Pre-print version of the article published in Animal (Cambridge Journals).
Volume 7. Issue 08. August 2013, pp 1374-1378.
http://dx.doi.org/10.1017/S1751731113000396

Oxidative Stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period

A. Abuelo*, J. Hernández, J.L. Benedito, C. Castillo.

Department of Animal Pathology. College of Veterinary Medicine. University of Santiago de Compostela. Campus Universitario s/n – 27002 Lugo (Spain).

*Corresponding author: Ángel Abuelo. E-mail: angel.abuelo@usc.es. Phone: +34 982822643; Fax +34 982822627

Pre-print version of the article published in Animal (Cambridge Journals).
Volume 7. Issue 08. August 2013, pp 1374-1378.
http://dx.doi.org/10.1017/S1751731113000396

Abstract

Oxidative stress plays a key role in the initiation or progression of numerous diseases and dairy cows undergo oxidative stress at the transition period. However, discrepancies between methodologies make it difficult to make comparisons between studies and therefore research on this topic may not be implemented in farms. This study aims to test under field conditions the use of an Oxidative Stress index (OSi) as a combined measurement through a ratio between pro- and antioxidants throughout the transition period in dairy farms. Serum samples of high yielding dairy cows were taken and markers of oxidative damage and antioxidant capacity were measured in 4 different production stages: (i) Late lactation (*LL*; -2 to -1 months); (ii) Prepartum (*PrP*; -1 month until parturition); (iii) Postpartum (*PsP*; delivery to +1 month) and (iv) Peak of Lactation (*PkL*; +1 to +2.5 months). Values were compared between production stages and against a metabolic baseline status (CTR, 4^{th} – 5^{th} month of gestation). Reporting for the first time in the literature values of the redox status for these cows with the lower metabolic demands available on high production commercial dairy herds. With the joint evaluation through the OSi differences were found, which were not present with the separate evaluation of pro- or antioxidants; supporting our hypothesis that the OSi indicates more accurately the oxidative status of the animals. It was also confirmed that dairy cows undergo oxidative stress after parturition and that antioxidant supplementation from one month before parturition until the peak of lactation may be needed to reduce the risk of OS.

Pre-print version of the article published in Animal (Cambridge Journals).
Volume 7. Issue 08. August 2013, pp 1374-1378.
http://dx.doi.org/10.1017/S1751731113000396

Keywords: Oxidative status; Peripartum period; Vitamin supplementation; Dairy cow; Antioxidants.

Implications

There is strong evidence that Oxidative stress (OS) plays a central role in the initiation, progression and maintenance of several pathologies. However, at present there is disagreement as to whether dairy cows suffer an oxidative challenge around parturition or not; in part due to the differences in the methodologies used. This study demonstrates that measurement of both pro- and anti-oxidants allows determination of an OS index that was utilized to quantify and demonstrate that the transition period is a time of increased oxidative stress in dairy cows. Moreover, the better timeframe for antioxidant supplementation, in terms of OS, was identified, in order to minimize the incidence of postpartum diseases.

Introduction

Oxidative Stress (OS) plays a key role in several pathological conditions connected with animal production, reproduction and welfare (Lykkesfeldt and Svendsen, 2007), and is attributable to an imbalance between oxidant and antioxidant substances in the body. OS can be particularly dangerous since no clinical symptoms are shown.

One of the most critical moments in dairy health, with consequences for production variables, is the transition period (Goff and Horst, 1997) when the capacity of antioxidant defenses is exceeded by the production of reactive oxygen substances (ROS). The influence of OS in ruminant health in this period is a relatively recent field of research but unfortunately, differences between

models and methodologies make it difficult to make meaningful comparisons (Celi, 2011) with practical conclusions.

