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Oxidative Stress (OS) and DNA Methylation Errors in Reproduction. A Place for a Support of the One Carbon Cycle (1-C Cycle) before Conception

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Abstract

Objective: To revisit and confirm the relationship between Oxidative stress (OS) and DNA methylation: two aspects of the same pathologic conditions leading to "delay/failure to conceive", but also to major gynecologic pathologies, polycystic ovarian syndrome and endometriosis. To demonstrate that a complete 1-Carbon (1-C) cycle support could help in avoiding transgenerational epigenetic failures, considering the environmental "pollution" aspect.

Methods: Combination of analysis of scientific literature on the biochemical aspects of oxidative stress, DNA methylation and gametes quality and our work on embryo metabolism. Analysis of the defenses of the embryo against the aggressions brought by endogenous and exogenous factors and impact on embryonic development and integrity of the conceptus.

Results: The 1-C cycle, and especially homocysteine recycling, is at the epicenter of the problem and the endocrine disruptors aggravate the negative impact as early as the preimplantation stages. A complete supplementation oriented towards a support of the 1-C cycle should be proposed during the preconception period. This should decrease the risks of pathologies for the conceptus and further on, minimize the risks of metabolic syndromes such as obesity and diabetes. Based on recent scientific observations, a decreased incidence of autism and other psychiatric disorders should be expected.

Conclusions: A total change in the paradigm in infertility concerning oxidative stress and epigenesis has to be understood. New pathophysiological consequences and therapeutic managements have to be drawn and followed.

Keywords: Oxidative stress, DNA methylation, Epigenesis, Gametes, Embryo, Metabolic disorders, 1 carbon cycle

Introduction

Hypo fertility or "delay/failure to conceive" is multifactorial but includes 2 major negative effectors: oxidative stress (OS) and alteration of the DNA methylation process. OS derives from an imbalance between free radicals generation and anti-oxidant protection. Reactive oxygen species (ROS) are generated endogenously (essentially at the level of mitochondria) and by exogenous factors (pollution of various origins and ionization). Proteins, lipids and DNA are the common targets of OS injuries affecting cell and tissues functions [1-3]. Clinical studies have demonstrated that environmental and dietary factors affect both gametes and embryo quality. DNA strands are modified by DNA adducts and abasic sites formation, the most common DNA insults. They can be also fragmented: the primary, secondary and tertiary structures of DNA helix are modified. In gametes and embryos OS-linked DNA damages, if left unrepaired, are obviously the most serious concerns due to possible transmission of mutations. DNA methylation is a biochemical pathway modifying gene expression without altering the underlying DNA sequences: it is involved in epigenesis. Methylation process is strongly and negatively affected by noxious environment such as pesticides and endocrine disruptors (EDs). Oxidative stress affects DNA methylation [4,5]. Methylation and protections against oxidative stress have in common the one carbon cycle (1-C cycle). This pathway drives to a certain extent the generation of glutathione and hypotaurine, universal free radical scavengers. Glutathione is a molecular masterpiece in preimplantation embryonic development. The 1-C cycle allows the regeneration of methionine, through

