Theriogenology 84 (2015) 1273–1282

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Antioxidant effect of crocin on bovine sperm quality and *in vitro* fertilization

V. Sapanidou [a], I. Taitzoglou [a], I. Tsakmakidis [b], I. Kourtzelis [c], D. Fletouris [d], A. Theodoridis [e], I. Zervos [a], M. Tsantarliotou [a,*]

[a] *Laboratory of Physiology, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*
[b] *Clinic of Farm Animals, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*
[c] *Laboratory of Genetics and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece*
[d] *Laboratory of Hygiene and Technology of Food Animal Origin, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*
[e] *Laboratory of Animal Production Economics, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*

ARTICLE INFO

Article history:
Received 4 March 2015
Received in revised form 1 July 2015
Accepted 1 July 2015

Keywords:
Crocin
Sperm
Embryo
Antioxidant
Oxidative stress

ABSTRACT

Reactive oxygen species (ROS) production above critical levels affects the genetic and functional integrity of spermatozoa by causing oxidative stress. Spermatozoa are susceptible to oxidative stress in terms of motility and fertilization capacity. Crocin (crocetin di-gentiobiose ester), a main constituent of *Crocus Sativus* L. (saffron), is known for its antioxidant activity by scavenging ROS, especially superoxide anion. The aim of the present study is to evaluate the effect of crocin on the quality characteristics of spermatozoa and fertilization rate. Frozen–thawed and washed spermatozoa from four different bulls were incubated with three different concentrations of crocin (0.5, 1, and 2 mM), for 120 and 240 minutes, in the presence of a negative control, and were evaluated in terms of motility, viability, acrosomal status, DNA fragmentation index, intracellular ROS, and lipid peroxidation. The most potent concentration of crocin (1 mM) was also added in the fertilization medium to test its impact on fertilization outcome. The results indicate that the incubation of spermatozoa with 1 mM of crocin resulted in a statistically significant lower production of ROS, lower lipid peroxidation and in better maintenance of motility, viability, and acrosomal integrity, with a very small number of fragmented cells, compared to the control and the other treated groups ($P < 0.05$). Crocin concentration of 1 mM resulted in a significant increase of blastocyst rate, compared to the control group ($P < 0.01$). These data indicate that crocin (1 mM) improves bovine sperm quality and its fertilization capability, directly and/or indirectly, by modulating ROS concentration.

1. Introduction

One of the most important factors contributing to poor semen quality is oxidative stress (OS). Oxidative stress is a condition associated with an imbalance between the production of reactive oxygen species (ROS) and the ability of a biological system to detoxify the reactive intermediates or easily repair the resulting damage [1]. In semen, potential sources of ROS are dead and abnormal/immature spermatozoa, as well as the leukocytes that are present in the ejaculate [2,3]. During IVF, the partial pressure of oxygen is much higher than the partial pressure of oxygen *in vivo* [4]. Spermatozoa generate superoxide anion and hydrogen peroxide, which are formed either spontaneously

* Corresponding author. Tel.: 0030 2310 999863; fax: 0030 2310 999861.
E-mail address: mtsant@vet.auth.gr (M. Tsantarliotou).

0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.07.005

or through the action of superoxide dismutase. Gametes and embryos are very vulnerable to OS, especially under *in vitro* conditions. It is suggested that mild and low OS may enhance the fertilizing potential by promoting hyperactivation, motility, and capacitation, through increased tyrosine phosphorylation [5,6]. Apart from that, ROS mediate crucial reproductive processes, such as sperm–oocyte interaction, implantation, and early embryo development [6]. An excess production of ROS is detected after cryopreservation and thawing or centrifugation; this affects not only sperm motility and its ability to fuse the oocyte but also DNA integrity and fertilizing capacity as well [7]. Spermatozoa are particularly susceptible to oxidative injury because of the abundance of plasma membrane polyunsaturated fatty acids (PUFAs) [8]. These unsaturated fatty acids provide fluidity that is necessary for sperm motility [6] and for membrane fusion events (e.g., acrosome reaction [AR] and sperm–egg interaction). However, the free radical attack and the ongoing lipid peroxidation (LPO) throughout the sperm plasma membrane result in accumulation of lipid peroxides on the sperm surface, loss of sperm motility [9], and oxidative damage to DNA [10]. There is evidence that a positive correlation between DNA damage, ROS generation, and apoptosis exists [7]. An early apoptotic feature reported in spermatozoa is the externalization of phosphatidylserine (PS) on the outer leaflet of the plasma membrane [11], which is associated with a decreased ability to fertilize [12], although Martin et al. [13] supported that PS exposure in human sperm is mainly related to the AR rather than apoptosis.

