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**ARAŞTIRMA MAKALESİ** **RESEARCH PAPER**

# Effect of Canary (*Serinus canary*) Semen Modified with Different Extenders on Fertilization in Artificial Insemination

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**Abstract:** In this study, the effect of Dulbecco's Phosphate Buffered Saline (DPBS) and Dulbecco's Modified Eagle's Medium (DMEM) extenders on artificial insemination was determined. For this purpose, the number of fertile eggs, the total number of eggs, the number of hatched eggs, and the number of hatchlings from fertile eggs were determined in the study. From these values, the fertility rate (%), egg hatch rate (%) and fertile egg hatch rate (%) were calculated. Twelve male and female canaries (Gloster breed; 1-2 years old) were used in each group. In the study, three groups, one control (C) and the other two treatment groups (T-1, T-2) were formed. Female canaries in group C were directly inseminated without using any extenders. Females in the T-1 group were diluted 1:1 with DPBS; females in the T-2 group were inseminated with semen diluted 1:1 with DMEM. Although there was a significant difference between the groups in terms of fertility rate ($P<0.05$), egg-laying rate and fertile egg-laying rate were not different in terms of parameters ($P>0.05$). The highest fertility rate (61.81%) was in group C; the lowest was in the T-1 (29.17%) group. ($P<0.05$). Using a DPBS extender, it was revealed that the fertility rate was negatively affected in insemination. Due to the limited number of studies on this subject, it is thought that it will be essential to investigate DPBS and DMEM diluents at different rates and methods in future studies

**Keywords:** Artificial insemination**;** bird semen, canary; extender.

# Suni Tohumlamada Farklı Semen Sulandırıcılarla Sulandırılan Kanarya (*Serinus canary*) Spermasının Yumurtalar Üzerine In-Vivo Etkileri

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**Öz:** Bu çalışmada, Dulbecco'nun Fosfat Tamponlu Salini (DPBS) ve Dulbecco'nun Modifiye Eagle's Medium (DMEM) sulandırıcılarının suni tohumlama üzerindeki etkisini belirlendi. Bu amaçla çalışmada döllü yumurta sayısı, toplam yumurta sayısı, kuluçkadan çıkan yumurta sayısı ve döllü yumurtalardan çıkan yavru sayısı belirlenmiştir. Bu değerlerden fertilite oranı (%), yumurtadan çıkış oranı (%) ve döllü yumurtadan çıkış oranı (%) hesaplandı. Her grupta 12 adet erkek ve dişi kanarya (Gloster cinsi; 1-2 yaşında) kullanıldı. Çalışmada biri kontrol (K) ve diğer iki tedavi grubu (T-1, T-2) olmak üzere üç grup oluşturuldu. K grubundaki dişi kanaryalar herhangi bir sulandırıcı kullanılmadan doğrudan tohumlandı. T-1 grubundaki dişiler DPBS ile 1:1 oranında seyreltildi; T-2 grubundaki dişiler DMEM ile 1:1 seyreltilmiş meni ile tohumlandı. Gruplar arasında fertilite oranı açısından önemli bir fark olmasına rağmen ($P<0.05$), yumurtlama oranı ve döllü yumurtlama oranı parametreler açısından farklı değildi ($P>0.05$). En yüksek fertilite oranı (%61,81) K grubundaydı; en düşük oran T-1 (%29,17) grubundaydı. ($P<0.05$). DPBS sulandırıcı kullanılarak tohumlamada fertilite oranının olumsuz etkilendiği ortaya çıktı. Bu konuda yapılan çalışmaların sınırlı sayıda olması nedeniyle gelecek çalışmalarda DPBS ve DMEM seyrelticilerinin farklı oran ve yöntemlerle araştırılmasının önemli olacağı düşünülmektedir.

**Anahtar kelimeler:** Suni tohumlama, kuş sperması, kanarya, sulandırıcı.

## INTRODUCTION

It has been reported that sperm storage temperature, storage time, and dilution rate have a decisive effect on spermatozoa viability (Dumpala et al., 2006). The diluted use of sperm increases the fertilization ability of female poultry by providing more prolonged survival of spermatozoa. In addition, male breeders are thought to reduce the maintenance cost because it allows more benefit from male animals by diluting the semen (Roiter and Konopleva, 2019). The importance of suitable diluent and cryoprotectant compatibility in the cryopreservation of bird semen has been emphasized (Hobbs et al., 2021). The extenders used for storing semen in poultry should be able to meet the metabolic needs of the semen of different animals (Donoghue and Wishat, 2000).

