Antioxidant effect of lutein protects against oxidative damage to porcine spermatozoa

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Abstract

Gavazza, M.; Marmunti, M.; Compagnoni M.; Palacios, A.: Antioxidant effect of lutein protects against oxidative damage to porcine spermatozoa. Rev. Vet. 30: 1, 7-11, 2019. Boar sperm is especially susceptible to peroxidative damage generated by reactive oxygen species (ROS). Chemiluminescence was initiated by incubating porcine semen in an in vitro ascorbate-Fe²⁺ system, a technique that allows the evaluation of oxidative stress in these cells. Lutein is known for its antioxidant effects, chemically it is a dihydric derivative of α-carotene and belongs to the group of xanthophylls. The main objective of this study was to investigate the antioxidant effect of lutein on boar spermatozoa. The effect of lutein was analyzed by two methods: 1) by adding lutein: the sperm samples were placed in an in vitro ascorbate-Fe²⁺ system, during 120 min at 37°C adding increasing amounts of lutein (50, 150 and 250 μg) per mg of protein and 2) by incubation with lutein in an in vitro ascorbate-Fe²⁺ system, for 120 min at 37°C, using spermatozoa obtained from porcine semen samples previously incubated with lutein (0.15 and 0.25 mg/ml) during 24 h at 15°C. In both methods a control group (without lutein) was used. Peroxidation was measured by chemiluminescence using a liquid scintillation counter, the light emission being quantified in cpm (counts per minute). Analyzing the effect of lutein by the two methods, it was observed that the total amount of cpm/mg of protein originated by chemiluminescence was lower in samples obtained from the lutein group than in the control group (without lutein). Total chemiluminescence (cpm total) was lower in samples obtained from the lutein group than in the control group (without lutein), with a significance of p<0.005. Percent inhibition of peroxidation was not concentration dependent. These results would demonstrate that lutein could act as an antioxidant that would protect the membranes of the sperm from oxidative damage.

Key words: porcine, spermatozoa, reactive oxygen species, chemiluminescence, lutein.

Resumen

Gavazza, M.; Marmunti, M.; Compagnoni M.; Palacios, A.: El efecto antioxidante de la luteína protege a los espermatozoides porcinos contra el daño oxidativo. Rev. Vet. 30: 1, 7-11, 2019. El esperma de cerdo es especialmente susceptible al daño peroxidativo generado por las especies reactivas de oxígeno (ERO). La quimioluminiscencia se inició incubando semen porcino en un sistema in vitro ascorbato-Fe²⁺, técnica que permite evaluar el estrés oxidativo en estas células. La luteína es conocida por sus efectos antioxidantes, químicamente es un derivado dihidrico del α-caroteno y pertenece al grupo de las xantofilas. El objetivo principal de este estudio fue investigar el efecto antioxidante de la luteína en espermatozoides de cerdos. El efecto de la luteína se analizó por dos métodos: 1) por adición de luteína: las muestras de espermatozoides se colocaron en un sistema in vitro ascorbato-Fe²⁺ dependiente, durante 120 min a 37°C adicionando cantidades crecientes de luteína (50, 150 y 250 μg) por mg de proteína y 2) por incubación con luteína: también en un sistema in vitro ascorbato-Fe²⁺ dependiente, durante 120 min a 37°C se utilizaron espermatozoides obtenidos de muestras de semen porcino previamente incubados con luteína (0,15 y 0,25 mg/ml) durante 24 h a 15°C. En ambos métodos se utilizó un grupo control (sin luteína). La peroxidación fue medida por quimioluminiscencia, utilizando un contador de centelleo líquido, siendo la emisión lumínica cuantificada en cpm (cuentas por minuto). Analizando el efecto de la luteína por los dos métodos anteriormente mencionados, se observó que la cantidad total de cpm/mg de proteína originada por quimioluminiscencia, fue menor en muestras obtenidas del grupo luteína que en el grupo control (sin luteína). Se observó que la quimioluminiscencia total (cpm totales) fue menor en muestras obtenidas del grupo luteína que en el grupo control.
Although, free radicals and ROS play major roles in re-
vision and prevents the progression of cataracts. It is
found in the mammalian spermatozoa membranes,
from three potential sources: sperm mitochondria, cy-
functions 1.

