Snail Mucus Enhances the Motility of Fresh Goat Semen Preserved in Egg Yolk Extender

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ABSTRACT

The effect of snail mucus on sperm motility (SM) and viability (% live-dead ratio) (LDR) of fresh goat semen extended in egg-yolk was evaluated. Semen was collected from four adult West African Dwarf (WAD) bucks using an electroejaculator under diazepam (0.3mg/kg) and ketamine (5mg/kg) anaesthesia. After collection, the semen were pooled together and divided into four different extenders comprising: (a) 20 mls of egg yolk (E); (b) 10 mls of egg yolk and 10 mls of 1% snail mucus (EM_1); (c) 5mls of egg yolk and 15mls of 1% snail mucus (EM_2) and (d) 20mls of 1% snail mucus (M) respectively at the rate of 5ml of extender to 0.5 ml of goat semen. Each part was again split into three parts and either refrigerated (5^{0}C), stored at room temperature (25^{0}C) or placed in water bath (37^{0}C) respectively. Both the SM and LDR were assessed at 0, 1, 3, 5, 12 and 24 hours of storage. The procedure was repeated four times and the means values determined. Data were compared using 2-way analysis of variance (ANOVA), with a 5% significance level. In this study, none of the extenders was able to maintain sperm motility for up to 12 hours. The SM was significantly ($P< 0.05$) higher in EM_1, than in other extenders. Only EM_1 extender was able to maintain the sperm motility above 50% for up to five hours of storage time. Similarly, the LDR was significantly ($P<0.05$) higher in EM_1 compared to other extenders. Also, none of the storage temperatures significantly ($P> 0.05$) influenced SM and LDR of the semen. It was therefore, concluded that addition of snail mucus to egg yolk improved the motility and percentage live-dead ratio of fresh goat semen for up to five hours, however this effect was dependent on the volume of the snail mucus added.

Key Words: Snail, mucus, goat, semen, motility, viability

INTRODUCTION

The preservation of mammalian sperm is a complex process that involves balancing many factors in order to obtain satisfactory results. The most common cryopreservation diluents used for goat semen contain either egg yolk or non–fat dried skim milk. However, the dilution of goat semen into diluents containing egg yolk or milk can be detrimental to the sperm cells (1). It was observed that egg yolk coagulated due to an enzyme of bulbourethral origin, known as egg yolk-coagulating enzyme (2). This enzyme hydrolyzes egg yolk lecithin into fatty acids and lysolecithin (3). This hydrolysis causes the sperm membranes to be more fusogenic thereby inducing the acrosome reaction (4) and chromatin condensation which is toxic to the sperm (5).

The conventional method of overcoming the harmful interactions of seminal plasma and egg yolk proteins is to dilute the goat semen sample in buffered diluents and then separate the seminal plasma from the sperm by centrifugation (6). However, this procedure is time consuming and may damage the sperm cells if improperly done. Although, the benefits of removing the seminal plasma from goat semen before dilution in egg yolk extender have been de-

scribed (4), other authors have reported positive results for sperm frozen without washing (7, 8). Storage of liquid semen at a temperature above 15^0C have been described as an alternative to the problem associated with egg yolk coagulating enzyme in goat semen preservation (9). The use of Tris-egg yolk cryopreservation diluents have also been recommended for goat semen preservation because of their ease of use (1). Despite these developments, it is quite evident that the search for the ideal diluents for preservation of both fresh and frozen goat semen is still ongoing as the diluents presently available are not satisfactory in all cases.

Glycosaminoglycans (GAGs) have been described as having the potentials of improving the quality of both fresh and frozen semen (10, 11) GAGs enhance sperm motility, calcium uptake, sperm capacitation and acrosome reaction (10). In addition, it increases *in vitro* fertilization rates of bull spermatozoa (12). Inclusion of synthetic heparin to boar and bull semen has been reported to increase the motility and capacitation of sperm *in vitro* (10, 13, and 14). Glycosaminoglycan was also reported to stabilize sperm with already declining motility (11).

