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INFERTILITY
REVIEW

Oxidative stress and alterations in DNA methylation: two sides of the same coin in reproduction

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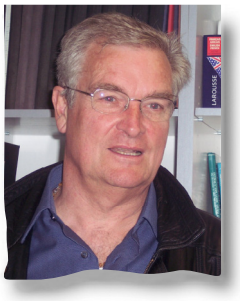

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Abstract The negative effect of oxidative stress on the human reproductive process is no longer a matter for debate. Oxidative stress affects female and male gametes and the developmental capacity of embryos. Its effect can continue through late stages of pregnancy. Metabolic disorders and psychiatric problems can also be caued by DNA methylation and epigenetic errors. Age has a negative effect on oxidative stress and DNA methylation, and recent observations suggest that older men are at risk of transmitting epigenetic disorders to their offspring. Environmental endocrine disruptors can also increase oxidative stress and methylation errors. Oxidative stress and DNA methylation feature a common denominator: the one carbon cycle. This important metabolic pathway stimulates glutathione synthesis and recycles homocysteine, a molecule that interferes with the process of methylation. Glutathione plays a pivotal role during oocyte activation, protecting against reactive oxygen species. Assisted reproductive techniques may exacerbate defects in methylation and epigenesis. Antioxidant supplements are proposed to reduce the risk of potentially harmful effects, but their use has failed to prevent problems and may sometimes be detrimental. New concepts reveal a significant correlation between oxidative stress, methylation processes and epigenesis, and have led to changes in media composition with positive preliminary clinical consequences.

KEYWORDS: 1-C cycle, endocrine disruptors, homocysteine, imprinting, methylation, oxidative stress

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Introduction

Oxidative stress

Generation of reactive oxygen species (ROS) is a normal feature of basal aerobic metabolism that supports life. They are active derivatives produced during the intermediate steps of oxygen reduction, which are catalyzed by small molecules such as iron and copper. Hydrogen peroxide, reactive nigrogen species and ROS function as important signalling molecules within cells, and are also necessary for the physiological processes of reproduction: ovulation (Shkolnik et al., 2011), capacitation (De Lamirande and Gagnon, 1995) and corpus luteum formation and function (Vu et al., 2012). Some ROS also act as messengers in cell signalling as dregulation of the redox status will disrupt signalling mechanisms. This has been shown in the case of endometriosis, in which ROS are involved in its progression (Ngô et al., 2009). Therefore, ROS play positive physiological roles, through involvement in signal transduction pathways. At the present time, more than 10 redox-sensitive transcription factors have been identified, affecting various kinases, cell growth and differentiation, either directly or indirectly (Ngô et al., 2009; Sun et al., 2016).

Oxidative stress is the result of an imbalance between ROS production and the defense mechanisms that protect against the generation of damage to biological materials, and to DNA in particular. Oxidative stress has been linked to numerous pathologies, to ageing in general, and to gamete quality, specifically. For these reasons, the effect of ROS on biological processes is considered to be mainly negative, via damage to lipids, proteins and DNA (more than 20 oxidation products, abasic sites, DNA adducts (Badouard et al., 2008; Zenzes, 2000). Left unrepaired, DNA damage may lead to mutagenesis and malignant cell transformation (cancer) (Bukhari et al., 2016; Katakwar et al., 2016). Lipid peroxidation causes deformation of cell structures, and this process is associated with anomalies in cell metabolite transport that can affect physiological function (Volinsky and Kinnunen, 2013).

Oxidative stress has been linked to the origin or recurrence of pathologies that are now increasing in incidence, such as endometriosis and polycystic ovary syndrome. In men, ejaculated sperm lack the capacity to repair DNA that has been damaged by oxidative stress. To reduce or even avoid ROS-related DNA damage, consumption of anti-oxidant products are proposed as a means of decreasing the burden of DNA repair. In the present systematic review, we discuss the reasons why current treatments given haphazardly in the population might not be appropriate during the reproductive years, and indeed may have potential risks. Traditional antioxidant supplementation has failed to prevent chronic diseases, and in some cases has been found to be detrimental (Bjelakovic et al., 2008).

Methylation

Methylation is a biochemical process involving the addition of methyl groups to lipids, proteins and DNA; DNA methylation occurs specifically on cytosine, to form 5-methyl cytosine, which is significantly involved in epigenesis and imprinting. It produces changes and modifications in gene expression without altering the underlying DNA sequences, and this process is driven mainly by external, environmental factors, or both. In addition, methylation of the histones associated with DNA can influence gene expression, as can other post-translational modifications to these proteins. Recent evidence has highlighted the negative effect of profound alterations in sperm DNA methylation on fertility, especially with increasing age; the effect is not limited to gametes, but also affects embryonic development and diseases in the offspring (Jenkins et al., 2014). In oocytes, maternal age induces dysregulation in DNA methylation (Hamatani et al., 2004) The effect of defective methylation can contribute to neuropsychiatric disorders, such as schizophrenia and autism (Jenkins et al., 2014; Menezo et al., 2015). Epigenetic changes also play an important role in many cancers, possibly originating from failures in epigenetic inheritance that are established in early development (Cheung et al., 2016; Mack et al., 2016). New concepts explore a significant correlation between oxidative stress, methylation and methylation–epigenetic deregulation (Hoffman, 2011; Maltseva et al., 2009; Ménézo et al., 2011; Menezo et al., 2015), the latter of these processes occurring increasingly with age (Jenkins et al., 2014). These concepts will be further developed here, with the aim of emphasizing the fact that the risks encountered are real. We also propose a few solutions that might contribute to minimizing potential problems for future generations. This study is based on a systematic review of the literature, together with some of our data concerning preimplantation embryo metabolism, and the one carbon cycle in particular.

Free radicals, reactive oxygen species and antioxidant status

Free radicals were recognized as entities by Fenton in 1894, but the chain reaction known as the Haber–Weiss Reaction was described by Haber and Willstatter in 1931:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH, \text{ then}$$
$$\bullet O_2 - + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$$

Fenton's discovery plays a major role in all aspects of our life and was, at least partly, a source for Denham Harman's free radical theory of ageing. Current evidence now confirms the prevalence and significance of free radicals in biology and medicine. Oxygen (O_2) is the most abundant molecule in biological systems: the addition of one electron to dioxygen (O_2) forms the superoxide anion radical (O_2°), the primary form of ROS. This can then be converted to the hydroxyl radical (OH°), peroxyl radical (ROO°), hydrogen peroxide (H_2O_2) or in some cases to nitrogen peroxides (NO_x). Free radicals can be generated through normal metabolic processes (via nicotinamide adenine dinucleotide phosphate [NADPH] and several other oxidases). Mitochondria are at the centre of these mechanisms. It is commonly accepted that a small percentage of mitochondrial oxygen consumption is involved in the formation of ROS. Mitochondria are maternally derived, and all of the consequences of mitochondrial impairment are driven by the oocyte, with an effect on embryo viability and development.

The origin of oxidative stress is partly endogenous, as mentioned earlier. It is now, however, commonly admitted that environment and lifestyle may increase levels of oxidative

stress. Smoking, pollution from pesticides, weed killers and endocrine disruptors (bisphenols and other plastic additives, parabens and phthalates) are the most common inducers of oxidative stress. All these compounds have some structural analogies with oestrogens and bind to oestrogen receptors, peroxisome proliferator-activated receptors, or both. Nutritional imbalance, food excesses, energy deprivation and excessive exercise also affect oxidative stress. Age increases basic oxidative stress, irrespective of gender.

Markers of oxidative stress

Markers of oxidative stress may be organic or inorganic. The most common organic markers are 8-oxo deoxyguanosine, a marker of DNA insult, and malondialdehyde, originating from lipid peroxidation. Several other lipid derivatives and DNA adducts have been described, but they are more difficult to analyse (Badouard et al., 2008). Inorganic markers include excessive levels of iron (Fe) and Copper (Cu). A high Cu/Zn ratio is a good indicator of oxidative stress (Brack et al., 2013, 2016).

