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Relations between the oxidative status, *mastitis*, milk quality and disorders of reproductive functions in dairy cows - a review

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Free radicals are natural final products of the intensive metabolism in cells located in organism high-yielding dairy cows. When the disturbing of homeostasis occurs, oxidative processes lead to oxidative stress which in high-yielding dairy cows cause inflammation of the mammary gland (*mastitis*). The inflammation can cause the reduction of milk yield and unfavourable changes in the milk composition, e.g. reduction in fat, casein proteins and calcium content with a simultaneous increase in the concentration of whey proteins, sodium and chlorine. Moreover, the activity of enzymes such a lipases, proteases, peroxides and the plasminogen in milk increases, negatively affecting its technological properties. In dairy cows the oxidative stress is associated with retaining placenta after calving and disrupting the activity of the *corpus luteum*, what affect the reproductive functions. The active immune response to inflammation leads to an increase in the secretion of other molecules having an adverse effect on embryo survival.

KEY WORDS: cell /cow /mammary gland /mastitis /milk production /oxidative stress / reactive oxygen species

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The organism of a high-yielding dairy cow is often in the state of disturbed homeostasis caused principally by the generation and accumulation of free radicals, which are natural and inevitable final products of the intensive metabolism in cells, created in the mitochondrial chain of the transport of electrons or as a result of NADPH stimulation [Valko *et al.* 2007]. Free radicals, coming from the molecular oxygen and the peroxide anion, are known as reactive oxygen species (ROS). Under conditions of homeostasis, the ROS are effectively neutralized by cellular defence mechanisms (enzymes) or non-enzymatic antioxidants.

In case of disrupted homeostasis oxidative processes are outweighing non-oxidizable processes. Such a situation is called the oxidizing stress. Even relatively slight changes in the level of ROS inside cells, affect the rate of metabolism, gene expression, post-translation protein alterations and participate in regulating the cycle of cell division and the programming of apoptosis [Śliwa-Jóźwik *et al.* 2002, Crujeiras *et al.* 2008]. The oxidative stress, first found in the organism of high-yielding dairy cows, causes disorders of the course of many physiological processes, resulting in a weakening of the immune system and thus leading to the appearance of various diseases. The ROS cause oxidation of DNA as a result of which some mutations my occur [Poulsen 2005].

In some cases the ROS cause the tearing of one or of both DNA threads, what inevitably leads to the death of the cell. Oxidative of proteins leads to the inactivation of enzymes, while oxidizing fats – to the disintegration of cell membranes. This way ROS, modifying biologically active substances (e.g. DNA, fatty acids, peptides and vitamins), lead to the destruction of cells and whole tissue structures [Poulsen 2005, Bagnicka *et al.* 2008, Jóźwik *et al*. 2010a,b, Litwińczuk *et al.* 2011]. The rate of these transformations is shown by the fact that in every cell, the DNA influenced by ROS is subject to 10^4-10^5 alterations/day, 99.9% of these changes being removed in the enzymatic way. Both the rate and the scope of damage in cells depends on the balance of pro-/non-oxidizable processes.

The results of experiments conducted over recent years show that the oxidative stress was the principal factor causing dysfunction of the immune system of the organism and impairing the response to inflammatory conditions, what in consequence leads to numerous diseases in cows, above all to the inflammation of the mammary gland *(mastitis),* especially in the perinatal period [Wilde 2006, Jóźwik *et al.* 2012a]. A substantial amount of organism' cells is characterized by a very high sensitivity to the oxidative stress, which causes both their necrosis (the gradual decline of cellular structures and the disintegration of the cell membrane) and apoptosis (programmed death). Under the oxidative stress, which leads to a reduced resistance to the invasion of pathogenic micro-organisms, the probability of appearance of inflammatory conditions and especially of *mastitis* increases.

Oxidative stress and *mastitis*

The specificity of the oxidative stress and its relation to *mastitis* and other diseases is a complex question, to which no simple answers have been found as yet. One of the symptoms of *mastitis* is an increased NO level and a reduced content of ascorbic acid in the blood serum of animals [Sordillo *et al.* 2009, Jóźwik *et al*. 2012b]. This is in accordance with Kleczkowski *et al*. [2005], who reported, compared to healthy animals, a significant reduction of the concentration of ascorbic acid in the blood serum and milk of cows with a subclinical form of *mastitis* caused by an infection with *Staphylococcus aureus*, *Staphylococcus agalactiae* or *E. coli*. Moreover, in the erythrocytes of cows with *mastitis* a higher level of hydroperoxide of lipids was observed. The reduced content of ascorbates and increased content of lipid *peroxidation* products indicates that there is a relation between *mastitis* and the oxidizing stress. Calving and early lactation also lead to increased *peroxidation* of lipids [Castillo *et al.* 2006], what indicates the appearance of the oxidative stress in this period. The relation between *mastitis* and the appearance of the oxidative stress was confirmed in an experiment on the triggering of this disease after the introduction of *E. coli* into the udder [Weiss *et al.* 2004].

