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## ► To cite this version:

Bernard Auroousseau, Dominique Gruffat, Denys Durand. Gestation linked radical oxygen species fluxes and vitamins and trace mineral deficiencies in the ruminant. *Reproduction Nutrition Development*, 2006, 46 (6), pp.601-620. 10.1051/rnd:2006045 . hal-00900649

**HAL Id: hal-00900649**

**<https://hal.science/hal-00900649>**

Submitted on 11 May 2020

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## Gestation linked radical oxygen species fluxes and vitamins and trace mineral deficiencies in the ruminant

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(Received 3 October 2005; accepted 18 September 2006)

**Abstract** – In mammals, radical oxygen species (ROS) are essential factors of cell replication, differentiation and growth (oxidative signal), notably during gestation, but are also potentially damaging agents. In Women, ROS play a role in remodeling of uterine tissues, implantation of the embryo, settlement of the villi and development of blood vessels characteristic of gestation. The body stores of vitamins and minerals of gestating females are used to keep ROS fluxes at a level corresponding to oxidative signals and to prevent an imbalance between their production and scavenging (oxidative stress), which would be detrimental to the mother and fetus. There is some evidence that, although based on different regulatory mechanisms, most of the effects of ROS reported in humans also occur in pregnant ruminant females, some of which have been actually reported. Many vitamins and trace elements have dual effects in the organism of mammals: (a) they are involved in the control of metabolic pathways or/and gene expression, (b) but most of the time they also display ROS trapping activity or their deficiencies induce high rates of ROS production. Deficiencies induce different disorders of gestation and can be induced by different kinds of stress. An example is given, corresponding to the decreased contents of cobalt of forages, when exposed to sustained heavy rains, so that the supply of vitamins B12 to the organism of the ruminant that grazes them is reduced and failure of gestation is induced. Outdoor exposure of ruminants to adverse climatic conditions by itself can increase the vitamin and trace element requirements. Adaptation of production systems taking into account these interactions between gestation and sources of stress or change of the quality of feeding stuffs as well as further developments of knowledge in that field is necessary to promote sustainable agricultural practices.

**gestation / ovine / bovine / vitamins / trace elements / antioxidant enzymes / radical oxygen species / radical phenomena**

### 1. INTRODUCTION

The present knowledge on the relationship between the physiological rate of ROS and healthy gestation as influenced by nutrition and the environment in ruminants is limited, so that farmers meet sometimes unexplained failure of reproduction.

The use of the more developed knowledge on physiological ROS production and the effects in pregnant women could help to understand and make the best of dispersed information on ROS production and effects in the course of gestation in ruminants. Different aspects are considered in this paper: ROS production, placental and fetal physiology, vitamin and micromineral element metabolism as well as the

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relationships between the environment and vitamin and mineral trace element intake, on the one hand, and outcome of gestation, on the other hand.

The thorough considerations of gestation in domestic animals that we develop address physiologic phenomena involved in oxidative signaling and risks of oxidative stress during gestation. Different sciences, i.e. physiology, veterinary and medical sciences, animal husbandry, agronomy and technology can participate in and benefit from new advances in this field. We already have given a functional review on these aspects [1].

## 2. OVERVIEW OF RADICAL PHENOMENA

Before handling our topic, it is necessary to help the reader with a general overview of ROS production and effects. A radical (reactive species) is formed when one atom of any molecule loses or gains one electron. Due to this unbalanced electronic configuration, radicals are extremely reactive with organic molecules. Two families of radicals, each including a large variety of species, can be distinguished: reactive oxygen species (ROS) and organic radicals. Organic radicals are large molecules that are very often integral parts of cell membranes. ROS originate in the organism from a large set of sources and their fate and their effects cover wide fields. Some very reactive radicals such as superoxide anion ( $O_2^{\bullet-}$ ) or hydroxyl radical ( $\bullet OH$ ) readily reacts with cell components only at the site of their production, while others such as hydrogen peroxide ( $H_2O_2$ ) or nitric oxide ( $\bullet NO$ ) can diffuse across membranes to appreciable distances where new radicals are induced [2, 3].

ROS can induce cascade propagation of oxidative reactions between molecules, followed by formation of new radical species and, eventually, an exponential am-

plification of rates of ROS production [4–8]. These chains of reactions are interrupted when the electronic disequilibrium of the radicals is corrected [9]. The correction of the disequilibrium is usually obtained by radical trapping by specialized components, specialized enzymes or non-specialized components or can also be obtained after condensation with another molecule to form a non reactive component [7, 10]. These different events following initial ROS formation are commonly referred to as radical chains [2]. We preferentially use the term radical phenomena, which refers more readily to an enlarged set of poorly unraveled phenomena: (a) a competitive equilibrium between ROS production and elimination by vitamins, specialized enzymes, and a large variety of unspecialized components, (b) oxidative alteration of the tissues of variable importance and (c) interacting loops between hormones, cytokines, growth factors, ROS fluxes and protein expression and activity.

The attack and alteration of cell constituents by ROS is the more commonly reported effects of radical phenomena and is considered as “oxidative stress”. Increasing rates of production of ROS relative to antioxidant defenses can induce increasing rates of oxidative alteration of lipids, proteins and DNA in the living organism, which can lead to pathologies or even achieve a non reversible state. For these deleterious aspects of ROS effects which are marginally related to our topic, we will direct readers to a small number of references only: two papers concerned with the alteration of proteins by ROS [6, 10] and two references more concerned with pathological effects of radical phenomena and with their effects on lipid peroxidation [2, 7].

The alterations of cell components obtained are:

- Lipid peroxidation and breakdown of fatty acid molecules and formation of lipid derived carbonyles (notably

malondialdehyde (MDA) and long chain fatty aldehydes), which are toxic when they reach high levels (notably through interaction with proteins) and fatty acid peroxides, which are non-radical components, but which, in interaction with free metal ions, can induce new radical chains.