Therefore it is necessary to provide useful OS markers that would help to analyze it with accuracy, in order to define protective nutritional strategies based on antioxidant supplementation. We consider that it is important to evaluate not only concentrations of oxidants and antioxidants separately, but also analyze their relationship through a proportion or ratio, because it is the imbalance between oxidants and the antioxidants that defines the concept of OS (Castillo *et al.*, 2005).

The first approach using this ratio was made in human medicine by Sharma *et al*. (1999). For dairy cattle, Celi (2011) in a review article proposed the use of the ratio pro-oxidants/antioxidants as an index indicative of an animal's risk to develop disease. Thus, an increase in the ratio indicates risk of OS due to increase in ROS production or defensive antioxidant consumption.

In line with these considerations, the approach we have used aims to test, under field conditions in a commercial dairy herd, the use of an Oxidative Stress index (OSi, based on the ratio between Reactive Oxygen Substances (ROS) and Serum Antioxidant Capacity (SAC), i.e. ROS/SAC), comparing the information given by this parameter with those given by ROS and SAC separately. Moreover, the usefulness of this index is evaluated in the most complex stage: around parturition (2 months prior parturition to 2.5 months after it) which includes the transition period.

Materials and Methods

This study involved the collection of serum samples at different stages of the transition from gestation to lactation in dairy cows in order to compare the

differences between these stages in terms of oxidative status. Permission for the procedures of the experiment was granted by the Bioethical Committee of the University of Santiago de Compostela (Spain)

Animals, nutrition and husbandry

The study was carried out on a commercial dairy herd located in Arzúa (Galicia, NW Spain), with an average 305 days normalized milk production of 10.235 kg/cow, and where economics play the main role in farm decisions. At this herd, we sampled fortnightly 25 cows from 2 months before the expected date of parturition until 2,5 months after it. The data obtained from 3 animals was discarded because one animal needed a caesarean section at calving, and another two of them developed clinical mastitis and left displacement of the abomasum respectively; therefore only data from cows (n=22) that had a normal calving and a healthy postpartum was included. Samplings took place between October and February, when the climate conditions (average (±s.d.) maximum temperature: 13.3ºC (±4.23); average (±s.d.) minimum temperature: 5.1ºC (±2.37) and average (±s.d.) relative humidity: 83.3% (±5.61)) are not supposed to increase the production of ROS due to heat stress (Bernabucci *et al.*, 2002).

As hitherto there are no published reference values for oxidative status biomarkers (Celi, 2011), it was necessary to establish a control group in order to have a baseline value to compare with those values obtained from transitional cows. This control group (CTR, n=40) was formed with animals between the 4th-5th month of pregnancy when neither lactation nor pregnancy were major metabolic burdens as described by Castillo *et al*. (2005).

During the study period all animals were kept under identical conditions. The diet for all cows consisted of a base ration fed as a daily total mixed ration (Table 1). Cows were dried off 60 days before the expected date of parturition and supplemented with a vitamin complex injection (see Table 1). Lactating cows were fed *ad libitum*, whereas dry cows were allowed only twice a day to feed, after every milking of lactating cows.

Blood samplings and groups

Blood samples were obtained by coccygeal venipuncture with evacuated tubes without anticoagulants, between 1800 and 1830 h. Tubes for serum collection were rapidly cooled on crushed ice and transported to the lab, where they were centrifuged at 2000×*g* for 20 min and the supernatant serum was frozen at -20ºC until analysis, within the first 3 months after collection.

In order to relate our results with the production stage of the transitional cows, the samplings of these animals were grouped ex post into four stages: (1) Late lactation (*LL*) from 2 to 1 months prior to parturition, (2) Prepartum (*PrP*) from -1 month until delivery, (3) Postpartum (*PsP*) from delivery to 1 month after parturition, and (4) Peak of Lactation (*PkL*) from +1 to +2,5 months after delivery. In each sampling, at least one control cow was also sampled, trying thereby to minimize any possible temporal effect.