After infecting the udder with both *E. coli* or endotoxins of *E. coli*, in the blood plasma and in the milk of cows increase the contents of nitrates and nitrites. Moreover, in case of the latter infection with endotoxins the content of these substances is higher than in case of infection of *E. coli.* [Komine *et al.* 2004].

When an inflammation occurs, products which can damage tissues gather in the organism. Among products secreted by *neutrophils* is the $NO^{\cdot}$, which causes an acute or chronic reaction [Lykkesfeldt and Svendsen 2007], with $O_2^{-\cdot}$ creating ONOO-. This association has a destructive effect on tissues. Also Wessely-Szonder *et al*. [2004] demonstrated, that animals suffering from *mastitis* produce considerable amount of NO and the myeloperoxidase enzyme*, i.e*. substances that together may lead to the formation of nitrotyrosine, which has the ability to disintegrate proteins.

In case of an infection of the mammary gland by pathogenic micro-organisms polymorphonuclear cells (PMN) are transferred from the blood to the inflammation sites in the udder, what in turn increases the somatic cell count [SCC] of milk. An excess of *neutrophils*, macrophages, cells from the epithelium of the glandular tissue, lymphocytes and eosinophils is recognized as a measure of the rate of infection of the mammary gland by micro-organisms causing an inflammatory condition [Knnapen *et al.* 1999]. Moreover, in an infected mammary gland the level of such cytokines as TNF-α, IL-1 β, IL-6, IL-8 and other molecules like the NO increases [Notebaerta *et al.* 2008]. So, *mastitis* is a disease which not only reduces cows' productivity, but also leads to deterioration of the milk chemical composition and quality. In many countries the SCC content is treated as a criterion of the hygienic quality of bulk milk and affects the price paid.

In Europe, according to the EEC directive No. 92/46 from April 1992, bulk milk, in which the SCC exceeds 400 thousand/ml, cannot be designated for consumption in liquid

form, while since 1998 it cannot be consumed under any form. In the USA the limit for SCC in the bulk milk amounts to 750 thousand, and in Canada to 500 thousand/ml. The SCC of milk is an indicator of the oxidizing stress, since it shows a positive correlation with the content of *malone dialdehyde* (MDA) – Suriyasathaporn *et al.* [2006]. PMN generate the forming of peroxides (O2-) which on one side destroy pathogenic micro-organisms and on the other oxidize lipids, thus generating the formulation of MDA. Among factors favouring the appearance of the oxidizing stress, the energy value of the diet, milk yield and condition of the cows are mentioned most often.

A lower energy value of the diet contributes to a decrease of the amount of free radicals being formed [Lopez-Lluch *et al.* 2006]. However, high-energy diets rich in starch, can increase the probability of the appearance of the oxidative stress [Sgorlon *et al.* 2008] and thus support the appearance of *mastitis*. Glukogenic diets lead to the high blood-glucose level, contributing to an increased production of ROS and the risk of the appearance of the oxidative stress. The high level of blood glucose is related to an increased GSH-Px activity, an increased level of products of the oxidation of fats, for which TBARS is an indicator (tiobarbituric acid reactive substances) and with a low level of FRAP (ferric ion reducing antioxidant power), which indicates a low level of antioxidants. An increased oxidative stress takes place also in case of the reduction of the body weight of cows, associated with the utilization of storage fat.

The mentioned relation is confirmed by a negative correlation between the α-tocopherol content and the content of non-esterified fatty acids (NEFA) – Hidgon and Frei [2003]. The frequent milking of cows favours the increase in the number of epithelium cells in the milk due to the local stimulation of the proliferation of cells and reduction of their destruction in the process of apoptosis [Stefanon *et al.* 2002]. The increased production of milk, being the result of an increased milking frequency, leads to an increased demand for energy, what in turn generates an increase in ROS..

