Oxidative status along different stages of pregnancy in dairy cows

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SUMMARY

Introduction: The oxidant-antioxidant balance play a crucial role in the development of a stable pregnancy in dairy cows. The oxidant system is represented by the reactive oxygen species (ROS), which are indispensable molecules in different processes, like embryo implantation, uterine and cervical modification during the last phase of gestation. On the other hand, it’s fundamental to counterbalance oxidative stress, mostly in periods in which their levels can reach particularly high concentrations such as during early and late pregnancy. This task is prerogative of the antioxidant system, a complex of vitamins, enzymes and oligoelements, capable of transforming oxygen radicals into non-radical compounds. Between them, β-carotene stands out mainly for its antioxidant skills, but also for the important role played during pregnancy.
Aim: This study aims to evaluate serum β-carotene and Reactive Oxygen Species concentrations along different stages of pregnancy (from 30-60 days to 240-270 days), in Friesian dairy cows.
Materials and methods: 80 healthy, pregnant Friesian cows were enrolled in this study and, based on gestational age, the cows were divided into 8 groups of 10 animals each. Blood was sampled and the obtained serum was used to dose β-carotene and ROS, by means of photometric analytical systems.
Results and discussion: Results show that blood β-carotene concentrations undergo a progressive, slow reduction from 30-60 days of pregnancy, until 180-210 days, when they significantly decreased, whereas ROS levels increase, especially at 180-210 days. Both the reduction of β-carotene and the rise of ROS can be dangerous during pregnancy. Indeed, the potential risk of oxidative stress in the periparturient cow and in its embryo has been described.
Conclusion: Therefore, the results of this study support the growing evidence that an adequate β-carotene integration in the diet of the parturient cow should be recommended in order to curb oxidative stress both in the mother and the fetus.

KEY WORDS
β-carotene; oxidative stress; pregnancy; dairy cow.

INTRODUCTION

Pregnancy is characterized by high energy requirements and an increase in oxygen consumption by both placenta and fetus[1]. It is precisely in such period that an increase in Reactive Oxygen Species (ROS) generation occurs due to both maternal and fetal metabolism[2].
Physiological ROS levels play a crucial role during pregnancy, modulating uterine function, luteal development, embryogenesis, embryonic attachment to the endometrium and both fetal and placental development. On the other hand, high ROS levels may represent a potential risk factor for both maternal and fetal health[2,3].
In order to reduce ROS-related damage, living organisms have developed a complex mechanism of antioxidant defense consisting of enzymes (superoxide-dysmutase, catalase, glutathione-peroxidase), vitamins (A and E) and some oligoelements (Copper, Zinc, Selenium, Manganese)[4].

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Retinol (Vit A) has in particular been defined as “scavenger”, i.e. a molecule endowed with the capability of reacting with free radicals so as to give rise to chemically stable byproducts[5]. Domestic herbivores do not take Vit A as such at pasture or with feeding, since this vitamin is present in plants only in its precursor form, carotenoids. β-carotene is, among carotenoids, the one which most transforms into Vit A[6], via activation of some enzymes located in the gut mucosa[7].
Vit A is essential for many biological functions to occur, such as cell growth and differentiation, epithelial protection, immune competence, eye function, besides playing a protective role against free radicals, oxidizing them. It has in vitro been demonstrated that Vit A enhances cell-to-cell communication, stimulating the synthesis of connexin, and therefore of gap-junctions[8], which are important, in the uterus, for labor induction. Deficits in Vit A and/or β-carotene levels around parturition have been associated with a reduction in reproductive performances and with an increase in uterine inertia, calving paresis, mastitis, placental retention, pathological mammary edema and late restoration of full cyclicity after calving[9,10].
Bovine dietary ration should include a daily β-carotene intake of 0.18 mg/kg B.W. (76 UI/kg B.W. of Vit A)[11]. β-carotene lev-

els ranging between 200 and 300 µg/100 ml should be considered normal; sub-normal levels should range between 100 and 200 µg/100 ml, whereas levels ranging between 9 and 100 µg/100ml indicate a deficit in β-carotene. As to Vit A, physiological values range between 25 and 80 µg/100 ml, sub-normal values range between 7 and 12 µg/100 ml and values lower than 7 µg/100 ml indicate a deficit in Vit A content[12].

Oxidative stress occurs when ROS generation is not adequately counterbalanced by antioxidant activity[13,14] and it is among the main factors inducing pregnancy alterations[15-17].

Given these premises, this study aims at evaluating blood β-carotene and ROS concentrations during pregnancy in dairy cows.

