selenium and Iodine Supplementation

Lipid peroxidation in cells supplemented with a combination of selenium (either NaSe or SeMet) and iodine was less than non-supplemented cells in both menadione and H_2O_2 treated cell. The greatest reduction was observed at 2.8 μM NaSe and 1 μM KI, and 3.2 μM SeMet and 1 μM KI (Fig. 9 and Supplementary Fig. 6).

In menadione treated cells, prior supplementation with 3.2 μM SeMet and 1 μM KI and 6.2 μM SeMet and 3 μM KI resulted in less lipid peroxidation compared with supplementation with SeMet alone ($P < 0.05$). Cells supplemented with 0.4 μM SeMet and 0.1 μM KI had decreased lipid peroxidation compared with those supplemented with 0.4 μM SeMet or 0.1 μM KI alone ($P < 0.05$).

In cells treated with H_2O_2, supplementation with the combination of 0.4 μM SeMet and 0.1 μM KI had a decreased amount of lipid peroxidation compared with those supplemented with 0.4 μM SeMet or 0.1 μM KI alone ($P < 0.05$). Lipid peroxidation of cells supplemented with 3.2 μM SeMet and 1 μM KI was lower than cells supplemented with 3.2 μM SeMet before H_2O_2 treatment ($P < 0.05$). Supplementation with 6.2 μM SeMet and 3 μM KI reduced lipid peroxidation and hence oxidative stress more than supplementation with SeMet alone ($P < 0.05$).

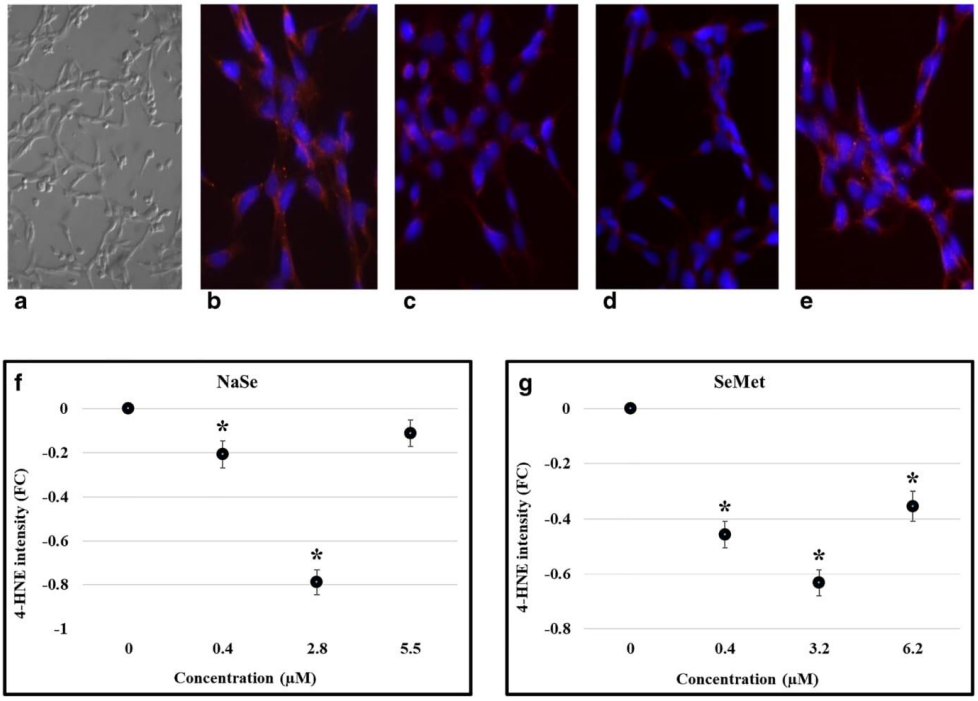

Fig. 4 Effect of selenium on lipid peroxidation of HTR8/SVneo cells. HTR8/SVneo cells were cultured with increasing concentration of NaSe (0–5.5 μM) for 24 h followed by another 24 h in DMSO (menadione vehicle). Cells were then fixed and stained with the primary polyclonal rabbit anti 4-hydroxynonenal antibody. After an overnight incubation at 4 °C, nuclei and membrane lipid peroxidation were labeled using 4′, 6-diamidino-2-phenylindole (nuclei: DAPI, blue) and secondary antibody of Alexa Fluor 555 goat anti-rabbit IgG (H + L) (4-HNE: red). From left: **a** cells in DMSO, image captured by bright field microscope, **b** cells supplemented with 0, **c** 0.4 μM, **d** 2.8 μM, and **e** 5.5 μM NaSe, immunofluorescence images captured by Invitrogen TM EVOS TM FL Auto II Imaging System. **f** Lipid peroxidation post NaSe supplementation ($n = 3$). **g** Lipid peroxidation post SeMet supplementation ($n = 3$). Data presented as a fold change (FC) relative to control ± standard error. Statistical significance was assessed using linear regression with log 2-transformed 4-HNE intensity to estimate fold change compared with controls followed by Tukey's post hoc multiple comparisons. *Denotes statistically different ($P < 0.05$) from control

Discussion

Humans obtain selenium in both inorganic and organic forms from their diet, so in this study, we have used sodium selenite (Na_2SeO_3: inorganic) and selenomethionine ($C_5H_{11}NO_2Se$: organic) to study the effects of selenium on placental cells. The current study, using HTR8/SVneo trophoblasts, showed that both inorganic and organic selenium compounds can protect placental cells against oxidative stress with increasing cell viability and reducing oxidative damage to membrane lipids. Previously, in vitro studies using BeWo, JEG-3, and Swan-71 cells showed that selenium supplementation can enhance cellular activity and specifically activity of enzymes involved in antioxidant defense such as glutathione peroxidase and thioredoxin reductase during oxidative stress [56–59]. Selenium concentrations used in previous studies were lower than those used in this study. Selenium is necessary for human reproduction and its deficiency, defined by lower selenium concentration either in the plasma, toenail, red blood cells, or hair, has been associated with a higher risk of pregnancy complications such as pregnancy-induced hypertension, preterm birth, miscarriage, gestational impaired glucose tolerance, and gestational diabetes mellitus [21, 22, 25, 26, 60–66]. Our findings emphasize the importance of selenium deficiency in cell death and oxidative damage, which is an intermediary step in the pathogenesis of pregnancy complications. The oxidative damage due to selenium deficiency is likely to be related to the reduction of antioxidant properties of selenoproteins such as glutathione peroxidases (I, II, III, IV, V, and VI), thioredoxin reductases (I, II, and III), and selenoproteins H, P, and W [14–20].

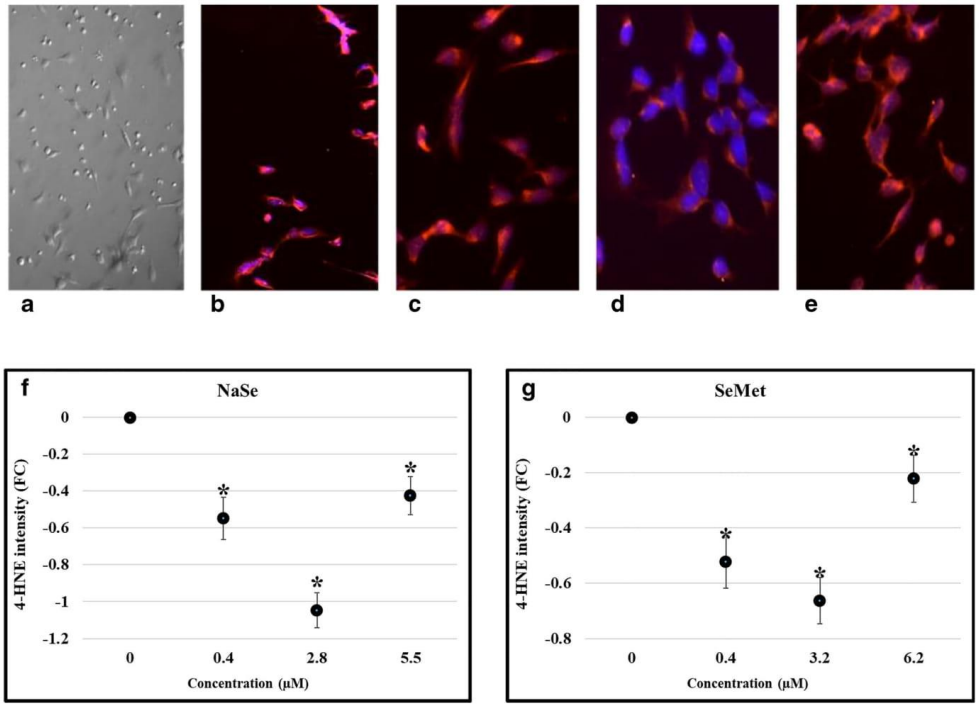

Fig. 5 Effect of selenium on lipid peroxidation after oxidative stress induction in HTR8/SVneo. HTR8/SVneo cells were cultured with increasing concentration of NaSe (0–5.5 µM) for 24 h followed by another 24 h in 15 µM menadione. Cells were then fixed and stained with the primary polyclonal rabbit anti 4-hydroxynonenal antibody. After an overnight incubation at 4 °C, nuclei and membrane lipid peroxidation were labeled using 4′, 6-diamidino-2-phenylindole (nuclei: DAPI in blue color) and secondary antibody of Alexa Fluor 555 goat anti-rabbit IgG (H + L) (4-HNE: red color). From left: **a** cells in menadione, image captured by bright field microscope, **b** cells supplemented with 0, **c** 0.4 µM, **d** 2.8 µM, and **e** 5.5 µM NaSe, immunofluorescence images captured by Invitrogen TM EVOS TM FL Auto II Imaging System. **f** Lipid peroxidation post-NaSe and menadione treatment ($n = 3$), **g** lipid peroxidation post SeMet and menadione treatment ($n = 3$). Data presented as a fold change (FC) related to control ± standard error. Statistical significance was assessed using linear regression with log 2-transformed 4-HNE intensity to estimate fold change compared with controls followed by Tukey's post-hoc multiple comparisons. *Denotes statistically different ($P < 0.05$) from control

This study showed that iodine deficiency is associated with a higher level of oxidative damage to cell membrane lipids, and iodine supplementation reduced lipid peroxidation in placental cells. It is known that iodine can reduce lipid peroxidation by reacting with double bonds of cell membrane polyunsaturated fatty acids to form iodolipids that are less reactive to ROS [50] and thus, may have antioxidant properties. Consistent with this, a study on BeWo and 3asubE choriocarcinoma cell lines showed a dose-dependent reduction in ROS production with iodine supplementation [67]. Also, perchlorate, an inhibitor of the iodine transporter (sodium iodide symporter), increases oxidative stress and decreases glutathione peroxidase 4 (GPX_4) mRNA and protein in BeWo cells [68]. A study among 212 pregnant women in different trimesters showed that mild iodine deficiency decreased concentrations of total antioxidant system and superoxide dismutase activity [41]. This study showed that iodine-deficient cells had greater lipid peroxidation that may be associated with a decreased capacity for antioxidant defense.

Interestingly, iodine supplementation reduced oxidative stress without impacting cell proliferation or viability. It is worth mentioning that the highest concentration of selenium in cells not treated with oxidative stress inducers had some cytotoxic effects, increased cell death, and lipid peroxidation while the same concentration was protective in cells exposed to oxidative stress. In addition, even the highest concentration of iodine reduced lipid peroxidation of cells during oxidative stress compared with vehicle control. These findings suggest

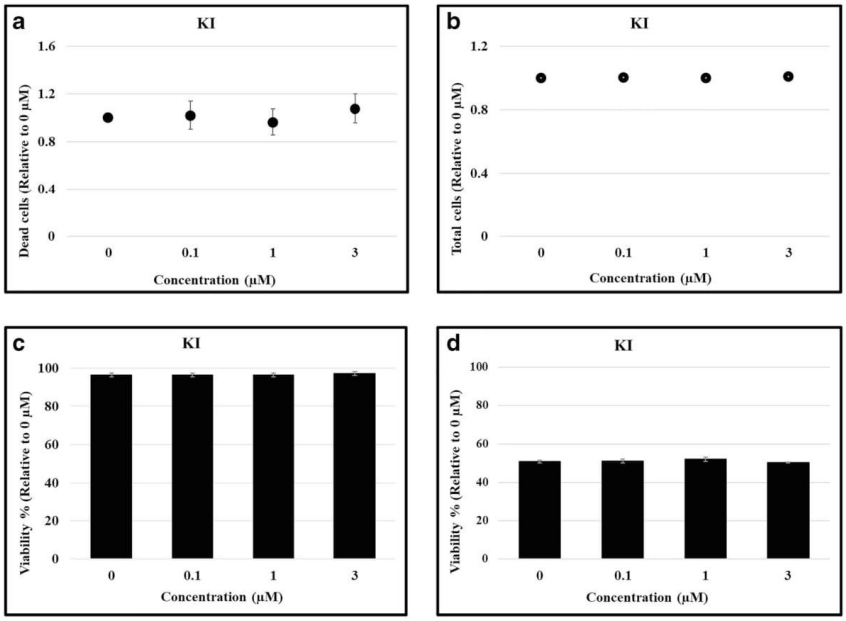

Fig. 6 Effect of iodine on dead cell, proliferation, and cell viability of HTR8/SVneo. HTR8/SVneo cells were cultured with 0, 0.1, 1, and 3 µM KI for 24 h followed by another 24 h in **a**, **b**, and **c** DMSO as the vehicle of menadione or **d** 15 µM menadione. After harvesting, dead cells, total cells (proliferation) and viability were assessed. Data presented as estimated marginal mean ± 95% confidence interval. Statistical significance was assessed using Poisson (or negative binomial as appropriate) mixed effects models. *Denotes statistically different ($P < 0.05$) from control. **a** Dead cell post potassium iodide supplementation in DMSO ($n = 3$), **b** Total cells (proliferation) post potassium iodide supplementation in DMSO ($n = 3$). **c** Cell viability percentage post potassium iodide supplementation in DMSO ($n = 3$). **d** Cell viability percentage post potassium iodide and menadione treatment ($n = 3$)

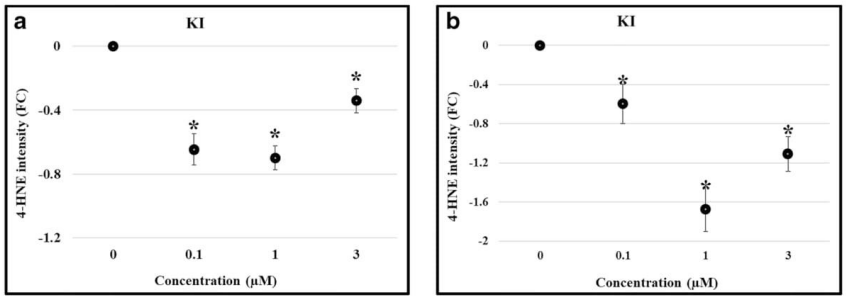

Fig. 7 Effect of iodine on lipid peroxidation of HTR8/SVneo cells. HTR8/SVneo cells were cultured with an increasing concentration of potassium iodide (0–3 µM) for 24 h followed by another 24 h in **a** DMSO or **b** 15 µM menadione. Cells were then fixed and stained with the primary polyclonal rabbit anti 4-hydroxynonenal (4-HNE) antibody. After an overnight incubation at 4 °C, nuclei and membrane lipid peroxidation were labeled using 4′, 6-diamidino-2-phenylindole (DAPI) and secondary antibody of Alexa Fluor 555 goat anti-rabbit IgG (H + L). Data presented as a fold change (FC) relative to the control ±standard error. Statistical significance was assessed using linear regression with log 2-transformed 4-HNE intensity to estimate fold change compared with controls followed by Tukey's post hoc multiple comparisons. *Denotes statistically different ($P < 0.05$) from control. **a** Lipid peroxidation post potassium iodide supplementation in DMSO ($n = 3$). **b** Lipid peroxidation post potassium iodide and menadione treatment ($n = 3$)

that cells exposed to oxidative stress may require increased amounts of selenium and iodine for survival.

Importantly, this study showed that supplementation to placental cells with the combination of selenium and iodine caused even greater protection against oxidative stress than supplementation with either one of them alone. This suggests a synergistic activity of selenium and iodine against oxidative stress.

Conclusion

Deficiency of selenium and iodine is associated with greater cell death and oxidative damage to cell membranes. Since oxidative stress plays a role in adverse pregnancy outcomes, deficiency of selenium and iodine may be involved in their pathogenesis. However, too much selenium in the absence of

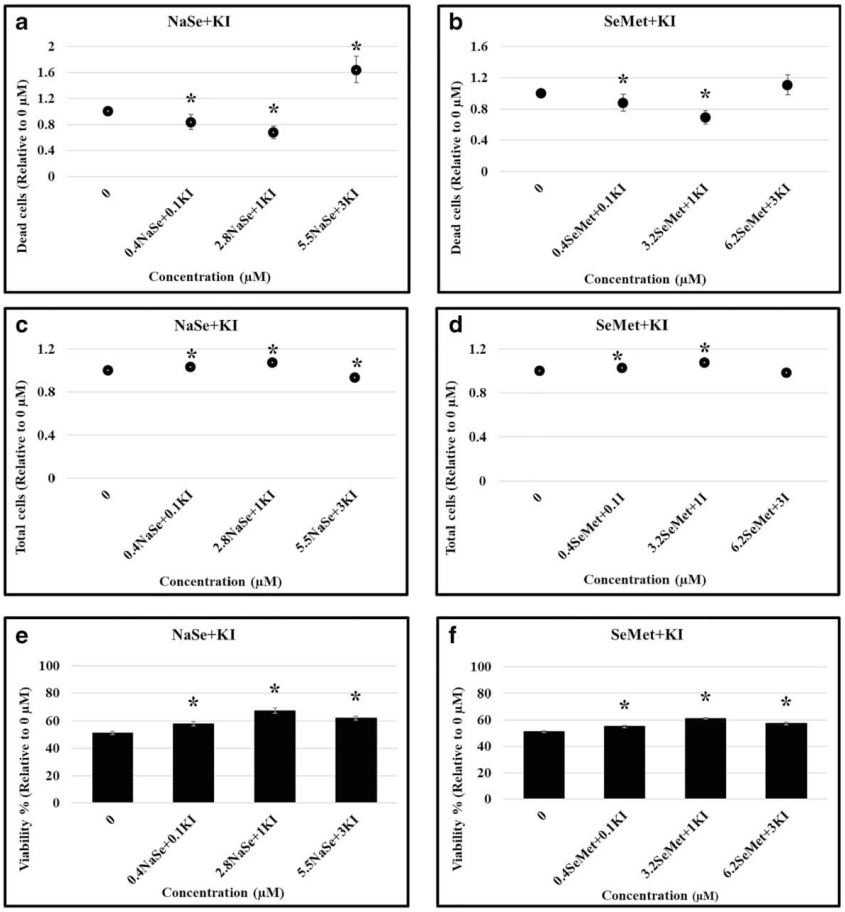

Fig. 8 Effect of selenium and iodine on dead cell, proliferation and cell viability of HTR8/SVneo cells. HTR8/SVneo cells were cultured with 0.4 µM NaSe and 0.1 µM KI, 2.8 µM NaSe and 1 µM KI, 5.5 µM NaSe and 3 µM KI or 0.4 µM SeMet and 0.1 µM KI, 3.2 µM SeMet and 1 µM KI, and 6.2 µM SeMet and 3 µM KI for 24 h followed by another 24 h in **a**, **b**, **c**, and **d** DMSO as the vehicle of menadione or **e** and **f** 15 µM menadione. After harvesting, dead cells, total cells (proliferation) and viability were assessed. Data presented as estimated marginal mean ± 95% confidence interval. Statistical significance was assessed using Poisson (or negative binomial as appropriate) mixed effects models. *Denotes statistically different ($P < 0.05$) from control. **a** Dead cells post NaSe + KI supplementation ($n = 3$), **b** Dead cells post SeMet + KI supplementation ($n = 3$). **c** Total cells (proliferation) post NaSe + KI supplementation ($n = 3$), **d** Total cells (proliferation) post SeMet + KI supplementation ($n = 3$). **e** Cell viability percentage post-NaSe + KI and menadione treatment ($n = 3$). **f** Cell viability percentage post SeMet + KI and menadione treatment ($n = 3$)

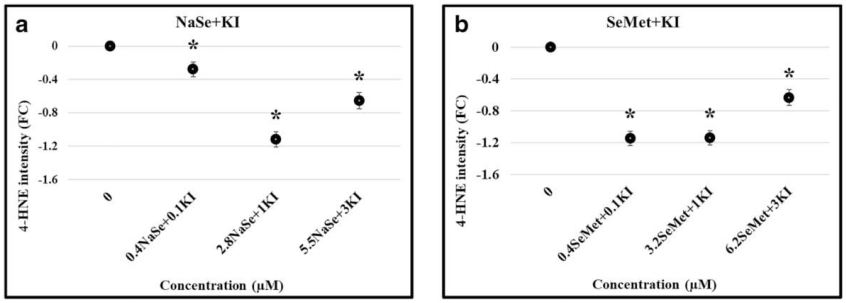

Fig. 9 Effect of selenium and iodine combination on lipid peroxidation of HTR8/SVneo cells. HTR8/SVneo cells were cultured with 0.4 μM NaSe and 0.1 μM KI, 2.8 μM NaSe and 1 μM KI, 5.5 μM NaSe and 3 μM KI or 0.4 μM SeMet and 0.1 μM KI, 3.2 μM SeMet and 1 μM KI, and 6.2 μM SeMet and 3 μM KI for 24 h followed by another 24 h in 15 μM menadione. Cells were then fixed and stained with the primary polyclonal rabbit anti 4-hydroxynonenal (4-HNE) antibody. After an overnight incubation at 4 °C, nuclei and membrane lipid peroxidation were labeled using 4′, 6-diamidino-2-phenylindole (DAPI) and secondary antibody of Alexa Fluor 555 goat anti-rabbit IgG (H + L). Data presented as a fold change (FC) related to control ±standard error. Statistical significance was assessed using linear regression with log 2-transformed 4-HNE intensity to estimate fold change compared with controls followed by Tukey's post hoc multiple comparisons. *Denotes statistically different ($P < 0.05$) from control. **a** Lipid peroxidation post NaSe+KI and menadione treatment ($n = 3$), **b** Lipid peroxidation post SeMet + KI and menadione treatment ($n = 3$)

oxidative stress can also be damaging. Further studies in placental tissues are needed to confirm this model and investigate potential mechanisms of these effects.

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Availability of Data and Material Data and material are available for data transparency.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Code Availability Not applicable.

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Chapter 4

Selenium and Iodine Supplementation Protects

First Trimester Human Placenta Against Oxidative Stress

"A person who never make a mistake never tried anything new!"
Albert Einstein

Statement of Authorship

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Contribution to the Paper	Contributed to designing the study, Performed tissue culture and collection and immunohistochemistry, contributed to LA ICP-MS, scanning placental explants and data analyses, drafted original manuscript		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Title: Selenium and iodine supplementation protect first trimester human placenta against oxidative stress

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Running title: Selenium and iodine reduce oxidative stress

Abstract

Study question: Can the micronutrients selenium, iodine and copper protect human first trimester placenta from oxidative stress in?

Summary answer: Selenium and iodine, alone or in combination, can protect first trimester human placenta against oxidative stress. Copper was not protective.

What is known already: Adequate maternal nutrition is vital for placental development. Poor placentation, imbalanced maternal micronutrient status and placental oxidative stress are associated with greater risk of pregnancy complications, which impact mother and infant health. Selenium, iodine and copper are essential micronutrients with key roles in antioxidant systems.

Study design, size, duration: First trimester (7-12 weeks of gestation) human placenta explants (n=15) were treated with a range of concentrations of selenium, iodine, selenium and iodine or copper. The concentrations represented deficient, physiological or high dose. After treatment, placenta explants were collected for assessment of proliferation, apoptosis and DNA damage.

Participants/materials, setting, methods: Micronutrient treated placenta explants (n=3) were harvested, fixed, processed and embedded in paraffin blocks to be used for laser ablation inductively coupled plasma-mass spectrometry (LA ICP-MS) to assess selenium and copper uptake. Additional placenta explants (n=12) were supplemented with sodium selenite (0, 0.8 or 1.6 µM), potassium iodide (0, 0.5 or 1 µM), a combination of sodium selenite and potassium iodide, or copper (II) sulphate (0, 20 or 40 µM) for 72 hours and 120 µM menadione or 480 µM antimycin for 24 hours to induce oxidative stress. Immunohistochemical labelling was performed for assessment of proliferation (Ki67), apoptosis (cleaved caspase-3), and DNA damage (8-hydroxy-2'-deoxyguanosine).

Main results and the role of chance: LA-ICP-MS element imaging showed that placenta explants could uptake selenium and copper from the media. Sodium selenite (1.6 µM) increased cell proliferation ($p < 0.05$). Sodium selenite and potassium iodide reduced DNA damage and apoptosis ($p < 0.05$). Following oxidative stress induction, a higher concentration of sodium selenite (1.6 µM) was needed to reduce DNA damage and apoptosis while both concentrations of potassium iodide (0.5 and 1 µM) were protective ($p < 0.05$). A

high concentration of copper (40 μM) increased apoptosis and DNA damage ($p < 0.05$) but this effect was no longer significant after induction of oxidative stress by menadione or antimycin ($p > 0.05$).

Limitations, reasons for caution: This study was only conducted in first trimester human placenta tissue therefore it is unknown if the impact of these micronutrients on oxidative stress in the placenta would be the same had the treatments occurred at different time points.

Wider implications of the findings: Excess oxidative stress deleteriously impacts human placental tissue resulting in increased apoptosis and oxidative damage to DNA molecules. Selenium and iodine, alone and in combination, can protect the placenta against oxidative stress. Therefore, an optimal maternal level of micronutrients may be essential for placenta health and hence offspring health.

Study funding/competing interest(s): This research was supported by National Health and Medical Research Council (NHMRC) GNT1161079 Targeting micronutrients to tackle pregnancy disorders: an integrated approach awarded to CTR, SJZ, TVP, JAG, SYL and TBM. CTR is supported by a NHMRC Investigator Grant GNT1174971 and a Flinders University Matthew Flinders Fellowship. The authors have no conflicts of interest.

Trial registration number: NA

Key words: copper; iodine; oxidative stress; placenta; selenium

Introduction

Maternal nutrition during early pregnancy is critical for fetal growth and development and can impact the future health of the offspring later in life [1,2]. A deficiency or excess in micronutrients such as selenium, copper, and iodine measured in maternal plasma, serum or urine are associated with a greater risk of pregnancy complications such as preeclampsia, gestational diabetes mellitus, spontaneous preterm birth and small-for-gestational age [3-7]. The detrimental effects of pregnancy complications are not limited to the pregnancy as their long-term consequences can impact the health of both mother and child later in life [8-10]. Thus, it is of paramount importance to determine the nutritional risk factors related to pregnancy complications and devise any potential preventive strategies.

The placenta is the key mediator of maternal nutrient supply to the fetus. Poor placental development, including incomplete remodelling of arteries during early pregnancy, has been associated with pregnancy complications like preeclampsia [11]. Partial remodelling of the maternal arteries causes hypoxia followed by reoxygenation which results in oxidative stress [12]. Oxidative stress is frequently associated with a range of pregnancy complications [13,14]. Oxidative stress is caused by accumulated reactive oxygen molecules and insufficient antioxidant activity [15,16], and results in damage of molecules such as lipids, proteins and DNA, thus tissue decay [16]. In addition, oxidative stress can increase apoptosis and this leads to high shedding of syncytial fragments into maternal blood resulting in a systemic inflammatory response, which is associated with pregnancy complications [17].

Micronutrients and oxidative stress are related through the antioxidant system [18,19]. For instance, glutathione peroxidase (GPx) and superoxide dismutase 1 (SOD1) are two antioxidant enzymes that require micronutrients for proper function [18,19]. GPx needs selenium and SOD1 requires copper or zinc to scavenge radicals and prevent oxidative stress

[18,19]. Studying micronutrient status in relation to placental development and oxidative stress may help elucidate the mechanisms involved in pregnancy complications. Although population studies have shown that selenium supplementation is associated with a lower incidence of preeclampsia and premature rupture of membranes [20-22], the impact of micronutrients on placental development is not fully understood. In addition, there is no information on potential interactions between selenium and other micronutrients. Iodine is an essential micronutrient and its deficiency during pregnancy puts both mother and offspring at a greater risk of pregnancy complications such as gestational hypertension, intrauterine growth restriction and preterm birth [7,23-27]. We recently showed that selenium and iodine deficiency are associated with lower cell proliferation and higher cell death and lipid peroxidation in HTR8/SVneo trophoblast cells [28]. In addition, individual or combined supplementation with selenium and iodine protected trophoblast cells against oxidative stress by enhancing cell viability and proliferation and reducing lipid peroxidation [28].

Copper is another essential component in a variety of metalloenzymes [29] including the antioxidant SOD1 enzyme [18]. High copper levels in maternal serum have been associated with a greater level of inflammation [3]. Investigating how copper may impact the placenta will help to understand its role and impact on pregnancy outcome. This study investigated how selenium, iodine and copper may impact oxidative stress response in first trimester human placenta by assessing their effect on proliferation, apoptosis, and DNA damage.

Materials and methods

Placenta explant tissue culture

First trimester (7-12 weeks' gestation; determined from last menstrual period) human placenta tissue samples (n=15) were collected with informed consent from women who

underwent elective surgical terminations at the Pregnancy Advisory Centre, Woodville, South Australia. Age and BMI Mean±SD of participants were 28.83 ± 7.18 year and 25.36 ± 3.38 kg/m^2 respectively. Ethnicity was mainly Caucasian. None of them reported a medical issue for the termination. Ethics approval was granted by the Queen Elizabeth Hospital Human Research Ethics Committee (HREC/16/TQEH/33).

Within an hour of termination, 10-15 mg of placenta tissue sections (3-4 pieces) were cultured on a pre-prepared gel base in each well of a 48-well plate. The gel base was Growth Factor Reduced Matrigel® (protein concentration: 3 mg/ml; Corning®) and 1X DMEM GlutaMAX™ media (Gibco® Life Technologies™) containing 10% v/v FBS (Sigma-Aldrich) and 1% v/v Antibiotic-Antimycotic (Life Technologies™) at pH 7.0. Culture conditions were maintained at 37ºC, 5% CO_2 and 1% O_2 for the duration of the experiments. Placenta explants were first cultured for 48 hours to enable regeneration of the syncytial layer. Explants were supplemented with sodium selenite (Sigma Aldrich®) (0, 0.8 or 1.6 µM), potassium iodide (Sigma Aldrich®) (0, 0.5 or 1 µM), copper (II) sulfate (Sigma Aldrich®) (0, 20 or 40 µM), a combination of sodium selenite and potassium iodide (0.8 µM sodium selenite and 0.5 µM potassium iodide), or sterile Milli-Q water for 72 hours with supplementation replenished every 24 hours. These concentrations represent the low, physiological and supraphysiological level as measured in maternal blood during pregnancy [3,30]. Explants were then treated with 120 µM menadione, 480 µM antimycin or 0.1% ethanol (vehicle for menadione and antimycin) for 24 hours. Menadione reduction can produce semiquinone radicals. Semiquinones are unstable and reactive substances that can make reactive oxygen species, therefore oxidative stress [31]. Antimycin can inhibit complex III in the mitochondrial respiratory chain, therefore reactive oxygen specious production increases and oxidative stress occurs [32]. Explants were harvested and fixed in 10% neutral buffered formalin for 2 hours at room temperature, then washed 3 times with

1X phosphate buffered saline (PBS) for 24 hours at 4°C and stored in 70% ethanol at 4°C until processing. Explants were processed and embedded in paraffin blocks for downstream assessments.

