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der Freien Universität Berlin

Untersuchungen zur Bestimmung der Antioxidativen Kapazität des Blutes von Milchkühen

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Investigations to determine the antioxidative capacity in the blood of dairy cattle

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at Freie Universität, Berlin

submitted by

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from Dair Assaneh-Irbid /Jordan

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Dedicated to

My beloved Mother

and all my family

Contents

1 Introduction…… 13

2 Literature review…….. 15

2.1 Free radicals……..…….... 15

2.1.1 Definition……..…… 15

2.1.2 Classification of free radicals……... 16

2.1.2.1 Reactive oxidative metabolites……...… 16

2.1.2.2 Reactive oxygen substances……...…… 17

2.1.2.3 Reactive nitrogen substances…….…… 20

2.1.2.4 Free radical like substances…… 20

2.1.2.5 Iron and other metal ions…… 21

2.1.3 Production of free radicals…… 23

2.1.4 Biological role of free radicals…….…… 25

2.2 Antioxidants..... 26

2.2.1 Definition and classification of antioxidants……..…….. 26

2.2.2 Enzymatic antioxidants…….…….……. 27

2.2.2.1 Superoxide dismutase……. 27

2.2.2.2 Glutathione peroxidase……..... 28

2.2.2.3 Catalase……... 29

2.2.3 Non-enzymatic protein antioxidants…….…… 29

2.2.4 Non-enzymatic low molecular weight antioxidants……. 29

2.2.4.1 Vitamin C…….……... 31

2.2.4.2 β-Carotene…….…….. 32

2.2.4.3 Vitamin A……...…… 33

2.2.4.4 Vitamin E…… 34

2.2.4.5 Selenium..... 36

2.2.4.6 Vitamin D……...…… 37

2.2.4.7 Glutathione……...…….. 38

2.2.4.8 Uric acid…….. 38

2.2.4.9 Albumin……...…….. 39
2.2.4.10 Bilirubin…….. 39
2.2.4.11 Antioxidant interactions……..…… 40
2.3 Oxidative stress..... 43
2.4 Oxidative system in health and diseas……. 43
2.4.1 Mastitis……. 43
2.4.2 Retained fetal membranes……..... 45
2.4.3 Fertility and infertility…….. 46
2.4.4 Immunity…….…….…… 47
2.4.5 The periparturient period…..……. 49
2.4.6 Milk and meat quality……... 52
2.4.7 Carcinogenesis…..……. 52
2.4.8 Aging…… 53
2.4.9 Ischemia Reperfusion Injury…… 54
2.5 Antioxidative capacity……...…….. 54
2.5.1 Definition…….. 54
2.5.2 Components…….…. 55
2.5.2.1 Compartmentation……...……... 55
2.5.2.2 Detoxification……...……..…… 55
2.5.2.3 Repair…….…….…… 55
2.5.2.4 Utilisation……... 56
2.5.3 Antioxidative capacity in the plasma……..…… 56
2.6 Measurement of antioxidative capacity…….…….. 57
2.6.1 In vitro tests……..…… 57
2.6.2 Photochemiluminescent detection……...…… 59
3 Materials and methods……..…… 61
3.1 Materials…….. 61
3.1.1 Animals……..……... 61
3.1.2 Blood sampling…….……... 61
3.2 Methods…….…….. 61
3.2.1 Reagents (ACW)……..…… 61
3.2.2 Reagents (ACL)…… 62
3.2.3 Preparation of the working solution (ACW)……..…….. 62
3.2.4 Preparation of the working solution (ACL)…….…… 62

3.2.5 Sample preparation (ACW)…… 63
3.2.6 Sample preparation (ACL)…… 63
3.3 Measuring principle…… 64
3.4 Calculating the antioxidative capacity 64
3.5 Statistics 64
4 Results…… 65
4.1 Water soluble antioxidative capacity (ACW)…… 65
4.1.1 Medium determination…… 65
4.1.2 The effect of time on the stability of ACW…… 68
4.1.2.1 Stability in the four different media…… 68
4.1.2.2 Stability under two different temperatures…… 71
4.1.2.3 Stability under five different temperatures…… 73
4.1.3 Stability under two different temperatures…… 77
4.1.4 Stability under five different temperatures…… 79
4.1.5 Centrifugation and time effect on ACW…… 83
4.1.6 Farm control…… 84
4.2 Fat soluble antioxidative capacity (ACL)…… 94
5 Discussion…… 95
5.1 Water soluble antioxidative capacity (ACW)…… 95
5.1.1 Medium determination…… 95
5.1.2 The effect of time on the stability of ACW…… 96
5.1.3 Temperature and ACW…… 97
5.1.4 Centrifugation and ACW…… 98
5.1.5 Farm control…… 98
5.2 Fat soluble antioxidative capacity (ACL)…… 100
5.3 General aspects and future work…… 100
6 Conclusion…… 101
7 Summary…… 102
8 Zusammenfassung…… 103
9 References…… 104

Abbreviations

AA	Ascorbic acid
ABTS	2, 2‘Azinobis (3
ACL	Antioxidative capacity in lipid
ACW	Antioxidative capacity in water
AO	Antioxidant
BAP	2, 2‘–azo-bis-(2-Amidinopropane)
BCS	Body condition score
CAD	Coronary artery disease
DMI	Dry matter intake
EDTA	Etheylindiamine tetraacetic acid
FF	Follicular fluid
GSSG	Glutathione disulfide
GSH	Glutathione
GSH-Px	Glutathione peroxidase
HO	Heme oxygenase
Hv	light/irradiation
HPLC	High performance liquid chromatography
IVF	In vitro fertilization
IVM	In vitro oocyte maturation
LA	Lipoic acid
L·+	Luminol-radical
L	Luminol
L*	Excited state
µl	Microliteres
µM	Micromole
NOS	Nitric oxide synthase
OF	Oxidised flavour
PMN	Polymorphonuclear neutrophils
PUFA	Poly unsaturated fatty acids
PCL	Photoinduced chemiluminescence

pp	Postpartum
Redo	Oxidation reduction
RFM	Retained fetal membranes
ROM	Reactive oxidative metabolites
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
SCC	Somatic cell count
Se	Selenium
TBARS	Thiobarbituric acid
TEAC	Trolox-Equivalent Antioxidant Capacity Assay
TRAP	Total radical antioxidant prameter
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
TPA	Tetradecanoyl phorbol acetate

1 Introduction

Living organisms are continuously exposed to reactive species since the generation of ATP from molecular oxygen demands electrons. This paradoxical need for a toxic source of energy molecule is central to the Life Sciences since organisms must continuously battle to keep an appropriate balance of prooxidants and antioxidants, which results in a status of good health and high milk production of cows.

Free radicals, which are reactive chemical species with an odd number of electrons inducing damage to lipids, proteins, carbohydrates and DNA, are involved in the aetiopathogenesis of civilization diseases such as atherosclerosis, cancer and diabetes. In physiological processes they are normally in a steady state with antioxidants. This antioxidative homeostasis is maintained by the antioxidative system of the organism, regulating absorption, synthesis, activation, release and excretion of exogenous and endogenous antioxidants (**Popov and Lewin, 2005**).

The recent growth in knowledge of free radicals and reactive oxygen species (ROS) in biology is producing a medical revolution that promises a new age of health. In fact, the discovery of the role of free radicals in chronic degenerative diseases is as important as the discovery of the role of microorganisms in infectious diseases (**Chance and Boveris, 1987)**.

During the last 3 decades, much attention has been focused on determining the mechanisms by which antioxidants protect cells from oxygen radicals and other activated oxygen species (**Malpes and Mason, 1989**).

Several methods have been developed to assess the total antioxidant capacity of human Serum or plasma because of the difficulty in measuring each antioxidant component separately and the interactions among different antioxidant components in the Serum or plasma (**Cao and Prior, 1998**). One of these methods is the method of photosensitized chemiluminscence (PCL) that allows the quantification of water -and lipid-soluble antioxidants using the PHOTOCHEM which was developed by Analytik Jena, Germany, which is used in this study to:

- Compare between different media (tubes) which are used for the transport of blood from the field to the laboratory or for immediate testing when the utilities are available and to choose the most suitable of these media.
- Evaluate the water- and lipid-soluble antioxidative capacity in the blood of dairy cattle.

- Evaluate the stability of water- and fat-soluble antioxidative capacity in the appropriate medium.
- Evaluate the reproducibility of this method in measuring water- and fat-soluble antioxidative capacity.

2 Literature review

2.1 Free radicals

The presence of free radicals in biological materials was discovered less than 50 years ago. Soon thereafter, it was hypothesized that oxygen radicals may be formed as by-products of enzymic reactions in vivo. In 1956, free radicals were described as a Pandora's Box of evils that may account for gross cellular damage, mutagenesis, cancer, and, last but not least, the degenerative process of biological aging. The science of free radicals in living organisms entered a second era after **McCord and Fridovich** (**1969**) discovered the enzyme superoxide dismutase (SOD), after that the roles of the different free radicals such as superoxide hydroxyl radical was discovered and more interest has been paid to this field since that time (**Dröge, 2002**).

2.1.1 Definition

A free radical is an atom, molecule, or compound that is highly unstable because of its atomic or molecular structure (i.e., the distribution of electrons within the molecule). As a result, free radicals are very reactive as they attempt to pair up with other molecules, atoms, or even individual electrons to create a stable compound. To achieve a more stable state, free radicals can steal hydrogen atoms from another molecule, bind to another molecule, or interact in various ways with other free radicals (**Wu and Cederbaum, 2003**).

Free radicals are highly reactive molecules or chemical species capable of independent existence with one or more unpaired electrons. The unpaired electron results in a species that is often highly reactive (**Darley-Usmar and Halliwell, 1996**). A subscript dot is used to denote a free radical. Examples include the oxygen centred radicals superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet -}$) (**Darley-Usmar and Halliwell, 1996**).

These compounds can react with enzymes, cell membranes and DNA and cause cell damage or cell death (**Weiss, 2005**).

Free radicals and reactive non-radical species derived from radicals exist in biological cells and tissues at low but measurable concentrations. Their concentrations are determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes, as illustrated schematically in Figure 1 (**Dröge, 2002**).

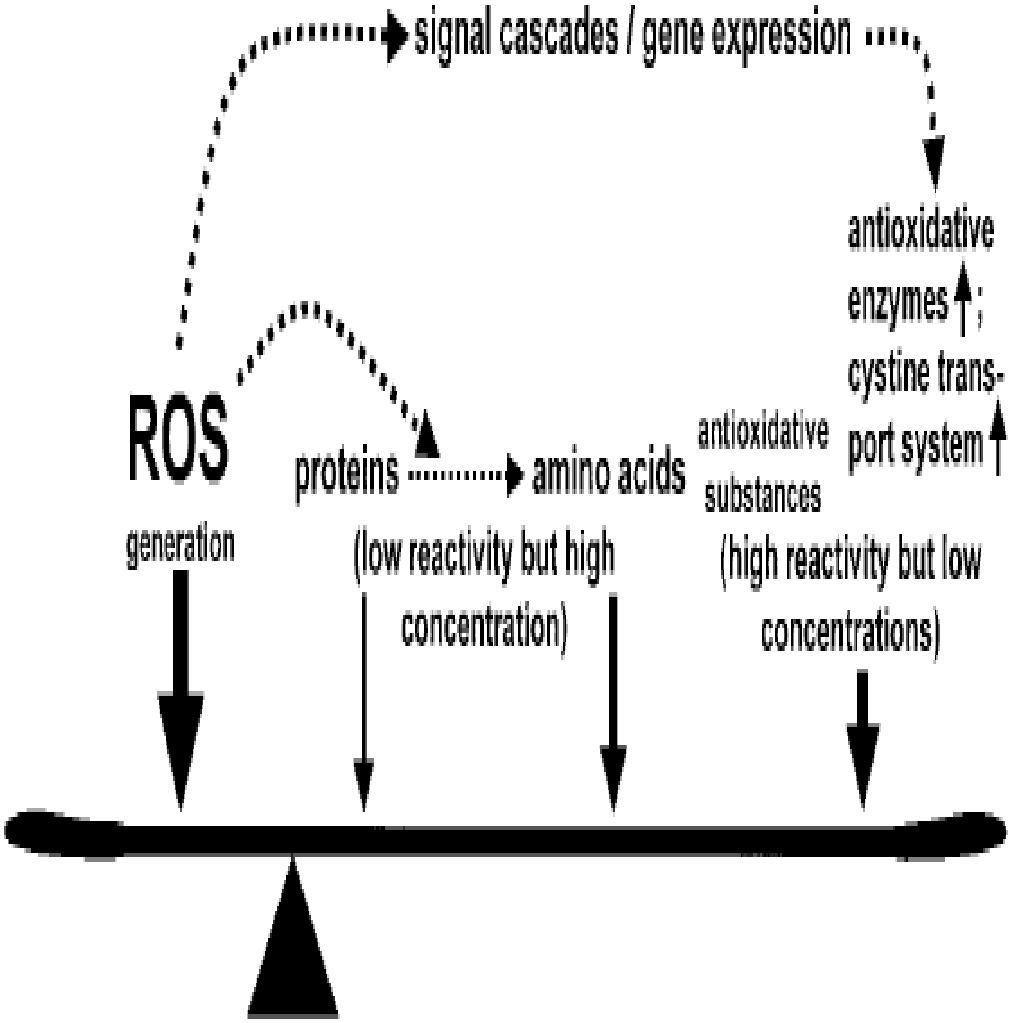

Figure1. Mechanisms of redox homeostasis balance between ROS production and various types of scavengers (Dröge, 2002).

2.1.2 Classification of free radicals

2.1.2.1 Reactive oxidative metabolites (ROM)

Biologists are using the term reactive oxidative metabolites to refer not only to oxygen radicals but also to a number of related species such as H_2O_2, and reactive nitrogen metabolites, which do not contain unpaired electrons but are often involved in the generation of free radicals and can be classified into:

- Reactive oxygen specie.

- Reactive nitrogen species.
- Free radical-like substances.
- Iron and other metal ions. (Table 1).

Table1. Free radicals and radical-like species (Darley-Usmar and Halliwell, 1996).

Reactive Oxygen species	
Radicals	**Non-radicals**
Superoxide ($O_2^{\bullet -}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl ($OH^{\bullet -}$)	Hypochlorous acid (HOCL)
Peroxyl ($RO_2^{\bullet}$)	Ozone (O_3)
Alkoxyl ($RO^{\bullet}$)	Singlet oxygen ($(^1O_2$)
Hydroperoxyl ($HO_2^{\bullet}$)	Peroxynitrit ($ONOO^-$)
Reactive Nitrogen Species	
Radicals	**Non-Radicals**
Nitric oxide ($NO^{\bullet -}$)	Nitrosyl(NO^+)
Nitrogen dioxide ($NO^{\bullet}{}_2^-$)	Noitrooxide (NO^-)
	Nitrous acid(HNO_2)
	Dinitrogen trioxide (N_2O_3)
	Dinitrogen tetraoxide (N_2O_4)
	Nitronium ion(NO_2^+)
	Peroxynitrite($ONOO^-$)
	Alkyl peroxynitrites (ROONO)

2.1.2.2 Reactive oxygen substances (ROS)

The term reactive oxygen metabolites has been applied to oxygen-cantered free radicals and their metabolites, they are unavoidable products of normal metabolic processes and are not always harmful (**Miller and Brzezinska-Slebodzinska, 1993**).

The major ROMs found in biological systems are superoxide, hydrogen peroxide, hydroxyl radical, and fatty acid radicals (**Weiss, 2005**) (Table1).

Oxygen is a vital substrate for aerobic energy generation in the biological systems of higher animals. However, small quantities of toxic substances known as free radicals are generated during this biochemical process. These compounds have enormous capacity to oxidize biological structures, notably the membranes rich in lipids and proteins, and depending on the degree of the damage, cause cellular necrosis (**Yonezawa et al., 2005**).

Oxygen-derived radicals are produced constantly through normal aerobic life. In the mitochondria, they are formed as oxygen is reduced along the electron transport chain. Also reactive oxygen species are a formed as intermediates in some of enzyme reactions. In some situations, oxygen radicals are overproduced:

- White blood cells such as neutrophils specialize in producing oxygen radicals, which are used in host defence to kill invading pathogens.
- Exposure of cells to abnormal situations such as hypoxia or hyperoxia generates abundant and often damaging reactive oxygen species, and some drugs.
- Ionizing radiation is well known to generate oxygen radicals within biological systems.
- $O_2^{\bullet -}$ production usually involves a one-electron reduction of molecular O_2. The negatively charged $O_2^{\bullet -}$ radical is unstable in aqueous solution (half-life of a few seconds) and is rapidly dismutated to H_2O_2. It is poorly cell membrane permeable and is generally restricted to the cell compartment in which it is produced. It can undergo several chemical reactions depending on the amount generated and the localization and proximity to other radicals and enzyme (**Jian-Mei and Shah, 2004**).

The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen ($^3O_2$), this process is mediated by enzymes such as NAD (P) H oxidases and xanthine oxidase or nonenzymically by redoxreactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. SODs convert superoxide enzymically into hydrogen peroxide. In biological tissues superoxide can also be converted nonenzymically into the non-radical species hydrogen peroxide and singlet oxygen ($^1O_2$). In the presence of reduced transition metals (e.g., ferrous or cuprous ions), hydrogen peroxide can be converted into the highly reactive hydroxyl radical ($OH^{\bullet}$) alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. In the glutathione peroxidase reaction glutathione is oxidized to glutathione disulfide, which can be converted back to glutathione by glutathione

reductase in an NADPH-consuming process. $O_2^{\bullet-}$can act both as an oxidant and as a reductant, and can give rise to other dangerously reactive substances (**Fridovich, 1989**).

In summary, $O_2^{\bullet-}$may

- Serve as a precursor for other ROS such as H_2O_2 and thereby act as a regulatory mediator in signalling processes leading to altered gene transcription and protein and enzyme activities (so-called "redox signalling").
- Rapidly inactivate $NO^{\bullet}$, thereby causing endothelial dysfunction.
- Cause oxidative damage of macromolecules, membranes, and DNA usually indirectly through the generation of more toxic (reactive) radicals such as $ONOO^{\bullet-}$ and $OH^{\bullet}$ (**Jian-Mei and Shah, 2004)** (Table 2).

Table 2. Characteristics of the main reactive oxygen species (ROS) (Ricardo et al., 2002).

ROS	Symbol	Characteristics
Superoxide	$O_2^{\bullet-}$	Intermediate in O_2 reduction to H_2O. Good reductant and bad oxidant, it is important because generates more ROS, such as $OH^{\bullet}$ and H_2O_2
Hydroxyl	$HO^{\bullet}$	The most powerful oxidant in biological systems, it is generated from Fenton and Haber-Weiss reactions
Peroxyl	ROO	Low oxidant ability, but high diffusibility
Alkoxyl	$RO^{\bullet}$	Medium oxidant ability with lipids
Hydrogen peroxide	H_2O_2	Originated from O_2 dismutation by the SOD enzyme
Hypochlorous acid	HCLO	Formed through Mieloperoxidase action, it is present in neutrophils on H_2O_2
Singlet oxygen	$^1O_2$	Molecularly excited oxygen through sunlight and radiation, highly oxidant

2.1.2.3 Reactive Nitrogen Species (RNS)

Reactive nitrogen species are highly reactive chemicals that contain nitrogen, having the ability to react easily with other molecules, resulting in potentially damaging modifications.
The radical ($NO^{\bullet -}$) is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine. This process is catalyzed by the enzyme Nitric oxide synthase (NOS). Depending on the microenvironment, $NO^{\bullet -}$ can be converted to various other reactive nitrogen species (RNS) such as nitrosonium cation (NO^{+}), nitroxyl anion ($NO^{\bullet -}$) or peroxynitrite ($ONOO^{-}$). Some of the physiological effects may be mediated through the intermediate formation of S-nitroso-cysteine or S-nitroso-glutathione (**Dröge, 2002**).
The small, light, and simple molecule nitric oxide ($NO^{\bullet -}$) was once regarded only as a noxious environmental pollutant in cigarette smoke, smog, and the exhaust from motorcars, destroying the ozone layer and causing acid rain. This negative reputation of $NO^{\bullet -}$ changed when in the 1980s several lines of research showed that $NO^{\bullet -}$ is an essential molecule in the physiology of the human body (**Ricciardolo et al., 2004**).
Since its discovery as a biologic messenger molecule just over a decade ago, nitric oxide ($NO^{\bullet -}$ has become well recognized for its participation in diverse biologic processes in nearly all aspects of life, including vasodilatation, bronchodilation, neurotransmission, inhibition of phagocyte and platelet aggregation, and antimicrobial activity (**Ricciardolo et al., 2004**).
Reactive nitrogen species are involved in inflammatory reactions, and are implicated as mediators of B-cell destruction (**Gille et al., 2002**) (Table. 1).