MATERIAL AND METHODS

Declarations Ethical Guidelines Committee

This study was performed in accordance with the ethical guidelines of the animal welfare committee and with the statute of the 'Aldo Moro' University of Bari, concerning the use of client-owned animals under clinical investigation. Procedures with animals were performed following good veterinary practice for animal welfare according to national laws in force (EU Directive 2010/63/EU; Italian Legislative Decree 116/92). Informed owner consent was obtained.

Procedure

80 healthy, pregnant Friesian cows were enrolled in this study. The cows were between their first and third lactation and had a BCS ranging between 3 and 3.5. The cows were kept in a dairy farm in the South of Italy, with a consistency of 530 animals and a mean milk production of 30 kg/day. The farm has installed a cooling system combining low pressure misting and forced ventilation, along the feeding line. The cows were under a semi-intensive breeding system and were fed pasture and unifeed (13 kg of concentrate, 6 kg of graminaceous forage and 10 liters of water/cow/day). Dried cows were fed pasture, 1 kg of supplementary feed for dried cows, dry forage and water *ad libitum*.

All cows received basal diet, with vitamins and minerals supplements, formulated to meet their requirements in different phase of lactation.

A complete clinical exam (rectal palpation and ultrasonography, SonoSite MicroMaxx Bothell WA, USA with linear probe set at 7.5 MHz), was used to check the gestational age, in accordance with Hughes and Davies[18].

Based on gestational age (expressed in days), all the cows enrolled in this study were divided into 8 groups of 10 animals each:

- **30-60**
- **60-90**
- **90-120**
- **120-150**
- **150-180**
- **180-210**
- **210-240**
- **240-270**

A venous blood sample taken from the coccygeal vein into vacutainer serum refrigerated tubes was used to dose β-carotene and ROS. Blood samples were taken at the same time in the morning from cattle before feeding.

Soon after blood sampling, centrifugation was performed at 3000xg for 10 minutes at room temperature (Centrifuge, XC-2000) and serum was stored in eppendorf (1.5 mL) until analytical determination.

β-carotene was dosed with ICheck™ Carotene Photometer (BioAnalyt, GmbH, Germany). Specifically, 0.4 mL of serum were taken with a graduate syringe and were put into a vial containing a patented solvent mixture (iEx™ Carotene vial extraction). Soon after, the vial with the sample was vigorously shaken for 10 seconds and then placed on a horizontal plane to allow the solution to sediment for 5 minutes until phase separation. Afterwards, the vial was placed in a photometer. The results are expressed in mg/L and enable the β-carotene content to be defined as: insufficient (<1.5 mg/L, equivalent to 150 µg/dL), marginal (1.5-3.5 mg/L, equivalent to 150-350 µg/dL) or optimal (>3.5 mg/L, equivalent to 350 µg/dL), with a *linear range* of 0.15-25.0 mg/L.

ROS serum concentrations were obtained by means of a photometric analytical system (FREE®, Diacron, Parma, Italy). FREE® measures reactive oxygen metabolites (ROMs), a variety of free radicals characterized by an odd number of electrons around the external orbital of oxygen. ROMs react with a chromogen which, if correctly buffered, forms a colored compound that can be measured photometrically (maximum absorbance peak at 505 nm). Once the absorbance value is determined, the instrument automatically converts the data into the appropriate arbitrary Carr Unit (1 U.Carr. = 0.08 mg H_2O_2/100 mL).

Statistical analysis

All data obtained underwent statistical analysis using the program IBM SPSS Statistics 19, through one way ANOVA and post hoc least significant difference test. Statistical significance was set at $p< 0.05$.

RESULTS

Serum concentrations (Media± s.d.) of β-carotene and ROS found in the pregnant cows at different gestational ages are shown on Table 1.

Table 1 - β-carotene (µg/dL) and Reactive Oxygen Species (ROS) (U.Carr) concentrations (Media±s.d.) in the different gestational ages in the dairy cow.

GESTATIONAL AGE (days)	β-carotene (µg/dL)	ROS (U.Carr)
30-60	196.25±31.12 A	94.56±5.87 D
60-90	195.00±24.59 A	100.89±8.55
90-120	174.50±25.40 A	119.25±14.58
120-150	174.63±19.65 A	120.85±8.25
150-180	173.75±13.76 A	126.96±11.56
180-210	171.38±44.21 A	121.87±8.36
210-240	109.13±23.56 B	128.59±10.23 E
240-270	107.88±12.28 B	147.25±15.26 E

The trend of β-carotene blood concentrations underwent a progressive, slow reduction throughout pregnancy duration, whereas ROS blood levels increased.