Spermatozoa are not heavily equipped with antioxidant systems, capable of protecting them from the overwhelming production of ROS. These limitations are due to the small volume of cytoplasm and the low concentration of scavenging enzymes [14]. Furthermore, the antioxidant systems of the seminal plasma are removed during assisted reproductive techniques (ART) [3].

Spermatozoa undergo the risk of OS, and many antioxidants have been used *in vitro* to maintain their integrity and functionality [15,16]. Several experimental and clinical studies on pathophysiology of OS and its impact on infertility have demonstrated the beneficial role of many antioxidants on sperm parameters and pregnancy rates (for review, see [15,17]). Enzymatic antioxidants, such as superoxide dismutase, and vitamins (e.g., vitamin E) are regarded as very efficient antioxidant agents to maintain a stable ratio between OS and the antioxidant capacity of spermatozoa [18]. A special group of antioxidants consists of plant-derived compounds, such as carotenoids. There is strong evidence that natural antioxidants, carotenoids included, may reduce or prevent many diseases that are ROS mediated (e.g., cancer, diabetes, varicocele) [19].

Carotenoids are equipped with an extensive system of conjugated double-edge bonds. They are regarded as one of the most efficient $^1O_2$ quenchers, as well as ROS scavengers operating in cellular lipid bilayers. Moreover, carotenoids offer a special protection against LPO [20].

Saffron (*Crocus sativus*, L.) is a natural food additive with a well-known antioxidant action [21] and multiple therapeutic properties that have been proved both *in vitro* and *in vivo* [22,23]. Saffron affects positively sperm morphology and motility in infertile men [24] and in mice [25].

Crocin (crocetin di-gentiobiose ester), a main constituent of saffron, is one of the few water-soluble carotenoids found in nature, which acts as an antioxidant by quenching free radicals, especially superoxide anion [26]. Under *in vitro* conditions, crocin had an ameliorative effect on postthawed sperm motility of red deer through an optimum level of ROS [27]. Crocin might affect sperm physiology through its protective antioxidant effect in the media of ART.

Although there is strong evidence that the use of antioxidant additives enhances sperm quality parameters [15–17,27], the effect of antioxidant supplementation in the IVF medium on fertilization rate and embryo quality remains controversial [28,29]. Therefore, the present study was undertaken to investigate for the first time whether supplementation of *in vitro* sperm preparation media with crocin can prolong thawed sperm quality characteristics over time, by preventing them from the oxidative attack in freeze–thawing procedures. Furthermore, crocin was tested as a beneficial antioxidant in the IVF medium on fertilization process in terms of embryo development rate.

2. Materials and methods

2.1. Experimental design

The semen used in the experiments originated from four different mature Simmental bulls of proven fertility, housed at the Center of Artificial Insemination of Thessaloniki (National Agricultural Research Foundation, Nea Ionia, Thessaloniki, Greece). The semen was collected with an artificial vagina at the same period of the year. The collected semen had greater than 70% initial motility, greater than 75% viability, and a total concentration of at least 4×10^9 spermatozoa/mL. The collection and freezing of semen were performed under commercial conditions. Semen was diluted with a commercial extender (20% Tris-egg yolk, 7% glycerol, 78-mM citric acid, 69-mM fructose, 50-μg tylosin, 250-μg gentamicin, 150-μg lincomycin, 300-μg spectinomycin in each mL of extended frozen semen) and packed into 0.5-mL plastic straws, each one containing approximately 50×10^6 spermatozoa/mL. The frozen straws were stored in liquid nitrogen (−196 °C). The experiments were conducted under the principles of Good Laboratory Practice. At the beginning of each experiment, one straw from each bull was thawed by immersion in distilled water (37 °C, 40 seconds). The straws were immediately pooled into a sterile plastic tube and were subsequently washed with the appropriate media for each assessment. All reagents were purchased from Sigma Aldrich Co. (Germany), unless otherwise specified.

Crocin (crocetin di-gentiobiose ester-17304) of high purity (>99%) was stored as a powder at +4 °C in the dark. The stock solution of crocin (10 mM) was prepared in water for embryo transfer (W1503), split in aliquots, and stored at −20 °C in the dark. In each experiment, a fresh diluted