It has been reported that a minimum of 2 µl semen can be obtained from songbirds. (Humann-Guilleminot et al.,2019). It has been stated that with an increase in the dilution rate, sperm functions also decrease. It was observed that the sperm motility rate increased during the mixing of semen with an extender and vigorous pipetting (Cramer et al., 2019). Sperm speed positively affects fertilization success (Lara et al., 2020).

The livestock industry needs to expand and develop artificial insemination practices because it allows animal producers to access breeding animals with superior genetic characteristics without having physically qualified fathers (Zambelli and Cunto, 2022). It has been reported that artificial insemination is a reproductive biotechnology technique widely used to produce bird species whose population is decreasing in nature. For this reason, it has been emphasized that the quality of semen is critical for fertilization. In addition, it was predicted that the success of artificial insemination with fresh semen could result in fertility close to natural mating (Piaček et al., 2020). The importance of using practices related semen diluted with semen extenders in artificial insemination has been emphasized (Shinde et al., 2013). To the dilution and storage of sperm play a decisive role in the success of artificial insemination in poultry (Pearlin et al., 2021). It has been observed that the effect of different extenders on the short-term storage of poultry semen can play a decisive role in artificial insemination (Mohan et al., 2015). Artificial insemination and cryopreservation of semen in poultry are essential for conserving endangered bird species. For this purpose, it has been stated that different techniques have been developed regarding the cryopreservation of semen and artificial insemination applications (Gee., 1995). It has been predicted that the dilution rate of semen used in artificial insemination in bird species is as low as possible, which may increase the success of insemination (Blanco et al., 2009). DMEM contains 4500mg glucose/L, 110 mg/L sodium pyruvate, and L-glutamine (Humann-Guilleminot et al., 2019). In studies on semen performance, extenders such as DMEM extenders are frequently used in addition to suitable temperature for semen. However, it has been reported that the efficiency of semen in the female reproductive tract may vary depending on the vaginal fluid, obstacles in the environment, or the content of the seminal fluid (Lupold et al., 2009). It has been stated that while determining the dilution rate of semen with DMEM in birds, it is decided according to the amount of semen taken individually (Mcdiarmid et al., 2022). In the study conducted with hummingbirds, PBS was preferred instead of DMEM because the sensitivity of semen may be different for different species. In the preliminary experiment, it was predicted that the two diluents could give similar results (Cramer et al., 2016). Commercial DPBS contains 8.12 g/L NaCl, 0.20 g/L KCl, 1.44 g/L Na2HPO4, and 0.20 g/L KH2PO4 (Almubarak et al., 2021). It has been reported that increasing the contents of extenders can positively affect semen parameters after freeze-thaw compared with DPBS (Hobbs et al., 2021). DMEM and DMSO are the most widely used media and cryoprotectant agents in various biotechnological reproduction techniques (Purohit et al., 2003).

## MATERIAL AND METHOD

***Ethics Committee Permission:*** Ethics committee approval of this study was obtained from Ondokuz Mayıs University Animal Experiments Local Ethics Committee (Date: 30.03.2023; Acceptance No: 2023/09).

***Experimental Plan:*** In the study, 12 pairs of Gloster canaries between the ages of 1-2 years were used in each group. Gloster canary is a small domestic show bird originating from England (Whiteaker, 1979). It has been reported that canaries can reach sexual maturity at 10 months of age (Coutteel, 2003). The canaries were housed singly in individual cages measuring 60 cm × 40 cm × 50 cm (Figure 1). Male and female birds were never combined during the study. It is known that photoperiod, food, temperature, and sound interactions with the opposite sex trigger sexual arousal in birds (Hudelson, 1996). Birds were sexually activated with a photoperiod of 16 h light and 8 h dark (Trivedi et al., 2006). Selected female canaries were divided into three groups: control (C), treatment 1 (T-1) and treatment 2 (T-2). The control group was determined as canaries artificially inseminated directly without the extender, canaries artificially inseminated with semen diluted with T-1 Dulbecco's Phosphate Buffer Saline (DPBS) solution, and birds artificially inseminated with semen diluted with T-2 Dulbecco's Modified Eagle Medium (DMEM).

