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# Oxidative state in the oestrus cycle of the buffaloes: a preliminary study

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Dear Editor and Reviewer,

thank You for the revision of our paper.

We are agree with the reviewer when he wrote that the “groups appear to be a thin sample”. We wanted to understand the trend of oxidative status in buffaloes, because we work in this field from many years. We enrolled a little sample, just to outline a first range of values, which have to be confirmed, expanding the samples. For this reason, we modified the title and added in conclusion (line 176-177) a sentence in which we specified that it is a preliminary study.

# Oxidative state in the oestrus cycle of the buffaloes: a preliminary study

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## ABSTRACT

The oxidative status, the relationship between oxidizing agents (free radicals) and antioxidant system, is able to influence the correct performance of the oestrous cycle. The aim of the experimentation was to define a first range of physiological values of reactive oxygen species (ROS) and the biological antioxidant potential (BAP), found during the phases of the buffalo estrous cycle. In this study, blood samples were taken from 30 buffaloes in various phases of the estrous cycle (oestrus, dioestrus, anoestrus), on whose serum by photometer, determinations of BAP and ROS have been carried out. The highest ROS values were detected during oestrus and this is due to the primary role they play in determining ovulation. The highest levels of BAP have been

found in dioestrus, when they perform protective action against oxidative damage in the ovaries and uterus.

Keywords: buffalo, oxidative status, oestrus, dioestrus, anoestrus.

# INTRODUCTION

Free radicals are molecules with one or more unpaired electrons on their last orbital, a condition that makes them highly reactive, so that they tend to subtract (Poston and Raijmakers, 2004) or donate an electron to a non-radical molecule (Hallywell, 1991), to achieve an electronic stability.

Two main categories of free radicals exist: Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Agarwal et al., 2005); both derive from oxidation (loss of electrons) or reduction (acceptance of electrons) reactions (Pourovà et al., 2010), occurring physiologically within the cells, during normal aerobic metabolic processes.

Free radicals play important roles in many biologic pathways, thus they are commonly found in blood. ROS are involved in respiratory brust (Saugstad, 2000; Bergendi et al, 1999), they are able to amplify inflammatory responses (Poston and Raijmakers, 2004) and appear to be involved in signal transduction in various biological processes (Saugstad, 2000; Fleury et al, 2002). However, when at high concentrations, they exert adverse effects in the cell, leading to lipid peroxidation, oxidation of proteins (with loss of their biological functions) and of nucleic acids (rupture of nucleotide filaments) (Valko et al., 2006).

Organisms have developed antioxidant defences in order to contain the potential adverse effects of ROS (Anderson and Phillips, 1999). These defences consist of exogenous components, taken with the diet, vitamin compounds, and not vitamin compounds (Liebler and Stratton, 1997), besides endogenous enzymatic and non-enzymatic antioxidants (Antolovich et al., 2002; Rahman, 2007; Uttara et al., 2009).

In reproduction, the ROS/antioxidants system modulates folliculogenesis, ovulation, formation and activity of the corpus luteum, luteolysis, early embryonic development, embryo implantation, initiation of parturition and placentation (Rizzo et al., 2012).

Studies performed in cows (Rizzo et al., 2007; Rizzo et al., 2009a), bitches (Rizzo et al., 2009b) and sheep (Rizzo et al., 2008) have shown that physiological ROS concentrations influence reproduction.

When ROS generation exceed antioxidant defences oxidative stress arises, leading to many diseases. In the ovary, high free radical levels inhibit oocyte development (Guerin et al., 2001), causing meiosis arrest, increase the percentage of degenerated oocytes (Tatemoto et al., 2000) as well as oocyte apoptosis (Liu et al., 2000). In addition, some authors have shown that oxidative stress is involved in the onset of follicular cysts, repeat breeder syndrome, mastitis, metritis and retained placenta in cattle (Kankofer, 2001; Miller et al., 2003; Rizzo et al., 2007; Rizzo et al., 2009a).

Given these premises and considering that, to the best of our knowledge, no data about the physiological range of blood ROS concentrations in the buffalo exist, this study aims to investigate, under physiological conditions, ROS serum concentrations in the buffaloes, during the different phases of the estrous cycle. Once established a physiological range, it could be helpful for diagnosing reproductive or other dysfunctions, in case they should lead to altered blood ROS concentrations.