– Protein peroxidation, namely accompanied by:

(a) Inactivation of sulfhydryles and modification of protein conformation, inactivation of varied enzymes and ion channels, formation of varied organic radicals.

(b) Formation of protein peroxides, which are non radical components and, like their fatty counterparts, can induce new radical chains.

(c) Formation of protein carbonyls when radical chains are interrupted. Protein carbonyls are non radical components which are devoid of further initiation of radical chains on their own. However, cells with high contents of these components display impaired metabolism and growth. They bear testimony of the intensity of biological oxidations.

(d) Scission of proteins into smaller parts or liberation of free amino acids.

(e) Alteration of some amino acids, formation of lipoprotein adducts (among which lipofuschins, non-radical components, bearing testimony of the intensity of radical phenomena, but which, nevertheless, increases the response to stress thereafter).

– Oxidative inactivation and mutation of DNA.

– Destruction of vitamins and other components involved in protecting cell components, etc.

The large interest casted on oxidative stress must not silence the fact that, as long as there is an equilibrium between the production and the elimination of ROS, they have for the most part a positive effect on the organism. This is because radical formation is, in the first place, an essential component of cell metabolism, taking part

in the catalysis of numerous enzymatic activities [2, 11] and in the fine control of cell metabolism through the control local redox potential [11, 12]. ROS can be induced, as second messenger, by cytokines, oncogenes, growth factors or hormones [11, 13–16]. ROS of external origin or resulting from some insult to the cells or from deviation of the usual metabolism of oxygen can mimic the effects of ROS induced as constitutive second messenger and can trigger activation of many transcription factors through the release of signaling proteins [11, 17, 18]. In this regard, T. Finkel [11] stated: “in many instances the production of reactive oxygen species is tightly regulated and their downstream targets exquisitely specific”.

From the above statements, it is clear that, in the cells, there is a continuous production and disposal of ROS, some of which are fully linked to cell signaling and have been referred to as “oxidative signals” [11, 18].

As long as the equilibrium between rate of production of ROS of different types and antioxidant defenses is not disrupted, one can consider the result as oxidative signaling, because some adaptations of gene expression and cell metabolism follow the challenge by increasing rates of ROS production. However, in this situation, some cell components (lipids, proteins, DNA) are steadily altered: there is also a mild oxidative stress. But, over a large range of rates of ROS production, the attack of the organism is fairly efficiently counteracted and DNA and membranes are repaired before consistent damages are inflicted to the cells [19–21].

Extensive knowledge on radical phenomena specifically associated to healthy gestation and embryogenesis has been obtained or inferred in humans or in laboratory animals [8, 22–24]. As in other physiological states, ROS can induce noxious effects and pathologies of gestation only when the equilibrium between the

production and the elimination of ROS is disrupted [5, 8, 22, 25]. In the fetus exposed to an episode of ischemia, alteration of DNA can increase, *in vivo*, by a factor from 12 to 128 [25]. Despite active repair activities [19], some of the damaged DNA is subject to permanent alterations which can induce cellular apoptosis or tissue necrosis [26, 27], fetal growth retardation [27], death of the embryo [28] or latent pathologies [29, 30], some of which break out when the organism faces new disequilibria between ROS production and elimination, notably during postnatal life.

### **3. ROS PRODUCTION IN GESTATING FEMALES**

#### **3.1. ROS production in the pregnant woman or gestating laboratory animal**

A large set of factors can induce high steady rates of ROS ( $\text{H}_2\text{O}_2$ ,  $\text{O}_2^{\bullet-}$  and  $\bullet\text{NO}$ ) from the time of ovulation up to delivery: high rates of estrogens [14, 31], cytokines, oncogenes and growth factor signaling [16], production by neutrophils [8, 32] or activation of membrane NADPH oxidases by contact between tissues from the embryo and mother [33]. High levels of  $\bullet\text{NO}$  are also observed in the circulation of pregnant women [5, 32] or gestating rats [27], notably produced by neutrophils together with  $\text{O}_2^{\bullet-}$ . ROS production by neutrophils during gestation do not bear any relationship to their usual function, adherence, chemotaxis or phagocytosis [32]. The interaction of the two ROS is one of the sources of  $\bullet\text{OH}$  and of peroxynitrite ( $\text{ONOO}^-$ ) in the circulation of the gestating female [5, 27]. In the rat, high rates of  $\text{O}_2^{\bullet-}$  (decreasing steadily from gestational day 12 to reach a 60% lesser level on day 16), of  $\text{H}_2\text{O}_2$  (with an increase between days 12 and 14 and a 63% decrease between days 14 and 16) and of  $\bullet\text{OH}$  (doubling between days 12 and 16) have also been recorded in *ex vivo* fetal homogenates [25].

#### **3.2. ROS production in the gestating ruminant**

The placenta in women or in murines are hemochorial while that observed in the ruminants is epitheliochorial, with some conjunctivochorial loci [34]. Despite these different types of placentation, several observations are in favor of some similarities between ROS phenomena in women and rats or in ruminants (cow, sheep). To figure what could happen at the beginning of gestation, we can consider the fact that, in the ewe or in the bovine, the blastocyst loses its zona pellucida respectively 7 or 10 days before implantation [34], whereas this phenomenon occurs at the moment of implantation in women. Therefore, an extended period of high rates of cell to cell contacts and triggering of NADPH oxidases and ROS fluxes is likely to occur in the ruminant. Moreover,  $\bullet\text{NO}$  radical production by peripheral bovine lymphocytes has been reported to be steadily increased by a factor of 3 between the non pregnant-state and the 7th day after conception and by a further factor of 1.5 between day 7 and days 90–120 of gestation [35]. A high expression of nitric oxide synthetase has also been reported in the endometrium and myometrium of the gestating ewe, up to the 140th day of gestation [36]. These observations infer that radical phenomena of physiological origin, although very likely subject to different mechanisms of control and different equilibria, are likely to display an equivalent intensity in the gestating ruminant as in monogastric species.