**Figure 1**. Canary breeding cages.

***Collecting Semen from Male Canaries:*** In the study, semen was collected from each male canary and evaluated. From male canaries that gained sexual activation, semen was taken every four days, and they were accustomed to give semen. Semen was collected from canaries using the cloacal massage method (Gee et al., 2004). The motility was examined immediately on semen collected individually from canaries (Schmoll and Kleven, 2016). In the examination of semen samples taken individually from male birds, it was seen that they had 70% and higher motility values. Contamination of semen used for artificial insemination with feces was avoided. Contaminated semen samples were not used for artificial insemination. Since the Gloster canary is a small bird, it gives an average of 2 µl of semen. No literature has been found regarding the appropriate insemination dose in canaries. The average semen volume in the canary was reported to be 10 µl (Gee et al., 2004). However, the semen volume collected from Gloster canaries was found to be well below average. Due to this situation, the collected semen is quickly affected by external temperature changes. It was concluded that it was negatively affected by the cannula passages used during sperm transfer. Therefore, the collected semen was evaluated as soon as it was collected without mixing. The semen density in 1 ml was calculated by the hemocytometer method by semen density examination in poultry (Bakst and Cecil, 1997). The density for Gloster canary semen collected at different times was $84x10^6$-$96x10^6$spp/ml. It was predicted that the average spermatozoa density in the semen diluted 1:1 and used was $42x10^5$-48 $x10^5$spp/µl (Figure 2).

***Insemination of Female Canaries:*** Nest material was given to female canaries showing signs of estrus. The birds were inseminated two days apart from the time they started nesting until the first egg was seen. Females in the control group were inseminated without dilution with 2 µl of semen from male birds. Artificial insemination practices in canaries were performed using the cloacal method (Blanco et al., 2009). To avoid the risk of contamination during insemination, the cloaca was controlled by gentle stimulation before insemination. A temperature value of 40°C was preferred and more successful in spermatozoa swimming speed and motility studies in songbirds (Kleven et al., 2009; Yang et al., 2019). The females in treatment 1 were inseminated with 4 µl of diluted semen by diluting 1:1 with DPBS solution heated to 40°C in a water bath. Females in treatment 2 were inseminated with 4 µl of semen diluted 1:1 in the same heated condition. Female birds in the control group were inseminated as soon as semen was taken from the male canaries. In all three applications, artificial insemination was performed without waiting for semen samples to be taken from canaries.

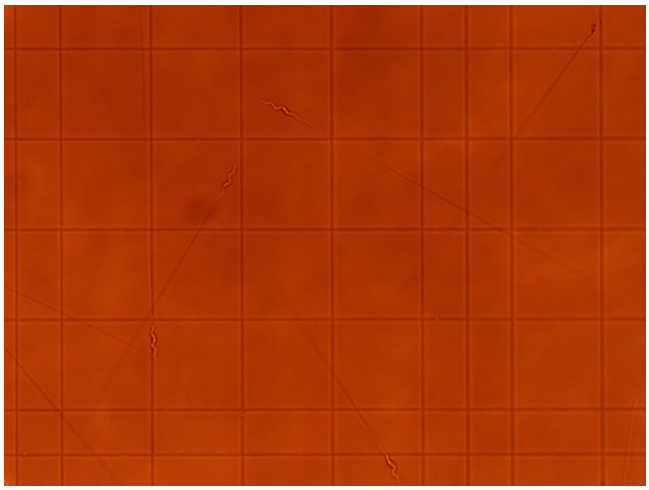

**Figure 2**. Canary spermatozoa density analysis by hemocytometer method.

***Egg Collection and Incubation Time:*** Each female canary was followed daily, and her eggs were collected in the morning. Eggs were placed under the birds that completed the egg-laying process. The aim was to ensure equal conditions between the eggs and observe the hatchlings in the same period. The egg incubation period is generally 14 days in canaries (Yenilmez, 2020). On the eighth day, the eggs were checked with the light method and fertile eggs were determined (Hall et al., 2023). Eggs with the prolonged egg-laying process were not intervened. The humidity required for hatching of the offspring should be between 60% and 80%. It has also been pointed out that high humidity can cause various health problems (Coutteel, 2003). In order to ensure optimum hatching conditions for the eggs, care was taken to keep the humidity between 60- 65% and the ambient temperature stable at 18-22ºC. Fertile eggs with delayed hatching were removed from the nest on the 18th day and evaluated as unsuccessful. Instant data were collected and recorded. The mother left the hatchlings alone (Figure 3). The study was repeated for each bird. When the baby birds were 30 days old, they were taken from the mother's side and placed in individual cages when they could self-sufficiently.