Analytical determinations

ROS were assayed as described by Trotti *et al*. (2002) using the spectrophotometric d-ROM test (Diacron International, Italy), which determines hydroperoxides (breakdown products of lipids as well as of other organic substrate, generated by the oxidative attack of ROS) through their reaction with

the chromogen N,N-diethylparaphenylenediamine. Results are expressed in arbitrary "Carratelli Units" (CarrU), where 1 CarrU is equivalent to the oxidizing power of 0.08 mg H_2O_2/dL. Intra- and inter-assay CV were 3.22 and 8.19% respectively.

Serum Antioxidant Capacity (SAC) was estimated with the OXY-Adsorbent Test (Diacron International, Italy) (Trotti *et al.*, 2001). This test exploits the capacity of a massive solution of hypochlorous acid (HClO) to oxidise the complete pool of antioxidants in serum (albumin, bilirubin, uric acid, thiol groups, vitamins, glutathione, glutathione peroxidase, superoxide dismutase, catalase, etc.). Thus SAC considers the cumulative action of all the antioxidants present in serum, rather than the simply sum of measurable antioxidants. Results are expressed as µmol HClO/mL. Intra- and inter-assay CV were 2.85 and 5.10% respectively.

Both variables were used previously in bovine studies and managed according to manufacturer's instructions. The Oxidative Stress index (OSi) was calculated as ROS/SAC; expressed as CarrU/(µmol HClO/mL). Both ROS and SAC were measured on an UV/VIS absorption spectrophotometer (Clima MC-15; RAL Técnica para el Laboratorio.).

Statistical procedure

Data for each parameter was checked for normal distribution with the Kolgomorov-Sminorv test. A repeated measurements ANOVA was used to compare means among the different stages of the transition period, with the individual cows as experimental unit. Following analysis of variance, significant inter-group differences were detected by Bonferroni test. To compare the means of each transitional stage with the mean of the CTR group the Student-*t*

test was used. The criterion for statistical significance was established at $P<0.05$. All statistical procedures were performed with the IBM SPSS v19.0 for Windows software package.

Results

Table 2 shows the results of the oxidative status markers in all the studied stages. Values in CTR-cows can be considered as the baseline values for dairy cows under field conditions, taking into account that they were obtained in animals with the theoretically lowest metabolic burdens than can be achieved in lactating cows in a commercial dairy farm. Thus, the values obtained at the transitional stages will be referred to CTR values.

Mean ROS values did not differ significantly between control and any stage of the transition period. ROS progressively increased from *LL* to *PsP* with a slight decrease in the next stage. Although values in *PkL* were higher than prior parturition, they didn't achieve statistical significance.

Similarly, mean OXY values did not statistically differ between CTR and transitional cows at any stage; nonetheless it can be noted that there was an increase in the antioxidant barrier in *PrP*, with a subsequent decrease after delivery, reaching the lowest activities at *PkL*.

Despite the lack of statistical significance in the differences among transitional stages and with the CTR group in either ROS or SAC separately, the evaluation of the oxidative status of the animals with the OSi found a significant difference between the means of OSi in lactating vs. dried animals. However, the values of CTR cows only differed significantly with the animals at *PkL*. It is noted that the values in dried animals were lower than in CTR ones; which were considered to be the baseline levels.

Pre-print version of the article published in Animal (Cambridge Journals).
Volume 7. Issue 08. August 2013, pp 1374-1378.
http://dx.doi.org/10.1017/S1751731113000396

Discussion

This experiment studied the differences between the separate and the joint evaluation of pro- and antioxidants at blood level throughout the transition period of dairy cows. Serum samples were taken at different stages of the transition from gestation to lactation and compared among them and a control group.

Pro-oxidants

Changes in ROS during the study were in accordance with previous reports that showed an increase in oxidant species after parturition, attributable to the metabolic challenges associated to this stage (Bernabucci *et al.*, 2005; Dobbelaar *et al.*, 2010). Furthermore, during lactation, energy partitioning associated to milk production contributes to maintain a metabolic stress, favoring high ROS production (Castillo *et al.*, 2006).