Antioxidant status

The total sum and the quality of defense against ROS is defined as the total antioxidant status. To balance the negative effect of free radical excess, organs are protected by compounds that neutralize them. This includes enzymes as well as non-enzymatic compounds. Small non-enzymatic molecules include vitamins A, E and C, but more importantly uric acid, cysteamine, pyruvate, hypotaurine and glutathione (GSH); the latter are most abundant in the genital tract, in gametes and in embryos (Guérin et al., 2001). Moreover, their oxidation products, taurine and oxidized glutathione are non-toxic compounds. The oxidation products of pyruvate, highly concentrated in the female genital tract, are acetate and carbon dioxide, naturally present and non-toxic. The same is not the case for vitamins A and C and uric acid, which can also be pro-oxidant, because after reduction of the oxidized compounds, vitamins C and E become in turn active as oxidants; therefore they can oxidize lipids and other compounds and then become reducing again. Several antioxidant enzymes protect against free radicals, such as copper and zinc superoxide The most important pathway, however, is the glutathione chain, which includes glutamine-cysteine synthetase, glutathione peroxidase and glutathione reductase. The glutathione oxidation–reduction ratio is a good marker of both oxidatives stress and antioxidant status (Brack et al., 2013). The question of gender-related resistance to oxidative stress has always been a subject of debate. Are there differences between men and women? This is no longer open to question: men and women have a different antioxidant status, with sensitivity and response to oxidative stress being better in women (Brack et al., 2016).

Oxidative stress and fertility

Oxidative stress and female fertility

The production of ROS is a necessary prerequisite for many essential aspects of female reproductive physiology, including ovulation and corpus luteum formation. It acts as a 'double edged sword', as perturbation of the physiological balance is a major source of problems: both oxidative and reductive stress can be potentially hazardous (Agarwal et al., 2005).

Ovary and oocytes

The effect of prenatal exposure to oxidative stress on the fetus and subsequent fertility of the offspring is an important concern. Although a potentially negative influence of oxidative stress on the fetal ovary during pregnancy has not been established, a strong correlation exists between oxidative stress and methylation and imprinting, related to homocysteine (Hcy) metabolism (Hoffman, 2011; Ménézo et al., 2011) (**Figure 1**). In germline cells, imprints are first erased, and then re-established in a gender-dependent fashion. Oxidative stress that disrupts methylation processes can certainly affect first imprint maintenance (preservation of the methyl marks on the corresponding DNA sequences), then its resetting in the embryo (re-acquisition of the methyl tags), with a later effect on the quality of the gametes. The role of oxidative stress in the cause of epigenetic defects is, therefore, of major concern.

It is now also clear that exposure to endocrine disruptors, such as BisPhenol A (BPA), diethylhexyl phthalate (DEHP), and dibutylphthalate (DBP) during fetal life may induce low birth weight and later onset of obesity, hyperglycaemia and other metabolic disorders (Manikkam et al., 2013; Song et al., 2015). In adults, the ovary is sensitive to oxidative stress: the relationship between oxidative stress, endometriosis and polycystic ovary syndrome is no longer in question. A component of plastics, DEHP inhibits the growth of cultured mouse antral follicles via an oxidative stress-dependent mechanism, and this inhibition can be prevented by thiol precursors of GSH (Mu et al., 2015). Endocrine disruptors (EDs) affect oocyte maturation and energy metabolism in the oocytes of large animals (Ambruosi et al., 2011). Urinary BPA concentration is inversely related to the number of oocytes retrieved per cycle. BPA induces a poor ovarian response to stimulation, although it is not certain whether this is linked to oxidative stress or to the continuous presence of BPA's oestrogen-like properties. The cause of idiopathic premature ovarian failure may involve oxidative stress (Kumar et al., 2010), a concept that reflects the resistance to stimulation observed in women undergoing assisted reproduction techniques (Mok-Lin et al., 2010).

Access to female gametes to study oxidative stress and DNA repair is limited, owing to the scarcity of material. The female gamete is unprotected during its periods of quiescence and follicular growth in the ovary (Lopes et al., 1998; Ménézo et al., 2010; Zenzes, 2000). Glutathione is a molecular 'masterpiece': FSH stimulates GSH synthesis, which counteracts ROS generation, thus inhibiting apoptosis and follicle atresia (Tsai-Turton and Luderer, 2006). During the stages of oocyte growth and final maturation, the oocyte stores mRNAs that code for protection against oxidative stress (El Mouatassim et al., 2000); these are translated in the preimplantation embryo until the time of transition from maternal to zygotic gene expression. The oocyte needs an environment rich in glutathione and its precursors (especially cysteine). A shortage of these in follicular and cumulus cells will have a negative effect on oocyte quality (Tsai-Turton and Luderer, 2006). Metabolism of thiols is again of major importance during this period (**Figure 1**). Glutathione is an important factor in oocyte cytoplasmic maturation and so-called 'oocyte competence',

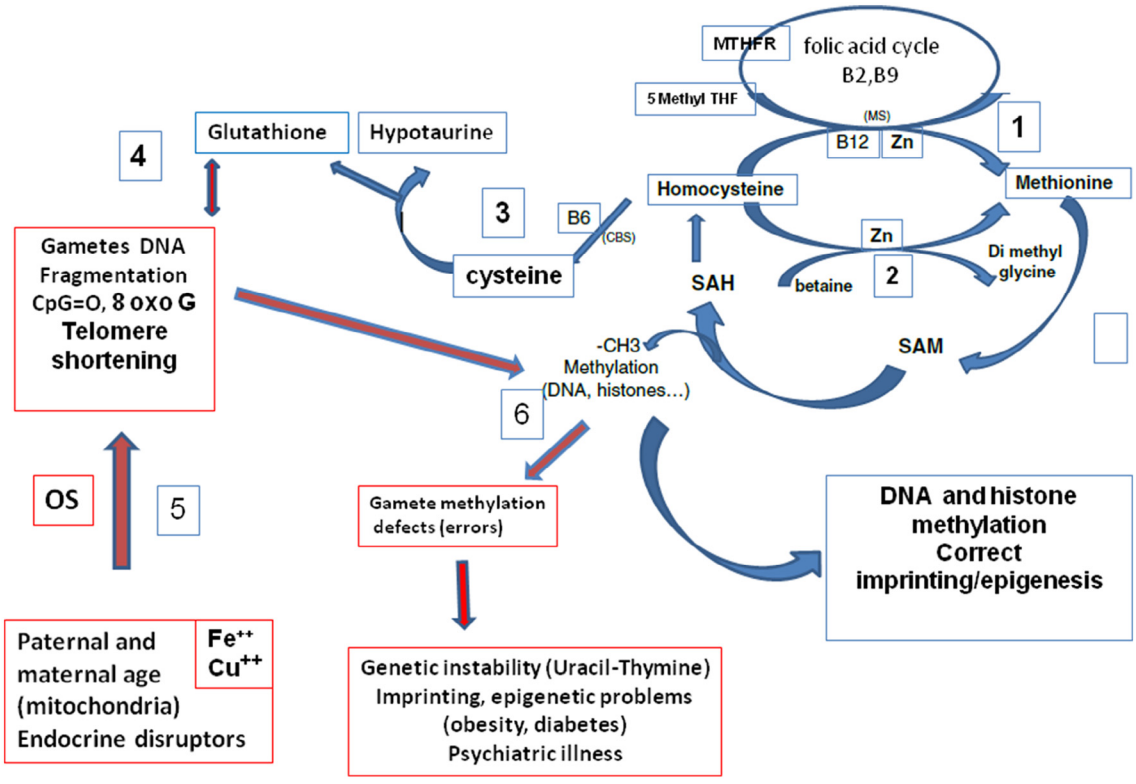

Figure 1 Interactions between oxidative stress, one-carbon cycle and methylation process. (1) One carbon cycle and folic acid cycle involving recycling of homocysteine to methionine. The folic acid cycle contains the MTHFR necessary for the formation of 5 Methyl THF, allowing the recycling; (2) the betaine-dimethyl-glycine pathway is marginally expressed in human oocytes and preimplantation embryo; (3) the cystathionine-beta-synthase pathway allows the formation of cysteine from homocysteine. This pathway is not expressed in the human oocyte and preimplantation embryo until maternal to zygotic transition; (4) cysteine is a precursor of glutathione and hypotaurine. Both are present in female genital tract. The Y counteract the damaging molecular action of reactive oxygen species; (5) Reactive oxygen species are generated by endogenous metabolic and exogenous factors. They alter the DNA. Age decreases the defense against reactive oxygen species. (6) DNA methylation is the crossroad. Impaired methylation will lead to genetic numerous major health problems. Cu, copper; FE, iron; MTHFR, methylene tetrahydrofolic acid reductase; OS, oxidative stress; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine.

as seen in sperm head swelling and in early embryo development up to the blastocyst stage. Its importance is even more crucial as the oocyte and preimplantation embryo have a poor capacity to recycle Hcy to form cysteine, a key molecule in glutathione (γ-L-Glutamyl-L-cysteinylglycine) formation (Ménézo et al., 2013) (**Figure 1**). The oocyte may be affected by DNA damage (Lopes et al., 1998; Zenzes, 2000); DNA benzopyrene-adducts can be found in the oocytes of heavy smokers.