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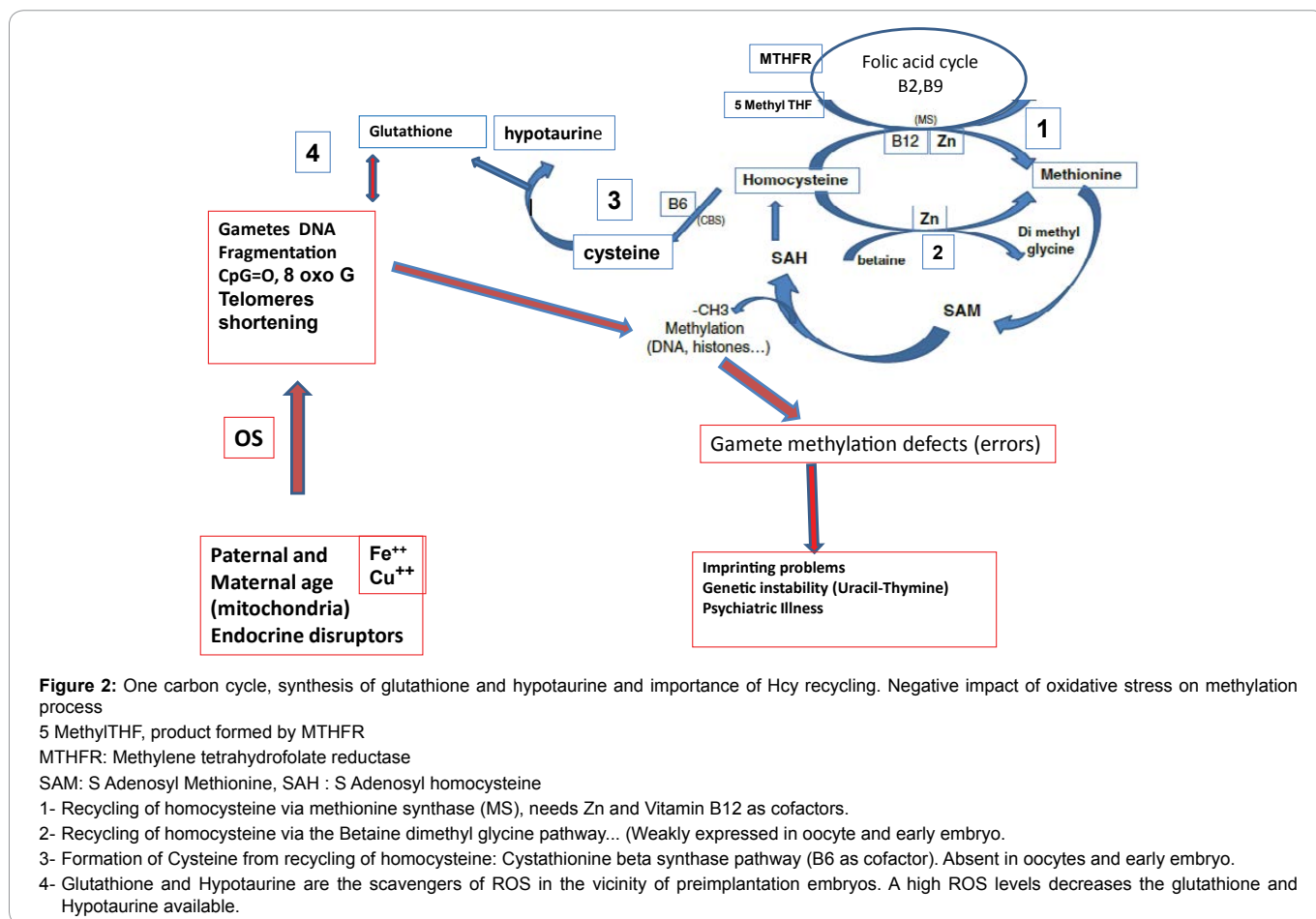
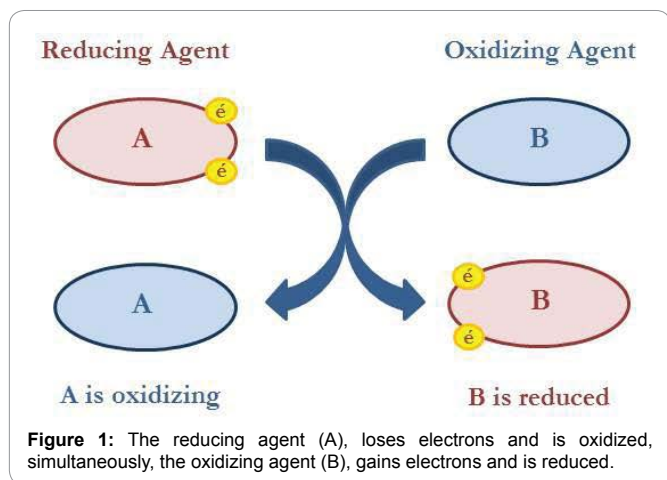
homocysteine recycling, thus stimulating S Adenosyl Methionine synthesis, the universal methylating agent. Homocysteine (Hcy) is a part of a vicious circle, cause and consequence of oxidative stress [6]. Hcy is a perturbator of methionine transport and methylation process in the embryo [7]. This molecule is strongly involved in numerous pathologies related to infertility but not only (brain, cardiac and various metabolic diseases and multiple cancers). Methylation errors contribute to altered DNA stability and transgenerational transmission of imprinting errors leading

to metabolic and psychiatric diseases [8-12].