2.1.2.4 Free radical-like substances

Several non-radical oxidants are important when considering oxidative modifications in the vessel wall. The most abundant of these is hydrogen peroxide (H_2O_2) derived from the action of oxidases such as glucose oxidase on O_2, or from the dismutation of $O_2^{\bullet -}$ (**Stocker and Keaney, 2003**) (Table 3).

Table 3. Examples of free radical-like substances (Modified from Stocker and Keaney, 2004).

Name	Function	Comments
Hydrogen peroxide	H_2O_2	A diffusible oxidant that is only a weak oxidizing agent and is generally poorly reactive. It may participate in cellular signalling and, in the presence of available transition metals, can give rise to $OH^{\bullet -}$.
Hypochlorite, hypochlorous acid	OCl^-, HOCl	Weak acid but strong oxidant. Reacts with Fe-clusters, metal ions held in proteins by thiolate ligands, heme, amino acid residues (methionine, cysteine) of proteins, and GSH. Can give rise to secondary, reactive species including chloramines and amino acidderived aldehydes.
Singlet oxygen	$^1O_2$	Reacts with other molecules chemically or by transfer of its excitation energy.
Ozone	O_3	Strong oxidant that attacks protein and lipids including cholesterol.

2.1.2.5 Iron and other metal ions

Iron can also damage tissues by catalysing the conversion of superoxide and hydrogen peroxide to free radical species that attack cellular membranes, proteins and DNA (**Gutteridge et al., 1982**). Proteins sequester iron to reduce this threat. Iron ions circulate bound to plasma transferrin, and accumulate within cells in the form of ferritin. Under normal circumstances, only trace amounts of iron exist outside these physiologic sinks. In the healthy state there is never an appreciable concentration of 'free iron' or iron chelated by low molecular wieght compounds. Any released Fe (II) is immediately chelated in cells by compounds such as citrate or adenosine diphosphate and this 'free iron or labile iron' could

participate in the Haber –Weiss chemistry, catalyzing the formation of the hydroxyl radical ($OH^{\bullet}$).

$O_2^{\bullet -} + Fe^{+3} + 2H^{+} \rightarrow O_2 + Fe^{+2}$

$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{\bullet} + HO^{-}$

The $OH^{\cdot}$ is capable of abstracting a hydrogen atom from polyunsaturated fatty acids (LH) to initiate lipid peroxidation.

$OH^{\cdot} + LH \rightarrow H_2O_2 + L^{\bullet}$

$L^{\cdot} + LH \rightarrow LOO^{\cdot}$

$OO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$

Once lipid hydroperoxides (LOOH) accumulate, free iron may directly initiate additional lipid peroxidation.

$Fe^{+2} + LOOH \rightarrow Fe + LO^{\cdot} + OH^{-}$

$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot}$ etc

The resulting accumulation of lipid hydroperoxides destroys membrane structure and function. The radical $OH^{\bullet}$ is very highly reactive and its estimated half-life in cells is only 10^{-9} seconds, and it can damage lipids, proteins, DNA, sugars and generally all organic molecules (**Nelson and McCord, 1998**).

Transition metal ions, especially iron and copper, are powerful promoters of oxidative damage in endothelial and other cells, because they can accelerate lipid peroxidation, and catalyze $OH^{\bullet}$ formation. But how do they become available in "catalytic" forms: organisms usually take great care in the handling of iron, using both transport (such as transferrin) and storage (such as ferritin and haemosiderin) proteins so as to minimize the amount of "catalytic" iron within cells and in extracellular fluids. The same is true of copper. This careful sequestration of transition metal ions is an important contribution to antioxidant defence. However, one consequence of excess formation of ROS or RNS is the release of catalytic iron and copper ions. Thus $O_2^{\bullet -}$ can mobilise iron from the storage ferritin, although

the amount of superoxide-releasable iron is small, and so ferritin bound iron is much safer than an equivalent amount of free iron. H_2O_2 can degrade haem proteins to release iron and haem proteins, both of which can catalyze lipid peroxidation, generating free radicals that propagate this process (**Darley-Usmar and Halliwell, 1996**).

The $OH^{\bullet -}$ is capable of abstracting a hydrogen atom from polyunsaturated fatty acids (LH) to initiate lipid peroxidation.

2.1.3 Production of free radicals

Aerobic life is characterized by a steady formation of reactive oxidative metabolites (ROM). These species include oxygen-derived free radicals: superoxide $O_2^{\bullet -}$, hydroxyl $OH^{\bullet}$ and nitric oxide $NO^{\bullet}$ and non-radical derivatives of oxygen: hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). The generation of ROS during the respiratory burst represents an important pathogenic mechanism for tissue damage and diseases associated with phagocytic infiltration. Phagocytic cells, predominantly polymorphonuclear leucocytes (PMNLs), when appropriately stimulated can release ROS. Fibroblasts, vascular endothelial cells and osteoclasts also produce ROS (**Baltacioglu et al., 2006**).

Free radicals are produced either by normal physiological processes or because of the influence of exogenous species. These exogenous species may be compounds that occur naturally in the biosphere (e.g. ozone, NO_2, ethanol, or tetradecanoyl phorbol acetate (TPA)), industrial chemicals that are purposefully synthesized by man (e.g. carbon tetrachloride) or xenobiotics that are inadvertently produced by man's activities (e.g. benzo (a) pyrene) (**Dröge, 2002**) (Table 4), (Figure 2).

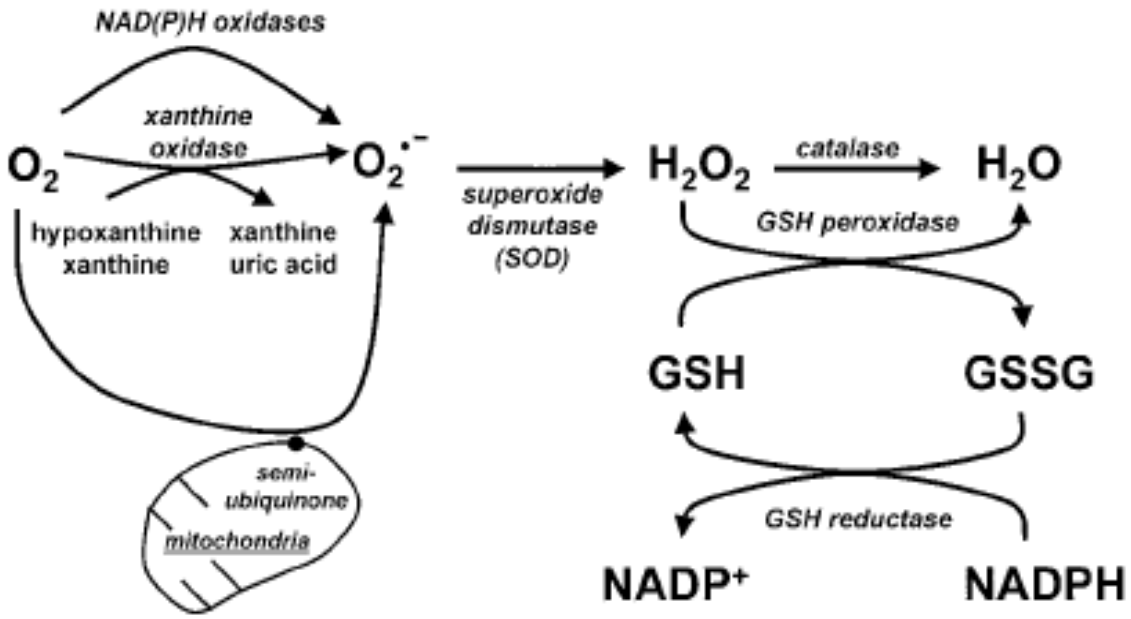

Figure2. Pathways of ROS production and clearance (Dröge, 2002).

Table 4. Some sources of free radicals.

Internal sources
Mitochondria
Phagocytes
Xanthine oxidase
Reactions involving iron and other transition metals
Arachidonate pathways
Peroxisomes
Exercise
Inflammation
Ischæmia/reperfusion
External sources
Cigarette smoke
Environmental pollutants
Radiation
Ultraviolet light
Certain drugs, pesticides, anaesthetics, and industrial solvents
Ozone

Normal cellular metabolism is a process of controlled electron flow that produces energy (ATP) and reducing equivalence to be used for cell synthetic processes (NADH and NADPH). Metabolic processes also produce free electrons (free radicals) which result in uncontrolled electron flow and may disrupt cell membranes, protein function, DNA structure and energy production (**Hurley and Doane, 1989**).

The mitochondrial respiratory chain can be a major source of $O^{\bullet -}$ (**Lenaz, 1998**). Potential sources of endothelial $O_2^{\bullet -}$ generation that are implicated in disease processes include mitochondria, xanthine oxidase (XO), uncoupled NO synthases, cytochrome P-450 enzymes, and NADPH oxidases. In addition, enzymes such as lipoxygenases may also generate $O_2^{\bullet -}$ (**Dröge, 2002**).

2.1.4 Biological role of free radicals

The presence of free radicals in biological materials was discovered less than 50 years ago it was hypothesized that oxygen radicals may be formed as by-products of enzymic reactions in vivo (**Dröge, 2002**).

Because free radicals are so reactive, and because their lifetime generally is very short, their very existence has often been clouded in acrimonious debate. The role of free radicals in biological systems was, if possible, even more controversial. In the early 1960s, in fact, most biochemists and biologists believed that free radicals were much too short-lived and uncontr-ollable to play any role in life processes. All of this was dramatically changed when **Joe Mc-Cord and Irwin Fridovich** reported the properties of the enzyme superoxide dismutase (SOD) in **1968** (**Pryor and Davies, 2005**).

Once generated free radicals interact with other molecules through redox reactions to obtain a stable electronic configuration. In a redox reaction, electron transference between the participating chemical species will take place. One loses free electrons (oxidation process) and the other gains them (reduction process). The oxidation of one chemical species implies the reduction of another. The molecule losing electrons is a reducing agent, while the molecule gaining electrons is an oxidant agent. When a free radical reacts with a non-radical molecule, it can loose or gain electrons or simply join the molecule. In any case the non-radical molecule turns into a free radical and chain reaction is triggered: one free radical generates another free radical. The reaction will stop only when two free radicals meet (**Richardo et al., 2002**).

Endothelial cells generate reactive oxidative metabolites (ROM), including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), NO, peroxynitrite ($ONOO^{\bullet -}$), hydroxyl radicals ($OH^{\bullet -}$), and other radicals (**Didion et al., 2002**).

The best known free radical produced by the vascular system is $NO^{\bullet}$, which plays a key role in vasodilatation and platelet aggregation/adherence (**Moncada and Higgs, 1995**).

Oxygen-derived free radicals play a role in a wide variety of pathological conditions in almost every animal species, especially in domestic animals. Besides the attack on cellular protein and nucleic acid, the pathogenic role of free radical is also ascribed to lipid peroxidation, which is a continuous biological process, highly detrimental to membrane structure and function (**Avellin, 1993**).

2.2 Antioxidants

2.2.1 Definition and classification

Antioxidants are molecules that can easily and harmlessly give up an electron. Nature produces an array of antioxidants to prevent free radical formation or to limit their damaging effects in cells. These include enzymes to decompose peroxides, proteins to bind transition metals, and other compounds that scavenge free radicals. The most important biological antioxidants are vitamins A, C, E and selenium, a key component of glutathione peroxidase. Vitamin A and or other carotenoids are abundant in many animal feeds and inexpensive to supplement. Vitamin C is produced naturally in the tissues of farm animals and thus is not routinely supplemented (**Berger, 2003**).

An oxidizable substrate might be a lipid, DNA, protein or any molecule found in vivo. Antioxidants are interrelated and may prevent oxidant damage in several ways: scavenging of ROS; decreasing the conversion of less reactive ROS to more reactive ROS; facilitating repair of damage caused by ROS; and providing an environment favourable for activity of other antioxidants (**Clarkson and Thompson, 2000**).

In general, the endogenous antioxidants can be divided into 3 major groups:

- Enzymatic antioxidants.
- Non-enzymatic protein antioxidants.
- Non-enzymatic low-molecular weight antioxidants.

2.2.2 Enzymatic antioxidants

The classic antioxidant enzymes are largely cell-associated proteins whose function is to maintain a reducing tone within cells; they may also be involved in the maintenance of extracelluar antioxidants (**Stocker and Keaney, 2003**).
Including SOD, GSH-Px and catalase represents the main form of intracellular antioxidant defence (**Bernabucciet al., 2005**) (Table 5).

Table 5. Primary antioxidant enzymes.

Component(Location in cell)	**Nutrients involved**	**Function**
Superoxide dismutase (cytosol)	Copper and zinc and Mn	An enzymes that converts superoxide to hydrogen peroxide
Glutathion peroxidase (cytosol)	Selenium	An enzyme that converts hydrogen peroxide to water
Catalase	Iron	An enzyme that converts hydrogen peroxide to water and oxygen

2.2.2.1 Superoxide dismutase

The first line of defence is composed of enzymes, such as superoxide dismutase (SOD), that catalyse the conversion of ROS to less reactive species (**Wall, 2002**).
There are three forms of SOD in mammalian systems: the copper-zinc (Cu, Zn-SOD), Mn-SOD, and extracellular SOD (EC-SOD). The copper-zinc enzyme is present in virtually all cells, where most of it is located in the cytosol, with some activity in lysosomes, peroxisomes, nucleus, and the space between inner and outer mitochondrial membrane. The copper ion functions in the dismutation reaction by undergoing alternate oxidation and reduction, Manganese-containing SOD is largely located in the mitochondria, is cyanide insensitive, and contributes 10% of total cellular activity. As indicated by its name, EC-SOD is an extracellular form of the enzyme. It also contains copper zinc and is a notable exception in that significant amounts of this antioxidant enzyme are present in the normal arterial wall outside cells (**Stocker and Keaney, 2003**).

Activities of EC-SOD within extracellular fluids are extremely low and of little biological relevance (**Baltacioglu et al., 2006; Chaudiere and Ferrari, 1999)**.

This enzyme is a metalloprotein present in aerobic cells and extracellular fluids. Its function is to catalyze superoxide dismutation into hydrogen peroxide, which does not require cosubstrates (**Bandyopadhyay et al., 1993; Chaudiere and Ferrari, 1999**).

For most mammals Cu, Zn-SOD activity is highest in liver tissues followed by kidney, heart, lung, and brain (**Halliwell and Guttridge, 1989**).

The significance of SOD in the aging process, in the etiology of certain diseases, and in ecology has been demonstrated in different systems. SOD levels have been manipulated by molecular techniques by the use of selective inhibitors, by hormones, and by nutritional means to study its functional role (**Ahmad, 1995**).

2.2.2.2 Glutathione peroxidase

H_2O_2 can also be destroyed by glutathione peroxidase which is considered as a selenium dependent peroxidase.

Glutathione peroxidase (GSH.Px) is one of the most important water-soluble antioxidants, not only because of its action as a scavenger, but also as an indispensable factor in proper catalytic action of some antioxidative enzymes. GSH properties are based on redox abilities of its thiol group, which can be easy oxidised and form glutathione disulfde (GSSG) (**Briviba and Sies, 1994**).

Reactions with protein radicals may lead to the formation of glutathione radicals and generated proteins (**Marciniak et al., 2005**). GSH.Px is involved in the detoxification of xenobiotics and ROS intermediates by its ability to react with electrophilic substrates. These reactions enable GSH.Px to protect thiol groups of biologically active proteins against peroxidative damage, as well as in the participation in termination of peroxidative chain reaction and repair of damaged molecules (**Marciniak, 2005**).

The enzyme is located in both the cytosol and mitochondria. There is a correlation between reduced plasma selenium and depressed glutathione peroxidase activities (**Lang et al., 1987**).

2.2.2.3 Catalase

One of the products of $O_2^{\bullet -}$dismutation is H_2O_2, H_2O_2 is detoxified by peroxidases and /or catalase (**Halliwell and Gutridge, 1989; Huang et al., 2005**), and catalase is one of the earliest identified enzymes and catalyses the following reaction:

$H_2O_2 \rightarrow H_2O + O_2$

Catalase is a hemoprotein that contains four heme groups widely distributed in the intracellular tissue concentrating in peroxisomes and mitochondria where it catalyzes hydrogen peroxide decomposition into water and oxygen. This function is shared with the enzyme glutathione peroxidase, which does not require cofactors (**Ricardo et al., 2002**). An important cofactor for the activity of catalase is Fe (**Bendich, 1993**).
Catalase (Fe) removes $O^{\bullet -}$ and H_2O_2 before they approach available promoters of Fenton chemistry (**Halliwell, 1987**).

2.2.3 Non-enzymatic protein antioxidants

Non-enzymatic antioxidants are primarily found in plasma. Total thiol groups of plasma represent the sulfhydryl groups of albumin-cysteine, and homocysteine. Protein sulfhydryl groups are considered a significant element of the extracellular antioxidant defence system against oxidative stress (**Bernabucci et al., 2005**).

2.2.4 Non-enzymatic low-molecular weight antioxidants

These antioxidants are found in plasma and in other extracellular fluids, in intracellular fluids, lipoproteins, and membranes. The non-enzymatic low-molecular weight antioxidants can be further subdivided into water-soluble and lipid-soluble antioxidants. Examples of water-soluble antioxidants are ascorbic acid, glutathione, and uric acid (**Bernabucci et al., 2005**).
Both water-soluble antioxidants and fat-soluble antioxidants are needed because free radicals are found in both areas of the cells. A free radical located in a cell membrane can not be

neutralised by an antioxidant located in the cytosol. Known antioxidant pathways suggest that the requirements of antioxidant nutrients are interrelated. A deficiency of one antioxidant may increase the requirement of another nutrient. However, a deficiency of a particular antioxidant nutrient cannot be alleviated fully by another nutrient (**Weiss, 2005**) (Table 6).

Table 6. Some of the antioxidant systems found in mammalian cells (modified from Weiss, 2005).

Component (location in cell)	**Nutrients Involved**	**Function**
Ferritin		Iron storage protein
Transferrin		Iron transport protein
Ceruloplasmin (water phase)	Copper	An antioxidant protein, may prevent copper and iron from participating in oxidation reactions
Albumin		Scavenger for $OH^{\bullet -}$, $LOO^{\bullet}$, HOCl etc
Catalase (cytosol)	Iron	An enzyme (primarily in liver) that converts hydrogen peroxide to water
Ascorbic acid (cytosol)	Vitamin C	Vitamin C reacts with several types of ROM
α-tocopherol (membranes)	Vitamin E	Breaks fatty acid peroxidation chain reactions
β -Carotene (membranes)	β-carotene	Prevents initiation of fatty acid peroxidation chain reactions

2.2.4.1 Vitamin C (Ascorbic acid, AA)

The most important antioxidant is ascorbic acid (AA). The compound remains an interesting topic of much scientific research even though it was discovered many years ago. The ability of AA to minimize harmful environmental influence on the metabolism is of special interest. AA protects DNA of the cells from the free radicals damage, prevents infections by strengthening cell membranes, and helps protecting phagocytic cells from oxidative damage (**Kleczkowski et al., 2005)**.

Domestic animals, including ruminants, have the ability to biosynthesize ascorbic acid in liver or kidney from glucose (**Eicher-Pruitt et al., 1992**). Ruminants are totally dependent on endogenous synthesis to meet their requirements of ascorbic acid, because all ascorbic acid derived from dietary sources is destroyed by rumen microorganisms (**Nickels, 1988**). The concentration of ascorbic acid is high in neutrophils, important immune cells with respect to mastitis and increase as 30-fold when neutrophils are stimulated (**Weiss, 2005**).

Consequently, the ascorbic acid supply for dairy cows is dependent on liver and kidney synthesis, and it may be compromised by any condition that decreases the availability of ascorbic acid precursors, such as glucose and galactose, which may result in insufficient synthesis of ascorbic acid. High producing dairy cows have an elevated demand for glucose by the mammary gland in order to produce lactose, so that these animals may synthesise less ascorbic acid than necessary to meet their requirements (**Santos et al., 2001**). However it was found that ascorbic acid concentration is independent on the number of lactations or the stage of lactation (**Santos et al., 2001**). Because of the rapid destruction by ruminal microflora and impaired synthesis ruminants can be prone to AA deficiency.