Micronutrient uptake

To evaluate if placenta tissue explants take up selenium and copper, three first trimester placentas from 7-12 weeks' gestation were cultured for 5 days including 48 hours syncytial layer regeneration (Jankovic-karasoulos et al, accepted pending revisions), and 72 hours of supplementation with (0, 0.8, 1.6 µM) sodium selenite or (0, 20, or 40 µM) copper (II) sulfate as above. Explants were then harvested, fixed and paraffin embedded. Tissue sections of 10 µm were placed on microscope slides. Sections were heated for 2 hours at 60°C, then dewaxed with 100% xylene and 100% ethanol and washed with PBS two times and finally air dried overnight. Standards were made by dissolving 10% gelatine (Sigma Aldrich®) in elements solutions of selenium and copper with 0, 1, 10, and 100 mM concentrations. Gelatine blocks were mounted in Tissue-Tek OCT Compound medium. Gelatine blocks were cut in 10 µm sections to match placenta explant sections using a cryomicrotome (Leica CM3050s Cryostat). After placing gelatine sections on the microscope slides, they were kept at -20°C. Gelatine sections were air dried overnight before laser ablation (LA) inductively coupled plasma-mass spectrometry (ICP-MS) analyses (Adelaide Microscopy, University of Adelaide), using an attached Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines with the following conditions: 23 µm spots size, 23 µm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for the following isotopes: ^{13}C, ^{23}Na, ^{24}Mg, ^{29}Mg, ^{31}P, ^{39}K, ^{43}Ca, ^{57}Fe, ^{65}Cu, ^{66}Zn and ^{77}Se. Only the elements of most interest (Se and Cu) are discussed in detail. Data was processed using the iolite data processing software, and concentrations were calculated and visualised relative to the

gelatine standards to form a 2D map of micronutrient concentration over a chosen surface of each placenta explant sample.

Assessment of proliferation, apoptosis and DNA damage

To assess the effect of supplementation on placental proliferation, apoptosis and DNA damage, explants from first trimester placentas (n=12) of 7-10 weeks' gestation were cultured as mentioned above. Tissue sections of 5 µm were placed on microscope slides for immunohistochemical labelling for assessment of proliferation (Ki67; Abcam®; ab16667), apoptosis (cleaved caspase-3; Cell Signalling Technology® ; CST.9661L) or DNA damage (8-hydroxy-2'-deoxyguanosine; Abcam®; ab48508) (Table 1). A Hamamatsu NanoZoomer Digital Pathology slide scanner was used to scan the stained sections. Eight areas per explant tissue, randomly chosen using NDP.view2 software, were used for quantification and statistical analyses. Positively stained cells per mm^2 / area were counted.

Table 2: Antibodies used for immunohistochemical labelling

Antibody	Dilution	Target Species	CAT #	Company	Diluent	Antigen Retrieval
Ki67	1/100	Rabbit	ab16667	Abcam®	5% Goat serum	Citrate buffer (10 mM Citric acid; pH 6.0; 10 min boiling in microwave, Sixth Sense, Whirlpool, VIC, Australia))
cleaved caspase-3	1/100	Rabbit	CST.9661L	Cell Signalling Technology®	5% Goat serum	Citrate buffer (10 mM Citric acid; pH 6.0; 10 min boiling in microwave)
8-hydroxy-2'-deoxyguanosine	1/200	Mouse	ab48508	Abcam®	5% Goat serum	Citrate buffer (10 mM Citric acid; pH 6.0; 10 min boiling in microwave)

Statistical analyses

Proliferation, apoptosis and DNA damage are expressed as a fold change (FC) relative to the control of each experiment ± standard error. To examine differences between micronutrient-treated and non-treated groups, Generalised Estimating Equations (GEE) were used with log2-transformed positive stain per area to estimate fold change compared to controls. Independence correlation structure was assumed for the GEE to account for measurements

from multiple regions per explant. Pre-specified post-hoc contrasts comparing individual sodium selenite and potassium iodide treatments with combination of sodium selenite and potassium iodide were performed with Bonferroni adjustment for multiple comparisons. P-values of less than 0.05 were considered significant. All analyses were performed using R version 3.5.3 or later.

Results

Confirmation of micronutrient uptake within the treated first trimester placenta explants

Qualitative assessment of micronutrient distributions with laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) element imaging showed that supplementation with selenium and copper increased the content of these micronutrients in the placenta explants confirming the micronutrient uptake by explants from the media supplemented with selenium and copper (Figure 1, Supplementary Figure 1 and 2). In all three placentas, highest uptake of selenium was found in tissues treated with 1.6 µM sodium selenate indicated by more red and yellow pixels in the LA-ICP-MS images (Figure 1D-F, Supplementary Figure 1D-F and Supplementary Figure 1J-L). In two placentas, it was apparent that with increasing selenium concentration, there was an increased uptake of selenium on the 2D map (Figure 1D-F and Supplementary Figure 1J-L). Assessment of tissues treated with copper (II) sulfate showed that control tissues had the lowest level of copper visualised by dark and light blue pixels. All tissues supplemented with 20 µM copper (II) sulfate and two tissues supplemented with 40 µM copper (II) sulfate (Figure 1J-L, Supplementary Figure 2D-F and Supplementary Figure 2J-L) had a higher content of copper compared to the control. This method was not able to assess iodine uptake but the evidence for selenium and copper uptake in the treated explants supports the likelihood of iodine uptake by the placenta tissues.

Apoptosis and DNA damage induction by menadione and antimycin

A dose response experiment (n = 3) was performed to find the optimal concentration of menadione and antimycin for inducing oxidative stress in first trimester human placenta tissue. The following concentrations of menadione (0, 60, 120, 240 µM) and antimycin (0, 240, 480, 960 µM) were tested. Immunohistochemical analysis for DNA damage using 8-hydroxy-2'-deoxyguanosine was used to select the optimal concentration that induced sufficient DNA damage compared to vehicle control in the absence of micronutrient supplementation. The concentrations selected were 120 µM for menadione and 480 µM for antimycin.

In the 12 first trimester placenta explants used in this study, immunohistochemistry analyses showed that compared to vehicle control (0.1 % ethanol) 120 µM menadione and 480 µM antimycin, in the absence of micronutrient supplementation, significantly increased the number of apoptotic cells and DNA damage ($p < 0.05$) (Figure 2 and Supplementary Figure 3). This confirmed that oxidative stress was induced in the cultured first trimester placenta explants and that this system could be used to assess whether micronutrient supplementation protects against induced oxidative stress.

Effect of selenium, iodine and copper supplementation on proliferation

Ki67 positivity was used to assess whether any of the micronutrients altered proliferation within the treated placenta explant tissues in the absence of oxidative stress. There were no changes in proliferation except for treatment with 1.6 µM sodium selenate which increased cell proliferation after 72 hours of supplementation (0.58, 95% CI: 0.25, 0.90) (Figure 3 A). When oxidative stress was induced, with menadione or antimycin, there were no changes in proliferation except for 1.6 µM selenium which again showed an increase in the number of

Ki67 positive cells per mm^2 after treatment with menadione (0.63, 95% CI: 0.31, 0.95) (Figure 3B), or antimycin (0.39, 95% CI: 0.11, 0.67) (Figure 3C) ($p < 0.05$).

Effect of selenium, iodine and copper supplementation on apoptosis

In the absence of oxidative stress, both 0.8 and 1.6 µM sodium selenite reduced apoptosis (-0.25, 95% CI: -0.44, -0.053 and -0.54, 95% CI: -0.83, -0.25, respectively) compared to control (sterile MilliQ water) (Figure 4A). Potassium iodide (0.5, 1 µM) significantly reduced apoptosis (-0.73, 95% CI: -1.06, -0.41 and -0.82, 95% CI: -1.18, -0.46, respectively) (Figure 4A). The combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide also reduced apoptosis compared to the vehicle control (-0.68, 95% CI: -0.96, -0.4) ($p < 0.05$) (Figure 4A). In addition, the effect of the combination of sodium selenite and potassium iodide significantly increased apoptosis compared to individual supplementation with 0.8 µM sodium selenite ($p < 0.05$) but not 0.5 µM potassium iodide. Treatment with 40 µM copper (II) sulfate significantly increased apoptosis (0.35, 95% CI: 0.11, 0.60) compared to control (Figure 4A).

Assessment of apoptosis following induction of oxidative stress by menadione showed that 1.6 µM sodium selenite (-1.04, 95%CI: -1.25, -0.84), both 0.5 µM (-1.34, 95%CI: -1.64, -1.04) and 1 µM potassium iodide (-1.28, 95%CI: -1.65, -0.91), and the combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide (-1.1, 95%CI: -1.32, -0.84) were associated with a significant reduction in apoptosis ($p < 0.05$) when compared to the menadione alone control (Figure 4B). Combination of sodium selenite and potassium iodide reduced apoptosis more than 0.8 µM sodium selenite ($p < 0.05$) but not more than potassium iodide (0.5 µM) alone (Figure 4B). Copper (II) sulfate did not significantly impact apoptosis ($p > 0.05$) (Figure 4B). Similar results were seen when oxidative stress was induced using antimycin (Figure 4C), that is, reduction in apoptosis with the higher dose of sodium selenite

(1.6 µM), both potassium iodide concentrations (0.5 and 1 µM) and sodium selenite and potassium iodide combination but no changes in apoptosis with copper (II) sulfate compared to the antimycin alone control (Figure 4C).

Effect of selenium, iodine and copper supplementation on DNA damage

Both concentrations of sodium selenite 0.8 µM (-0.4, 95% CI: -0.6, -0.16) and 1.6 µM (-0.65, 95% CI: -1.27, -0.02), potassium iodide 0.5 µM (-0.42, 95% CI: -0.83, -0.02), 1 µM (-0.43, 95% CI: -0.85, 0.01), and the combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide (-0.73, 95% CI: -1.18, -0.29) reduced DNA damage compared to control (sterile MilliQ water) following 72 hours of treatment ($p < 0.05$) (Figure 5A). However, the effect of the combination of sodium selenite and potassium iodide was not significantly different from individual supplementation with sodium selenite or potassium iodide ($p > 0.05$) (Figure 5A). There was a significant increase in DNA damage in cells treated with 40 µM copper (II) sulfate (0.63, 95% CI: 0.17, 1.09) (Figure 5A).

Assessment of DNA damage after oxidative stress induction with menadione showed that 1.6 µM sodium selenite (-0.92, 95% CI: -1.44, -0.41), both concentrations of potassium iodide: 0.5 µM (-0.87, 95% CI: -1.49, -0.24), 1 µM (-0.85, 95% CI: -1.42, -0.27) and the combination of sodium selenite and potassium iodide (-0.95, 95% CI: -1.41, -0.48) resulted in a significant reduction in DNA damage compared to the menadione alone control while copper (II) sulfate did not have any significant effect ($p > 0.05$) (Figure 5B). While the combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide significantly reduced DNA damage after menadione treatment, there was no effect with the individual supplementation of 0.8 µM sodium selenite ($p > 0.05$). In addition, no significant difference was found between supplementation with 0.5 µM potassium iodide and the combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide ($p > 0.05$) (Figure 5B). Similar results

were seen in DNA damage assessment of placenta explants treated with antimycin (Figure 5C).

Discussion

Selenium supplementation increased proliferation and reduced DNA damage and apoptosis in the absence or presence of oxidative stress. This can be explained by the effect of selenium on the cell cycle and the antioxidant system [33,34]. Selenium stimulates transition from Gap 2 to mitosis phase in cell cycle and this increases cell division or proliferation [34]. The cell cycle is precisely monitored and damaged DNA molecules can be detected at several checkpoints [35]. Although checkpoints in different cell cycle phases may work differently, the ultimate outcome is if the DNA molecule is not repaired the cell containing damaged DNA cannot proliferate and undergoes apoptosis [35]. GPx 4 and TRx are seleno-antioxidant enzymes that can protect cells against oxidative damage such as DNA damage, therefore reducing apoptosis [36-38]. Notably, when placenta explants were treated with menadione or antimycin the lower concentration of sodium selenite could not reduce DNA damage or apoptosis. This suggests that under conditions of oxidative stress, cells may require a higher concentration of selenium for protection against oxidative damage.

This study showed that potassium iodide could protect from oxidative damage to DNA molecules and reduced apoptosis. This is consistent with previous findings showing the protective effect of potassium iodide against oxidative stress in a placenta cell line [28,39]. Systemically, iodine is a radical scavenger as seen by its ability to metabolise H_2O_2 in thyroid hormone production [40]. The exact mechanisms by which iodine interacts with the antioxidant system in combating oxidative stress has not been clearly defined but studies have shown that iodine has antioxidant properties such as increasing total antioxidant status of human serum [41]. Iodine sufficient pregnant women (>150 µg/l urinary iodine

concentration) had higher activity of the enzyme SOD compared to iodine deficient women [24]. Interestingly, the combination of sodium selenite and potassium iodide resulted in a greater reduction in DNA damage in antimycin treated cells, compared to either micronutrient alone. This suggests that combining iodine and selenium may provide a stronger protection to the placenta against oxidative stress. Iodide and selenium can form selenenyl iodide that is a substrate for placental TRx and may increase antioxidant activity of TRx [42], thereby reducing DNA damage and apoptosis.

In a prospective cohort study of 1065 pregnant Australian women, a high level of maternal plasma copper was associated with higher plasma C-reactive protein (CRP) concentrations [3]. CRP is an inflammatory biomarker that increases in oxidative stress [43] and its higher levels are associated with a greater incidence of pregnancy complications such as preterm birth [44] and preeclampsia [45]. The results of this study showed that a high concentration of copper increased DNA damage and apoptosis. Excess intake of copper may increase production of reactive oxygen species and diminish antioxidant defence systems [46], resulting in DNA damage and apoptosis. Interestingly, in menadione or antimycin induced oxidative stress the toxic effect of excess copper was not apparent. To remove reactive molecules and protect the cells against oxidative stress, the antioxidant system is overexpressed and the activity of enzymes such as SOD1 increases [47]. Therefore, these enzymes may require higher levels of cofactors including copper. This avoids copper being accumulated and causing toxic effects.

In this *in vitro* study, gestational age did not make any difference in the effect of micronutrients on apoptosis and DNA damage. However, since oxygen pressure and levels of antioxidant enzymes may change from 7 to 10 week's [48] further research with more explants from different gestational ages is needed.

A strength of this study was the use of laser ablation inductively coupled plasma-mass spectrometry to show that first trimester human placental explants can take up selenium and copper from the media. To the best of our knowledge, this is the first study that has directly shown that selenium and copper supplementation increases their content and distribution in cultured placental explants. This method cannot confirm iodine uptake. Additionally, this is the first study that has investigated the effect of micronutrients on placental explants' proliferation, apoptosis and DNA damage in the absence or presence of oxidative stress, lending support to the important role of maternal micronutrient intake on placental development and hence pregnancy health.

In conclusion, selenium and iodine supplementation may protect the first trimester human placenta against oxidative stress. An excess intake of copper is related to oxidative damage to DNA and apoptosis. Since oxidative stress is involved in pregnancy complications, an optimal level of micronutrients may help to ensure healthy placental development and hence reduce the incidence of adverse pregnancy outcomes.

Authors' roles

The study was designed by N.H., T.B-M., C.T.R., T.J-K., J.A.G., A.V.P, S.J.Z., and performed by N.H. and T.B-M. Tissue collection was performed by D.M. Tissue culture and harvest was done by N.H. LA ICP-MS was performed by S.G. and N.H. Immunohistochemistry was optimised, performed and quantified by N.H., T.B-M., C.R. and A.L. Statistical analyses were performed by N.H. and S.Y-L.L. The original manuscript was drafted by N.H. All authors critically reviewed and revised the manuscript and approved the final version.

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Conflict of interest

The authors have no conflicts of interest.

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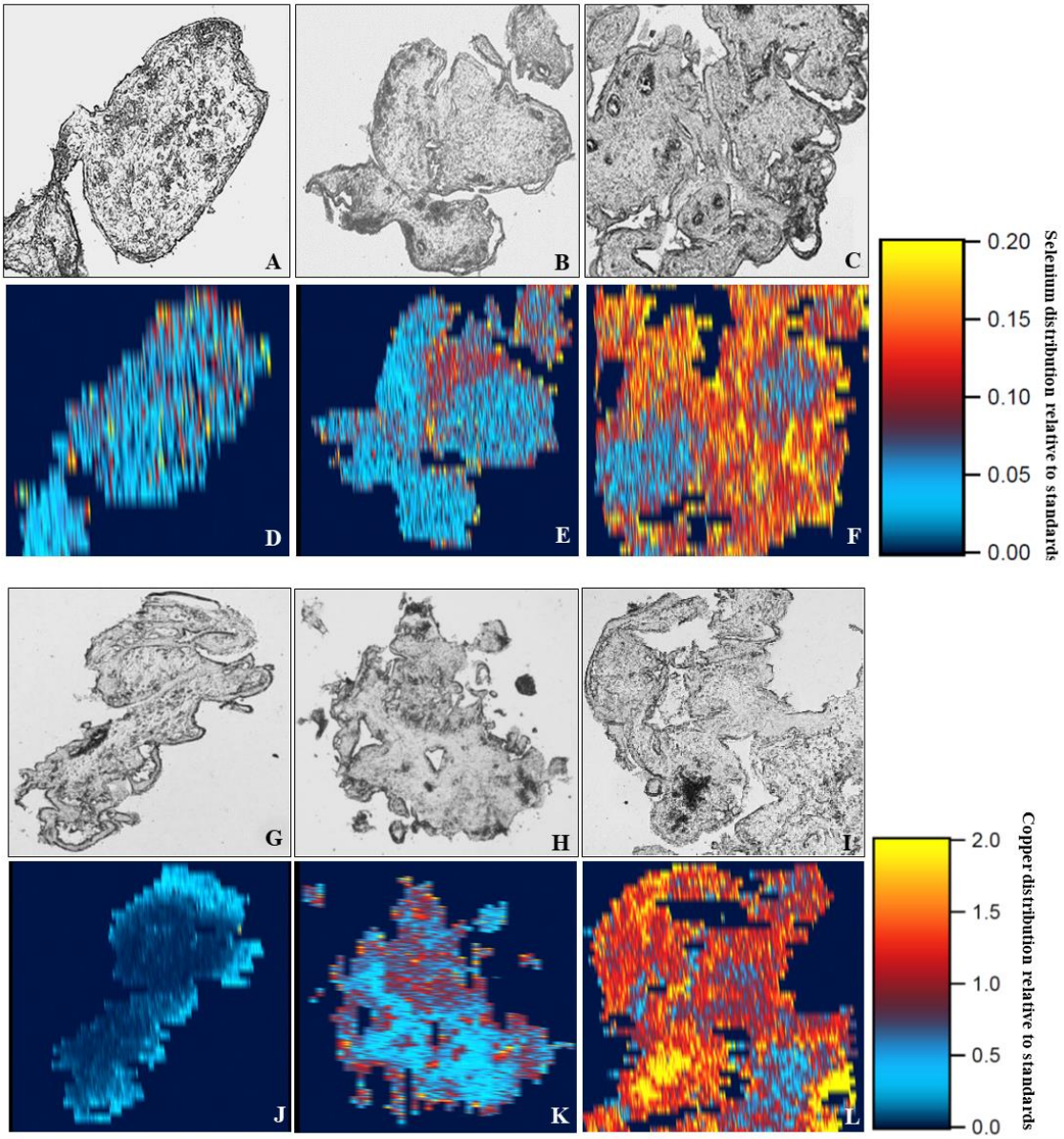


Figure 1. Selenium and copper uptake in first trimester human placental explants

Placenta explants (7-12 weeks of gestation) were supplemented with sodium selenite (A & D: sterile MilliQ water, B & E: 0.8 µM or C & F: 1.6 µM) or copper (II) sulfate (G & J: sterile MilliQ water, H & K: 20 µM or I & L: 40 µM) for 72 hours. Tissue sections (10 µm) were placed on microscope slides. Standards were made by dissolving 10% gelatine in 0, 1, 10, and 100 mM solutions of selenium and copper. Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) analyses on the standard gelatine and placental explant sections were analysed using a Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines: 23 µm spots size, 23 µm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for each isotope. Data was processed using the iolite data processing software, and element intensity calculated relative to the gelatine standards to form a 2D map of micronutrient distribution over a chosen surface of each placental explant sample.

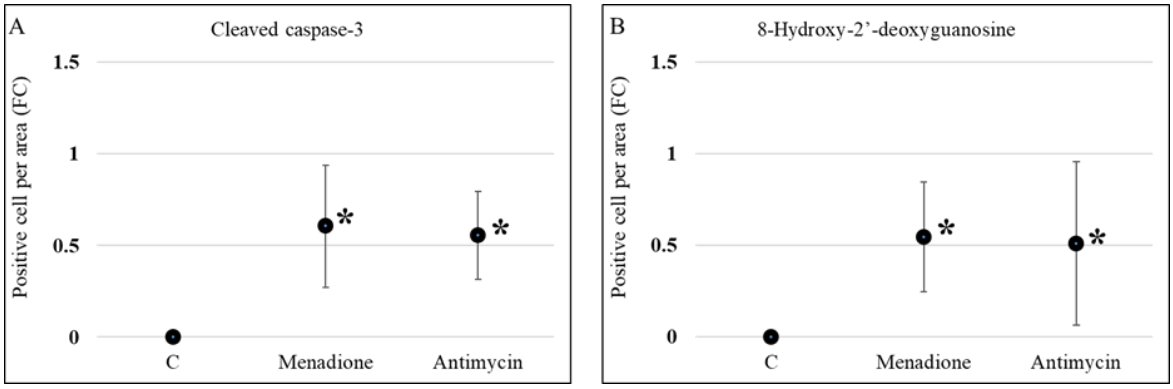
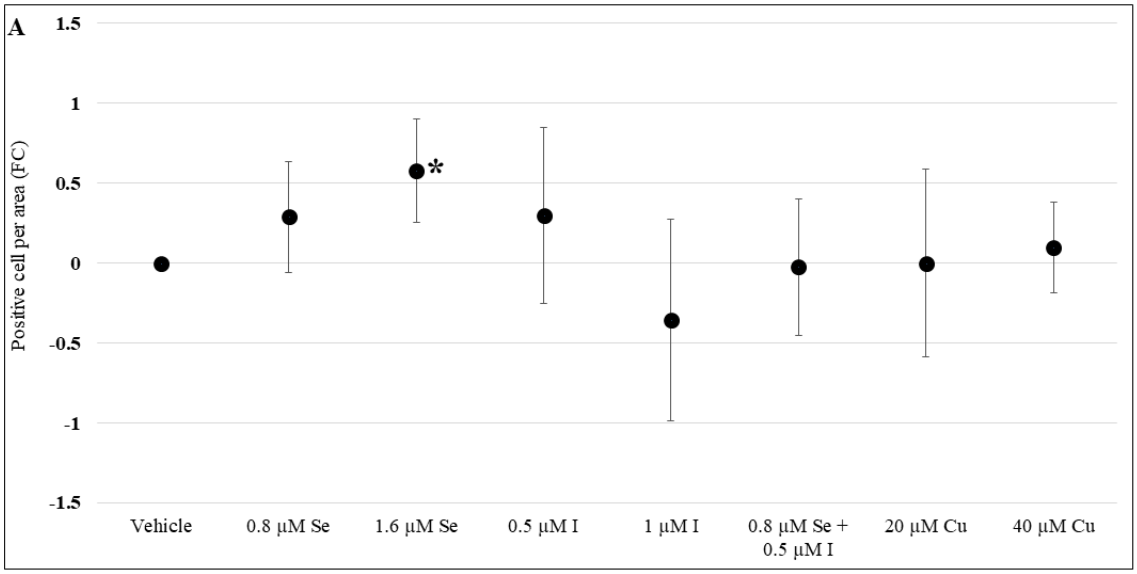
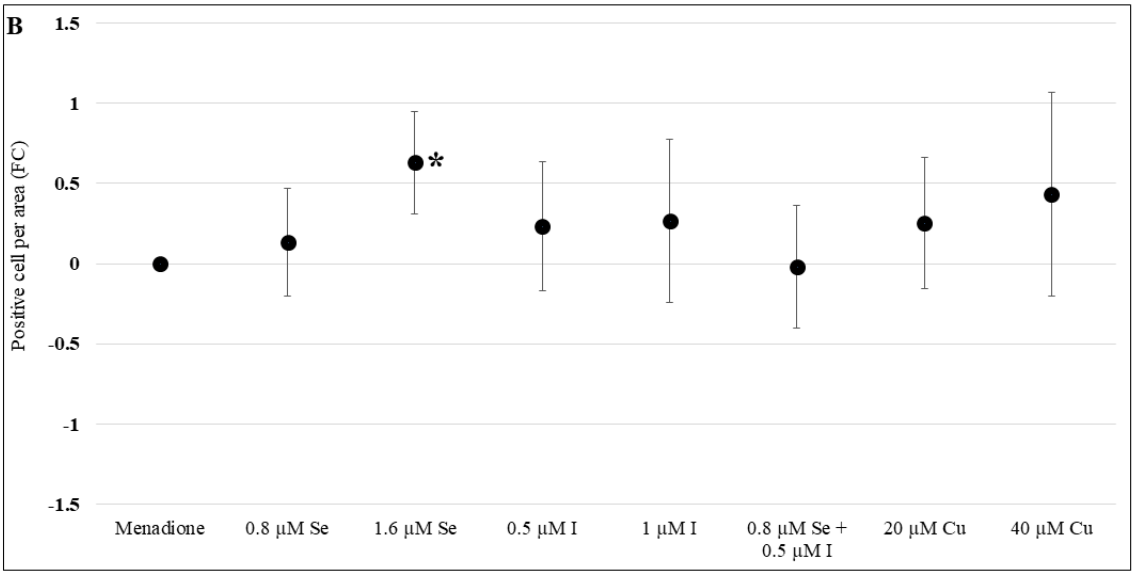


Figure 2. Effect of menadione and antimycin on apoptosis and DNA damage in first trimester human placental explants

Explants from 7-12 weeks of gestation (n=12) were cultured for 5 days in media with 10% v/v FBS and 1% v/v Antibiotic-Antimycotic and then treated with 120 µM menadione, 480 µM antimycin or 0.1% ethanol (vehicle control) for 24 hours. Immunohistochemical labelling for apoptosis (cleaved caspase-3) or DNA damage (8-hydroxy-2'-deoxyguanosine) was performed. Eight randomly selected regions per explant were used for quantification and statistical analyses. Data are presented as a fold change (FC) relative to control ± standard error. Statistical significance was assessed using Generalised Estimating Equations with independence correlation structure with log 2-transformed positive stain per area to estimate fold change compared to controls followed by pre-specified post-hoc Bonferroni adjusted multiple comparisons. * Indicates statistically different ($P < 0.05$) from control.


