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The development of a Comprehensive Index (Oxiscore) to assess the Oxidative Status in Periparturient Dairy Cows

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ABSTRACT

This study introduces an Oxiscore for dairy cows by combining various oxidation and antioxidant markers to measure oxidative stress in clinically healthy periparturient dairy cow. The Oxiscore is defined by subtracting a 'damage score' from a 'protection score'. To obtain the 'damage score', levels of determinable reactive oxygen metabolites and thiobarbituric acid reactive substances in plasma are combined. To obtain a 'protection score', levels of superoxide dismutase in plasma, selenium-dependent glutathione peroxidase in both plasma and erythrocytes (packed cell volume) and thiol groups in erythrocytes are combined. No dietary antioxidants, like α-tocopherol, or pro-oxidants, like iron, were taken into account. Data from sixteen periparturient dairy cows were used. From these cows, blood samples were taken from the jugular vein at day 30.4 ± 2.0, 24.7 ± 2.4, 17.6 ± 2.2, 10.8 ± 2.2, and 3.9 ± 1.6 antepartum and at 4, 11, 18, 25, and 30 days postpartum. The Oxiscore showed a significant decrease ($P = 0.046$) only in cows with a mean body condition score above 2.5 between 4 days antepartum and 25 days postpartum. Further, the Oxiscore is significant higher ($P = 0.002$) in cows with a non esterified fatty acid concentration above 0.4 mmol/L than cows with concentrations below 0.4 mmol/L, but the difference between cows with a β-hydroxybutyrate concentration higher and lower than 0.9 mmol/L was not significant.

Regression analysis showed that none of the single markers contributed significantly to the Oxiscore consistently at all sampling days. Due to the small test population and physiological variation of the markers in these healthy subjects the predictive value of their contributions to the Oxiscore requires further study, in which healthy animals that have oxidative stress, healthy animals that do not have oxidative stress and animals that develop disease should be included for validation.Also, reference values of the various biomarkers have to be determined. This would facilitate the determination of the sensitivity of the

individual markers and of the Oxiscore in the detection of changes in the oxidative status and thus the value of the Oxiscore in practice.

KEY WORDS

Dairy cattle, periparturient period, oxidative stress, body condition score

INTRODUCTION

The periparturient period of dairy cows involves major metabolic changes that are associated with lactogenesis. The onset of lactogenesis results in an increased metabolic rate and consequently, amongst other events, in an increased release of reactive oxygen species (**ROS**) during the periparturient period (Halliwell and Gutteridge, 2007). Reactive oxygen species is a term of a group of oxygen free radicals and their metabolites that includes oxygen-centered radicals like the superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and some non-radical derivates of oxygen like hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). The hydroxyl radical is a highly reactive metabolite and causes a free-radical chain reaction. Reactive oxygen species can damage cell structures when they are excessively generated for a prolonged time. Under normal physiological conditions, a complex network of several antioxidants and antioxidant enzymes scavenges these ROS. An imbalance in favour of the production of ROS relative to available antioxidants results in oxidative stress (Sies, 1991; Castillo et al., 2005). When this occurs, progressive oxidation of lipids, proteins and DNA may occur and thereby result in a situation that harms health. Direct effects on health include peroxidative changes in cell structures and cell membranes, indirect effects include consumption of reducing equivalents which can interfere with important metabolic functions. Severe oxidative stress can also result in cell apoptosis (Miller et al., 1993; Davies, 2000; Halliwell and Gutteridge, 2007).

The nature and severity of oxidative stress can be determined by direct or indirect measures of oxidant and antioxidant activity. Several methods have been developed to measure biomarkers of peroxidation damage, the concentration of antioxidants and/or the activity of antioxidant enzymes (Celi, 2011).

Thiobarbituric Acid Reactive Substances (**TBARS**) represent a group of lipid peroxidation products including malondialdehyde (**MDA**) and are considered indicators of

oxidative stress when measured in plasma (Halliwell and Chirico, 1993). Elevated levels of TBARS in periparturient cows were reported by Bernabucci et al. (2005). Also, d-ROM levels were shown to continue to increase even after three weeks postpartum which suggests that the antioxidative capacity in dairy cows around calving seems to be insufficient to counteract the increase in d-ROM supply (Castillo et al., 2005; Bernabucci et al., 2005; Celi et al., 2012).

Several enzymes and proteins act as antioxidants:

- Superoxide dismutase (**SOD**) is an enzyme that converts the $O_2^{\cdot -}$ oxidant into O_2 and H_2O_2. Hydrogen peroxide has limited chemical reactivity but could act as a metabolic signal, possibly by oxidizing certain protein thiol groups (Halliwell and Chirico, 1993). Activity of SOD increases after calving in dairy cows (Gaál et al., 2006; Colitti and Stefanon, 2006), but this increase was not observed by Dobbelaar et al. (2010).
- Selenium-dependent glutathione peroxidase (**GSH-Px**) reacts with H_2O_2 and hydroperoxides, oxidizing glutathione to glutathione disulfide (Halliwell and Chirico, 1993; Davies, 2000).
- Thiol groups (**Gr-SH**) in plasma represent sulfhydryl groups of albumin, l-cysteine and homocysteine. These non-enzymatic protein antioxidants are considered an important part of the extracellular antioxidant defence system (Ueland et al., 1996; Sen and Packer, 2000).

Apart from these antioxidants, numerous dietary antioxidants exist, like vitamin E and β-carotene. The administration of vitamin E in order to manipulate the oxidative status in dairy cows has resulted in different results. It was hypothesized that supplementation of vitamin E and Se could reduce oxidative stress and consequently improve health in high-producing dairy cows (Spears, 2000) and supplementing vitamin E may improve overall health and fertility in dairy cows (Miller et al., 1993; Baldi et al., 2000). It is reported that supplementation of β-

carotene and vitamin E intramuscularly to postpartum dairy cows, resulted in lower serum determinable reactive oxygen metabolites (**d-ROM**, test by Diacron) concentrations on day of delivery (Rizzo et al., 2013). However in another study, oral vitamin E supplementation to periparturient heifers did not reduce d-ROM concentration in serum (Dobbelaar et al., 2010). In supplementation studies, individual status with regard to the supplement and other antioxidants is often neglected.