In particular, levels of blood β-carotene content remained stable from 30-60 days of pregnancy, until 180-210 days, when they significantly decreased. Conversely, blood ROS levels slightly increased from 30-60 days of pregnancy to 180-210 days, when they underwent a sharp increase until term. As to β-carotene, values were marginal, i.e. they ranged between 150 and 350 µg/dL, until 180-210 days of pregnancy; afterwards, their concentration declined to insufficient levels (<150 µg/dL).

DISCUSSION AND CONCLUSION

β-carotene plays a pivotal role in the delicate and complex phase of the beginning of gestation, in cows; this vitamin is necessary for protecting luteal parenchyma from oxidative damage and promoting luteal functional[16] as well as embryonic implantation and development[19].

Moreover β-carotene is crucial for maintaining adequate blood progesterone (P4) levels; this vitamin in fact enhances pituitary luteinizing hormone (LH) secretion, leading to an increase in gonadic P4 synthesis. Furthermore β-carotene protects the activity of the enzyme P450 side chain cleavage (*scc*), which, in turn, leads to the transformation of cholesterol into P4, in steroidogenic cells[20]. These are the reasons why β-carotene blood levels are required to be constantly high throughout pregnancy.

β-carotene blood content should also be kept constantly sustained in the last pregnancy period, i.e. close to term, since it has been demonstrated *in vitro* that carotenoids promote cell to cell communication, stimulating connexin synthesis and an increase in gap-junctions, necessary for uterine contraction to occur.

On the contrary, in this study, a sharp decrease in β-carotene blood levels occurred between 210 and 270 days of pregnancy. This decrease could be due both to the shift to the dry period and to a likely high β-carotene consumption in an attempt to counteract the oxidative stress known to occur at the feto-maternal interface as pregnancy goes on and the placenta gets older[1,21].

Pregnancy is known, in fact, to be characterized by sustained ROS generation, from its beginning, until term[16,17,21]. During pregnancy embryonic and fetal growth are associated with an increase in both placental and maternal metabolism which, in turn, leads to an increase in ROS generation, mainly deriving from an increase in lipid peroxidation, and, potentially, to oxidative stress[22].

At the beginning of gestation, when implantation occurs, the increase in reactive oxygen species is functional to embryonic development and growth, since ROS mediate embryonic physiological signaling pathways; furthermore ROS play a pivotal role in promoting neaoangiogenesis and apoptosis, thus enhancing the invasion of the maternal tissues, necessary for a physiological implantation to take place[23,24].

Notwithstanding these important functions, the first period of gestation is very critical, since the embryo may easily become prone to oxidative damage. It has in fact been shown that oxidative stress, if not properly counterbalanced, may impair gamete and embryo viability, leading to the arrest of embryo development to two cell stage[16] .

As pregnancy went on, maternal β-carotene and ROS blood concentrations showed an opposite trend, the former decreasing, the latter increasing, strengthening the hypothesis that the increase in ROS generation due to embryonic and fetal growth as well as to the accelerated placental metabolism might have been adequately kept under control by the reducing effect of the investigated antioxidant vitamin.

Starting from the period between 90 and 120 days of gestation, when β-carotene and ROS mean blood levels were 174.50±25.40 µg/dL and 119.25±14.58 U. Carr, respectively, a plateau phase was observed, lasting until 180-210 days of gestation, i.e. through mid-gestation, in which, only a progressive fetal growth occurs, without the occurrence of crucial events such as implantation or calving.

In the last phase of gestation, the highest ROS blood levels were found, compared to all the other phases investigated. This is an expectable event, in accordance with numerous research works, both in human and veterinary medicine, showing that in the last third of pregnancy an increase in reactive oxygen species occurs. This increase is mediated by a progressive increase in estrogen secretion by the maternal organism, which, in turn, promotes a massive arrival of leukocytes (mainly PMN and macrophages) in the utero-cervical tissue, as well as the release of an amount of pro-inflammatory cytokines, functional to trigger uterine contraction and cervical ripening and remodeling[25-29] .

In this study, the statistically significant increase in ROS found at term, might have depleted maternal blood β-carotene sources which, were found to be almost half compared to what had been observed at the beginning of pregnancy.

Many Authors described the potential risk of oxidative stress in the periparturient cow as being an excess of reactive oxygen species and, if not properly counterbalanced, a potential harmful factor contributing to the development of mastitis or cystic ovarian disease (COD)[16,26,29,30].

The results of this study support the growing evidence that an adequate β-carotene integration in the diet of the parturient cow should be recommended in order to curb the dangerous effect of uncontrolled ROS generation and oxidative stress both in the mother and the unborn.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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