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## EGG YOLK AND LDL: POSSIBILITIES FOR ARTIFICIAL INSEMINATION IN EQUINES

### YEMA DE HUEVO Y LDL: POTENCIALIDADES PARA LA INSEMINACION ARTIFICIAL EN EQUINOS

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## ABSTRACT

The world horse industry exerts an important role as a job and income generation source. Reproductive technologies arises as an important tool in the service of world equine growth. Artificial insemination (AI) is perhaps the biotechnology with greater impact on equine breeding; a stallion can leave hundreds of offsprings over his reproductive life if AI is efficiently used. In some countries, egg yolk is frequently used as part of equine seminal extenders. The egg yolk provides the spermatozoa "resistance factors" when it is added. The protective fraction of the egg yolk probably is the low density lipoproteins (LDL). Several studies have reported successful results with the addition and replacement of egg yolk by LDL. There are many citations about the use of egg yolk in seminal extenders for stallion's cooled and frozen semen, and in the equine reproduction practice. The egg yolk dilutors are used with good fertility results. New research is needed for the better understanding of the protective effects of egg yolk and the LDL for stallion semen. The LDL would be a great solution for dilutors to artificial insemination in horse. This review discusses the use and the advantages of egg yolk and LDL as constituents of equine semen extenders.

**Key words:** Egg yolk; LDL, semen, equine.

## RESUMEN

La industria equina ejerce un importante papel como fuente generadora de empleo y renta. Las biotecnologías de la reproducción constituyen una valiosa herramienta para la mejora mundial en la especie equina. Dentro de las técnicas se encuentra la inseminación artificial (IA), que probablemente es la biotecnología con mayor impacto en la equino-cultura, una vez que un garañón pueda producir centenas de productos de buena calidad a lo largo de su vida reproductiva. En algunos países la yema de huevo es utilizada como medio de dilución para semen equino, porque puede proporcionar a los espermatozoides "factores de resistencia". Los efectos protectores de este medio probablemente sean ejercidos por las lipoproteínas de baja densidad (LDL). Diversos estudios han relatado el suceso cuando substituyen yema de huevo por LDL. También existen trabajos orientados a la utilización de yema de huevo como constituyente del medio de dilución para semen de garañones conservados a temperaturas de refrigeración y congelación. Se requiere de nuevas investigaciones para entender los mecanismos protectores de la yema del huevo y las LDL para el semen del garañón. El objetivo de la presente revisión fue contextualizar sobre la utilización de la yema de huevo y las LDL como medio de dilución del semen equino, pudiéndose esta última, constituir en una gran solución como medio de dilución en la inseminación artificial de esta especie animal.

**Palabras clave:** Yema de huevo, LDL, semen, equino.

## INTRODUCTION

The world horse industry exerts an important role as a job and income generation source. Biotechnology reproduction arises as an important tool in the service of world equine growth, as an instrument of direct genetic improvement. Given the advantages offered by artificial insemination (AI), this is perhaps the biotechnology with greater impact on equine breeding, because a stallion can leave hundreds of offsprings over his reproductive life if AI is efficiently used (1).

Artificial insemination in horses is widely practiced throughout the world, and the most commonly way used in this species is by cooling and transporting semen to the mare's location (1). It has been suggest that apparently, the countries that mostly use the AI method of cooled transported semen are the United States, followed by Brazil (2).

The transport of equine semen, is not in itself a new technique, and may even have been responsible for the first citation in literature involving AI in domestic animals; the Arabians texts from the year 1322 reported a Chieftain that would have ordered

his warriors to collect semen from a stallion of a rival tribe to perform the insemination of one of his mares (3).

For many decades, the development and use of AI in the equine species was restricted because many breeders' associations did not allow the use of the technique (4). Recently the laws in many countries have become more flexible, allowing the registration of foals generated by this biotechnology, having a major impact on the world's horse industry, mainly USA (1), Europe (5) and Brazil (2).

However, not all stallions present satisfactory fertility rates after the cooling and transport of its semen, due to the low sperm quality (with low sperm motility or normal motility with low fertility) (1,6). Alternatives have been proposed for improving the results of cooled semen transport, such as: centrifugation prior cooling to remove seminal plasma; collection of fractionated sperm; the use of different cooling rates as well as the use of several seminal extenders (1,6-10).

The search for a seminal extender has been the focus of several papers (2,7,9,11), however in accordance to Amann and Pickett (12) and Silva Filho (8) the formulation of an ideal extensor will yet be the target of many researches.

According to other authors (3,4,13,14), the most used extender in the world is based on skim milk dried-glucose proposed by Kenney et al (11). In countries such as Germany (15), Japan (16,17) and Brazil (7,18-20) the egg yolk is widely use in equine seminal extenders.

The main objective of this paper is to review the potential uses of egg yolk and egg yolk lipoproteins as main components of extenders used in the artificial insemination equine industry.