Although several antioxidant micronutrients have been reported to affect the immune system in various ways, deficiencies of those do not necessarily results in disease or an increased susceptibility to disease (Spears, 2000). Responses to nutrient antioxidant supply on periparturient diseases and disorders are not consistent. Some studies showed no effect of supplementation on incidence of placenta retention (Ishak et al., 1983; Hidiroglou et al., 1987) whereas other studies showed significant reductions or a tendency of a reduced risk on retention of placenta (Miller et al., 1993; LeBlanc et al., 2002). Meta-analysis of over 40 studies on the effects of vitamin E supplementation during the dry period on the risk of retained foetal membranes (**RFM**) indicated that supplementation does result in a reduction of the incidence of RFM (Bourne et al., 2007). A significant association between vitamin E supplementation and a decreased incidence of mastitis has been described in a study with 3 groups of 22 cows which were fed different dietary levels of vitamin E (Weiss et al., 1997), whereas in another study there was no significant association between serum vitamin E (α-tocopherol) concentration and the risk of mastitis (LeBlanc et al., 2004). Adverse effects of vitamin E supplementation have been reported in a study with 5 farms. Cows had a high vitamin E status prior to the study trial (Bouwstra et al., 2010). Vitamin E supplemented to dairy cows to obtain high (>3 μg/mL) or medium (2-3 μg/mL) α-tocopherol levels in serum at calving resulted in a 4 times lower incidence of mastitis compared to the low α-tocopherol (< 2 μg/mL) group (Politis et al., 2012).

The presence of hydroperoxides, amongst others, indicates peroxidation of lipids (Halliwell and Chirico, 1993). Löhrke et al. (2005) reported that the concentration of serum lipid hydroperoxides in low-density lipoprotein was positively correlated with milk production, which suggests that milk production level may contribute to the level of oxidative processes. Polyunsaturated fatty acids (PUFAs) are particularly susceptible to peroxidation due to their number of double bonds. When the process of lipid peroxidation is initiated, it can result in a chain reaction whereby hydroperoxides are formed (Gutteridge, 1995). Lipid supplements that are partially resistant to biohydrogenation in the rumen have been developed and result in elevated PUFAs in plasma and milk (Loor et al., 2002). Also, concentrations of PUFAs in milk were increased when extruded flax seed was used in the diet (Neveu et al., 2013). This suggests that diets that contain more rumen protected PUFAs lead to higher concentrations of PUFAs and should probably contain more livid soluble antioxidants as well. Lipid peroxides are fairly stable molecules but their decomposition is catalyzed by transition metals and metal complexes. Superoxide reduces certain ferric chelates. This results in reduced iron complexes (Fe^{2+}) which, in turn, react with lipid peroxides to give alkoxyl radicals. These radicals cause a chain reaction of lipid peroxidation (Halliwell and Chirico, 1993; Gutteridge, 1995). Fe is therefore considered a dietary pro-oxidant. Sordillo and Raphael (2013) and Bernabucci et al. (2005) discuss the association between postpartum lipid mobilization and oxidative stress. Markers of lipid mobilization are non-esterified fatty acids (NEFA) and, as the case may be, β-hydroxybutyrate (b-HBA). Increased plasma NEFA concentrations can increase lipid hydroperoxide formation and enhanced uptake of NEFA by the liver is accompanied by an increase in peroxisomal oxidation (Sordillo and Raphael, 2013). Moreover, increased concentrations of NEFA and b-HBA in cows with a higher BCS and BCS loss were accompanied by higher TBARS and d-ROM values (Bernabucci et al.,

2005). However, Dobbelaar et al. (2010) did not find a significant correlation between NEFA and d-ROM.

To obtain an estimation of the oxidative status, several oxidant and antioxidant markers in plasma and erythrocytes can be measured. In a recently published review, Celi (2011) discusses the usefulness of various biomarkers of oxidative stress. The methods used to measure these markers differ per individual marker and have both advantages and disadvantages in applicability, specificity and sensitivity.

Some tests are supposed to measure the total antioxidant capacity of biological samples. The ferric reducing ability of plasma (FRAP) assay is based on a coloured ferrous-tripyridyltriazine complex which forms in a reduction of ferric to ferrous ion at low pH. FRAP values are obtained by comparing the absorbance change with mixtures with known concentrations of ferrous ions (Benzie and Strain, 1996). It has the disadvantage that not every free radical reduces ferric and GSH is not measured, but has the advantage that serum dilution effect is not seen. The trolox equivalent antioxidant capacity assay is based on scavenging of radical anions and has been altered to prevent overestimation of antioxidant capacity (van den Berg et al., 1999). It is a fast and simple method, but results vary with sample dilution and the used antioxidant may interact with the solvent molecules. The total radical-trapping antioxidant parameter is a method in which the loss of fluorescence, due to peroxidation, of a certain protein is monitored (Ghiselli et al., 1995). It gives an idea of the free radical formation but may not trap all types of free radicals. The biological antioxidant potential test covers a wide variety of antioxidants and is based on the same principle as the FRAP assay. It is a simple method, but it can be performed in plasma and serum samples only. Hyperlipemic samples can cause underestimation of results and it is unknown what the influence of dietary antioxidants is. Reactive oxygen species are measured by spectrometry and is simple and fast. The d-ROM test detects derivatives of ROS and possibly not all ROS.

None of these tests includes all oxidative and antioxidant markers. In conclusion, Celi (2011) states that no single measurement can adequately describe oxidative stress due to the different advantages and disadvantages.

Veglia et al. (2006) developed a procedure to compute a comprehensive index (OXY-SCORE) of oxidative stress in humans which considers both oxidative and antioxidant markers. Levels of the oxidative markers were combined to form a "damage score" and the levels of the antioxidant markers were combined to form a "protection score". The OXY-SCORE was obtained by subtracting the protection score from the damage score and the result supposedly reflects the oxidative stress status of the patient. By combining multiple measures their aim was to obtain a score with higher sensitivity to physiological and pathological alterations than individual markers (Veglia et al., 2006). In a recent published paper, a new tool, the Oxidative stress index (OSi), has been presented to assess redox status in dairy cattle using the ratio of d-ROM and serum antioxidant capacity (Abuelo et al., 2013). Serum antioxidant capacity (SAC) was estimated with a test that exploits the overload capacity of a solution of hypochlorous acid to oxidize the whole serum sample and uses an indicator to convert the capacity to a number, via photometric measurement. When d-ROM and SAC were used separately no significance change of the oxidative status was identified, but when using the OSi it was found that dairy cows were classified to have oxidative stress in the periparturient period (Abuelo et al., 2013).

The aim of this study is to develop an Oxiscore based on clinically healthy dairy cows in the periparturient period. By combining several markers, it could be more sensitive than individual markers in the detection of differences in the oxidative status. Hereafter it can be used in another dataset to be validated and to test whether the Oxiscore is better able to distinguish stressed and unstressed animals due to oxidative processes compared to individual markers.