Oxidation is a Fine-Tuned Regulatory Process in Cell Biochemistry

The “Superoxide theory of oxygen toxicity”, [13] brought a crucial advancement in the free radicals biochemistry and their roles in cell signaling, aging and diseases generation. The oxidation process and its reactions, namely *redox reactions*, provide the working substrate to a number of biochemical pathways in cells. Chemically, the *redox reactions* are events of gain-loss of electrons. When an *oxidizing agent* oxidizes other substances, its oxidative state decreases; symmetrically, when a *reducing agent* reduces other substances and loses electrons, its oxidative state increases [14] (Figure 1). This is a typical feature for the reaction involving vitamins C and E: antioxidant and then pro-oxidant compounds.

Nitric oxide (NO), Superoxide ion (O₂⁻), Hydroxyl (OH[•]), Peroxyl (ROO[•]) and Alkoxyl (RO[•]) are radical compounds containing one or more unpaired electrons within shells around the atomic nucleus. Any free radical containing oxygen is to be considered as ROS. At physiologic levels, ROS are regulatory mediators in signaling processes and in physiological events such as capacitation, ovulation and corpus luteum function. At supra-physiologically high concentrations, they induce inflammation and *in fine* deregulate transcription factors and cell division leading to oncogenesis [15]. The free radicals scavengers, balancing the ROS action are glutathione (GSH) and hypotaurine (HTau)



in the surrounding of the gametes and the early embryo. These two molecules are linked to the 1-carbon cycle (Figure 2) and homocysteine recycling. An antioxidant “back up” is also realized by several superoxide dismutases’ [16].

Major ROS sources: general considerations

Endogenous fonts: The major endogenous source of ROS is the mitochondria. Over- generation of ROS increases through the oxidative phosphorylation metabolic pathway (OXPHOS) under pathological conditions such as altered membrane integrity, high levels of circulating glucose and aging [17] Whatever the organ, an excessive metabolism of glucose will lead to increased ROS generation and its cohort of detrimental effects.

Exogenous fonts: Ionizing and non-ionizing irradiations, air pollutants such as car exhausts, cigarette smoke, and industrial contaminants and important sources of ROS. A large variety of xenobiotics, toxins, pesticides, herbicides, altered food (containing aldehydes, oxidized fatty acids, and transition metals) are noxious and inducers ROS linked toxicity

Oxidative Stress and Hypo fertility

Oxidative stress is now considered as one of the major features in hypo-fertility [18]. However, It is usually difficult to establish a strict correlation between serum classical markers of OS and infertility. The ovary and the testis seem at this point quite “autonomous”. Moreover, in recent studies [19,20], no correlations were observed between severe pathologies and low circulating levels of “classical reducing vitamins” such as vitamins A, C and E or Selenium (Se). The interesting markers seem to be rather organic and inorganic: Zn (Zinc), Fe (iron) and Cu (copper), glutathione, and uric acid. Reducing thiols and the Cu/Zn ratio were the only reliable markers of the endogenous OS. The circulating OS markers are gender dependant.

Gametes and embryos

Due to the scarcity of the female material available, the knowledge is less important for the female gamete than for the sperm.

Sperm: The oxidases: xanthine, glycolate, amine, membrane NADPH oxidases and OXPHOS generate ROS. ROS are the major effectors of sperm DNA fragmentation, with apoptosis and immaturity [21-23] mitochondrial activity is high in sperm, in order to promote a correct motility. ROS induce peroxidation of the membrane lipids which affect motility [24,25] OS insults lead to the generation of DNA strand breaks, abasic sites (Apyriminic/apyrimidinic, APsites) and formation of DNA base adducts such as 8-hydroxyl-2'-deoxyguanosine (8-OH-dG) and other molecules linked to nutrition and occupational exposure to toxicants [26]. More than twenty DNA base oxidation products have been described. Guanine is a major target for DNA oxidation, with the formation of 8-OH deoxyguanosine. Guanine is highly represented in telomeres (TTAGGG). Telomere length is a marker of reproductive age/capacity [27]: there is a strong correlation between telomere shortening and sperm DNA fragmentation. OS, beside its role in sperm DNA fragmentation affects, to a certain extent, sperm nucleus decondensation, thus affecting nucleus tertiary structure. A correct tertiary structure is mandatory in order to avoid delay in the pronucleus formation and further on, in the first embryonic cleavages. These two negative impacts have

a harmful effect on preimplantation and even late embryonic development. The oocyte is rather well equipped for repairing DNA fragmentation but not for fixing tertiary structure [28]. The sperm DNA damages must therefore be regarded as a potential risk factor for the development of abnormal human embryos: they may lead to mutations if left unrepaired by the oocyte and then during the first cell divisions.