Guanine is a major target for DNA oxidation, with the formation of 8-OH deoxyguanosine. Guanine is highly represented in telomeres, as a highly repetitive, non-coding terminal nucleotide sequence (TTAGGG) that protects the chromosome from DNA decay and from chromosome–chromosome fusion. Therefore, oxidative stress also damages telomeres directly and accelerates telomere shortening, which causes defects in meiosis, fertilization and embryo development, all of which can lead to infertility (Keefe and Liu, 2009). Telomere length is a marker of reproductive ageing (Kalmbach et al., 2013). Oocyte telomeres begin to shorten during fetal oogenesis, and continue to shorten in the adult ovary, related to oxidative stress and to genotoxic insults. Women who have experienced assisted reproduction technique failures have shorter oocyte telomeres than those who conceived successfully (Keefe et al., 2007).

The female gamete has an important capacity for repairing ROS-linked DNA insults (Ménézo et al., 2007a, 2007b, 2010). The repair systems may often be redundant, but the oocyte also has to deal with sperm-associated DNA damage after fertilization. An important factor in DNA repair is APEX/Ref-1 (apurinic and apyrimidinic sites), which is highly expressed in the oocyte (El-Mouatassim et al., 2007) associated with thioredoxin, which is also expressed in oocytes during up-regulation of redox potential and in response to ROS (Hedley et al., 2004). It is estimated that around 2 million or more DNA repair events, linked not only to ROS insults, must be carried out during the first embryonic cell cycle (Ménézo et al., 2010). Ovulation is another example of an ovarian process that is regulated by ROS whereby ROS levels rise and antioxidant levels fall in response to the pre-ovulatory LH surge (Shkolnik et al., 2011).

Polycystic ovarian syndrome

Polycystic ovary syndrome is a common endocrine disorder of genetic and environmental cause, characterized by insulin

resistance and dysregulation, hyperandrogenism and often obesity. The FSH–LH ratio is usually inverted owing to an increase in circulating LH. Serum levels of anti-Müllerian hormone and most of the androgens are elevated. Chronic anovulation, menstrual irregularity, or both, are often observed. A definitive link exists between oxidative stress and polycystic ovary syndrome. Total antioxidant status is decreased, and two strong markers of oxidative stress, malonedialdehyde (MDA) and carbonylated proteins are elevated (Fenkci et al., 2003). Carbonylation is currently used as a marker for irreversible protein and non-enzymatic oxidative damage (Dalle-Donne et al., 2006). Notably, serum Hcy levels are increased (Moti et al., 2015), and Hcy generates strong perturbations in methylation processes in oocytes and early embryos (Huffman et al., 2015; Ménézo et al., 1989). Polycystic ovary syndrome affects fertility severely through menstrual deregulation, poor oocyte quality and an increased incidence of spontaneous abortion.

Endometriosis

Sampson's proposal of retrograde menstruation and implantation is the commonly accepted theory about the cause of endometriosis (Sampson, 1927). Endometrial cells travel back through the fallopian tube via retrograde menstruation. The process causes chronic inflammation, with production of ROS. Endometriotic cells display higher endogenous oxidative stress, with an increase in ROS and nitric oxide production as well as alterations in ROS detoxification pathways (Ngô et al., 2009). Serum levels of superoxide dismutase decrease and lipid peroxidation increases (Liu et al., 2013; Singh et al., 2013). Moreover, a strong correlation exists between ROS levels and patterns of cell proliferation. Follicular fluid Hcy concentrations are significantly elevated in patients with endometriosis (Ebisch et al., 2006).

Oxidative stress and male fertility

The negative effect of ROS on human spermatogenesis is clear (Tremellen, 2008): the contribution of ROS to male infertility is estimated to be between 30 and 80%. *In vivo*, however, ROS also have a physiological role in spermatogenesis, as they are involved in padlocking the protamines that condense sperm DNA. Sperm DNA decay analysis (fragmentation and decondensation) and its importance in the reproductive process is based upon the pioneering work of Evenson et al. (1980) on sperm chromatin structure. DNA fragmentation provides an indirect estimation of ROS insults, representing an analysis of their consequences, but several other genetic and biochemical factors may also marginally induce fragmentation, such as infection, faulty apoptosis and topoisomerase dysfunction (Sakkas et al., 2003). Initially, ROS are generated from sperm metabolism via mitochondrial activity, but the environment, including endocrine disruptors, also acts as a major source; ROS cause DNA fragmentation and decreased sperm fertilizing ability (Lopes et al., 1998). According to Evenson et al. (2002), the critical threshold for numbers of sperm with DNA fragmentation is 25%. If only 75% of cells are without DNA damage, then this is insufficient to allow normal fertility. This means that the sperm cells have already initiated a process of DNA degradation that is inevitably deleterious. A proposal of 25% as a critical threshold is probably merely the 'tip of the iceberg'. Therefore, basal levels of damage will be amplified during transit through the female tract *in vivo* (Bungum et al., 2007). Guanine is the most sensitive to oxidation, and as expected, there is a good correlation between 8-oxo deoxyguanosine, the most important product of DNA oxidation, and sperm DNA fragmentation (Oger et al., 2003), as this process often leads to abasic site generation. Therefore, sperm cell telomeres with TTAGGG repeats are highly sensitive to oxidation and, crucially, the association between short telomeres and sperm DNA fragmentation has been clearly demonstrated (Rodríguez et al., 2005).

More than 10 oxidation products of DNA bases are currently found in sperm, in addition to DNA adducts (Ménézo et al., 2010). Some of them arise from a reaction with 4-hydroxy-2-nonenal, the main aldehyde compound released during lipid peroxidation along with MDA, or after occupational exposure to vinyl chloride (Badouard et al., 2008). Generation of abasic sites is also an important ROS-driven insult. It is currently known that epididymal and ejaculated sperm have no DNA repair capacity, but even if (polyA)mRNAs coding for DNA repair are present in ejaculated sperm, such as APEX/Ref1 (El-Mouatassim et al., 2007), they will be translated in the oocyte only after sperm DNA swelling. Sperm DNA swelling allows liberation and translation of the mRNAs, which cannot occur when the sperm nucleus is in its condensed state. Then APEX, in association with thioredoxin (Hedley et al., 2004), will also control the redox status of transcription factors such as fos, jun, hypoxia inducing factor1-alpha and P53 (Kelley and Parsons, 2001). A possible role for these enzymes as an auto-repair system in the oocyte at the time of fertilization cannot be excluded. Originating from lipid peroxidation, MDA is present in seminal plasma and is also a marker of immaturity linked to an excess of polyunsaturated fatty acids, which are more sensitive to peroxidation (Aitken et al., 2006). Reaching the site of fertilization during sperm ascent in the female genital tract may also be a hazard, as mentioned earlier: sperm with a high DNA fragmentation index are less capable of achieving an ongoing pregnancy after artificial insemination with husband's sperm than after IVF or intracytoplasmic sperm injection (Bungum et al., 2007). Transit through the female genital tract, if associated with inflammation or infection, increases ROS-linked risks (Bałajewicz-Nowak, 2011); therefore, sperm cells 'washed' free from seminal plasma, either *in vivo* or *in vitro*, rapidly undergo ROS-linked DNA damage (Muratori et al., 2003). High levels of Hcy are associated with low sperm quality. This is demonstrated clearly in patients who carry isoforms of methyl tetrahydrofolate (MTHFR) 677 C-T and methionine synthase (MS A2756G) (Liu et al., 2012); MTHFR is the enzyme involved in the mandatory transformation of folic acid to 5-methyl Tetrahydrofolate, the active methyl donor. Folic acid is completely ineffective without this transformation (reduction). In the presence of vitamin B12, methionine synthase allows the methyl group on Hcy to be condensed, forming methionine (**Figure 1**). The presence of the above-mentioned isoforms results in a restricted capacity for recycling Hcy to methionine. About 20–30% of the general population is known to carry these isoforms.