Cattle can produce ascorbic acid in the liver except during the first few weeks of life. Cow's milk is a poor source of ascorbic acid and its amount in milk (1-2 mg/100 ml) is not adequate to fulfil the requirements of 1-week-old calves, and if no supplement is added to colostrum or milk, the ascorbic acid level in plasma decreases considerably in few days, calves therefore require exogenous ascorbic acid (Sahinduran and Alby, 2004).

Vitamin C plays a potential role as a stress-relieving nutrient as it has recently been characterized as one of the anti-stress substances (**Afify and Makled, 1995**). The synthesis of ascorbic acid by farm animals is reduced or may cease during stress caused by disease, vaccination, higher temperature, overcrowding or physical activity (**Verde and Piquer, 1986**).

Ascorbic acid is the most important antioxidant in extracelluar fluids (**Chew, 1995**). It is thought to be important in optimum functioning of the immune system through enhancement of neutrophil production and also through protection against free radical damage. Vitamin C is found in high concentrations in blood leukocytes (**Moser, 1987**). The protective effect of vitamin C may in part be mediated through its ability to reduce circulating glucocorticoids (**Degkwitz, 1987**).

Vitamin C supplementation resulted in a 78% decrease in the susceptibility of lipoproteins to mononuclear cell mediated oxidation (**Rifici and Khachadurian, 1993**).

Ascorbic acid is very high in phagocytic cells with these cells using free radicals and other highly reactive oxygen containing molecules to help kill pathogens that invade the body. In the process, however, cells and tissues may be damaged by these reactive species. Ascorbic acid helps to protect these cells from oxidative damage (**McDowell, 2002**).

2.2.4.2 β-Carotene

Carotenoids are red and yellow pigments naturally occurring in all photosynthetic plants and organisms. Of the more than 600 characterized compounds, less than 10% can serve as Precursors of vitamin A, β-Carotene the most commonly available carotenoid in human diets, also is the major carotenoid precursor of vitamin A. Recent work has shown that β -Carotene is an efficient quencher of singlet oxygen and can function as an antioxidant (**Bendich, 1993**).

The members of the retinoid family play a fundamental role as the regulators of cell growth, embryonic morphogenesis and differentiation in many types of cells, through a series of oxidative reactions (**Morriss-Kay and Ward, 1999; Ross et al., 2000**).

The mechanism by which carotenoids regulate immunity largely are unclear, the most widely recognised mechanism of carotenoids is its antioxidant function (**Krinsky, 1989**).

β–Carotene possesses potent activities that scavenge singlet oxygen and quench peroxyl radicals, especially under low oxygen tensions (**Burton and Oliver, 1978**). β-Carotene has significant antioxidant properties and effectively quenches singlet oxygen free radicals (**Mascio et al., 1991**).

Cows fed supplemental β-Carotene plus vitamins A have decreased milk SCC during lactation, and a lower incidence of new intramammary infections during the early dry period (**Michal et al., 1994**).

It has been suggested that oestrus indications in the cows that were fed feed rich in β-Carotene became more indicative, the rate of pregnancy increased and cystic ovary incidence was reduced. According to the observations of **Michal et al. (1994)** protective effects of β-Carotene may be mediated through its immunoregulatory role. Indeed, β Carotene increased blood and milk phagocyte killing ability as well as peripheral blood lymphocyte proliferation in peripartum dairy cows in vitro. Similar in vivo studies in dairy cattle are not available.

2.2.4.3 Vitamin A

Vitamin A cannot quench singlet oxygen and has less antioxidant activity than the other antioxidant nutrients discussed, however, it is important to the immune system (**Bendich, 1993**).

Deficiency of vitamin A in cows may cause infertility, abortion, retained placenta, blind foetus, and irregularity in sexual cycle, suboestrus, anoestrus, delayed ovulation and increase in the rates of endometritis (**Hemken and Bremel, 1982**).

Vitamin A has been called the anti-infective vitamin and its deficiency has been associated with increased risk of infections. Vitamin A is critical for the development and functioning of T and B lymphocytes. Thus, low vitamin A status understandably results in a reduction of cell mediated immune responses and decreased specific antibody responses following immunization. Even marginal deficiency, with no clinical signs of deficiency, decreased immune responses to vaccines and production of pathogen-specific antibodies (**Bendich, 1993**).

Vitamin A is necessary for maintenance of skeletal muscle and epithelial tissue as well as for normal immune function, vision, growth, and spermatogenesis. Vitamin A is clearly present at the ovarian level and in steroidogenesis. Higher vitamin A concentrations are found in non-atretic follicles and this might indicate a role of vitamin A in follicular development (**Charles et al., 2005**).

2.2.4.4 Vitamin E

Vitamin E is one of four fat-soluble vitamins required by all mammals. Natural forms of vitamin E are synthesized in plants and are comprised of a group of related compounds, the tocopherols and tocotrienols, which demonstrate various degrees of biological activity (**Brigelius-Flohe and Traber, 1999**).

The primary role of vitamin E in biological systems is that of an antioxidant. The vitamin neutralizes free radicals and prevents oxidative damage of intramembranous and intracellular lipids. Although vitamin E content varies throughout the body, the highest concentrations occur within tissues of high lipid content (e. g. adipose and liver) (**Combs, 1991**). Vitamin E is primarily present and active within cell membranes and cellular organelles that possess high oxidation-reduction capabilities, specifically microsomes and mitochondria (**McCay et al., 1981**).

Vitamin E deficiency, like selenium deficiency, causes retained placenta and mastitis in dairy cattle. Plasma concentrations of α-tocopherol, the most biologically active form of vitamin E, start to decline at 7–10 days before parturition, reach their lowest concentrations at 3–5 days after calving, and then start increasing. Newborn calves rely on colostrum for vitamin E and increase of vitamin E intake during the prepartum period elevates vitamin E in the colostrum. Thus, if maternal diets are limited in vitamin E, the intake of vitamin E by calves might not be adequate to provide a sufficient deposition in newly formed membranes to prevent free radical-initiated peroxidative changes (**Kumagai and Chaipan, 2004**).

There are four different isomers of tocopherol, alpha, beta, gamma and delta: the alpha form is the most potent tocopherol. One of the most characterised features of α- tocopherol is its ability to prevent the initiation and propagation of lipid peroxidation by scavenging free radicals (**Warren et al., 1992)**.

α- tocopherol is the most prevalent and exhibits the highest degree of biological activity of the naturally occurring forms of vitamin E (**LaRoche, 1994**).

Calves are born with a very low blood Serum concentration of vitamin E as its transplacental transmission is limited. Vitamin E concentrations increase as the calf ages. The main sources of vitamin E for neonates are colostrum and milk. Vitamin E concentrations in milk are 6-7 times higher than in later-produced milk (**Pavlata et al., 2005**).

Since vitamin E acts as a tissue antioxidant and aids in quenching free-radicals produced in the body, any infection or other stress factors may exacerbate depletion of the limited vitamin E stores from various tissues. The protective effects of vitamin E on animal health

may be involved with its role in reduction of glucocorticoids, which are known to be immunosuppressive. Vitamin E also most likely has an immune enhancing effect by virtue of altering arachidonic acid metabolism and subsequent synthesis of prostaglandin, for cows fed dietary treatments with low or intermediate thromboxanes and leukotrienes. Under stress conditions, increased levels of these compounds by endogenous synthesis or exogenous entry may adversely affect immune cell function (**Hadden, 1987**).

Dairy cattle consuming stored forages are often low in vitamin E unless supplemented, and vitamin E deficiencies are frequently observed during the periparturient period. The periparturient period is (**Smith et al., 1997**) associated with increased incidence of mastitis in dairy herds (**Smith et al., 1984**).

White muscle disease is a classic sign of a clinical deficiency of vitamin E. More recently, the incidence of reproductive disorders (predominantly retained fetal membranes) and mastitis has been related to vitamin E intake. The supplementation of approximately 1000IU/d of vitamin E (usually all –rac-tochophryl acetate) to dry cows when adequate Se is supplemented reduces the incidence of RFM in some but not all studies (**Weiss, 1998**).

There is clear evidence that Vitamin E deficiencies may lead to alterations in the synthesis of steroid hormones and the prostaglandins (**Smith et al., 1997).**

Vitamin E is an important component of maternal colostrum. Because α-tocopherol does not cross the placenta in appreciable amounts, the calf is born with very limited stores of vitamin E instead, the calf is dependent upon colostral intake to obtain vitamin E after birth. Colostrum normally contains much more vitamin E than milk and is intended to be the first source of vitamin E for the calf. However, vitamin E content of colostrum is usually low unless the cow is provided supplemental dietary vitamin E (**Quigley, 2001**).

The first direct evidence that deficiencies in vitamin E and Se are related to mammary health was reported nearly a decade ago (**Hogan et al., 1993**) (Figure 3).

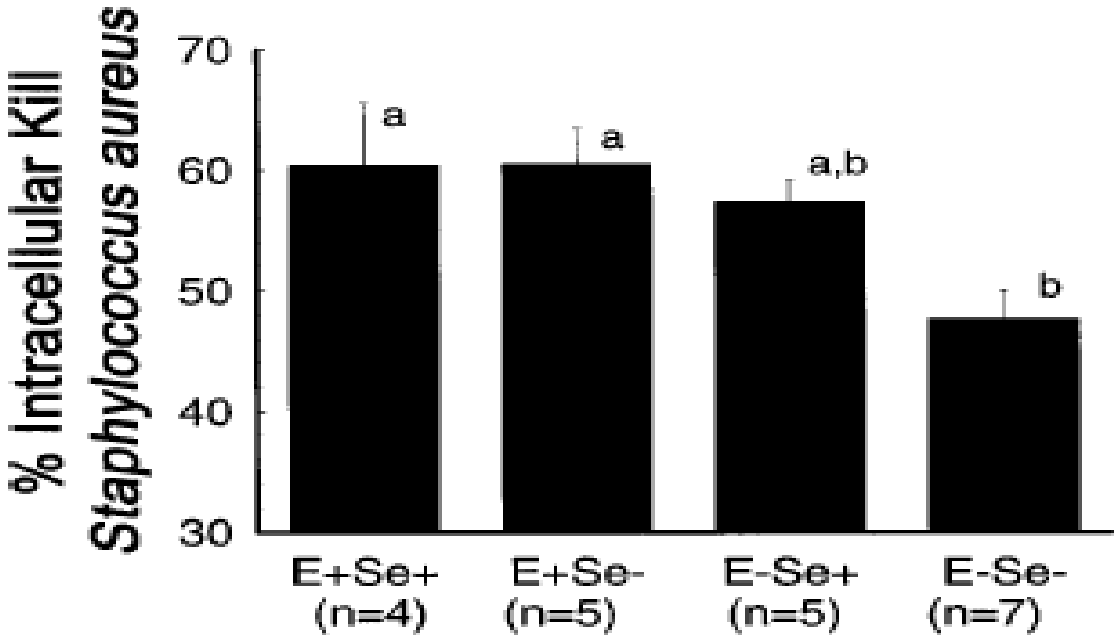

Figure 3. Percentage of intracellular kills of Staphylococcus aureus in blood neutrophils from cows fed diets supplemented (+) or unsupplemented (–) with Vitamin E (E), Se, or both a, b Means with different superscripts differ (p < .05).(Smith et al., 1997).

2.2.4.5 Selenium

Selenium, as a component of enzyme glutathione peroxidase, has a primary role in destroying reactive oxygen species that inevitably form in three major ways. First by protecting the integrity of the pancreas, allowing normal vitamin E (fat) digestion to take place, second by reducing the amount of peroxides attacking the cell membranes by way of glutathione peroxidase, and third by aiding in the retention of vitamin E in the blood (**Hatfield et al., 1999**). Selenium is a cofactor in a number of enzyme systems, especially glutathione peroxidase (**Lang et al., 1987**).

Selenium and vitamin E used separately are able to mitigate the severity of the clinical symptoms of mastitis (**Smith et al., 1997**) and to shorten their effects; used together, they are even more efficacious. Selenium in feed has been shown to shorten the duration of mastitis and to lessen its symptoms, especially Escherichia coli, but not that caused by Staphylococcus aureus. Selenium and vitamin E supplementation curtail the prevalence of

infections by environmental pathogens and staphylococci during calving and the incidence of clinical mastitis during the first lactation (**Jukola et al., 1996**).

Se plays a role in protecting leukocytes and macrophages during phagocytosis, the mechanism whereby animals immunologically kill invading bacteria. Both vitamin E and Se may help these cells to survive the toxic products that are produced in order to effectively kill ingested bacteria (**McDowell, 2002**), (Figure 4).

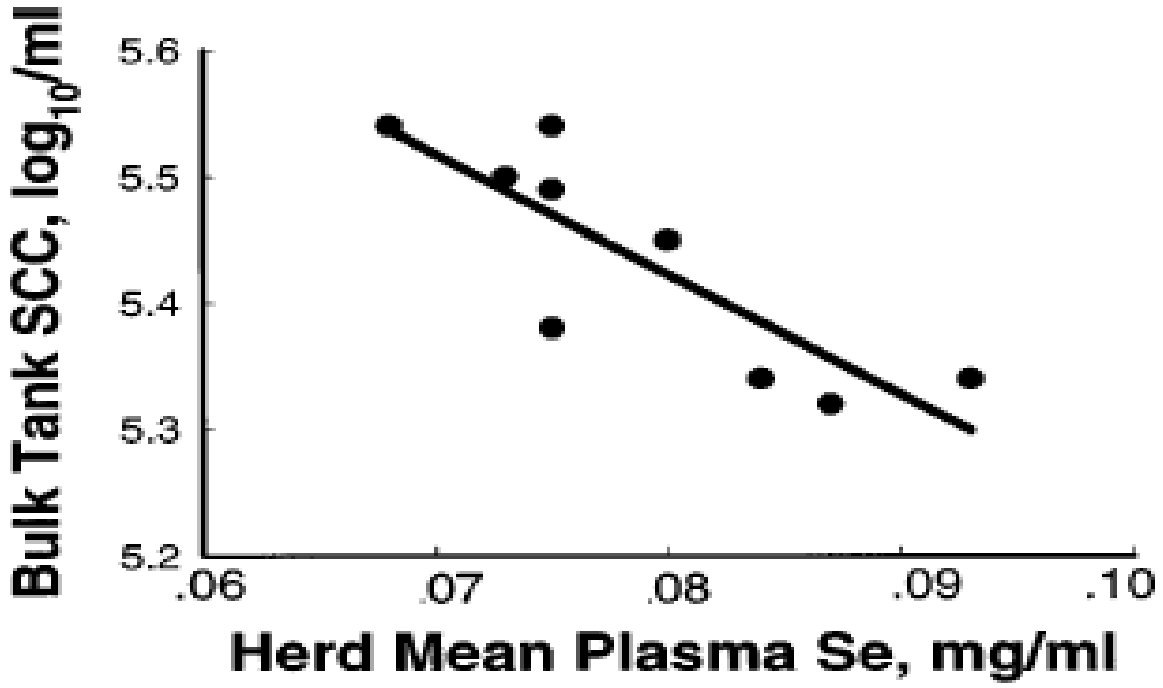

Figure 4. Relationship between herd means Se concentration in plasma and bulk tank milk somatic cell count (SCC). (Smith et al., 1997).

2.2.4.6 Vitamin D

Vitamin D, one of the fat soluble antioxidants, is usually taken up through feed. Hay and other sun-dried forages contain appreciable quantities of vitamin D, and cows exposed to sunlight can synthesize vitamin D. Vitamin D also is involved with immune function.

Increased lymphocyte proliferation was observed when Jersey cows were infused subcutaneously with 50 mg of 1, 25-dihydroxyvitamin D/d for 7 d (**Weiss, 1998**).
Vitamin D is involved with calcium and phosphorous homeostasis and immunity. Hay and other sun-dried forages contain quantities of vitamin D, and cows exposed to sunlight can synthesize vitamin D. The NRC committee (1989) stated that quantitative requirements for vitamin D are not well defined for dairy cows (**Weiss, 1998**).

2.2.4.7 Glutathione

Glutathione is among the most important antioxidants in biological systems, it is a tripeptide composed of the amino acids glutamate, cysteine, and glycine (**Cadenas and Packer, 2002**). Glutathione is mainly synthesized de novo within the liver (**Bernabucci et al., 2005**).
Its reduced form is a tripeptide (GSH) that has variable tissue distribution, it has a low molecular weight and is the most abundant thiolic compound in mammalian cells (**Powers and Lennon, 1999**). Due to its chemical properties, it reacts with several oxidant compounds, such as hydrogen peroxide, superoxide, hydroxyl and reactive carbon species (**Yu, 1994; Halliwell and Guttridge, 1984**). It can also reduce tocopheroxyl free radicals and dehydroascorbate to their original forms.

2.2.4.8 Uric acid

It was proposed that uric acid may be an important antioxidant in humans. This hypothesis is supported by the ability of uric acid to scavenge hydroxyl radicals, singlet oxygen, and oxo-heme oxidants (**Kirk and Mason, 1987**).
Uric acid has been traditionally considered as an end product of purine metabolism, its function as an intra- and extracellular biological antioxidant has been accepted recently. It seems to prevent vitamin C oxidation and forms complexes with Fe and Cu (**Ricardo et al., 2002**).

2.2.4.9 Albumin

Albumin, a single polypeptide with 585 amino acids, is a major plasma protein, responsible for binding and transport of many biologically active molecules. Albumin is a well-characterized product of the liver, and albumin is synthesized largely in the liver, although non-hepatic expression has been documented in several other tissues including mouse retina (**Shamay et al., 2005**).

Albumin has the ability to rapidly bind with Cu. Thus it acts as a good scavenger of Cu ions, in turn it could minimize the generation of the potent $OH^{\cdot}$ radical and reduce the threat for lipid peroxidation (**Sami, 1995**).

2.2.4.10 Bilirubin

One of the most important bile pigments found in the Serum of domestic animals is bilirubin, which is derived from haemoglobin. Bilirubin exists in tow forms as a protein-bound substance in plasma and as a conjugate known as blirubin glucoronide.

Heme oxygenase (HO) is the rate-limiting enzyme of blirubin production. It is a microsomal enzyme, present in both central and peripheral tissues, that converts heme to biliverdin and CO (**Maines, 1988**). Biliverdin is subsequently reduced to bilirubin by the cytosolic enzyme biliverdin reductase (**Yamaguchi et al., 1994**).

The importance of blirubin as an effective antioxidant was also proven by **Dennery et al, (1995**). Bilirubin is a strong reducing agent and a potential physiological antioxidant (**Stocker and Keaney, 2003**).

It has been suggested that bilirubin may have a protective role in the atherosclerotic process (**Mayer, 2000**). For many years, the bile pigment bilirubin was considered a toxic waste product formed during heme catabolism (**Mayer, 2000)**.

Bilirubin has been suggested to function as a physiological antioxidant as it efficiently protects human albumin-bound fatty acid from peroxyl-radical-mediated oxidation in vitro and, when incorporated into liposomes, can act as a chain breaking antioxidant which is as efficient as α- tocopherol (**Neuzil et al., 1993**).

In several studies it was found that different circulating forms of bilirubin are powerful antioxidants: Free bilirubin, albumin-bound bilirubin, conjugated bilirubin, and

unconjugated bilirubin were all noted to be effective scavengers of peroxyl radicals and to be able to protect human LDL against peroxidation (**Mayer, 2000**).

2.2.4.11 Antioxidant interactions

A large body of evidence suggests that a high degree of interactions exists among endogenous and exogenous antioxidants. The ability of one antioxidant to regenerate another oxidised species is common (**Buttner, 1993**). For example, vitamin E can be regenerated by vitamin C from tocopheryl by the donation of one hydrogen atom (**Tanaka et al., 1997**). Cooperation between various antioxidants might provide greater protection against oxidative stress than any antioxidant alone. It is believed that vitamin E and selenium have a synergistic effect therefore they are usually supplied in combination. Also vitamin E can protect against selenium deficiency. Vitamin E can protect β -Carotene from oxidation and can spare its action (Figure 5).