A
1.5
1
0.5
0
-0.5
-1
-1.5
Positive cell per area (FC)
*
Vehicle
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu


B
1.5
1
0.5
0
-0.5
-1
-1.5
Positive cell per area (FC)
*
Menadione
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu

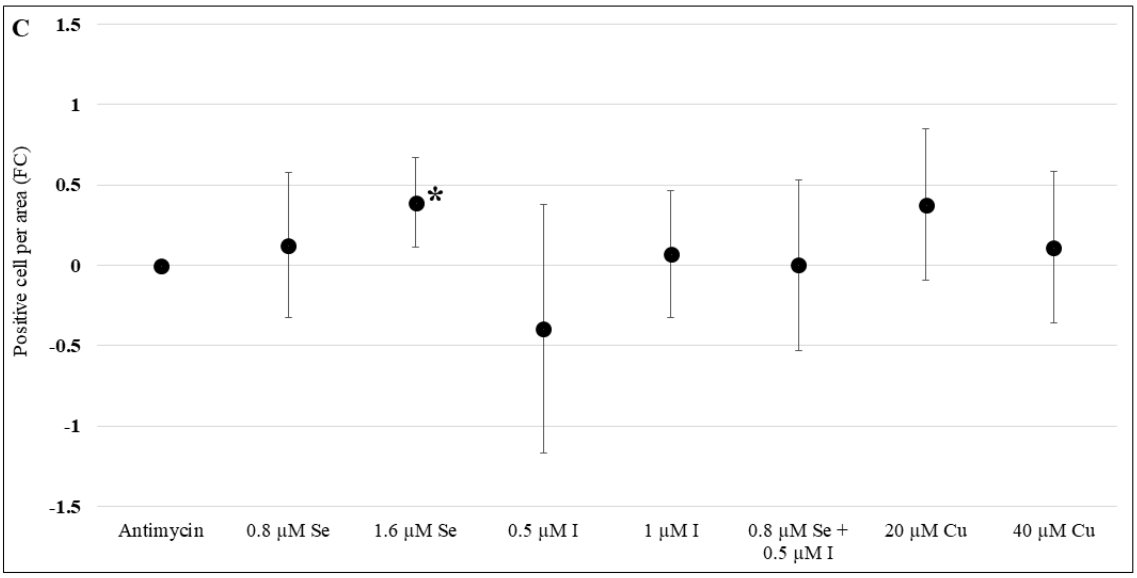
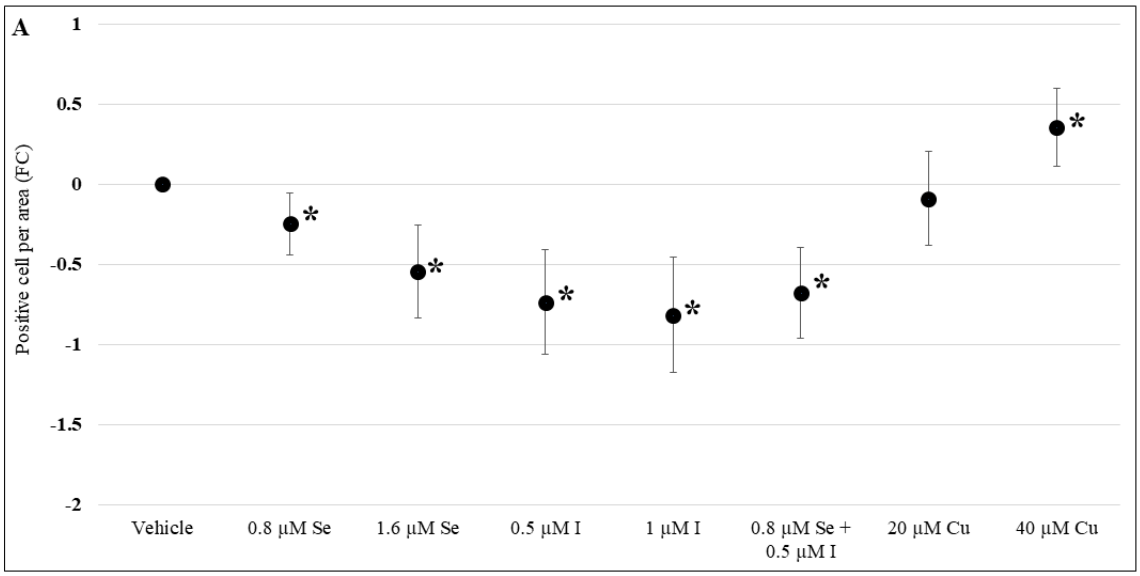
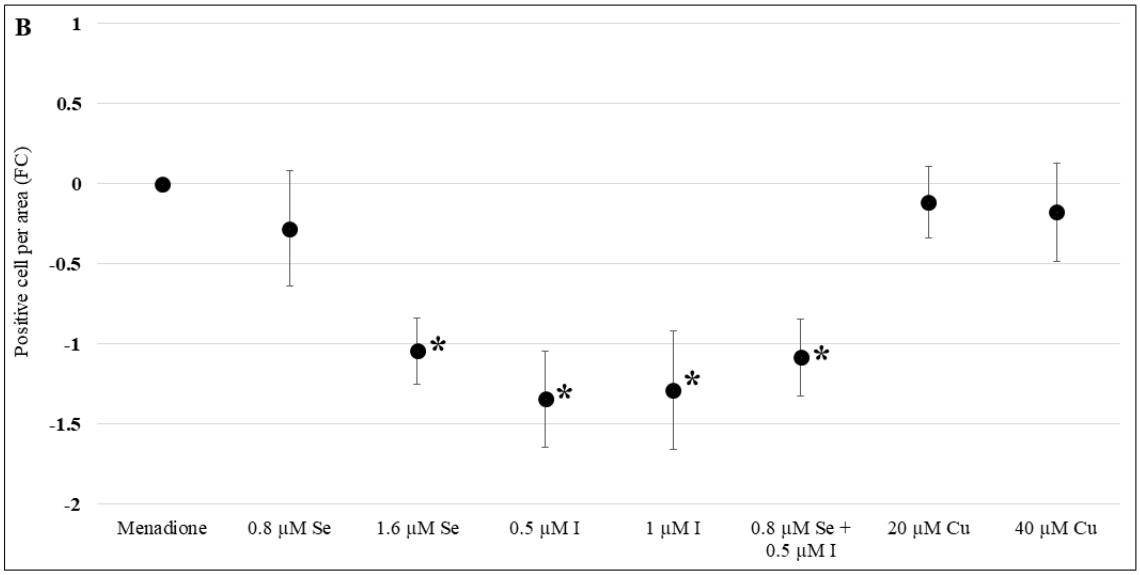

C
1.5
1
0.5
0
-0.5
-1
-1.5
Positive cell per area (FC)
*
Antimycin
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu

Figure 3. Effect of selenium, iodine and copper supplementation on proliferation in first trimester placental explants

Placenta explants from 7-12 weeks of gestation (n=12) were cultured for 48 hours for syncytial regeneration followed by supplementation with sodium selenite (0, 0.8 or 1.6 µM), potassium iodide (0, 0.5 or 1 µM), combination of sodium selenite and potassium iodide (0.8 µM sodium selenite and 0.5 µM potassium iodide), or copper (II) sulfate (0, 20 or 40 µM) for 72 hours with supplementation replenished every 24 hours. (A) Placental explants were harvested following 72 hours of supplementation and proliferation assessed by immunolabelling for Ki67. In addition, after 72 hours supplementation, placental explants were treated with B) 120 µM menadione or C) 480 µM antimycin for 24 hours to induce oxidative stress and then assessed for proliferation. Eight randomly selected regions per explant were used for quantification and statistical analyses. Data presented as a fold change (FC) relative to control ± standard error. Statistical significance was assessed using Generalised Estimating Equations with independence correlation structure with log 2-transformed positive stain per area to estimate fold change compared to controls followed by pre-specified post-hoc Bonferroni adjusted comparisons. * Indicates statistically different ($P < 0.05$) from vehicle control (C).


A
Positive cell per area (FC)
1
0.5
0
-0.5
-1
-1.5
-2
Vehicle
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu


B
Positive cell per area (FC)
1
0.5
0
-0.5
-1
-1.5
-2
Menadione
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu

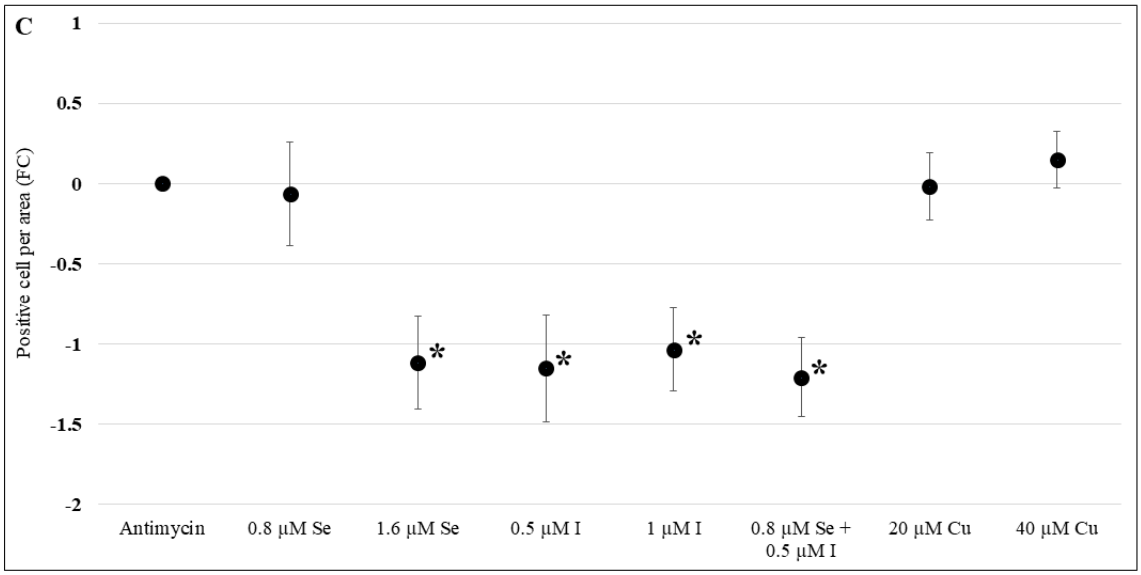
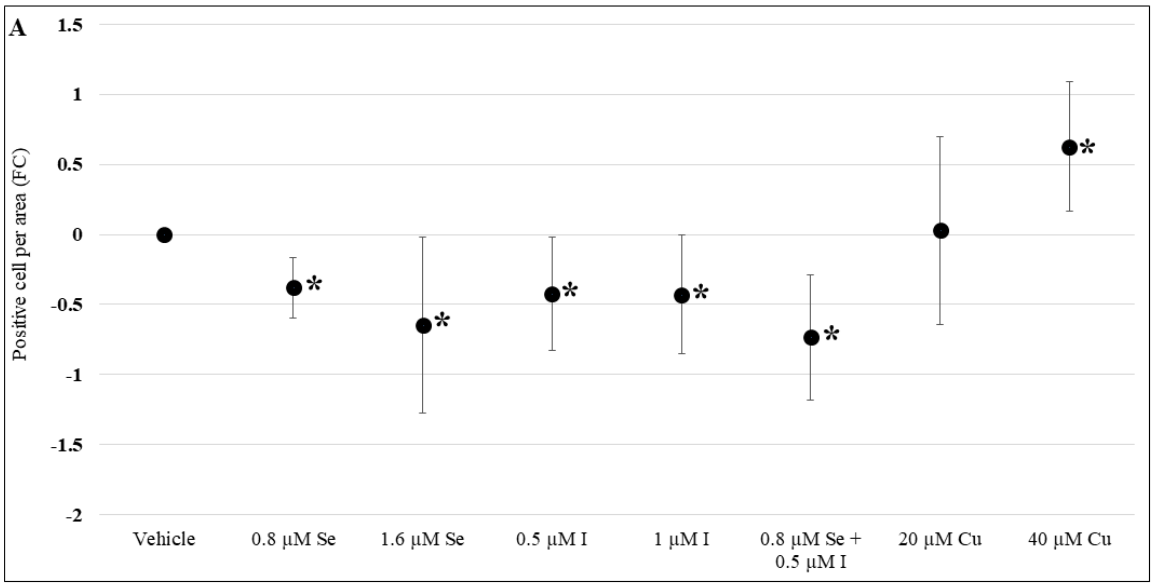
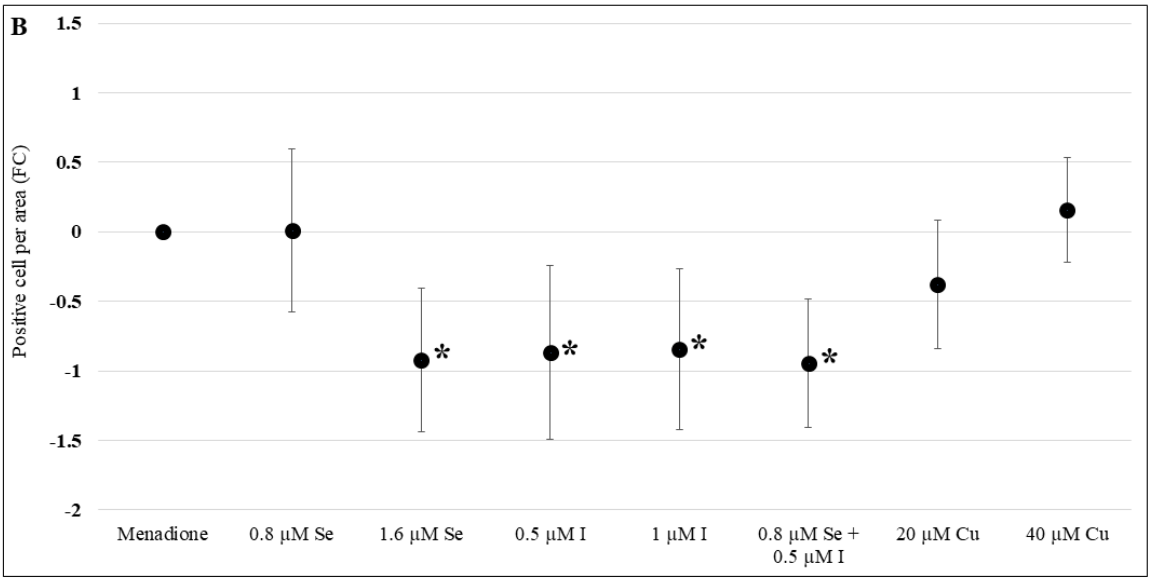

C
Positive cell per area (FC)
1
0.5
0
-0.5
-1
-1.5
-2
Antimycin
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu

Figure 4. Effect of selenium, iodine and copper supplementation on apoptosis in first trimester placental explants

Placenta explants from 7-12 weeks of gestation (n=12) were cultured for 48 hours for syncytial regeneration followed by supplementation with sodium selenite (0, 0.8 or 1.6 µM), potassium iodide (0, 0.5 or 1 µM), combination of sodium selenite and potassium iodide (0.8 µM sodium selenite and 0.5 µM potassium iodide), or copper (II) sulfate (0, 20 or 40 µM) for 72 hours with supplementation replenished every 24 hours. (A) Placental explants were harvested at the end of 72 hours supplementation and apoptosis assessed by immunolabelling for cleaved caspase-3. In addition, after 72 hours supplementation, placental explants were treated with B) 120 µM menadione or C) 480 µM for 24 hours to induce oxidative stress and then assessed for apoptosis. Eight randomly selected regions per explant were used for quantification and statistical analyses. Data presented as a fold change (FC) relative to control ± standard error. Statistical significance was assessed using Generalised Estimating Equations with independence correlation structure with log 2-transformed positive stain per area to estimate fold change compared to controls followed by pre-specified post-hoc Bonferroni adjusted comparisons. * Indicates statistically different ($P < 0.05$) from control.


A
Positive cell per area (FC)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
Vehicle
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu


B
Positive cell per area (FC)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
Menadione
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu

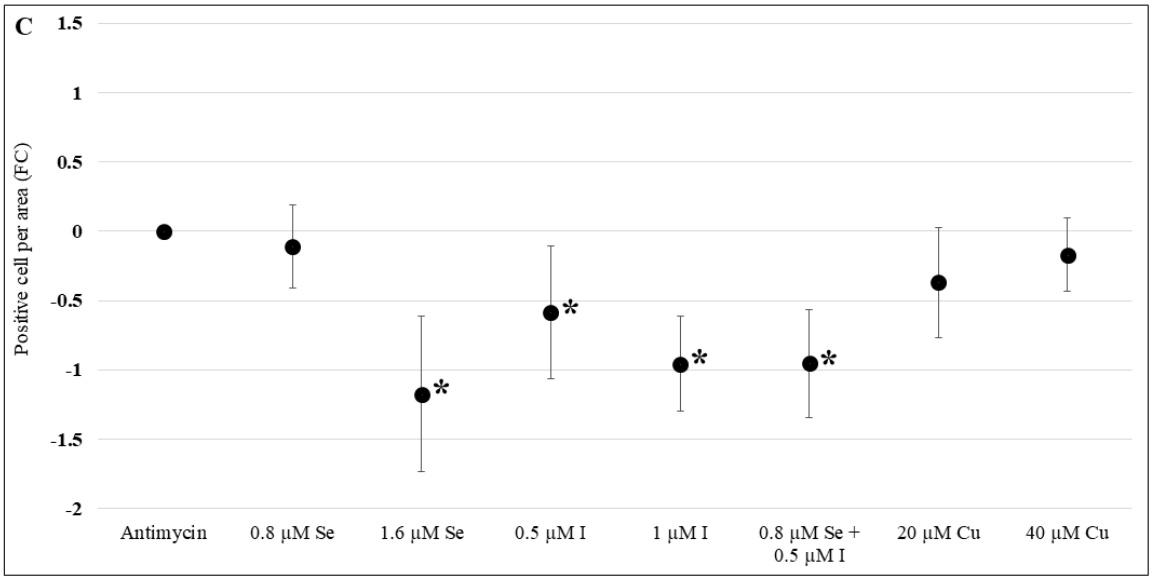
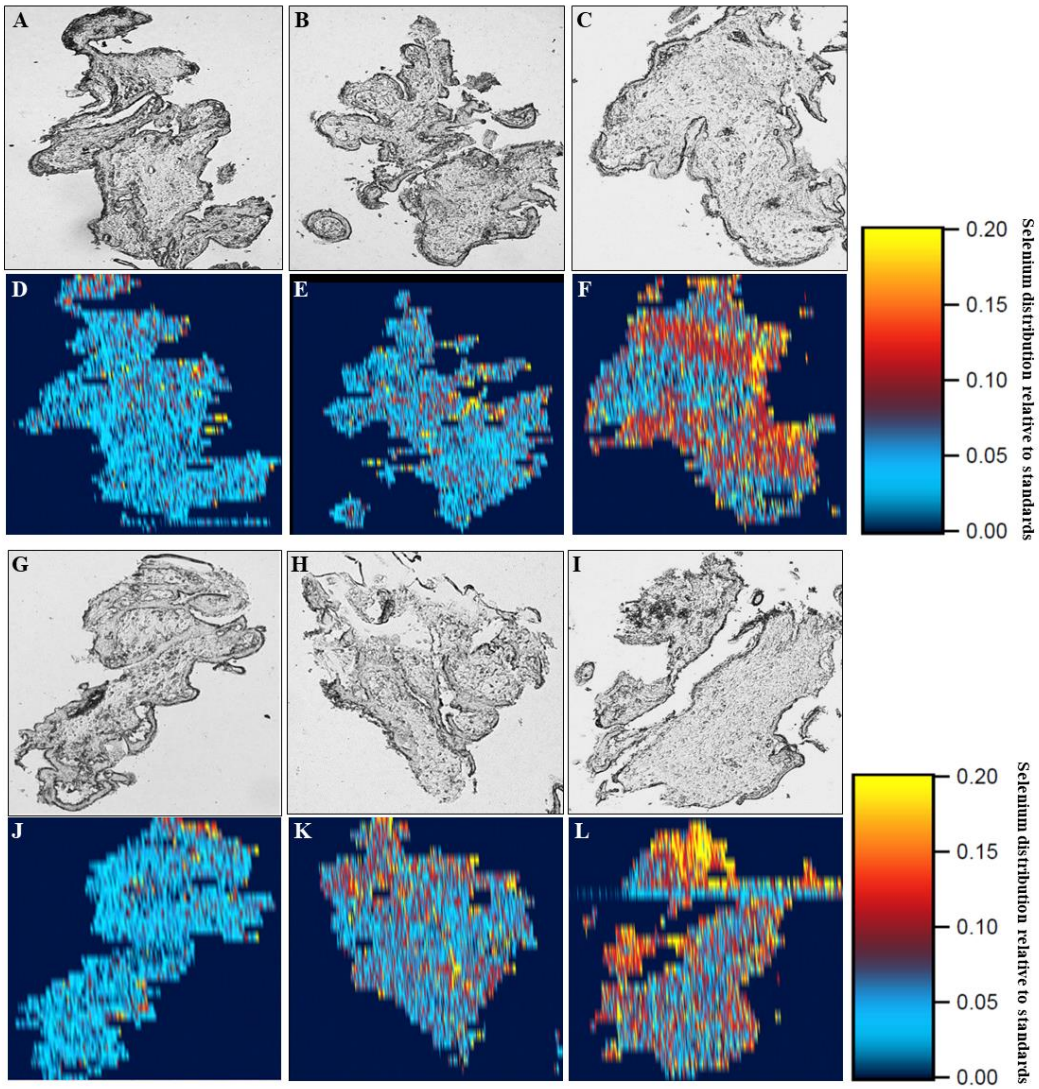
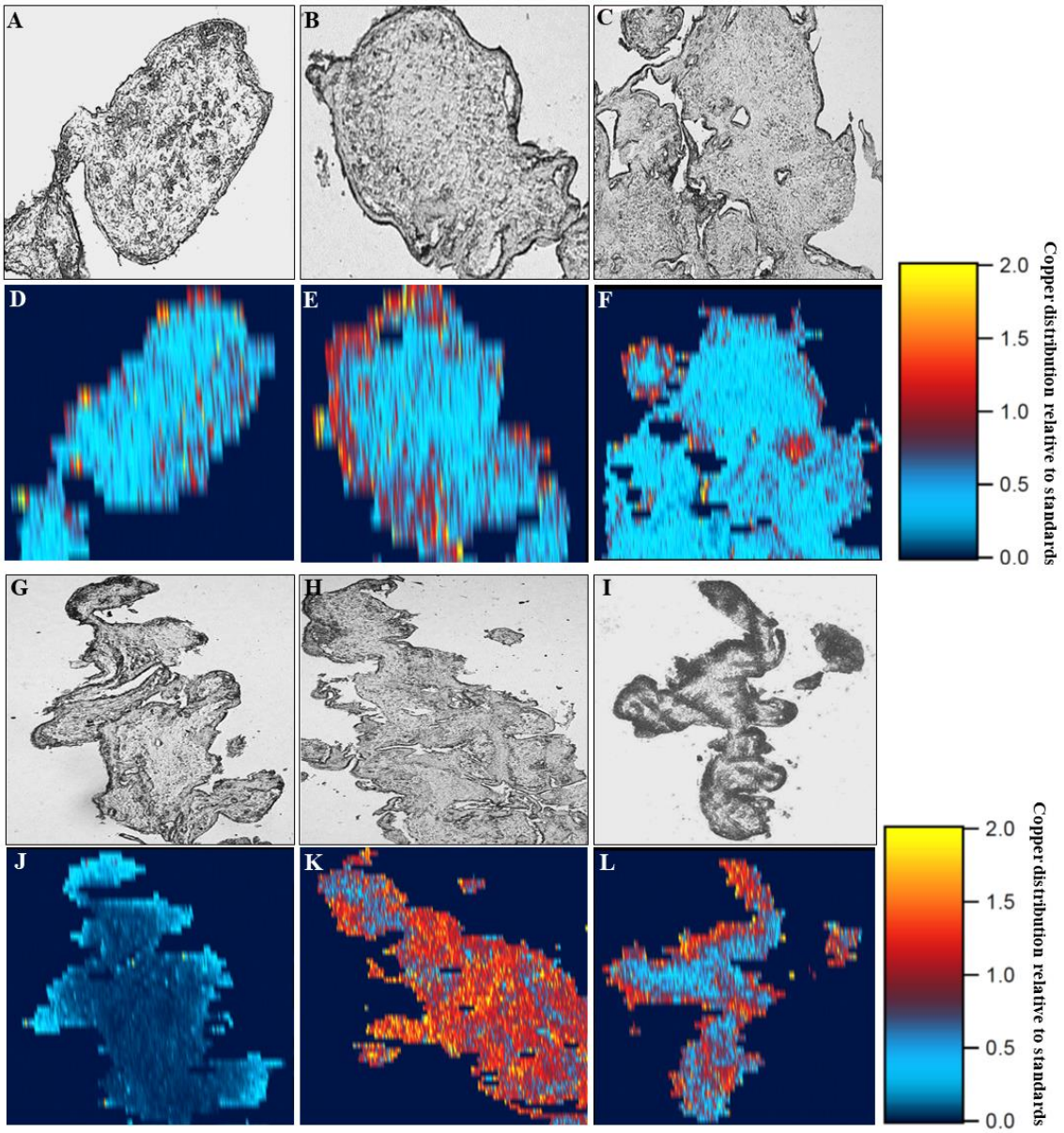

C
Positive cell per area (FC)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
Antimycin
0.8 µM Se
1.6 µM Se
0.5 µM I
1 µM I
0.8 µM Se + 0.5 µM I
20 µM Cu
40 µM Cu

Figure 5. Effect of selenium, iodine and copper supplementation on DNA damage in first trimester human placental explants

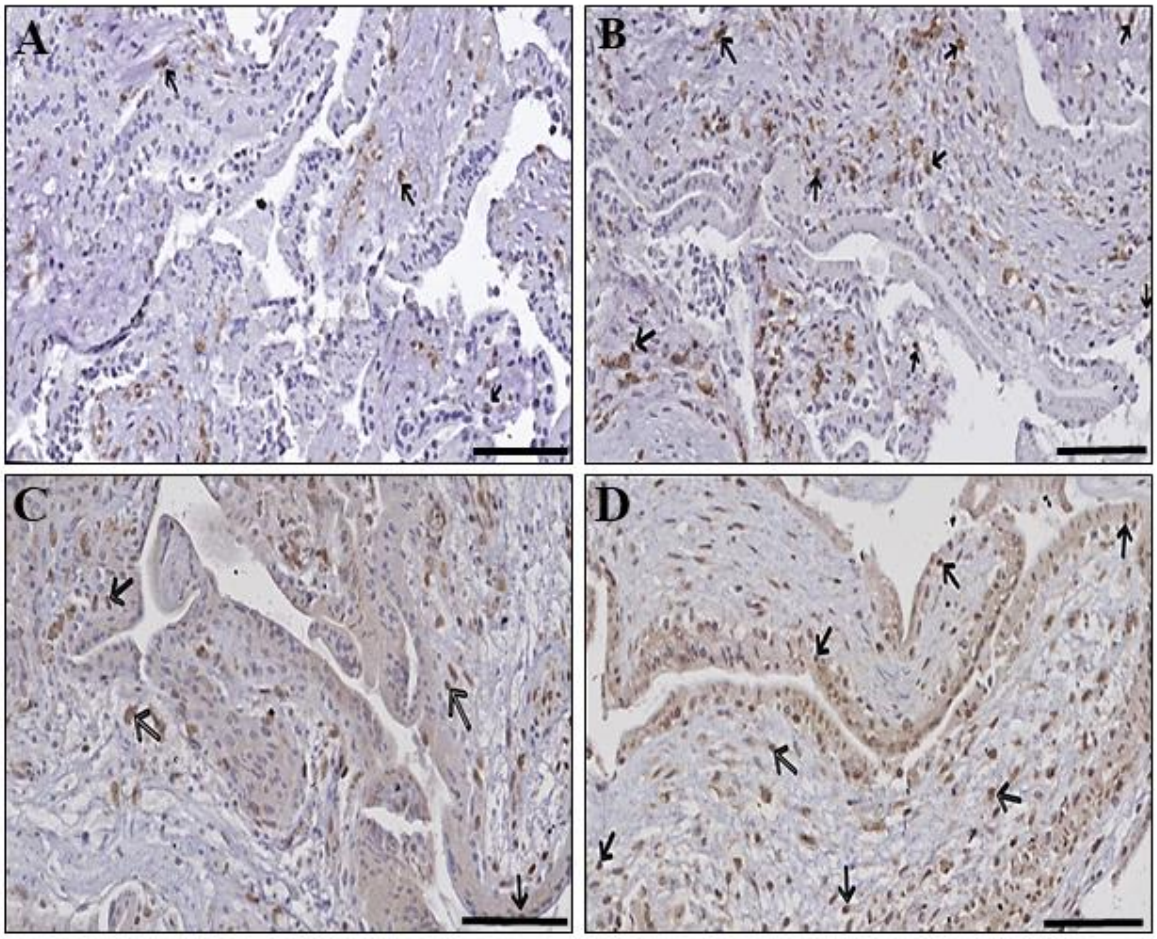
Placenta explants from 7-12 weeks of gestation (n=12) were cultured for 48 hours for syncytial regeneration followed by supplementation with sodium selenite (0.8 or 1.6 µM), potassium iodide (0.5 or 1 µM), combination of sodium selenite and potassium iodide (0.8 µM sodium selenite and 0.5 µM potassium iodide), or copper (II) sulfate (20 or 40 µM) for 72 hours with supplementation replenished every 24 hours. (A) Placental explants were harvested following 72 hours of supplementation and DNA damage assessed by immunolabelling for 8-hydroxy-2'-deoxyguanosine. After 72 hours of supplementation, placental explants were treated with B) 120 µM menadione or C) 480 µM for 24 hours to induce oxidative stress and then assessed for DNA damage. Eight randomly selected regions per explant were used for quantification and statistical analyses. Data presented as a fold change (FC) relative to control ± standard error. Statistical significance was assessed using Generalised Estimating Equations (GEE) with independence correlation structure with log 2-transformed positive stain per area to estimate fold change compared to controls followed by pre-specified post-hoc Bonferroni adjusted comparisons. * Indicates statistically different ($P < 0.05$) from control.



Supplementary Figure 1. Selenium uptake in first trimester human placental explants
Placenta explants from 7-12 weeks of gestation were supplemented with sodium selenite (A & D & G & J: sterile MilliQ water, B & E & H & K: 0.8 µM or C & F & I & L: 1.6 µM) for 72 hours. Tissue sections were cut at 10 µm and placed on microscope slides. Standards were made by dissolving 10% gelatine in solutions of selenium and copper with 0, 1, 10, and 100 mM concentrations. Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) analyses on the standard gelatine and placental explant sections were analysed at Adelaide Microscopy, University of Adelaide, using a Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines: 23 µm spots size, 23 µm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for each isotope. Data was processed using the iolite data processing software, and element intensity calculated relative to the gelatine standards to form a 2D map of micronutrient distribution over a chosen surface of each placental explant sample.



Supplementary Figure 2. Copper uptake in first trimester human placental explants
Placenta explants from 7-12 weeks of gestation were supplemented with copper (II) sulfate (A & D & G & J: sterile MilliQ water, B & E & H & K: 20 µM or C & F & I & L: 40 µM) for 72 hours. Tissue sections were cut at 10 µm and placed on microscope slides. Standards were made by dissolving 10% gelatine in solutions of selenium and copper with 0, 1, 10, and 100 mM concentrations. Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) analyses on the standard gelatine and placental explant sections were analysed at Adelaide Microscopy, University of Adelaide, using a Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines: 23 µm spots size, 23 µm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for each isotope. Data was processed using the iolite data processing software, and element intensity calculated relative to the gelatine standards to form a 2D map of micronutrient distribution over a chosen surface of each placental explant sample.



Supplementary Figure 3. Apoptosis and DNA damage induction by menadione in first trimester human placental explants

First trimester placenta tissues were cultured for 5 days in media including 10% v/v FBS and 1% v/v Antibiotic-Antimycotic and then treated with A) and C) 0.1% ethanol (vehicle control) or B) and D) 120 µM menadione for 24 hours. Immunohistochemical labelling for A) and B) apoptosis (cleaved caspase-3) or C) and D) DNA damage (8-hydroxy-2'-deoxyguanosine) was performed. Stained slides were scanned with a Hamamatsu NanoZoomer Digital Pathology slide scanner and NDP.view2 software were used for analyses. Black arrows indicate positively stained cells. Scale bar is 100 µm.

Selenium and iodine supplementation protect first trimester human placenta against oxidative stress

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Subject Section:	Early Pregnancy

Selenium and iodine supplementation protect first trimester human placenta against oxidative stress

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Running title: Selenium and iodine reduce oxidative stress

Abstract

Study question: Can the micronutrients selenium, iodine and copper protect human first trimester placenta from oxidative stress in?

Summary answer: Selenium and iodine, alone or in combination, can protect first trimester human placenta against oxidative stress. Copper was not protective.

What is known already: Adequate maternal nutrition is vital for placental development. Poor placentation, imbalanced maternal micronutrient status and placental oxidative stress are associated with greater risk of pregnancy complications, which impact mother and infant health. Selenium, iodine and copper are essential micronutrients with key roles in antioxidant systems.

Study design, size, duration: First trimester (7-12 weeks of gestation) human placenta explants (n=15) were treated with a range of concentrations of selenium, iodine, selenium and iodine or copper. The concentrations represented deficient, physiological or high dose. After treatment, placenta explants were collected for assessment of proliferation, apoptosis and DNA damage.

Participants/materials, setting, methods: Micronutrient treated placenta explants (n=3) were harvested, fixed, processed and embedded in paraffin blocks to be used for laser ablation inductively coupled plasma-mass spectrometry (LA ICP-MS) to assess selenium and copper uptake. Additional placenta explants (n=12) were supplemented with sodium selenite (0, 0.8 or 1.6 μM), potassium iodide (0, 0.5 or 1 μM), a combination of sodium selenite and potassium iodide, or copper (II) sulphate (0, 20 or 40 μM) for 72 hours and 120 μM menadione or 480 μM antimycin for 24 hours to induce oxidative stress. Immunohistochemical labelling was performed for assessment of proliferation (Ki67), apoptosis (cleaved caspase-3), and DNA damage (8-hydroxy-2'-deoxyguanosine).