Spermatozoa have longer telomeres but, in common with the female gamete, ROS have a negative effect on their telomeres (as mentioned earlier, oxidation of guanine is an important perturbation factor). Shorter telomeres in sperm may

be responsible for male infertility to some extent (Thilagavathi et al., 2013). All of the observations made about the effect of environment and lifestyle on female fertility are also valid for male fertility. Recreational drugs, smoking, alcohol and obesity impair male fertility. The fetal testis is a major target for endocrine disruptors: herbicides, pesticides, PCBs, plasticizers (Gaspari et al., 2012; Sultan et al., 2001). These can induce a syndrome known as testis dysgenesis syndrome, associated with hypospadias, low sperm counts, testicular cancer, cryptorchidism and ambiguous genitalia. The effect of these compounds seems to be less significant in the adult testis; however, a strong negative correlation has been established between sperm quality and the level of phthalates found in the urine (Bloom et al., 2015).

Oxidative stress and the preimplantation embryo

Embryo biotechnology applied to assisted reproduction in ruminants and in the human has contributed enormously to our knowledge about embryo biochemistry and metabolism. The dry weight of a human preimplantation embryo can be estimated to be between 75 and 100 ng (Ménézo et al., 2006): therefore, the risks and consequences of ROS-induced damage on such a small amount of material are immediate, with effects on lipids, proteins, genomic DNA (only a few copies) and on mtDNA, impairing mitochondrial function. It is important to note here that oocytes undergo an age-related decrease in mRNA storage (Hamatani et al., 2004). This age-related global decline in transcript abundance affects methylation machinery, mitochondrial function and defense against ROS at a time when global defense against oxidative stress is already decreasing.

According to El Mouatassim et al. (2000) and Guérin et al. (2001), the in-vivo site of fertilization is highly protected against ROS. Antioxidant compounds are present in large amounts and in various forms in the environment surrounding oocytes and sperm. In the mouse, a burst of ROS occurs at the time of fertilization, and again at the G2/M phase of the second cell cycle (Nasr-Esfahani et al., 1992; Nasr-Esfahani and Johnson, 1991). Follicular and tubal fluid surrounding the early embryo have high levels of enzymatic and non-enzymatic antioxidants such as hypotaurine, ascorbic acid, superoxide dismutase, glutathione peroxidase and γ-glutamyl-cysteine synthetase (Guérin et al., 2001). The tubal epithelium can specifically synthesize hypotaurine via the cysteine sulphinate pathway (Guérin and Ménézo, 1995). Hypotaurine is an interesting molecule: taurine, its product of oxidation, is not pro-oxidant (unlike some vitamins) and it also has important properties as an osmolyte, but taurine is not an anti-oxidant *per se*.

Two major proteins, transferrin and ceruloplasmin, are present in tubal fluid (Menezo and Laviolette, 1972). These proteins regulate circulating levels of two divalent cations that induce oxidative stress via the Haber Weiss–Fenton reaction: iron (transferrin) and copper (ceruloplasmin). Embryos are highly sensitive to oxidative stress and, in most mammalian species, glycolysis and oxidative phosphorylation (OXPHOS) generate the adenosine triphosphate (ATP) necessary for embryo development, but also produce ROS. High levels of glucose increase ROS production by the embryo (Jiménez et al., 2003). This is important in diabetic patients, in whom preimplantation embryo development can be significantly and negatively affected through ROS-linked apoptosis (Ávila et al., 2015; Jiménez et al., 2003). In human IVF, an excess of glucose *in vitro* can produce monozygotic twinning through polarized apoptosis in the inner cell mass (Ménézo and Sakkas, 2002). The female embryo seems to be more effectively protected through the X-linked inhibitor of apoptosis (*XIAP*) gene. ROS are not only of mitochondrial origin (OXPHOS); NADPH, xanthine and amine oxidases also generate ROS (0_2^- and consequently hydrogen peroxide). Amine oxidase is involved in catabolism of spermine and spermidine, both highly active in the preimplantation embryo (Ménézo et al., 2013). Adiponectin is a pleiotropic hormone secreted from adipose tissue that mediates some of the negative effects of obesity. Tubal adiponectin, like carnitine, can help the embryo to increase lipid metabolism, decreasing glucose requirements (Ménézo et al., 2013; Montjean et al., 2010). Embryonic lipid metabolism provides larger quantities of ATP with lower levels of free radical generation. We found that adiponectin receptor 2 is highly expressed, and receptor 1 is poorly expressed in the human oocyte (Ménézo, unpublished observations).

Glutathione is a universal free radical scavenger. After fertilization, glutathione is immediately mobilized through two ATP-dependent steps: γ-glutamylcysteine synthetase and glutathione synthetase, both associated with cysteine availability. Glutathione mobilization is mandatory for sperm head swelling and formation of a fully developed male pronucleus (Luberda, 2005). It is also necessary for up-regulation of glucose metabolism and increased activity of the pentose phosphate pathway, which influences the initiation of the first S-phase in both the male and the female pronuclei, as well as during embryo development up to the blastocyst stage (Furnus et al., 2008). The effect of glutathione mobilization on further embryonic development is immediate, resulting in an increased rate of blastocyst formation and increased cell number per blastocyst (Furnus et al., 2008). The pentose phosphate pathway synthesizes C_5 sugars and also allows regeneration of NADPH, necessary for thioredoxin reductase activity. Thioredoxin is vital for all cells. Transcripts for six members of the peroxiredoxin family, for 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase have been detected in the oviduct and oocyte (El Mouatassim et al., 2000). This is of major importance in relation to Hcy recycling, which is necessary for correct imprinting processes, and for synthesis of thymidine via MTHFR (Furnus et al., 2008). This is particularly important in human oocytes, as the cystathionine-beta-synthase pathway for recycling of Hcy is absent in the human oocyte and early preimplantation embryo until the stage of maternal to zygotic transition (Ménézo et al., 2013). Embryos have a high capacity for DNA repair, especially of ROS-linked damage. This capacity, however, is finite and decreases with female age (Hamatani et al., 2004). The products of degradation then have to be removed, a process that involves sanitizing the endogenous pool in order to avoid re-incorporation of the damaged (oxidized) bases: 'defective bricks build a defective house'.

Methylation

'Methylation helps give life, and it can take it away. In fact, without methylation there would be no life at all.' Craig Cooney (Vanyushin, 2005).