A new glycosaminoglycan has been isolated from the internal mucus of the land helix *Archatina fulica* (15, 16). Analysis of the mucus showed that it is composed mainly of water (95%), electrolytes, mucus glycol conjugates, lecithin and haemocyanin (17). It was reported to be similar to but distinct from heparin (15). These glycosaminoglycans have been reported to be involved in binding, uptake and transport of divalent cations, uptake and retention of water, as well as, in the mobility of the snail (15). In addition, the proteoglycans and glycosaminoglycans in the snail mucus have been reported to function as glyconutrients, enzymes and signals to cells.

With the abundance of glycosaminoglycans in the snail mucus and the potential role of GAG in *in vitro* sperm preservation, it is hypothesized that inclusion of snail mucus to egg-yolk extenders will improve the condition of semen preservation and the attending increase in fertility. The aim of this study therefore, was to evaluate the effect of inclusion of different amounts of snail mucus in egg yolk extender on the motility and viability of goat semen. We also evaluated the effect of three different storage temperatures on the motility and viability of goat semen preserved in egg-yolk extender with varying amount of snail mucus.

MATERIALS AND METHODS

Animals

Four sexually matured West African Dwarf bucks with average weight (15.2 ± 2.6 kg) and age ranging between 2-3 years were used for this study. They were purchased from the local market and housed in individual pens of the small ruminants housing unit of the Department of Veterinary surgery and Reproduction in University of Ibadan. They were fed with concentrates and fresh elephant grass and supplied with clean water *ad libitum*. In addition, they were conditioned for the experiment two weeks prior to the commencement of this study during which they were dewormed with ivermectin (Ivomec®, Hoescht, Germany) at a dose rate of 1 mg/100kg body weight. Also, 5% oxytetracycline injection (Oxytetracyclina®, Invesa, Netherlands) at dose rate of 5mg/kg body weight was administered for 5 days.

Snail mucus

Shells of matured land snail (*Achatina marginata*) were opened at the apex and a spirally coiled rod inserted to remove the fleshy body from the excretory parts. The fleshy parts were then placed in about 250 ml of distilled water and washed several times until the mucus was completely washed off. Washings from several snails were then pooled together in a plastic bucket and precipitated using chilled acetone and then lyophilized. The lyophilized flakes of mucus were then pulverized into fine powder and stored in an air-tight container until used. When used, a 1 % solution of the snail mucus was prepared by dissolving 10 grams of the pulverized powder in one litre of distilled water.

Semen collection

Semen was collected using 25 voltage electroejaculator under anaesthesia induced by the combination of 0.5% diazepam (Trazinite®, Ningbo Pharmaceuticals, China) at the rate of 0.3 mg/kg and 5% ketamine hydrochloride (Ketmin®, Laborate Pharmaceuticals, India) at the rate of 5 mg/kg. Semen was collected from each buck once weekly. For semen collection, each buck was aseptically prepared prior to collection by clipping the preputial hair, then the preputial sheath and penis were washed with Chlorhexidine (Purit, Saro life Care Limited, Lagos, Nigeria) and distilled water. A graduated collection tube with a plastic funnel was washed, dried and insulated with Styrofoam. During the buck preparation

for semen collection, feaces were manually evacuated from the rectum with gloved hands, the rectal probe was lubricated with non-perfumed Vaseline and inserted into the buck's rectum and the other end of the probe was connected to the electroejaculator and the voltages applied and increased in pulses (0-5V) intermittently not exceeding 5V. An average of 0.6 ml was collected from each buck.

Semen analysis

The semen collected from the four goats were pooled together to eliminate goat effect and placed in a water bath at 37^{0}C and examined macroscopically and for the volume, color and consistency. In addition, an aliquot was taken from the pooled semen and immediately analyzed for gross and individual subjective motility.

Design

After determining the spermiogram of the pooled semen, the rest of the semen was divided into 4 parts of 0.5 mls each and diluted with 5 mls of each of the extenders. Each extender contained either 20 mls of egg yolk (E), 10mls of egg yolk and 10mls of 1% snail mucus (EM_1), 5mls of egg yolk and 15 mls of 1% snail mucus (EM_2), or 20mls of 1% snail mucus (M) to which 80mls of citrate buffer was added to make a 100ml solution. Each diluted fraction was further split into three parts for storage at 5^{0}C, room temperature (25^{0}C) and hot water bath (37^{0}C). Equal aliquots were taken from each diluted fractions and placed in plastic vials. Each fraction was evaluated at 0 hr 1 hr, 3 hr, 5 hr, 12 hr and 24 hr respectively. The whole procedure was repeated four times to obtain five replicates for each extender.