Oocytes: In women, the oocytes are not protected during their quiescent (dormant) life and during their growth. DNA decays seem equally shared between male and female gametes [21]. Little information is available on the ‘oxidative stress’ impact on the oocyte DNA: the damaging effect of the tobacco smoke results in DNA adduct accumulation (benzopyrene) [29]. The human oocyte expresses most DNA repair genes to support the early preimplantation phase of the embryo development and up to blastocyst formation [29,30]. This repair activity significantly limits the DNA damage, the high expression of mismatch repair genes (MMR genes) in metaphase II oocytes suggests that this pathway is directed towards repair rather than apoptosis: a kind of “species salvation”. DNA repair mechanisms are involved in base methylation/demethylation and are of extreme importance for the oocyte and then developing embryos until maternal to zygotic transition (MZT). The DNA repair capacity and defense against ROS is finite and decreases with maternal age [31]. The genes coding for mitochondrial function, oxidative damages and stress-responses, DNA methylation, genome and chromosome stability follow the same concomitant decreased expression patterns. Therefore the age-associated meiotic defects observed in oocytes from older patients might be linked to oxidative stress, aberrant DNA damage response and chromosome fusion: this naturally leads to miscarriages and infertility [32]. Once the repair capacity is over-whelmed, one of two events can occur: apoptosis leading to embryo developmental arrests, or tolerance a kind of ‘unrepaired DNA tolerance’ producing a gene mutation, a source of genetic deregulations and then pathogenesis. The site of fertilization is highly protected against ROS *in vivo*. Follicular and tubal fluid surrounding the early embryos have high levels of enzymatic and non-enzymatic antioxidants, such as hypotaurine and ascorbic acid, SODs, glutathione peroxidase and γ -glutamyl-cysteine synthetase [16]. Fertilization process induces a burst in oocyte glutathione content necessary for pronuclei formation and further on for the first cleavages. . However, the dry weight of a human preimplantation embryo can be estimated as 75-100 nanograms: thus it must be considered that the risks of ROS induced decays on such a small amount of material are immediate.

Two major pathologies strongly linked to OS, Polycystic ovarian syndrome and endometriosis affect severely female fertility first via damages to oocyte and then implantation problems [33].

Epigenetic/DNA Methylation Perturbations: Role in Physiopathology

DNA methylation is involved in regulation of important cellular processes such as gene transcription, genomic imprinting and chromosome stability but also gene inactivation: the most common is X-chromosome inactivation. DNA methylation (and the associated process of histones modifications) gained considerable attention during the last ten years due to its major effect on epigenetic transmission. Epigenetic mechanisms can modify the expression of specific genes without changing the underlying DNA sequence. These

modifications are transgenerationally transmitted by modifications of the germ cell through methylation erasing/resetting.

Molecular basis of epigenesis

DNA methylation: In mammals, DNA methylation occurs at the 5'-position of cytosine residues, mainly within CpG dinucleotides, 60-80% of which are methylated within the promoter regions of genes. Methylation of CpG dinucleotides within the promoter regions leads to silencing of transcription process and is mediated by modifications in the condensation status. Furthermore, the DNA methylation process is catalyzed by DNA methyltransferases (DNMTs). They are classified as "de novo" DNMTs, namely those methylating specific chromosomal sequences during early embryogenesis, and "maintenance" DNMTs that faithfully restore the methylation patterns after each DNA replication cycle. The DNA methylation is of major importance in gametogenesis as primordial germ cells, when entering the developing gonads, undergo a process of DNA de-methylation. Then this process will be subsequently reversed in the prenatal life in males and during post-natal follicle development in females. Histone methylation (H3K9 for example) is linked to DNA methylation at imprinting control regions in mammals [34].