Methylation is a key biochemical process that adds methyl groups covalently to several molecules: lipids, proteins and DNA. The universal cofactor for methylation is S-adenosyl methionine (SAM), which forms S-adenosyl Hcy once the target molecule has been methylated (**Figure 1**). S-adenosyl Hcy is then hydrolysed to Hcy. The oocyte and early embryo have a full capacity to synthesise SAM (Ménézo et al., 1989, 2013) and all the intermediary metabolites involved in the methylation process, all of the enzymes involved in each step being expressed (Benkhalifa et al., 2010). Hcy must be recycled to methionine, as it is a strong inducer of methylation failure, especially in the preimplantation embryo (Ménézo et al., 1989). Methylation is key to epigenetic processes, with consequences related to histone modification and chromatin remodelling. Methylation of DNA and its associated histones modifies chromatin structure and thus the function of the genome. Methylation is one of several biochemical modifications of histones (others include acetylation), especially of lysine residues on histone H3 (H3Ks). Epigenetic modifications result in mitotically and meiotically heritable changes in gene expression, without changing the DNA base sequence. When located at a site close to or in a gene promoter, DNA methylation usually represses gene transcription; DNA methylation, therefore, plays an important role in mammalian development. This process is not only involved in genomic imprinting, gene inactivation and epigenesis, but also in carcinogenesis (for review see Schübeler, 2015). The target for DNA methylation is predominantly cytosine, and is effected enzymatically via DNA methyl transferases (DNMTs) to 5-methylcytosine. De-novo methylation is carried out by DNMT3A and DNMT3B (known to be post-transcriptionally regulated by microRNAs); global methylation maintenance is catalyzed by DNMT1 after replication. The mRNAs coding for DNMTs (especially DNMT1) are highly expressed in human oocytes and preimplantation embryos (Huntriss et al., 2004; Ménézo et al., 2007a). DNA is methylated at the level of CpG sites, where cytosine is followed by guanine; CpG islands (or CG islands) are regions with a high frequency of CpG sites (clusters with at least 200 bp, and a GC percentage greater than 50%). CpG islands represent 1–2% of the genome. Differentially, methylated regions are CpG-rich sequences of DNA that have different methylation patterns compared with other samples. An estimated 29,000 CpG islands are located in the vicinity of promoters, regulatory elements and transcription binding sites for housekeeping and other important genes. In these regions, where methylation has inactivation and suppression roles, the methylation state reaches 60–80%. It is not yet clear how many CpGs participate in genomic regulation. In addition to DNA methylation, histone acetylation co-contributes to regulation of gene silencing via modification of chromatin structure (Pedone et al., 1999). The methylation status of CpG islands is generally low and stable through multiple cell divisions. Physiological differences can occur according to the type of tissue, during varying periods of growth and development, or according to the gender origin of the genes during imprinting or inactivation processes (e.g. X-inactivation). Some imprinted genes can have a transitory dual expression during embryonic development, depending upon the organ and the age of the embryo, indicating an 'imprinting plasticity' (Buckberry et al., 2012; Murphy et al., 2012). Demethylation is accomplished either by direct removal of 5-methylcytosine, after transformation to thymine followed by an excision repair mechanism, or, more frequently, by demethylation of 5-methylcytosine and elimination of the products formed by DNA repair.

During parental imprinting, DNA methylation is controlled by imprinting control regions. This epigenetic form of gene regulation leads to monoallelic expression depending on parental origin. Epigenetic marks (methylation) include both DNA and histone methylation at the level of the imprinting control region. DNA methylation might be a prerequisite for the acquisition of repressive histone marks. The male and female genomes are complementary rather than equivalent, and epigenetic heritable changes in gene expression are the result of activation and deactivation of genes without alteration of the underlying DNA sequence.

Epigenetic mechanisms can be affected by several factors and processes, including ovarian stimulation in assisted reproduction techniques (Huffman et al., 2015) and environmental influences, such as chemicals, drugs and pharmaceuticals, ageing and diet, on both development *in utero* and during childhood. Epidemiological studies have shown that nutritional and environmental disturbance during early life can modify basic housekeeping metabolism, leading to metabolic diseases (obesity and diabetes) in later life via modifications in DNA methylation (Hogg et al., 2012; Vickers, 2014). The first cited example is the 'Dutch Famine' (1944–1945): individuals exposed prenatally to food shortage were found to have a reduced methylation status on the imprinted *IGF2* gene several decades later. This reinforces the concept that development *in utero* is a critical period for establishing or maintaining epigenesis. Methyl groups present in some dietary sources can tag DNA and activate or repress genes. Histone modification occurs when epigenetic factors that bind to histone 'tails' alter the extent to which DNA is wrapped around histones, affecting the availability of genes in the DNA to be activated. All of these biochemical factors and processes, especially during embryogenesis and early life, can influence future health, possibly resulting in cancer, autoimmune diseases, mental disorders (autism and schizophrenia), or diabetes and other metabolic illnesses (Kawai et al., 2015; Sandovici et al., 2012; Soubry et al., 2015). The resulting epigenome of a cell is highly dynamic, determined by a complex additive interplay of genetic and environmental factors. Although imprinting-related DNA methylation is stable, this is not the case for the process of epigenesis, which shows high plasticity and a high degree of inter-individual variations. Whatever the process, ageing has a negative effect on global DNA methylation (Richardson, 2003). Epigenetic changes play an important role in normal regulation of gene expression: this means that their alterations can significantly contribute to cancer, frequently associated with DNA methylation changes. These changes include both hypomethylation and DNMT3-related hypermethylation, which is often associated with micro RNA expression. Silencing of tumour suppressors by CpG island promoter hypermethylation is a strong generator of breast cancer, affecting genes such as *BRCA1* (Esteller, 2005).

Methylation in gametes and during early embryogenesis

DNA methylation is of major importance in gametogenesis, as primordial germ cells undergo a process of DNA

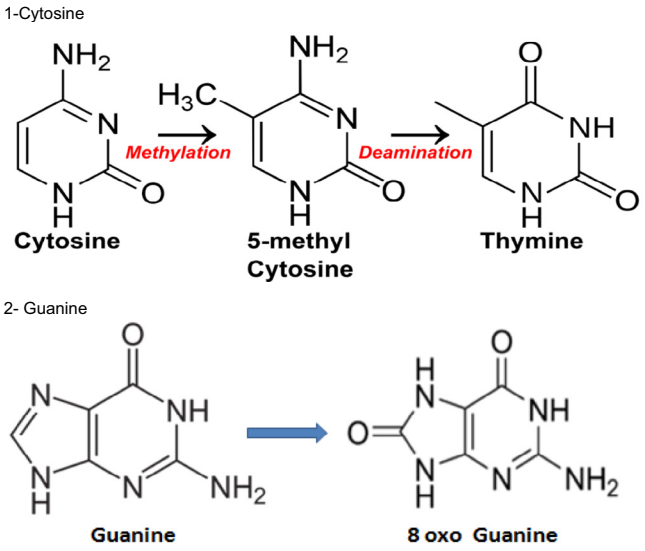

Figure 2 Oxidation products of cytosine and guanine (CpG islands are important for imprinting processes, related to oxidative stress and methylation). Note that Guanine is the base that is most sensitive to oxidation.

de-methylation when they enter the developing gonads (imprint erasure) (Lees-Murdock and Walsh, 2008). This process is subsequently reversed during prenatal life in men, and during post-natal follicle development in women (Lees-Murdock and Walsh, 2008). Epigenetic marks acquired in the germ line drive the allele-specific expression of specific parentally 'imprinted' genes in the embryo. Although global demethylation seems to be a normal feature of preimplantation development, however, mass spectrometry reveals no decrease in the quantity of 5 Methyl cytosine (5M-C) in the mouse embryo, even immediately after genomic activation (Okamoto et al., 2016). This is an important observation, suggesting that demethylation is balanced by active maintenance of methylation, involving a high level of DNMTs 1 and 3 activity and the presence of the methyl-CpG binding proteins (Huntriss et al., 2004). This process is probably of major importance during early preimplantation development, considering the risk of perturbation via endocrine disruptors (now generally present in the human body) on the one hand, and the small mass of the embryo on the other hand (**Figure 2**).

Sperm methylation

The milestone work of Kobayashi et al. (2007) and Marques et al. (2004) established a strong link between errors in sperm DNA methylation and male hypofertility. Age and lifestyle factors, such as food overconsumption (especially sugars), sedentary occupation, alcohol and recreational drug abuse, severely increase these negative methylation and epigenetic differences in paternal sperm (Stuppia et al., 2015), leading to increased risks of varying pathology (Feinberg et al., 2015; Jenkins et al., 2014). In the rat, plastic-derived endocrine disruptors induce transgenerational sperm epigenetic modifications, controlled by methylation levels in regions of DNA methylation (Manikkam et al., 2013). Transgenerational pubertal abnormalities, testicular disease and obesity are observed as a result.