fatty acids, sensitive to oxidation reactions; DNA is
in restoring a balance between ROS generation and
that herbal products can also boost male reproductive
scavenging activities. There are emerging evidences
produce lutein, it is included in food supple-
derived from three potential sources: sperm mitochondria, cy-
tosolic L-amino acid oxidases, and plasma membrane
seminal quality for a limited time. In recent years, work
high proportion of polyunsaturated fatty acids present
in its membranes. ROS are naturally formed as a by-
product of normal oxygen metabolism and are involved
in physiological sperm functions. When the balance
between ROS production and detoxification, its neu-
ralization by the antioxidants (Ax) is interrupted, ex-
duce lipid peroxidation that, in turn, disrupts membrane
characteristics that are critical for the maintenance of
sperm function 18, including the capacity to fertilize
an egg. Furthermore, the lipid aldehydes generated as
consequence of lipid peroxidation bind to proteins in
the mitochondrial electron transport chain, triggering
yet more ROS generation in a self-perpetuating cycle.

The boar spermatozoon is especially susceptible
to peroxidative damage generated by ROS, due to the
high proportion of polyunsaturated fatty acids present
in its membranes. ROS are naturally formed as a by-
product of normal oxygen metabolism and are involved
in physiological sperm functions. When the balance
between ROS production and detoxification, its neu-
ralization by the antioxidants (Ax) is interrupted, ex-
cess ROS creates an oxidative stress, which blocks the
cellular metabolism and decreases sperm motility. The
addition of Ax in refrigeration could minimize sperm
damage generated by ROS 21.

The measurement of the light emission from a
chemical reaction is very analytically useful because,
under appropriate experimental conditions, the light
output is directly related to the analytical concentra-
tion, thus allowing a precise and sensitive quantitative
analysis. In addition, light emission is usually repre-
sented by steady-state kinetics, which simplifies sam-
ple handling and measurement procedures. Chemilu-
 control (sin luteína), con una significancia de p<0,005. Los porcentajes de inhibición de la
peroxidación no fueron dependientes de la concentración. Estos resultados demostrarían que
la luteína podría actuar como un antioxidante que protegería las membranas de los esperma-
untozoides del daño oxidativo.

**Palabras clave:** porcino, espermatozoides, especies reactivas de oxígeno, quimioluminis-
cencia, luteína.

**INTRODUCTION**

Severe oxidative stress progressively leads to cell
dysfunction and ultimately cell death. Oxidative stress
is defined as an imbalance between pro-oxidants and/
or free radicals on the one hand, and anti-oxidizing sys-
tems on the other. The oxygen required for living may
indirectly be responsible for negative effects; these del-
eterious effects are due to the production of free rad-
cals, which are toxic for the cells (superoxide anions,
hydroxyl radicals, peroxy radicals, hydrogen peroxide,
hydroperoxides and peroxinitrite anions) 7.

Free radical attacks are responsible for cell dam-
age and the targeted cells are represented by the cell
membranes, which are particularly rich in unsaturated
fatty acids, sensitive to oxidation reactions; DNA is
also the target of severe attacks by reactive oxygen spe-
cies (ROS) 7.

High amounts of polyunsaturated fatty acid are
found in the mammalian spermatozoa membranes,
thereby making them susceptible to lipid peroxidation.
Although, free radicals and ROS play major roles in re-
production, they are strongly associated with oxidative
stress. Notably, antioxidant such as vitamin E and C,
carotenoids and carnitine have been found beneficial
in restoring a balance between ROS generation and
scavenging activities. There are emerging evidences
that herbal products can also boost male reproductive
functions 1.

Lutein is a dihydroxylated derivative of α-carotene,
a chemical compound belonging to the group of xan-
thophylls. It is a yellow pigment found in plants, algae,
photosynthetic bacteria and egg yolk. Because animals
do not produce lutein, it is included in food supple-
ments as an antioxidant.

Along with zeaxanthin are found in the ocular mac-
ula, however, these compounds are not transformed
into retinol. It is related to the reduction of degenera-
tion of the ocular macula, having as effect a better
vision and prevents the progression of cataracts. It is
also considered as a skin filter for light of shorter wave-
length (blue and violet) 6.

Oxidative stress plays a major role in the life and
death of mammalian spermatozoa. These gametes are
professional generators of ROS, which appear to derive
from three potential sources: sperm mitochondria, cy-
otosolic L-amino acid oxidases, and plasma membrane
nicotinamide adenine dinucleotide phosphate oxidases.