Antioxidants

Unlike other studies (Sharma *et al.*, 1999) that used the Biological Antioxidant Potential (BAP) as an estimation of antioxidant capacity, we decided to estimate the SAC by the plasma barrier to oxidation (OXY-Adsorbent test). This was because in addition to the “scavengers” antioxidants (those determined by BAP), this test can also measure the so called “shock adsorbers”, i.e. all the antioxidants not active from the chemical point of view but able to “plug” the oxidant action of reactive oxygen substances. This provides information on the structural component of the antioxidant barrier of which the period of recovery is relatively slow, in comparison with antioxidants of lower weight, or at least a more rapid turnover, included in the BAP

203 determination. Therefore we are measuring the cumulative capacity of the
204 antioxidant defense against a particular oxidant aggression (how the animal has
205 been accumulating and disposing its antioxidants reserves to specific,
206 predictable and expected situations such as delivery and early lactation) rather
207 than measure the short-term antioxidants at the time of sampling.

208 The prepartum period is characterized by a depleted antioxidant status
209 and, consequently, OS (Bernabucci *et al.*, 2002 and 2005), and therefore these
210 cows were supplemented with a vitamin complex before parturition, which is a
211 recommended practice to minimize the risk of postpartum diseases (Politis,
212 2012). This fact prevents us from getting the clear picture of the natural cycle of
213 OSi; but, on the other hand, shows the oxidative status that might be observed
214 in dairy cows in commercial farms. Under these conditions, the slightly increase
215 in SAC values at *PrP* can be considered as result of the preventive vitamin
216 complex administration (Dobbelaar *et al.*, 2010). The small drop at *PsP* is not
217 only the consequence of the utilization of antioxidants in colostrum production
218 (Goff and Horst, 1997), but also the consequence of antioxidant consumption, in
219 an attempt to cope with the metabolic production of oxidants. As lactation
220 progresses, antioxidants continue to decline due to the depletion of fat-soluble
221 antioxidants by milk in combination with their consumption by endogenous ROS
222 production.(Castillo *et al.*, 2005 and 2006).

223 *Oxidative Stress index*

224 Currently there it is not agreement on whether dairy cows undergo OS
225 during the transition period or not. Previous studies suggested that dairy cows
226 experience OS during the peri-partum period (Bernabucci *et al.*, 2005, Castillo
227 *et al.*, 2005). However, in contrast with these studies some authors do not

report an oxidative challenge in the periparturient period (Wullepit *et al.*, 2009, Dobbelaar *et al.*, 2010).

While with a separate evaluation of our results of ROS and SAC, apparently these cows didn't undergo OS, since no significant difference was found between the studied stages neither for ROS nor for SAC; when the oxidative status is studied by a combined evaluation of pro- and antioxidants with the OSi, statistical differences between the foregoing and subsequent parturition stages were found, suggesting that, in fact, these cows experienced an oxidative challenge after parturition. This finding suggests that it may be a better practice to evaluate jointly both oxidants and antioxidants rather than separately, since OS could be either a consequence of an excessive production of ROS production and/or a decrease in the body antioxidant defense and therefore these parameters are strictly interdependent.

This agrees with a study in human medicine, in which the relationship between the level of OS and pathology was higher when oxidants and antioxidant defense measurements were combined as a ratio (Sharma *et al.*, 1999). However, care must be taken when interpreting these results, taking into account the higher variance associated with the base measurements. Marked individual variations were already reported for other oxidative status biomarkers in periparturient dairy cows, with many factors influencing it (Castillo *et al.*, 2005 and 2006).