Oocyte methylation

In mouse and bovine oocytes, DNA methylation and imprinting is established in an oocyte size-dependent manner, linked to oocyte developmental stage and maturity (Hiura et al., 2014; O'Doherty et al., 2012). Ovarian stimulation has a negative influence on epigenetic settings (Huffman et al., 2015). This can be linked to Hcy levels: a negative correlation exists between follicular fluid Hcy concentration and the maturity and quality of the oocyte (Berker et al., 2009; Boxmeer et al., 2009; Ebisch et al., 2006, 2007; Szymański and Kazdepka-Ziemińska, 2003). Follicular fluid Hcy levels are higher after ovarian stimulation. Hcy affects epigenetic regulation through disturbance of methylation. At physiological concentrations, it acts as an 'uncertain/unreliable' partner in regulating general development (Ménézo et al., 1989). In some cases, Hcy can also induce hypermethylation through feedback effects on DNA methyltransferase activity. It is important to emphasize here that the oocyte and early embryo has a poor capacity for recycling Hcy. Moreover, methionine and Hcy compete for the same transporter for entry into the oocyte and early embryo. The process of methylation (maintenance) is crucially important during preimplantation development (Arand et al., 2015; Ménézo et al., 1989; Okamoto et al., 2016). We observed that ethionine and aza cytidine, strong inhibitors of methylation, have a rapid and dramatic degenerative effect on preimplantation mouse embryos, even at very low concentrations (Ménézo et al., 1989). Two separate dynamic processes are involved: global demethylation of the genome, as well as the opposite, maintenance of methylation (especially for the imprinted genes). Methylation restarts at the time of the first process of differentiation, the blastocyst stage. Paternal genome demethylation takes place at the time of pronucleus formation, and occurs more slowly for the female genome. Demethylation can be passive (at each division) or active. When active, ten–eleven–translocation proteins seem to participate in DNA demethylation. They can convert 5mC into 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine through oxidation reactions (Mohr et al., 2011). These modified bases may represent intermediates in the process of DNA demethylation. An active demethylation process is also mediated via methyl-CpG binding domains (MBDs) and DNA glycosylase (Thymine DNA glycosylase). In the human oocyte, MBD4 and methyl-CpG-binding-domain-4-DNA-glycosylase are highly expressed, at 500 times and 15 times the background signal, respectively. In agreement with others (Huntriss et al., 2004), we found that expression of the mRNA coding for the enzyme DNMT1, responsible for DNA methylation maintenance, is one of the most significant transcripts found in the oocyte (1000 times background). This suggests that methylation maintenance is not a marginal process during this period of preimplantation embryo development. DNMT3A and B are expressed at a lower level (100 times background signal). It must be emphasized here that mRNAs stored in the human oocyte are the reserves for early development up to D3 (4 to 8-C stage). In the mouse, rapid cleavage *in vitro* is a sign of an abnormal DNA methylation process (Market Velker et al., 2012). Embryonic DNA sequence variations in the gene

encoding DNMT3L are associated with abnormal paternal DNA methylation (Kobayashi et al., 2009). It now seems clear that human assisted reproduction techniques have a negative effect on DNA methylation (Hiura et al., 2014; Song et al., 2015), more in relation to technical aspects than to the quality of the gametes and cause of infertility. Comparison of the placenta derived from births conceived through assisted reproduction techniques with that from children conceived *in vivo* reveals consistent and significant differences in CpG methylation sites (Katari et al., 2009; Song et al., 2015). Endocrine disruptors have an equally deleterious effect, in both men and women. In animal models, transgenerational epigenetic modifications lead to polycystic ovarian syndrome, primordial follicle loss and obesity, and also to testicular disease and pubertal anomalies in men (Manikkam et al., 2013).

Oxidative stress and epigenetic alterations

OS impairs sperm DNA methylation (Tunc and Tremellen, 2009). An important feature to bear in mind is that methylation occurs on CpG islands. Guanine is the nucleotide base that is most sensitive to oxidation (leading to the formation of 8 oxo deoxyguanosine), but cytosine (C) can also be altered, leading to formation of 5-OH C, 5,6-diOH C, C glycol (Ménézo et al., 2010) (**Figure 2**). 5-hydroxymethylcytosine is a physiological product of oxidation, and is a prerequisite for DNA demethylation. The formation of thymine by deamination of 5-MeC, 8-oxoG or 5-MeC to 5HmC is even more important, as it significantly inhibits binding of the methyl CpG-binding domain-proteins (MBDs) to the corresponding CpGs. The affinity of DNA for DNMT3A is also then weakened, leading to poor methylation. Moreover, the resulting over-expression of DNMTase activity could result in randomly increased cytosine methylation at non-CpG sites. Base oxidation in the CpG sites modifies the interactions between the CpG sites and the transcription factors (**Figure 3**) (Dattilo et al., 2016). DNA oxidative damage, therefore, may result in heritable epigenetic changes via modifications to chromatin organization (Donkena et al., 2010; Maltseva et al., 2009), aberrant hypermethylation of some gene promoter regions and global hypo-methylation. CpG islands are mutation hotspots (Wachsman, 1997); ROS-induced abnormal DNA methylation pattern alterations are implicated in malignant transformation and progression of numerous tumors (O'Hagan et al., 2011; Valinluck et al., 2004). An oxidative product of cytosine is 5HmC; abnormal uncontrolled 5HmC levels can induce active aberrant DNA demethylation processes.

Two important observations can be made about the relationship between oxidative stress and transmission of epigenetic disorders: obesity is a major source of oxidative stress, and paternal obesity jeopardizes the health of offspring (McPherson et al., 2014). Paternal lifestyle influences embryo development and has a further influence on the future lifelong health of children (Stuppia et al., 2015). Oxidative stress during pregnancy alters fetal epigenetic patterns and will later

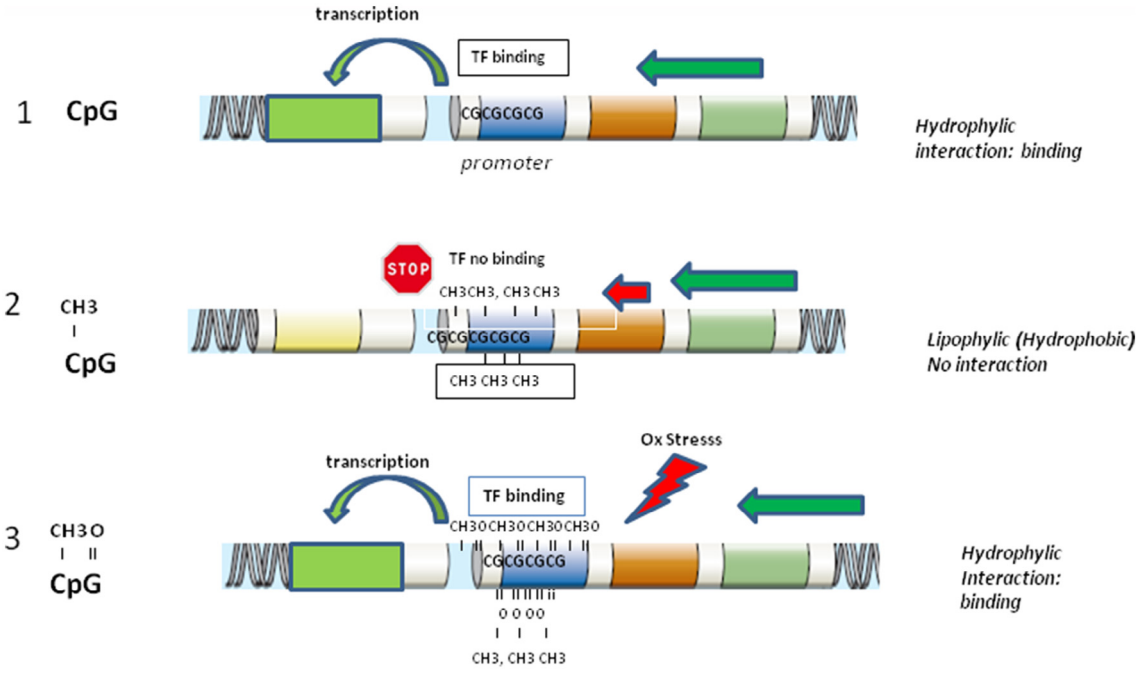

Figure 3 Effect of DNA methylation on transcription of some (imprinted and epigenetically modified) genes (simplified). Methylation modifies the interaction with DNA (Van der Waals and coulombic interactions) with the transcription factor (TF). Oxidation of the bases (G) at CpG sites modifies totally the interactions and restores the affinity between the TF and the DNA (see also Dattilo et al., 2016). (1) No modification on the promoter, the transcription factor binds, and the gene is transcribed downstream. An expression (in green) is expected; (2) methylation changes the promoter to an hydrophobic status: no binding, no transcription downstream: no expression (yellow); (3) oxidation of the methylated locus at guanine level: the promoter returns to a hydrophylic state: binding of the transcription factor and gene (in green) expression expected.