MATERIALS AND METHODS

Data for our study were obtained from a subset of 16 healthy dairy cows from a previously published study (Bernabucci et al., 2005). In that study, blood samples were taken from the jugular vein at day 30.4 ± 2.0, 24.7 ± 2.4, 17.6 ± 2.2, 10.8 ± 2.2, and 3.9 ± 1.6 antepartum and at 4, 11, 18, 25, and 30 days postpartum. 5 cows were not sampled at the first day of sampling which results in less number of data (N=11).

From these data, three summary indexes of oxidative status were calculated:

1) A score of oxidative damage which is made up of the summed values of d-ROM and TBARS in plasma.
2) A protection score of antioxidant capacity which is composed of the summed values of GSH-Px in plasma (**GSH-PxPl**), GSH-Px in packed cell volume (**GSH-PxPCV**), Gr-SH in plasma and SOD in PCV.
3) The Oxiscore, which is defined by subtracting the oxidative damage score from the score of antioxidant capacity.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics 17.0. The criterion for statistical significance was $P \leq 0.05$.

Histograms have been plotted and a Shapiro-Wilk test was calculated to test if the values of the different markers were normally distributed on each sampling day. When the value of test statistic W was below 0.9 this variable was considered not being normally distributed. Determinable reactive oxygen metabolites, GSH-PxPCV, SOD and Gr-SH appeared to be normally distributed on each sampling day, but GSH-PxPl and TBARS were not normally distributed. To obtain normally distributed data, GSH-PxPl and TBARS were transformed using a Log-transformation as this provided the most normally distributed

variables compared to other transformations. After this transformation only 1 sampling day of GSH-PxPl and 3 sampling days of TBARS were not considered to be normally distributed (Shapiro-Wilk test statistics 0.7-0.8) but were still used in the tests statistics due to the small sample size.

Different components were measured using different units. Therefore variables differ in measurement units and variability. To compare and combine these variables they had to be standardized by creating standard scores (z-scores) of each value. Z-scores have a mean of 0 and a standard deviation of 1. Z-scores were created using the usual formula:

$$z_{ab} = \frac{(x_{ab} - \mu_b)}{\sigma_b}$$

Where z_{ab} and x_{ab} are the standardized score and the original measure, respectively. μ_b and σ_b are the mean and standard deviation of all values of variable *b*, respectively. Normally, when making comparisons between healthy and non-healthy individuals, the mean and standard deviation of the healthy population would have been used (Veglia et al., 2006).

Per sampling day, means were calculated of each marker (N varied from 8-16). The means of the standardized pro-oxidant markers d-ROM and (log)TBARS were aggregated to create a 'damage score' (DS) and standardized anti-oxidant markers (log)GSH-PxPl, SOD, GSH-PxPCV and Gr-SH were aggregated to create a 'protection score' (PS). The Oxiscore was obtained by subtracting the DS from the PS.

Graphs were plotted to determine how the different variables changed during the transition period and a scatterplot was plotted to relate the Oxiscore to those variables. T-tests have been calculated to test if the Oxiscore was significantly different between sampling days in the periparturient period.

Regression analysis, of each sampling day with the Oxiscore as the dependent variable and the six other variables as independent variables, was used to predict the contribution to the Oxiscore. A stepwise (forward) method has been chosen so that only significant variables

would be used in the final model. Missing values were excluded list wise and values of probability to enter (PIN) and probability to remove (POUT) were 0.05 and 0.10 respectively.

Bernabucci et al. (2005) showed that cows with a higher body condition score (**BCS)** were more prone to oxidative stress. Therefore cows were also divided into two groups based on their mean BCS during the periparturient period: LowBCS with a mean BCS of <2.5 (n=8) and HighBCS with a mean BCS of >2.5 (n=8). The Oxiscores of LowBCS and HighBCS were plotted together. T-tests have been calculated to test if changes of the Oxiscore were significant at different periods of time in the periparturient period.

Sordillo and Raphael (2013) discuss the interrelationship between metabolic stress, lipid mobilization and oxidative stress. Increased plasma NEFA concentrations can increase lipid hydroperoxide formation and enhanced uptake of NEFA by the liver is accompanied by an increase in peroxisomal oxidation (Sordillo and Raphael, 2013). Also, Bernabucci et al. (2005) reported that d-ROM values were higher in high BCS cows which also had increased concentrations of b-HBA and NEFA. Van Knegsel et al. (2007) studied the effect of glucogenic, lipogenic and mixed diets on these metabolic markers. Cut-off values used in this study were based on the results of their mixed diet. For NEFA 0.4 mmol/L and for b-HBA 0.9 mmol/L were chosen. Oxiscores for NEFA concentrations above 0.4 mmol/L (highNEFA) and for NEFA concentrations below 0.4 mmol/L (lowNEFA) were plotted together and Oxiscores for b-HBA concentrations above 0.9 mmol/L (highB-HBA) and for b-HBA concentrations below 0.9 mmol/L (lowB-HBA) were plotted together. T-tests have been calculated to test if the Oxiscore was significantly different between these groups.

RESULTS

The Oxiscore for HighBCS and LowBCS during the periparturient period are shown in figure 1. T-test shows a significant decrease ($P = 0.046$) in the Oxiscore in the HighBCS group between day 4 antepartum (mean = 1.22) and day 25 postpartum (mean = -1.34) as is presented in Table 1. The differences between the HighBCS group and the LowBCS group are not significant at any sampling day.

A forward regression analysis has been used to predict the contribution of one or more variables to the Oxiscore on each sampling day during the periparturient period. This analysis shows that 30 and 25 days antepartum, d-ROM and SOD are the variables that contributed significantly to the Oxiscore. On day 18 and 11 days antepartum, GrSH and GSH-Px (PCV) contributed significantly to the Oxiscore, whereas at day 4 antepartum and day 4 postpartum none of the variables contributed significantly. At days 11 and 18 postpartum, TBARS and GSH-Px (PCV) contributed significantly and later postpartum multiple variables contributed significantly, but SOD (day25) and TBARS (day30) the most, with the highest adjusted R^2. Details about the regression analysis are shown in table 2.

Means of highNEFA and lowNEFA cows are shown in figure 2. Means of highB-HBA and lowB-HBA cows are shown in figure 3. T-test shows a significant difference ($P = 0.002$) between the Oxiscore of the highNEFA (mean 1.32) and lowNEFA (mean -0.23) from 4 days antepartum to 30 days postpartum.