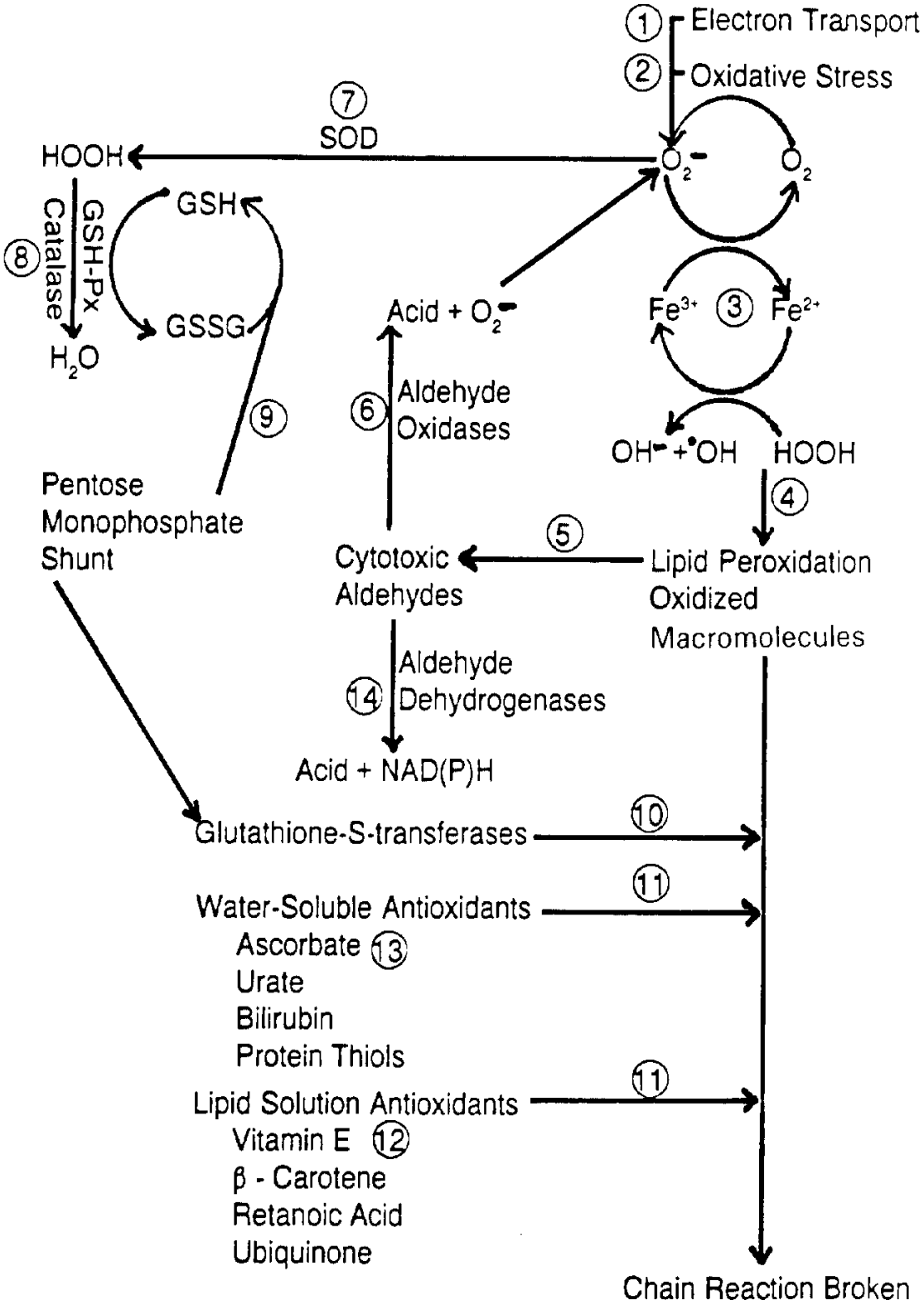

Figure 5. Systems for protection against reactive oxidative stress (Miller and Brzezinska-Slebdzinska 1993):

1) Superoxide is generated during normal metabolism.

2) Exogenous contributors to oxidative stress include dietary imbalances, disease, environmental pollutants, and solar radiation.

3) Superoxide reduces Fe^{3+}, enabling it to enter into Fenton-type reactions, which produce hydroxyl radical.

4) The extremely reactive hydroxyl radical attacks macromolecules and initiates peroxidative chain reactions.

5) Cytotoxic aldehydes are end products of lipid peroxidation.

6) When tissues are disrupted, aldehyde dehydrogenases are converted to aldehyde oxidases, which generate superoxids.

7) Superoxide dismutases (Mn, Cu, and Zn) convert superoxide to peroxides. This conversion retards reduction of Fe^{3+} to Fe^{2+}, which catalyzes formation of $OH^{\bullet}$.

8) Catalase and glutathione peroxidase (Se) convert peroxides to compounds that do not participate in Fenton-type reactions. Reduction of peroxides is accompanied by oxidation of reduced glutathione.

9) Reduced glutathione can be regenerated from glutathione disulfide (GSSG) by reducing equivalents from NADPH, which is generated by the pentose monophosphate shunt.

10) Glutathione S-transferases conjugate glutathione with peroxy radicals, this pathway may be more active when it is deficient in Se or vitamin E. The resulting destruction of glutathione increases consumption of reducing equivalents, thus competing with other metabolic pathways that depended on NADPH.

11) Chain-breaking antioxidants interrupt peroxidative chains initiated by reactive oxygen metabolites that escaped enzymatic degradation.

12) Vitamin E serves as a chain-breaking antioxidant by reacting directly with free radicals. Although vitamin E is consumed when free radicals are quenched reducing equivalents are conserved in comparison with glutathione S-transferases serving as chain breakers.

13) Vitamin C, in addition to regenerating vitamin E and possibly also glutathione, can act in its own right as a water-soluble antioxidant.

14) Aldehyde dehydrogenases convert aldehydes to less toxic products.

2.3 Oxidative stress

Oxidative stress is one type of stress that athletes of any species deal with. Exercise increases oxygen uptake. As oxygen is used to help produce energy in the mitochondria, intermediates are produced called reactive oxidative metabolites (ROM). These ROM normally are not a problem in the resting body because of the antioxidant defence system in place to combat an overproduction. However, sometimes ROM can become overwhelming to the antioxidant defence system and pose potential problems to cellular lipids, proteins and DNA, this is called oxidative stress (**Clarkson and Thompson, 2000**). It has been found that oxidative stress also contributes to degenerative changes including aging, cancer, and chronic fatigue, as well as other problems during exercise.

Oxidative stress was described as a disturbance in the pro-oxidant/antioxidant balance in favour of the former. This original denotation has been modified since to the more refined definition of "imbalance between oxidants and antioxidants in favour of the oxidants potentially leading to damage". This definition accounts for some important operational considerations. For example, an oxidative challenge or a loss of antioxidants alone does not constitute. Oxidative stress, however, if increased formation of oxidant(s) is accompanied by a loss of antioxidant(s) and/or accumulation of oxidized forms of the antioxidant(s), oxidative stress is approached **(Stocker and Keaney, 2003**).

2.4 Oxidative system in health and disease

2.4.1 Mastitis

Mastitis is one of the most costly diseases in the dairy industry (**Hidiroglour et al., 1997).** Mastitis continues to be an economically vital disease all over the world. Due to its anatomic topography, the udder is exposed to environmental effects, leading to inflammatory and non-inflammatory diseases. The disease in small ruminants is very important because of its high mortality rate on acute and peracute forms even though it is seen relatively rarely (**Cetin, 2003**).

The mechanisms by which inflammation cause damage to mammary gland tissue during mastitis is still not fully understood. It is well known that inflammatory reactions, in which

vascular permeability increases and leukocyte migration occurs involve free radicals, such as $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet -}$ (**Cetin et al., 2003**).

On well managed farms, approximately 50 cases of clinical mastitis can be expected per 100 cows (assuming 305 d lactation). The total costs associated with clinical mastitis range from about $ 100 to $140 per case. An inflammatory response occurs after a pathogen invades the mammary gland. Substantial amounts of ROM are produced by certain types of immune cells during the inflammatory response to assist those cells in killing the pathogen. When those cells contain adequate amounts of antioxidants, the concentrations of ROM are kept in check, which allows those cells to kill additional bacteria before the immune cell is killed. When the antioxidant capacity is limited, the lifespan of those immune cells is reduced and the infection can become established or the severity of the infection can increase (**Weiss, 2005**).

Antioxidant supplementation could decrease the duration, incidence, and severity of clinical mastitis (**Smith et al., 1997; Smith et al., 1984)**. Antioxidant nutrition is an important part of coliform mastitis prevention because of the critical role of these micronutrients in mammary resistance to this disease (**Bowers, 1997; Smith et al., 1997; Smith et al., 1984)**. Supplemental vitamin E and /or Se has been shown to reduce prevalence and severity of mastitis and reduce SCC (**Malbe et al., 1995; Smith et al., 1984;Weiss et al., 1997; Wichtel et al., 1994**).

Table 7: Summary of micronutrient effects on mammary gland immunity (Sordillo et al., 1997).

Micronutrient	Observation
Se	Decreased efficiency in neutrophils function. Improved bactericidal capabilities of neutrophils. Decreased severity and duration of mastitis
Vitamin E	Increased neutrophil bactericidal activity Decreased incidence of clinical mastitis
Vitamin A	Decreased SCC. Moderated glucocorticoid levels
Beta Carotene	Increased bactericidal function of phagocytes. Increased mitogen-induced proliferation of lymphocytes
Cu	Deficiency decreased neutrophil killing capability. Deficiency increased susceptibility to bactericidal infection
Zn	Deficiency decreased leukocyte function. Deficiency increased susceptibility to bacterial infection

2.4.2 Retained fetal membranes

Various surveys report that about 9% of all calving in the U.S. resulted in retained fetal membranes (RFM).The estimated total cost associated with RFM range from about $100 to $280/case (**Weiss, 2005**).

Accumulating evidence strongly suggests that in many cases, RFM is an oxidative stress disease. The vitamin C concentration in maternal and fetal placental tissue is about 50% lower when cows have RFM than when they not have (**Kankofer, 2001**).

Retention of fetal membranes in cows, which is one of the most important postpartum disorders, had been connected with the imbalance between production and neutralisation of

ROS. Retained fetal membranes can, in addition to cost of treatment, lower milk yield, market value, and productive life of the cow and result in indirect costs that are difficult to quantify. The well documented reduction in incidence of RFM when the nutrients used for antioxidant defence, vitamin E and Se, are supplemented peripartum suggests that the etiology of this disorder may involve oxidative stress. Possible relationships between oxidative stress and periparturient disorders of dairy cows have been reviewed (**Kankofer, 2001**).

The experiments on the supplementation of the diet of prepartum cows with vitamin E and selenium showed lower incidence of retained placenta than in unsupplemented animals (**Gwazdauskas et al., 1979**).

The properties of some antioxidant compounds such as GSH may also point toward a role of this compound as one of the members of nonenzymatic antioxidative defence mechanism in processes of releasing or retaining the placenta. GSH levels and GSH-Px activity in red blood cells of cows during the last 6 weeks of pregnancy and parturition were determined by **Brzezinska-Slebodzinska et al. (1994**). GSH levels and GSH-Px activity were lower in cows with retention of fetal membranes as opposed to without, with a tendency to increase towards delivery (**Kankofer, 2001**).

Possible relationships among oxidative stress, antioxidant nutrients, and performance of periparturient dairy cows have been reviewed. The occurrence of RFM increases the incidence of other diseases and reduces reproduction and milk production. Thus, an increase in antioxidant defence and a reduction in the incidence of RFM would be beneficial (**Campell and Miller, 1998**).

2.4.3 Fertility and infertility

Successful fertilization and implantation rely on complex and progressive interactions between the maternal genital tract, gametes and fertilized oocytes. The oviducts function as a sperm reservoir, a site of male gamete selection and a site of fertilization in cows and other species. Although reactive oxygen species (ROS: H_2O_2, $O_2^{\bullet -}$, $OH^{\bullet}$, $NO^{\bullet}$) are known to play an important role in male fertility/infertility, in vitro oocyte maturation (IVM), in vitro fertilization (IVF) and in vitro embryo development, little is known about the control of ROS levels by antioxidants in the oviduct in vivo (**Lapointe and Bilodeau, 2003**).

Productive consequences of free radical damage include disruption in function of spermatozoa (**Griveau et al., 1995**) and preimplantation embryos (**Fujitani et al., 1997**). Antioxidant status may be one determinant of reproductive function in dairy cattle. Administration of vitamin E or the combination of vitamin E and selenium has been reported to reduce the incidence of postpartum reproductive disorders such as retained fetal membranes, metritis, and cystic ovaries (**Paula-Lopes et al., 2003; Are´chiga et al., 1994**)

The effects of ROS such as the superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) on sperm functions are beneficial in some cases and detrimental in others. H_2O_2 blocks the motility of bovine sperm in vitro and ROS decrease sperm-oocyte penetration and block sperm-egg fusion in mice. However, binding of sperm to the zona pellucida is promoted by low levels of ROS and is inhibited by antioxidants. Thus, the way the female tract controls the generation of ROS could be a determining factor in successful fertilization and subsequent implantation (**Lapointe and Bilodeau, 2003**).

The balanced presence of reactive oxygen species and antioxidants has a positive impact on sperm functions, oocyte maturation, fertilization and embryo development in vitro. The mammalian oviduct is likely to provide an optimal environment for final gamete maturation, sperm-egg fusion and early embryonic development (**Lapointe and Bilodeau, 2003**).

2.4.4 Immunity

The immune system is responsible for protection against infection by pathogens such as bacteria, viruses, and protozoan parasites. Pathogens, recognized as invaders of the body and as “non-self”, are then destroyed by immune cells and their secretions. Antioxidants can play a role in enhancing the functions of the immune system for example carotenoids can enhance immune functions independently of any provitamin A activity. The mechanisms of immunoenhancement may include the capacities of a number of carotenoids to quench free radicals and singlet oxygen (**Bendich, 1993**).

The reduced concentration of antioxidants on the other hand affects the immune system and phagocytic activity of cells and results in an increase in the incidence of mastitis and puerperal diseases in pregnancy, delivery, and post-partum periods (**Goff and Stabel, 1990; Hidiroglou, 1995**).

Since vitamin E acts as a tissue antioxidant and aids in quenching free-radicals produced in the body, any infection or other stress factors may exacerbate depletion of the limited

vitamin E stores from various tissues. The protective effects of vitamin E on animal health may be involved with its role in reduction of glucocorticoids, which are known to be immunosuppressive. Vitamin E also most likely has an immune enhancing effect by virtue of altering arachidonic acid metabolism and subsequent synthesis of prostaglandin, for cows fed dietary treatments with low or intermediate thromboxanes and leukotrienes. Under stress conditions, increased levels of these compounds by endogenous synthesis or exogenous entry may adversely affect immune cell function (**Hadden, 1987**).

Vitamin A has been called the anti-infective vitamin for many decades. Overt vitamin A deficiency has been associated with increased risk of infections. Vitamin A is critical for the development and functioning of T and B lymphocytes. Thus, low vitamin A status understandably results in a reduction of cell-mediated immune responses and decreased specific antibody responses following immunization (**Bendich, 1993**) (Table 7).

Table 8. Effects of vitamin supplementation on immune responses.
DTH = Delayed-type hypersensitivity; IL-2 = interleukin-2; NK = natural killer.
(Bendich, 1993).

Oral supplement	Duration/Days	Immune effects
Vitamin E, 800 IU	30	Enhanced DTH, IL-2, and proliferation
β-Carotene, 30 mg	70	Prevented UV-induced depression in DTH in young adults
β-Carotene, 45 to 60 mg	60	Increased markers for helper T cells, NK cells, and IL-2 receptors
Vitamin A, 800 1U	28	Increased markers for helper T cells and total T cells; enhanced pro-liferation
Multivitamin mineral sup-plement	16	Enhanced DTH. Enhanced proliferation

2.4.5 Periparturient period

The transition period for dairy cows is characterized by increased risk of several metabolic and infectious diseases. One important causal factor is impaired immune function in peripartum cows (**Mallard et al., 1998**), and cowś vitamin A and vitamin E status are component factors in immune function (**NRC, 2001**).

The transition period is particularly important for health and subsequent performance of dairy cows, which are exposed to drastic physiological changes and metabolic stress. Relationships between BCS and incidence of metabolic diseases have been exhaustively reported. It has been hypothesized that an involvement of oxidative stress during transition period is the etiology of some diseases and disorders in dairy cows (**Bernabucci et al., 2005**).

Peripartum immunosuppression is multifactorial but is associated with endocrine changes and decreased intake of critical nutrients (**Goff and Horst, 1997**). Circulating concentrations of vitamins A and E decrease around calving (**Goff et al., 2002**). Decreased phagocytosis and intracellular killing by neutrophils occur in parallel with decreased DMI, and decreased circulating vitamin E (α-tocopherol) concentration (**Hogan et al., 1992**). Vitamin E is a fat-soluble membrane antioxidant that enhances the functional efficiency of neutrophils by protecting them from oxidative damage following intracellular killing of ingested bacteria (**Herdt and Stowe, 1991**).

It was stated that vitamin A and E, and β-Carotene levels decreased in pregnant cows, reaching the minimum values at the birth period (Figure 6), and started to reincrease in the post-partum period. The decrease resulted from the utilization of the compounds for the colostrum and milk synthesis accordingly to the growing of the foetus. The reduced concentration of antioxidants affects the immune system and phagocytic activity of cells and results in an increase in the incidence of mastitis and puerperal diseases in pregnancy, delivery, and post-partum periods (**Daniel et al., 1991**).

Cows fed supplemental β-Carotene plus vitamins A have decreased milk SCC during lactation (**McDowell, 2002**). Optimal blood concentrations of antioxidants may be greater during periods of stress, such as parturition. Plasma vitamin E concentrations in dairy cows are normally lowest when neutrophils functions are depressed during the periparturient period. The decrease in plasma α-tocopherol during the periparturient period is related to changes in consumption of vitamin E and to decreased transport capacity for the vitamin in plasma (**Hogan et al., 1993**).

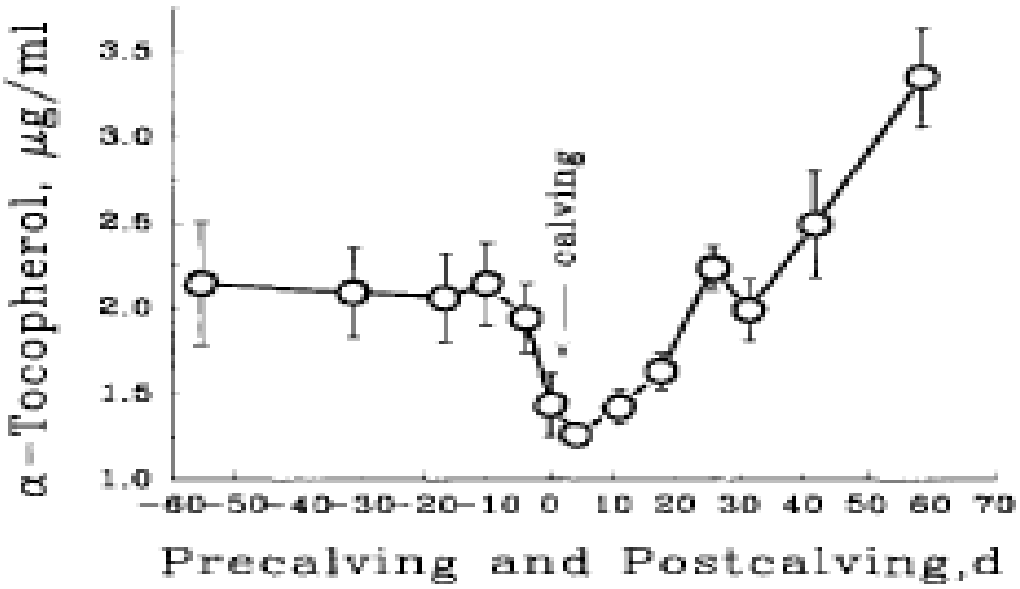

Figure 6. Changes in plasma α-tocopherol values during the dry period and early lactation in cows (Hogan et al., 1993).