**Figure 3**. Day-old canary chick (3rd and 20th days).

***The Evaluation of Eggs:*** The results were evaluated as fertility, egg laying, and fertile egg hatch rates. The ratios of these parameters were calculated with the help of the following formulas (RAKHA et al., 2016).

$$Fertility\ rate\ (\%) = \frac{number\ of\ fertile\ eggs}{total\ number\ of\ eggs} * 100$$

$$Fertile\ egg\ laying\ rate\ (\%) = \frac{number\ of\ hatchlings}{total\ number\ of\ eggs} * 100$$

$$Egg\ hatching\ rate\ (\%) = \frac{number\ of\ hatchlings}{fertile\ egg\ number} * 100$$

***Statistical Analysis:*** The distribution of data in terms of fertility rate, fertile egg laying rate, egg hatching rate was determined according to Skewness and Kurtosis. Since Skewness and Kurtosis values were in the range of (-1.5)- (+1.5) according to Tabachnick and Fidell (2013), the data showed a normal distribution. Therefore, parametric testing was performed.

Analysis of variance one-way Analysis of Variance (ANOVA) and comparisons between groups (Duncan's test) were performed using the SPSS 22.0 package programs. The effects (significance) of the groups were evaluated at P<0.05 level (IBM., Corp., 2011). In addition, the number of animals used in the study was determined by G*Power analysis (Faul et al., 2007).

## RESULTS

Data regarding fertility rate, fertile Egg laying rate, fertile egg hatching rate show a normal distribution (Table 1).

**Table 1.** Distribution of the data (Skewness and Kurtosis).

| Parameters | Skewness | Kurtosis |
|---|---|---|
| Fertility rate (%) | -0.571 | -0.230 |
| Fertile Egg laying rate (%) | -0.041 | -1.064 |
| Fertile egg haching rate (%) | -0.737 | -0.800 |

The effects of different extenders on canary egg fertilization success (fertility rate, egg laying rate and fertile egg hatch rate) are given in Table 2. Although there was a significant difference between the groups in terms of fertility (P<0.05), there was no significant difference between the groups in terms of hatching rate and hatching rate of fertile eggs.

**Table 2.** Effect of different extenders on canary egg fertilization success (N=12).

| Gruplar | Parameters | | |
|---|---|---|---|
| | Fertility (%) | Hatching (%) | Hatchability of fertile eggs (%) |
| **C** | 61.81[a] | 47.92 | 75.69 |
| **T-1** | 29.17[b] | 22.22 | 52.78 |
| **T-2** | 43.19[ab] | 34.86 | 58.33 |
| **SEM** | 5.297 | 4.629 | 7.420 |
| **P** | 0.036 | 0.073 | 0.433 |

a, b: The differences between the means shown with different letters in the same column are significant (P<0.05). C: Control, T-1 (Treatment-1): DPBS (Dulbecco's Phosphate Buffer Saline), T-2 (Treatment-2): DMEM (Dulbecco's Modified Eagle Medium), SEM: Average of standard errors

In this study, the fertilization success of female canaries inseminated with canary semen diluted with two different extenders was compared by comparing egg parameters. Artificial insemination practices with diluted semen were less successful than canaries inseminated with undiluted fresh semen. When the diluents used were compared, there was no statistical difference. However, the fact that the fertilization rate of the DMEM extender was numerically higher than that of the DPBS extender suggested that the content of the extender may be effective in artificial insemination.

It is thought that the success of artificial insemination after cryopreservation of grouse semen may be related to the fructose content in extenders (Rakha et al., 2016). It has been observed that the quality of semen in birds is decreased when only phosphate-buffered saline (PBS) solution is used instead of an extender containing additional nutrients such as DMEM to dilute semen. In addition, it was determined that the motility of spermatozoa diluted with DMEM was high (Cramer et al., 2019). When the motile spermatozoa ratio was examined, it was reported that the DMEM extender was more successful. This situation is thought to be because the components (seminal fluid proteins, sugars, ions) in the DMEM extender are essential in affecting sperm performance (Cramer et al., 2019). Although the T-2 group of diluents used in our study was numerically higher than the T-1 diluted ones in terms of fertility rate, it was not statistically significant compared to group C. The numerically higher values of DMEM diluent data coincide with the current study.