Main results and the role of chance: LA-ICP-MS element imaging showed that placenta explants could uptake selenium and copper from the media. Sodium selenite (1.6 μM) increased cell proliferation ($p < 0.05$). Sodium selenite and potassium iodide reduced DNA damage and apoptosis ($p < 0.05$). Following oxidative stress induction, a higher concentration of sodium selenite (1.6 μM) was needed to reduce DNA damage and apoptosis while both concentrations of potassium iodide (0.5 and 1 μM) were protective ($p < 0.05$). A high concentration of copper (40 μM) increased apoptosis and DNA damage ($p < 0.05$) but this effect was no longer significant after induction of oxidative stress by menadione or antimycin ($p > 0.05$).

Limitations, reasons for caution: This study was only conducted in first trimester human placenta tissue therefore it is unknown if the impact of these micronutrients on oxidative stress in the placenta would be the same had the treatments occurred at different time points.

Wider implications of the findings: Excess oxidative stress deleteriously impacts human placental tissue resulting in increased apoptosis and oxidative damage to DNA molecules. Selenium and iodine, alone and in combination, can protect the placenta against oxidative stress. Therefore, an optimal maternal level of micronutrients may be essential for placenta health and hence offspring health.

Study funding/competing interest(s): This research was supported by National Health and Medical Research Council (NHMRC) GNT1161079 Targeting micronutrients to tackle pregnancy disorders: an integrated approach awarded to CTR, SJZ, TVP, JAG, SYL and TBM. CTR is supported by a NHMRC Investigator Grant GNT1174971 and a Flinders University Matthew Flinders Fellowship. The authors have no conflicts of interest.

Trial registration number: NA

Key words: copper; iodine; oxidative stress; placenta; selenium

Introduction

Maternal nutrition during early pregnancy is critical for fetal growth and development and can impact the future health of the offspring later in life (Grieger and Clifton, 2014, Tahir, et al., 2019). A deficiency or excess in micronutrients such as selenium, copper, and iodine measured in maternal plasma, serum or urine are associated with a greater risk of pregnancy complications such as preeclampsia, gestational diabetes mellitus, spontaneous preterm birth and small-for-gestational age (Charoenratana, et al., 2016, Mistry, et al., 2008, Molnar, et al., 2008, Wilson, et al., 2018a, Wilson, et al., 2018b). The detrimental effects of pregnancy complications are not limited to the pregnancy as their long-term consequences can impact the health of both mother and child later in life (Leeson, 2013, Neiger, 2017, Pisaneschi, et al., 2013). Thus, it is of paramount importance to determine the nutritional risk factors related to pregnancy complications and devise any potential preventive strategies.

The placenta is the key mediator of maternal nutrient supply to the fetus. Poor placental development, including incomplete remodelling of arteries during early pregnancy, has been associated with pregnancy complications like preeclampsia (Falco, et al., 2017). Partial remodelling of the maternal arteries causes hypoxia followed by reoxygenation which results in oxidative stress (Roberts and Escudero, 2012). Oxidative stress is frequently associated with a range of pregnancy complications (Fujimaki, et al., 2011, Wiktor, et al., 2004). Oxidative stress is caused by accumulated reactive oxygen molecules and insufficient antioxidant activity (Lobo, et al., 2010, Shackelford, et al., 2000), and results in damage of molecules such as lipids, proteins and DNA, thus tissue decay (Lobo, Patil, Phatak and Chandra, 2010). In addition, oxidative stress can increase apoptosis and this leads to high shedding of syncytial fragments into maternal blood resulting in a systemic inflammatory response, which is associated with pregnancy complications (Ishihara, et al., 2002).

Micronutrients and oxidative stress are related through the antioxidant system (Friedmann Angeli and Conrad, 2018, Mondola, et al., 2016). For instance, glutathione peroxidase (GPx) and superoxide dismutase 1 (SOD1) are two antioxidant enzymes that require micronutrients for proper function (Friedmann Angeli and Conrad, 2018, Mondola, Damiano, Sasso and Santillo, 2016). GPx needs selenium and SOD1 requires copper or zinc to scavenge radicals and prevent oxidative stress (Friedmann Angeli and Conrad, 2018, Mondola, Damiano, Sasso and Santillo, 2016). Studying micronutrient status in relation to placental development and oxidative stress may help elucidate the mechanisms involved in pregnancy complications. Although population studies have shown that selenium supplementation is associated with a lower incidence of preeclampsia and premature rupture of membranes (Rayman, et al., 2015, Rayman, et al., 2014, Tara, et al., 2010), the impact of micronutrients on placental development is still unknown. In addition, there is no information on

potential interactions between selenium and other micronutrients. Iodine is an essential micronutrient and its deficiency during pregnancy puts both mother and offspring at a greater risk of pregnancy complications such as gestational hypertension, intrauterine growth restriction and preterm birth (Borekci, et al., 2009, Charoenratana, Leelapat, Traisrisilp and Tongsong, 2016, Cuellar-Rufino, et al., 2017, Dillon and Milliez, 2000, Gulaboglu, et al., 2007, Vidal, et al., 2014). We recently showed that selenium and iodine deficiency are associated with lower cell proliferation and higher cell death and lipid peroxidation in HTR8/SVneo trophoblast cells (Habibi, et al., 2020). In addition, individual or combined supplementation with selenium and iodine protected trophoblast cells against oxidative stress by enhancing cell viability and proliferation and reducing lipid peroxidation (Habibi, Jankovic-Karasoulos, Leemaqz, Francois, Zhou, Leifert, Perkins, Roberts and Bianco-Miotto, 2020).

Copper is another essential component in a variety of metalloenzymes (Fontecave and Pierre, 1998) including the antioxidant SOD1 enzyme (Mondola, Damiano, Sasso and Santillo, 2016). High copper levels in maternal serum have been associated with a greater level of inflammation (Wilson, Bianco-Miotto, Leemaqz, Grzeskowiak, Dekker and Roberts, 2018a). Investigating how copper may impact the placenta will help to understand its role and impact on pregnancy outcome. This study investigated how selenium, iodine and copper may impact oxidative stress response in first trimester human placenta by assessing their effect on proliferation, apoptosis, and DNA damage.

Materials and methods

Placenta explant tissue culture

First trimester (7-12 weeks' gestation) human placenta tissue samples (n=15) were collected with informed consent from women who underwent elective pregnancy terminations at the Pregnancy Advisory Centre, Woodville, South Australia. Ethics approval was granted by the Queen Elizabeth Hospital Human Research Ethics Committee (HREC/16/TQEH/33).

Within an hour of termination, 10-15 mg of placenta tissue sections (3-4 pieces) were cultured on a pre-prepared gel base in each well of a 48-well plate. The gel base was Growth Factor Reduced Matrigel® (protein concentration: 3 mg/ml; Corning®) and 1X DMEM GlutaMAX™ media (Gibco® Life Technologies™) containing 10% v/v FBS (Sigma-Aldrich) and 1% v/v Antibiotic-Antimycotic (Life Technologies™) at pH 7.0. Culture conditions were maintained at 37°C, 5% CO_2 and 1% O_2 for the duration of the experiments. Placenta explants were first cultured for 48 hours to enable regeneration of the syncytial layer. Explants were supplemented with sodium selenite (Sigma Aldrich®) (0, 0.8 or 1.6 µM), potassium iodide (Sigma Aldrich®) (0, 0.5 or 1 µM), copper (II) sulfate (Sigma Aldrich®) (0, 20 or 40 µM), a combination of sodium selenite and potassium iodide (0.8 µM sodium selenite and 0.5 µM potassium iodide), or sterile Milli-Q water for 72 hours with

supplementation replenished every 24 hours. These concentrations represent the low, physiological and supraphysiological level as measured in maternal blood during pregnancy (Pan, et al., 2019, Wilson, Bianco-Miotto, Leemaqz, Grzeskowiak, Dekker and Roberts, 2018a). Explants were then treated with 120 μM menadione, 480 μM antimycin or 0.1% ethanol (vehicle for menadione and antimycin) for 24 hours. Explants were harvested and fixed in 10% neutral buffered formalin for 2 hours at room temperature, then washed 3 times with 1X phosphate buffered saline (PBS) for 24 hours at 4^{o}C and stored in 70% ethanol at 4^{o}C until processing. Explants were processed and embedded in paraffin blocks for downstream assessments.

Micronutrient uptake

To evaluate if placenta tissue explants take up selenium and copper, three first trimester placentas from 7-12 weeks' gestation were cultured for 5 days including 48 hours syncytial layer regeneration, and 72 hours of supplementation with (0, 0.8, 1.6 μM) sodium selenite or (0, 20, or 40 μM) copper (II) sulfate as above. Explants were then harvested, fixed and paraffin embedded. Tissue sections of 10 μm were placed on microscope slides. Sections were heated for 2 hours at 60^{o}C, then dewaxed with 100% xylene and 100% ethanol and washed with PBS two times and finally air dried overnight. Standards were made by dissolving 10% gelatine (Sigma Aldrich®) in elements solutions of selenium and copper with 0, 1, 10, and 100 mM concentrations. Gelatine blocks were mounted in Tissue-Tek OCT Compound medium. Gelatine blocks were cut in 10 μm sections to match placenta explant sections using a cryomicrotome (Leica CM3050s Cryostat). After placing gelatine sections on the microscope slides, they were kept at -20^{o}C. Gelatine sections were air dried overnight before laser ablation (LA) inductively coupled plasma-mass spectrometry (ICP-MS) analyses (Adelaide Microscopy, University of Adelaide), using an attached Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines with the following conditions: 23 μm spots size, 23 μm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for the following isotopes: ^{13}C, ^{23}Na, ^{24}Mg, ^{29}Mg, ^{31}P, ^{39}K, ^{43}Ca, ^{57}Fe, ^{65}Cu, ^{66}Zn and ^{77}Se. Only the elements of most interest (Se and Cu) are discussed in detail. Data was processed using the iolite data processing software, and concentrations were calculated and visualised relative to the gelatine standards to form a 2D map of micronutrient concentration over a chosen surface of each placenta explant sample.

Assessment of proliferation, apoptosis and DNA damage

To assess the effect of supplementation on placental proliferation, apoptosis and DNA damage, explants from first trimester placentas (n=12) of 7-12 weeks' gestation were cultured as mentioned above. Tissue sections of 5 μm were placed on microscope slides for immunohistochemical labelling

for assessment of proliferation (Ki67; Abcam®; ab16667), apoptosis (cleaved caspase-3; Cell Signalling Technology® ; CST.9661L) or DNA damage (8-hydroxy-2'-deoxyguanosine; Abcam®; ab48508) (Table 1). A Hamamatsu NanoZoomer Digital Pathology slide scanner was used to scan the stained sections. Eight areas per explant tissue, randomly chosen using NDP.view2 software, were used for quantification and statistical analyses. Positively stained cells per mm^2 / area were counted.

Table 1: Antibodies used for immunohistochemical labelling

Antibody	Dilution	Target Species	CAT #	Company	Diluent	Antigen Retrieval
Ki67	1/100	Rabbit	ab16667	Abcam®	5% Goat serum	Citrate buffer (10 mM Citric acid; pH 6.0; 10 min boiling in microwave, Sixth Sense, Whirlpool, VIC, Australia))
cleaved caspase-3	1/100	Rabbit	CST.9661L	Cell Signalling Technology®	5% Goat serum	Citrate buffer (10 mM Citric acid; pH 6.0; 10 min boiling in microwave)
8-hydroxy-2'-deoxyguanosine	1/200	Mouse	ab48508	Abcam®	5% Goat serum	Citrate buffer (10 mM Citric acid; pH 6.0; 10 min boiling in microwave)

Statistical analyses

Proliferation, apoptosis and DNA damage are expressed as a fold change (FC) relative to the control of each experiment ± standard error. To examine differences between micronutrient-treated and non-treated groups, Generalised Estimating Equations (GEE) were used with log2-transformed positive stain per area to estimate fold change compared to controls. Independence correlation structure was assumed for the GEE to account for measurements from multiple regions per explant. Pre-specified post-hoc contrasts comparing individual sodium selenite and potassium iodide treatments with combination of sodium selenite and potassium iodide were performed with Bonferroni adjustment for multiple comparisons. P-values of less than 0.05 were considered significant. All analyses were performed using R version 3.5.3 or later.

Results

Confirmation of micronutrient uptake within the treated first trimester placenta explants

Qualitative assessment of micronutrient distributions with laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) element imaging showed that supplementation with selenium and

copper increased the content of these micronutrients in the placenta explants confirming the micronutrient uptake by explants from the media supplemented with selenium and copper (Figure 1, Supplementary Figure 1 and 2). In all three placentas, highest uptake of selenium was found in tissues treated with 1.6 μM sodium selenate indicated by more red and yellow pixels in the LA-ICP-MS images (Figure 1D-F, Supplementary Figure 1D-F and Supplementary Figure 1J-L). In two placentas, it was apparent that with increasing selenium concentration, there was an increased uptake of selenium on the 2D map (Figure 1D-F and Supplementary Figure 1J-L). Assessment of tissues treated with copper (II) sulfate showed that control tissues had the lowest level of copper visualised by dark and light blue pixels. All tissues supplemented with 20 μM copper (II) sulfate and two tissues supplemented with 40 μM copper (II) sulfate (Figure 1J-L, Supplementary Figure 2D-F and Supplementary Figure 2J-L) had a higher content of copper compared to the control. This method was not able to assess iodine uptake but the evidence for selenium and copper uptake in the treated explants supports the likelihood of iodine uptake by the placenta tissues.

Apoptosis and DNA damage induction by menadione and antimycin

A dose response experiment (n = 3) was performed to find the optimal concentration of menadione and antimycin for inducing oxidative stress in first trimester human placenta tissue. The following concentrations of menadione (0, 60, 120, 240 μM) and antimycin (0, 240, 480, 960 μM) were tested. Immunohistochemical analysis for DNA damage using 8-hydroxy-2'-deoxyguanosine was used to select the optimal concentration that induced sufficient DNA damage compared to vehicle control in the absence of micronutrient supplementation. The concentrations selected were 120 μM for menadione and 480 μM for antimycin.

In the 12 first trimester placenta explants used in this study, immunohistochemistry analyses showed that compared to vehicle control (0.1 % ethanol) 120 μM menadione and 480 μM antimycin, in the absence of micronutrient supplementation, significantly increased the number of apoptotic cells and DNA damage ($p < 0.05$) (Figure 2 and Supplementary Figure 3). This confirmed that oxidative stress was induced in the cultured first trimester placenta explants and that this system could be used to assess whether micronutrient supplementation protects against induced oxidative stress.

Effect of selenium, iodine and copper supplementation on proliferation

Ki67 positivity was used to assess whether any of the micronutrients altered proliferation within the treated placenta explant tissues in the absence of oxidative stress. There were no changes in proliferation except for treatment with 1.6 μM sodium selenate which increased cell proliferation after 72 hours of supplementation (0.58, 95% CI: 0.25, 0.90) (Figure 3 A). When oxidative stress was induced, with menadione or antimycin, there were no changes in proliferation except for 1.6 μM

selenium which again showed an increase in the number of Ki67 positive cells per mm^2 after treatment with menadione (0.63, 95% CI: 0.31, 0.95) (Figure 3B), or antimycin (0.39, 95% CI: 0.11, 0.67) (Figure 3C) ($p < 0.05$).

Effect of selenium, iodine and copper supplementation on apoptosis

In the absence of oxidative stress, both 0.8 and 1.6 µM sodium selenite reduced apoptosis (-0.25, 95% CI: -0.44, -0.053 and -0.54, 95% CI: -0.83, -0.25, respectively) compared to control (sterile MilliQ water) (Figure 4A). Potassium iodide (0.5, 1 µM) significantly reduced apoptosis (-0.73, 95% CI: -1.06, -0.41 and -0.82, 95% CI: -1.18, -0.46, respectively) (Figure 4A). The combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide also reduced apoptosis compared to the vehicle control (-0.68, 95% CI: -0.96, -0.4) ($p < 0.05$) (Figure 4A). In addition, the effect of the combination of sodium selenite and potassium iodide significantly increased apoptosis compared to individual supplementation with 0.8 µM sodium selenite ($p < 0.05$) but not 0.5 µM potassium iodide. Treatment with 40 µM copper (II) sulfate significantly increased apoptosis (0.35, 95% CI: 0.11, 0.60) compared to control (Figure 4A).

Assessment of apoptosis following induction of oxidative stress by menadione showed that 1.6 µM sodium selenite (-1.04, 95%CI: -1.25, -0.84), both 0.5 µM (-1.34, 95%CI: -1.64, -1.04) and 1 µM potassium iodide (-1.28, 95%CI: -1.65, -0.91), and the combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide (-1.1, 95%CI: -1.32, -0.84) were associated with a significant reduction in apoptosis ($p < 0.05$) when compared to the menadione alone control (Figure 4B). Combination of sodium selenite and potassium iodide reduced apoptosis more than 0.8 µM sodium selenite ($p < 0.05$) but not more than potassium iodide (0.5 µM) alone (Figure 4B). Copper (II) sulfate did not significantly impact apoptosis ($p > 0.05$) (Figure 4B). Similar results were seen when oxidative stress was induced using antimycin (Figure 4C), that is, reduction in apoptosis with the higher dose of sodium selenite (1.6 µM), both potassium iodide concentrations (0.5 and 1 µM) and sodium selenite and potassium iodide combination but no changes in apoptosis with copper (II) sulfate compared to the antimycin alone control (Figure 4C).

Effect of selenium, iodine and copper supplementation on DNA damage

Both concentrations of sodium selenite 0.8 µM (-0.4, 95% CI: -0.6, -0.16) and 1.6 µM (-0.65, 95% CI: -1.27, -0.02), potassium iodide 0.5 µM (-0.42, 95% CI: -0.83, -0.02), 1 µM (-0.43, 95% CI: -0.85, 0.01), and the combination of 0.8 µM sodium selenite and 0.5 µM potassium iodide (-0.73, 95% CI: -1.18, -0.29) reduced DNA damage compared to control (sterile MilliQ water) following 72 hours of treatment ($p < 0.05$) (Figure 5A). However, the effect of the combination of sodium selenite and potassium iodide was not significantly different from individual supplementation with sodium

selenite or potassium iodide ($p > 0.05$) (Figure 5A). There was a significant increase in DNA damage in cells treated with 40 μM copper (II) sulfate (0.63, 95% CI: 0.17, 1.09) (Figure 5A).

Assessment of DNA damage after oxidative stress induction with menadione showed that 1.6 μM sodium selenite (-0.92, 95% CI: -1.44, -0.41), both concentrations of potassium iodide: 0.5 μM (-0.87, 95% CI: -1.49, -0.24), 1 μM (-0.85, 95% CI: -1.42, -0.27) and the combination of sodium selenite and potassium iodide (-0.95, 95% CI: -1.41, -0.48) resulted in a significant reduction in DNA damage compared to the menadione alone control while copper (II) sulfate did not have any significant effect ($p > 0.05$) (Figure 5B). While the combination of 0.8 μM sodium selenite and 0.5 μM potassium iodide significantly reduced DNA damage after menadione treatment, there was no effect with the individual supplementation of 0.8 μM sodium selenite ($p > 0.05$). In addition, no significant difference was found between supplementation with 0.5 μM potassium iodide and the combination of 0.8 μM sodium selenite and 0.5 μM potassium iodide ($p > 0.05$) (Figure 5B). Similar results were seen in DNA damage assessment of placenta explants treated with antimycin (Figure 5C).

Discussion

Selenium supplementation increased proliferation and reduced DNA damage and apoptosis in the absence or presence of oxidative stress. This can be explained by the effect of selenium on the cell cycle and the antioxidant system (Trigona, et al., 2006, Zeng, 2009). Selenium stimulates transition from Gap 2 to mitosis phase in cell cycle and this increases cell division or proliferation (Zeng, 2009). The cell cycle is precisely monitored and damaged DNA molecules can be detected at several checkpoints (Chao, et al., 2017). Although checkpoints in different cell cycle phases may work differently, the ultimate outcome is if the DNA molecule is not repaired the cell containing damaged DNA cannot proliferate and undergoes apoptosis (Chao, Poovey, Privette, Grant, Chao, Cook and Purvis, 2017). GPx 4 and TRx are seleno-antioxidant enzymes that can protect cells against oxidative damage such as DNA damage, therefore reducing apoptosis (Matsushita, et al., 2015, Muri, et al., 2018, Selenius, et al., 2012). Notably, when placenta explants were treated with menadione or antimycin the lower concentration of sodium selenite could not reduce DNA damage or apoptosis. This suggests that under conditions of oxidative stress, cells may require a higher concentration of selenium for protection against oxidative damage.

This study showed that potassium iodide could protect from oxidative damage to DNA molecules and reduced apoptosis. This is consistent with previous findings showing the protective effect of potassium iodide against oxidative stress in a placenta cell line (Habibi, Jankovic-Karasoulos, Leemaqz, Francois, Zhou, Leifert, Perkins, Roberts and Bianco-Miotto, 2020, Olivo-Vidal, et al.,

283 2016). Systemically, iodine is a radical scavenger as seen by its ability to metabolise H_2O_2 in thyroid
284 hormone production (Smyth, 2003). The exact mechanisms by which iodine interacts with the
285 antioxidant system in combating oxidative stress has not been clearly defined but studies have shown
286 that iodine has antioxidant properties such as increasing total antioxidant status of human serum
287 (Winkler, et al., 2000). Iodine sufficient pregnant women (>150 μg/l urinary iodine concentration)
288 had higher activity of the enzyme SOD compared to iodine deficient women (Cuellar-Rufino,
289 Navarro-Meza, Garcia-Solis, Xochihua-Rosas and Arroyo-Helguera, 2017). Interestingly, the
290 combination of sodium selenite and potassium iodide resulted in a greater reduction in DNA damage
291 in antimycin treated cells, compared to either micronutrient alone. This suggests that combining
292 iodine and selenium may provide a stronger protection to the placenta against oxidative stress. Iodide
293 and selenium can form selenenyl iodide that is a substrate for placental TRx and may increase
294 antioxidant activity of TRx (Mugesh, et al., 2003), thereby reducing DNA damage and apoptosis.

295 In a prospective cohort study of 1065 pregnant Australian women, a high level of maternal plasma
296 copper was associated with higher plasma C-reactive protein (CRP) concentrations (Wilson, Bianco-
297 Miotto, Leemaqz, Grzeskowiak, Dekker and Roberts, 2018a). CRP is an inflammatory biomarker that
298 increases in oxidative stress (Cottone, et al., 2006) and its higher levels are associated with a greater
299 incidence of pregnancy complications such as preterm birth (Lohsoonthorn, et al., 2007) and
300 preeclampsia (Cebesoy, et al., 2009). The results of this study showed that a high concentration of
301 copper increased DNA damage and apoptosis. Excess intake of copper may increase production of
302 reactive oxygen species and diminish antioxidant defence systems (Yu, et al., 2008), resulting in DNA
303 damage and apoptosis. Interestingly, in menadione or antimycin induced oxidative stress the toxic
304 effect of excess copper was not apparent. To remove reactive molecules and protect the cells against
305 oxidative stress, the antioxidant system is overexpressed and the activity of enzymes such as SOD1
306 increases (Soto, et al., 2014). Therefore, these enzymes may require higher levels of cofactors
307 including copper. This avoids copper being accumulated and causing toxic effects.

308 A strength of this study was the use of laser ablation inductively coupled plasma-mass spectrometry
309 to show that first trimester human placental explants can take up selenium and copper from the media.
310 To the best of our knowledge, this is the first study that has directly shown that selenium and copper
311 supplementation increases their content and distribution in cultured placental explants. This method
312 cannot confirm iodine uptake. Additionally, this is the first study that has investigated the effect of
313 micronutrients on placental explants' proliferation, apoptosis and DNA damage in the absence or
314 presence of oxidative stress, lending support to the important role of maternal micronutrient intake
315 on placental development and hence pregnancy health.

316 In conclusion, selenium and iodine supplementation may protect the first trimester human placenta
317 against oxidative stress. An excess intake of copper is related to oxidative damage to DNA and
318 apoptosis. Since oxidative stress is involved in pregnancy complications, an optimal level of
319 micronutrients may help to ensure healthy placental development and hence reduce the incidence of
320 adverse pregnancy outcomes.

321 **Authors' roles**

322 The study was designed by N.H., T.B-M., C.T.R., T.J-K., J.A.G., A.V.P, S.J.Z., and performed by
323 N.H. and T.B-M. Tissue collection was performed by D.M. Tissue culture and harvest was done by
324 N.H. LA ICP-MS was performed by S.G. and N.H. Immunohistochemistry was optimised, performed
325 and quantified by N.H., T.B-M., C.R. and A.L. Statistical analyses were performed by N.H. and S.Y-
326 L.L. The original manuscript was drafted by N.H. All authors critically reviewed and revised the
327 manuscript and approved the final version.

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338 **Conflict of interest**

339 The authors have no conflicts of interest.

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Figure Legends

Figure 1. Selenium and copper uptake in first trimester human placental explants

Placenta explants (7-12 weeks of gestation) were supplemented with sodium selenite (A & D: sterile MilliQ water, B & E: 0.8 µM or C & F: 1.6 µM) or copper (II) sulfate (G & J: sterile MilliQ water, H & K: 20 µM or I & L: 40 µM) for 72 hours. Tissue sections (10 µm) were placed on microscope slides. Standards were made by dissolving 10% gelatine in 0, 1, 10, and 100 mM solutions of selenium and copper. Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) analyses on the standard gelatine and placental explant sections were analysed using a Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines: 23 µm spots size, 23 µm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for each isotope. Data was processed using the iolite data processing software, and element intensity calculated relative to the gelatine standards to form a 2D map of micronutrient distribution over a chosen surface of each placental explant sample.

Figure 2. Effect of menadione and antimycin on apoptosis and DNA damage in first trimester human placental explants

Explants from 7-12 weeks of gestation (n=12) were cultured for 5 days in media with 10% v/v FBS and 1% v/v Antibiotic-Antimycotic and then treated with 120 µM menadione, 480 µM antimycin or 0.1% ethanol (vehicle control) for 24 hours. Immunohistochemical labelling for apoptosis (cleaved caspase-3) or DNA damage (8-hydroxy-2'-deoxyguanosine) was performed. Eight randomly selected regions per explant were used for quantification and statistical analyses. Data are presented as a fold change (FC) relative to control ± standard error. Statistical significance was assessed using Generalised Estimating Equations with independence correlation structure with log 2-transformed positive stain per area to estimate fold change compared to controls followed by pre-specified post-hoc Bonferroni adjusted multiple comparisons. * Indicates statistically different ($P < 0.05$) from control.

Figure 3. Effect of selenium, iodine and copper supplementation on proliferation in first trimester placental explants

Placenta explants from 7-12 weeks of gestation (n=12) were cultured for 48 hours for syncytial regeneration followed by supplementation with sodium selenite (0, 0.8 or 1.6 µM), potassium iodide (0, 0.5 or 1 µM), combination of sodium selenite and potassium iodide (0.8 µM sodium selenite and 0.5 µM potassium iodide), or copper (II) sulfate (0, 20 or 40 µM) for 72 hours with supplementation replenished every 24 hours. (A) Placental explants were harvested following 72 hours of supplementation and proliferation assessed by immunolabelling for Ki67. In addition, after 72 hours

494 supplementation, placental explants were treated with B) 120 μM menadione or C) 480 μM antimycin
495 for 24 hours to induce oxidative stress and then assessed for proliferation. Eight randomly selected
496 regions per explant were used for quantification and statistical analyses. Data presented as a fold
497 change (FC) relative to control ± standard error. Statistical significance was assessed using
498 Generalised Estimating Equations with independence correlation structure with log 2-transformed
499 positive stain per area to estimate fold change compared to controls followed by pre-specified post-
500 hoc Bonferroni adjusted comparisons. * Indicates statistically different ($P < 0.05$) from vehicle
501 control (C).

502 **Figure 4. Effect of selenium, iodine and copper supplementation on apoptosis in first trimester**
503 **placental explants**
504 Placenta explants from 7-12 weeks of gestation (n=12) were cultured for 48 hours for syncytial
505 regeneration followed by supplementation with sodium selenite (0, 0.8 or 1.6 μM), potassium iodide
506 (0, 0.5 or 1 μM), combination of sodium selenite and potassium iodide (0.8 μM sodium selenite and
507 0.5 μM potassium iodide), or copper (II) sulfate (0, 20 or 40 μM) for 72 hours with supplementation
508 replenished every 24 hours. (A) Placental explants were harvested at the end of 72 hours
509 supplementation and apoptosis assessed by immunolabelling for cleaved caspase-3. In addition, after
510 72 hours supplementation, placental explants were treated with B) 120 μM menadione or C) 480 μM
511 for 24 hours to induce oxidative stress and then assessed for apoptosis. Eight randomly selected
512 regions per explant were used for quantification and statistical analyses. Data presented as a fold
513 change (FC) relative to control ± standard error. Statistical significance was assessed using
514 Generalised Estimating Equations with independence correlation structure with log 2-transformed
515 positive stain per area to estimate fold change compared to controls followed by pre-specified post-
516 hoc Bonferroni adjusted comparisons. * Indicates statistically different ($P < 0.05$) from control.
517 **Figure 5. Effect of selenium, iodine and copper supplementation on DNA damage in first**
518 **trimester human placental explants**
519 Placenta explants from 7-12 weeks of gestation (n=12) were cultured for 48 hours for syncytial
520 regeneration followed by supplementation with sodium selenite (0.8 or 1.6 μM), potassium iodide
521 (0.5 or 1 μM), combination of sodium selenite and potassium iodide (0.8 μM sodium selenite and 0.5
522 μM potassium iodide), or copper (II) sulfate (20 or 40 μM) for 72 hours with supplementation
523 replenished every 24 hours. (A) Placental explants were harvested following 72 hours of
524 supplementation and DNA damage assessed by immunolabelling for 8-hydroxy-2'-deoxyguanosine.
525 After 72 hours of supplementation, placental explants were treated with B) 120 μM menadione or C)
526 480 μM for 24 hours to induce oxidative stress and then assessed for DNA damage. Eight randomly
527 selected regions per explant were used for quantification and statistical analyses. Data presented as a

fold change (FC) relative to control ± standard error. Statistical significance was assessed using Generalised Estimating Equations (GEE) with independence correlation structure with log 2-transformed positive stain per area to estimate fold change compared to controls followed by pre-specified post-hoc Bonferroni adjusted comparisons. * Indicates statistically different ($P < 0.05$) from control.

Supplementary Figure 1. Selenium uptake in first trimester human placental explants

Placenta explants from 7-12 weeks of gestation were supplemented with sodium selenite (A & D & G & J: sterile MilliQ water, B & E & H & K: 0.8 µM or C & F & I & L: 1.6 µM) for 72 hours. Tissue sections were cut at 10 µm and placed on microscope slides. Standards were made by dissolving 10% gelatine in solutions of selenium and copper with 0, 1, 10, and 100 mM concentrations. Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) analyses on the standard gelatine and placental explant sections were analysed at Adelaide Microscopy, University of Adelaide, using a Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines: 23 µm spots size, 23 µm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for each isotope. Data was processed using the iolite data processing software, and element intensity calculated relative to the gelatine standards to form a 2D map of micronutrient distribution over a chosen surface of each placental explant sample.