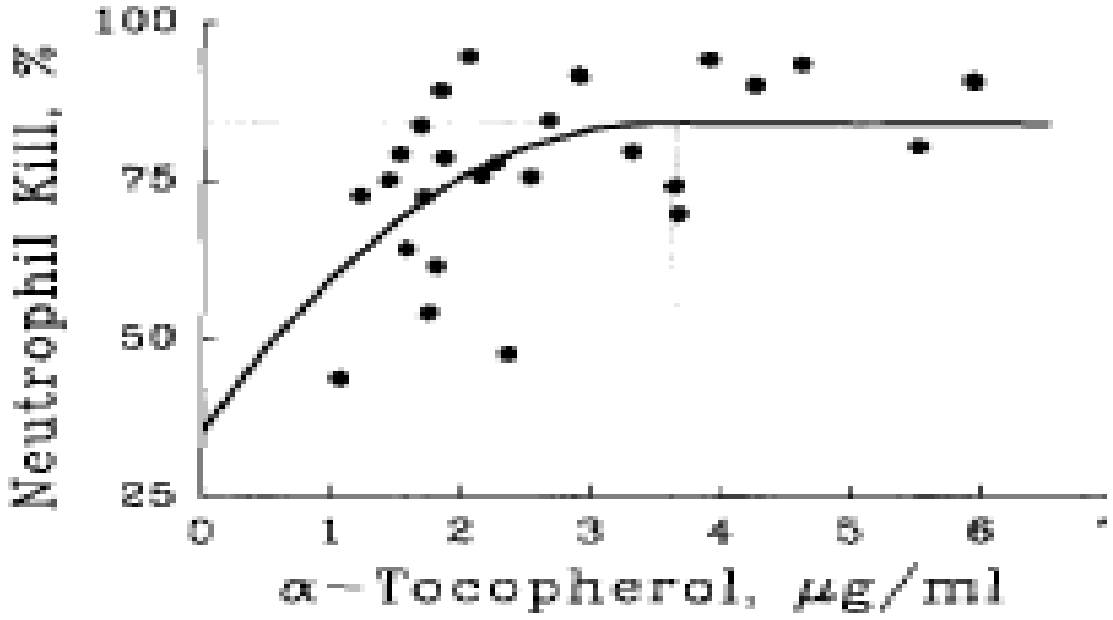

Figure 7. Relationship between intracellular kill of bacteria by neutrophils and plasma α- tocopherol at calving (Hogan et al., 1993).

Concentration of plasma α-tocopherol typically decreases 7 to 10 d prior to calving and remains low during the first 2 to 3 wk of lactation, even when the dietary vitamin E offered to cows is constant throughout this period (Figure 6) (**Hogan et al .,1993**).

2.4.6 Milk and meat quality

Milk quality is usually defined in terms of mastitis. Milk with a low somatic cell count (SCC) and visibly normal appearance (no clots) is considered high quality. Most fluid milk is judged to have a good flavour up to 14 d of storage but off-flavuor (OF) of milk is still an important problem, In certain situations, OF can be detected in milk almost immediately following milking. Some antioxidants (for example, Cu) can increase susceptibility to oxidized flavour development, others reduce susceptibility. Milk with high concentrations of Cu is extremely susceptible to the development of OF, especially if the milk also is high in polyunsaturated fatty acids (**Weiss, 2005**).

Lipid oxidation is a major cause of deterioration in the quality of muscle foods. Oxidation leads to the production of off-flavours and odours, reduction of polyunsaturated fatty acids, fat-soluble vitamins and pigments, lower consumer acceptability, and the production of compounds such as peroxides and aldehydes which may be toxic. Lipid oxidation is a free-radical-mediated process which occurs in raw muscle, and especially in cooked muscle. The process is believed to be initiated at the membrane level owing to the oxidation of the highly unsaturated membrane lipids (**Morrissey et al., 1994**).

Naturally occurring antioxidants such as α-tocopherol protect membrane lipids, the development of rancidity in meat can be minimized effectively with the use of dietary antioxidants.

2.4.7 Carcinogenesis

Oxidative damage may occur and this appears to be important in contributing to several pathological conditions including carcinogenesis, and free radicals may react with DNA causing reversible and irreversible damage, leading to mutation, carcinogenesis or cell death (**Floyd, 1990)**.

There has been a strong association between oxygen free radicals and cancer development, perhaps since the demonstration that ionizing irradiation caused cancer. Although the direct role of oxygen-free radicals in carcinogenesis has not yet been proven, a stronger correlation Will be apparent as more information becomes available. Recent observations present a clearer view of the many possible mechanisms where oxygen-free radicals may influence cancer development (**Floyd, 1990**).

A critical factor in mutagenesis is cell division. When the cell divides, an unrepaired DNA lesion can give rise to a mutation. Thus an important factor in mutagenesis, and therefore carcinogenesis, is the cell division rate in the precursors of tumour cells. Stem cells are important as precursor cells in cancer because they are not on their way to being discarded. Increasing their cell division rate would increase mutation (**Bruce et al., 1993**).

Antioxidants can defend against oxidative stress by scavenging free radicals and interrupting free radical induced chain reactions. Antioxidant vitamins have been shown to effectively prevent induced tumours in animals, including hormonally mediated tumours **(Hennekens, C. H. 1997).**

2.4.8 Ageing

In 1956, Harman proposed the free radical theory of ageing. The basic idea behind this theory was the assumption that ageing results from random deleterious effects to tissues brought about by free radicals. Support for this concept came from the observed acceleration of some features of ageing following radiolytic radical generation by body radiation with X-rays (**Nohl, 1993**).

The degenerative diseases associated with ageing include cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts. The functional degeneration of somatic cells during ageing appears, in good part, to contribute to these diseases. The relationship between cancer and age in various mammalian species illustrates this point. Oxidative damage to DNA, proteins, and other macromolecules accumulates with age and has been postulated to be a major, but not the only, type of endogenous damage leading to ageing. Superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet -}$), which are mutagens produced by radiation, are also by-products of normal metabolism. Lipid hydroperoxides, lipid alkoxyl and peroxyl radicals, and enals (**Bruce et al., 1993**).

Research over the past 40 years has led to a greater understanding of the aging process. Energy production occurs in the mitochondria and it is these energy generators that become less efficient as we age, producing greater numbers of mutagenic oxygen radicals (**Ames, 2003**).

Production of reactive species, including free radicals, is an integral part of human metabolism. Because of the high potential to damage vital biological systems, reactive species have now been incriminated in aging and in more than 100 disease states (**Cao and Prior, 1998**).

2.4.9 Ischemia-reperfusion Injury

Ischemia-reperfusion injury is a term that encompasses the tissue and cellular damage that occurs when inadequate blood is supplied to a region of the body, followed by the resumption of blood flow. Inadequate perfusion leads to a lack of oxygen, depletion of high-energy molecules (such as ATP) and build up of toxic metabolites (**Wall, 2002**).

2.5. Antioxidative capacity

2.5.1 Definition

According to **Paszkowski and Clarke (1996)** Total Antioxidative Capacity (TAC) is defined as a measure of overall free-radical scavenging potential

Antioxidants in plasma can be classified into two groups namely the

- water-soluble
- lipid soluble.

The water-soluble antioxidants include ascorbic acid or vitamin C, uric acid, protein thiols, and bilirubin. Plasma also contains very low levels of glutathione, which is a major intracellular antioxidant.

The lipid-soluble antioxidants comprise of α and γ tocopherol, ubiquinol, lycopene, β Carotene and some other carotenoids and oxycaroteniods (**Motchnik et al., 1994**).

2.5.2 Components

Mechanisms of antioxidant protection can be classified into four categories:

- Compartmentation
- Detoxification
- Repair
- Utilization

2.5.2.1 Compartmentation

Compartmentation is both the spatial separation of potentially harmful but essential substrates (e.g. storage of iron in ferritin) and cell and tissue distribution of antioxidative compounds and serves the prevention of uncontrolled oxidation (**Popov and Lewin, 1999**).

2.5.2.2 Detoxification

Detoxification is the most significant mechanism from the therapeutic point of view because it results in the breakdown of free radicals to non-toxic substances (oxygen, water), thus protecting from many diseases. Detoxification of oxidative molecules i.e. radicals and peroxides, is ensured by enzymatic and non-enzymatic substances. The detoxification enzymes are present intra- and extracellularly and protect cells from the destructive side effects of free radical chain reaction (**Popov and Lewin, 1999)**.

2.5.2.3 Repair

Antioxidants can be repaired by reverting the original changes due to free radicals when they are still reversible (**Jovanovic and Simic, 2000**; **Popov and Lewin, 1999).** Protection mechanism whereby further degradation of denatured and peroxidized potentially toxic proteins and lipids occurs (**Popov and Lewin, 1999**). Total antioxidant capacity is therefore the total ability of the body to protect itself from destructive side effects of physiological metabolism.

2.5.2.4 Utilisation

Utilization is considered as a secondary antioxidant protection mechanism whereby further degradation of denatured and peroxidized potentially toxic proteins and lipids occurs (**Popov and Lewin, 1990**).

2.5.6 Antioxidative capacity in the plasma

The antioxidant defence system comprises a number of interconnected, overlapping components that include both enzymatic and non-enzymatic factors. Vitamin E, the major lipidsoluble antioxidant, protects against lipid peroxidation. Vitamin C can quench free radicals as well as singlet oxygen and can also regenerate the reduced antioxidant form of vitamin E. Together with uric acid, carotenoids, flavonoids and ubiquinol, these antioxidants make up the total antioxidative capacity (TEAC) in plasma (**Wouters-Wesseling et al., 2003**).

2.6 Measurement of the antioxidative capacity

2.6.1 In vitro tests

A broad variety of in vitro techniques has been developed for the detection of antioxidants which are based on the ability of compounds to scavenge peroxyl radicals. These methods are based on the direct interaction with reactive molecules or on the reactivity with metal ions and the effects are monitored by chemical measurements (in many cases by spectrophotometry). Examples are determination of peroxyl radical scavenging (trichlormethyl peroxyl or alkoxyl peroxyl radical. The ORAC assay (oxygen radical absorbance assay), the PLC test (Photochemoluminescence assay). Different forms of the TEAC test (2,2'-azino-bis /3-etyhlbenzthioazoline-6-sulfonic acid radical ABTS+/metmyoglobin), including the TROLOX (a specific form of TEAC with manganese dioxide), the TOSCA (total antioxidant scavenging assay), the DPPH test (diphenyl-1-1-picrylhydrazyl assay) , the TRAP (total radical antioxidant parameter), or the FRAP method (ferric reducing ability of, the TBARS (thiobarbituric acid reactive substances) assay is based on the measurement of malondialdehyde (MDA) which is formed as a consequence of the lipid peroxidation and can be conducted with subcellular membrane preparations or intact cells, prevention of formation of MDA can be used to assess antioxidant properties (**Hoelzel et al., 2005**).

The ORAC assay is considered to have a high specificity using a physiological important radical (**Cao and Prior, 1998**). The use of various extraction techniques in the ORAC assay enables separate estimates of aqueous and lipid-soluble antioxidant capacities to be made. In addition, different sources of radicals can be used (**Ou et al., 2002**). An advantage of the ORAC assay is that it combines both time and degree of inhibition of radical generation because it takes the oxidation reaction to completion and uses the area under the curve to quantify the antioxidant capacity (**Cao et al., 1993**)

The TEAC assay uses only the degree of free-radical inhibition at a fixed time to determine antioxidant capacity and does not take the duration of inhibition into account, which may result in underestimation of antioxidant capacity. This assay has been criticised because a non-physiological radical is used and because of dilution effects. However, commercial TEAC assay kits are available and the assay is relatively fast (**Cao and Prior, 1998**).

The TRAP assay involves the initiation of lipid peroxidation by generating water-soluble peroxyl radicals and is sensitive to all known chain breaking antioxidants, but it is relatively

complex and time-consuming to perform, requiring a high degree of expertise and experience. However, the TRAP assay has been criticized as employing an unphysiological oxidative stress (water-soluble peroxyl radicals), but the method can be adapted to use lipidsoluble initiators (**Prior et al., 2005**) (Table 8).

Table 9. Different tests used for measuring the antioxidative capacity (Lewin Popov, 2000)

Author	Radical – generator	Radical detector	Measuring time
Emanuel et al.,1961	Methyl oleate +O_2	Peroxide	12-16 h
Stocks et al., 1974	Brain homogenate +O_2	O_2 Consumption	1 h
Frank et al., 1982	Oil+ O_2	Electr. Conductivity	1-3 min
Wayner et al., 1985	ABAP	O_2-Consumption	30-60 min
Popov t eal., 1999	Luminol + UV-A	Chemolumineszenz	1-3 min
Niki et al., 1985	ABAP	O_2 Consumption	30-60 min
Klebanov et al., 1988	egg yolk + $Fe2^+$	Chemiluminescence	10-20 min
Miller et al., 1993 TEAC-Test	ABTS+Peroxidase+ H_2O_2	VIS spectrophotometry	5 min
Nakano et al., 1994	Meth-Hb	Luminescence, O_2	20-40 min
Ghiselli et al., 1995 TRAP-Test	ABAP	Fluorescence, R-Phycoerythrin	20-40 min
Saramet et al., 1996	Luminol +H_2O_2	Chemiluminescence	10-20 min

2.6.2 Photochemiluminescent detection of antiradical activity (PCL).

Figure 8: The Photochem.

Increasing attention is being paid to the investigation of antioxidant substances and their regulating mechanisms, considering the multitude of biologically relevant enzymatic and non-enzymatic antioxidants. It is not easy to ascertain them or their importance under defined pathologic conditions, simple, fast, reliable and informative methods are needed to measure and compare different antioxidants and thus to determine their relevance and significance of measurement in clinical practise (**Lewin and Popov, 1994**).

In general, measurement of antioxidant properties of a substance can be performed in a system containing a free radical generator and a detector which indicates changes of the measuring signal in the presence of the antioxidant. In a newly developed device, the Photochem photochemiluminometer, free radicals are generated photochemically by UV irradiation of a photosensitizer solution and are registered chemiluminometrically after the transport of irradiated solution to the measuring cell of the chemiluminometer.

When the assay mixture contains an antioxidant (e.g. plasma sample), it consumes the free radicals, leaving fewer available for reaction with the detecting substance. Depending on the measuring mode of the Photochem (ACW, water-soluble or ACL, lipid-soluble antioxidants) the process is recorded as a retarded or inhibited rise of the emission. The antioxidant capacity (ACW or ACL) of a sample is expressed in equivalent concentrations of the standard compounds ascorbic acid or α-tocopherol, respectively. Luminol is used both as a photosensitizer and as a detecting substance for free radicals (**Popov and Lewin, 1999**).

The principle of PCL is based on an UV-A light (365 nm)-induced photochemical reaction, consisting of two steps:

1. Absorption of light, substrate excitation: $S + h\nu \; S^*$

2. Generation of free radicals and/or singlet oxygen.

Thermally initiated decay of water and fat-soluble azo-compounds is used in this equipment as the source of free radicals.

Irradiation of the solution of a photosensitizer takes place in a vessel (1) by means of a low pressure mercury lamp (2) having a narrow spectral band with a maximum at 360 nm. The assay mixture consists of 0.1 mol/1 carbonate buffer, pH 10.8. 0.1 mmol/l Na-EDTA and 30 μumol/1 luminol. During the measurement, a continuously irradiated solution is transported with the help of a peristaltic mini pump (4) from the vessel (1) into the measuring cell (5) of a chemiluminometer (6). The signal is registered and evaluated with a computer (7) (**Popov and Lewin, 1994**) (Figure 8).

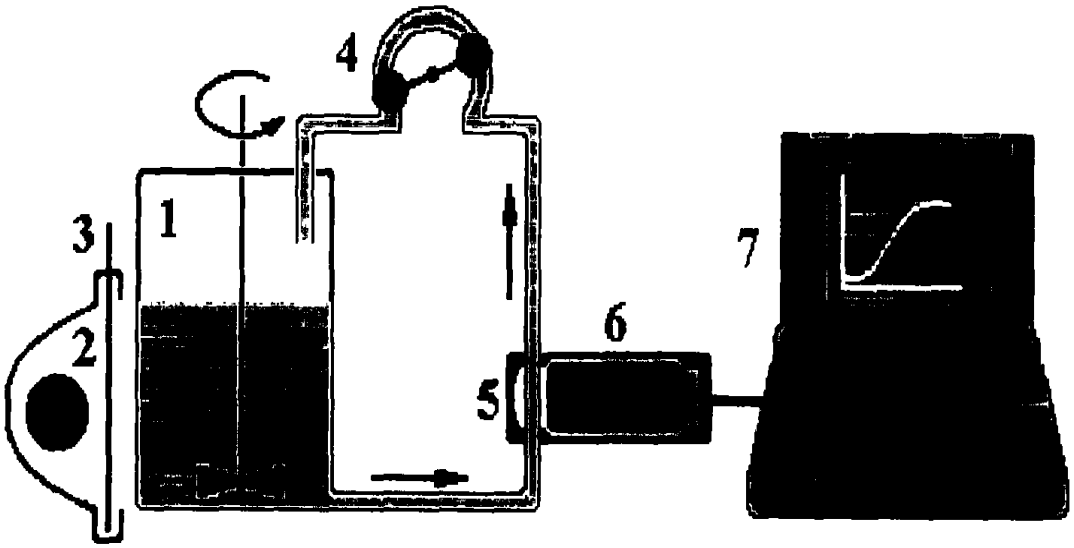

Figure 8.

Scheme of the apparatus for measuring of photoinduced chemiluminescence. (1) vessel for UV irradiation of a test solution: (2) low pressure mercury lamp,(3) shutter: (4) peristaltic mini-pump;(5) flow cell of a chemduminometer: (6) photomultiplier: (7) computer (Popov and Lewin, 1994).

3 Materials and Methods

3.1 Materials

3.1.1 Animals

Cows were enrolled in this study by the Clinic of Cattle, Faculty of Veterinary Medicine, Freie Universität, Berlin, Germany when they were admitted to the clinic as well as cows already hospitalised in the University clinic, from farms under Faculty study from August 2005 to December 2006, and from other farms under university study.

3.1.2 Blood sampling

Blood samples were taken from the middle coccygeal vein or jugular vein using different kinds of tubes according to the experiment need These tubes contained sometimes anticoagulants (EDTA, Li heparin, and NaF-LiH), and mostly were without anticoagulant (Serum tubes).

3.2 Methods

The method used for the determination of the antioxidative capacity (both the water soluble antioxidative capacity ACW and lipid soluble antioxidative capacity ACL) in this study is the method described by **Popov and Lewin (1994**) which is a method for testing and quantification of non-enzymatic antioxidants, such as ascorbic acid and uric acid and of polycomponent systems like plasma. This system is based on a photochemical generation of free radicals combined with their chemiluminescent detection.

3.2.1 Reagents (ACW)

For the determination of the water soluble antioxidative capacity the following reagents were used

- Reagent 1: ACW-Diluent (sample solvent).
- Reagent 2: Reaction buffer.

- Reagent 3: Stock solution (Photo sensitizer and detection reagent), 250µl/vial.
- Reagent 4: Calibration standard for the quantification of water soluble antioxidants in equivalents of ascorbic acid.

3.2.2 Reagents (ACL)

- Reagent 1: methanol.
- Reagent 2: reaction buffer.
- Reagent 3: (Photosensitizer and detection reagent), 250µ/vial.
- Reagent 4: calibration standard for the quantification of lipid soluble antioxidants in equivalents of Trolox.

3.2.3 Preparation of the working solution (ACW)

- Reagent 1 is ready for use.
- Reagent 2 is ready for use.
- Reagent 3 working solution: The vial containing reagent3 stock solution was thawed (the vials must be preserved frozen) and 750 µl of reagent 2 was added to the vial, the vial was briefly mixed on vortex.
- Reagent 4- stock solution: 490 µl of reagent 1 was added to the vial, then 10µl concentrated 95-97% H_2SO_4 was added to the vial containing reagent4, then the vial was mixed on vortex for 20-30 seconds.
- Reagent 4- working solution: Reagent 4 stock solution was diluted 1:100 with reagent 1 (e.g. 10 µl reagent 4 stock solution + 990 µl reagent 1).

3.2.4 Preparation of the working solution (ACL)

- Methanol is used as reagent 1.
- Reagent 2 is ready for use.

- Reagent 3- working solution: the vial containing reagent 3 stock solution was thawed and 750 µl of reagent 2 was added, the contents of the vial were then mixed briefly on vortex.
- Reagent 4- stock solution: 500µl of reagent 1 was added to the vial containing reagent 4 and then mixed on vortex for 20-30 seconds.
- Reagent 4 stock solution was then diluted -1:100 with reagent 1 (e.g.10µl reagent 4 stock solution + 990 µl reagent 1).