DISCUSSION

The aim of this study was to develop an Oxiscore for clinically healthy dairy cows in the periparturient period by combining multiple markers of oxidative status and which can be validated in other studies. It should discriminate healthy and non-healthy individuals as far as the oxidative status is affecting that particular disease or is affected by that disease. Such a comparison has been made in a study on humans with healthy subjects and subjects with coronary artery disease (Veglia et al., 2006). We have followed a similar methodology to develop an Oxiscore for cows. Variables in this Oxiscore were the plasma markers: d-ROM, TBARS, Gr-SH and GSH-Px and erythrocytes markers (measured in packed cell volume): SOD and GSH-Px. Combining these markers could provide a more powerful index in the evaluation of oxidative status than individual markers.

The Oxiscore shows a significant decrease only in the HighBCS group, between 4 days antepartum and 25 days postpartum. This could implicate that periparturient cows with a higher BCS are more prone to oxidative stress. This corresponds with a study of Dobbelaar et al. (2010). Cows in the HighBCS group had a mean BCS of 2.9 which cannot be considered to be overconditioned or obese, but still, studies in humans have reported that a high body mass index is associated with oxidative stress (Trevisan et al., 2001; Keaney et al., 2003).

None of the single markers contributed significantly to the Oxiscore at all sampling days. The aim of this analysis was to find out whether one of the markers would contribute significantly to the Oxiscore on multiple sampling days and therefore would be the most useful marker to be measured in practice. A multivariate repeated measure regression analysis would be a more appropriate method but is difficult to apply appropriately in SPSS. Due to the small test population and physiological variation of these markers in our healthy subjects the predictive value of these significant contributions to the Oxiscore can not be established. It would be useful to have the Oxiscore applied to larger number of cows and to compare

groups of clinically healthy animals that are stressed, clinically healthy animals that are not stressed and animals that develop disease.

Bernabucci et al. (2005) reported that d-ROM is positively related to b-HBA and NEFA, but Dobbelaar et al. (2010) did not confirm this result. In further studies, it might be useful to include analysis of NEFA and b-HBA to gain a better understanding of the relation between lipid mobilization and oxidative stress (Sordillo and Raphael, 2013).

Oxidative stress is a young field of research in dairy cows. In a recently published review, Celi (2011) describes the various biomarkers of oxidative stress that are being measured in ruminant medicine. Many methods have been developed to measure these biomarkers and are still being altered to obtain more accurate measurements. The procedure of these measurements differs in applicability, specificity and sensitivity. Other methods than methods used by us, are known to measure oxidative damage markers or antioxidant status but still would only be able to explain part of the Oxiscore. In conclusion, Celi (2011) states that no single measurement can adequately describe oxidative stress. Also, Veglia et al. (2006) showed that combining different markers leads to a higher sensitivity than individual markers. This justifies the selection of multiple markers for the Oxiscore.

No clinical diseases or disorders around calving of these cows were reported so no comparison could be made between healthy and diseased cows as has been done in humans between healthy humans and humans with coronary artery disease (Veglia et al., 2006).

A range of markers was measured, but we selected 6 markers in accordance with markers of another available dataset so that the Oxiscore can be validated in the near future. More markers could provide a stronger score by gaining more information about the contribution of different markers to the Oxiscore. Also other tissues such as liver and ovarian follicles can be examined (Dobbelaar et al., 2010) to discover if different tissues have a similar oxidative status and what value it has for this field of research.

Supplementation of dietary antioxidants has been reported to affect the oxidative status (Miller et al., 1993; Spears, 2000; Rizzo et al., 2013). Vitamin E supplementation results in higher vitamin E (α-tocopherol) levels in blood (Politis et al., 2004; Dobbelaar et al., 2010). It is considered to be an important lipid soluble antioxidant (Baldi, 2005). All cows in this study were fed the same total mixed ratio diet ad libitum, but no dietary antioxidants, like vitamin E, were measured. Therefore, the contribution of dietary antioxidants to the Oxiscore could not be taken into account. This also implicates that different measurements between individuals are not influenced by different dosages of dietary antioxidants in the feed.

To combine the different variables and values to obtain this Oxiscore the variables were standardized. The obtained values of this standardization are expressed in units of the standard deviation of the original values of that variable. And though the Oxiscore is all about the relative contribution of each variable, a difference of 1 unit in one variable could implicate a small absolute change in the original measurement whereas the same difference of 1 unit for another variable could implicate a large absolute change in the original measurement (Larsen and Marx, 2011). The implications in respect to this study are unknown. To increase the accuracy of the validation of this Oxiscore, weighted values, if possible, should therefore be used in future studies. In order to do that, future studies also have to determine the reference values of the various biomarkers. Further, it will be essential to develop a manner to define the activity of these biomarkers in the same way or to convert different measurement units to the same unit.

CONCLUSIONS

An Oxiscore for the healthy periparturient dairy cow has been developed including 4 plasma markers, GSH-PxPl, Gr-SH, d-ROM, TBARS and 2 erythrocyte (packed cell volume) markers, GSH-PxPCV and SOD.

The Oxiscore does not differ significantly in any period of time during the periparturient period, but shows a significant decrease in the HighBCS group (BCS above 2.5) between 4 days antepartum and 25 days postpartum. This could implicate that cows with a higher BCS are more prone to oxidative stress. The differences between the HighBCS group and the LowBCS group are not significant at any sampling day.

Regression analysis showed that none of the single markers contributed significantly to the Oxiscore consistently at all sampling days. At different sampling days, different markers contribute the most to the Oxiscore. Further, the Oxiscore is significant higher (P = 0.002) in cows with a non esterified fatty acid concentration above 0.4 mmol/L than cows with concentrations below 0.4 mmol/L, but the difference between cows with a β-hydroxybutyrate concentration higher and lower than 0.9 mmol/L was not significant.

This study does not provide a sound basis for a comprehensive index to assess the oxidative status in periparturient dairy cows. Due to the small test population and physiological variation of the markers in these healthy subjects the predictive value of their contributions to the Oxiscore requires further study, in which healthy animals that have oxidative stress, healthy animals that do not have oxidative stress and animals that develop disease should be included for validation. Also, reference values of the various biomarkers have to be determined. This would facilitate the determination of the sensitivity of the individual markers and of the Oxiscore in the detection of changes in the oxidative status and thus the value of the Oxiscore in practice.

References

1. Abuelo, A., J. Hernández, J. L. Benedito and C. Castillo. 2013. Oxidative stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period. Animal. 7:1374-1378.