Supplementary Figure 2. Copper uptake in first trimester human placental explants

Placenta explants from 7-12 weeks of gestation were supplemented with copper (II) sulfate (A & D & G & J: sterile MilliQ water, B & E & H & K: 20 µM or C & F & I & L: 40 µM) for 72 hours. Tissue sections were cut at 10 µm and placed on microscope slides. Standards were made by dissolving 10% gelatine in solutions of selenium and copper with 0, 1, 10, and 100 mM concentrations. Laser ablation inductively coupled plasma-mass spectrometry (LA-ICP-MS) analyses on the standard gelatine and placental explant sections were analysed at Adelaide Microscopy, University of Adelaide, using a Resolution 193 nm excimer laser ablation system coupled to an Agilent 7900x ICP-MS. Samples were ablated with a series of parallel lines: 23 µm spots size, 23 µm/s speed, 10 Hz repetition rate and a fluence of ~1 J/cm^2. Intensity of micronutrients were recorded as counts per second for each isotope. Data was processed using the iolite data processing software, and element intensity calculated relative to the gelatine standards to form a 2D map of micronutrient distribution over a chosen surface of each placental explant sample.

562 **Supplementary Figure 3. Apoptosis and DNA damage induction by menadione in first trimester**
563 **human placental explants**
564 First trimester placenta tissues were cultured for 5 days in media including 10% v/v FBS and 1% v/v
565 Antibiotic-Antimycotic and then treated with A) and C) 0.1% ethanol (vehicle control) or B) and D)
566 120 μM menadione for 24 hours. Immunohistochemical labelling for A) and B) apoptosis (cleaved
567 caspase-3) or C) and D) DNA damage (8-hydroxy-2'-deoxyguanosine) was performed. Stained slides
568 were scanned with a Hamamatsu NanoZoomer Digital Pathology slide scanner and NDP.view2
569 software were used for analyses. Black arrows indicate positively stained cells. Scale bar is 100 μm.
570

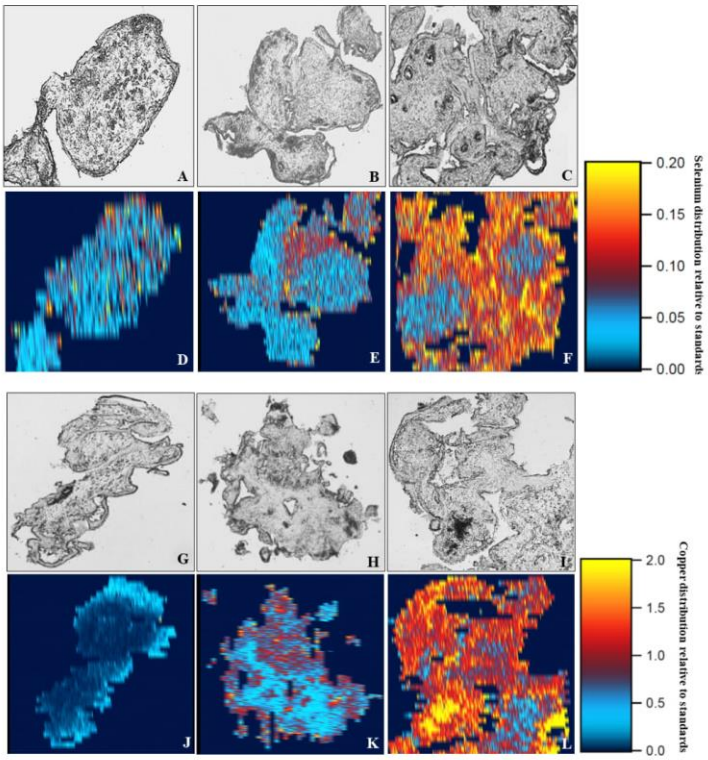

Figure 1

294x314mm (96 x 96 DPI)

Figure 2

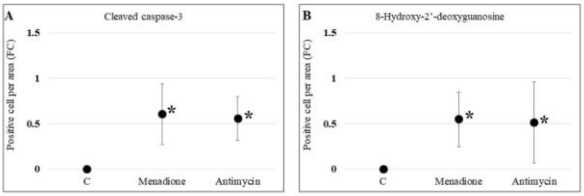

Figure 2

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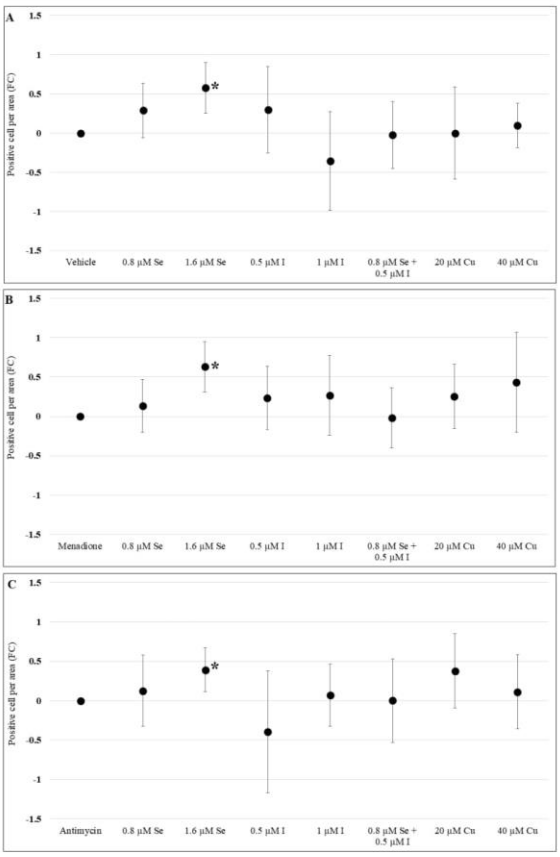

Figure 3

290x436mm (96 x 96 DPI)

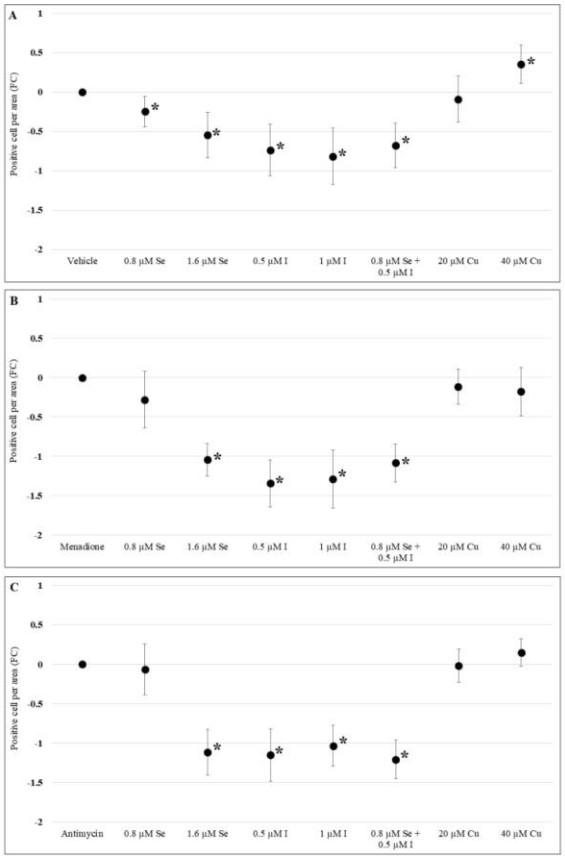

Figure 4

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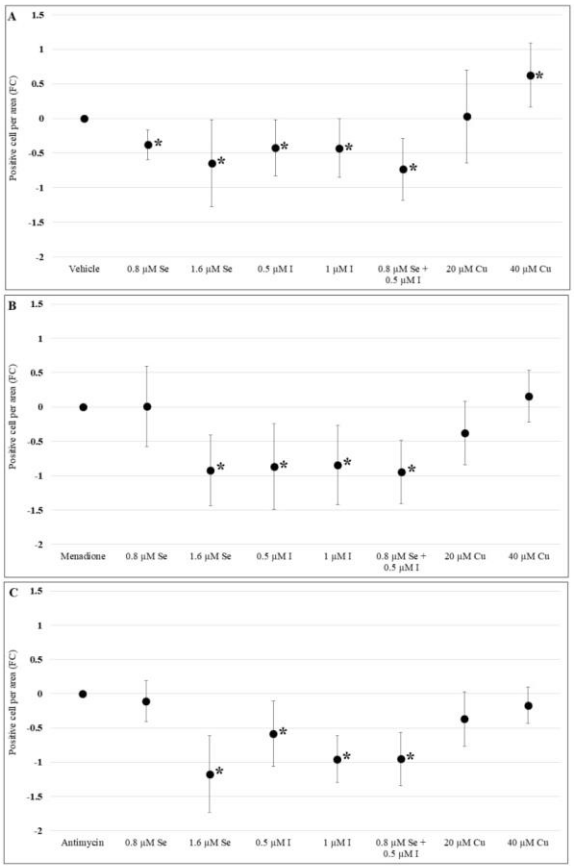

Figure 5

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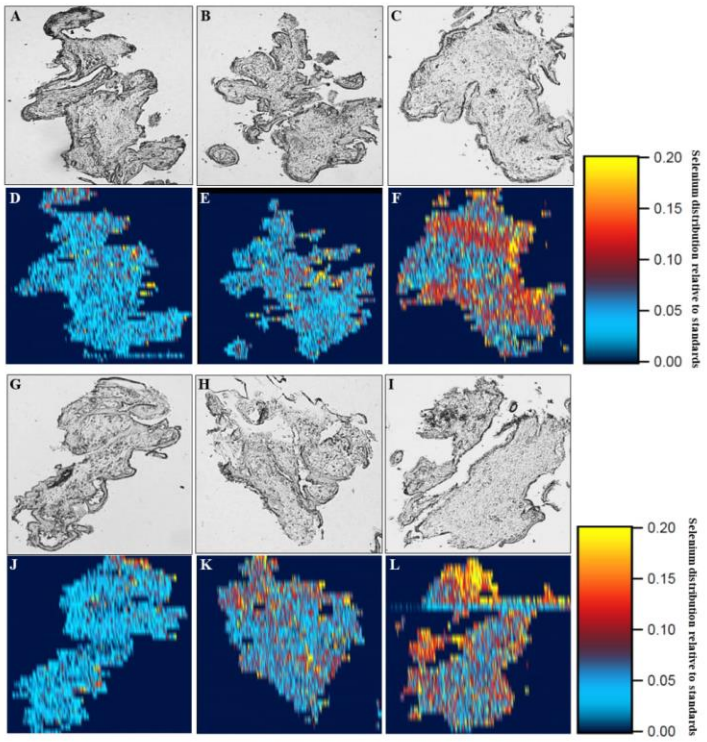

Supplementary Figure 1

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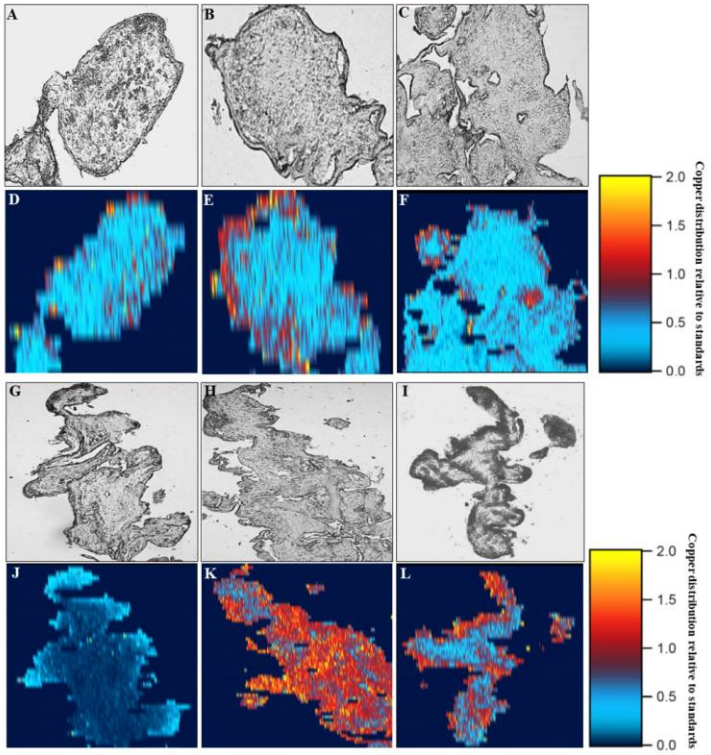

Supplementary Figure 2

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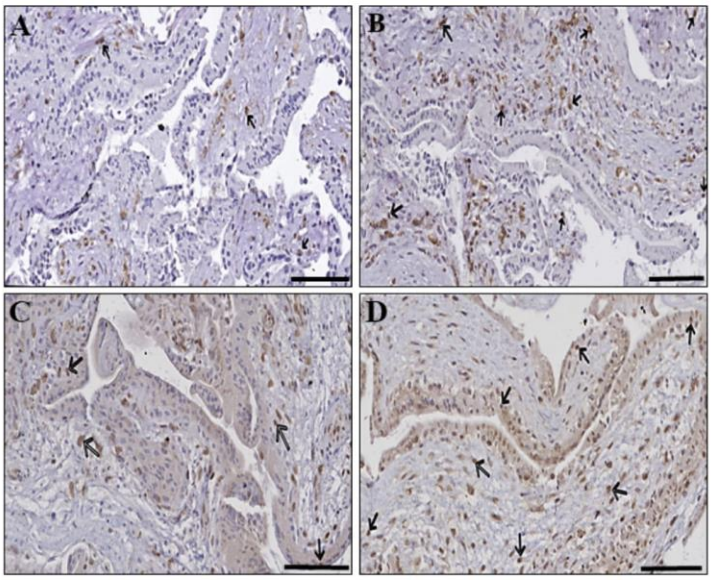

Supplementary Figure 3

164x133mm (96 x 96 DPI)

Chapter 5

Reduced Buffy Coat Mitochondrial DNA Content is Associated with a Greater Risk of Pregnancy Complications

"Never give up on what you really want to do.
The person with big dreams is more powerful than one with all the facts."
Albert Einstein

Title: Reduced buffy coat mitochondrial DNA Content is associated with a greater risk of pregnancy complications

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Abstract:

Mitochondrial DNA content (mtDNAc) is a biomarker of mitochondrial oxidative phosphorylation. Associations between mtDNAc and oxidative stress and inflammation in aging and chronic diseases are frequently reported. Oxidative stress and inflammation also contribute to pregnancy complications. Micronutrients are required for optimal mitochondrial structure and function and are also essential to support pregnancy health. To determine if mtDNAc is associated with pregnancy outcome and maternal micronutrient status, samples and data of 317 pregnant women from a prospective cohort study were used. Relative mtDNAc was measured in buffy coat using real-time quantitative PCR. Regression analyses were performed to examine the relationship between mtDNAc and pregnancy outcomes and maternal factors. Greater mtDNAc was associated with lower risk of any

pregnancy complication (OR 0.04, 95% CI: 0.00, 0.34). Every 1% increase in plasma C-reactive protein concentration was associated with an estimated 5% reduction (95% CI 1% to 9% decrease) in mtDNAc. There was no association between maternal micronutrient status and mtDNAc. Reduced mtDNAc was associated with a greater risk of pregnancy complications but not with maternal micronutrient status.

Keywords: mitochondrial DNA copy number, Pregnancy complication, inflammation, CRP, Oxidative stress, Micronutrient

1 Introduction

Mitochondria are essential for cell survival with continuous supply of adenosine triphosphate (ATP) [1]. They also are involved in promoting apoptosis and necrosis [2], modulating Ca^{2+} signalling, steroidogenesis, redox control and innate and adaptive immunity [2,3]. Of high importance, mitochondria can impact fertility [4]. The human mitochondrial genome is an extra nuclear DNA built of 16,569 DNA base pairs [5] and altered mitochondrial DNA content (mtDNAc) is often used as a biomarker of oxidative stress [6]. Changes in mtDNAc can reflect mitochondrial dysfunction and impaired oxidative phosphorylation [7]. Altered mtDNAc has been reported in aging [8] and chronic diseases such as Parkinson's disease, coronary heart disease, cardiovascular disease and metabolic syndrome [6,9-11]. Oxidative stress and inflammation contribute to aging [8] and chronic diseases [6,9,10], as well as pregnancy complications [12]. However, research on mtDNAc in relation to pregnancy outcomes is scarce and controversial [13-16].

Pregnancy complications including preeclampsia, preterm birth, and small for gestational age increase morbidity and mortality both prenatally and later in life for both mother and child [17-20]. Pregnancy complications have been associated with poor maternal nutrition [20-22] that also associates with low birth weight, insulin resistance, and cognitive

dysfunction in offspring [23-25]. A balanced supply of nutrients is necessary for mitochondrial structure and function [21]. A variety of nutrients such as B vitamins, coenzyme Q10, selenium, zinc and vitamin E are involved in mitochondrial electron transport chain and their primary or secondary deficiency may result in mitochondrial dysfunction such as bioenergetics failure [21]. This study aimed to investigate whether there was an association between buffy coat mtDNAc and pregnancy outcome or maternal micronutrient status.

2 Results

Participant characteristics reported at 15 ± 1 weeks' gestation are summarized in Table 1. For 1% increase in C-reactive protein (CRP) concentration there was an estimated 5% decrease in mtDNAc at 15 ± 1 weeks' gestation (95% CI 1% to 9% decrease) (Figure 1). Figure 2 shows the mean ± SD of mtDNAc for the different pregnancy outcomes. Complicated pregnancy (mean ± SD: 0.29 ± 0.10), preeclampsia [Median (IQR): 0.24 (0.17, 0.29)], small for gestational age [Median (IQR): 0.25 (0.19, 0.31)], and spontaneous preterm birth [Median (IQR): 0.22 (0.15, 0.30)] had lower mean ± SD of mtDNAc compared to healthy pregnancy [Median (IQR): 0.313 (0.25, 0.37)] ($p < 0.05$) (Figure 2). Higher mtDNAc was associated with a lower risk of any pregnancy complication, preeclampsia, small for gestational age and spontaneous preterm birth adjusted for maternal age and BMI, although the effect was very small (Figure 3). Maternal age, BMI, smoking, alcohol intake, micronutrient status, and folate supplement intake were not significantly associated with mtDNAc at 15 ± 1 weeks' gestation ($p > 0.05$) (Figure 4).

Table 1. Participant characteristics of 317 nulliparous pregnant women

Characteristics	N (%) or Median (IQR)	
Age (*y*)	22 (19, 26)	
Body Mass index (*kg/m^2*)	25.20 (22.23, 29.78)	
Ethnicity		
-Caucasian	299 (94.3)	
-Other (including Asian, Indian and African)	18 (5.7)	
Alcohol drinking	Yes	No
-3 months pre-pregnancy	163 (51.4)	154 (48.6)
-First trimester	122 (38.5)	195 (61.5)
-15 ± 1 week'	9 (2.8)	308 (97.2)
Smoking	Yes	No
-Pre-pregnancy	156 (49.2)	161 (50.8)
-First trimester	150 (47.3)	167 (52.7)
-15 ± 1 week'	91(28.7)	226 (71.3)
Folate supplement intake	Yes	No
-Pre-pregnancy	112 (35.3)	205 (64.7)
-First trimester	269 (84.9)	48 (15.1)
-15 ± 1 week'	235 (74.1)	82 (25.9)
Pregnancy Outcome		
-Uncomplicated	168 (52.9)	
-SGA	48 (15.1)	
-GHT	34 (10.7)	
-PE	21 (6.6)	
-SPTB	21 (6.6)	
-GDM	11 (3.4)	
-Placental Abruption	9 (2.8)	
Fetal sex		
-Female	167 (53)	
-Male	150 (47)	
Maternal plasma micronutrient concentration		
-Selenium (*µMol/L*)	0.89 (0.81, 1.01)	
-Copper (*µMol/L*)	30.90 (21.84, 28.41)	
-Zinc (*µMol/L*)	9.72 (8.53, 10.87)	
-Iron (*µMol/L*)	16.58 (12.74, 20.86)	
-Calcium *(mMol/L)*	2.07 (1.98, 2.14)	
Maternal CRP (*µg/ml*)	12.24 (5.68, 21.38)	

CRP, C-reactive protein; GHT, Gestational Hypertension; GDM, Gestational Diabetes Mellitus; PE, Preeclampsia; SGA, Small for Gestational Age; SPTB, Spontaneous Preterm Birth

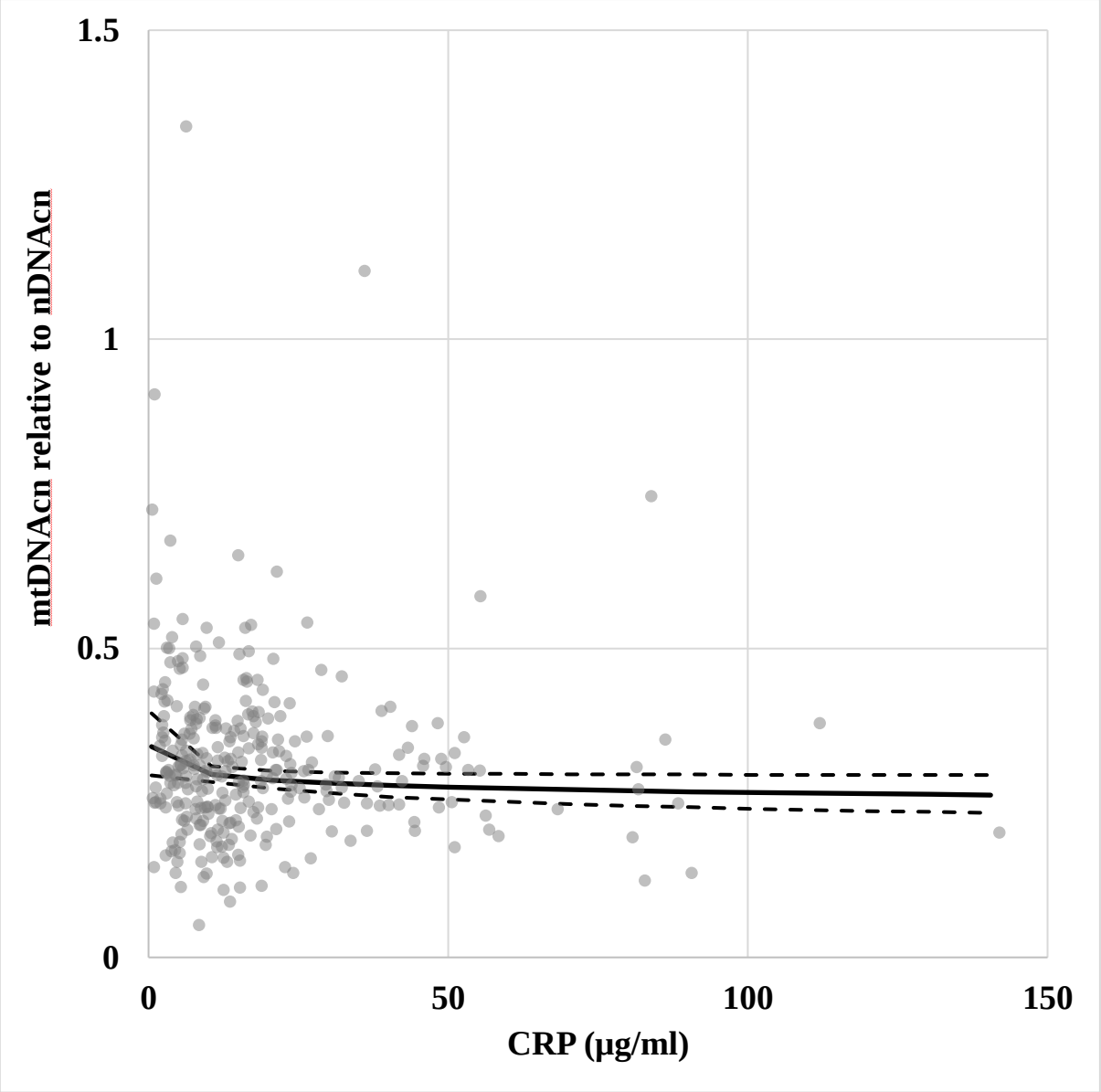


Figure 1. Association between maternal CRP concentration and buffy coat mitochondrial DNA content. For every 1% increase in CRP there was an estimated 5% decrease (95% CI 1% to 9% decrease) in mtDNAc. Log transformed CRP and mtDNAc data were used in linear regression analysis. Mitochondrial DNA content (mtDNAc) was calculated relative to nuclear DNA copy number (nDNAcn). Solid line indicates the geometric mean of mtDNAc and dotted lines show the corresponding 95% CI.

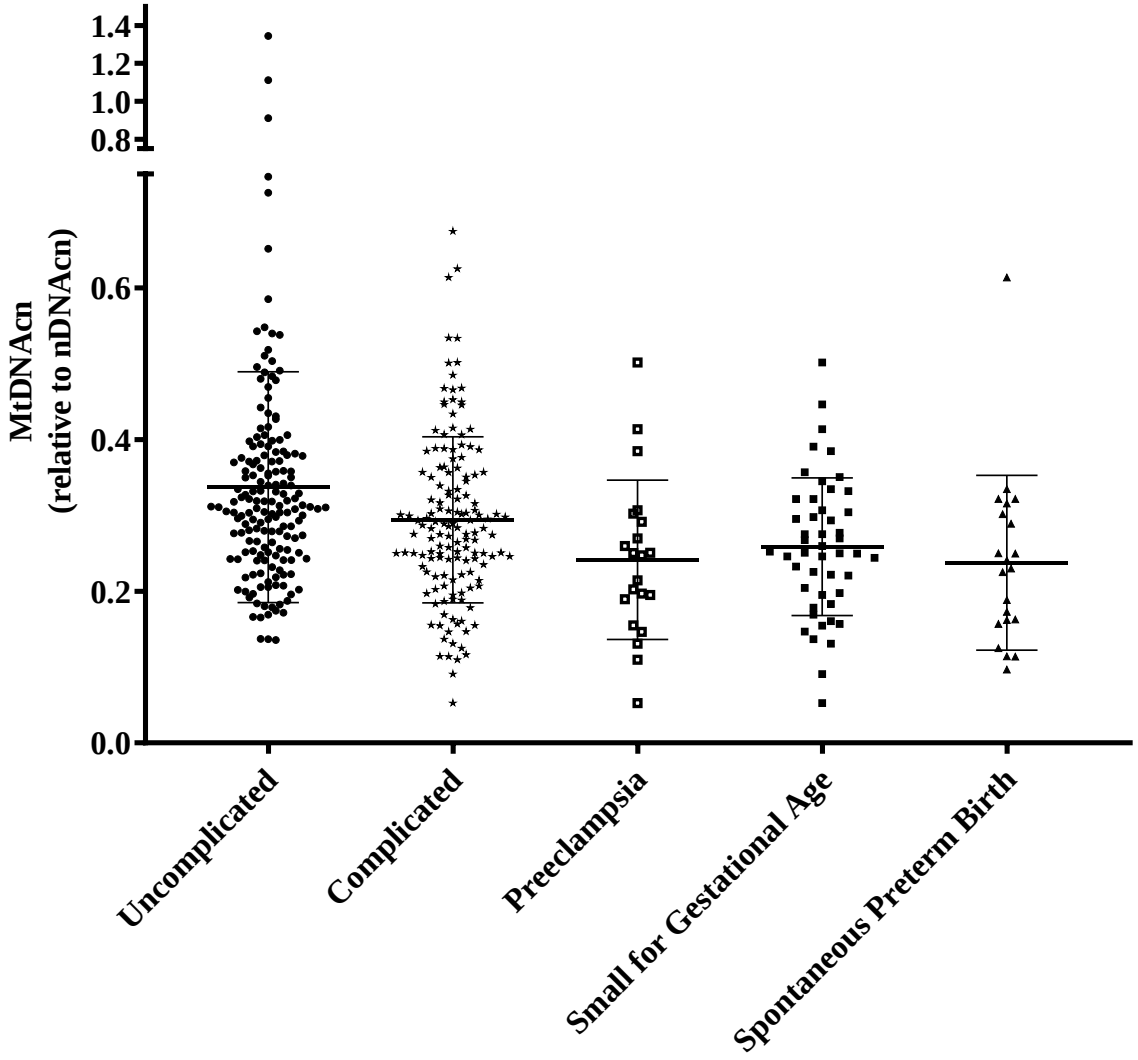


Figure 2. Buffy coat mitochondrial DNA content and pregnancy outcomes (mean ± SD). There was a significant difference between mitochondrial DNA contentof uncomplicated pregnancy [Median (IQR): 0.313 (0.25, 0.37)] with mitochondrial DNA content of any pregnancy complication including SGA, GHT, PE, SPTB, GDM and placental abruption [Median (IQR): 0.28 (0.22, 0.35)], preeclampsia [Median (IQR): 0.24 (0.17, 0.29)], small for gestational age [Median (IQR): 0.25 (0.19, 0.31)], spontaneous preterm birth [Median (IQR): 0.22 (0.15, 0.30)] ($p < 0.05$). Mitochondrial DNA content (mtDNAc) was calculated relative to nuclear DNA copy number (nDNAcn).