Gametes and embryo

Sperm: First of all, DNA methylation process regulates the tertiary structure of the sperm nucleus: this avoids delays in sperm head swelling of the nucleus, the formation of the male pronucleus and prevents delays in the first embryo division and, further on, developmental arrests [35]. The strong link between sperm male hypofertility and DNA methylation errors has been described only recently [36,37]; these failures can affect DNA regions at the vicinity of promoters, regulatory elements and transcription binding sites, for housekeeping and other important genes. Age and lifestyle severely increase these

negative methylation/epigenetic errors in paternal sperm [38] leading to increased risks of various pathologies [39]. Abnormal DNA methylation profiles in pathologic sperm lead to a decrease in pregnancy rates [36] MTHFR (Methylene tetrahydrofolate reductase) isoforms (C677C-T and 1298A-C) in male partners induce recurrent miscarriages. Pesticides, phthalates and other plastics derived endocrine disruptors affect severely sperm methylation pattern. They generate transgenerational sperm epigenetic modifications, methylation patterns, [10] leading to pubertal abnormalities, testis disease and obesity. In general pesticides may lead to TDS: testis dis-genesi syndrome.

Oocytes: DNA methylation is established in a size dependent manner in bovine and mouse. Assisted reproductive technology has allowed a better access to human oocytes and thus a better basic knowledge. Controlled ovarian stimulation alone modifies the epigenetic settings [40,41]. This is linked to the increased level of homocysteine in follicular fluid [42]. Hcy enhances the sensitivity of granulosa cells to FSH, which is positive in term of CHO (and number of oocytes retrieved) and follicle growth (via an increase FSHR expression), but negative in term of oocyte quality. This is also consistent why the negative link between the qualities of the oocyte retrieved during ART and the presence of a MTHFR isoforms in the female partner. As mentioned earlier, Hcy is a strong perturber of methylation (hyper or hypo-methylation): it competes with methionine for transport into oocytes and preimplantation embryos [7] and hinders the formation of S-Adenosyl methionine, the universal methylation agent. The most important pathway for Hcy recycling is the one carbon cycle (1-C cycle), which requires vitamins B2, B9 (folic acid) and B12. The second Hcy recycling mechanism is the cystathionine beta synthase pathway (CBS), requiring vitamin B6: it leads to the formation of cysteine (and then glutathione and/or hypotaurine: Figure 3). This pathway is not expressed in human oocyte. The early human embryo has so a limited capacity to recycle homocysteine. It is now suggested that ART *per se*