### **4. ORIGIN AND INTEREST OF ROS PRODUCTION DURING GESTATION**

#### **4.1. Origin of ROS production during gestation**

The usefulness of ROS in gestating rats has been brought into evidence [23, 31].

We have already listed several sources of ROS formation in the placenta and fetus in Section 3.1. More sources of ROS have been described, such as the triggering of radical phenomena by changes in oxygen partial pressures [22,24,37,38] or synthesis of hormones [5]. ROS can also be formed after electron escape from the mitochondria of proliferating cells, favored by high rates of multiplication and metabolism and increased activity of mitochondrial superoxide dismutase (Mn-SOD) [39, 40] or, in human placental mitochondria, after interaction between NADPH and iron [41]. Other sources of ROS are increased activities of NADPH oxidases by contact between tissues from the embryo and mother or between replicating cells [33], interactions between estrogens and minerals [42] or triggering of NADPH peroxidases associated to the membranes inside the cells [43].

Rates of  $\bullet\text{OH}$  fluxes deserve consideration since they have been reported to increase in the 16 day old rat fetus, while  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$  fluxes subside [25]. To explain these high  $\bullet\text{OH}$  concentrations in placental and fetal tissues observed in the rat and expected in the other species (actually reported in pathological states),  $\text{H}_2\text{O}_2$  could diffuse across the membranes and react with redox minerals (mainly Fe) in the cytoplasm [44], at the level of the nuclear membrane [43] or within the nucleus [45]. Direct formation of  $\text{O}_2^{\bullet-}$  production at the level of the nuclear membrane through an NAD(P)H dependent mechanism is possibly of major importance and can end in the interaction of this radical with iron to produce  $\bullet\text{OH}$  [43]. More means of  $\bullet\text{OH}$  formation can be recruited, such as interactions between  $\text{H}_2\text{O}_2$  or  $\text{O}_2^{\bullet-}$  and iron [17] or interactions between various peroxides of proteins, lipids or catechols and redox minerals [2, 10, 42, 46]. In those instances, high rates of storage of iron occur in the placenta and in the fetus observed in various species including the bovine, starting

from the early stages of pregnancy [47] and can be released through many different mechanisms [48]. The  $\bullet\text{OH}$  radical seems essential in several pathways of signal transduction, although it also induces risks of severe oxidative stress [42, 43, 49].

#### 4.2. Oxygen partial pressure as a factor of ROS production during gestation

Environmental oxygen partial pressure of living organisms is one of the basis of formation of ROS, notably at the level of placental tissue [22, 24, 37, 38]. It has been reported that oxygen partial pressure displays positive effects on embryo development only within a narrow range and that an oxidative stress can occur, with possibly potential lethal effects, below (slowing down of ATP synthesis) or above this range (direct peroxidation of membranes due to an excess of oxygen and radical chain initiation) [37]. In vitro, deviation of the oxygen partial pressures from the normal can induce oxidative stress and end embryo-toxicity if antioxidant enzymes (AOE) or antioxidant components are not provided [28, 50, 51]. During in utero embryo development, embryopathies occur, apparently due to oxygen induced ROS production, but in less well characterized circumstances [28, 50, 52].

A good knowledge of variations of oxygen partial pressure in different parts of the reproductive tract and conceptus has been obtained in the monogastric, including the woman. Before implantation, the oxygen partial pressure in the lumen of the uterus settles between 20 and 37 mm Hg, regardless of the species of mammals considered [53, 54]. However, from in vitro studies, it appears that, inside the cells of the embryo, the oxygen partial pressure is 10 to 5 times lower than outside the cells, so that the induced rate of ROS production is lower than that expected from



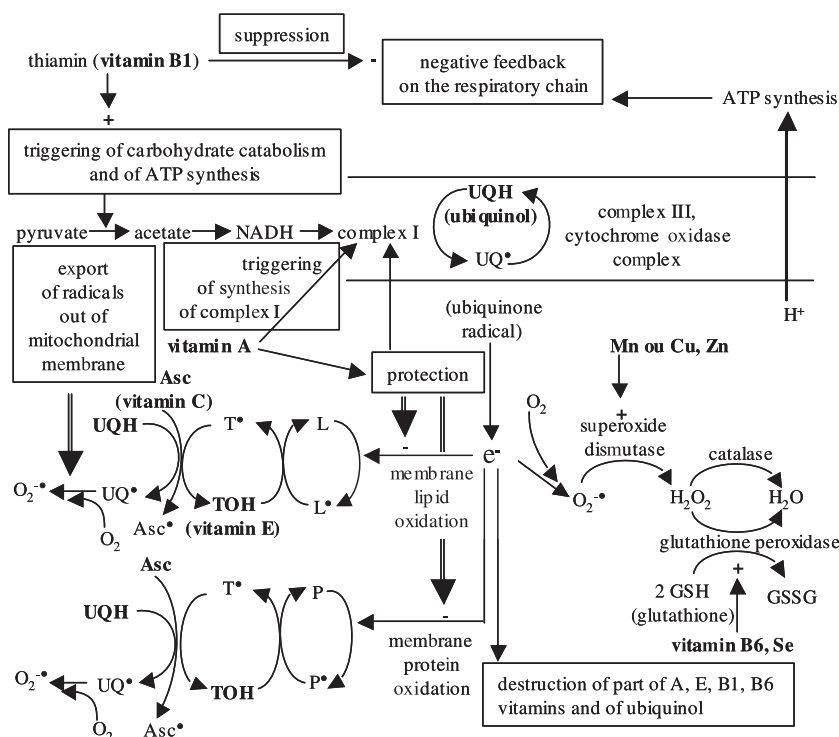
the external oxygen partial pressure [50]. At implantation, oxygen partial pressure at the level of the cytotrophoblasts is thus of the order of 14 mm Hg, which corresponds to a level of 2% of oxygen in the atmosphere, i.e. a condition of hypoxia which is likely to trigger cell multiplication and inhibit differentiation [38, 52]. In the woman, invasion of spiral arteries by incompletely differentiated trophoblasts with slow motility occludes the tip of maternal vessels and limits maternal blood flow into the placenta [22, 38]. Inefficient invasion of the spiral arteries can induce an unresolved placental oxidative stress and lead to pre-eclampsia [5, 8, 22]. Due to the occlusion of the maternal vessels by trophoblasts, the initial gradient of oxygen partial pressure between the mother (where it amounts to around 50–100 mm Hg) and the embryo (where it amounts to around 14 mm Hg) is maintained after the 8th week of gestation and evolves slowly to 39.6 mm Hg at a gestational age of 10 weeks [38, 52, 54], the complete transformation of the spiral arteries being achieved only between weeks 18 and 20 of gestation [55]. In the conceptus, the low initial oxygen partial pressure evolves only slowly between the 10th and 16th week of pregnancy from 64.3 to 73.2 mm Hg at the level of decidua, from 25.6 to 56.2 mm Hg at the level of the placenta, and, remains unchanged during this interval at the level of the coelomic fluid, i.e. between 20.0 and 18.2 mm Hg [54]. The initial preservation of relatively low oxygen partial pressure at the level of the placenta and fetus in the human caused by the occlusion of the maternal vessels is matched in the ruminant by the occurrence of several membranes between the blood of the dam and of the fetus, which also preserve a relatively low oxygen partial pressure at the level of the placenta and fetus, so that the patterns of ROS induced by oxygen in the fetuses of the two species are likely to share similar intensities.