3.2.5 Sample preparation (ACW)

Water-soluble compounds should be dissolved with reagent 1 and when necessary diluted within the range of the calibration curve. The sample should be put on a vortex before being measured with the Photochem.

Table 10. Composition of the water-soluble reaction sample (pipetting scheme, all volumes in µl)

Reagent	1	2	3	4
Blank	1500	1000	25	0
Calibration	1500-x	1000	25	x
Measurement	1500-y	1000	25	y

*At least 2 blank measurements were made.

*x: for the calibration of the curves 5µl, 10µl, 20µl and 30µl of reagent 4 were used, sometimes 40, 50, depending on the antioxidative capacity of the sample tested.

*y: for the testing of samples the quantity used varied according to the antioxidant content of the sample tested.

3.2.6 Sample preparation (ACL)

Lipid-soluble samples should be dissolved with reagent 1 and when necessary diluted within the range of the calibration curve. The sample should mixed on a vortex before being measured with the Photochem.

Table 11. Composition of the fat-soluble reaction sample (pipetting scheme, all volumes in µl)

Reagent	1	2	3	4
Blank	2300	200	25	0
Calibration	2300-x	200	25	x
Measurement	2300-y	200	25	y

*At least 2 blank measurements were made.

*x: for the calibration of the curves 5µl, 10µl, 20µl and 30µl of reagent 4 were used, sometimes 40, 50, depending on the antioxidative capacity of the sample tested.

*y: for the testing of samples the quantity used varied according to the antioxidant content of the sample tested.

3.3 Measuring principle

Free radicals are being produced by irradiation of a photosensitizer (dye) substance. These radicals are partially eliminated from the sample after they react with the antioxidants normally present in the sample, the remaining radicals in the measuring cell cause luminescence to the detector substance. In this way the antioxidant capacity of the sample is being determined. Quantification of the antioxidative capacity of the sample is determined by comparison with the standard (calibration curves are constructed using ascorbic acid in case of ACW measurement, and trolox in case of ACL measurement).

3.4 Calculating the antioxidative capacity

Concentration (µg/ml)= (Ammount*Dilution*M) / p

Ammount: the result in nmol.

P: pipeted volum of the sample (µl).

M: molar weight, in case of ACW= 176,13, in case of ACW=250,3.

Dilution: by 1:10, dilution= 10.

3.5 Statistics

Data were analysed using SPSS V12 for windows, one-way ANOVA, and mean value comparison (T test by paired samples).

4 Results

4.1 Water soluble antioxidative capacity (ACW)

4.1.1 Medium determination

The first step in this study was to choose a medium in which blood samples will be preserved, and therefore the stability of the water-soluble antioxidative capacity was tested in different media under room temperature conditions.

The four media used are:

- Serum tubes (without anticoagulant).
- Tubes containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant.
- Tubes containing sodium fluoride (NaF) and lithium heparin as anticoagulant.
- Tubes containing lithium heparin (LiH) an anticoagulant.

The blood samples were taken, kept in the refrigerator for 30 minutes, centrifuged for 10 minutes, 4000 R/min and then tested under room temperature conditions (+25C˚) in one hour interval in order to determine the stability of the water-soluble antioxidative capacity. The first measurement was done after one hour because of technical reasons (it took sometime time withdraw the samples, transport them to the laboratory, and to centrifuge the samples which was sometimes repeated more than one time).

As seen in (Table 12) the ACW values in the Serum tubes were the highest among the four media used, and the lowest were achieved using EDTA containing tubes. The ACW values in NaF-LiH containing tubes were higher than in LiH containing tubes.

Table12. Comparison between the ACW values in the different media/original values after 1 hour.

Medium	n	Mean Value	s	s_x
Serum	10	13.3	4.27	1.35
EDTA	10	7.0	2.87	0.91
NaF-LiH	10	10.1	3.49	1.10
LiH	10	8.0	2.61	0.83

*Dependant variable ACW (µg/ml)
*n: number of tested samples
*Mean value, standard deviation, standard failure of the original values was calculated

By comparing the ACW values it is noticed that they are arranged as follows: Serum> NaF-LiH> LiH> EDTA where the difference between the ACW values is statistically significant when compared with the other media (Table 13).

Table 13. Comparison between ACW values in the different media after 1 hour

Medium (I)	Medium (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
Serum	EDTA	10	6.3	1.42	0.002	3.06	9.49
	NaF	10	3.1	0.95	0.009	0.99	5.28
	LiH	10	5.3	1.38	0.004	2.14	8.36
EDTA	Serum	10					
	NaF	10	-3.1	0.93	0.008	-5.25	-1.03
	LiH	10	-1.0	0.63	0.140	-2.45	0.41
NaF	Serum	10					
	EDTA	10					
	LiH	10	2.1	1.07	0.079	-0.30	4.53
LiH	Serum	10					
	EDTA	10					
	NaF						

* Means difference is significant $p \leq 0.05$

*Dependant variable ACW (µg/ml)

After two hours the results remained the same, the ACW values remained arranged in this order: Serum> NaF-LiH> LiH> EDTA (Table12). By comparing the ACW values with each other it is clear that the ACW values in the Serum tubes were the highest and the ACW values in the EDTA containing tubes were the lowest where the differences were statistically significant in both cases (Table 14).

Table 14. Comparison between the different media/original values after 2 hour.

Medium	n	Mean value	s	s_x
Serum	10	13.3	3.92	1.24
EDTA	10	6.3	3.10	0.98
NaF	10	10.0	4.37	1.38
LiH	10	7.9	2.72	0.86

*Dependant variable ACW (µg/ml)

Table 15. Comparison between the different media after 2 hours.

Medium (I)	Medium (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
Serum	EDTA	10	7.1	1.29	0.000	4.13	9.98
	NaF	10	3.4	1.08	0.013	0.91	5.81
	LiH	10	5.4	1.41	0.004	2.24	8.62
EDTA	Serum	10					
	NaF	10	-3.7	0.87	0.002	-5.65	-1.74
	LiH	10	-1.6	0.54	0.014	-2.84	-0.41
NaF	Serum	10					
	EDTA	10					
	LiH	10	2.1	1.03	0.075	-0.25	4.39
LiH	Serum	10					
	EDTA	10					
	NaF						

* Means difference is significant $p \leq 0.05$
*Dependant variable ACW (µg/ml)

The same results were also achieved after three hours (Tables 16 and 17)

Table 16. Comparison between the different media/original values after 3 hours.

Medium	n	Mean value	s	s_x
Serum	10	13.8	5.16	1.63
EDTA	9	5.9	2.75	0.92
NaF	10	10.0	3.90	1.23
LiH	10	7.8	2.62	0.83

*Dependant variable ACW (µg/ml)

Table 17. Comparison between the different media after 3 hours.

Medium (I)	Medium (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
Serum	EDTA	9	8.5	1.49	0.000	5.12	11.97
	NaF	10	3.8	0.84	0.002	1.86	5.67
	LiH	10	6.0	1.64	0.005	2.26	9.67
EDTA	Serum	9					
	NaF	9	-4.7	0.92	0.001	-6.86	-2.64
	LiH	10	-2.2	0.66	0.011	-3.68	-0.65
NaF	Serum	10					
	EDTA	9					
	LiH	10	2.2	1.16	0.091	-0.43	4.83
LiH	Serum	10					
	EDTA	10					
	NaF						

* Means difference is significant $p \leq 0.05$
*Dependant variable ACW (µg/ml)

4.1.2 The effect of time on the stability of ACW

4.1.2.1 Stability in the four different media

Blood samples were taken using the four different media (Serum, EDTA, NaF LiH, and LiH), kept in the refrigerator for 30 minutes, then centrifuged for 10 minutes, 4000R/min, and tested in one hour intervals. Because of technical reasons, the first measurement of the ACW was possible after one hour.

As seen Tables 18 and 19 there was no statistically significant decrease noticed in the ACW values in the blood samples kept in the Serum tubes within three hours, where the difference between the ACW values was not significant between the different times (Table 19).

Table 18. Stability of ACW in Serum tubes within 3 hours/original values

Time (hr)	n	Mean value	s	s_x
1	10	13.3	4.27	1.35
2	10	13.3	3.92	1.24
3	10	13.8	5.16	1.63

*Dependant variable ACW (µg/ml)

Table. 19. Stability of ACW in Serum tubes within 3 hours

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	2	10	-0.1	0.58	0.926	-1.37	1.26
	3	10	-0.5	0.77	0.523	-2.27	1.24
2	1	10					
	3	10	-0.5	0.75	0.555	-2.16	1.24
3	1	10					
	2	10					

* Means difference is significant $p \leq 0.05$
*Dependant variable ACW (µg/ml)

In the samples kept in the EDTA containing tubes there was a signinifcant decrease in the ACW values which started after one hour from the first measurement and continued over the three hours (Tables 20).The difference between the different times was significant (Table 21).

Table 20. Stability of ACW in EDTA containing tubes within 3 hours/original values

Time (hr)	n	Mean value	s	s_x
1	10	7.0	2.87	0.91
2	10	6.3	3.10	0.98
3	9	5.9	2.75	0.92

*Dependant variable ACW (µg/ml)

Table 21. Stability of ACW in EDTA tubes within 3 hours

Time (I)	Time (J)	n	Mean Difference (I-J)	s_x	p	95% Confidence Interval	
						Lower limit	Upper Limit
1	2	10	0.7	0.43	0.125	-0.24	1.70
	3	9	1.7	0.47	0.007	0.59	2.75
2	1	10					
	3	9	0.9	0.20	0.002	0.42	1.33
3	1	9					
	2	9					

* Means difference is significant $p \leq 0.05$
*Dependant variable ACW (µg/ml)

In the blood samples kept in NaF-LiH containing tubes the ACW values remained without a significant change during the test (Tables 22 and 23).

Table 22. Stability of ACW in NaF-LiH containing tubes within 3 hours/original values

Time	n	Mean value	s	s_x
1	10	10.1	3.49	1.10
2	10	10.0	4.37	1.38
3	10	10.0	3.90	1.23

*Dependant variable ACW (µg/ml)

Table 23. Stability of ACW in NaF-LiH containing tubes within 3 hours

Time (I)	Time (J)	n	Mean Difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	2	10	0.2	0.57	0.776	-1.13	1.46
	3	10	0.1	0.55	0.838	-1.13	1.36
2	1	10					
	3	10	-0.1	0.41	0.900	-0.98	0.87
3	1						
	2						

* Means difference is significant $p \leq 0.05$
*Dependant variable ACW (µg/ml)

In the samples kept in LiH containing tubes similar results to NaF-LiH containing tubes were noticed where there was no significant change in the ACW values within three hours (Tables 24 and 25).

Table 24. Stability of ACW in LiH containing within 3 hours/ original values

Time	n	Mean value	s	s_x
1	10	8.0	2.61	0.83
2	10	7.9	2.72	0.86
3	10	7.8	2.62	0.83

*Dependant variable ACW (µg/ml)

Table 25. Stability of ACW in LiH containing tubes within 3 hours

Time (I)	Time (J)	n	Mean difference	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	2	10	0.1	0.41	0.770	-0.80	1.05
	3	10	0.1	0.41	0.770	-0.80	1.05
2	1	10					
	3	10	0.1	0.30	0.796	-0.60	0.76
3	1	10					
	2	10					

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

4.1.2.2 Stability under tow different temperatures

Blood samples taken using Serum tubes were centrifuged for 10 minutes, 4000 R/min after being kept in the refrigerator for 30 minutes, then divided in tow groups. The first group was kept under room temperature (+25C˚) and the second was kept in the refrigerator (+4 C˚). The first measuring was done after one hour because of technical reasons, then every tow hours the ACW was measured in both groups.

As seen in (Table 26) there was a decrease in the ACW values under both tested temperatures after tow hours but the difference between the tow temperatures was not significant (Table 27).

Table 26. Comparison between the stability of ACW under room and refrigerator (+4C°) conditions after 2 hours/ original values

Condition	Time	n	Mean value	s	s_x
Room(first value)	1	10	14.3	3.62	1.14
Room	2	10	13.4	3.73	1.18
+4C	2	10	13.3	3.31	1.05

*Dependant variable ACW (µg/ml)

Table 27. Comparison between the stability of ACW under room (+25C°) and refrigerator (+4C°) conditions after 2 hours

Mean difference	n	s_x	p	95% Confidence interval	
				Lower limit	Upper limit
0.0	10	0.51	0.977	-1.14	1.17

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

After four hours the decrease in the ACW values continued in both temperatures (2.18µg/ml under room temperature, 1.97µg/ml in the refrigerator) (Table 28), by comparing the tow temperatures together the difference was not significant (Table 29).

Table 28. Comparison between the stability of ACW under room (25C°) and refrigerator (+4C°) conditions after 4 hours/ original values

Condition	Time	n	Mean value	s	s_x
Room	4	10	12.18	3.67	1.16
+4C	4	10	12.33	3.40	1.07

*Dependant variable ACW (µg/ml)

Table 29. Comparison between the stability of ACW under room (25C°) and refrigerator (+4C°) conditions after 4 hours

Mean difference	n	s_x	p	95% Confidence interval	
				Lower limit	Upper limit
-0.1	10	0.35	0.70	-0.93	0.63

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

Ther same results were noticed after 6 hours (Tables 30 and 31).

Table 30. Comparison between the stability of ACW under room (25C°) and refrigerator (+4C°) conditions after 6 hours/ original values

Condition	Time	n	Mean value	s	s_x
Room	6	10	11.7	3.69	1.17
+4C	6	9	11.6	2.82	0.94

*Dependant variable ACW (µg/ml)

Table 31. Comparison between the stability of ACW under room (25C°) and refrigerator (+4C°) conditions after 6 hours

Mean difference	n	s_x	p	95% Confidence interval	
				Lower limit	Upper limit
-0.3	9	0.53	0.552	-1.57	0.90

*The mean difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

The same results were noticed after 8 hours (Tables 32 and 33).

Table 32. Comparison between the stability of ACW under room (25C°) and refrigerator (+4C°) conditions after 8 hours/ original values

Condition	Time	n	Mean value	s	s_x
Room	8	9	11.2	3.60	1.20
+4C	8	9	10.8	3.06	1.02

*Dependant variable ACW (µg/ml)

Table 33. Comparison between the stability of ACW under room and refrigerator (+4C) conditions after 8 hours

Mean difference	n	s_x	p	95% Confidence interval	
				Lower limit	Upper limit
0.3	10	0.47	0.496	-0.76	1.43

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

4.1.2.3 Stability under five different temperatures within 168 hours

In this experiment blood samples were taken using Serum tubes, kept for 30 minutes in the refrigerator and then centrifuged for 10 minutes, 4000R/min. The outcoming Serum was divided into five groups, which were kept at different temperatures (room temperature (+25C°), +4C°, -20C°, -24C° and under liquid nitrogen, further each of these groups was also

divided to be tested after 24 hours and 168 hours.The first measurement was done after one hour because of technical reasons (Table 34).

Table 34. ACW values at room temperature after one hour

	n	Mean value	s	s_x	Minimum	Maximum
ACW(µg/ml)	10	8.42	3.56	1.13	5.12	16.25

*Dependant variable ACW (µg/ml)

In the samples kept at under room temperature (+25C˚) there was a statistically significant decrease noticed after 24 hours wheras the samples kept under other temperatures (+4C˚, -20C˚, -24C˚ and under liquid nitrogen) remained without a statistically noticed decrease. In the samples kept under +4C˚, compared with the samples kept under liquid nitrogen conditions, there was a significant decrease (Tables 35 and 36).

Table 35. Stability of the ACW value after 24 h under the five different temperatures /original values

Temperature	n	Mean value	s	s_x
+25C˚	10	5.2	3.02	0.95
+4C˚	10	7.1	3.36	1.06
-20C˚	10	8.4	3.02	0.95
-24C˚	10	7.0	3.64	1.15
Liquid nitrogen	10	8.4	2.33	0.74

*Dependant variable ACW (µg/ml)

Table 36. Stability of the ACW value after 24 h under the five different temperatures

Temperature (I)	Temperature (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
+25C°	+4C°	10	-1.8	0.51	0.006	-2.98	-0.68
	-20C°	10	-3.1	0.79	0.003	-4.89	-1.31
	-24C°	10	-1.8	0.59	0.015	-3.12	-0.44
	Liquid nitrogen		-3.2	0.43	0.000	-4.13	-2.20
+4C°	+25C°						
	-20C°	10	-1.3	0.85	0.17	-3.20	0.66
	-24C°	10	0.1	0.48	0.919	-1.03	1.13
	Liquid nitrogen	10	-1.3	0.58	0.046	-2.64	-0.03
-20C°	+25C°						
	+4C°						
	-24C°	10	1.3	0.79	0.130	-0.47	3.11
	Liquid nitrogen	10	-0.1	0.70	0.932	-1.64	1.52
-24C°	+25C°						
	+4C°						
	-20C°						
	Liquid nitrogen	10	-1.4	0.72	0.088	-3.02	0.25
Liquid nitrogen	+25C°						
	+4C°						
	-20C°						
	-24C°						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

After 168 hours the decrease in the ACW values in the samples kept at the room temperatures (+25C°) continued and was significant under both room (+25C°) and refrigerator (+4C°) temperatures. The highest values were measured under liquid nitrogen conditions (Tables 37 and 38).

Table 37. Stability of the ACW value after 168 h under the five different temperatures /original values

Temperature	n	Mean value	s	s_x
+25C°	10	3.5	2.52	0.80
+4C°	10	5.4	2.65	0.84
-20C°	10	7.3	2.58	0.82
-24C°	10	7.2	2.75	0.87
Liquid nitrogen	10	8.4	2.60	0.82

*Dependant variable ACW (µg/ml)

Table 38. Stability of the ACW value after 168 h under the five different temperatures

Temperature (I)	Temperature (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower Limit	Upper limit
+25C°	+4C°	10	-1.9	0.91	0.064	-3.99	0.14
	-20C°	10	-3.8	1.27	0.015	-6.70	-0.95
	-24C°	10	-3.7	1.30	0.018	-6.69	-0.80
	Liquid nitrogen	10	-4.9	1.36	0.006	-7.97	-1.81
+4C°	+25C°						
	-20C°	10	-1.9	0.68	0.021	-3.44	-0.35
	-24C°	10	-1.8	0.67	0.024	-3.34	-0.30
	Liquid nitrogen	10	-3.0	0.83	0.006	-4.83	-1.09
-20C°	+25C°						
	+4C°						
	-24C°	10	0.1	0.29	0.799	-0.59	0.74
	Liquid nitrogen	10	-1.1	0.37	0.018	-1.89	-0.23
-24C°	+25C°						
	+4C°						
	-20C°						
	Liquid nitrogen	10	-1.1	0.37	0.014	-1.99	-0.30
Liquid nitrogen	+25C°						
	+4C°						
	-20C°						
	-24C°						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

4.1.3 Stability under tow different temperatures

Blood samples taken using Serum tubes were centrifuged for 10 minutes, 4000 R/min after being kept in the refrigerator for 30 minutes, then divided in tow groups. The first group was kept under room temperature (+25C°) and the second group was kept in the refrigerator ($^{+}$4 C°). The first examination was done after one hour because of technical reasons, then every tow hours the ACW was examined in both groups.

There is a clear decrease that starts after one hour from the first measurement, which is statistically significant for the ACW values in the samples kept under room temperatures over the eight hour test time (Tables 39 and 40).

Table 39. ACW stability under room temperatures within/ original values

Time	n	Mean value	s	s_x
1	10	14.3	3.62	1.14
2	10	13.4	3.73	1.18
4	10	12.2	3.67	1.16
6	10	11.7	3.69	1.17
8	9	11.2	3.60	1.20

*Dependant variable ACW (µg/ml)

Table 40. ACW stability at room temperature within 8 hours

Time (I)	Time (J)	Mean difference (I-J)	n	s_x	p	95% Confidence interval	
						Lower limit	Upper Limit
1	2	1.0	10	0.33	0.018	0.20	1.71
	4	2.1	10	0.42	0.001	1.20	3.08
	6	2.6	10	0.48	0.00	1.51	3.67
	8	2.8	9	0.51	0.001	1.61	3.96
2	1						
	4	1.2	10	0.39	0.014	0.30	2.06
	6	1.6	10	0.38	0.002	0.79	2.49
	8	2.1	9	0.48	0.002	0.98	3.20
4	1						
	2						
	6	0.5	10	0.19	0.040	0.03	0.88
	8	0.8	9	0.28	0.023	0.14	1.42
6	1						
	2						
	4						
	8	0.4	9	0.29	0.166	-0.23	1.11
8	1						
	2						
	4						
	6						

*Means difference is significant $p \leq 0.05$
*Dependant variable ACW (µg/ml)

In the samples kept under +4C° temperatures the same result was noted where the decrease started after one hour and continued over the eight hours of the test (Tables 41 and 42).