# MATERIALS AND METHODS

This study was carried out between July 2017 and April 2018, in the farm "The Park", located in San Giovanni Rotondo, in the province of Foggia, Apulia Region, Italy.

The farm has a consistency of about 500 animals, including an average of 180 lactating ones, receiving a diet consisting of unifeed, deriving from corn silage, oat hay, lucerne hay, corn flour, soybean meal, cotton, crushed barley, beet pulp, vitamin and trace elements supplements.

Female buffaloes (3-5 year old), in postpartum from at least 100 days, with a daily milk production of about 10 kg and weighting 600 to 700 Kg were enrolled in this study.

The animals underwent a complete clinical examination, including genital tract inspection (rectal palpation), necessary to rule out eventual diseases and to detect the specific phase of the oestrous cycle of each buffalo. The detection of a tonic uterus, with a follicle of about 1.5 cm in diameter, with tense and floating wall and a corpus luteum in regression in the ovary, let define oestrus.

The buffaloes with a hypotonic uterus, a corpus luteum of approximately 1.5-2 cm in diameter and follicles <1 cm were referred to as being in the luteal phase (El-Shahata and Kandilb, 2012; Verma et al., 2018).

Upon complete clinical exmination examination, 30 healthy buffaloes, with a BCS of 3.5 to 4 on a scale from 1 to 5 (Anitha et al., 2011), were chosen for this study, of which 10 were in oestrus, 10 in dioestrus and 10 in anoestrus.

All the buffaloes underwent blood collection from the coccygeal vein using vacutainer serum-tubes chilled in advance.

The samples were transferred on ice to the laboratory of the Obstetrics section at the Department of Veterinary Medicine of Bari, where they were centrifuged at 1600xg for 10 minutes at + 4 ° C. The sera obtained were stored in 1.5 mL eppendorf and frozen at -20 ° C until analytical determinations.

Biological antioxidant potential (BAP test) and oxidative potential (dROMs-test reactive metabolites) were determined on the sera, through mono ready test photometer dedicated for use Free Carpe Diem ® (Diacron International, Grosseto, Italy).

dROMs-test, known as d-ROMs, allows to determine the concentration of Reactive Oxygen Metabolites (ROMs) in a biological sample, particularly hydroperoxides, deriving from the oxidative attack of many biochemical substrates (glycids, lipids, amino acids , proteins, nucleotides,

etc.). The results of d-ROMs test are expressed in arbitrary units, the Carratelli Units or U.CARR, where 1 U.CARR. equals 0.08 mg $H_2O_2$/dL.

As to BAP test, it is based on the ability of serum to reduce a given oxidizing substrate, suitably chosen based on its redox potential. Measuring the "dynamic" or "biologically active" component of the blood antioxidant barrier, provides a global measurement of many antioxidants (bilirubin, uric acid, vitamins C and E).

The results of BAP test are expressed as micromoles (µmol) of reduced iron per liter (L) of sample.

All the results are shown as mean ± SD. They were compared using SPSS 19 statistical program (IBM, NY). Particularly, one-way ANOVA with post-hoc LSD test was used for inter-group determinations. A value of $P<0.05$ was set as statistically significant.

# RESULTS

The results (mean ± SD) of ROS and BAP serum concentrations in oestrus, dioestrus and anoestrus are shown in Table 1.

| | **oestrus** | **dioestrus** | **anoestrus** |
|---|---|---|---|
| **ROS** (U.CARR) | 101.8 ± 6.03<br>a | 88.40 ± 3.47<br>b | 87.10 ± 11.84<br>b |
| **BAP** (µMmol / L) | 2072.71 ± 94.50<br>c | 2207.68 ± 111.90<br>d | 2200.16 ± 133.11<br>d |

**Table 1**: Mean ± SD concentrations of Reactive Oxygen Species (U.CARR) and Blood Antioxidant Potential (µmol/L) in buffaloes in the different phases of the cycle: oestrus, dioestrus and anoestrus. a, b: $P<0.01$; c, d: $P<0.05$

The highest ROS and BAP concentrations were observed in oestrus (101.8 ± 6:03 U. CARR) and dioestrus (2207.68 ± 111.90µmol/L), respesctively.