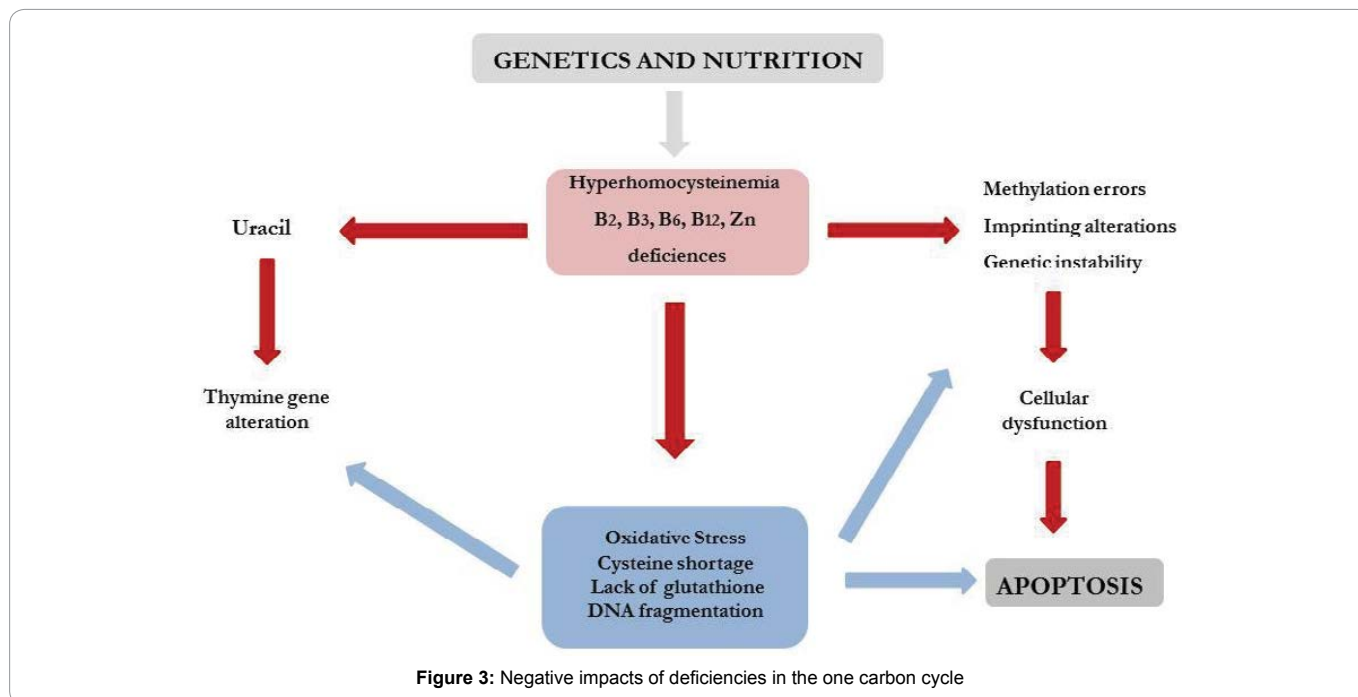


Figure 3: Negative impacts of deficiencies in the one carbon cycle

has a deleterious impact on DNA methylation of the embryos conceived *in vitro* [43,44]. DNA methylation patterns differ in placenta and umbilical blood samples obtained from children born by IVF, when compared with naturally conceived children [45]. In rodents, maternal exposure to plastics derived endocrine disruptors (BPA, DEHP and BBP) alters genomic imprinting. This leads to epigenesis-linked transgenerational transmission of obesity, reproductive diseases and sperm epimutations.

Anti-Oxidants and Prevention of Subfertility

A significant lifestyle factor affecting fertility is the nutrition. Diet is a source of exogenous vitamins and oligo-elements. It is often tempting, in order to decrease the OS decays, to propose complements. The "classical antioxidant treatments" given irrespectively of clinically quantified deficits, are potentially perturbing. In women, consumption of classical antioxidants has no beneficial effect and some adverse effects have been described in several clinical trials [46]. Over-consumption may be deleterious, moreover vitamin deficiencies are very rare and their use should be re-evaluated [19,20].

Vitamin C, E, and A, Selenium (Se), CoQ10

Vitamins A, C, E deficiencies are rarely observed even in the most serious pathologies [19]. Although several studies demonstrated some beneficial effects of antioxidants on semen quality and viability, others failed to confirm these results. In sperm if these compounds are apparently effective in reducing sperm DNA fragmentation, they also induce decondensation of sperm nuclei. This negatively affects sperm nucleus tertiary structure thus inducing delays in fertilization process and then cell divisions. Vitamin C has a potential denaturizing action, via an opening of the protein disulfide bond. In no case, vitamin C is a Panacea [47]. These vitamins may have hazardous effects in cancer [48,49].

Selenium is often presented as an anti-aging compound. The first point is that Se deficiency is very rare in the general population. In woman, a high level of Se is rather the sign of an oxidative stress [20]: There are gender differences in circulating Se. In men the optimum concentrations range is in semen, between 50 and 70 µg/ml; if not in this range the quality of sperm is altered moreover if selenium supplementation increases the serum values, it does not modify the testicular values [50]. At high doses Se significantly reduces the sperm motility through a modification of the metabolism of thyroid hormones.

Deficiency of the Co-enzyme Q10 (coQ10) is occasional and occurs exceptionally in case of recessive autosomal mutations, in cancer or in case of neuro-vegetative disorders such as diabetes. A double-blinded randomized study [51] showed no improvement in sperm parameters; in some cases, extended treatment inhibited the production of spermatozoa, down to 10% of the original count. However, in females Coenzyme Q10 restores oocyte mitochondrial function and could counteract, to a certain extent, reproductive aging [52].

Zinc, Glutathione and hypotaurine

Zinc is a micro nutriment present in meat and seafood. According to the CDC, the National Center for Health Statistics (NCHS), 15% of the Caucasian population suffers from Zn deficiency. Zn is a cofactor for more than 80 metal-enzymes involved in DNA

transcription. It is mandatory in reproduction, by stabilizing the genome, a fundamental aspect in gametogenesis and early embryogenesis. In animal models, preconception zinc deficiency compromises oocyte epigenetic programming and disrupts post-implantation embryo development [53]. Zinc concentrations are lower in seminal plasma of men with idiopathic sub fertility. It is commonly admitted that oral intake of zinc usually improves sperm parameters.