It is predicted that excessive dilution of semen may negatively affect the success rate in artificial insemination applications performed by diluting bird semen (Mohan et al., 2018). A study on rooster semen used a dilution ratio of 1:2. In the study, it was observed that there was a serious decrease in the fertilization rate of older females (Hassan, 2009). In our study, young birds aged 1-2 years were preferred in order to eliminate age-related problems and use homogeneous materials. Since the negative effects of excessive dilution are known, the dilution ratio of Gloster canary semen was determined as 1:1. In our study, the instantaneous motility values in

diluted semen were above 70% for both groups, which is promising. However, it should not be forgotten that the density per unit area decreases in semen diluted 1:1. Additionally, the adaptation of diluted sperm to the environment in the female reproductive tract is unknown. More studies are needed on this subject. In addition, it is thought that external factors such as not being able to collect sperm from each man at the same time, the individual reaction of the semen to the diluent, ambient temperature and humidity may affect the success of insemination.

A study on raptors stated that artificial insemination with fresh semen could provide a fertility rate close to that of natural mating. In addition, it was predicted that semen quality may be directly related to the success of artificial insemination (Piaček et al., 2020). Although the animal material and artificial insemination methods in the study are different, when the fertility rate is evaluated, it overlaps with the control group in our current study. In our study, it is seen that the success rate of fresh semen is higher than both extender groups. However, in the current study, it was reported that artificial insemination yielded results close to the fertility rate achieved with fresh semen. It is estimated that, in addition to individual differences due to race, the difficulties encountered in semen collection and artificial insemination due to the small size of the canary and the negativities resulting from the low semen volume obtained may be effective here. It is thought that more successful results can be obtained when the study is conducted at lower dilution rates.

It has been revealed that the quality of the semen extender, the storage temperature of the collected semen, and the quality of the semen significantly affect the fertilization rate in artificial insemination in poultry (Mohan et al., 2015). Observing the effects of different extenders on artificial insemination in the research is consistent with our study. Additionally, the importance of the semen extender used was emphasized. Although the DMEM diluent appears numerically positive when compared to the DPBS diluent, no statistically significant difference was found.

A study on the Amazon parrot (Amazona guildingii) reported that the average spermatozoa density was 12000. However, the semen densities of the study parrots were between 500 and 110500 (Fischer et al., 2020). In a study on budgerigars (Melopsittacus undulatus), semen density was expressed as $100x10^6$/ml (Madeddu et al., 2022). The semen density calculation used in this study coincides with the calculation method used for canaries (Bakst and Cecil, 1997). It has been reported that birds generally have $2x10^9$-$10x10^9$ spermatozoa density (Gee et al., 2004). It was emphasized that budgerigar has 5.4+1.1 µl volume and $2.5x10^7$spp/ml average spermatozoa. In addition, it has been stated that the average volume of canary semen is 10 µl (Gee et al., 2004). However, there is no literature on semen density in canaries. In our study, the semen density for the Gloster canary was determined as $84x10^6$-$96x10^6$spp /ml. The average canary semen density diluted 1:1 was calculated in the range of $42x10^6$-$48x10^6$spp /ml (Bakst and Cecil, 1997). Although the mean canary semen volume was expressed as 10 µl, Gloster canary was found to have an average semen volume of 2 µl. This situation is thought to be because the Gloster canary is smaller than many other domesticated bird species.

## CONCLUSION

The diluted use of semen makes it possible to benefit more from semen. In addition, semen can be diluted and stored for longer time. In our study investigating the effects of different extenders on artificial insemination in canary semen, the effects of extenders on artificial insemination were revealed. In the study, it was found statistically significant that the fertility rate was higher in the artificial insemination application compared with the groups using the diluent in the insemination without the use of diluent. In the study, it is estimated that the low percentage of hatching rate of canaries may be due to individual defects such as nest construction defects of female canaries, inability to turn eggs sufficiently in the nest, and early hatching of female canaries. The research revealed that it is important that the semen taken from Gloster canaries is small in quantity and is rapidly affected by temperature differences in the external environment. The success of artificial insemination is directly related to the personal skill of the practitioner and the method he uses. In addition, the diluent used and the dilution rate are other significant factors. As a result, it is predicted that more successful results can be obtained using different dilution rates and application methods for artificial insemination in canaries.

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