2. Baldi, A. 2005. Vitamin E in dairy cows. Livest. Prod. Sci. 98:117-122.

3. Baldi, A., G. Savoini, L. Pinotti, E. Monfardini, F. Cheli and V. D. Orto. 2000. Effects of vitamin E and different energy sources on vitamin E status, milk quality and reproduction in transition cows. Journal of Veterinary Medicine Series A. 47:599-608.

4. Benzie, I. F. F. and J. J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant power": The FRAP assay. Anal. Biochem. 239:70-76.

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

6. Bourne, N., R. Laven, D. C. Wathes, T. Martinez and M. McGowan. 2007. A meta-analysis of the effects of vitamin E supplementation on the incidence of retained foetal membranes in dairy cows. Theriogenology. 67:494-501.

7. Bouwstra, R. J., M. Nielen, J. A. Stegeman, P. Dobbelaar, J. R. Newbold, E. H. J. M. Jansen and T. van Werven. 2010. Vitamin E supplementation during the dry period in dairy cattle. part I: Adverse effect on incidence of mastitis postpartum in a double-blind randomized field trial. J. Dairy Sci. 93:5684-5695.

8. Castillo, C., J. Hernandez, A. Bravo, M. Lopez-Alonso, V. Pereira and J. L. Benedito. 2005. Oxidative status during late pregnancy and early lactation in dairy cows. The Veterinary Journal. 169:286-292.

9. Celi, P. 2011. Biomarkers of oxidative stress in ruminant medicine. Immunopharmacol. Immunotoxicol. 33:233-240.

10. Celi, P., M. Merlo, O. Barbato and G. Gabai. 2012. Relationship between oxidative stress and the success of artificial insemination in dairy cows in a pasture-based system. The Veterinary Journal. 193:498-502.

11. Colitti, M. and B. Stefanon. 2006. Effect of natural antioxidants on superoxide dismutase and glutathione peroxidase mRNA expression in leukocytes from periparturient dairy cows. Vet. Res. Commun. 30:19-27.

12. Davies, K. J. A. 2000. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life. 50:279-289.

13. Dobbelaar, P., R. J. Bouwstra, R. M. A. Goselink, R. Jorritsma, J. J. G. C. van den Borne and E. H. J. M. Jansen. 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. J. Dairy Sci. 93:3103-3113.

14. Gaál, T., P. Ribiczeyné-Szabó, K. Stadler, J. Jakus, J. Reiczigel, P. Kövér, M. Mézes and L. Sümeghy. 2006. Free radicals, lipid peroxidation and the antioxidant system in the blood of cows and newborn calves around calving. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 143:391-396.

15. Ghiselli, A., M. Serafini, G. Maiani, E. Azzini and A. Ferro-Luzzi. 1995. A fluorescence-based method for measuring total plasma antioxidant capability. Free Radical Biology and Medicine. 18:29-36.

16. Gutteridge, J. M. 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin. Chem. 41:1819-1828.

17. Halliwell, B. and S. Chirico. 1993. Lipid peroxidation: Its mechanism, measurement, and significance. The American Journal of Clinical Nutrition. 57:715S-724S.

18. Halliwell, B. and J. M. C. Gutteridge. 2007. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, UK.

19. Hidiroglou, M., A. J. Mcallister and C. J. Williams. 1987. Prepartum supplementation of selenium and vitamin E to dairy cows: Assessment of selenium status and reproductive performance. J. Dairy Sci. 70:1281-1288.

20. Ishak, M. A., L. L. Larson, F. G. Owen, S. R. Lowry and E. D. Erickson. 1983. Effects of selenium, vitamins, and ration fiber on placental retention and performance of dairy cattle. J. Dairy Sci. 66:99-106.

21. Keaney, J. F., M. G. Larson, R. S. Vasan, P. W. F. Wilson, I. Lipinska, D. Corey, J. M. Massaro, P. Sutherland, J. A. Vita and E. J. Benjamin. 2003. Obesity and systemic oxidative stress: Clinical correlates of oxidative stress in the framingham study. Arterioscler. Thromb. Vasc. Biol. 23:434-439.

22. Larsen, R. J. and M. L. Marx. 2011. An Introduction to Mathematical Statistics and its Applications. 5th ed. Pearson Education, Inc., Boston, United States of America.

23. LeBlanc, S. J., T. F. Duffield, K. E. Leslie, K. G. Bateman, J. TenHag, J. S. Walton and W. H. Johnson. 2002. The effect of prepartum injection of vitamin E on health in transition dairy cows. J. Dairy Sci. 85:1416-1426.

24. LeBlanc, S. J., T. H. Herdt, W. M. Seymour, T. F. Duffield and K. E. Leslie. 2004. Peripartum serum vitamin E, retinol, and beta-carotene in dairy cattle and their associations with disease. J. Dairy Sci. 87:609-619.

25. Löhrke, B., T. Viergutz, W. Kanitz, F. Becker, K. Göllnitz, A. Hurtienne and F. J. Schweigert. 2005. Relationship between oxidant stress and milk productivity in dairy cows. Berliner Und Münchener Tierärztliche Wochenschrift. 118:265-269.

26. Loor, J. J., J. H. Herbein and T. C. Jenkins. 2002. Nutrient digestion, biohydrogenation, and fatty acid profiles in blood plasma and milk fat from lactating holstein cows fed canola oil or canolamide. Anim. Feed Sci. Technol. 97:65-82.

27. Miller, J. K., E. Brzezinska-Slebodzinska and F. C. Madsen. 1993. Oxidative stress, antioxidants, and animal function. J. Dairy Sci. 76:2812-2823.

28. Neveu, C., B. Baurhoo and A. Mustafa. 2013. Effect of feeding extruded flaxseed with different forage:Concentrate ratios on the performance of dairy cows. J. Dairy Sci. 96:3886-3894.

29. Politis, I., I. Bizelis, A. Tsiaras and A. Baldi. 2004. Effect of vitamin E supplementation on neutrophil function, milk composition and plasmin activity in dairy cows in a commercial herd. J. Dairy Res. 71:273-278.

30. Politis, I., G. Theodorou, A. D. Lampidonis, A. Kominakis and A. Baldi. 2012. Short communication: Oxidative status and incidence of mastitis relative to blood α-tocopherol concentrations in the postpartum period in dairy cows. J. Dairy Sci. 95:7331-7335.

31. Rizzo, A., M. Pantaleo, M. Mutinati, G. Minoia, C. Trisolini, E. Ceci and R. L. Sciorsci. 2013. Blood and milk oxidative status after administration of different antioxidants during early postpartum in dairy cows. Res. Vet. Sci. 95:1171-1174.