lead to cardiovascular disease (Ávila et al., 2015). In the mouse, spermatoza-induced oxidative damage leads to sex-related glucose and fat-related metabolic pathologies in offspring (Lane et al., 2014). Human maternal diabetes represents an important source of oxidative stress, and increases the risk of autism disorders in the infants (Xiang et al., 2015); epigenetic changes certainly seem to play a role in these disorders (Loke et al., 2015).

The deleterious effect of Hcy during ovarian stimulation (Ebisch et al., 2006) confirms a correlation between oxidative stress and effectors of methylation. Therefore, controlled ovarian stimulation increases the level of Hcy, with an associated inhibition of oxidative stress and methylation (Ménézo et al., 1989) in the follicular fluid, and strongly induces epigenetic alterations in the zygotes (Huffman et al., 2015). Some assisted reproduction technique procedures do apparently increase imprinting and DNA methylation errors (Song et al., 2015). A high level of ROS depletes endogenous glutathione, and excess Hcy, that is not recycled, prevents the regeneration of methionine, affecting SAM formation for methylation and also the formation of cysteine, precursor of glutathione (**Figure 1**), a typical 'vicious circle' situation. In addition, abundant quantities of ROS can reduce the expression of the gene for a major antioxidant enzyme, glutathione-s-transferase P1 by inducing the methyl-binding proteins, histone deacetylases, and DNA methyltransferase complex to methylate the promoter (Donkena et al., 2010). Expression of glutathione-s-transferase P1 is low in oocytes, unlike histone deacetylases and methyl-binding proteins, which are highly expressed universally. In mitochondria, oxidative stress and DNA methylation are correlated with ageing (Zinovkina and Zinovkin, 2015). Paternal age induces a lesser resistance to oxidative stress, leading to increased sperm DNA fragmentation (strongly linked to oxidative stress) and alteration in methylation status (Richardson, 2003). After assisted reproduction techniques, this may lead to an increase in neurological disorders among the infants conceived (Evenson et al., 2014), an obvious consequence of epigenetic dysfunction (Jenkins et al., 2014; Menezo et al., 2015). A recent study (Amar et al., 2015) compared two groups of men who showed a high (>20%) sperm DNA fragmentation index (highly correlated with oxidative damage). The first treatment consisted of 1-C cycle support only (treatment A), and the second was a sequence of a 'strong' classical antioxidant mixture followed by 1-C cycle support (treatment B). Both treatments were compared with a control group receiving no treatment. Both treatments significantly decreased the Sperm Decondensation Index (treatment A: $P < 0.003$; treatment B: $P < 0.001$ versus control group). The sequential treatment (B) with strong 'classical antioxidants', however, gave only marginal improvement, which was non-significant compared with treatment with 1-C cycle support alone (A). Similar results were reported by Cornet et al., 2015 (**Table 1**) in patients waiting for a next assisted reproduction technique treatment, treated for at least 3 months with one carbon cycle support (Procrelia ®, Laboratoire Nurilia) compared with control (no treatment). A second interesting observation noted that the SDI is directly related to nuclear decondensation using the classical anti-oxidant mixture, i.e. selenium, vitamins C, A and E (Ménézo et al., 2007b). Supporting the 1-C cycle has a direct positive effect on sperm nucleus condensation (Dattilo et al., 2014). In another study that included 173 women with at least 3 years' primary infertility and at least one failed assisted reproduction technique treatment, supplements that support the 1-C cycle were given for 4 months before starting assisted reproduction technique treatment (Cornet et al., 2015). The control group received no treatment. A high spontaneous pregnancy rate was observed in the treated group (30%), with an overall pregnancy rate (including assisted reproduction technique procedure) of 45% (40% delivery rate), compared with 13.7% and 10.9%, respectively, in the control group. It was not possible, however, to determine whether the improvement was directly related to decreased oxidative stress in the treated patients. In contrast, certain dietary polyphenols that have a strong 'reducing' capacity affect DNA methylation by inhibiting DNA methyltransferase activity, via S-adenosyl-L-Hcy generation (Fang et al., 2007). It is commonly accepted, however, that polyphenols usually reverse adverse epigenetic regulation (Russo et al., 2015).

Table 1 The effect of one-carbon-cycle support treatment for at least 3 months, between two assisted reproduction technique attempts, on sperm DNA fragmentation and achievement of pregnancy.[a]

	Treated	*Control*
Nuimber of patients	95	84
Mean sge (years) (SD)	34.7 (4.3)	37.8 (6.7)
DFI before treatment	27 (6.8)	26.9 (4.5)
DFI after treatment	17.3 (4.5)[b]	25.9 (4.4)
Pregnancies	49[c] (52%)	23[d] (27.4%)
Deliveries	45 (47%)	18 (21%)

DFI, DNA fragmentation.
[a]Control: no treatment.
[b]$P = 0.001$ for comparison with DFI before treatment.
[c]Eight spontaneous, 41 after assisted reproduction techniques.
[d]0 spontaneous; 23 after assisted reproduction techniques.

Environmental factors, especially plastic derived endocrine disruptors (BPA, DEHP and DBP), generate oxidative stress and induce epigenetic modifications via anomalous DNA methylation (Cooney, 2007; Cooney et al., 2002; Manikkam et al., 2013; Menezo et al., 2015). Some of the most important pathological aspects identified as being due to alterations in DNA methylation and imprinting and those linked to ROS are presented in **Figure 4**. Supporting the one carbon cycle by administering methyl donors can restore DNA methylation to some extent, and thus avoid epigenetic variations in offspring (Cooney et al., 2002; Dolinoy et al., 2007). Folate depletion during pregnancy and lactation reduces genomic DNA methylation in the adult offspring: folate is a key metabolite at the centre of Hcy recycling to methionine. According to O'Neill (1998), folic acid (B9) is mandatory as a methyl donor during early embryonic development, participating in at least two pathways: synthesis of thymine and recycling of Hcy, independent of low anti-oxidant capacity (Koyama et al., 2012). All of this information confirms that the process of DNA methylation is clearly involved in epigenetic pathologies. From a heuristic point of view, all of the enzymes involved in the folate pathway are highly expressed in the oocyte and then in the preimplantation embryo: that is, methyltetrahydrofolate dehydrogenase 1 (NADP dependent), MTHFR 5,10-methyl tetrahydrofolate