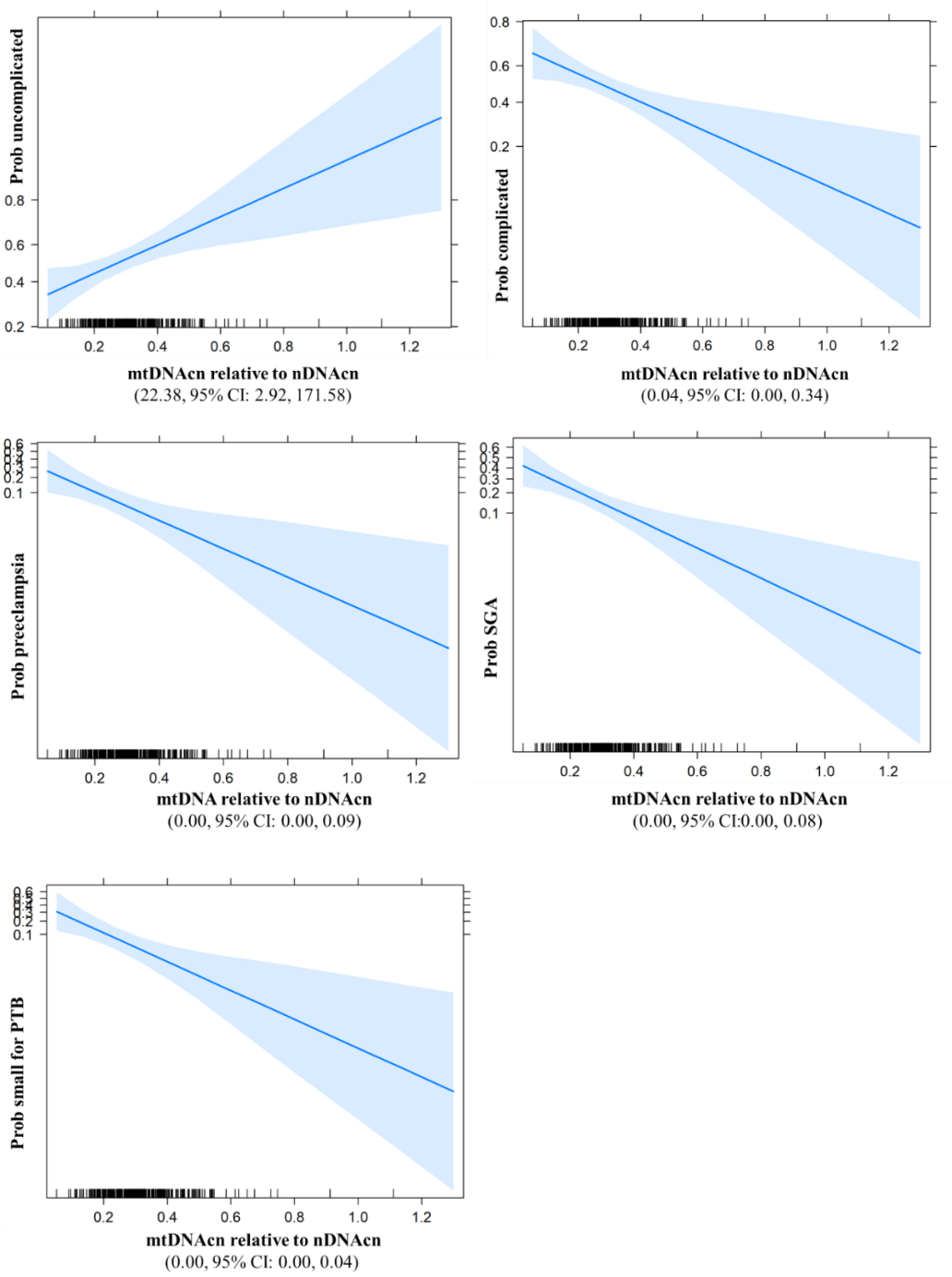
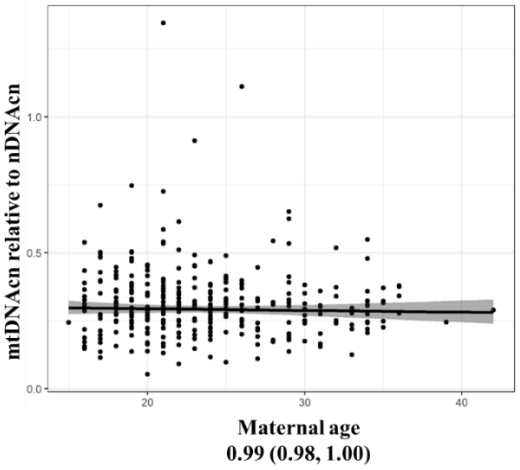
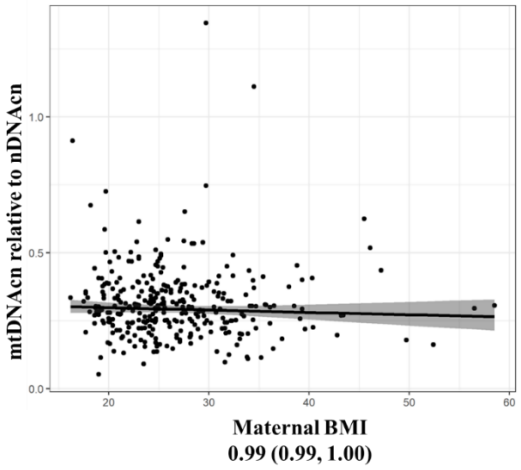
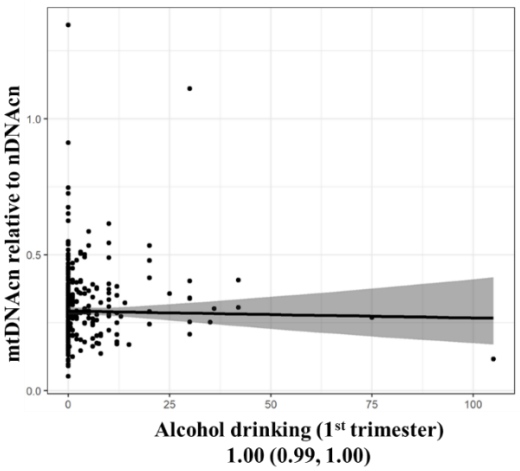
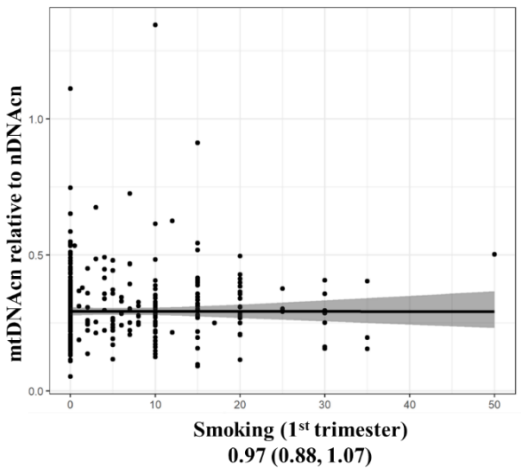


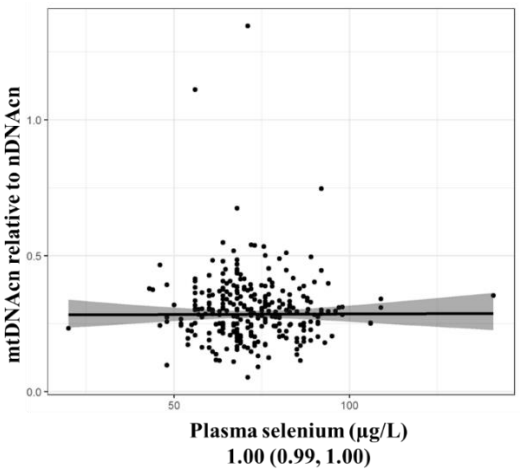
Figure 3. Predicted probability of pregnancy outcomes for a range of mitochondrial DNA content relative to nuclear DNA copy number, estimated from Logistic regressions. Higher mitochondrial DNA content was associated with lower risk of complicated pregnancies including SGA, GHT, PE, SPTB, GDM and placental abruption as all together as well as preeclampsia, spontaneous preterm birth (PTB), and small for gestational age (SGA), individually. However, the effect was minimal.

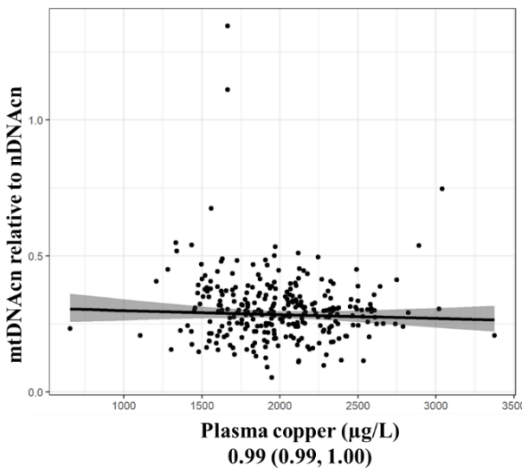

mtDNAcn relative to nDNAcn
Maternal age
0.99 (0.98, 1.00)


mtDNAcn relative to nDNAcn
Maternal BMI
0.99 (0.99, 1.00)


mtDNAcn relative to nDNAcn
Alcohol drinking (1st trimester)
1.00 (0.99, 1.00)


mtDNAcn relative to nDNAcn
Smoking (1st trimester)
0.97 (0.88, 1.07)


mtDNAcn relative to nDNAcn
Plasma selenium (µg/L)
1.00 (0.99, 1.00)


mtDNAcn relative to nDNAcn
Plasma copper (µg/L)
0.99 (0.99, 1.00)

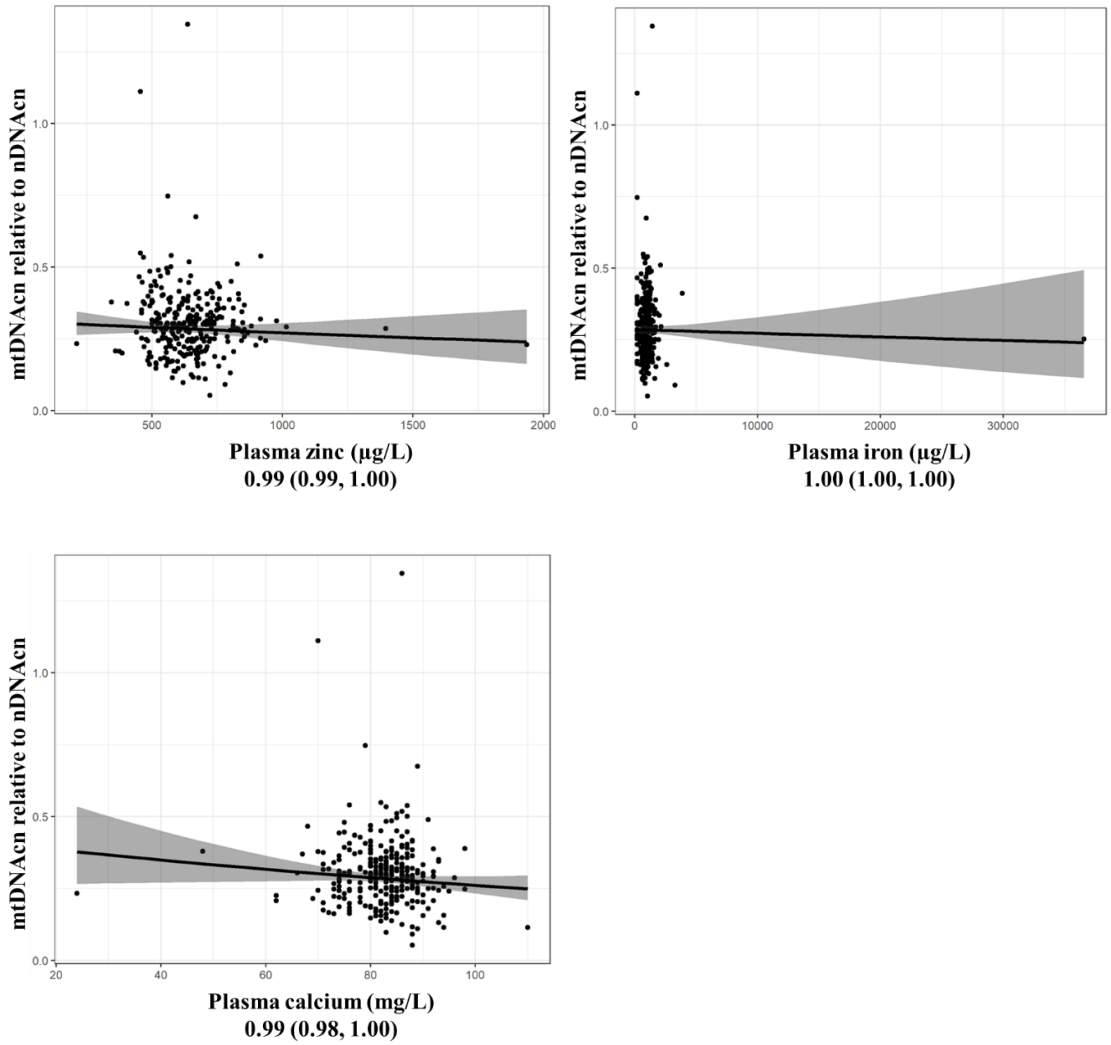


Figure 4. Association between buffy coat mitochondrial DNA content and maternal factors. Mitochondrial DNA content (mtDNAc) was calculated relative to nuclear DNA copy number (nDNAcn). Linear Regressions were used to test the association between maternal characteristics and mtDNAc, adjusted for maternal age and BMI except for age and BMI characteristics. Data are presented as a ratio of geometric mean and corresponding 95% CI.

3. Discussion

In this study, increasing CRP was associated with mtDNAc reduction. Studies in young [26], adult and old [27,28] populations have also demonstrated an inverse association between mtDNAc and CRP. The latter is a marker of inflammation and innate immune response [29]. Higher CRP levels are associated with oxidative stress [30] and an increased risk of pregnancy complications such as preterm birth and preeclampsia [31,32]. The contribution of oxidative stress in the pathogenesis of pregnancy complications is increasingly recognised

[12]. The mitochondrial DNA and electron transport chain are in close proximity to each other [33]. The mitochondrial electron transport chain is a source of free radicals production [34] and accumulation of free radicals results in oxidative stress that can cause DNA damage and mutation in mitochondrial DNA, and thus reduce DNA copy number [33-37]. Indeed, mtDNAcn reduction is a biomarker of mitochondrial dysfunction caused by inflammation and oxidative stress [35].

Pregnancy is energetically demanding due to new tissue synthesis, placental growth and function and fetal growth. The mitochondria play a fundamental role in providing this energy [38]. A high-energy requirement can increase electron transport chain activity and DNA replication in mitochondria [39]. Thus, mtDNAc increases during states of high-energy requirement [39,40]. Lower buffy coat mtDNAc was associated with a higher risk of pregnancy complications which is consistent with previous studies that have shown that reduced mtDNAc in peripheral blood cells was associated with gestational diabetes mellitus, preeclampsia and preeclampsia with intrauterine growth restriction [13,14]. In contrast, Williams *et al*. (2013) and Qiu *et al*. (2012) measured mtDNAc in whole blood collected at delivery and reported a higher mtDNAc in preeclamptic participants and in those who experienced placental abruption associated with preeclampsia [15,16]. The inconsistencies may be due to differences in samples since whole blood and buffy coat are not the same cell populations, which may contribute to a different mitochondrial DNA profile. Maternal whole blood contains cell free fetal DNA [41] and preeclampsia is associated with a higher concentration of cell free fetal DNA compared to an uncomplicated pregnancy [42]. This fetal DNA may contribute to the increased mtDNAcn seen in these studies. Also, Williams *et al*. (2013) and Qiu *et al*. (2012) collected whole blood at delivery while sampling time was 15 ±1 weeks' in our study, and first trimester [13] and 32-36 weeks' gestation [14] in

other studies. This suggests that more research is needed in this area assessing different stages of pregnancy to determine if mtDNA is a good biomarker of pregnancy outcome.

Our data did not show any association between micronutrient status or folate supplement intake and mtDNAc. Previously, a study among 108 pregnant women revealed that maternal supplementation with either iron and folic acid or multiple micronutrients for a duration ranging from 20 to 197 days was associated with increased mtDNAcn [38]. Since the Adelaide SCOPE participants were from a low socioeconomic status population and socioeconomic status affects dietary intake and maternal nutritional status [43-46] more research in groups with diverse nutritional status is essential to elucidate the potential association between maternal micronutrient levels and mtDNAc.

There were limitations with sample type, cohort size and time point measurement in this study. We measured mtDNAc in buffy coat, which may not be the ideal sample type for this purpose. Buffy coat contains different cell types that may have different mtDNAc and change in different pregnancy outcomes. Although cohort size was larger or close to the other studies, it might not be large enough to investigate all differences as some pregnancy complications had a small sample size. We only measured mtDNAc at one time point and mtDNAc may alter during pregnancy. Large cohort studies to measure mtDNAc in specific cells at a series of time points may provide a more comprehensive information in this field.

4. Material and method

4.1 Study participants

Participants were nulliparous women with a singleton pregnancy who were recruited to the Screening for Pregnancy Endpoints in Adelaide (SCOPE Adelaide) study between September 2005 and September 2008 at the Lyell McEwin Hospital, Adelaide, Australia [47]. Peripheral blood samples were collected at 15 ± 1 weeks' gestation from 317 pregnant

women after written informed consent [47]. Ethics approval was granted by the Queen Elizabeth Hospital Human Research Ethics Committee (approval no: REC 1712/5/2008).

4.2 Data Collection

Maternal demographic characteristics, physical measurements and information on smoking and alcohol consumption were collected by a research midwife who interviewed participants at 15 ± 1 weeks' gestation [48]. Plasma C-reactive protein (CRP) [49] and micronutrient status [50] were measured at 15 ± 1 weeks' gestation and pregnancy complications were assessed at the end of pregnancy[48].

4.3 DNA extraction, measurement of genomic and mitochondrial DNA content

DNA was extracted from buffy coat from EDTA peripheral blood samples using QIAamp 96 DNA blood kit, QIAGEN, USA [48]. DNA concentration and purity were assessed using a NanoDrop One UV-Vis Spectrophotometer (Thermo Fisher Scientific™). DNA samples with A260/280 ratio of 1.8-2.0 were considered acceptable. mtDNAc was measured relative to nuclear DNAcn using the Bio-Rad CFX96 Real-time PCR detection system (Bio-Rad, Hercules, CA) and SsoFast EvaGreen Supermixes (Bio-Rad®) and calculated with ΔΔCq equation. Real-time quantitative polymerase chain reactions (qPCR) were performed in triplicate in 384-well plates with adding 20 ng DNA per well. Forward and reverse primers for mitochondrial DNA were h-mtDNA-F (D41 [nucleotide 3254–3277]): 5'CGAAAGGACAAGAGAAATAAGG3' and h-mtDNA-R (D56 [nucleotide 3126–3147]): 5'CTGTAAAGTTTTAAGTTTTATGCG3', respectively. Correspondingly, HBGF: 5'GCTTCTGACACAACTGTGTTCACTAGC3' and HBGR: 5'CACCAACTTCATCCACGTTCACC3' have been used as forward and reverse primers for nuclear DNA. Primer pair efficiencies were assessed using a five-point 1:10 serial

dilution starting with 10ng/μL of three different cell line DNA samples (R^2=0.996, E=96.63%). Product size of h-mtDNA and HBG primers were 152 and 120 bp, respectively. Specificity of the PCR products and absence of primer dimer were ensured by melt curve analysis and agarose gel electrophoresis of PCR products. PCR cycling conditions were: 98.0°C for 2 min, followed by 40 cycles of 5 s at 98.0°C and 10 s at 57.5°C, followed by melt curve analysis from 65.0°C to 95.0°C with a 0.5 increment for 5 s.

4.4 Statistical analyses

The relationship between mtDNAc and CRP was investigated using linear regression, with mtDNAc and CRP log-transformed to satisfy model assumptions, and results are reported as ratio of geometric means per 1% increase in CRP. Logistic regression was used to compare mtDNAc between uncomplicated pregnancy and pregnancy complications, adjusting for maternal age and BMI. Results are reported as odds ratios with corresponding 95% confidence intervals. The predicted probability of pregnancy complications was also obtained for a range of mtDNAc, averaged across maternal age and BMI, to assist with visualisation of the effect of mtDNAc. To assess associations between mtDNAc and maternal age, BMI, status, SES, smoking, CRP, and fetal sex linear regression was performed. mtDNAc was log-transformed to approximate normality, and results are reported as ratio of geometric means with corresponding 95% confidence intervals. P-values of less than 0.05 were considered significant. All analyses were performed using R version 3.6.3 or later.

5 Conclusion

Reduced mitochondrial DNA content was associated with a greater risk of pregnancy complications but because the effect was small, it is not a good biomarker for predicting pregnancy complications. Although this may support the notion of mitochondrial

dysfunction in pregnancy complications future large scale cohort studies targeting mitochondrial biogenesis and function at different time points of pregnancy, as well as experimental research investigating mitochondrial DNA content in plasma and the placenta help to understand if mitochondria DNA content can be an early biomarker of pregnancy complications better.

6 Acknowledgment

We acknowledge Ms Sandy Khor and Prof. Rachel A. Burton for technical assistance with qPCR setup.

7 Funding

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8 Conflict of Interest

The authors have no conflicts of interest.

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Chapter 6

Maternal Diet and Offspring Telomere Length: a Systematic Review

"Persistence in scientific research leads to what I call instinct for truth."

Louis Pasteur

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Contribution to the Paper	Contributed to designing the review, extracting papers from search engines, screening studies, data extraction and drafting original manuscript		
Overall percentage (%)	70%		
Certification:	This paper is a systematic review that I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Lead Article

Maternal diet and offspring telomere length: a systematic review

Nahal Habibi, Tina Bianco-Miotto, Yan Yin Phoi, Tanja Jankovic-Karasoulos, Claire T. Roberts, and Jessica A. Grieger

Context: *Many studies assert a negative influence of inappropriate maternal diet and nutritional status during pregnancy on offspring, not only in utero but throughout life, because of the role in the programing of noncommunicable diseases. Telomere length is a biomarker of aging, and shorter telomeres are associated with chronic disease later in life. Maternal nutrition and nutritional status may be an important determinant of offspring telomere length.* ***Objective:*** *A systematic review was conducted to determine the effect of maternal nutrition and nutritional status in pregnancy on offspring telomere length.* ***Data Sources:*** *This systematic review was conducted according to PRISMA guidelines. Database searches of PubMed, CINAHL, Scopus, Medline, and Web of Science were performed.* ***Study Selection:*** *Included studies assessed the association between maternal nutrition (dietary intake and nutritional status) during pregnancy and offspring telomere length measured in cord blood, serum, plasma, and peripheral blood mononuclear cells.* ***Data Extraction:*** *Three authors screened and determined the quality of the articles; disagreements were resolved by a fourth author. All authors compared the compiled data.* ***Results:*** *Seven studies were extracted and evaluated. Studies comprised a double-blind placebo-controlled trial ($n = 1$), prospective cohort studies ($n = 5$), and a cross-sectional study ($n = 1$). Higher circulating maternal folate and 25-hydroxyvitamin D_3 concentrations, along with higher maternal dietary caffeine intakes, were associated with longer offspring telomere length, whereas higher dietary intake of carbohydrate, folate, n-3 polyunsaturated fatty acids, vitamin C, or sodium was not.* ***Conclusion:*** *The limited but suggestive evidence highlights the need for further research to be conducted in this area, particularly longitudinal studies involving larger cohorts of pregnant women.*

Systematic review registration: *PROSPERO registration no. CRD42019136506*

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Key words: cord blood, folate, maternal diet, maternal nutritional status, offspring, pregnancy, telomere length, vitamin D.

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INTRODUCTION

Optimal nutritional supply to the developing fetus is paramount to achieving appropriate fetal growth and development. Nutrition prior to[1,2] and during early pregnancy[3,4] may impact offspring health and future disease. A dietary pattern containing several protein-rich food sources, fruit, and some whole grains is associated with reduced likelihood of preterm delivery.[1] Following a Mediterranean dietary pattern is associated with lower risk of maternal gestational diabetes and atopy in offspring.[5] Comparatively, unhealthy dietary patterns characterized by high intakes of refined grains, processed meat, and foods high in saturated fat or sugar are associated with lower birth weight and a higher risk of preterm birth.[6] Exposure to famine and maternal overweight and obesity during pregnancy is associated with increased risk for later-life cardiovascular disease, type 2 diabetes, and cancer in offspring.[7] Low maternal micronutrient concentrations have also been associated with greater risk of developing pregnancy complications,[8,9] as well as offspring insulin resistance, cognitive dysfunction, and lower birth weight.[10–12] The critical effect of maternal nutrition on offspring health and disease risk is encompassed by the developmental origins of health and disease hypothesis, which describes that fetal exposure to nutritional imbalances in utero can program the fetus to develop chronic diseases later in life.[13]

Several markers are used to predict future risk of chronic disease. One such marker is telomere length.[14] Telomeres are noncoding double-stranded tandem repeats of [5′-(TTAGGG) n-3′] deoxyribonucleic acid (DNA) sequences at the ends of chromosomes. Their main function is to provide genome stability by protecting DNA from decay or fusion with other chromosomes.[14] In normal somatic cells, telomeres shorten with each subsequent cell division until they reach a critical short length.[15] This results in DNA damage, interruption to cellular function, chromosomal fusion, and cell senescence.[15,16] In adults, there are clear and consistent associations between shorter telomeres and development of chronic diseases such as cancer, cardiovascular disease, and type 2 diabetes,[15–17] as well as all-cause mortality.[18]

Maternal exposures in pregnancy are critical predictors of telomere length in offspring.[19] There have been some studies in pregnancy associating psychosocial stress, gestational diabetes, and tobacco exposure with shorter telomeres measured in the cord blood of newborns.[20–26] The authors of the present study recently showed that children of mothers who had metabolic syndrome in pregnancy had shorter salivary telomere lengths, at age 10 years, than children whose mothers did not have metabolic syndrome during pregnancy.[27]

Given the important links between maternal diet and offspring health, it is plausible that maternal nutrition could play a key role in offspring telomere length. Recently, Gorenjak et al[19] reviewed the potential impact of some maternal factors (including circulating dietary measures) on telomere length, but there has been no comprehensive systematic review assessing the effect of maternal dietary intake on offspring telomere length. Using PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analysis) guidelines (Appendix S1; *please see the Supporting Information online*), this review will answer the following research question: Does an association exist between maternal nutrition/nutritional status and offspring telomere length? The aim of the current review, therefore, was to systematically summarize the literature regarding the association between maternal nutrition (dietary intakes, supplements, and nutritional biomarkers in blood, plasma, and serum) and offspring telomere length.

METHODS

Search strategy

Potentially relevant studies were identified via electronic searches of the following databases: Medline, PubMed, Scopus, Web of Science, and Cumulative Index to Nursing and Allied Health Literature (CINAHL). Furthermore, the references of relevant review articles were manually searched for studies that met the inclusion criteria. The search strategy for each database was chosen to ensure all relevant literature was captured, with N.H. and J.A.G. pilot testing various key words. The search strategy included terms such as pregnancy, maternal, nutrition, diet, telomere length, and offspring (Table 1). Inclusion criteria were full–text articles, published in English, and published in academic journals, and only published content was examined (ie, not conference abstracts). The search was performed by N.H. and J.A.G. in January 2020 and the search was updated in July 2020 (no limits on time frame). All citations were firstly imported into an Endnote file and duplicates removed, and then exported into a Rayyan software database for blind screening.[28]

The review authors independently screened the titles and abstracts yielded by the search against the inclusion criteria. N.H. screened all titles/abstracts and T.J.-K. and Y.Y.P. screened half each. A 10% sample of all excluded articles was cross-checked by J.A.G. Articles were identified as "exclude" (did not meet inclusion criteria), "maybe" (a review of the full-text

Table 1 **Search strategy**

Database	Search terms
PubMed	(("pregnancy"[mh] OR "mothers"[mh] OR pregnan*[tiab] OR maternal*[tiab] OR mother*[tiab]) AND ("nutritional status"[mh] OR "diet"[mh] OR "diet, food, and nutrition"[mh] OR nutrition*[tiab] OR diet*[tiab] OR food*[tiab])) AND ("telomere"[mh] OR "telomere shortening"[mh] OR "telomere homeostasis"[mh] OR telomere*[tiab])
CINAHL	(MH pregnancy+ OR TI pregnan* OR AB pregnan* OR TI maternal OR AB maternal) AND (MH nutrition+ OR MH "maternal nutritional physiology+" OR TI nutrition* OR AB nutrition* OR TI diet* OR AB diet* OR TI food* OR AB food*) AND (MH telomere OR TI telomere* OR AB telomere*)
Scopus	("maternal diet" OR "pregnancy" OR "food" OR "maternal" OR "maternal nutrition" AND "telomere length")
Medline	((Pregnancy or Mothers) and (Nutritional Status or Diet or Food) and Telomere).af. (ALL FIELDS)
Web of Science	("pregnancy" OR "pregnant" OR "maternal") AND ("maternal diet" OR "food" OR "maternal nutrition" OR "nutrition") AND "telomere length")

article was required), and "include" (required confirmation of inclusion following review of the full-text article). Copies of the full-text papers were obtained and reviewed for each of the "included" and "maybe" studies. Reference lists were checked for each of the included articles as well as articles that were read as full text but not included in the review. Studies were excluded if they did not meet the inclusion criteria. Following screening, the "blind" setting of the software tool Rayyan was turned off in order to resolve any potential conflicts. Seven articles were identified, with no disagreement among the authors of the present study.

Eligibility criteria

All potentially eligible studies identified were assessed for inclusion by the research team, using the PICO (population-intervention–comparator–outcome) criteria (Table 2). "Population" referred to the following: pregnant women of any age and any gestational stage, not restricted to pregnant women with specific diseases or conditions. "Intervention" referred to the following: randomized controlled trials (RCTs) that assessed foods or supplements, or observational studies assessing maternal dietary intake or nutrient status (the exposure) at any point in gestation. "Comparator" referred to the following: for RCTs, the comparator was a control or placebo group; for observational studies, the comparator was nonexposed controls. "Outcome" referred to the following: offspring telomere length, measured in DNA obtained from cord blood, saliva, serum, plasma, or peripheral blood mononuclear cells. The outcome was not restricted to type of method of assessment or type of telomere measurement (eg, relative telomere length [= T/S ratio: ratio of telomeric DNA to a single-copy gene] or absolute base-pair length). There was no prioritization in terms of how telomere length was measured and assessed, nor on how the biological specimen or its assessment was collected. Studies assessing alcohol intake, contaminants in food/water, and environmental/food toxins (eg, cadmium, lead, mercury) were excluded.

Data extraction and synthesis

Data extraction of the included articles was first pilot-tested using various headings, but finalized into an Excel spreadsheet that included the following: author; year; country; study design; sample size; data collection and timing of maternal dietary exposure; maternal characteristics; data collection and timing of offspring outcome; offspring characteristics; adjustment for confounders; and results. N.H. and Y.Y.P. carried out the data extraction following training by J.A.G., to ensure consistency. The results of the data extraction are shown in Table 3.

A systematic narrative synthesis is provided with information presented in the text and tables to summarize and explain the characteristics and findings of the included studies. The narrative synthesis explores the relationship and findings both within and between the included studies, in line with the guidance from the Centre for Reviews and Dissemination.[36] All studies, regardless of overall level of bias, were synthesized.

Assessment of risk of bias

Study quality for the RCT was assessed by the primary reviewers (N.H. and J.A.G.) using Cochrane's Collaboration tool for assessing risk of bias for randomized trials.[37] Two investigators (N.H. and Y.Y.P.) independently appraised the risk of bias of included observational studies using the ROBINS-I (risk of bias in nonrandomized studies of interventions) tool.[38] The ROBINS-I tool was developed to assess risk of bias in the results of nonrandomized studies that compare the health effects of 2 or more interventions. Designs of nonrandomized studies include observational studies (cohort or case-control) and quasi-randomized studies.[38] The ROBINS-I tool evaluates the risk of bias in estimates of the comparative effectiveness of safety (benefit or harm) of an intervention from studies that did not use randomization to allocate interventions. The ROBINS-I tool includes domains that cover

Table 2 **PICOS criteria for inclusion of studies**

Parameter	Criterion
Population	Pregnant women of any age and any stage of gestation, not restricted to pregnant women with specific diseases or conditions
Intervention	Food and supplement interventions in RCTs, maternal dietary intake or nutrient status (the exposure) at any point in gestation in observational studies
Comparison	For RCTs, the comparator was a control or placebo group; for observational studies, the comparator was non-exposed controls, or a pre-exposure group for pre-/post-designs
Outcome	Offspring telomere length at birth and beyond, measured in DNA obtained from cord blood, saliva, serum, plasma, or PBMCs. No priority for how telomere length was measured and assessed, nor on how the biological specimen or its assessment was collected. Studies assessing alcohol intake, contaminants in food/water, and environmental toxins (eg, cadmium, lead) were excluded.
Study design	RCTs, observational studies

Abbreviations: PBMC, peripheral blood mononuclear cell; RCT, randomized controlled trial.

confounders, selection, classification of interventions, deviations, missing data, measurement of outcomes, and selection of the reported results, with signaling questions to facilitate judgments about the risk of bias. The categories for risk of bias judgments are "low risk," "moderate risk," "serious risk," and "critical risk," with an overall level of risk of bias across all domains. The overall risk of bias was based on the authors' judgment regarding both the severity of bias and relative consequence of bias in each domain.

