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Sperm–egg penetration assay assessment of the contraceptive effects of glycerol and egg yolk in rooster sperm diluents

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ABSTRACT

Glycerol (GLY) and egg yolk (EY) are good cryoprotectants of avian and mammalian sperm, but in birds, they strongly inhibit the eventual fertilization of ova. Using the sperm penetration (SP-holes) assay and fertility trials, the present study investigates (1) the possible mechanism by which this contraceptive effect occurs in chickens and (2) the maximum concentrations of GLY and EY tolerated by fresh rooster sperm. Seventy Black-Barred Andaluza hens (five per treatment) were inseminated four times (twice per week) with 0.1 mL of fresh semen from roosters of the same breed diluted 1:1 (v:v) with Lake and Ravie medium containing different concentrations of GLY or EY. No adverse effects on acrosome integrity, sperm motility, or viability were seen with any concentration of GLY or EY. The number of SP-holes on perivitelline layer samples taken from above the germinal disc became progressively lower at GLY concentrations of 1.5% or greater ($P > 0.05$). No holes caused by sperms were seen in unfertilized eggs. The corresponding fertility results showed similar reductions when the GLY concentration was 1.5% or greater. No changes in the number of SP-holes were seen with increasing EY concentrations (0%–7.5%), nor were any differences in fertility observed, except for a reduction when 15% EY was used. The results therefore reveal that GLY affects the transit of sperms through the oviduct in their attempt to reach the infundibulum area, limiting their access to the ovum perivitelline layer. Egg yolk had no such effect, nor did it influence acrosome reaction capacity; its mechanism of contraceptive action therefore remains unknown. The maximum GLY and EY concentrations tolerated by the rooster sperm were 0.75% and 7.5%, respectively.

1. Introduction

The cryopreservation of sperm requires the use of cryoprotectants that minimize cell damage during cooling and freezing and thawing [1]. Glycerol (GLY), one of many permeating cryoprotectants, is generally thought to be the most effective at protecting avian and mammalian sperm cells against cold shock and is the least toxic toward them [2–6]. Egg yolk (EY) also helps in protecting spermatozoa during cooling [7], freezing and thawing [8]. The synergistic effect afforded by EY and GLY when used together allows even higher postthaw sperm survival rates to be achieved, as seen in bovines [9]. Egg yolk is, therefore, routinely included as an additive in semen cryopreservation protocols [8].

Although the use of GLY in rooster sperm diluents is associated with high frozen-thawed sperm motility and

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viability, its use has been shown to have contraceptive effects in birds, leading to low fertility rates [10]. A number of protocols have therefore been developed to eliminate any GLY from frozen-thawed semen before insemination [10–15]. However, the damage caused by exposing poultry sperm to GLY [16–18], the consequence of chemical toxicity and osmotic shock [19,20], cannot be reversed by simply removing the cryoprotectant. In addition, GLY might alter the functionality of the avian genital tract by changing its osmolality, the properties of the luminal fluid, or the pattern of cilia motion [10]. Certainly, it has been reported in chicken that GLY exerts some form of contraceptive effect at the uterovaginal junction [21–24].

The use of EY also reduces eventual fertility rates in chicken [25–28]. A recent study reported that diluting rooster semen with chicken EY protected the sperm well from cold shock during cooling and during the freezing and thawing process, but that this sperm could fertilize no ova. This suggests EY also has a contraceptive effect in the hen genital tract [29].

Unfortunately, the true mechanisms underlying the contraceptive effects of both GLY and EY remain unknown [1,29]. The sperm–egg penetration (SP-holes) assay, however, could be useful in investigating them. The SP-holes are used to determine the number of spermatozoa that bind to and penetrate the inner perivitelline layer of an ovum before its fertilization, and this indicates an adequate acrosome reaction. Certainly, it is useful for determining the fertilizing capacity of sperm from male birds [30]. Studying SP could improve our knowledge of the ability of GLY- and EY-exposed spermatozoa to traverse the uterovaginal junction and show a successful acrosome reaction.