Semen evaluation

Both the progressive motility (SM) and percentage live-dead ratio (LDR) were determined for each split fractions of the extended semen. Progressive motility and the live dead ratio were determined subjectively by two blind assessors, after which the average score for the assessors was determined as described (3). Percentage live – dead ratio (LDR) was evaluated using Eosin-Nigrosin stain (9) and was expressed as the number of live cells out of one hundred cells that were counted.

Data analysis

Data were expressed as mean ± standard deviation. Both the sperm motility and the percentage live – dead ratio were compared using Wilcoxon sign rank test. Comparison was made between the different temperatures and the type of extender using two way analysis of variance (ANOVA). P value < 0.05 was considered significant in all cases.

RESULTS

At refrigeration temperature (5^{0}C), the percentage sperm motility decreased significantly (P<0.05) in goat semen preserved in (EM_2) media (80% to 0%), while the motility was

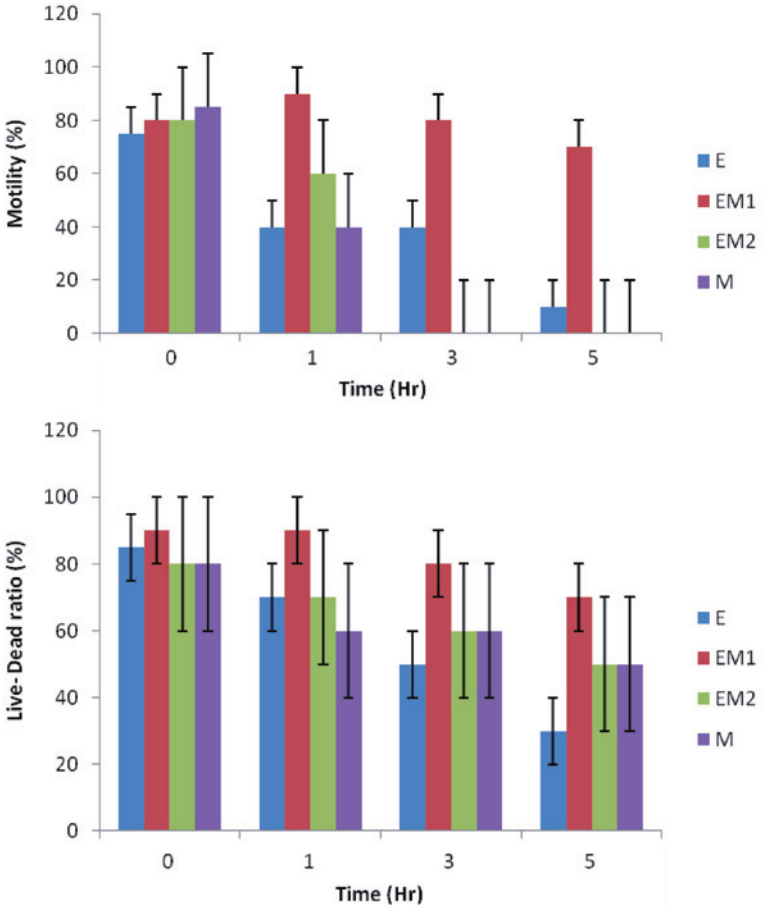

Fig 1a & b: Sperm motility (SM) and Live – Dead ratio (LDR) of goat semen preserved either in 20mls of egg yolk (E); 10mls of egg yolk and 10mls of snail mucus (EM_1), 5mls of egg yolk and 15mls of snail mucus (EM_2) and 20mls of snail mucus (M) respectively and stored either at 5^{0}C. P < 0.05

well maintained in (EM_1) media throughout the 5 hour period (90% to 70%) (Fig.1a). The motility of the goat semen preserved in (E) media maintained motility for 5 hours although at a very low rate (75% to 10 %). The percentage live-dead ratio was significantly higher (P< 0.05) in goat semen preserved in (EM_1) media for up to five hours of storage (90% to 75%) (Fig. 1b). All of the media maintained at least 50 percent live spermatozoa for the 5 hour duration except (E), in which the percentage live spermatozoa was about 30 percent at five hours of preservation. None of the extender maintained sperm motility by twenty four hours of storage.