RESULTS

A total of 413 studies were identified from the 5 databases (Figure 1); of these, 87 articles were excluded because they were duplicate publications, book sections, or not published in English. After screening of the titles and abstracts, 306 further articles were excluded. Full-text versions of 20 articles (including 10 reviews) were then retrieved for detailed review. Three studies were removed as they did not meet the inclusion criteria (2 of these studies investigated toxic metals and one other examined maternal supplementation during and after pregnancy); the remaining 10 studies were excluded following screening of their references (Figure 1). Thus, 7 articles were included in the final systematic review.

The characteristics of the 7 included studies are summarized in Table 3[29–35]; the review involved 1 cross-sectional study, 5 observational studies, and 1 double-blind RCT. Sample sizes ranged from 57 to 229, and the RCT comprised 98 pregnant atopic women with a 12-year follow-up of their children. The included studies assessed maternal dietary intake and nutritional status during pregnancy – dietary caffeine (n = 1), dietary vitamin C and sodium (n = 1), dietary folate equivalents (n = 1), dietary n-3 polyunsaturated fatty acids (n-3 PUFAs) (n = 1), energy and macronutrient intake (n = 2), serum folate (n = 1), serum vitamin D (n = 1), and serum n-3 PUFA (n = 1) – and their association with telomere length measured in cord blood or non-fasting venous blood in offspring aged up to 12 years.

Risk of bias

The included RCT was rated overall as of moderate quality (Table 4).[34] While it rated low for selection bias, reporting bias, performance bias, and attrition bias, it was unclear who had measured the outcomes and whether they were blinded for data collection (high risk of detection bias). Overall, the risk of bias in the observational studies (Table 5) was low in 2 studies,[31,33] moderate in 1 study,[35] and serious in 3 studies.[29,30,32] All studies reported a low risk of bias in the measurement of outcome domain, whereas 4 studies scored moderate for selection bias[29,30,32,35] and 3 for missing data.[30,33,35] There was serious risk of bias for the domain classifications of intervention and selection of the reported results in 2 studies,[29,32] and serious risk of confounding bias in 3 studies.[29,30,32] Maternal age, maternal BMI, and infant sex and birth weight were included as confounders in 2 studies,[31,33] whereas the study by Entringer et al[35] only included maternal BMI and infant sex and birth weight. There was inconsistency across all studies regarding other confounders, and the studies by Myers et al[30] and Griffin et al[29] did not report on any confounding variables.

Maternal dietary determinants of offspring telomere length

Table 3 describes the characteristics of the included studies. In the double-blind, placebo-controlled, parallel-group study, women were randomized to 4 g/d of n-3 PUFA (n = 52) or control (olive oil capsules, n = 46) before 20 weeks' gestation, up until delivery.[34]Eighty-three women completed the study (n = 40 in the intervention group; n = 43 in control). Supplementation had no effect on telomere length in offspring at birth (measured in cord blood) or at age

Table 3 **Maternal dietary determinants of offspring telomere length**

Author, year Country	Country	Study design	Sample size	Data collection and timing of maternal dietary exposure	Maternal characteristics	Data collection and timing of offspring outcome	Offspring characteristics	Adjustment for confounders	Results
Griffin et al (2020)[29]	USA	Prospective cohort study	57 mother-newborn pairs	Caffeine intake measured using FFQ, which was completed before hospital discharge. Women enrolled at labor.	Mean (SD) - Age: 25.5 (5.2) y - BMI, % Normal/underweight: 24.8% Overweight: 17.5% Obese: 57.9% - Ethnicity, % White: 35.7% Black: 30.4% Hispanic: 25.0% Other: 8.9%	At delivery, cord blood DNA for measurement of relative telomere length (T/S ratio)	Mean (SD): - Gestational age: 39.5 (± 1.3) wk - Birth weight: 3381.4 (± 509.1) g - T/S ratio: 0.9 (± 0.2)	None reported	1) No association between mean caffeine consumption (117 mg) and T/S ratio 2) Higher caffeine intake (>300 mg or >200 mg) was associated with longer telomere length (both β=0.0042; 95%CI, 0.002–0.008; $P<0.05$)
Myers et al (2019)[30]	USA	Prospective cohort study.	64 healthy mother-newborn pairs	Maternal vitamin C and sodium intake measured using the DHQ. Women recruited upon admission into labor and delivery, but unclear when dietary assessments were measured.	Age: 57.81% <27 y and 42.19% ≥28 y	At delivery, cord blood buffy-coat DNA for measurement of relative telomere length (T/S ratio)	None reported	None reported.	1) Association between maternal vitamin C intake and newborn TL (OR 1.003; 95%CI, 1.001–1.005). 2) Association between maternal sodium intake and newborn TL (OR 0.999; 95%CI, 0.999–0.999).
Kim et al (2018)[31]	South Korea	Cross-sectional study.	106 healthy mother-newborn pairs	Fasting maternal blood $25(OH)D_3$ concentration, dietary intake of energy, macronutrients, and DFE. Collected in the third trimester of pregnancy.	Mean (SD): - Age: 32.69 ± 2.85 y - Prepregnancy BMI: 21.80 ± 2.01 kg/m^2 - $25(OH)D_3$: median IQR: 14.21 (11.16–18.18) ng/mL - Energy intake: 1818 ± 307 kcal/d - Macronutrients (% total energy): protein (16.2 ± 6.5); CHO (62.1±8.1); fat (21.77±6.21) DFE: 566.8 (459.4–706.8) μg/d	At delivery, cord blood buffy-coat DNA for measurement of relative telomere length (T/S ratio)	Mean (SD): - Birth weight: 3424.5 ± 370.1 g - T/S ratio: 1.61 (1.24–2.16)	Maternal age, BMI, leukocyte telomere lengths, WBC count, glycosylated hemoglobin level, health behaviors, nutritional intakes, newborn's sex and birth weight	Positive association between maternal energy intake and newborn TL (r=0.22, P=0.03) and maternal $25(OH)D_3$ concentrations with newborn TL (r = 0.72, $P<0.01$).
Salihu et al (2018)[32]	USA	Prospective cohort study.	62 healthy mother-newborn pairs	Maternal CHO and saturated fat intake measured using the DHQ. Collected before discharge from the hospital.	Mean (SD): *High CHO group* - Age: 25.8 ± 5.1 y - BMI: 33.8 ± 9.0 kg/m^2 - Weeks' gestation: 39.6 ± 1.3 d *Adequate CHO group* - Age: 23.9 ± 5.0 y - BMI: 37.2 ± 9.3 kg/m^2 - Weeks' gestation: 39.4 ± 1.3 d *High fat group* - Age: 26.1 ± 5.5 y - BMI: 35.6 ± 5.8 kg/m^2 - Weeks' gestation: 39.7 ± 1.3 d *Adequate fat group* - Age: 25.0 ± 4.9 y - BMI: 34.0 ± 10.7 kg/m^2 - Weeks' gestation: 39.4 ± 1.3 d	At delivery, cord blood buffy-coat DNA for measurement of relative telomere length (T/S ratio)	No difference in birth weight or head circumference between neonates across maternal diet groups	Maternal SES, delivery characteristics that caused a change of ≥10% in magnitude of association between predictor variables and outcome	High fat consumption during pregnancy was associated with shorter fetal T/S ratio ($P<0.05$), (95%CI, -3.71×10^{-3}, -1.44×10^{-3})

(continued)

Table 3 **Continued**

Author, year Country	Country	Study design	Sample size	Data collection and timing of maternal dietary exposure	Maternal characteristics	Data collection and timing of offspring outcome	Offspring characteristics	Adjustment for confounders	Results
Yeates et al (2017)[33]	Seychelles	Cohort study.	229 mother-newborn pairs	Nonfasting maternal serum PUFA. Collected at 28 weeks' gestation.	Mean (SD): - Age, 27.2 ± 5.93 y; BMI at enrollment, 25.77 ± 6.38 kg/m^2 - Weeks' gestation: 38.75 ± 1.34 d - Serum n-3 PUFA: 0.03 ± 0.01 g/L - Serum n-6 PUFA: 1.2 ± 0.2 g/L - Serum n-6 : n-3 PUFA ratio: 40.2 ± 11.7 - TL (T/S ratio): 0.64 ± 0.11	At delivery, cord blood leukocyte DNA and nonfasting blood at age 5 y Measurement of relative TLs (T/S ratios)	Mean (SD): - Birth weight: 3.24 ± 0.47 kg *Age 5 y:* - BMI: 14.96 ± 1.98 kg/m^2 - Plasma AA: 0.05 ± 0.01 g/L - Plasma EPA+DHA: 0.04 ± 0.01 g/L - Plasma AA : DHA ratio: 1.51 ± 0.34 - Cord T/S ratio: 1.18 ± 0.5 - T/S ratio: 0.71 ± 0.1 - Telomere attrition rate (T/S ratio): 0.47 ± 0.14	Maternal age, BMI, smoking during pregnancy (yes or no), alcohol consumption during pregnancy (yes or no), child's sex, birth weight, gestational age, SES	No association between prenatal n-3 PUFA, prenatal n-6 PUFA, or prenatal n-6 : n -3 PUFA ratio with TL in cord blood or in blood at age 5 y
See et al (2016)[34]	Australia	Double-blind, placebo-controlled, parallel-group study.	98 pregnant atopic women at <20 weeks' gestation	Maternal blood collected at 30 and 36 weeks' gestation and 6 weeks postnatally for measurement of erythrocyte DHA and EPA. Randomized to 4 g/d of n-3 PUFA or olive oil control, from 20 weeks' gestation to delivery. 40 women completed the intervention and 43 completed the control.	Mean (SD): *n-3 PUFA group:* - Age: 31.0 ± 4.0 y - Prepregnancy BMI: 23.7 ± 3.8 kg/m^2 - Weeks' gestation: 74.7 ± 8.3 d *Control group:* - Age: 32.4 ± 3.5 years - Prepregnancy BMI: 24.1 ± 4.0 kg/m^2 - Weeks' gestation: 274.2 ± 7.8 d	At delivery, cord blood erythrocyte fatty acids (DHA & EPA) At delivery, absolute TL, DHA, and EPA extracted from red blood cell membranes assayed by gas chromatography	Mean (SD): *n-3 PUFA group:* - Birth weight : 3502.7 ± 342.1 g - Birth length: 50.5 ± 2.0 cm - Head circumference: 35.2 ± 1.3 cm *Control group:* - Birth weight: 3430.1 g ± 371.8 g - Birth length: 49.7 ± 1.9 cm - Head circumference: 34.7 ± 1.2 cm	Gender, maternal age, and cigarette smoke exposure during pregnancy	No difference in TL at birth (150.6 ± 1.4 vs 156.7 ± 1.4 kb/genome) or at age 12 y (153.1 ± 1.6 vs 174.8 ± 1.4 kb/genome). No change over time between groups.
Entringer et al (2015)[35]	USA	Prospective cohort study.	119 healthy mother-newborn pairs	Nonfasting maternal serum folate concentrations, collected in the first trimester.	Mean (SD): - Age: 24.5 ± 4.5 y - Prepregnancy BMI: 27.5 ± 7.3 kg/m^2 - Weeks' gestation: 9.5 ± 2.1 d - Total folate: 29.5 ± 10.9 ng/mL - 5MeTHF: 17.2 ± 7.3 ng/mL - 5Fo-THF: 10.5 ± 6.9 ng/mL	At delivery, cord blood leukocyte DNA for relative TLs (T/S ratios)	Mean (SD): Birth weight: 3206 ± 521 g	Maternal SES, race/ethnicity, prepregnancy BMI, gestation, birth weight, infant's sex, obstetric complications	1) A 10 ng/mL increase in total folate associated with a 5.8% increase in median TL (95%CI, 0.5%–11.3%) 2) Approximately 10% shorter median TL in newborns of mothers in lowest quartile of total folate (equivalent to ∼1000 bp) vs newborns of mothers in highest folate quartile (95%CI, 1.5%–14.3%) 3) A 10 ng/mL increase in 5MeTHF associated with a 7.7 % increase in median TL (95%CI, 0.3%–15%) 4) No association between maternal levels of 5Fo-THF and newborn TL

Abbreviations: AA, arachidonic acid; CHO, carbohydrate; DFE, dietary folate equivalent; DHA, docosahexaenoic acid; DHQ, Dietary History Questionnaire-1; EPA, eicosapentaenoic acid; 5Fo-THF, 5-formyltetrahydrofolate; 5MeTHF, 5-methyltetrahydrofolate; FFQ, food frequency questionnaire; 25(OH)D_3, 25-hydroxyvitamin D3; IQR, interquartile range; n-3 PUFA, omega-3 polyunsaturated fatty acid; OR, odds ratio; SD, standard deviation; SES, socioeconomic status; TL, telomere length; T/S, ratio of telomeric DNA to a single-copy gene; WBC, white blood cell.

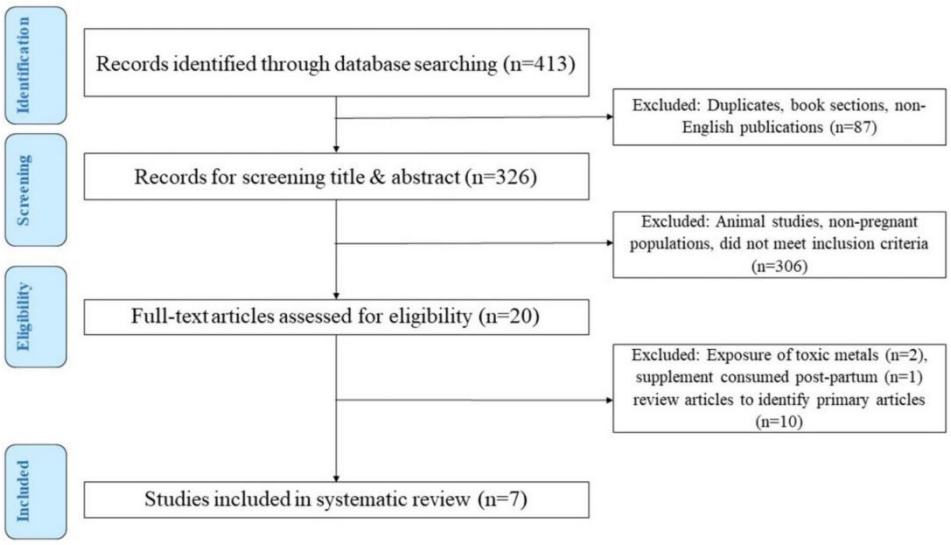

Figure 1 **Flow diagram of the literature search process.** A total of 413 studies were identified from the 5 databases searched. Eighty-seven articles were removed because they were duplicates, book sections, or not published in English. After screening of the titles and abstracts, another 306 articles were excluded because they examined nonpregnant populations or were animal studies. Ten reviews were removed after screening of their references. Three further studies were removed after reading the full-text versions as they did not meet the inclusion criteria. Thus, 7 studies were included in the final systematic review

12 years (measured in a fasting blood sample) compared with control.[34]

In 57 socioeconomically disadvantaged mother-newborn pairs from the United States, caffeine intake, assessed via a food frequency questionnaire at the end of pregnancy, was not associated with newborn telomere length, measured in cord blood.[29] However, when categorized as consuming >200 mg/d caffeine or >300 mg/d caffeine, telomere length was longer ($\beta = 0.0042$; 95%CI, 0.002–0.008).[29]

In the first nutrition cohort study of mothers and children of the Seychelles Child Development Study, maternal n-3 PUFA status measured at 28 weeks' gestation and at delivery was not associated with telomere length measured in cord blood or in nonfasting blood of the 5-year-old children.[33,35]

Maternal serum total folate and its two fractions, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate, were assessed in 119 folate-replete women in the first trimester of gestation.[35] The results of this prospective cohort study demonstrated that newborns of mothers in the lowest folate quartile had shorter median telomere length by approximately 10% (95%CI, 1.5%–14.3%) than newborns of mothers in the highest folate quartile.[35] Maternal 5-formyltetrahydrofolate level was not associated with newborn telomere length. However, for every 10 ng/mL increase in maternal 5-methyltetrahydrofolate, there was a 7.7% (95%CI, 0.3%–15%) increase in newborn median telomere length. Additionally, for every 10 ng/mL increase in maternal serum total folate, there was a 5.8% (95%CI, 0.5%–11.3%) increase in newborn median telomere length.[35] In contrast, in a cross-sectional study of 106 healthy pregnant women in the third trimester of pregnancy, dietary intake of dietary folate equivalents, measured by a 24-hour recall, was not associated with fetal telomere length.[31] However, a positive correlation between maternal blood vitamin D, 25-hydroxyvitamin D3 ($25[OH]D_3$), and newborn leukocyte telomere length ($\beta = 0.33$; $P < 0.01$) was reported.[31]

In a prospective cohort study among 64 women, recruited upon admission into labor and delivery, Myers et al[30] reported an association between dietary intake of vitamin C and fetal telomere length (odds ratio, 1.00; 95%CI, 1.001–1.005) and sodium and fetal telomere length (odds ratio, 0.99; 95%CI, 0.999–0.999).

Two studies reported on maternal macronutrient intakes and telomere length.[31,32] Kim et al[31] found a

Table 4 **Quality assessment for the randomized control trial[34] using the revised Cochrane risk of bias tool for randomized trials[37]**

Domain	Description	Assessment (high, low, or unclear risk of bias)	Comments
Selection bias: random sequence generation	Described the method used to generate the allocation sequence in sufficient detail to allow an assessment of whether it should produce comparable groups.	Low	The study was single-centered and stratified by parity (no previous term child birth vs one or more), prepregnancy BMI, age, and maternal allergy (allergic rhinitis or asthma) with block randomization (1 : 1).
Selection bias: allocation concealment	Described the method used to conceal the allocation sequence in sufficient detail to determine whether intervention allocations could have been foreseen before or during enrollment.	Low	Concealment of allocation process was described. Double blinded.
Reporting bias: selective reporting	Stated how the possibility of selective outcome reporting was examined by the authors and what was found.	Low	Significant results reported. Nonsignificant interactions not reported but unlikely to have significantly influenced the study conclusions.
Other bias: other sources of bias	Any important concerns about bias not addressed above.	High	Selection bias due to the inclusion of women with atopy.
Performance bias: blinding (participants and personnel)	Described all measures used, if any, to blind study participants and personnel from knowledge of which intervention a participant received. Provided any information relating to whether the intended blinding was effective.	Low	Double blinded.
Detection bias: blinding (outcome assessment)	Described all measures used, if any, to blind outcome assessors from knowledge of which intervention a participant received. Provided any information relating to whether the intended blinding was effective.	Unclear	Not reported who measured outcomes and whether they were blinded for data collection.
Attrition bias: incomplete outcome data	Described the completeness of outcome data for each main outcome, including attrition and exclusions from the analysis. Stated whether attrition and exclusions were reported, the numbers in each intervention group (compared with total randomized participants), and reasons for attrition/exclusions where reported.	Low	Losses to follow-up were disclosed and the analyses were conducted using maximum likelihood estimation, which handles missing data by using complete and incomplete data as well as intraclass correlation to estimate the most likely mean for the whole sample at each time point.

weak positive association between intake of energy in the third trimester and fetal telomere length measured in cord blood ($r = 0.22$; $P = 0.03$). However, protein, carbohydrate, or fat intake, classified as percentage of total energy intake, was not associated with fetal telomere length.[31] Comparatively, in a prospective cohort study of 62 women, high maternal fat consumption, assessed by the Dietary History Questionnaire-1 before discharge from hospital, was found to be associated with shorter telomere length measured in umbilical cord blood (95%CI, -3.71×10^{-3} to -1.44×10^{-3}), while maternal carbohydrate consumption was not associated with fetal telomere length.[32]

DISCUSSION

This is the first systematic review to examine the effect of maternal nutrition (diet and nutritional status) during pregnancy on offspring telomere length. Higher circulating maternal folate and $25(OH)D_3$ concentrations, and higher maternal dietary caffeine intakes, were associated with longer offspring telomere length, whereas

Table 5 **Quality assessment for the nonrandomized studies using the ROBINS-I (risk of bias in nonrandomized studies of interventions) assessment tool[38]**

First author	Confounders	Selection bias	Classification of intervention	Deviations	Missing data	Measurement of outcome	Selection of the reported results	Overall risk of bias
Griffin et al (2020)[29]	Serious	Moderate	Serious	Unclear	Low	Low	Serious	Serious
Myers et al (2019)[30]	Serious	Moderate	Moderate	Low	Moderate	Low	Low	Serious
Salihu et al (2018)[32]	Serious	Moderate	Serious	Unclear	Low	Low	Serious	Serious
Kim et al (2018)[31]	Low	Low	Low	Unclear	Unclear	Low	Low	Low
Yeates et al (2017)[33]	Low	Low	Low	Low	Moderate	Low	Low	Low
Entringer et al (2015)[35]	Low	Moderate	Low	Unclear	Moderate	Low	Low	Moderate

higher dietary intake of carbohydrate, folate, n-3 PUFA, vitamin C, or sodium was not.

One study assessed maternal daily intake of energy, macronutrients, and folate with a single-day self-reported 24-hour dietary recall in Korean women.[31] Maternal energy intake, but not macronutrients and folate, was found to be associated with newborn telomere length.[31] However, the mean daily energy intake was 1818 kcal/d, which is much lower than the energy requirements for Korean pregnant women aged 30–49 years in the third trimester of pregnancy (2350 kcal/d).[39] Different macro- and micronutrients are substrates or cofactors for DNA synthesis; thus they are essential for DNA homeostasis and telomere integrity.[40] In the study by Ma et al,[41] although an experienced clinical dietitian advised women on how to recall foods and beverages using a 24-hour dietary recall, one day's dietary intake may not provide sufficient data and may underestimate energy intake. Dietary assessment using three 24-hour dietary recalls – two weekday and one weekend day – enhances the accuracy of energy intake and reduces underreporting.[41] Therefore, to investigate the association between maternal dietary factors and offspring telomere length, assessment of more than one day's dietary intake may be necessary, particularly in studies involving small sample sizes.

Despite the lack of association with dietary folate intake,[31] Entringer et al[35] reported a positive association between maternal total serum folate concentration and newborn telomere length. Previous studies in nonpregnant adults have demonstrated positive associations between plasma folate concentrations and telomere length.[42–44] Related, in nonpregnant adults, folate and vitamin B12 may delay the aging process by preventing a reduction in relative telomere length.[45,46] Folate provides precursors for nucleotide synthesis, and low folate availability causes nucleotide imbalances and subsequent DNA damage.[47] Hence, folate may influence telomere length through its effect on telomeric DNA.[47,48] Additionally, folate can impact telomere length via epigenetic regulation through DNA methylation[49]; folate deficiency leads to decreased thymidylate synthesis, deoxyribonucleotide pool imbalance, and uracil misincorporation into DNA strands during replication and repair processes.[42]

In pregnancy, maternal supply of folate is critical for fetal development and for DNA synthesis and cell proliferation.[50] Moreover, telomere length is heritable and newborn telomere length may be important for long-term health effects.[51,52] It is surprising that this review identified only 2 studies relating to maternal folate and telomere length,[33,35] and no studies assessed the impact of maternal folic acid supplementation. Further dietary and mechanistic studies relating to maternal folate status and offspring telomere length are warranted. Additionally, as the study by Entringer et al[35] was carried out in a folate-replete population, identifying whether folic acid supplementation – in initially deplete populations – further impacts telomere length would add to the existing literature and mechanisms regarding DNA synthesis and repair and telomere biology.

Higher maternal circulating 25(OH)D_3 concentrations were found to be associated with longer telomere length in neonates.[31] Although some factors, including skin color, clothing, season, and latitude, can affect vitamin D concentrations, many pregnant women in different regions of the world are at risk of vitamin D deficiency.[53–57] The association reported between maternal 25(OH)D_3 and telomere length may be related to the protective role of vitamin D in reducing inflammation and oxidative stress.[58–61] Lower 25(OH)D_3 concentration in cord blood is associated with higher C-reactive protein in newborns, indicating increased inflammation,[62] and cord blood vitamin D level depends

on maternal vitamin D status.[63] Given the supporting mechanisms regarding vitamin D and reduced oxidative stress and inflammation, it appears that lower maternal vitamin D status contributes to shorter telomeres in the offspring.

Salihu et al[32] reported that higher maternal fat intake is associated with shorter telomere length in newborns. Dietary intakes were collected using the Dietary History Questionnaire-1. Seventy-nine percent of women (n = 49) consumed a high intake of carbohydrate, classified as more than the 175 g/d dietary reference intake, and 37% (n = 23) were high fat consumers, with a fat intake of more than 35 g/d. Higher intake of saturated fatty acids is related to increased oxidative stress and inflammation, which can shorten telomeres.[64,65] However, it was unclear whether total fat or saturated fat intake was assessed as there were inconsistencies throughout the presented data on what was assessed. In addition, the authors state that they used the upper cutoff point of the acceptable macronutrient distribution range for total fat, and defined high fat intake as consumption of more than 35 g/d for pregnant women aged 18–50 years. While they reported that this was based on the Institute of Medicine's dietary reference intake for pregnant women aged 18–50 years, there is currently no dietary reference intake value for daily fat intake, but rather an acceptable macronutrient distribution range – of 20%–35% of total energy.[66] Thus, the upper cutoff point at 35% of daily total energy is different from 35 g/d for a pregnant woman, likely indicating that the authors miscalculated high fat intake in their women. As such, whether there is a true association between fat consumption and fetal telomere length is unclear. Furthermore, the sample size was small (n = 62) with women birthing at a hospital serving a community of low socioeconomic status.[21] This calls into question both the validity and generalizability of the study findings.

Myers et al[30] assessed dietary intake of vitamin C and sodium using the Dietary History Questionnaire-1. It was reported that higher maternal vitamin C intake and lower sodium intake were associated with longer offspring telomere length. However, the reported odds ratios were 1.00, with extremely tight confidence intervals involved in telomere attrition, and may reduce generalizability to other populations. Griffin et al[29] reported that higher maternal caffeine intake (>200 mg/d) was associated with longer telomere lengths in cord blood. While this may contradict public health messages to consume lower intakes of caffeine during pregnancy,[67] the literature on caffeine intake and telomeres in adults is inconsistent.[68–70] Further studies, involving larger sample sizes, and using a higher-quality methodological approach, are needed to support or refute the earlier findings.

Some studies have shown that a higher level of inflammation – as indicated by higher levels of C-reactive protein, fibrinogen, interleukin-6, or tumor necrosis factor–alpha – is associated with shorter telomere length.[71–73] Oxidative stress can also contribute to telomere shortening by causing oxidative damage to guanosine mainly at the GGG sequence in the telomere setting.[74,75] Folate,[76] vitamin D,[58–61,77] vitamin C,[78–80] and n-3 PUFA[81–83] can protect cells against oxidative stress and inflammation, while high consumption of saturated fat[64,65] is associated with higher oxidative stress and inflammation, which subsequently reduces telomere length. Therefore, it is not surprising that folate and vitamin D are associated with longer telomeres; however, additional studies to support these findings are needed.

Finally, it is worth noting the study quality and risk of bias in these studies. The studies by Myers et al,[30] Salihu et al,[32] and Griffin et al[29] were identified as having an overall serious risk of bias. Importantly, confounding variables were either not included[29,30] or were reported in an ambiguous manner.[32] It is imperative that confounding variables are considered when conducting epidemiology studies as these variables may suggest an association where none exists or mask a true association. Another 3 studies[31,33,35] all included a range of covariates, but none included paternal factors, which have also been associated with offspring telomere length.[84] In particular, men who are older have sperm with longer telomeres, and their offspring also have longer leukocyte telomere lengths.[84,85] Given that telomere length shortens with age in most proliferating tissues,[86] and paternal age appears to be an important determinant of offspring telomere length, further research investigating the combined effects of maternal and paternal exposures, and the plausible effect of folic acid on offspring telomere length, will be valuable. Lastly, missing data was often not reported on in these studies, with the measure of deviations often unclear. This highlights the need for transparency to improve the quality of research reporting and increase the usefulness of the research findings.

CONCLUSION

Findings from the 7 identified studies showed that higher circulating maternal folate and 25(OH)D_3 concentrations, along with higher dietary caffeine intake, were associated with longer offspring telomere length, whereas higher dietary intake of carbohydrate, folate, n-3 PUFA, vitamin C, or sodium was not. The limited but suggestive evidence highlights the need for further

research in this area, particularly longitudinal studies involving larger cohorts of pregnant women. Such studies will help identify maternal nutritional determinants and the impact of offspring telomere length as a marker of future chronic disease.

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Supporting Information

The following Supporting Information is available through the online version of this article at the publisher's website.

Appendix S1 **PRISMA 2009 checklist**

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Chapter 7

Summary, Limitations and Future Directions

"Once you stop learning, you start dying."

Albert Einstein

Thesis Summary

Thesis Summary

The work presented in this thesis has demonstrated that: selenium and iodine, alone and in combination are protective against oxidative stress in placental cells and tissue, increased inflammation is associated with reduced mitochondrial DNA (mtDNA) content, lower maternal buffy coat mtDNA content in first trimester is associated with risk of pregnancy complications, and exploring the effect of maternal nutrition on offspring telomere length, as a marker of future health, is needed.

In Australia, one in four first pregnancies become complicated with preeclampsia, spontaneous preterm birth, intrauterine growth restriction, or gestational diabetes mellitus. The detrimental effects of pregnancy complications can last after childbirth and their long-term consequences can increase morbidity and mortality for both mother and offspring, mainly due to an increased risk of chronic diseases later in life. An association between oxidative stress and pregnancy complications has frequently been shown. A defective placenta with partial remodelling of the arteries causes hypoxia followed by reoxygenation that results in high reactive oxygen species production and ultimately oxidative stress. Therefore, investigations into the effects of different factors on placental development and oxidative stress should be of high importance in research on pregnancy complications.

A balanced maternal diet during pregnancy is critical for fetal growth and development as well as their health throughout life because of the role of in utero exposures in the programming of chronic diseases. Because of the fundamental role of micronutrients in the antioxidant system, maternal intake of micronutrients may be associated with oxidative stress during pregnancy. Maternal intake may also impact offspring telomere length, a biomarker of chronic diseases. It is also important to investigate how maternal nutrient levels

may influence mtDNA content because the mitochondria is the power house of the cell and its dysfunction is related to oxidative stress.