#### 4.3. ROS formation in the mitochondria of placental and fetal tissues

At the different stages of pregnancy a high energy metabolic rate is observed [56], but is automatically followed by a high flux of ROS, originating notably from the mitochondria [25, 39, 40]. Several features specific to embryogenesis increase mitochondrial production of  $O_2^{\bullet-}$  and  $H_2O_2$ , since, as opposed to the situation in the adult, an initial imbalance between enzymatic production of electrons (NAD dehydrogenase and succinate deshydrogenase), on the one hand, and the ability of the respiratory chain to use them for ATP synthesis (cytochrome oxidase), on the other hand, are seen in the fetus [37].

More mechanisms are involved in the control of ROS production at the level of the mitochondria (Fig. 1) and are of major importance for the positive outcome of gestation. In this respect, vitamin B1 plays a major role and its deficiency is lethal during the early part of gestation [57]. This vitamin affords some protection to the early embryo both because it prevents an extensive use of electrons for ROS production after releasing the feedback of ATP on its synthesis [58] and because it traps ROS efficiently [59].

In women, vitamin A deficiency during specific states of pregnancy is lethal and a deficiency in the control of the development of different organs linked to a lack of expression of some genes has been described [29]. However, vitamin A deficiency is also followed, in rats, by an increase of malondialdehyde (a marker of peroxidation of lipids) and of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (a marker of the peroxidation of DNA) and by decreases of the mitochondrial GSH content and GSH/GSSG ratio [60]. Vitamin A can detoxify organic peroxides *in vitro* [61], but is extensively catabolized in the process. This suggests that ROS trapping could be the origin of the momentary



**Figure 1.** Control of radical phenomena originating from mitochondria (L = lipids, L• = lipid radical, P• = protein radical, UQ• = ubiquinone radical, T• = tocopheryl radical, Asc• = ascorbyl radical).

decrease of vitamin A stores in the rat fetus between days 10-14, at the time of induction of organ differentiation [62] and of the synergic effect with vitamin E in ROS trapping [21]. Vitamin A also negatively regulates the TNF- $\alpha$  pathway and affords protection of the placenta against ROS by that mean [63].

In vitro, supplements of vitamin E have also been reported to protect the integrity of the human placental mitochondria through a dose dependent inhibition of lipid peroxidation [41]. In this study, a synergy was observed between the antioxidant potential of vitamins E and C, independently of the  $\text{Fe}^{3+}/\text{Fe}^{2+}$  ratio, and was thought to be related to recycling of vitamin E by vitamin C. In vivo sparing

of vitamin E by vitamin C, GSH, lipoic acid or ubiquinol has been confirmed in laboratory animals, involving or not enzymatic activities for the recycling of the tocopheroxy radical [64].

In the mouse, a mutation replacing ubiquinone, UQ, the cofactor of the mitochondrial respiratory chain, by demethoxyubiquinone has been shown to reduce by 35% the mitochondrial oxygen consumption and to induce an arrest of the development of the embryo [65]. Moreover, in cell cultures, targeting a ubiquinone derivative to the mitochondria inhibited lipid peroxidation of the organelle's membrane as well as apoptosis [66], while endogenous ubiquinol also protects both lipids and proteins of beef heart



sub-mitochondrial particles against oxidation [46].

In rat embryo culture, embryopathy can be induced by a high concentration of oxygen (20%) which can increase the rate of  $H_2O_2$  formation (+180%) and decrease the GSH content in the cells [28]. Two enzymes associated with ROS trapping by GSH, glutathione peroxidase (Gpx) and glutathione reductase (Gred), which could help to maintain the GSH content of the cells, are respectively dependent on vitamin B6 [67] and B2 [68]. The two vitamins undergo extensive oxidative degradation after ROS trapping [69, 70] and the depletion of the mitochondrial content of the 2 vitamins, can aggravate the detrimental effect of high rates of ROS production. Another enzyme of GSH metabolism, phospholipid hydroperoxide glutathione peroxidase (PHGpx or Gpx-4) is essential to avoid the death of the embryo [71]. Mitochondrial PHGpx is essential to inhibit the peroxidation of mitochondrial phospholipids and the release of apoptotic factors from the mitochondria [72]. PHGpx has been shown to display a synergic effect with vitamin E on the protection of PUFA: it eliminates the lipid hydroperoxides formed from the reaction of lipid hydroperoxyl radicals with the vitamin before any reinitiation of peroxidation [73]. Two other AOE, namely Mn-SOD [74] and catalase (CAT) [40] are also important to prevent detrimental increases of rates of ROS production in the fetal mitochondria.