Similarly, the percentage motile sperm of semen preserved at room temperature (25^{0}C) significantly (P< 0.05) decreased in all the media used except in the mucus (EM_1) media (Fig. 2a). Goat semen preserved in (M) was able to maintain motility for 5 hours, although at a very low rate (75% to 20%). Sperm motility appeared to be higher in goat semen preserved in (M) (85% - 20%) than those preserved in (E) (75% - 5%). Overall, the (EM_1) (90 % to 70 %) has the overall highest motility compared to the other three media tested. Also, the percentage live spermatozoa was significantly higher (P< 0.05) in semen preserved in (EM_1) than the other three media (Fig. 2b). In addition, the percentage live sperm was higher in goat semen preserved either in (M) (80% to 50%) or (EM_2) (80% to 50%) compared to those preserved in (E) (85% to 30 %) between 1-5 hours of semen preservation.

The sperm motility and viability of goat semen preserved in four different media in a water bath (37^{0}C) is shown in Fig. 3a and b. None of the extenders was able to maintain

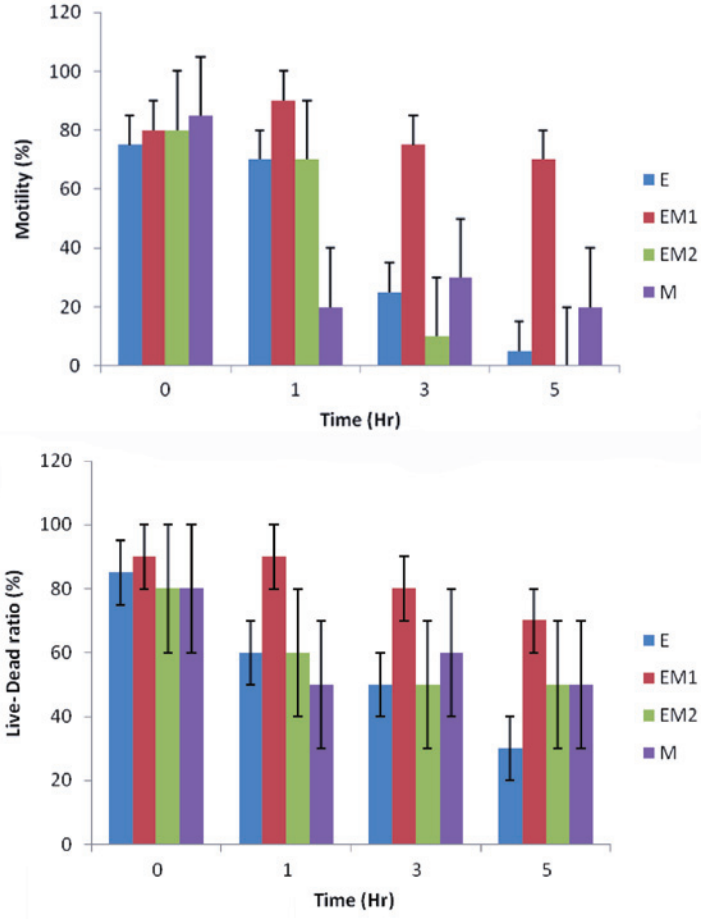

Fig 2a & b: Sperm motility (SM) and Live – Dead ratio (LDR) of goat semen preserved either in 20mls of egg yolk (E); 10mls of egg yolk and 10mls of snail mucus (EM_1), 5mls of egg yolk and 15mls of snail mucus (EM_2) and 20mls of snail mucus (M) respectively and stored either at bench (25^{0}C). P < 0.05