**The egg yolk and LDL.** Pioneer studies conducted by Bogart and Mayer (21), clearly demonstrate the greatest sensitivity of stallion spermatozoa to handling and storage, and that the spermatozoa can acquire "resistance factors" when egg yolk is added to extenders. These experiments show the important elucidation of the factors of the protection of egg yolk for equine semen, relative to the cold shock, and capacity for prolonged preservation of the extender with egg yolk.

The fatty acid composition of the egg yolk from hens fed with a corn soybean diet, corresponds to 35% saturated fatty acids, 45% monounsaturated fatty acids and 20% polyunsaturated fatty acids (22). Lipids are the primary components of egg yolk (about 65% of dry matter). They are composed of triglycerides (65%) phospholipids (29% out of which 86% are phosphatidylcholine and 14% phosphatidylethanolamine) cholesterol (5%), and free fatty acids (<1%) (23).

An extender with a chemically defined protein base has been the target of several researches as there are disadvantages to using egg yolk or milk because they are organic products and they vary widely in their composition from one batch to another. With this in mind researchers have identified and used, in seminal extenders, the protective fraction of the milk, as being the

native phosphocaseinate (9), and that of the egg yolk, as being the low density lipoproteins (LDL) (24). The egg yolk, as demonstrated by Pace and Graham (25), can contain substances able to interfere with cellular respiration and subsequently can lead to motility loss. Thus the purification of the protective portion would bring benefits to the process of sperm conservation, as the undesired effects would be removed and only the LDL portion would be used in extenders (26).

Several studies have reported successful results with the addition and replacement of egg yolk by LDL in the semen freezing process of different species, like bull (26,27), ram (28) and dogs(29). Similar studies in the ram (30) and in the Tomcat (31) have reported the use of LDL for cooling semen at 5° C. Unfortunately there are few studies involving the addition of LDL for conservation of stallion semen (32).

The LDL is the portion of the egg yolk with major emulsification capacity, representing around 2/3 of its solid content, and is part of the soluble fraction of the yolk called plasma (33). It has an average density of 0.982g/ml, a spherical format with 17 to 60 nm in diameter, with a lipid layer comprising triglycerides and cholesterol, which are surrounded by a film of phospholipids and protein. The phospholipids play a key role in the stability of the LDL structure because the forces of association between molecules are essentially hydrophobic (23,33). The LDL contains between 83 - 89% lipids and 11 - 17% protein. The LDL are composed of approximately 69% triglycerides, 26% phospholipids and 5% cholesterol (22).

The LDL is considered responsible for the cold shock protection factors presented by Bogart and Mayer (21), and reaffirmed by Pace and Graham (25). It is not known for sure by which mechanism the LDL protects the spermatozoa at low temperatures. According to the recent studies, with cryopreserved bovine semen, conducted by Manjunath et al (34) and Bergeron et al (35), there is an interaction between proteins expressed in the bull's seminal plasma and the egg yolk LDL (35). The bull expresses in the seminal plasma lipid-binding-proteins

(BSP: BSP-A1/A2, BSP-A3 and BSP-30-kDa). These BSPs induce the removal of cholesterol and phospholipids from the spermatid membrane, thus inducing spermatid capacitation (35). The addition of egg yolk in the freezing extenders causes interaction between LDL and BSP, thus making the membrane stable to the process of cooling and freezing (36).

**Dilutors and egg yolk.** Phillips (37) was the first to report the use of egg yolk based extenders for bulls' semen while Lardy and Phillips (38) were the first to cite the use of the egg yolk component in seminal extenders of horses. Apparently Berliner (39) was the first to use egg yolk based extenders in jacks and stallions semen in a horse breeding center in the state of Mississippi, USA. Since then, many papers have been published, and there have been several citations about the use of egg yolk, as a constituent of the extenders used for stallions cooled and frozen semen, in the equine reproduction industry (7,15,18,40, 41).

The extender of Nagase and Niwa (42) modified by Silva Filho et al (7) (lactose-egg yolk) without glycerol, is widely used in Brazil with good fertility results, equivalent to or above the Kenney extender (11) (skimmed milk dried-glycose) and the glycine-egg yolk extender (18-20, 43-49). After the good results obtained with the stallion semen, the dilutor has been used in donkeys, also showing good results (50). Experiments were conducted by Silva Filho and colleagues (44) and compared pregnancy rates between the use of lactose-egg yolk dilutor after transport and insemination on the stud, against fresh semen *in natura*, under the same conditions, transported and used on the spot of harvest. In this experiment they evaluated 101 cycles of 64 Mangalarga Marchador mares, and obtained the best results with the diluted semen both transported (MSP-1 container) and not transported. The pregnancy rates post the third cycle with diluted shipped semen was 83.88% (25/30 cycles); and for the raw semen (*in natura*) shipped 87.5% (7/8) and for the raw semen (*in natura*) without shipment 89.47% (34/38).