The oxidative stress created via these sources ap-
ppears to play a significant role in driving the physiologi-
minescence has been widely used as an indicator of the formation of reactive oxygen species in whole cells and organs, allowing the study of a number of pathophysiological conditions related to oxidative stress.\(^{18}\)

The purpose of this study was to analyze the susceptibility to non-enzymatic peroxidation by the in vitro addition of different doses of the vegetable antioxidant lutein on porcine spermatozoa and incubate semen samples previously with the vegetable antioxidant lutein and them subject the spermatozoa to a peroxidation process.

**MATERIALS AND METHODS**

**Chemicals.** Bovine serum albumin (BSA) (Fraction V) was obtained from Wako Pure Chemical Industries, Japan. L (+) ascorbic acid was from Merck Laboratories. Lutein was kindly supplied by Sigma Laboratory. All other reagents and chemicals were of analytical grade from Sigma.

**Animals, handling, evaluation and processing of ejaculates.** Procedures involving animals were in accordance with the recommendation of the Bioethics Committee of UNLP (National University of La Plata). The fresh boar semen samples (n = 6) kept at 15°C were obtained from stallions of the Faculty of Veterinary Sciences UNLP and of different commercial establishments in the province of Buenos Aires, Argentina.

Sperm rich ejaculate fractions (cross-breeding) obtained from three boars were collected by gloved-hand technique and filtered with gauze during collection and evaluated for conventional semen characteristics. Ejaculates that showed progressive motility >70%, viable cells >70%, normal spermatozoa >80%, and normal acrosomal integrity >80% were used.

Semen concentration was calculated by a manual count of sperm cells on a hemacytometer Bürker chamber. After evaluation, semen was diluted 1:4 in a commercial extender and transferred to 100 ml tubes, containing a dose of 6 billion spermatozoa each, cooled to 17°C, and sent by mail, packaged in insulated containers. The extended semen arrived at the Reproduction Lab., Faculty of Veterinary Sciences (La Plata, UNLP) the day after collection.

At the laboratory, it was evaluated for semen quality: 1) percentage of progressively motile spermatozoa placed under a coverslip in the center of a prewarmed 37°C plate; 2) percentage of viable cells, by eosin-nigrosin staining; 3) percentage of normal spermatozoa morphology and of acrosomal integrity, by viewing wet mounts of extended semen fixed in buffered 8% glutaraldehyde solution under a phase contrast microscope at a magnification of 1000x.

An aliquot of each of the kept at 15°C boar samples were sent from the Reproduction Lab. to the Biochemical Laboratory (Faculty of Veterinary Sciences, UNLP).

**Preparation of fresh boar semen samples.** An aliquot (1 ml) of semen of each samples was centrifuged at 800x g for 10 min, sperm pellets were separated, and washed by resuspending in PBS (phosphate buffer salinum) and recentrifuging (three times). After the last centrifugation, 1 ml of deionized water was added to spermatozoa and they were snap-frozen and stored at −20°C and used within a week of its preparation, after one cycle of freezing and thawing. All operations were performed at 4°C.

**Non-enzymatic spermatozoa peroxidation.** Chemiluminescence and peroxidation were initiated by adding ascorbate-Fe\(^{2+}\) to spermatozoa preparations.\(^{23}\) Spermatozoa samples (1 mg of protein) were incubated at 37°C with 0.01 M phosphate buffer (pH 7.4), 0.4 mM ascorbate, final volume: 1 ml.

Phosphate buffer is contaminated with sufficient iron to provide the necessary ferrous or ferric iron for peroxidation (final concentration in the incubation mixture was 2.15 \(\mu\)M)\(^{19}\). Pellets were incubated in vitro with increasing amounts of lutein (0.05 to 0.25 mg/ml) per mg of sperm protein.

In addition, samples of porcine semen previously incubated with lutein (0.15 and 0.25 mg/ml) were incubated for 24 h and maintained at 15°C. In all cases, spermatozoa preparations which lacked ascorbate-Fe\(^{2+}\) (control) were carried out simultaneously.

Chemiluminescence was measured as counts per min (cpm) in a liquid scintillation analyzer Packard 1900 TR. Membrane light emission was determined over 120 min period, and recorded as cpm every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein.

**Protein determination.** Proteins were determined by the method of Lowry et al. (1951) using BSA as standard.\(^ {14}\)

**Statistical analysis.** The data were subjected to the Student’s t-test. Data were expressed as mean ± SD. The 0.05 level was selected as the point of minimal statistical significance. Statistical criterion for significance was selected at different p values and indicated in each case.