32. Sen, C. K. and L. Packer. 2000. Thiol homeostasis and supplements in physical exercise. Am. J. Clin. Nutr. 72:653s-669s.

33. Sies, H. 1991. Oxidative stress: From basic research to clinical application. Am. J. Med. 91:31S-38S.

34. Sordillo, L. M. and W. Raphael. 2013. Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. Veterinary Clinics of North America: Food Animal Practice. 29:267-278.

35. Spears, J. W. 2000. Micronutrients and immune function in cattle. Proc. Nutr. Soc. 59:587-594.

36. Trevisan, M., R. Browne, M. Ram, P. Muti, J. Freudenheim, A. Carosella and D. Armstrong. 2001. Correlates of markers of oxidative status in the general population. American Journal of Epidemiology. 154:348-356.

37. Ueland, P. M., M. A. Mansoor, A. B. Guttormsen, F. MÃ¼ller, P. Aukrust, H. Refsum and A. M. Svardal. 1996. Reduced, oxidized and protein-bound forms of homocysteine and other aminothiols in plasma comprise the redox thiol Statusâ€"A possible element of the extracellular antioxidant defense system. J. Nutr. 126:1281S-1284S.

38. van den Berg, R., G. R. M. M. Haenen, H. van den Berg and A. Bast. 1999. Applicability of an improved trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem. 66:511-517.

39. van Knegsel, A. T. M., H. van den Brand, J. Dijkstra, W. M. van Straalen, R. Jorritsma, S. Tamminga and B. Kemp. 2007. Effect of glucogenic vs. lipogenic diets on energy balance, blood metabolites, and reproduction in primiparous and multiparous dairy cows in early lactation. J. Dairy Sci. 90:3397-3409.

40. Veglia, F., G. Cighetti, M. De Franceschi, L. Zingaro, L. Boccotti, E. Tremoli and V. Cavalca. 2006. Age- and gender-related oxidative status determined in healthy subjects by means of OXY-SCORE, a potential new comprehensive index. Biomarkers. 11:562-573.

41. Weiss, W. P., J. S. Hogan, D. A. Todhunter and K. L. Smith. 1997. Effect of vitamin E supplementation in diets with a low concentration of selenium on mammary gland health of dairy cows. J. Dairy Sci. 80:1728-1737.

Table 1. T-test for two paired samples shows a significant decrease in the Oxiscore of high body condition group (HighBCS)[1] from 4 days antepartum (a.p.) (mean = 1.22) to 25 days postpartum (p.p.) (mean = -1.34).

Oxiscore	Paired Differences					t	df	Sign. (2-tailed)
	Mean diff.	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Day 4 a.p. – Day 25 p.p.	2.555	2.980	1.053	0.064	5.046	2.425	7	0.046

[1] HighBCS: N=8, mean body condition score of whole periparturient period > 2.5

Table 2. Multiple forward regression analysis in the periparturient period with the Oxiscore as the dependent variable and with all standardized variables, determinable reactive oxygen metabolites (ZROM), thiobarbituric acid reactive substances (logZTBARS), glutathione peroxidase in plasma (logZGSH-PxPl), glutathione peroxidase in packed cell volume (ZGSH-PxPCV), thiol groups (ZGr-SH) and superoxide dismutase (ZSOD) as independent variables. Criteria: probability to enter and probability to remove were 0.05 and 0.10, respectively. Day of calving is set on 0. On day -4 and day 4 no variables contributed significantly to the Oxiscore and data of these days were therefore omitted in this table.

			Unstandardized coefficients		Standardized coefficients		
Day	Model	Variable	B	Std. Error	Beta	Sig.	Adjusted R^2
-30	1	(Constant)	0.038	0.316		0.911	0.745
		ZROM	-1.784	0.500	-0.899	0.038	
	2	(Constant)	-0.070	0.055		0.338	0.992
		ZROM	-1.481	0.091	-0.747	0.004	
		ZSOD	0.636	0.064	-0.459	0.010	
-25	1	(Constant)	0.652	0.465		0.204	0.421
		ZROM	-1.365	0.522	-0.703	0.035	
	2	(Constant)	0.581	0.229		0.044	0.860
		ZROM	-2.101	0.299	-1.082	0.0004	
		ZSOD	1.828	0.381	-0.739	0.003	
-18*	1	(Constant)	-.147	0.808		0.864	0.512
		ZGrSH	1.296	0.423	0.757	0.018	
	2	(Constant)	0.597	0.482		0.262	0.851
		ZGrSH	1.160	0.236	0.677	0.003	
		ZGSH-PxPCV	1.482	0.360	0.567	0.006	
-11	1	(Constant)	1.030	0.526		0.097	0.505
		ZGSH-PxPCV	1.748	0.612	0.759	0.029	
11	1	(Constant)	1.141	0.595		0.077	0.318
		(log)ZTBARS	-1.443	0.526	-0.605	0.017	
	2	(Constant)	0.401	0.499		0.437	0.615
		(log)ZTBARS	-1.582	0.397	-0.664	0.002	
		ZGSH-PxPCV	2.070	0.622	0.554	0.006	
	3	(Constant)	-0.095	0.335		0.781	0.847
		(log)ZTBARS	-1.402	0.254	-0.588	0.0002	
		ZGSH-PxPCV	2.133	0.393	0.572	0.0002	
		ZSOD	0.911	0.209	0.464	0.001	
18	1	(Constant)	-0.237	0.390		0.555	0.540
		(log)ZTBARS	-1.458	0.375	-0.761	0.003	
	2	(Constant)	-0.250	0.307		0.461	0.677
		(log)ZTBARS	-1.284	0.303	-0.670	0.003	
		ZGSH-PxPCV	0.855	0.360	0.400	0.039	

Day	Model	Variable	Unstandardized coefficients		Standardized coefficients		
			B	Std. Error	Beta	Sig.	Adjusted R^2
25**	1	(Constant)	-0.554	0.376		0.163	0.366
		ZSOD	0.892	0.287	0.639	0.008	
	2	(Constant)	-0.305	0.344		0.361	0.515
		ZSOD	0.931	0.252	0.664	0.003	
		ZROM	-0.783	0.341	-0.414	0.039	
	3	(Constant)	-0.126	0.279		0.660	0.698
		ZSOD	1.314	0.236	0.942	0.0001	
		ZROM	-1.021	0.280	-0.540	0.003	
		ZGSH-PxPCV	0.872	0.292	0.516	0.011	
30	1	(Constant)	-0.229	0.550		0.684	0.364
		(log)ZTBARS	-1.642	0.547	-0.640	0.010	
	2	(Constant)	0.085	0.401		0.863	0.678
		(log)ZTBARS	-1.797	0.391	-0.700	0.001	
		ZROM	-1.680	0.454	-0.564	0.003	
	3	(Constant)	-0.057	0.222		0.802	0.903
		(log)ZTBARS	-1.651	0.217	-0.643	0.00001	
		ZROM	-2.034	0.258	-0.683	0.00008	
		(log)ZGSH-PxPl	1.278	0.239	0.467	0.0002	

* (log)ZTBARS was included in model 3 but is not shown because of its limited contribution to the Oxiscore (R^2 change = 0.073).