## 5. INTEREST OF ROS PHENOMENA IN THE GESTATING MAMMAL

### 5.1. Interest of ROS phenomena in the gestating mammal: implantation

As reported in the previous section, the exposure of the blastocyst or the embryo to oxygen induces risks of oxidative stress and of gestation failure or pathologies.

However, our topic is centered on normal gestation where high physiological rates of ROS production have been reported in the uterine wall (Section 3). In the rabbit, after a contact is established between the cells of the blastocyst and of the uterus, an extensive membrane lipid peroxidation occurs as a result of ROS exposition, and phospholipase A2 is triggered, notably by lipoperoxides, and leads to an intensive degradation of membrane phospholipids [75]. In the human, high rates of thiobarbituric acid reactive substances (TBARS) have also been reported and bare testimony of high rates of lipid peroxidation during the first term of healthy gestation, both at the level of the placenta or in the fetal liver [76]. The precise time scale of events is not known, but the high physiological rates of ROS production and related responses (notably lipid peroxidation) are very likely to induce uterus oedema and to increase degradation of the extracellular matrix, fluidity of the membranes and fusion between cells from the outer blastocyst layer (at the syndesmochorial loci) or between the cells from the surface of the endometrium and of the fetus [23, 52]. They are also thought to facilitate the formation of the decidua, the remodeling of placental tissues and the development of specific blood vessels, as well as trophoblast invasion [38, 52]. The effects of the vascular endothelium growth factor (VEGF) on permeability and angiogenesis were established a long time ago: after further investigation, it appears that VEGF induces ROS formation as a second messenger to induce a cascade of reactions leading to the final reported effect [11]. ROS also increase membrane permeability, which can allow ameliorated import of nutrients (at least into the placenta, through non specific increase of permeability) as well as export of altered components from the cells [8, 52].

An apparented type of event has been described in the bovine, with trophoblast giant cells, migration into the endometrium

and fusion with uterine epithelial cells [77]. Keeping in mind that the peculiarities of each species do not preclude a large common set of metabolic events leading to ROS production in ruminant animals, women or rodents, the numerous results obtained in the monogastric species give us some general clues on the ROS flux origins and effects.

### **5.2. Interest of ROS phenomena in the gestating mammal: hyperplasia**

The regulation of cellular hyperplasia by ROS has been described *in vitro* on cultured cells [26, 40]. Cellular proliferation is slow when ROS levels are low and increases with increasing levels of ROS until achievement of a threshold, after which proliferation is blocked and replaced by differentiation, apoptosis or necrosis. In these studies, removal of ROS from the incubation medium is also able to decrease their positive (moderate ROS levels) or adverse effects (high ROS levels) on cellular proliferation.

### **5.3. Interest of ROS phenomena in the gestating mammal: differentiation**

Different examples of the induction of differentiation by hormones, cytokines, oncogenes or growth factors have been obtained, but further studies underline the absolute requirement of ROS signaling to obtain these effects [40, 78]. The precise circumstances and mechanisms and the cascade of regulatory factors involved in the differentiation of the embryo have not been fully elucidated and involve paradoxical issues, but a change in the redox potential in the cytoplasm, obtained mainly through increased oxidation of GSH, is usually observed at the time of gene induction [12, 79]. A so-called oxidative burst drastically increasing ROS fluxes has been

repeatedly observed at the time of the swing from hyperplasia to differentiation [11, 22, 80], notably in the embryo, being triggered by a brisk increase of oxygen partial pressure allowed by the disconnection of the trophoblastic plugs [22].

### **5.4. Gene signaling and cell motility at the time of implantation**

Up to the time of blastocyst implantation, the low oxygen partial pressure found at the level of the cytotrophoblasts corresponds to a level of 2% of oxygen. Under this low oxygen partial pressure, hypoxia triggers transcription and stabilization of hypoxia inducible factor-1, HIF-1 [81], apparently as a result of a low rate of  $H_2O_2$  released at the level of complex III of the respiratory chain [82]. HIF-1 is involved in placentation and slow migration of the cytotrophoblasts into the spiral arterioles, where they are progressively exposed to higher oxygen partial pressure [22, 38, 54]. Above a level of 40 mm Hg (6% oxygen),  $O_2^{\bullet -}$  and  $\bullet OH$  radicals are formed, HIF-1 is submitted to proteolysis [22, 38, 81] and the change in the equilibrium between the different oncogenes in invasive cytotrophoblasts induces them to fully differentiate, to acquire more motility, to invade the maternal blood vessels more deeply [38], to release the occlusion of the maternal arteries and to convert the maternal arteries into distended utero-placental ones which allow an increase of the blood flow to the conceptus [22, 38].

The functionality of the interrelations between other genes, such as AP-1, and ROS on the development of the placenta and their changes with other metabolic features are not fully solved and will not be dealt with here. As in the human, proteins of the *cfos/jun* family are expressed in the ovine trophoblasts and, prior to the implantation of the embryo: they are expressed, together with the ruminant

specific interferon- $\tau$  [83], at a time when extensive growth and remodeling of the blastocyst occur [84]. Along with the scarce data on ROS fluxes, the effects of cell to cell contact or cell fusion already discussed, this additional observation plays in favor of a large common set of features of gestation in ruminants and monogastrics.

### 5.5. Mn- and Cu/Zn-SOD induction and control

We will focus on a complete chain of events involved in some aspects of the regulation of differentiation and growth (Fig. 2) and which makes obvious the involvement of physiological, non detrimental, high ROS fluxes in these changes. A cascade of events thus starts from ROS production in the mitochondria, includes the TNF - NF- $\kappa$ B pathways and ends in the induction of Mn- and Cu/Zn-SOD, which confers adaptation of the cells and of the organism to sustained episodes of oxidative stress [12, 39, 85].