Weiss et al (47) in a comprehensive report under field practice conditions, with mares of the Crioulo breed, the second main race of horses from Brazil with next to 300 thousand animals registered, compared the effect of inseminations with fresh semen diluted with lactose-egg yolk extender, against fresh semen *in natura*. They performed artificial insemination in only one cycle of 142 and 160 mares respectively, and fertility rates were found to be similar. For diluted semen was 74.6% (106/142 cycles) and for fresh semen *in natura* 74.3% (119/160 cycles).

Silva Filho (8) made the comparison between fresh semen *in natura* and fresh diluted semen, using the following extenders: 1) skimmed milk dried-glycose, 2) lactose - egg yolk and 3) glycine - egg yolk. They performed AI of 42 crossbred Breton and Campolina breed mares, in 64 cycles, not getting differences between extenders and *in natura* semen. Respectively for Dilutor 1 81.25% (13/16 cycles); Dilutor 2 70.59% (12/17) and for the Dilutor 3 56.25% (9/16).

In an experiment conducted under field practice conditions in Brazil (18), the researchers compared the pregnancy rates after AI with fresh semen diluted in skimmed milk-glycose, and semen diluted in lactose-egg yolk transported at 15 - 20°C. Seventy nine mares and fillies of the Mangalarga Marchador breed were used showing similar pregnancy rates among the extenders (71.05% and 68.29% for skimmed milk extender and lactose-egg yolk respectively).

Using the lactose egg yolk dilutor, Carvalho et al (43) made the comparison of different inseminating doses for semen transported for a short period of time of 2 hours at 20°C, in a container called MSP -2. They carried out the insemination of 92 Mangalarga Marchador mares and fillies, using inseminating doses of: < 250 millions progressive motile spermatozoa, 250 to 350 million progressive motile spermatozoa, and > 350 million progressive motile. There were no statistical differences between the various concentrations, with a lower numerical tendency towards the higher doses. Sperm

number  $< 250 \times 10^6 = 36\%$  (9/25 cycles); sperm number  $250-350 \times 10^6 = 44.12\%$  (15/34 cycles); sperm number  $> 350 \times 10^6 = 54.76\%$  (23/42 cycles).

Silva Filho et al (19) compared two extenders, glycine – egg yolk and lactose-egg yolk extenders. They performed artificial insemination in 108 cycles of 62 mares of the Mangalarga Marchador breed. Semen was extended and transported at  $20^\circ\text{C}$  in the MSP-2 container. Conception and pregnancy rates between extenders showed very similar results. Diluitor 1 on stud farm 1 =  $48\%$  (12/25 cycles)/ Diluitor 01 on stud farm 2  $63.33\%$  (19/30 cycles); Diluitor 2 Nagase in the stud farm 1 =  $41.67\%$  (10/24 cycles)/ Diluitor 2 in stud 2 =  $51.72\%$  (15/29 cycles).

Lima et al (20) analyzed reproductive efficiency data in two Mangalarga Marchador stud farms in the state of Minas Gerais, Brazil, with 125 mares of various ages and reproductive categories. The inseminations were performed with the same protocol extending the stallion's semen with lactose-egg yolk and transported in a MSP-2 container at  $20^\circ\text{C}$ , with the inseminating dose of 300 million of sperm with progressive motility. The mares were inseminated: T1-one AI / cycle  $51.43\%$  (18/35); T2 two AI / cycle  $48.89\%$  (22/45); T3 - three or more AI / cycle  $47.50\%$  (19/40). Effects of the number of inseminations per cycle over pregnancy rates were not observed.

The egg yolk presents other disadvantages in the constitution of the dilutors such as the turbidity that hampers the seminal assessment process by conventional microscopy (8). Some attempts have been made to carry out the clearing of the egg yolk through different techniques, but without

achieving the same success of *in natura* yolk egg (14). The addition of orvus-es-paste (OSP), a substance with emulsifying properties, was performed by Martim et al (40). This extender became very popular given the success on fertility rates, in addition to better clearance and visualization under a microscope.

Canisso et al (48) performed the addition of the OSP to the original dilutor (42), in the same proportion (0.04 ml OSP/20 egg yolk ml) used by the authors earlier. Through fertility tests in 60 mares Campolina breed, with inseminations performed until 6 hours after ovulation with at least 300 million of sperm with progressive motility, they compared the effect of Nagase and Niwa's (42) dilutor and that of Martim et al (40), in donkey semen freezing. The pregnancy rates at 13 days were  $54\%$  and  $53\%$  respectively without statistical differences between the Extenders, and also obtaining better spermatoc visualization on the common optical microscope.

## CONCLUSIONS

The egg yolk has been successfully applied to the horse breeding industry. The raw diluted, cooled and frozen preserved with egg yolk present fertility rates equal either superior to those with skimmed milk base dilutors. Generally, the most useful concentration of egg yolk is twenty per cent. The LDL is protective fraction of egg yolk, the use of this fraction it might be provide a suitable sperm protection. New research is needed for better understanding the mechanisms of action of egg yolk and LDL for stallion semen dilutor. The LDL would be a great solution for dilutors to artificial insemination in horse.

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