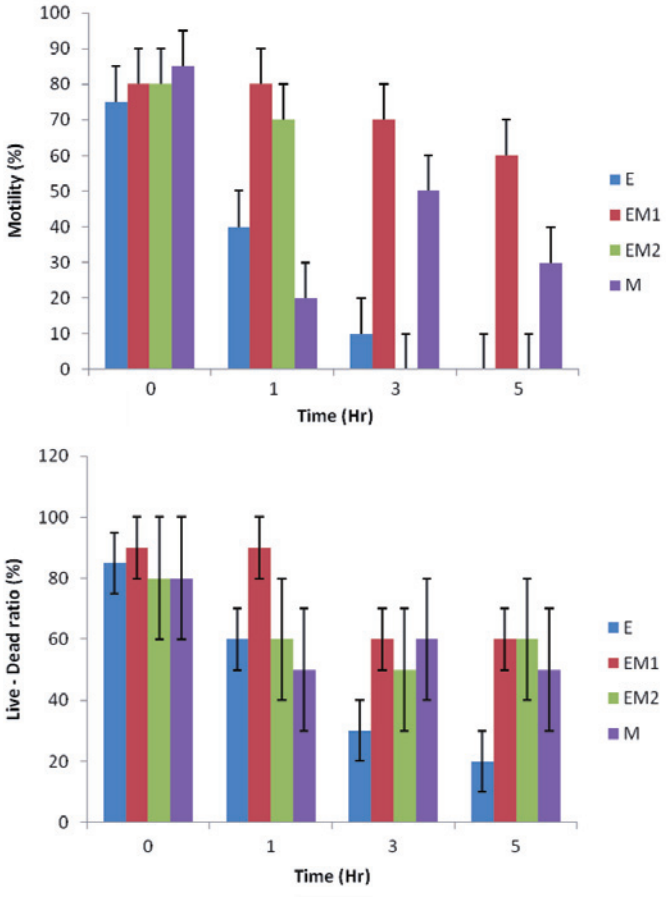

Fig 3a & b: Sperm motility (SM) and Live – Dead ratio (LDR) of goat semen preserved either in 20mls of egg yolk (E); 10mls of egg yolk and 10mls of snail mucus (EM_1), 5mls of egg yolk and 15mls of snail mucus (EM_2) and 20mls of snail mucus (M) respectively and stored either at 37^{0}C. P < 0.05

sperm motility for up to 24 hours, while only egg-yolk (E) and egg-yolk-mucus (EM_1) were able to maintain sperm motility for up to 3 and 5 hours of semen preservation respectively. At 1, 3 and 5 hours of semen preservation, the sperm motility was significantly ($P < 0.05$) higher in goat semen preserved in (EM_1) compared to other media used. Similarly, the live-dead ratios tended to decrease with time in all the media used while the live–dead ratios were significantly higher ($P < 0.05$) in semen preserved with (EM_1).

DISCUSSION

In this study, the goat semen was found to be creamy in colour, while the volume was 2.5 ml. In addition both the gross and subjective motility were scored to be +3 and 90% respectively. The addition of snail mucus to egg-yolk based extender was observed to sustain sperm motility and viability of goat semen for up to five hours. However, the effect was dependent on the ratio of snail mucus and egg yolk as well as to the storage temperature. This trial is the best of our knowledge the first study to describe the effect of snail mucus on *in vitro* sperm motility and live-dead ratio in goats.

Goat semen preserved in egg-yolk extender has been reported to have declining motility due to the presence of egg yolk coagulating enzyme (EYCE) in the seminal plasma of the semen (3). In this study, the motility of the goat semen was maintained for up to 5 hours in most of of the extenders. For both EM2 and M, motility was only maintained for one hour. The reason for the declining motility may be due to the toxic effect of EYCE on the egg yolk. However, it is possible that addition of equal volume of snail mucus to egg-yolk may reduce this effect since the semen preserved in EM_1 were able to maintain motility of over 60% for up to five hours.

The snail mucus is a visco-elastic substance secreted by granules within the snail's body and localized on the outer surface as a result of exposure to stress. In the snail, the mucus has been described to be responsible for the uptake of divalent cations and for the mobility of the snail itself (15). In addition, snail mucus has also been found to regulate sperm competitions in these gastropods (18). In this study, addition of snail mucus to egg-yolk extender was found to maintain *in vitro* motility and live-dead ratio of fresh goat semen.

The rheological measurement of snail mucus (19) showed that they are elasto-visco-plastic in nature with characteristics of a physically cross-linked gel below the yield points and a strongly rate dependent apparent viscosity. This viscosity is due to high amount of glycosaminoglycans in the mucus (20). The implication of the elastovicosity is its effect on the miscibility with the sodium citrate buffer and egg yolk. Thus, the mixture had to be pre-warmed at 37^0C to ensure proper mixing. In addition, the high elastoviscosity may underestimate the sperm motility through the mucus network. This may explain the low motility observed in the study when the volume of the snail mucus added was greater than 10 mls. However, the high viscosity of snail mucus may also protect the spermatozoa against cold shock (21). This might explain why the motility and live-dead ratio parameters were higher in (EM_1) than with (E).