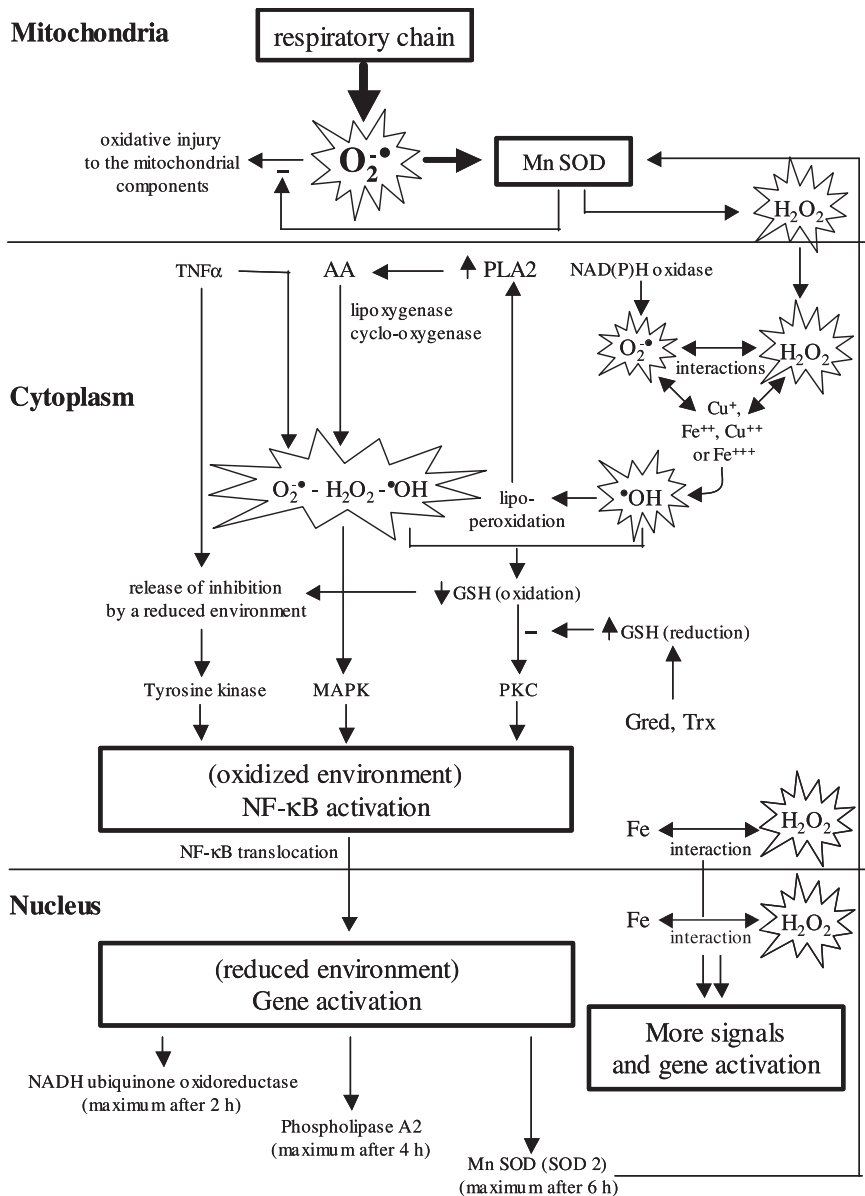
In the monogastric, after establishment of a fetal blood vessel network, gradients of oxygen partial pressure within the physiological range of 5-30 mm Hg, become steeper and more complex within the fetus [80]. Gradients of hormones and nutrients, notably retinoic acid [86], are also observed, but are out of the scope of our paper and have not been considered in this review.

Different factors of regulation are involved in the expression of Cu/Zn- and Mn-SOD, but the regulation of the expression of the 2 enzymes can be differentially modulated by ROS fluxes: sustained fluxes of ROS per se induce transcription of Cu/Zn-SOD, while a short exposure to ROS also induces, per se, transcription of Mn-SOD [87].

It is always difficult to sort out the early events and their chronology or to

sort out the controlling factor from the accompanying ones. However, at the time of appearance of the 2 antioxidative enzymes, relatively high rates of fluxes of  $O_2^{\bullet-}$ ,  $H_2O_2$  and  $\bullet OH$ , seemingly formed inside the cells, at the level of all sub-cellular organelles, have been reported in 12 to 16 day old rat embryos and display different timely patterns of changes [25]. Cu/Zn-SOD does appear earlier than Mn-SOD in the rat fetus [88], at a time when  $O_2^{\bullet-}$  fluxes are at their maximum [25]. Mn-SOD and mitochondria, where Mn-SOD is strictly located, follow parallel initial appearance, inhibition or loss [89,90]. Due to the development of Cu/Zn-SOD, the very active and deleterious  $O_2^{\bullet-}$  radical is dismutated into  $H_2O_2$  (40% decrease and 40% increase of their respective rates of production [25]).  $H_2O_2$  can then diffuse to the site of its action and is more readily a signaling molecule [7, 78]. The decrease of  $O_2^{\bullet-}$  concentrations in the cells can change the effects of this radical from an initial deleterious effect [89,90] into an oxidative signal [78]. Mitochondria remain functional and allow cell growth only when it is equipped with sufficient Mn-SOD to dismutate mitochondrial  $O_2^{\bullet-}$  into  $H_2O_2$  [39].