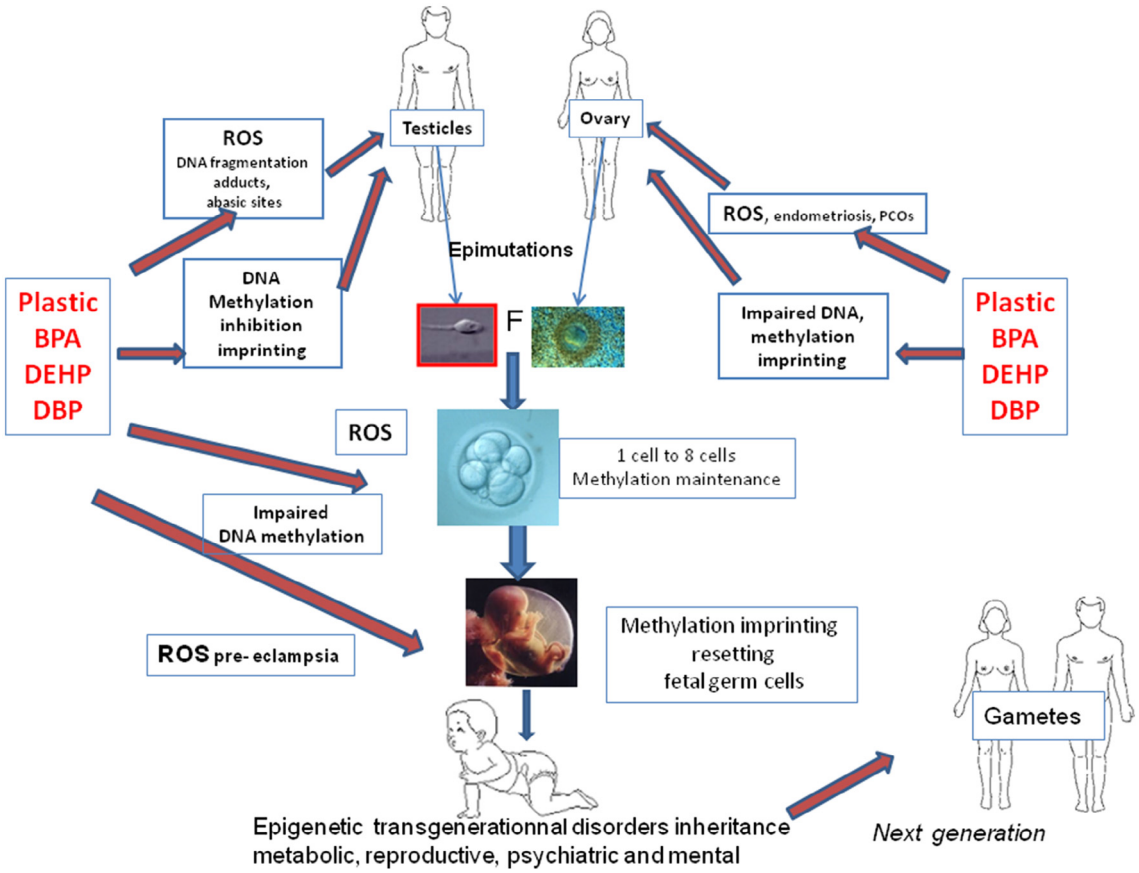

Figure 4 The main pathological consequences of DNA methylation, imprinting errors and reactive oxygen species-linked deleterious perturbations. BPA, bisphenol A; DBP, dibutylphtalate; DEHP, diEthylHexylphtalate; ROS, reactive oxygen species.

reductase; MTR 5-methyltetrahydrofolate-Hcy transferase; MTRR methyltetrahydrofolate-Hcy methyltransferase reductase (Benkhalifa et al., 2010). Moreover, the human oocyte has a high level of both folate receptor 1 expression (300 times background signal) and folate transporter member 1, also known as SLC19A1 (660 times background), indicating that there is a high level of trafficking around this molecule before and after genomic activation (Ménézo et al., 2013), as well as in human embryonic stem cells (Steele et al., 2005). This is an important feature, as the cysteine beta synthase pathway is absent before the maternal to zygotic transition; this renders Hcy recycling somewhat 'fragile', and fully dependent on the methionine synthase pathway (**Figure 1**), as the betaine dimethylglycine pathway is poorly expressed.

In conclusion, a redox state that is perturbed has a negative effect on ATP production, and this not only induces a shift in metabolism (OXPHOS), but also affects gene expression (Harvey et al., 2002). Redox status must be finely tuned and balanced, as reducing molecules can also have pro-oxidative properties when isolated from the complex environment that exists *in vivo*. It has become increasingly obvious that ART technology can have an effect on imprinting, and the techniques used should be improved (Hiura et al., 2014; Song et al., 2015). In our opinion, there are two major areas for improvement. The natural milieu for gametes and embryos contains redundant and overlapping ROS-protective systems that cannot be mimicked *in vitro*, but nonetheless efforts should be made to optimize media for manipulation of gametes and embryos. According to Muratori et al. (2003), current sperm washing media induce ROS, and Martín-Romero et al. (2008) reported that culture media for oocytes and embryos can generate ROS in isolation, in the absence of gametes and embryos. Culture media should be supplemented with compounds such as hypotaurine, which is present in the oviduct as a natural antioxidant. Suppositions that are aberrant and unfounded should be avoided. Although it has often been said that rapid cleavage of a human embryo is a marker of good quality, the exact opposite is the case in mouse embryos, where rapid cleavage jeopardizes correct imprinting (Market Velker et al., 2012). This may also be related to another feature of culture medium: based on observations made in the mouse system, Lane et al. (2001) suggested that essential amino acids should be removed from culture media during the first few days of human embryo culture, a proposal that was adopted by many companies who manufacture commercial culture media. This means that cysteine and methionine, the latter being involved in imprinting through the formation of SAM, are absent from many commercial sources of culture media. Methionine is not only essential for imprinting, but also has other important roles in cell metabolism, such as synthesis of polyamines that are important in early embryogenesis via SAM (spermine and spermidine) (Ménézo et al., 2013). The concept surrounding the role of essential amino acids is highly misleading and potentially harmful in terms of imprinting and epigenesis. Culture

media should contain compounds such as folic acid that support the one carbon cycle, bearing in mind the high level of trafficking that surrounds this molecule in the early embryo. Similarly, media should be supplemented with precursors of glutathione such as cysteine (another 'essential amino acid'), a glutathione does not pass through the cell membrane. Zinc is also essential as it stabilizes DNA, and prevents oxidative stress by capturing superoxide and hydroxyl radicals through its involvement in metallothioneins and zinc superoxide dismutase (present in oocytes and early embryos); it is also a strong catalytic contributor to the 1-C cycle.

Oxidative stree is clearly far from marginal in reproduction, and represents an important burden, potentially leading to recurrent spontaneous abortions (Simşek et al., 1998) and pre-eclampsia (Burton and Jauniaux, 2004). It is tempting to reduce the negative charge by treating patients pre-conception as well as before and during assisted reproductino techniques with 'antioxidant' supplements that contain several vitamins (A, E, C) and minerals such as selenium. This practice is potentially dangerous (Bjelakovic et al., 2007; Brack et al., 2016) inefficient (Ménézo et al., 2010), or both, for both men and women. Moreover, oxidative stress status differs according to gender (Brack et al., 2016). For example, although it has been established that 15% of the white people have a zinc deficiency, this is definitely not the case for selenium, whatever the population tested (Brack et al., 2013). New theories about Hyc recycling and methylation now have a solid scientific basis. The pathways involved may also influence a large number of pathologies, including vascular and kidney diseases and endothelial damage (Hoffman, 2011), as well as gametogenesis. Oxidative injury is exacerbated by Hcy. Moreover, Hcy allows optimal synthesis of hypotaurine and glutathione, two major effectors in protection against ROS, with no side-effects. With embryogenesis, of greatest concern is the weak capacity of oocytes to recycle Hcy and regenerate cysteine for glutathione synthesis. On this basis, supplementation with N-acetyl cysteine might be a logical strategy. In addition, although folic acid (Vitamin B9) is a recommended supplement immediately before and during the early stages of pregnancy, preliminary studies suggest that supplements should also contain B2, B6 and B12, which are necessary for methionine synthesis (Amar et al., 2015; Cornet et al., 2015). In view of the negative effect of the environment on gametogenesis and on epigenesis in general, supplementation that includes all of these should be recommended. Treating problems in reproduction requires a rigid scientific approach rather than the haphazard therapies that have often been applied to date. 'Optimization processes' in infertility must be handled with care, given our increasing knowledge about transgenerational health problems via epigenetic modifications, whether or not linked to 'modern' environmental conditions.

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