Of particular interest is the observation that in peak lactation, when theoretically the cow is metabolically adapted to milk production (Castillo *et al.*, 2006), there is the maximum risk for OS, with values of the OSi significantly higher than CTR cows. This reason can be attributed not only to the slight

253 decrease in ROS, as the results of a lesser metabolic burden, but also to the
254 larger decrease in SAC. For these reasons, and although no clinical symptoms
255 of disease were observed in the studied animals, these findings suggest us the
256 convenience of extending antioxidant supplementation from the dry period until
257 peak of lactation.

258 **Conclusions**

259 Under the conditions of this study, the Oxidative Stress index (OSi)
260 provides an objective assessment of the relationship between oxidants and
261 antioxidants, not seen by the determination of both components separately.

262 In addition, baseline levels of oxidative status biomarkers under field
263 conditions for commercial high yielding dairy cows are reported, which will bring
264 a step forward their applicability in farms. It was also found that dairy cattle
265 show an increase in the levels of oxidative stress after parturition, and hence to
266 develop preventive actions that would minimize the effects of production
267 diseases after parturition, further studies should study the effects of antioxidant
268 supplementation from one month prior parturition until the peak of lactation.

269 **Acknowledgments**

270 This study was supported by the Xunta de Galicia (Spain)
271 (10MRU261004PR). The funding source played no role in the design of study,
272 collection, analysis and interpretation of data, or preparation or approval of the
273 manuscript. A. Abuelo is a FPU fellowship holder (Ref. AP2010-0013) from the
274 Spanish Ministry of Education. The authors thank Lucía Casanova Iglesias for
275 her expert technical assistance.

Preliminary data of this study was presented as an oral communication at the 6th European Congress of Bovine Health Management, held in Liège (Belgium) in September 2011.

References

Bernabucci U, Ronchi B, Lacetera N and Nardone A 2002. Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. Journal of Dairy Science 85, 2173-2179.

Bernabucci U, Ronchi B, Lacetera N and Nardone A 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. Journal of Dairy Science 88, 2017-2026.

Castillo C, Hernández J, Bravo A, López-Alonso M, Pereira V and Benedito JL 2005. Oxidative status during late pregnancy and early lactation in dairy cows. The Veterinary Journal 169, 286-292.

Castillo C, Hernández J, Valverde I, Pereira V, Sotillo J, López Alonso M and Benedito JL 2006. Plasma malonaldehyde (MDA) and total antioxidant status (TAS) during lactation in dairy cows. Research in Veterinary Science 80, 133-139.

Celi P 2011. Biomarkers of oxidative stress in ruminant medicine. Immunopharmacology and Immunotoxicology 33, 233-240.

Dobbelaar P, Bouwstra RJ, Goselink RMA, Jorritsma R, van den Borne JJGC and Jansen EHJM 2010. Effects of vitamin E supplementation on and the association of body condition score with changes in peroxidative biomarkers and antioxidants around calving in dairy heifers. Journal of Dairy Science 93, 3103-3113.

Goff JP and Horst RL 1997. Physiological changes at parturition and their relationship to metabolic disorders. Journal of Dairy Science 80, 1260-1268.

Lykkesfeldt J and Svendsen O 2007. Oxidants and antioxidants in disease: Oxidative stress in farm animals. The Veterinary Journal 173, 502-511.

Politis I 2012. Reevaluation of vitamin E supplementation of dairy cows: bioavailability, animal health and milk quality. Animal 6, 1427-1434.

Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ and Agarwal A 1999. The reactive oxygen species vs. total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Human Reproduction 14, 2801-2807.

Trotti R, Carratelli M, Barbieri M, Micieli G, Bosone D, Rondanelli M and Bo P 2001. Oxidative stress and a thrombophilic condition in alcoholics without severe liver disease. Haematologica 86, 85-91.

Wullepit N, Raes K, Beerda B, Veerkamp RF, Fremaut D and De Smet S 2009. Influence of management and genetic merit for milk yield on the oxidative status of plasma in heifers. Livestock Science 123, 276-282.