Selenium and iodine are essential micronutrients and their deficiency has been associated with a greater incidence of pregnancy complications in population studies. Besides, selenium is a part of antioxidant enzymes and selenodeiodinases. Selenodeiodinases are involved in iodine metabolism suggesting the potential cooperation between selenium and iodine in biochemical reactions inside the cell and tissues. Copper is an essential micronutrient that plays a pivotal role in superoxide dismutation in antioxidant defence system, but its high level in maternal specimens was associated with greater risk of inflammation. Inflammation and oxidative stress can create a defective ongoing cycle in pregnancy complication initiation and progression. Therefore, three chapters of this thesis (2, 3 & 4) discuss the role of selenium, iodine, their combination, and copper in placental development and oxidative stress.

We comprehensively summarised the evidence that shows the importance of adequate selenium and iodine during pregnancy and their effects on offspring health, as well as their association with pregnancy complications (Chapter 2). Then, the potential synergistic role of selenium and iodine in protecting cells against oxidative stress in the placenta was hypothesized (Chapter 2). This highlighted the necessity of further study on the effect of selenium and iodine separately and in combination on placental cells and tissues.

While a deficiency of selenium and iodine in HTR-8/SVneo placental cells was associated with a higher level of oxidative damage, their supplementation increased cell viability and protected cell integrity assessed by reducing lipid peroxidation of cell membranes (Chapter 3). Although individual iodine supplementation was not associated with a significant change in cell viability, supplemented cells with the combination of selenium and iodine had greater

viability compared to selenium treated cells. In addition, a combination of selenium and iodine provided a greater reduction in lipid peroxidation than their individual supplementation. These findings suggest a synergistic effect of selenium and iodine in combating oxidative stress in HTR-8/SVneo cells. A high concentration of selenium increased cell death and lipid peroxidation but was no longer toxic under conditions of induced oxidative stress. On that note, cells exposed to oxidative stress may need increased amounts of micronutrients to survive (Chapter 3).

Our findings were reproducible in first trimester human placenta (Chapter 4). Due to the contribution of copper in the antioxidant system and report that high maternal copper levels are associated with greater oxidative stress and inflammation we explored the role of copper in relation to oxidative stress in the placental tissue as well. We used laser ablation inductively coupled plasma-mass spectrometry to see if there was uptake of the micronutrients used to supplement the cultured placenta tissues. To the best of our knowledge, this study was the first to show the direct assessment of micronutrient uptake in the placental explants. Selenium and copper content were higher in supplemented placental tissues compared to vehicle control confirming micronutrient uptake in first trimester placenta explants upon supplementation of the culture media (Chapter 4).

Similar to HTR-8/SVneo cells, oxidative stress caused higher apoptosis and DNA damage in selenium and iodine deficient placental explants. Selenium supplementation increased proliferation and reduced DNA damage and apoptosis. Iodine protected placental explants against oxidative damage with reducing apoptosis and DNA damage. In oxidative stress induced tissues, a lower concentration of selenium was not protective suggesting a higher demand of selenium under oxidative stress conditions. In addition, a combination of low doses of selenium and iodine provided a significant protection against oxidative stress, perhaps because iodine may increase the antioxidant effect of selenium by enhancing its

availability to antioxidant enzymes such as thioredoxin reductase. A high concentration of copper was toxic to the placenta explants measured by increased DNA damage and apoptosis. However, such an effect was not apparent under conditions of oxidative stress suggesting a higher consumption of copper in oxidative stress, perhaps by superoxide dismutase, which is an antioxidant enzyme. Therefore, an optimal level of micronutrients are essential to protect the placenta from oxidative stress (Chapter 3 & 4).

Adverse maternal exposures can shorten offspring telomere length, which is associated with a higher incidence of chronic disease later in life. A number of nutrients affects telomere length possibly through mechanisms such as inflammation, oxidative stress, DNA damage, and DNA methylation. We only found a few studies on this important topic and were unable to make a clear conclusion (Chapter 6). However, higher maternal circulating folate and 25(OH)D3 and dietary caffeine intake were associated with longer offspring telomere length, while dietary intake of carbohydrate, folate, n-3 PUFA, vitamin C or sodium was not. This is a big gap in this field and further research including longitudinal studies in large cohorts of pregnant women are required (Chapter 6).

The fact that pregnancy complications affect maternal and offspring health during their entire life lends support to the need to find early biomarkers of pregnancy complications so that prevention strategies can be used in early pregnancy. Changes in mtDNA content can reflect mitochondrial dysfunction, inflammation and oxidative stress in aging, chronic diseases such as Parkinson's, coronary heart disease, cardiovascular disease and metabolic syndrome. We found that reduced buffy coat mtDNA content in early pregnancy was associated with inflammation and a greater risk of pregnancy complications however; because of weak power of prediction, it is not a suitable biomarker (Chapter 5). Additionally, we investigated how maternal micronutrient levels may be associated with mtDNA content. Mitochondria in mammalian cells provide the greatest amount of energy from

macronutrients. Pregnancy has a high level of energy demand due to increased metabolism and new tissue synthesis. Micronutrients are essential for mitochondrial oxidative phosphorylation. Therefore, micronutrient deficiencies may impair mitochondrial energy production. We did not find any association between buffy coat mtDNA content and maternal micronutrients status. However, further larger cohort studies using maternal plasma and placenta can help to investigate the potential association more comprehensively (Chapter 5).

In summary, we reviewed the literature on selenium and iodine and highlighted the need to investigate their potential synergistic effect on placental health and oxidative stress (Chapter 2). We demonstrated that selenium and iodine individually and in combination are protective against oxidative stress in a placental cell line (Chapter 3) and first trimester human placenta explants, while a high concentration of copper increased oxidative damage (Chapter 4). Therefore, a balanced concentration of micronutrients is vital to maintain placental health and combat oxidative stress (Chapter 3 and 4). We determined that reduced buffy coat maternal mtDNA content in early pregnancy is associated with a greater risk of pregnancy complication but not maternal micronutrient status (Chapter 5). We highlighted that further research, including longitudinal studies investigating the association between maternal diet during pregnancy and offspring telomere length is required because of the importance of this association and the lack of work in this field (Chapter 6).

Limitations and Future Directions

1- We assessed the effect of three essential micronutrients on placental health and oxidative stress. Including more micronutrients can provide a more comprehensive knowledge of their effects and potential interaction or synergistic effects toward the oxidative stress and placental health.

2- Oxidative stress and inflammation have a positive feedback loop. Investigation of the effect of micronutrients on inflammation and other potential mechanisms in pregnancy complications will enhance our understanding of the effect of maternal nutrition on pregnancy complications.

3- We could only explore the effect of micronutrients on oxidative stress in first trimester human placenta. Continued research on a group of micronutrients in the placenta samples from other trimesters can address the potential differences across gestation.

4- Using laser ablation inductively coupled plasma-mass spectrometry in the assessment of micronutrients uptake is novel. Further research including coupling this method with immunohistochemistry or immunofluorescence labelling of different placental cell types as well as quantifying micronutrients uptake in the placental explants opens a new avenue in studies related to micronutrients and the placenta.

5- We measured mitochondrial DNA content in buffy coat of 317 samples. Mitochondrial DNA content assessment in large scale cohort studies using plasma and the placenta samples may help to determine if mitochondrial DNA content is associated with maternal factors such as micronutrient status and can be an early biomarker of pregnancy complications.

6- We found that investigation of the association between maternal diet and offspring telomere length is essential and currently lacking. Exploring this association with further research including large cohort studies may help to develop an early biomarker to identify children at risk and intervene for chronic disease prevention or for closer monitoring if they are at higher risk.

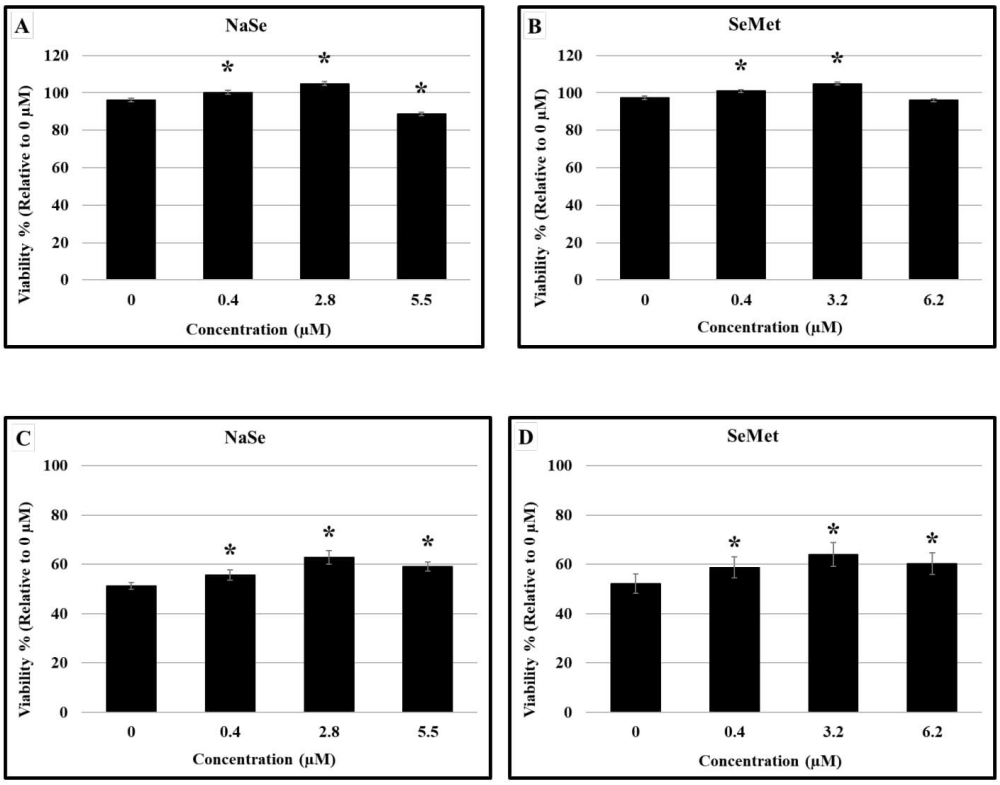
7- Mitochondrial DNA content and telomere length may change across gestation. Perhaps this is one of the reasons that the comparison of current findings with previously published studies are not completely possible. There is not enough research that shows their alteration during healthy and complicated pregnancy. This can be addressed by assessment at different time points during pregnancy (ie. different trimesters).

Appendices

Appendix I

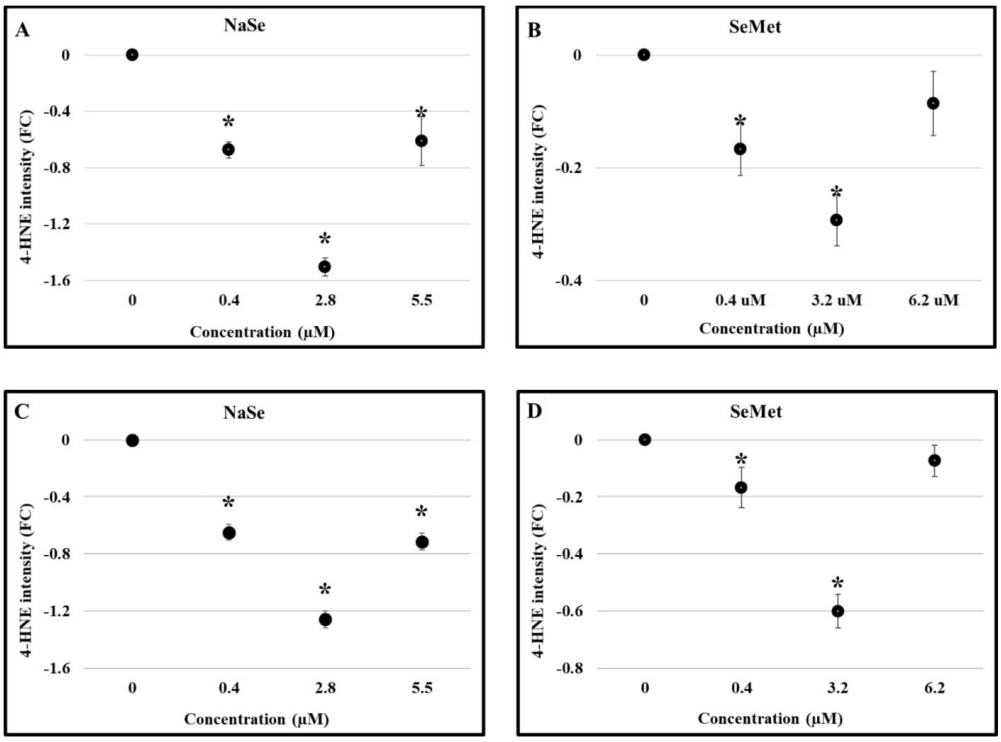
Electronic supplementary material of Chapter 3 from journal website

Supplementary data:



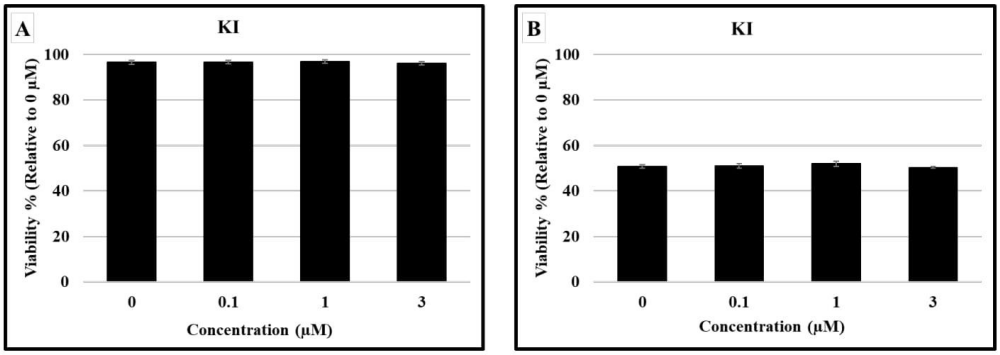
Supplementary Fig 1. Effect of selenium on HTR8/SVneo cell viability

HTR8/SVneo cells were cultured with 0, 0.4, 2.8, and 5.5 µM NaSe or 0, 0.4, 3.2, and 6.2 µM SeMet for 24 hours followed by another 24 hours in A) and B) water or C) and D) 40 µM H_2O_2. Data presented as estimated marginal mean ± 95% confidence interval. Statistical significance was assessed using Poisson (or negative binomial as appropriate) mixed effects models. *Denotes statistically different ($P < 0.05$) from control. A) Cell viability percentage post NaSe supplementation in water (n=3), B) Cell viability percentage post SeMet supplementation in water (n=3). C) Cell viability percentage post NaSe and H_2O_2 treatment (n=3), B) Cell viability percentage post SeMet and H_2O_2 treatment (n=3).



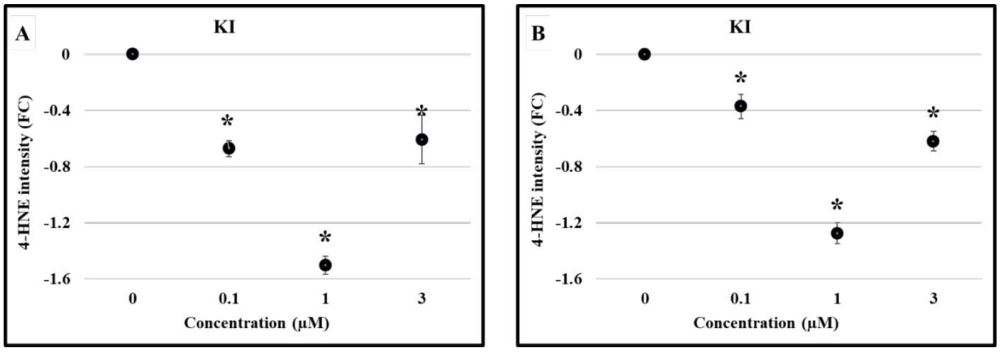
Supplementary Fig 2. Effect of selenium on lipid peroxidation after oxidative stress induction by H_2O_2

HTR8/SVneo cells were cultured with increasing concentration of NaSe (0-5.5 µM) or SeMet (0-6.2 µM) for 24 hours followed by another 24 hours in A) and B) water or C) and D) 40 µM H_2O_2. Cells were then fixed and stained with primary polyclonal rabbit anti 4-hydroxynonenal antibody. After an overnight incubation at 4°C, nuclei and membrane lipid peroxidation were labelled using 4', 6-diamidino-2-phenylindole and secondary antibody of Alexa Fluor 555 goat anti-rabbit IgG (H+L). Data presented as a fold change (FC) related to control ± standard error. Statistical significance was assessed using linear regression with log 2-transformed 4-HNE intensity to estimate fold change compared to controls. This followed by Tukey's post-hoc multiple comparisons. *Denotes statistically different ($P < 0.05$) from control. A) Lipid peroxidation post NaSe supplementation in water (n=3), B) Lipid peroxidation post SeMet supplementation in water (n=3). C) Lipid peroxidation post NaSe and H_2O_2 treatment (n=3), B) Lipid peroxidation post SeMet and H_2O_2 treatment (n=3).



Supplementary Fig 3. Effect of iodine on cell viability of HTR8/SVneo cells

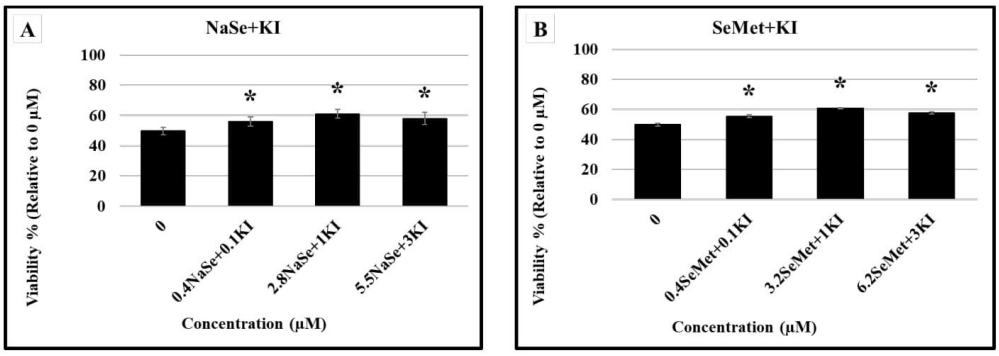
HTR8/SVneo cells were cultured with 0, 0.1, 1, and 3 µM potassium iodide for 24 hours followed by another 24 hours in A) water as the vehicle of H_2O_2 or B) 40 µM H_2O_2. After harvesting, cell viability was assessed. Data presented as estimated marginal mean ± 95% confidence interval. Statistical significance was assessed using Poisson (or negative binomial as appropriate) mixed effects models. *Denotes statistically different ($P < 0.05$) from control. A) Cell viability post potassium iodide supplementation in water (n=3), B) Cell viability post potassium iodide and H_2O_2 treatment (n=3).



Supplementary Fig 4. Effect of iodine on lipid peroxidation of HTR8/SVneo cells

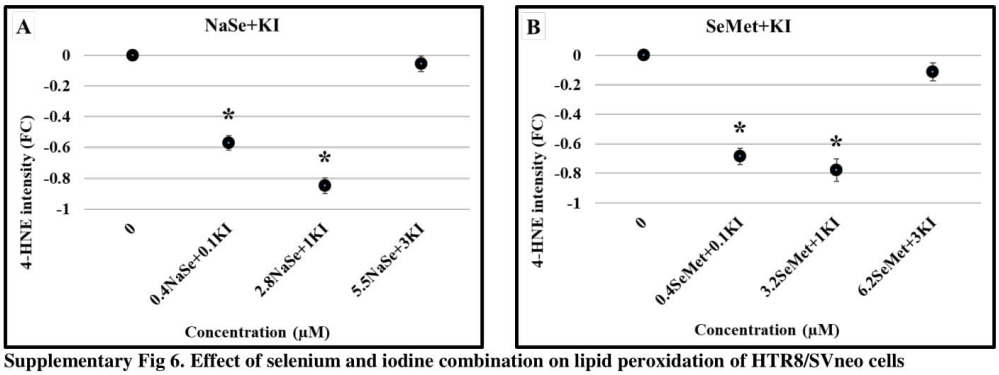
HTR8/SVneo cells were cultured with increasing concentration of potassium iodide (0-3 µM) for 24 hours followed by another 24 hours in A) water as the vehicle of H_2O_2 or B) 40 µM H_2O_2. Cells then have been fixed and stained with primary polyclonal rabbit anti 4-hydroxynonenal antibody. After an overnight incubation at 4°C nuclei and membrane lipid peroxidation were labelled using 4', 6-diamidino-2-phenylindole and secondary antibody of Alexa Fluor 555 goat anti-rabbit IgG (H+L). Data presented as a fold change (FC) related to control ± standard error. Statistical significance was assessed using linear regression with log 2-transformed 4-HNE intensity to estimate fold change compared to controls. This followed by Tukey's post-hoc

multiple comparisons. *Denotes statistically different ($P < 0.05$) from control. A) Lipid peroxidation post potassium iodide supplementation in water (n=3), B) Lipid peroxidation post potassium iodide and H_2O_2 treatment (n=3).



Supplementary Fig 5. Effect of selenium and iodine combination on HTR8/SVneo cell viability

HTR8/SVneo cells were treated with 0.4 µM NaSe and 0.1 µM KI, 2.8 µM NaSe and 1 µM KI, 5.5 µM NaSe and 3 µM KI or 0.4 µM SeMet and 0.1 µM KI, 3.2 µM SeMet and 1 µM KI, and 6.2 µM SeMet and 3 µM KI for 24 hours followed by another 24 hours in 40 µM H_2O_2. After harvesting cell viability was assessed. Data presented as estimated marginal mean ± 95% confidence interval. Statistical significance was assessed using Poisson (or negative binomial as appropriate) mixed effects models. *Denotes statistically different ($P < 0.05$) from control. A) Cell viability percentage post NaSe+KI and H_2O_2 treatment (n=3), F) Cell viability percentage post SeMet+KI and H_2O_2 treatment (n=3).



Supplementary Fig 6. Effect of selenium and iodine combination on lipid peroxidation of HTR8/SVneo cells

HTR8/SVneo cells were treated with 0.4 µM NaSe and 0.1 µM KI, 2.8 µM NaSe and 1 µM KI, 5.5 µM NaSe and 3 µM KI or 0.4 µM SeMet and 0.1 µM KI, 3.2 µM SeMet and 1 µM KI, and 6.2 µM SeMet and 3 µM KI for 24 hours followed by another 24 hours in 40 µM H_2O_2. Cells then have been fixed and stained with primary polyclonal rabbit anti 4-hydroxynonenal antibody. After an overnight incubation at 4°C nuclei and membrane lipid peroxidation were labelled using 4', 6-diamidino-2-phenylindole and secondary antibody of Alexa Fluor 555 goat anti-rabbit IgG (H+L). Data presented as a fold change (FC) related to control ± standard error. Statistical significance was assessed using linear regression with log 2-transformed 4-HNE intensity to estimate fold change compared to controls. This followed by Tukey's post-hoc multiple comparisons. *Denotes statistically different ($P < 0.05$) from control. A) Lipid peroxidation post NaSe+KI and H_2O_2 treatment (n=3), B) Lipid peroxidation post SeMet+KI and H_2O_2 treatment (n=3).

Appendix II

Placenta Collection Protocol (Chapter 4)

1) Use a scalpel blade to cut open the specimen sock/collection container to access the placenta

2) Take 3-4 petri dishes (eg. 2 bottoms and a lid) and fill with cold 0.9% NaCl

3) Using forceps, move pieces/chunks of tissue onto the first petri dish and wash out excess blood by moving tissue back and forth in the solution. Move the washed tissue into next petri dish of 0.9% NaCl and repeat (so it undergoes 3-4 washes depending how bloody the sample is)

4) Label tube with:

- Date

- Gestational age

- Study code (PAC###)

5) Place all samples on ice in esky and transport to AHMS

Appendix III

Harvesting Placenta Explants (Chapter 4)

1. Remove the media that is covering the explants

2. Using an appropriate pipette gently suck the explant and place into a cassette.

3. Place the cassette into 10% Formalin for 2-4 hours.

4. Transfer the cassette to 1x PBS for 24 hours @ 4°C.

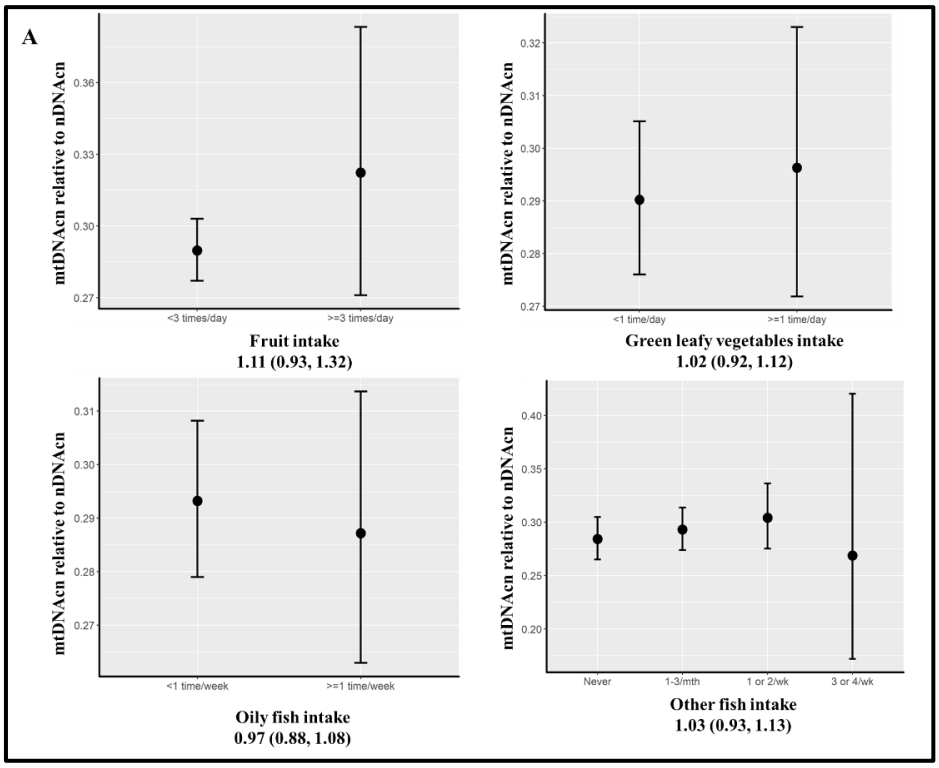
5. Repeat step 4

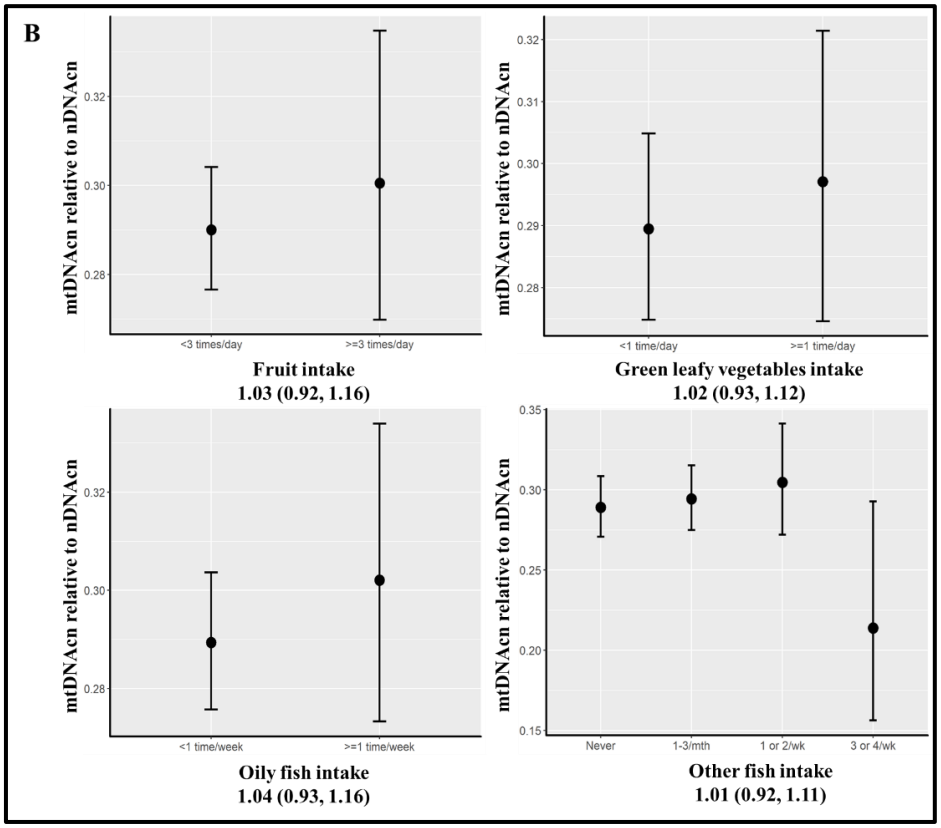
6. Repeat step 4 again

7. Once you have harvested all of your explants place your cassettes in 70% ethanol for minimum 2 hours @ 4°C and then take to histology for embedding (cassettes can stay in 70% ethanol for days).

Appendix IV

Assessment of association between dietary intake and mitochondrial DNA content (Chapter 5)





Supplementary Figure 1. Association between buffy coat mitochondrial DNA content and pre-pregnancy and pregnancy maternal dietary intake. A research midwife collected dietary intake of the (A) one month prior to conception and (B) during pregnancy with food frequency questions for fruit, green leafy vegetables, and fish at 15 ± 1 weeks'. Mitochondrial DNA copy number (mtDNAcn) was calculated relative to nuclear DNA copy number (nDNAcn). Linear Regressions were used to test the association between maternal dietary and mtDNAc, adjusted for maternal age and BMI. There was no association between maternal dietary intakes and mtDNc. Data are presented as a ratio of geometric mean and corresponding 95% CI.

Appendix V

PRISMA checklist (Chapter 6)

topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Cover page, p1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Cover page, p1 and 2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Main document p1 and 2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Main document p3 and table 2
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	Main document p2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Main document p3, 4 and table 2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Main document p2, 3, 4 and table 1
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Main document p2 and table 1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Main document p2-5and Figure 1

Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Main document p4 and 5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	Main document, p2 and table 2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Main document, p4 and 5 and table 4 and 5
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	Main document, p5 and 6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	NA

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Main document, p5 and 6 and table 4 and 5
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	NA
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Main document p3 and Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 4 and 5
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Main document, p5

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			and 6 and table 4 and 5
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Main document, p5-7 and table 3
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	NA
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Main document, p5 and 6 and table 4 and 5
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	Main document, p8-11
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	NA
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	Main document, p11
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	NA

1. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *BMJ (Clinical research ed).* 2009;339:b2535-b2535.