The results of many experiments indicate the existence of a negative relation between the level of milk production and the net energy balance in the organism of cows, susceptibility to diseases and their reproductive traits [Verkamp *et al.* 2003]. High-yielding dairy cows, particularly in the *perinatal* period, are susceptible to various health disorders. Physiological stress appearing during this period, associated with a drastic diversification of the secretory parenchyma, intensive growth of the mammary gland and the synthesis of milk, increases the demand for energy and oxygen [Gitto *et al.* 2002]. In turn, this increased demand is accompanied by an increased number of reactive oxygen forms what, in case of their overproduction, leads to the oxidative stress. A negative correlation exists between the available amount of antioxidants in the organism of the cow and the frequency of *mastitis*.

In the organism of the cow the non-oxidizable functions are performed also by vitamin A and β-carotene. However, reports concerning the relation between the blood plasma level of vitamin A and β-carotene in cows and the frequency of *mastitis* are not univocal and majority of them indicate that such a relation does not exist. Also, an addition of Cu to the diet of cows increases their resistance to *mastitis*. Scaletti *et*

al. [2003] showed that after infecting the mammary gland with *E. coli* the number of somatic cells and the total number of bacteria in milk decreased. Vitamin C, an *endogenic* antioxidant synthesised in the liver, also affects both frequency of *mastitis* and severity of its symptoms [Weiss and Hogan 2007].

It is suggested, that the etiology of *mastitis* is associated with the oxidative stress, which may be expressed either by the FRAP (ferric ion reducing antioxidant power) indicator in the blood plasma, or by the milk production and content of lactose of it [Bansal *et al.* 2005]. Both the FRAP indicator and the content of lactose in milk show the significance of antioxidants in retaining the health status of the cow mammary gland. A positive relation between the milk lactose content and the health of the mammary gland was demonstrated among others by Bansal *et al*. [2005]. A low lactose content indicates an infection in the udder. The correlation coefficient, calculated on the basis of the results reported by the authors cited, between the SCC and the content of lactose amounted to -0.25. Also, Chawla and Kaur [2004], showed that the elevated content of antioxidants in the blood of cows to a considerable degree protected them from metabolic diseases, including *mastitis*. This is confirmed by the coefficients of correlation between the SCC and the real milk yield, the ECM production and producton of milk fat, amounting to -0.22, -0.23 and -0.21, respectively.

According to Oltenacu and Algers [2005], the selection for high milk yield contributes to the reduction of the adaptation ability of contemporary high-yielding cows.

Oxidative stress and milk yield and quality

Due to the fact that a close correlation exists between the oxidative status and *mastitis* in cows a superiority of oxidizing processes can indicate a subclinical inflammation of the mammary gland. Depending on the size of the inflammation one may observe a reduction in milk yield and unfavourable changes in the milk composition [Jóźwik *et al.* 2004, Strzałkowska *et al.* 2009ab]. In the milk of cows afflicted with *mastitis* one may observe a reduction in fat, casein proteins and calcium content with a simultaneous increase in the concentration of whey proteins, sodium and chlorine. Moreover, the activity of enzymes from the group of lipases, proteases, oxidases and the plasminogen increases in the milk, negatively affecting its technological properties and eating quality of milk products [Sawa *et al.* 2008, Strzałkowska *et al.* 2010].

In such milk also bacterial enterotoxins may be found. The oxidation processes occurring in milk, beside lowering its nutritive value, have a negative effect on organoleptic parameters, principally taste, and cause the inactivation of many biologically active ingredients contained in it. As a rule, the increase in the number of somatic cells and bacteria in milk accompanies the inflammation of the mammary gland; both these indicators, as mentioned earlier, play a key role in the assessment of milk quality [Sawa *et al.* 2008, Bagnicka *et al.* 2011].

The most neuralgic period covers the first three weeks after calving, during which cows are especially exposed to the udder inflammation. Disorders appearing during that time can lead not only to a reduction of milk yield, but also adversely affect the health of the entire organism [Weiss 2005, Jóźwik 2010c].

The intensive metabolism observed during this period is accompanied by an increase in the amount of ROS generated, which when appearing in excess in relation to the non-oxidizable potential, can damage cells and tissues [Sordillo 2009].

In recent years attempts are made at finding indicators for the evaluation of commercial milk quality that would be: (1) more precise than the SCC criterion applied hitherto, (2) would enable a quick evaluation of the nutritive value and quality of milk, and (3) characterized by a superior quality and fulfilling the systematically growing consumer's demands [Weiss 2005, Litwińczuk *et al*. 2011].

Oxidative stress and reproduction

Only a few papers are available at present on the relation between the oxidative stress and reproduction functions of cows.