The snail mucus is composed mainly of water (96.5%), electrolyte, mucus glycoconjugates and proteoglycans. The glycosaminoglycans in the snail mucus are highly soluble in aqueous solution and are important as glyconutrients, enzymes and signals to cells (15). In the snail, the internal mucus provides the developing embryo with protein and galactogen (20). In this study, either snail mucus or its addition to egg yolk supported buck semen preservation for up to five hours with (EM_1) being the one that presented the best results of sperm motility and viability. The positive effect of the snail mucus as an additive to egg yolk based extender on sperm motility may be multifactorial including supply of glycoproteins and preservation of the spermatozoa membrane.

Several factors play roles in maintaining the quality of semen during storage. It is known that sperm motility and fertilizing ability of undiluted semen stored *in vitro* gradually reduces within one hour post collection (22). It has been reported the that percentage of sperm motility decreases at temperatures higher than the subject's body temperature, while the motility is better maintained at lower temperatures (23). In this particular study, the percentage sperm motility of goat semen was not significantly affected by the temperature of semen preservation as earlier reported but was better preserved at a temperature of 25^0C. The exact reason for this is unknown; however there may be specie specific variation in the semen responses to temperature changes. Although it would have been expected that the motility of the spermatozoa preserved at 5^0C should be lower than those preserved at 25^0C or 37^0C, the exact reason for the motility to be higher in semen preserved at 25^0C than those preserved at 37^0C is not known.

Throughout their journey in the female genital tract, in-

seminated spermatozoa are exposed to intraluminal fluid which affects sperm function (24). Glycosaminoglycans such as hyaluronan, heparin sulphate, chondroitin sulphate and dermatan sulphate have been identified in the female reproductive tract (25, 26). Important aspects of sperm functions such as motility and capacitation appeared to be mediated at least partially through hyaluronan (27). Addition of hyaluronan to fresh and frozen – thawed human semen improved motility and delayed cryocapacitation (28). In this study, the addition of 10 mls of snail mucus to egg-yolk extender was observed to maintain fresh goat semen up to five hours. In addition, goat semen preserved in (EM_1) was observed with higher motility and percentage of live-dead ratio compared with those preserved in egg yolk (E). This finding suggested that snail mucus glycoproteins contain glycosaminoglycans which may be related structurally and/or biologically to either hyaluronan or heparin sulphate. Previous physicochemical analysis of mucus of *Achatina fulica* showed that the mucus is similar to heparin but distinct from it (15). The result of this trial further suggested that snail mucus may be added to fresh or frozen thawed semen to improve the motility and hence improved fertility.

Finally, the addition of 15 mls of snail mucus to 5 mls of egg-yolk (EM_2) produced lower sperm motility and percentage of live spermatozoa of the goat semen between 1-5 hours of storage time compared to when 10 mls volume addition of snail mucus was added to 10 mls of egg yolk (EM_1) suggesting that the effects of snail mucus on sperm motility might be dependent on the concentration of the snail mucus. It thus appears that the optimal inclusion of snail mucus to egg yolk extender in order to maintain good sperm motility is one part of egg yolk to one part of snail mucus. Although the exact mechanism for this negative effect is not known, study in rats showed that addition of 1250 μg/ml of hyaluronan to rat semen was found to decrease sperm motility compared with 750 μg/ml (28), suggesting that increasing the inclusion levels of glycosaminoglycans to semen sample beyond a particular level will result in a negative effect.

In conclusion, the result of this study suggested that the addition of snail mucus to egg yolk maintained the motility of fresh goat semen at 50 % and live dead ratio at 70% for up to five hours alone. Longer duration of storage resulted in further decline in the sperm motility and percentage live cells, with the values almost zero at 12 hours of storage. This effect was found to be dependent on the concentration of the snail mucus where higher doses may have negative effect on sperm motility. It is yet to be determined whether similar effect will be obtained when thawed frozen semen is used. In addition, the fertility rate of does inseminated with semen preserved in egg yolk-snail mucus extender needs to be investigated in a further study to determine the suitability of snail mucus as a semen extender inclusion for the preservation of goat semen.

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