** (log)ZTBARS, (log)ZGSH-PxPl and ZGr-SH were included in further models but are not shown because of their limited contribution to the Oxiscore (R^2 change < 0.075).

FIGURES

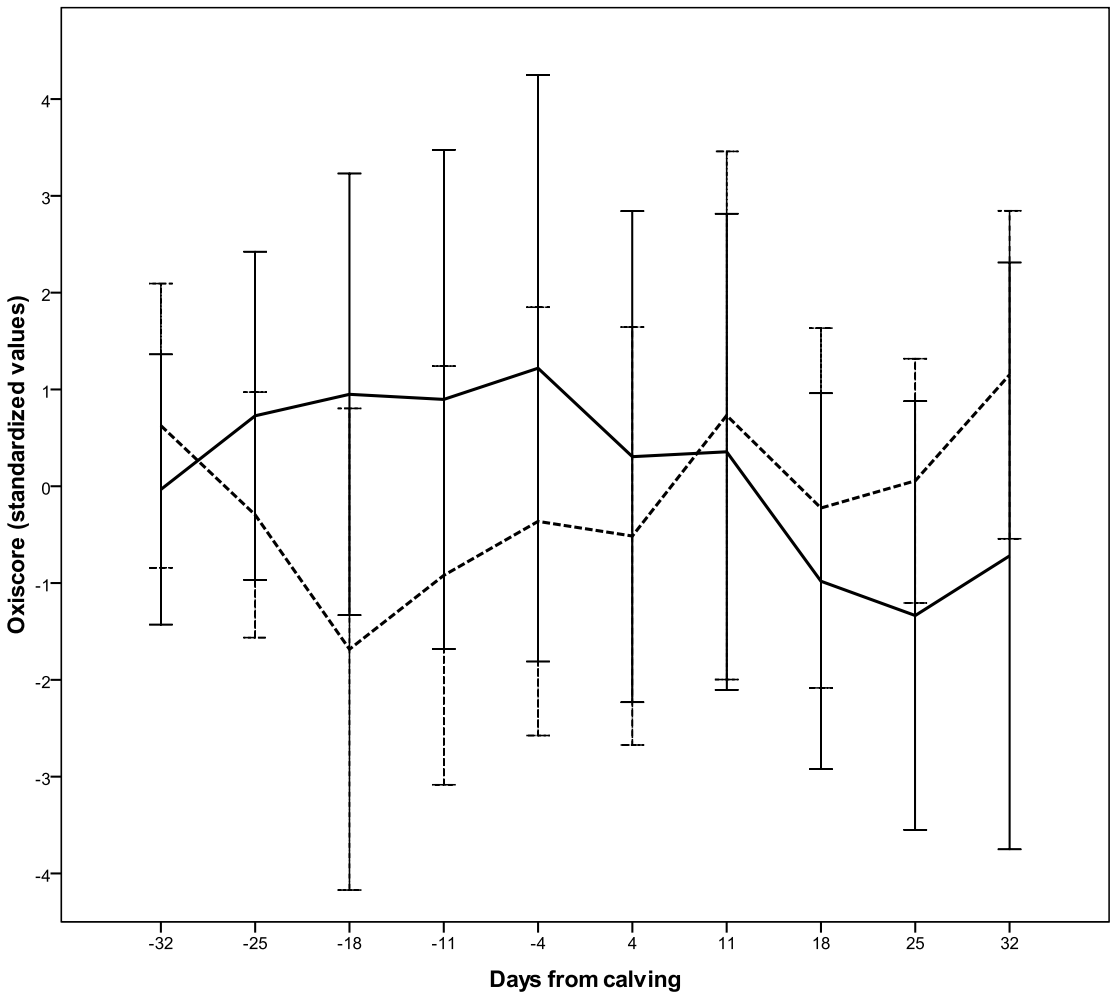

Figure 1 The Oxiscore in the periparturient period of HighBCS* (——) and LowBCS (- - -) ± standard deviation. The decrease between day -4 and day 25 of the HighBCS group is significant (P = 0.046).**
*** HighBCS = Body condition score of >2.5**
****LowBCS = Body condition score of <2.5**