Pre-print version of the article published in Animal (Cambridge Journals).
Volume 7. Issue 08. August 2013, pp 1374-1378.
http://dx.doi.org/10.1017/S1751731113000396

Table 1

Ingredients and chemical composition of the diet supplied in the present study

Diet composition *(kg DM/cow per day)* [†] [‡]	
Total dry matter offered	21.7
Corn silage	5.1
Grass silage	4.8
Concentrate[∂]	11.6
Vitamin/mineral premix[θ]	0.2
Nutrient analysis	
Dry matter (%)	47.3
Crude protein (% DM)	17.8
Neutral detergent fibre (% DM)	30.6
Acid detergent fibre (% DM)	16.4
Starch (% DM)	31.2
Ether extract content (% DM)	4.4
Ashes (% DM)	7.3
PDIE (g/kg DM)	133.5
PDIN (g/kg DM)	130.9
Milk fodder units (UFL/kg DM)	0.94

DM: dry matter; PDIE: protein supplied when energy is limited in the rumen; PDIN: protein supplied when nitrogen is limited in the rumen. UFL: 'Unité Fouragère Lait'. UFL is the net energy for lactation equivalent to 1 kg standard air-dried barley.'

[†] The diet was fed as a total mixed ration. Lactating cows were fed *ad libitum*, whereas dried cows had only access to the feedbunk twice a day; although water and straw were available without restriction.

[‡]15 days before expected parturition the cows received a vitamin complex injection (Hipravit-AD_3E-Forte® Hipra Laboratories) at a dose of 0.10 mL/kg BW, containing each mL 75000 IU of cholecalciferol, 50 mg of α-tocopherol acetate and 500000 IU of vitamin A.

[∂]Concentrate composition (% as fed): rapeseed meal (26.2), corn (20.0), wheat DDGs (15.9), soybean meal (11.5), calcium soap (3.2), sugarcane (1.6), bicarbonate (1.6), calcium carbonate (0.9) and sodium chloride (0.8).

[θ]Contained: 14% Ca, 4% P, 6% Na, 5% Mg, 650000 IU/kg vitamin A, 130000 IU/kg vitamin D3, 2600 IU/kg vitamin E, 9700 ppm Zn (oxide), 8100 ppm Mn, 8100 ppm Fe, 2000ppm Cu, 100ppm I, 40 ppm Cu, 40 ppm Se and 30 ppm Mo.

Pre-print version of the article published in Animal (Cambridge Journals). Volume 7. Issue 08. August 2013, pp 1374-1378.
http://dx.doi.org/10.1017/S1751731113000396

Table 2

Mean values of oxidative status markers throughout the studied stages.

Parameter	Units	Transitional stages (n=22)				*CTR (n=40)*	rmse	*P-value*
		LL	***PrP***	***PsP***	***PkL***			
ROS	*CarrU*	121.4	129.8	153.0	145.1	135.6	35.3	0.074
SAC	*µmol HClO/mL*	481.1	516.8	489.8	425.8	480.0	117.8	0.169
OSi	*CarrU/(µmol HClO/mL)*	0.26^{a}	0.26^{a}	0.34^{bc}	0.37^{c}	0.30^{ab}	0.10	0.003

ROS: Reactive oxygen substances; SAC: Serum Antioxidant Capacity; OSi: Oxidative Stress index. *CTR:* control cows (between the 4th and 5th month of gestation); *LL:* late lactation (between 2 and 1 month before parturition); *PrP*: prepartum (from 1 month before parturition until delivery); *PsP*: postpartum (from delivery until 1 month after calving); *PkL*: peak of lactation (from 1 month after parturition until peak lactation). rmse: root mean squared error.

A repeated measurements ANOVA with cow as experimental unit was used to compare the means within the transitional stages, whereas the means of these stages were compared with the means of the CTR group through the Student-*t* test. Means with different superscript alphabets within rows are significantly different (P<0.05).