Thereafter, Mn-SOD expression and activity in differentiated cells appear to be controlled by a loop of events related to the equipment of the cells with mitochondria and Mn-SOD. As reported in Section 4.1 several means can explain the increase of  $\bullet OH$  fluxes reported in the rat fetus towards the 16th day of gestation [25]. After being formed, the  $\bullet OH$  radical seems to alter the redox status of GSH or sulfur-proteins at the site of its formation as a major mean of control of gene expression [12]. The hypothesis of a prominent role of  $H_2O_2$  diffusion out of the mitochondria or into the nucleus is suggested by the loss of effect of  $H_2O_2$  on gene expression after strong chelation of the redox minerals within the cells or by the absence of effect of  $\bullet OH$  when  $H_2O_2$  is reacted with free Fe added



**Figure 2.** Selected aspects of the involvement of radical phenomena in the differentiating embryo. ( $O_2^{\bullet-}$ : superoxide radical,  $H_2O_2$ : hydrogen peroxide,  $\bullet OH$ : hydroxyl radical, Mn-SOD: Mn superoxide dismutase, PLA2: phospholipase A2, AA: arachidonic acid, Gred: glutathione reductase, Trx: thioredoxin reductase).

to a cell homogenate [44, 45]. Suppression of ROS fluxes can suppress the expression of both Mn- and Cu/Zn-SOD [78]. Moreover, the coordination of the whole set of regulatory events and its relation to oxygen partial pressure can be altered by variation of the cellular contents of the  $\cdot\text{NO}$  radical, because this compound inhibits HIF-1 $\alpha$  stabilization under hypoxia [52] or its oxidation under normal oxygenation [91], or, again, triggers GSH synthesis to change the redox environment and the control of gene expression [15].

A large set of factors can be recruited by different means and are involved in the regulation of the expression of both enzymes and other proteins: TNF $\alpha$ , NF- $\kappa$ B, cytokines, protein kinases, AP-1 [12, 78, 85, 87, 92]. The mechanistic aspects of the regulation of gene expression by those factors are well known, but not the precise functionality aspects of these convergent or alternative pathways of control, with regards to the relation with ROS fluxes and to the time pattern of embryo differentiation and development.

### 5.6. Defense against ROS and the outcome of gestation

The placenta and fetus must be protected to avoid any lethal effect of the high rates of ROS production as already mentioned in Section 4.3. A limited survey of the literature points to evidence of dual roles of several components, mineral trace elements or vitamins, both as factor of control of the metabolism and as radical trapping agents.

GSH production and metabolism are of the utmost importance to prevent pathologies of gestation [5, 8, 28], both for the radical scavenging properties of GSH [12, 28] and for its role in the control of the redox status inside the cells and the control of gene expression [12, 79]. Another radical trapping component, ubiquinone, which is

also essential for energy metabolism [65] has also been reported to be essential for gestation outcome or viability of stem cells in the mouse.

Protection of cell structures is also afforded by a set of enzymes specialized in defense against ROS: Mn-SOD, Cu/Zn-SOD, CAT, Gpx, Gred and PHGpx have already been listed. An extracellular SOD (EC-SOD) [93], a ubiquitous thioredoxin [12, 20] and peroxiredoxin, an enzyme controlling ROS concentration at the level of the nuclear membrane [49], are also important in the same respect. The seven enzymes, the activities of which are dependent on mineral trace elements, are essential for the positive outcome of the reproduction both in monogastrics and in ruminants [29, 49, 50, 71, 94–96].

Different vitamin deficiencies have also been reported to impair the outcome of pregnancies or gestation, some of which seem clearly related to the detrimental ROS effect: vitamin E in women [29] or vitamin C in the sow [97]. In pre-eclampsia prone women, significant prevention of the pathology is obtained after supplementation with a mixture of vitamin E and C between the 16th and 22nd weeks of pregnancy [98]. Other studies do not include any explanation for gestation failure with vitamin A in women [29], vitamin B1 in the rat [57], vitamin B2 in women [99], vitamin B9 in women [100] or in the pig [101] or vitamin B12 in the ewe [88, 102]. Vitamin B2, which seems essential for the health of the fetus or adult [103], displays paradoxical ROS trapping activity [69] or, when added into cell culture medium, pro-oxidative activity [104]. These findings underline that we need to learn more on the mechanisms of vitamin action and dual potential. Conflicting observations are obtained on the role of vitamin B1 deficiency in gestation failure: secretion of pituitary hormones or estrogen do not seem to be impaired despite a loss of appetite in gestating rats [57], while this vitamin can be

recruited and activated in cells of the pituitary or blood vessel walls scavenging damaged cell components after an episode of ischemia [105] and is also an efficient ROS trapping component [59]. Increased rates of ROS fluxes have been reported in vitamin A, B2, B6, B9 or B12 deficiencies [60, 94, 106–108] which, together with the ROS trapping ability of these compounds [21, 60, 61, 69, 70, 109, 110], points to some relationship to ROS phenomena, in addition to their effect on the control of gene expression and metabolism [29, 65, 67, 68, 110–112].

Satisfaction of the requirements of the gestating female must be worked out in relation to long term effects of transitory deficiencies. This statement is based on a study where an episode of vitamin B12 deficiency in young ewe lambs displayed long term effects, only corrected by a long period of supplementation [76]. Ewes raised on marginally Co deficient pastures and receiving Co supplements continuously during two consecutive year seasons (calculated to meet gestation requirements), compared to ewes raised in the same environment and receiving the same level of Co supplements 5 months before the second breeding season and throughout gestation, displayed a higher numerical productivity (116 against 83 lambs for 100 ewes), and their lambs displayed significantly higher live-weight gains between 8 and 14 weeks of age (+38%) [76]. In this experiment, the marginally Co deficiency during grazing was induced by heavy rains observed during gestation, with a drop of about 40% of the Co content of grass, as previously reported by others [113]. Another study brought into evidence year to year variations of cobalt status in ewe lambs reared outdoors, with significant decreases of circulating levels (–33%) or hepatic stores (–25%) of vitamin B12 during periods of the years where increased rainfall occur and significant decrease of growth under a threshold level [114]. Thus

exogenous circumstances such as periods of heavy rain can decrease the satisfaction of the vitamin B12 requirements of the gestating females after decreasing the Co content of the grazed forage. The Co content of the conserved forage grown and harvested after periods of heavy rains can also be decreased under such circumstances. In addition, direct loss of vitamins and mineral trace elements from the organism of the animals can occur during exposure to an external stress (such as for example, a climatic stress), since MS-Co(II), an inactivated form of vitamin B12 produced by oxidation of MS-Co(I) in the course of enzymatic methionine synthesis, can be oxidized by ROS [110, 115]. Indeed, inactivation of vitamin B12 by •NO [115] has been reported and is involved in pernicious anemia in humans, after disturbing both methionine synthesis and carbon flow through the folate pathway [116].