Table 41. Stability of the ACW under +4C° condition within 8 hours/ original values

Time	n	Mean value	s	s_x
2	10	13,3	3,30	1,05
4	10	12,3	3,39	1,07
6	9	11,6	2,82	0,94
8	9	10,8	3,06	1,02

*Dependant variable ACW (µg/ml)

Table 42. Stability of the ACW under +4C° condition within 8 hours

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	2	10	1	0,33	0,016	0,23	1,71
	4	10	2	0,47	0,002	0,92	3,06
	6	9	2,5	0,56	0,002	1,16	3,77
	8	9	3,1	0,70	0,002	1,50	4,74
2	1	10					
	4	10	1,0	0,42	0,039	0,07	1,97
	6	9	1,5	0,50	0,016	0,36	2,68
	8	9	2,1	0,62	0,010	0,66	3,50
4	1	10					
	2	10					
	6	9	0,4	0,31	0,244	-,32	1,10
	8	9	1,1	0,38	0,022	0,20	1,95
6	1	10					
	2	10					
	4	10					
	8	8	0,6	0,41	0,071	-0,01	1,83
8	1						
	2						
	4						
	6						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

4.1.4 Stability under conditions of five different temperatures

In this experiment blood samples were taken using Serum tubes, kept for 30 minutes in the refrigerator and then centrifuged for 10 minutes, 4000r/min, the outcoming Serum was divided into five groups, which were kept at different temperatures (room temperature (+25C°), +4C°, -20C°, -24C° and under liquid nitrogen, each of these groups was also divided to be tested after 24 hours and 168 hours.The first measurement was done after one hour because of technical reasons.

As seen in Tables 43 and 44 there was a statistically significant decrease in the ACW in the samples kept under room temperatures after 24 hours. Also there was adecrease after 168 hours.

Table 43. Stability of ACW at room temperature (+25C°) within 168 h/ original values

Time	n	Mean value	s	s_x
1	10	8.4	3.56	1.12
24	9	5.8	2.56	0.85
168	7	4.9	1.16	0.44

*Dependant variable ACW (µg/ml)

Table 44: Stability of ACW at room temperature (+25C°) within 168 h

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	24	9	3.0	0.60	0.000	1.70	4.24
	168	10	2.2	0.73	0.020	0.43	3.98
24	1						
	168		-0.2	0.43	0.720	-1.21	0.89
168	1						
	24						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

The same result was noticed in the samples kept in the refrigerator (+4C°), where the decrease started after 24 hours (Tables 45 and 46).

Table 45. Stability of ACW at refrigerator temperature (+4C°) within 168 h/ original values

Time	n	Mean value	s	s_x
1	10	8.4	3.55	1.12
24	10	7.1	3.36	1.06
168	10	5.0	2.65	0.84

*Dependant variable ACW (µg/ml)

Table 46. Stability of ACW at refrigerator temperature (+4C°) within 168 h

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	24	10	1.35	0.58	0.044	0.04	2.66
	168	10	3.02	1.05	0.019	0.634	5.40
24	1						
	168	10	1.67	0.79	0.064	-0.12	3.45
168	1						
	24						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

In the samples kept under -20C°, -24C° and liquid nitrogen no statistically significant change in the water-soluble antioxidative capacity was observed within the 168 hours of the study (Tables 47, 48, 49, 50, 51 and 52).

Table 47. Stability of ACW at -20C° within 168 h/ original values

Time (hr)	n	Mean value	s	s_x
1	10	8.4	3.55	1.12
24	10	8.4	3.02	0.95
168	10	7.3	2.58	0.82

*Dependant variable ACW (µg/ml)

Table 48. Stability of ACW at -20C° within 168 h

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	24	10	0.1	0.54	0.890	-1.15	1.31
	168	10	1.1	0.69	0.140	-0.45	2.69
24	1						
	168	10	1.0	0.68	0.160	-0.50	2.58
168	1						
	24						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

Table 49. Stability of ACW at -24C° within 168 h/ original values

Time	n	Mean value	s	s_x
1	10	8.4	3.55	1.12
24	10	7.0	3.64	1.15
168	10	7.2	2.75	0.87

*Dependant variable ACW (µg/ml)

Table 50. Stability of ACW at -24C° within 168 h

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	24	10	1.4	0.71	0.080	-0.21	3.01
	168	10	1.2	0.54	0.050	-0.01	2.41
24	1						
	168	10	-0.2	0.60	0.740	-1.57	1.16
168	1						
	24						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

Table 51. Stability of ACW in liquid nitrogen within 168 h/ original values

Time	n	Mean value	s	s_x
1	10	8.4	3.55	1.12
24	10	8.4	2.33	0.74
168	10	8.4	2.60	0.82

*Dependant variable ACW (µg/ml)

Table 52. Stability of ACW in liquid nitrogen within 168 h

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	24		0.0	0.57	0.970	-1.26	1.30
	168		0.1	0.62	0.930	-1.34	1.46
24	1						
	168		0.0	0.42	0.930	-0.93	1.01
168	1						
	24						

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

4.1.5 Centrifugation and time effect on ACW

Blood samples were taken using Serum tubes and kept in the refrigerator (+4 C˚) for 30 minutes. The first measurement of the ACW was possible after one hour because of technical reasons, in tow hours interval a blood sample was centrifuged for 10 minutes, 4000R/min and the water soluble antioxidative capacity was measured to test the stability of ACW.

As seen in Tables 53 the ACW values ranged from 11.1µg/ml after one hour to 10.2 µg/ml in the samples tested after 6 hours. The difference was statistically not significant (Table 54).

Table 53. Comparison of the ACW stability within 6 hours/ original values

Time	n	Mean value	s	s_x
1	10	11.1	4.40	1.39
2	10	10.9	4.39	1.39
4	10	10.8	4.24	1.34
6	10	10.2	4.70	1.49

*Dependant variable ACW (µg/ml)

Table 54. Comparison of the ACW stability within 6 hours

Time (I)	Time (J)	n	Mean difference (I-J)	s_x	p	95% Confidence interval	
						Lower limit	Upper limit
1	2	10	0.2	0.22	0.387	-0.30	0.71
	4	10	0.3	0.37	0.369	-0.49	1.18
	6	10	1.0	0.35	0.024	0.16	1.76
2	1	10					
	4	10	0.1	0.22	0.530	-0.36	0.65
	6	10	0.8	0.32	0.044	0.03	1.48
4	1	10					
	2	10					
	6	10	0.6	0.49	0.245	-0.50	1.71
6	1	10					
	2	10					
	4	10					

*Means difference is significant $p \leq 0.05$
*Dependant variable ACW (µg/ml)

4.1.6 Farm control

Blood samples were taken in Serum tubes from farms in which different postpartum times were tested. For this the cows were divided into four groups to measure the water soluble antioxidative capacity. Blood samples were centrifuged for 10 minutes, 4000 r/min after being kept for 30 minutes in the refrigerator (+4C°). Because of technical reasons the first test was done after one hour.

The animals were divided as follows:

- Group a: -3-0 weeks postpartum.
- Group b: 0-3 weeks postpartum.
- Group c: 3-5 weeks postpartum.
- Group d: 5-17 weeks postpartum.

As seen (Tables 55 and 56) in group a (-3-0 weeks pp) the ACW values were quiet different from each other in the different farms, varying from 9.2 µg/ml in farm 1 to 15.1 in farm 4.

Table 55. Comparison between ACW values in five farms in group a/ Original values

Farm	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
					Lower limit	Upper limit		
1	10	9.2	2.58	0.82	7.3	11.00	4.24	12.91
2	10	15.1	3.80	1.20	12.37	17.80	5.75	19.74
3	10	11.9	3.50	1.11	9.40	14.440	7.44	17.36
4	10	15.1	3.14	1.00	12.90	17.38	7.53	18.47
5	10	12.1	3.00	0.90	10.00	14.28	7.80	15.38

*Dependant variable ACW (µg/ml)

*F= 5.34, p= 0.002

Table 56. Comparison between ACW values in five farms in group a

Farm (I)	Farm (J)	Mean difference (I-J)	s_x	p	95% Confidence interval	
					Lower limit	Upper limit
1	2	-5.9	1.44	0.000	-8.84	-3.02
	3	-2.7	1.44	0.065	-5.64	0.17
	4	-6.0	1.44	0.000	-8.89	-3.08
	5	-3.0	1.44	0.045	-5.89	-0.07
2	1	5.9	1.44	0.000	3.02	8.84
	3	3.2	1.44	0.032	0.29	6.10
	4	-0.1	1.44	0.971	-2.96	2.85
	5	3.0	1.44	0.047	0.04	5.86
3	1	2.7	1.44	0.065	-0.17	5.64
	2	-3.2	1.44	0.032	-6.10	-0.29
	4	-3.2	1.44	0.029	-6.15	-0.34
	5	-0.2	1.44	0.867	-3.15	2.66
4	1	6.0	1.44	0.000	3.08	8.89
	2	0.1	1.44	0.971	-2.85	2.96
	3	3.2	1.44	0.029	0.34	6.15
	5	3.0	1.44	0.043	0.10	5.91
5	1	3.0	1.44	0.045	0.07	5.89
	2	-3.0	1.44	0.047	-5.86	-0.04
	3	0.2	1.44	0.867	-2.66	3.15
	4	-3.0	1.44	0.043	-5.91	-0.10

*Means difference is significant p≤ 0.05

*Dependant variable ACW (µg/ml)

In group b (0-3 weeks pp) the values also were different from each other in the different farms but there was an increase in the ACW values noticed in all farms under study (Tables 57 and 58).

Table 57. Comparison between ACW values in five farms in group b/ original values

Farm	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
					Lower limit	Upper limit		
1	8	10.1	2.41	0.85	8.13	12.16	6.40	12.30
2	8	16.6	1.89	0.67	15.03	18.19	12.88	18.41
3	10	17.1	4.71	1.49	13.73	20.46	8.77	25.00
5	10	14.0	1.67	0.53	12.83	15.23	10.70	16.12

*Dependant variable ACW (µg/ml)

*F= 5.34, p= 0.002

Table 58. Comparison between ACW values in five farms in group b

Farm (I)	Farm (J)	Mean difference (I-J)	s_x	p	95% Confidence Interval	
					Lower limit	Upper Limit
1	2	-6.5	1.51	0.000	-9.54	-3.40
	3	-7.0	1.43	0.000	-9.86	-4.04
	5	-3.9	1.43	0.010	-6.80	-0.98
2	1	6.5	1.51	0.000	3.40	9.54
	3	-0.5	1.43	0.738	-3.39	2.43
	5	2.6	1.43	0.080	-0.33	5.49
3	1	7.0	1.43	0.000	4.04	9.86
	2	0.5	1.43	0.738	-2.43	3.39
	5	3.1	1.35	0.030	0.32	5.81
5	1	3.9	1.43	0.010	0.98	6.80
	2	-2.6	1.43	0.080	-5.49	0.33
	3	-3.1	1.35	0.030	-5.81	-0.32

*Means difference is significant p≤ 0.05

*Dependant variable ACW (µg/ml)

In group c (3-5 weeks pp) there was a clear increase in the ACW values in all farms reaching a maximum value of 21.1 µg/ml in farm 3. all of the values were quiet different from each other (Tables 59 and 60).

Table 59. Comparison between ACW values in five farms in group c/ original values

Farm	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
					Lower limit	Upper limit		
1	7	11.1	2.94	1.11	8.33	13.77	4.54	13.22
2	10	19.9	3.05	0.97	17.69	22.05	16.61	25.68
3	10	21.1	9.84	3.11	14.02	28.11	13.12	46.00
4	8	14.6	4.09	1.45	11.14	17.98	7.00	18.82
5	10	14.29	1.27	0.40	13.3705	15.2015	12.45	17.00

*Dependant variable ACW (µg/ml)

*F= 5.34, p= 0.002

Table 60. Comparison between ACW values in five farms in group c

Farm (I)	Farm (J)	Mean difference (I-J)	s_x	p	95%-Confidence interval	
					Lower limit	Upper Limit
1	2	-8.8	2.63	0.002	-14.13	-3.50
	3	-10.0	2.63	0.000	-15.33	-4.69
	4	-3.5	2.76	0.212	-9.09	2.08
	5	-3.2	2.63	0.226	-8.55	2.08
2	1	8.8	2.63	0.002	3.50	14.13
	3	-1.2	2.39	0.62	-6.02	3.63
	4	5.3	2.53	0.042	0.19	10.43
	5	5.6	2.39	0.024	0.76	10.41
3	1	10.0	2.63	0.000	4.69	15.33
	2	1.2	2.39	0.620	-3.63	6.02
	4	6.5	2.53	0.014	1.39	11.62
	5	6.8	2.39	0.007	1.95	11.60
4	1	3.5	2.76	0.212	-2.08	9.09
	2	-5.3	2.53	0.042	-10.43	-0.19
	3	-6.5	2.53	0.014	-11.62	-1.39
	5	0.3	2.53	0.915	-4.85	5.39
5	1	3.2	2.63	0.226	-2.08	8.55
	2	-5.6	2.39	0.024	-10.41	-0.76
	3	-6.8	2.39	0.007	-11.60	-1.95
	4	-0.3	2.53	0.915	-5.39	4.85

*Means difference is significant $p \leq 0.05$

*Dependant variable ACW (µg/ml)

In group d (5-17 weeks pp) the ACW values continued their increase to reach 21.9 μg/ml in farm 3 (Tables 61 and 62).

Table 61. Comparison between ACW values in five farms in group d/ original values

Farm	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
					Lower limit	Upper limit		
1	9	9.9	2.41	0.80	8.01	11.72	6.00	12.66
2	10	18.0	5.61	1.77	14.03	22.05	10.60	31.39
3	10	21.9	5.39	1.71	18.05	25.76	10.16	29.26
4	10	17.6	4.31	1.36	14.54	20.70	14.34	27.81
5	3	13.6	0.63	0.36	12.08	15.20	12.96	14.20

*Dependant variable ACW (μg/ml)

*F= 9.092, p= 0.000

Table 62. Comparison between ACW values in five farms in group d

Farm (I)	Farm (J)	Mean difference (I-J)	s_x	p	95% Confidence Interval	
					Lower limit	Upper Limit
1	2	-8.2	2.08	0.000	-12.39	-3.96
	3	-12.0	2.08	0.000	-16.25	-7.82
	4	-7.8	2.08	0.001	-11.97	-3.54
	5	-3.8	3.02	0.219	-9.89	2.34
2	1	8.2	2.08	0.000	3.96	12.39
	3	-3.9	2.02	0.064	-7.97	0.24
	4	0.4	2.02	0.839	-3.69	4.52
	5	4.4	2.98	0.149	-1.64	10.44
3	1	12.0	2.08	0.000	7.82	16.25
	2	3.9	2.02	0.064	-0.24	7.97
	4	4.3	2.02	0.041	0.18	8.38
	5	8.3	2.98	0.009	2.22	14.30
4	1	7.8	2.08	0.001	3.54	11.97
	2	-0.4	2.02	0.839	-4.52	3.69
	3	-4.3	2.02	0.041	-8.38	-0.18
	5	4.0	2.98	0.19	-2.06	10.02
5	1	3.8	3.02	0.219	-2.34	9.89
	2	-4.4	2.98	0.149	-10.44	1.64
	3	-8.3	2.98	0.009	-14.30	-2.22
	4	-4.0	2.98	0.190	-10.02	2.06

*Means difference is significant p≤ 0.05

*Dependant variable ACW (μg/ml)

In Tables 63, 64, 65, 66, 67, 68, 69, 70, 70 and 72 the different postpartum groups were compared with each other in the different farms.

In farm 1 (Tables 63 and 64) the highest ACW value was noticed in group c (3-5 weeks pp) and the lowest was noticed in group a (-3-0 weeks pp).

Table 63. Comparison between the different postpartum groups in farm 1/ original values

Group	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
					Lower limit	Upper limit		
a	10	9.2	2.58	0.81	7.31	11.00	4.24	12.91
b	8	10.1	2.41	0.85	8.13	12.16	6.40	12.30
c	7	11.1	2.94	1.11	8.33	13.77	4.54	13.22
d	9	9.9	2.41	0.80	8.01	11.72	6.00	12.66

*Dependant variable ACW (µg/ml)

*F=0.764, p= 0.523

Table 64. Comparison between the different postpartum groups in farm 1

Group (I)	Group (J)	Mean difference (I-J)	s_x	p	95% Confidence Interval	
					Lower limit	Upper limit
a	b	-1.0	1.22	0.425	-3.48	1.51
	c	-1.9	1.27	0.145	-4.49	0.69
	d	-0.7	1.18	0.554	-3.12	1.71
b	a	1.0	1.22	0.425	-1.51	3.48
	c	-0.9	1.33	0.500	-3.63	1.81
	d	0.3	1.25	0.825	-2.27	2.83
c	a	1.9	1.27	0.145	-0.69	4.49
	b	0.9	1.33	0.500	-1.81	3.63
	d	1.2	1.30	0.367	-1.46	3.84
d	a	0.7	1.18	0.554	-1.71	3.12
	b	-0.3	1.25	0.825	-2.83	2.27
	c	-1.2	1.30	0.367	-3.84	1.46

*Means difference is significant p≤ 0.05

*Dependant variable ACW (µg/ml)

In farm 2 (Tables 65 and 66) it was the highest ACW values were in group c (5-17 weeks pp) and the lowest values in group a (-3-0 weeks pp). The ACW values in group d were higher than in group a and b.

Table 65. Comparison between the different postpartum groups in farm 2 /original values

pool	Group	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
						Lower limit	Upper limit		
15	a	10	15.1	3.79	1.20	12.37	17.80	5.75	19.74
27.37	b	8	16.6	1.89	0.67	15.03	18.19	12.88	18.41
26.68	c	10	19.9	3.05	0.97	17.69	22.05	16.61	25.68
20.87	d	10	18.0	5.60	1.77	14.03	22.05	10.60	31.39

*Dependant variable ACW (µg/ml)

*F= 2.687, p= 0.062

Table 66. Comparison between the different postpartum groups in farm 2

Group (I)	Group (J)	Mean difference (I-J)	s_x	p	95% Confidence interval	
					Lower limit	Upper limit
a	b	-1.5	1.86	0.416	-5.30	2.25
	c	-4.8	1.75	0.010	-8.34	-1.23
	d	-3.0	1.75	0.101	-6.51	0.61
b	a	1.5	1.86	0.416	-2.25	5.30
	c	-3.3	1.86	0.088	-7.03	0.52
	d	-1.4	1.86	0.448	-5.20	2.35
c	a	4.8	1.75	0.010	1.23	8.34
	b	3.3	1.86	0.088	-0.52	7.03
	d	1.8	1.75	0.303	-1.73	5.39
d	a	3.0	1.75	0.101	-0.61	6.51
	b	1.4	1.86	0.448	-2.35	5.20
	c	-1.8	1.75	0.303	-5.39	1.73

*Means difference is significant p≤ 0.05

*Dependant variable ACW (µg/ml)

The same result noticed for farm 2 was noticed in farm 3, where the ACW vales ranged from 11.9µg/ml 3 weeks antepartum to 21.9µg/ml 17 weeks postpartum (67 and 68).