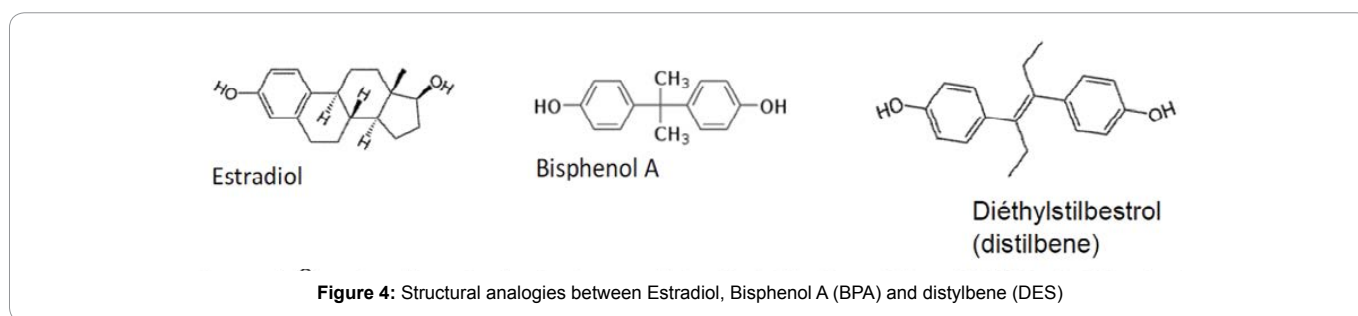
Glutathione is a tripeptide composed of glutamate, cysteine and glycine (L-γ-glutamyl-L-cysteinyl-glycine), universal free radical scavenger. It is synthesized in two consecutive steps catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. It cannot be provided by oral way as it is hydrolyzed and destroyed in the stomach and intestine; moreover it does not pass the cell membranes. Injection of glutathione is poorly efficient. Cysteine can be provided in order to increase the glutathione synthesis *in vivo*: it is usually added under its more stable form N acetyl cysteine (NAC). NAC improves significantly ovulation and pregnancy rates in PCOs patients [54]. It refrains also the progression of endometriosis [55]. Hypotaurine (HTau) is a major anti-oxidant of the female genital tract [56], secreted by the tubal epithelium, but it is not present in seminal plasma. The embryo does not possess the CSD (Cysteine sulfinate decarboxylase) necessary for its synthesis. HTau is never present in the "common" antioxidant cocktails, probably because of its high price.

The 1-C cycle support

Homocysteine (Hcy) is recycled into methionine *via* the one carbon cycle. Low concentrations of folate and B12 in sperm are detrimental for sperm concentration and DNA stability [57,58]. Cysteine can be generated from Hcy via the cystathionine betasynthase pathway (CBS), a derivation of the 1-c cycle (Figure 2). Cysteine can be used for the synthesis of hypotaurine and glutathione (Figure 2). However the CBS pathway is not expressed in the oocyte and the early embryo [59]. The negative impact of a subnormal Hcy recycling during the conception process is presented in Figure 3; Folic acid is commonly proposed in women, during the preconception period and during the first trimester of pregnancy to prevent several birth defects. It is surprising that folic acid alone is proposed: moreover it is commonly admitted that 15% of the women suffer MTHFR isoforms; the most common is the 677C-T. These isoforms are less efficient in transforming Folic acid to 5 MethylTHF the efficient molecule for Hcy recycling. For these patients folic acid may be totally counterproductive due to a feedback effect, blocking the folic acid cycle. However it was recently demonstrated that folic acid given during preconception can decrease to a certain extent the risk of autism in the infant [60]. This is in total agreement with the relation between methylation processes occurring during early life and some psychiatric disorders in infants [61]. In mice, a complex maternal methyl supplement (containing B9 and B12) affects positively DNA methylation in pups. It is also evident that a controlled nutrition allows a better level of (B) vitamins in blood, a decreased level of homocysteine and as a result, a better quality of the gametes [62]. The beneficial effect of supplementation based on support of the 1-Carbon cycle on both male and female fertility in patients having failed previous ARTs treatments has been recently reported [63]. The experimental groups received B vitamins + Zn versus a control group receiving no complements. High pregnancy and delivery rates were observed in the treated group of women (Table 1).