The concentrations of GLY and EY suitable for adding to freezing media, i.e., those that provide the best protection possible against sperm cryoinjury while avoiding a strong contraceptive effect, also need to be established. This is particularly true when dealing with local or endangered breeds [31]. The assay of SP-holes could also be useful in this respect.

Using both of SP-holes assay and fertility trials, the aim of the present work was to investigate (1) the possible mechanisms behind the contraceptive effects of GLY and EY in chickens and (2) the maximum concentrations of GLY and EY tolerated by fresh rooster sperm without adversely affecting any of the sperm functions.

2. Materials and methods

2.1. Experimental birds

Black-Barred Andaluza chickens (15 males and 70 females), all one year old at the beginning of the experiment, were housed under natural photoperiod and temperature conditions in sand-floor pens with a partial roof cover at the El Encín Research Station (Madrid, Spain, 40°31′N). These birds were raised as part of the genetic resources conservation program of the Spanish National Institute for Agricultural and Food Research [32,33]. All birds were fed a commercial diet containing 16% CP, 2700 kcal of ME/kg, 3.5% Ca, and 0.5% available P over the entire experimental period. All handling procedures were approved by the Spanish National Institute for Agriculture and Food Research and Technology (INIA) Ethics Committee and were performed in accordance with the Spanish Policy for Animal Protection RD1201/2005 which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

2.2. Semen collection

Semen was collected in 15-mL graduated centrifuge tubes (Sterilin; Sterilin Ltd., Newport, UK) using the massage technique [34]. Pools of semen from all 15 roosters were made on each occasion. These were immediately divided into equal aliquots, as required by each experiment, and diluted 1:1 (v:v) at field temperature with a medium composed of sodium glutamate (1.92 g), glucose (0.8 g), magnesium acetate $4H_2O$ (0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone (relative molecular mass, Mr = 10,000; 0.3 g), and H_2O (100 mL; final pH 7.08; final osmolality, 343 mOsm/kg; hereinafter referred to as Lake and Ravie [L&R] medium [11]). Different concentrations of GLY (final concentration of 0.0%–10.0%) or EY (final concentration of 0.0%–15.0%) were added to this medium, as required by each experiment. The diluents were prepared in the laboratory using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma–Aldrich Co. (St. Louis, MO, USA).

2.3. Sperm evaluation

All semen analyses were made in duplicates for each treatment within 1 hour of semen collection. Osmolality was measured in the semen samples diluted with the L&R medium containing the different concentrations of GLY and EY, using an Advanced Micro Osmometer Model 3300 (Advanced Instruments Inc., Norwood, MA, USA; Table 1). Sperm concentration and motility were assayed as described elsewhere [35] using a computer-aided sperm analysis system coupled to a Nikon Eclipse model 50i phase-contrast microscope (negative contrast) and using the Sperm Class Analyzer v.4.0. software (Microptic S.L., Barcelona, Spain) with settings adjusted to detecting avian spermatozoa (A = 5 μm^2; curvilinear velocity [VCL] = 10–100 μm/s). As required, sperm samples were diluted over the range 1:41 to 1:61 (v:v) in the L&R medium and loaded onto a warmed (38 °C) 20-μm Leja 8-chamber slide (Leja Products B.V., Nieuw-Vennep, the Netherlands). The percentage of motile spermatozoa and the percentage showing progressive motility (spermatozoa swimming forward quickly in a straight line) were recorded. Sperm movement characteristics, VCL, straight-line velocity (VSL), average path velocity (VAP), and beat cross

Table 1
Osmolality of semen diluted 1:1 (v:v) with Lake and Ravie medium containing different concentrations of glycerol (GLY) or chicken egg yolk (EY).

Final GLY concentration (%)	0	0.75	1.5	3	4	5.5	10
Osmolality (mOsm/kg)	365	442	530	741	858	1138	1987
Final EY concentration (%)	0	0.75	1.5	3	5	7.5	15
Osmolality (mOsm/kg)	335	339	339	340	341	337	339