A second level of the effects of vitamin deficiencies encountered in the course of pregnancy or gestation concerns the adverse effects on the health of the mother [96, 117] and on the development and viability of the new-born. Thus, the growth and resistance of the newborn can thus be lowered after a period of deficiency in one or several vitamins or trace mineral elements, i.e. vitamin B12 [102, 118], vitamin E [119], copper [120] in ovines or after a period of deficiency of selenium (Se) in bovines [121] or in ovines [122]. Some of these consequences are linked to the fact that newborn animals usually face a period of high demand immediately after birth, caused notably by a brisk change of the oxygen partial pressure at the level of lungs and other sources of ROS production. In this respect, a lack of folic acid could be involved in the poor vitality of some newborn lambs or calves, despite the fact that no folic acid deficiency has been reported in gestating ruminants. Folic acid requirements are usually thought to be easily met by synthesis in the rumen, where



it requires less Co than vitamin B12 to achieve a maximum rate of synthesis [123]. However, the rate of synthesis of folic acid could be reduced to a large extent when the ingested grass has lost a large part of its Co content under heavy rain, and, since this vitamin is a ROS trapping component [109], it could be further destroyed by ROS in ruminants exposed to stress. In young heifers, circulating folic acid level is relatively low and this vitamin is apparently more quickly disposed of than in older animals [124, 125], while supplements of this vitamin can significantly increase the rate of growth of young ruminants, suggesting that the requirements are very often not met at the time of birth [126]. Folic acid supplementation of gestating cows increases folic acid levels in the blood of newborn animals by 24% and folic acid levels in colostrum by 54% [127] and could be of relevance in preventing postnatal pathologies of their progeny. Similarly, the 3 week delay necessary between Se supplementation and the expected increase in Gpx activity does not allow the newborn deficient in Se to face ROS exposure, while an increase of the supply in utero (supplementation of the ewe before mating) provides the newborn lamb with the desired Gpx activity at birth [122]. Similarly, Gpx is increased or not at calving whether the cows receive supplemental Se during the last 7 weeks or 4 weeks of gestation [121]. This latency of the effects of supplementation could also be of relevance to earlier periods of gestation, when the protection of the maternal and fetal organisms are required and extensive oxidative destruction of the different AO components (including vitamins) or enzymes could deplete the cell contents below the threshold value necessary for their role in the control of specific gene induction.

Several situations have been reported where the pathologies induced by vitamin or mineral trace element deficiencies during fetal life or growth could not read-

ily be cured later. These long lasting effects have received little attention because they are very likely due to the interactions between several deficiencies. Thus, Cu deficiency during pregnancy has been shown to induce postnatal malfunctions [96]. In rodents, sub-optimal Zn or Cu nutrition during gestation also induces postnatal malfunctions as well as long lasting immunological and biochemical impairments in the adult [30]. Since part of the mechanisms of induction of the effects of deficiencies are very likely similar in the embryo or during postnatal life, an illustration of the interaction between several factors was obtained in young growing lambs [128]. In this study prevention of abnormality of bone marrow or other health defects were observed only in a group of lambs continuously fed a diet sufficient both in vitamins E and B12. After a 9 month period of either vitamin E or vitamin B12 deficiency, a period of 13 to 17 months on a sufficient diet failed to correct the induced health troubles. More evidence of such long lasting effects, have also been seen in the young child: the remanent effects of a vitamin B12 deficient diet between birth and 6 years of age were still obvious after an equilibrated regimen between the ages of 6 and 12 years [129].

## 6. CONCLUSIONS

The increasing interest for radical phenomena during the last 20 years has taught us that the supply of oxygen to the organisms of mammals, essential for energy metabolism, also raises some risks of damaging peroxidative insults and induced pathologies. During gestation and embryogenesis, the organism of the mother and embryo are the site of physiological high rates of ROS production, primarily involved in cell signaling and control of the development of the fetus: replication,

differentiation and maturation of the fetal cells and organs.

However, exaggerated rates of production of ROS can be involved in paradoxical effects and can lead to breakdown of vitamins and mineral trace-elements associated to enzymes specialized in the defense against ROS and can end in peroxidation of cell components and induction of pathologies and gestation failure.

Such episodes of exaggerated rates of production of ROS are induced after exposition of breeding females to different stresses, and seem to be more easily prevented by equilibrated provision of different vitamins or mineral trace elements than by single supplementation. Among the different types of stress, climatic stress has been reported to display major adverse effects on health and reproduction of grazing ruminant animals, directly by increasing production of ROS in the organism, or indirectly, because the quality of fresh or conserved forage can be lowered. Notably, a reduction in forage cobalt content has thus been reported under conditions of heavy sustained rain, leading to vitamin B12 deficiencies.

Large fields of precise knowledge on all those aspects are lacking. However, apart from the necessary veterinarian care, production systems could be adapted to try to anticipate the different sources of vitamin and mineral trace-element deperdition, notably the hazardous conditions of climatic changes, and the induction of reproduction failure due to deficiencies, decreased viability of the newborn or of subsequent growth, so as to promote sustainable agricultural practices.

## ACKNOWLEDGEMENTS

The authors want to acknowledge the thorough work of criticism of the reviewers who thus took a major part in the final field of this review.

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