Statistically significant differences between oestrus vs dioestrus and oestrus vs anoestrus were found for ROS ($P < 0.01$) whereas for the BAP, the differences among the same groups showed a significance of $P < 0.05$.

# DISCUSSION

This study, even performed on a limited number of animals, is the first, to our best knowledge, to show a range of reference for physiological serum Reactive Oxygen Species (ROS) and Blood Antioxidant Potential (BAP), in the different phases of the estrous cycle, in buffaloes.

The results obtained in this study show that serum ROS levels, in buffaloes, are higher than those reported in the bovine species (Rizzo et al., 2007; Rizzo et al 2009a). It is conceivable that this finding may be related to inadequate feeding management (Bertoni et al., 2001). On the other hand, BAP levels are similar to those found in cattle (Rizzo, 2018 unpublished thesis, University of Bari Aldo Moro). This last datum is also confirmed by studies in which blood antioxidant concentrations (Glutathione peroxidase and superoxide dismutase) are similar to those of cattle (Morgante et al., 2001).

The greater rusticity of the buffalo, compared to the cow, could explain the findings of this study, i.e. ROS concentrations higher in buffaloes than in cows, with a similar BAP, in healthy conditions.

As to the different phases of the oestrous cycle, the highest serum ROS concentrations found in oestrus, could be interpreted with the knowledge that ROS play pivotal roles in this phase, in the ovary. In fact, they take part to the rupture of the preovulatory follicle walls (Jozwik et al., 1999), stimulating the apoptosis of granulosa cells and inducing, thereby, ovulation (Kodaman and Behrman, 2001; Shkolnik et al., 2011). Many literary data prove that the ovary itself produces ROS, in order to properly undergo ovulation. Ovulation was seen to be prevented if ROS generation is

148 counteracted by antioxidants (Miyazaki et al., 1991); in line with Miyazaki et al. (1991), Rizzo et al
149 (2009a) found lower ROS concentration in ovarian cystic fluid than in follicular fluid and suggested
150 that the reduced ROS concentrations had been unable to determine the rupture of the follicular wall,
151 having led to cystic formation.
152 As to the buffaloes enrolled in this study, in contrast to the high estrous ROS concentrations, a low
153 BAP was detected in the same phase. At this stage, antioxidants have the task of ensuring oocyte
154 protection from oxidative damage, until reaching the pre-ovulatory ovarian follicle stage and
155 improve the quality of the gametes (Sugino et al., 2000; Fujii et al., 2005), without, however,
156 interfere with the prime function of apoptosis induced by ROS, essential for ovulation (Sato et al.,
157 1992; Miyamoto et al., 2010).
158 During the luteal phase, however, a decline in ROS concentrations and a concurrent increase in
159 antioxidants was registered. The opposite trend observed, besides being in line with the functional
160 antagonism of ROS and BAP (Peltier and Smullan, 2006; Jones et al., 2008), might be explained
161 with the importance of antioxidants (mainly β–carotene) (Rizzo et al., 2013) in the luteal phase,
162 when they are necessary for a secretion of progesterone (Kato et al., 1997).
163 As to anoestrus, the concentrations of ROS and BAP were lower than the ones encountered in the
164 oestrus and dioestrus, a sign that, in these farming conditions, the antioxidant system is both
165 endogenous and exogenous (vitamin intake in the diet), and is able to keep under control the
166 oxidative status.
167 During dioestrus, a decrease in ROS and an increase in BAP levels were registered; this datum let
168 hypothesize that when BAP is high, it efficacely counteracts ROS generation (Peltier and Smullan,
169 2006; Jones et al., 2008), besides stimulating luteal progesterone secretion (Kato et al., 1997).
170 In this study, the high BAP levels detected in the luteal phase are in accordante with literature and
171 are related to the high luteal antioxidant content (mainly β-carotene).
172 Concluding, the results of this study, together with the cited literary data let infer that the ovary
173 might be a primary ROS source as a function of its activity and, at the same time, it is protected by

an adequate antioxidant system against the oxidative damage, contributing to a balance between oxidant and antioxidant activity.

This was a preliminary investigation. Further studies are needed to confirm the obtained range and to evaluate the trend of oxidative status under pathological conditions.

## ETHICAL APPROVAL

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee for animal experimentation, No. prot. 27/18).

## CONFLICT OF INTERST

The authors declare that they have no conflict of interest.

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