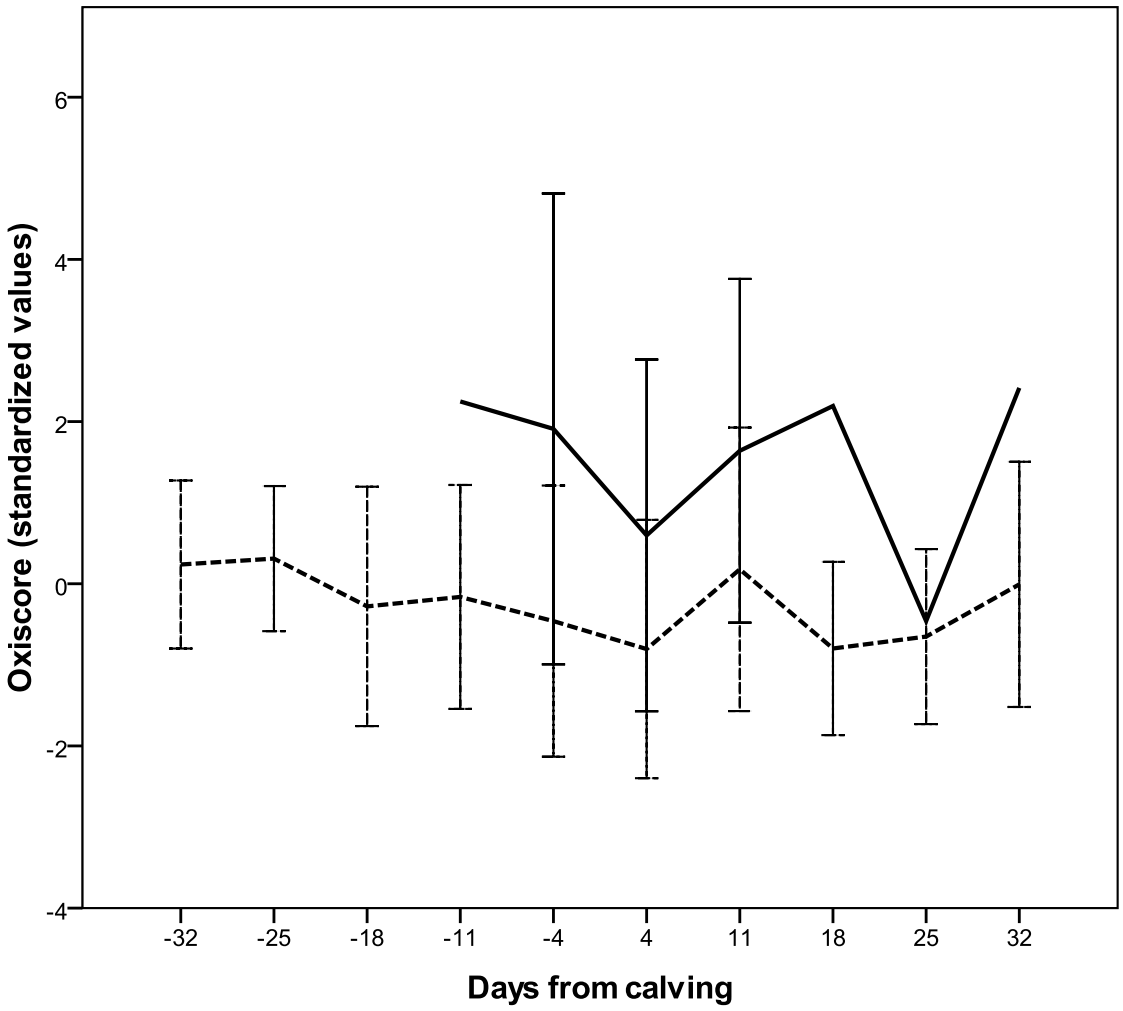

Figure 2 The Oxiscore in the periparturient period of cows with plasma non-esterified fatty acids (NEFAs) concentrations above 0.4 mEq/L (——) and NEFAs concentrations below 0.4 mEq/L (- - -) and 95% confidence intervals

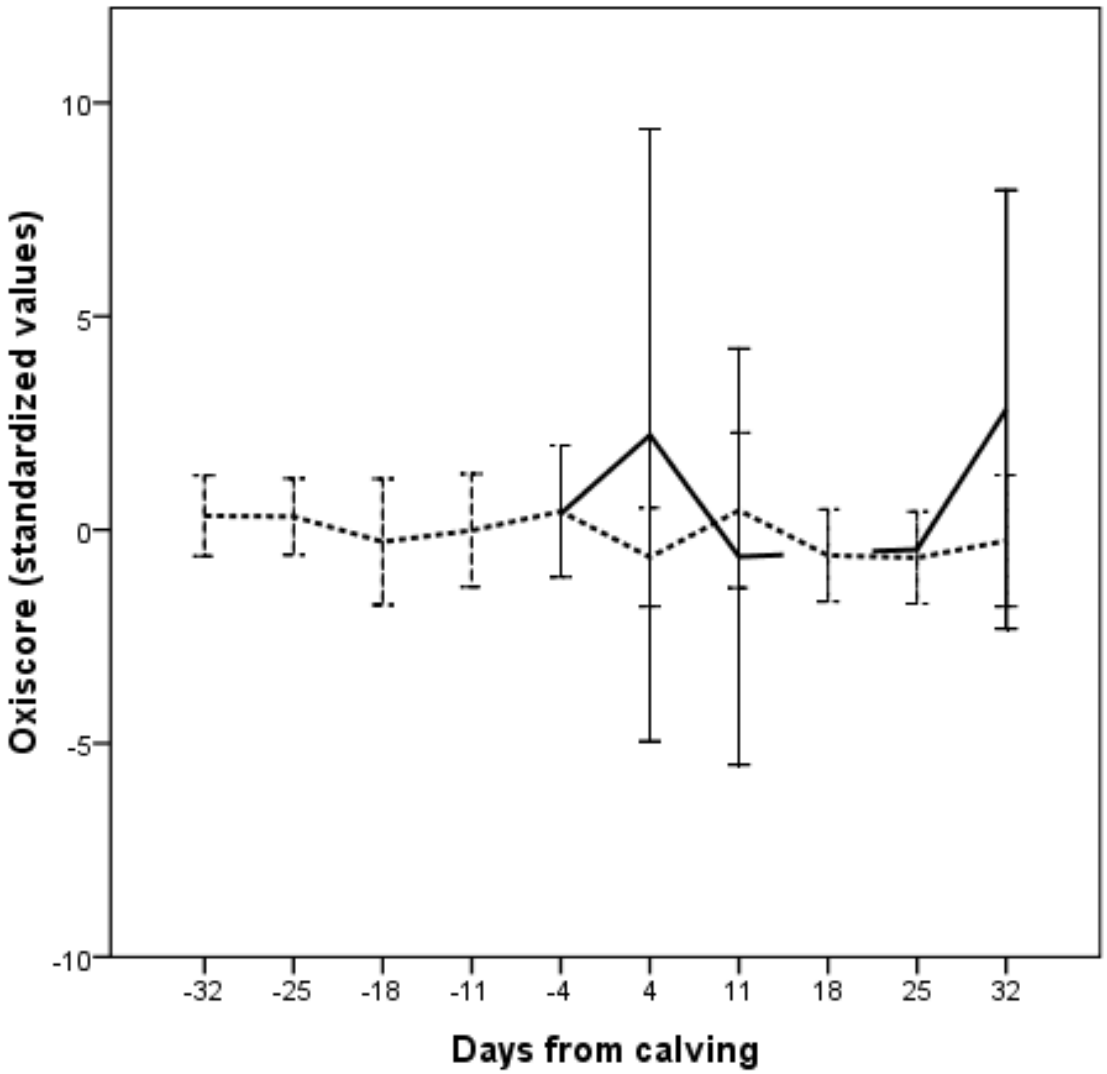

Figure 3 The Oxiscore in the periparturient period of cows with plasma β-hydroxybutyrate (b-HBA) concentrations above 0.9 mmol/L (——) and plasma b-HBA concentrations below 0.9 mmol/L (- - -) and 95% confidence intervals