In dairy cows the oxidizing stress is associated with retaining placenta after calving, *oedema* of the udder and *mastitis*, what may indirectly affect the reproductive functions. Earlier Miller *et al*. [1993] showed that deficiency of antioxidants results in a lower muscle tone and thus poorer contraction of the uterus, what inhibits the transport of semen to the oviduct and causes a retained placenta. ROS, which are produced in steroidogenic cells and mononuclear phagocytes of the *corpus luteum*, affect luteolysis [Sugino 2006]. Moreover, ROS suppress the P4 synthesis through the inhibition of P450scc cytochrome, limiting the intracellular transport of cholesterol to mitochondria and injuring receptors of LH hormone [Sugino 2006]. Because ROS and SOD are present in the *corpus luteum*, there is a high probability that these substances are acting as local adjusters of the function of the *corpus luteum*.

In case of a redox balance disorder, the appearing metabolic stress can disrupt the activity of the *corpus luteum*, leading to premature luteolysis [Sugino 2006]. One of the reasons for an increased ROS content in rats with a delusion pregnancy and receiving PGF2 α is a decrease of the expression of Zn-SOD in the *corpus luteum* in the disappearance phase [Shimamura *et al.* 1995]. PGF2 α may also contribute to the increase in the ROS amount, since they stimulate the oxidation of lipids [Tanaka *et al.* 2000]. A hypothetical probability exists that also macrophages, at the moment of their activation, produce ROS and damage cells. An increase in their numbers may be observed at the *corpus luteum* regression stage, together with an increased production of cytokines which are suppressing the P4 production by the *corpus luteum* [Sugino 2006].

Development of embryos under oxidative stress

There is little information in the literature available on the relation between the oxidative stress and embryo mortality. ROS is generated during the process of normal embryo metabolism. Preventive action against their production and their destructive effect on cells and tissues is related to the defence mechanisms located both inside embryos (among others from SOD and GSH-Px) and outside them, in the environment of the oviduct with transferrin and ascorbic acid [Guerin *et al.* 2001]. However, in the situation of a constant high production of ROS during the embryo development, the probability of the appearance of the oxidative stress is high and it may lead to various damages of the embryos [Guerin *et al.* 2001].

ROS can prevent the preimplantation of embryos by increasing the lipid oxidation and DNA fragmentation [Takahashi *et al.* 2000] and by changing the structure and functions of enzymes or mitochondria, what may lead to the death of embryos [Agarwal *et al.* 2005]. The oxidizing stress can affect the mortality of embryos by triggering inflammations in the organism. This is confirmed by observations conducted on cows afflicted with *mastitis*, in which the response to an inflammatory condition or the immune reaction of the organism lead to the embryo death [Hansen *et al.* 2004]. The loss of embryos takes place as a result of the activation of many metabolic pathways, which interrupt the reproductive axis at several points, including the pituitary-subthalamic axis, ovaries, oocytes and embryos.

The main reason for embryo mortality that is related to *mastitis* is the increase in the production of cytokines, both in the mammary gland and in other tissues (in response to the signal obtained from the mammary gland), which modulate reproductive functions at several levels [Hansen *et al.* 2004]. On the one hand the active immune response to inflammation leads to an increased production of cytokines, which in turn increase the secretion of other molecules having an adverse effect of embryo survival [Hansen *et al.* 2004], on the other the elimination of a bacterial infection by way of phagocytosis results in an activation of n*eutrophils*, which once stimulated release of ROS to eliminate strange pathogens. In such a situation the lack of selective actions ROS may result also in the destruction of the host tissues [Lykkesfeldt and Svendsen, 2007].

Oxidative stress can be induced also by thermal stress. The increased environmental temperature causes an increase in free radicals created in the embryo organism, which delay or block their development [Sakatani *et al.* 2008]. Bernabucci *et al*. [2002] found more oxidative and non-oxidizable biomarkers in erythrocytes of cows calving in the summer, compared with those calving in spring. Also Di Trana *et al*. [2006], found a higher concentration of ROS in the blood plasma of goats during the summer period compared to the spring.

Oxidative stress, especially during the perinatal period, affects not only *mastitis*, but causes other disturbances in the health of cows. Placing the principal stress to the increase of milk production on the farms increases the susceptibility of cows to metabolic stress and diseases associated with it. This results in an increased demand for antioxidants.

A more comprehensive understanding of the relation between the redox status of the organism and the occurrence of *mastitis* could favour the elaboration of a more effective strategy for the prevention of diseases.

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