**RESULTS**

**Effect of lutein by addition to the in vitro system ascorbate-Fe\(^{2+}\) on spermatozoa peroxidation.** Spermatozoa incubation in the presence of ascorbate-Fe\(^{2+}\) resulted in membrane peroxidation as evidenced by light emission (chemiluminescence) when the control and ascorbate-Fe\(^{2+}\) groups were compared. Values were 339.66 ± 72.01 in the control group while 535.33 ± 65.45 in the ascorbate-Fe\(^{2+}\) group.

After incubation of spermatozoa in an ascorbate-Fe\(^{2+}\) system at 37°C for 120 minutes in the presence of increasing amounts of lutein (50 \(\mu\)g, 150 \(\mu\)g and...
250 µg) per mg protein, the cpm originated from the light emission was lower in the lutein group than in the ascorbate-Fe²⁺ group. Values were 535.33 ± 65.45 in the ascorbate-Fe²⁺ group while with the addition of 0.05, 0.15 and 0.25 mg of lutein were 304 ± 17.68, 301.33 ± 30.44 and 310 ± 20.85 respectively, the significance was p<0.005 (Figure 1).

Spermatozoa obtained from porcine semen incubation with lutein: chemiluminescence. The values of light emission of spermatozoa obtained from porcine semen incubated with lutein (Figure 2) showed statistically differences (p<0.005), when the spermatozoa control group (ascorbate-Fe²⁺ without lutein) were compared with the spermatozoa incubated group with 0.15 and 0.25 mg/ml of lutein.

DISCUSSION

Peroxidation is recognized as a harmful process for spermatozoa, leading to loss of motility and reduced fertilization capacity in spermatozoa of many species, including man 2. Peroxidation occurs spontaneously in mammalian spermatozoa 12 and is greatly improved in human subfertile ejaculates 4 or in stored semen of birds 2, 22.

It is believed that the mechanisms by which ROS disrupt sperm function imply the peroxidation of the polyunsaturated fatty acids present in the sperm plasma membrane and this process plays an important role in the pathophysiology of male infertility 2. ROS increase the fragmentation of DNA 2, modify the cytoskeleton 13, affect the development of the sperm axon 10 and inhibit sperm fusion.

Porcine semen has high levels of unsaturated fatty acids. In previous studies we have shown that these fatty acids exposed to an in vitro system, in the presence of ascorbate, were vulnerable to peroxidation. In the presence of lycopene, a natural antioxidant that has a high in vitro ability to bind to singlet oxygen, a protective effect against peroxidation was observed, since the main unsaturated fatty acids were not affected by this process.

The main objective of this study was to investigate the antioxidant effects of lutein on boar spermatozoa. In vitro peroxidation research is desirable for the elucidation of possible peroxide formation mechanisms in vivo 4, since membrane composition causes vulnerability to peroxidative degradation 17.

Although important studies have been carried out to characterize changes in the structure, composition and physical properties of membranes undergoing oxidation 5, 15, 16, 20, it is necessary to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against the harmful effects produced by reactive species of oxygen and other free radicals.

The actual evidence shows that lutein has an effective antioxidant activity 11, with lutein being able to improve the biochemical parameters and reduce the formation of inflammatory cytokines, thus avoiding oxidative stress.

In conclusion, our results agree with the hypothesis that lutein could protect the membranes from oxidative damage.

Figure 1. Effect of lutein by addition to the in vitro system ascorbate-Fe²⁺ on spermatozoa peroxidation. Results are expressed as mean ± SD of six independent experiments. The values of light emission showed statistically differences (*p<0.005), when the control (without ascorbate) and ascorbate-Fe²⁺ (with ascorbate-Fe²⁺) spermatozoa membrane groups were compared. Similarly, differences (*p<0.005) were found when was compared ascorbate-Fe²⁺ with ascorbate and antioxidant addition (0.05, 0.15 and 0.25 mg/ml) spermatozoa membrane groups.

Figure 2. Spermatozoa obtained from porcine semen incubation with lutein: chemiluminescence. Results are expressed as mean ± SD of six independent experiments. After non enzymatic peroxidation, the values of light emission showed statistically differences (*p<0.005) between the lutein incubated spermatozoa membrane group compared with the control + ascorbate group (non incubated).
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