	Mean age	AMH	Pregnancies	Miscarriages	Deliveries
Treated(100 patients)	32.5 (4.1)	2.3(1.5)	45 (45%)	5 (11%)	40 (40%)
P		0.12	0.001		
Control(73 patients)	34.7(5.3)	2.4(2.6)	10 (13.7%)	2 (20%)	8 (10%)

Table 1 : Influence of a 1-C nutraceutical support in women after an IVF attempt failure and waiting for another attempt. The control group did not receive any treatment. Polycystic ovarian syndrome (PCOS) and endometriosis distribution similar in both groups (+/- 50%) All the parameters for the male partner are within the normal ranges. AMH: Anti Mullerian hormone ($\mu\text{G/L}$).



This is no really surprising that methyl donor can counteract negative problems linked to Endocrine disruptors [64]: it has to be emphasized that BisPhenols and phtalates are now always found in urine of patients undergoing ART treatments [65,66] (Figure 4).

Conclusions

The negative impact of Oxidative stress on reproduction is no longer a matter for debate. Redox status must be finely tuned and balanced and it is not less obvious that the “classical” antioxidant preparations including vitamin C, E and A and some minerals like Se, Cu and Fe are poorly efficient if not harmful [20,48]. Moreover the OS markers are gender dependent. OS impact on gametogenesis, induces sooner or later metabolic pathologies, such as diabetes and obesity in the progeny [67]. A part of the huge increase in diabetes prevalence (X4 since1980, according to the 2016 WHO statistics) has be attributed, in part, to the negative impact of endocrine disruptors on epigenesis/imprinting and not only to high food consumption, lifestyle and pollution *via* endocrine disruptors (EDs) currently increase the risks. EDs are true inducers of OS *via* their affinity with the estrogen receptors (ERs) and PPARs (peroxisome proliferator-activated receptors): this leads to hormonal and/or metabolic diseases. DNA methylation errors lead to similar metabolic disorders in the progeny. Homocysteine accumulation is at the epicenter of these 2 concerning problems [6,59,61]: it exacerbates oxidative injury. Oxidation of cytosine and Guanine at the levels of the CpG sites is another major link. Lifestyle and nutrition have an impact on the progeny [68] *via* alteration of gamete quality but later on, during embryogenesis, at the time of epigenesis maintenance and resetting . The number of children diagnosed for autism (ASD) has increased at a concerning rate (1 out 68 children in the US). This aspect is more than probably linked to exogenous agressions leading to OS and methylation patterns alterations [69]. Diabetes, characterized by a high basic OS, increases the risk of Autism syndrome disorders: this reinforces the link between OS and DNA methylation. Increased ASD incidence is also observed in infants of patients in their 40s, with lower defenses against ROS, consulting for infertility. Moreover Assisted reproductive technology ART has a direct effect on OS and imprinting, and the techniques used should be improved [43,44]. Efforts should be

made to optimize Controlled ovarian stimulation protocols and media for manipulation of gametes and embryos. New theories regarding homocysteine recycling and methylation now have a solid scientific basis. Homocysteine recycling allows optimal synthesis of hypotaurine and glutathione, two major effectors in protection against ROS, with no side effects. In terms of embryogenesis, of great concern is the weak capacity of oocytes to recycle homocysteine and regenerate Cysteine for glutathione synthesis. On this basis, supplementation with Cysteine or more stable derived compounds, such as N acetyl Cysteine, might be a logical strategy. In addition, although folic acid (Vitamin B9) is a recommended supplement in the pre-conception period and after, preliminary studies suggest that supplements should also contain B2, B6 and B12, which are necessary for methionine synthesis [64]. BPA is also found in urine of nearly all women consulting for fertility problems [70] and probably in the whole population. In view of the negative impact of environment on gametogenesis and on epigenetic process in general, supplementation that includes all of these should be recommended, pre and post conception, during pregnancy. This could minimize adverse neurological problems in the infant. Treating problems in reproduction requires a rigid scientific approach rather than the haphazard therapies that have often been applied to date.

Disclosure of conflict of interest

The authors declare no conflict of interest. YM and MC are members of the “oxidative stress College”, a nonprofit scientific society. ES is a MS fellow from the University of Bari (Italy).

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Compliance with Ethical Standards

All the works presented here have been already published. To the authors’ knowledge, all the studies presented have received ethical approvals.

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