Table 67. Comparison between the different postpartum groups in farm 3/ original values

Group	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
					Lower limit	Upper limit		
a	10	11.9	3.50	1.11	9.39	14.39	7.44	17.36
b	10	17.1	4.71	1.49	13.73	20.46	8.77	25.00
c	10	21.1	9.84	3.11	14.02	28.11	13.12	46.00
d	10	21.9	5.39	1.70	18.05	25.76	10.16	29.26

*Dependant variable ACW (µg/ml)

*F= 5.218, p= 0.004

Table 68. Comparison between the different postpartum groups in farm 3

Group (I)	Group (J)	Mean difference (I-J)	s_x	p	95% Confidence interval	
					Lower limit	Upper limit
a	b	-5.2	2.83	0.074	-10.95	0.54
	c	-9.2	2.83	0.003	-14.91	-3.43
	d	-10.0	2.83	0.001	-15.75	-4.27
b	a	5.2	2.83	0.074	-0.54	10.95
	c	-4.0	2.83	0.170	-9.71	1.77
	d	-4.8	2.83	0.098	-10.55	0.93
c	a	9.2	2.83	0.003	3.43	14.91
	b	4.0	2.83	0.170	-1.77	9.71
	d	-0.8	2.83	0.768	-6.58	4.90
d	a	10.0	2.83	0.001	4.27	15.75
	b	4.8	2.83	0.098	-0.93	10.55
	c	0.8	2.83	0.768	-4.90	6.58

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml)

In farm 4, on the other hand the highest ACW values were noticed in group d (5-17 weeks pp) and the lowest were noticed in group a (Tables 69 and 70).

Table 69. Comparison between the different postpartum groups in farm 4/ original values

Greoup	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
					Lower limit	Upper limit		
a	10	15.1	3.14	.993	12.89	17.38	8	18
c	7	15.6	2.94	1.111	12.92	18.36	10	19
d	10	17.6	4.31	1.361	14.54	20.70	14	28

*Dependant variable ACW (µg/ml)

*F= 1.315, p= 0.287

Table 70. Comparison between the different postpartum groups in farm 4

Group (I)	Group (J)	Mean difference (I-J)	s_x	p	95% Confidence interval	
					Lower limit	Lower limit
a	c	-0.5	1.76	0.779	-4.14	3.14
	d	-2.5	1.60	0.134	-5.79	0.82
c	a	0.5	1.76	0.779	-3.14	4.14
	d	-2.0	1.76	0.272	-5.62	1.66
d	a	2.5	1.60	0.134	-0.82	5.79
	c	2.0	1.76	0.272	-1.66	5.62

*Means difference is significant p≤ 0.05

*Dependant variable ACW (µg/ml)

In farm 5 the highest ACW values were noticed in group c (3-5 weeks pp) and the lowest in group a (-3-0 weeks pp) (Tables 71 and 72).

Table 71. Comparison between the different postpartum groups in farm 5/ original values

Pool porobe	Group	n	Mean value	s	s_x	95% Confidence interval		Minimum	Maximum
						Lower limit	Upper limit		
13.38	a	10	12.1	3.00	0.95	9.99	14.28	7.80	15.38
13.13	b	10	14.0	1.67	0.53	12.83	15.23	10.70	16.12
13.63	c	10	14.3	1.28	0.40	13.37	15.20	12.45	17.00
11.14	d	3	13.6	0.63	0.36	12.08	15.20	12.96	14.20

*Dependant variable ACW (µg/ml)

*F= 2.202, p= 0.109

Table 72. Comparison between the different postpartum groups in farm 5

Group (I)	Group (J)	Mean difference (I-J)	s_x	p	95% Confidence interval	
					Lower limit	Upper limit
a	b	-1.9	0.92	0.047	-3.77	-0.02
	c	-2.2	0.92	0.026	-4.03	-0.28
	d	-1.5	1.35	0.273	-4.26	1.25
b	a	1.9	0.92	0.047	0.02	3.77
	c	-0.3	0.92	0.782	-2.13	1.62
	d	0.4	1.35	0.774	-2.37	3.15
c	a	2.2	0.92	0.026	0.28	4.03
	b	0.3	0.92	0.782	-1.62	2.13
	d	0.6	1.35	0.635	-2.11	3.40
d	a	1.5	1.35	0.273	-1.25	4.26
	b	-0.4	1.35	0.774	-3.15	2.37
	c	-0.6	1.35	0.635	-3.40	2.11

*Means difference is significant p≤ 0.05
*Dependant variable ACW (µg/ml

4.2 Fat soluble antioxidative capacity (ACL)

For the determination of the ACL using the Photochem, the first step was the appropriate preparation of the samples.

Blood samples were taken using Serum tubes, were kept in the refrigerator for 30 minutes and then centrifuged for 10 minutes, 4000 r/min. The first measurement was possible after one hour because of technical reasons.

The Serum samples were first prepared in the following way:

100ml Serum+10ml distilled water+200ml Methanol+400ml N-Hexan

in order to extract as much as possible of the fat soluble antioxidants like vitamin A, vitamin E, vitamin D, steroids, alpha napthol, and ubiquinone.

First methanol was added to the Serum sample, and shaken with the hand for two minutes, then distilled water and N-hexan was added, and all contents were mixed on the vortex for two minutes, then the contents of the tube were centrifuged 4000R/min for 5 minutes.

In the tube there were two layers of alcohol with a layer that separated them which is the water layer.

The lower layer contained methanol and Serum, the upper contained hexan and the fat soluble antioxidants.

The contents of the upper layer were tested using the photochem under a nitrogen gas tent, which served to prevent the oxidation of antioxidants due to atmospheric oxygen.

This part of the study was stopped because of:

- The high costs of the nitrogen gas tent.
- Extraction of the fat-soluble antioxidants using this method takes long time.
- Processing the N-Hexan extract was not possible using the Photochem, because the pump system was not suitable. After a few measurements it turned out that the apparatus was defect, after repeated repairing of the apparatus, it was decided to stop this part of the study.

5 Discussion

5.1 Water soluble antioxidative capacity

5.1.1 Medium determination

In the current study attempts were first made to choose between different media used to preserve blood samples taken in the field, and to determine in which of these media is the water soluble antioxidative capacity (ACW) is most stable, because sometimes it takes long time for the blood samples taken in the field to be transported to the laboratory where different biochemical tests are made, in this time during transport the samples underly many factors that have an effect on the content of the samples under study like light, temperature, and time itself.

There are no studies related directly to choosing the proper medium for preserving blood samples when the water soluble antioxidative capacity will be tested. In this study the highest values were achieved using Serum samples, and this was the reason why Serum tubes were chosen in the rest of the study.

In this study four different media were tested, and compared with each other, the measured ACW values in the different media were arranged as follows:

Serum> NaF-LiH> LiH> EDTA

Serum tubes do not contain an anticoagulant, the results showed no significant decrease in the ACW values within three hours. A decrease might have happened in the first hour before the ACW values were measured, because it is well known that antioxidants, especially ascorbic acid which comprises a part of the water soluble antioxidants are sensitive to light.

As NaF has a toxic effect, the NaF tubes contained also LiH which has an anticoagulant effect which might play a role together with the toxic effect of NaF in paralysing red blood cells. The action of red blood cells on the antioxidants present in the sample stoped, which appear later in the test, where the values achieved using these tubes come in the second place after Serum and NaF-LiH tubes.

LiH on the other hand acts as anticoagulant that gives red blood cells the chance to live longer than in Serum tubes, during this time they consume antioxidants which might be the reason why the results using these tubes are lower than in Serum tubes. In Human medicine EDTA which is an acid containing four carboxylic acid groups, and two amine groups with ion-pair electrons that chealate calcium, and several other metal ions. Calcium is important

for a wide range of enzyme reactions of the coagulation cascade, and its removal irreversibly prevent blood clotting within the collection tubes, through this action of EDTA on red blood cells the red blood cells have the chance to live longer, and consume the antioxidants. Before the samples are tested.

Plasma tubes were used for the determination of the antioxidative capacity from human blood by **Popov and Lewin, (2005)** using the Photochem. Whereas by spectrophotometry method blood samples were kept in heparin containing tubes to determine the antioxidative capacity from mares (**Gorecka et al, 2002**). Tubes containing ethylenediamine-tetraacetic acid dipotassium salt (EDTA-K2) were used by **Rezaei and Naghadeh, (2006)** to evaluate the antioxidant status and antioxidative stress in cattle naturally infected with Theileria annulata.

It is not advised to use EDTA containing tubes because of the low measured ACW values, it is advised to use Serum tubes for the purpose of measuring the water soluble antioxidative capacity, as a second choice NaF LiH, and LiH has the advantage, that mostly it needs to be centrifuged only one time. LiH is used as the medium of choice, because the samples are tested immediately, but in case of veterinary medicine, immediate test of samples is not possible, because samples taken by veterinarians in the field need to be transported to the laboratory first, for this reason it is better to use Serum samples kept under low temperatures (lower than -20C˚).

5.1.2 The effect of time on the stability of ACW

Regarding the effect of time on the stability of water soluble antioxidative capacity which is composed mainly of ascorbic acid. It is noticed that the ACW values are stable in the Serum tubes, NaF-LiH and LiH containing without a significant change within three hours. In this study the first measurement was possible only after one hour which was the time needed to withdraw the samples, keep them in the refrigerator for 30 minutes and the centrifugation time. Weather the decrease in the ACW values happened during this time (antioxidants are sensitive to light) or weather the ACW values are really stable in these tubes is unclear.

In the second part of the study, there is a decrease in the ACW values, in the Serum samples kept under room temperature (+25C˚) that starts after tow hours. Individual variations between the animals in this study are to be considered, because blood samples were taken from different animals, suffering from different diseases in different disease stages

(sometimes after reception of the animal, or after treatment of the animal), the antioxidative status of every individual animal is different, depending on the health and nutritional status.
In the EDTA containing tubes the decrease continues over the three hours for reasons that might be related to the anticoagulant action of EDTA. In the NaF-LiH and LiH tubes antioxidants are consumed by the red blood cells, and are under the effect of light and temperature before centrifugation, but it seems that the red blood cells are not so active like in EDTA containing tubes.
In the Serum samples blood cells are physiologically not functional, but in the first hour before the first measurement is done, blood cells take the antioxidant, which prevent or help these antioxidants against the effect of light, after that when the sample is centrifuged, these antioxidants are released, and this might be the reason why the ACW values in these tubes are higher, after centrifugation there is decrease in the ACW values in the Serum tubes.
these results are similar to the results reported by (**George et al., 1995**) who reported that under -70C° ascorbic acid is stable over 4 years in human plasma.

5.1.3 Temperature and ACW

Regarding the effect of temperature on the stability of ACW, it was found that the water-soluble antioxidative capacity is most stable under liquid nitrogen with no decrease within 168 hours, and the highest decrease was under room temperature conditions, and in the refrigerator +4C°. The ACW values were also stable under -24C° and -20C° conditions within 168 hours.
Similar results were found by (**Haying et al., 2003**) who reported that the concentration of vitamin C did not change during the 6-day storage at -20 C° in the plasma of fattening cattle treated with dithioerythritol (DTE), but the vitamin C level in untreated samples decreased significantly (P<0.05) during storage at -20C°. Also (**George et al., 1995**) reported that under -70C° ascorbic acid is stable over 4 years in human plasma.
Margolis and Duewer (1996) demonstrated that total ascorbic acid (TAA, the sum of ascorbic acid and dehydroascorbic acid) in properly prepared human plasma is stable at -70 C° at least 6 years when preserved with dithiothreitol. TAA in human plasma or Serum preserved with metaphosphoric acid degrades slowly, at a rate of no more than 1% per year.
It is believed that the biochemical changes, that samples undergo, which in turn have an effect on the antioxidant contents of the samples is stopped under low temperatures (-20C°, -24C° and liquid nitrogen), which might have been the reason behind the stability of water

soluble antioxidative capacity in the samples kept under these temperatures in this study. These effects and changes could not be stopped under room temperature conditions and under +4C˚ conditions.

5.1.4 Centrifugation and ACW

Regarding the effect of centrifugation on the stability of water-soluble antioxidative capacity, the results showed that the ACW values of blood samples kept in Serum tubes without centrifugation are stable within 6 hours. Red blood cells take antioxidants and die in the samples kept in Serum tubes, and through this action, antioxidants are protected from the effects of light, temperature, but after centrifugation these antioxidants are released once again to be measured by the Photochem.

5.1.5 Farm control

The status of good health and high milk production of cows result from the balance between prooxidative and antioxidative processes in the body fluids, and in the cells. Oxidative stress can be stopped by defense activity of antioxidative system that consists of antioxidants inhibiting activated molecules. The most important antioxidant is ascorbic acid (**Kleczkowski et al., 2005**).

Regarding farm control, where different postpartum times in different farms were tested, for measuring the water soluble antioxidative capacity, it was noticed that the ACW is lowest 3 weeks before parturition in group a, and increases gradually to reach the highest values after 17 weeks of parturition in group d. The antioxidative capacity of animals in groups b and c are higher than those in group a. There are differences between th farms, which might be the result of different management systems, and different feeding . Considering ascorbic acid as the major component of the water soluble antioxidants, it was reported by **Santos et al., (2001),** that the concentration of plasma ascorbate is not affected by the stage of lactation, and those plasma ascorbate concentrations for primiparous cows were similar to those observed for multiparous, with no interaction between stage of lactation and number of lactations.

It is known that reactive oxygen metabolites are produced continuously by normal metabolic processes, but the rate of production might be increased markedly under diverse conditions of increased metabolic demand. The metabolic demands imposed on the cow by colostrum production and the onset of lactation are high (**Castillo et al., 2004**).

Haliloglu et al., (2002) found that vitamin C levels were 5.09±0.28 µg/ml in the 5^{th}-6^{th} months of pregnancy, in the research made by **Yldiz et al., (2005)** it was found that the level of vitamin C reached the maximum level in the 1^{st} month of pregnancy, and the minimum level at the delivery, he also noticed that that the concentration of ascorbic acid at the delivery period was lower than in the 1^{st}, 2^{nd} and 5^{th} months of pregnancy. Such a difference in the concentration of an important vitamin which is a component of the water soluble antioxidants at the delivery period might be the result of the transport of vitamin C and other nutrients from the blood into the colostrum, the stress caused by giving birth causes also a decrease in the antioxidative capacity of the animal (**Yldiz et al., 2005)**. By comparing between some biochemical parameters it was found by (**Karapehlüvan et al., 2007**), that there is a higher concentration of uric acid, and glutathione in the Serum of healthy calves than in the Serum of calves infected with ring worm, based on this information, taking into consideration that uric acid, and glutathione are components of the water soluble antioxidants, it is expected that the water soluble antioxidative capacity of cows around parturition will be lower than of cows not suffering from the stress caused by this stage.

In the Serum of cows suffering from mastitis stress, it was found by **Kleczkowski et al., (2005**) that there is a decrease in the concentration of ascorbic acid as a result of the stress caused by mastitis.

It was also stated that vitamin A and E, and β-Carotene levels decreased in pregnant cows, reaching the minimum values at the birth period (**Daniel et al., 1991**). **Herdt and Stowe, (1991)** reported that the concentration of vitamin E reaches its minimum concentration around parturition. **Hogan et al., 1993** reported that the concentration of α- tocopherol increases gradually after parturition.

Management of farms is not the same, the farms in this study were managed in different ways by different farmers, who use different feed rations, and this might be the reason behind the differences in the ACW values measured in the different farms. The blood samples were taken in Serum tubes from the animals in the farm, and then transported to the laboratory to be tested, the transport time ranged from 6-7 hours, so there is a decrease of the ACW values, which can not be measured, that happens during transport time

Depending on the results of this study, in the farm control section, it has been shown that the ACW values reflect the physiological status of the animals under study, and that the ACW values give an indication of the overall physiological status of the cow. Using pool probes is also a usefull means to estimate the physiological, and stress status of a whole farm.

5.2 Fat soluble antioxidative capacity (ACL)

It was not possible to measure the fat soluble antioxidative capacity using the Photochem because of long time needed for the extraction of the fat soluble antioxidants, and high costs. It is better to measure the concentration of each component of the ACL separately, which is the standard method used in laboratories.

5.3 General aspects and future work

Measuring of the water soluble antioxidative capacity (ACW) gives an idea of the physiological status of dairy cattle, it shows weather the animals are under stress or not, this picture can be complemented by measuring other biochemical parameters, like vitamin A, vitamin E. Weather it is possible to adapt this method to dairy cattle to make it one of the routine tests depends on the stability of the antioxidants in the Serum sample, and on the conditions under which these samples are transported to the laboratory, together with other biochemical parameters the ACW value can give a good picture of the health status of dairy cattle.

6 Conclusion

Based on this study, the following points can be concluded

- The water soluble antioxidative capacity (ACW), measured in different media (tubes) using the photochem, is not stable, and there exists variation in the values depending on the tubes. The ACW values are arranged in this manner: Serum> NaF-LiH> LiH > EDTA. For the measurement of ACW values from dairy cattle blood samples Serum tubes are most suitable.
- The water soluble antioxidative capacity (ACW), measured in Serum tubes is most stable without statistical detectable changes, under liquid nitrogen, at -24C°, -20C° within 168 hours, and is not stable when measured under room temperature conditions (+25C°), or at +4C°. In these cases there is a decrease, that is first noticed after one hour from the first measurement.
- The water soluble antioxidative capacity reflects the internal physiological status of dairy cattle, it was noticed that the ACW values around parturition were lower than the values 17 weeks postpartum, the values starting to increase gradually after parturition.
- ACW values are variable in different farms, depending on the management system in each farm.
- Measurement of the fat soluble antioxidative capacity is not possible by the photochem, it is better to measure the different components of the ACL separately.

7 Summary

Investigations to determine the antioxidative capacity in the blood of dairy cattle

Water and lipid-soluble antioxidative capacities were evaluated from blood samples taken from dairy cows in different lactation stages, and from diseased and healthy dairy cattle.

Blood samples using different media were taken, from dairy cattle in different lacatation stages, diseased, and healthy, in the clinic of cattle, faculty of veterinary medicine, Free university, Berlin. The first step was evaluation of the stability of the water soluble antioxidative capacity in different media used, using the photochem, the Serum samples were choosen because of the high values achieved using these tubes.

After that, the stability of ACW was tested using Serum tubes under different temperatures within a specific period, the samples kept under liquid nitrogen, -24C°, -20C° were stable within 168 hours, and the samples kept under room temperature (+25C°), and +4C° were not stable, the first changes were noticed after 2 hours.

Different postpartum times were tested, the ACW values reflect the internal physiological status of the animals. And animals suffering from stress around delivery have lower values than cows in the other groups. Different farms are also compared

It was not possible to measure the lipid soluble antioxidative capacity, because of a failure in the tubules in the apparatus.

8 Zusammenfassung

Untersuchungen zur Bestimmung von Antioxiddativen Kapazität im Blut von der Milchkuh

Als erstes erfolgte die Auswertung des Wassers, und der Lipid-löslichen Antioxidativenkapazität der Blutproben, die von den kranken und gesunden Milchkühen im unterschiedlichen Laktationsstadien entnommen werden..

Die Blutproben wurden mittels unterschiedlichen Medien (Röhrchen) aus der Klauentiere Klinik der freien Universität Berlin entnommen. Die Blutproben wurden mit hilfe von dem Photochem getestet. Die Serumröhrchen erzielten die höchsten Werte wobei die EDTA-Röhrchen die niedrigsten Werte erzielt haben.

Der zweite Schritt wurde die Stabilität der wasserlöslichen Antioxidativenkapazität in den Serumröhrchen ausgewertet. die auf Grund ihrer hohen Werte verwendet wurden. Infolgedessen wurde die Stabilität von ACW in den Serumröhrchen unter unterschiedlichen Temperaturen innerhalb einer spezifischen Zeit geprüft.

ACW Werte von Blutproben, die im flüssigem Stickstoff, unter -24C°, und unter -20C° aufbewahrt wurden, waren stabil innerhalb von 168 Stunden, und die ACW Werte von den Proben, die unter Raumtemperatur (+25C°), und unter +4C° aufbewahrt wurden, haben Veränderungen gezeigt, die nach zwei Stunden zu beachten waren.

Serumproben von Kühen, im unterschiedlischen Postpartumzeiten, von unterschiedlichen Bestände wurden entnommen, und getestet. Die ACW Werte stellen das innere Physiologische Status von dem Tier dar.

Es wurde in diesem Studie nachgewiesen, dass Kühe um die Geburtszeit relativ niedrige ACW- Werte als Tiere, die weit von diesem Zeitpunkt waren, haben. Dabei stiegen die ACW- Werte nach dem Geburt an.

Messung von den lipid löslischen Antioxidativenkapazität war auffallend nich möglisch, aufgrund zeitaufendige Extraktion. N-Hexan Extract könnte Im Gerät nicht verarbeitet werden. Nach einigee Messläufen war das Gerät defekt, nach wiederholte Reparatur wurde beschlossen die Messung einzustellen.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den

Hayajneh Firas Mahmoud