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The Effects of Mesoporous Silica Nanoparticles on Spermatological Parameters of Merino Ram*

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Abstract: In recent years, nanotechnology has emerged as a rapidly advancing field with diverse applications across various sectors including materials science, electronics, healthcare, aerospace, environmental sustainability, defense, biotechnology, agriculture, and food industries. Mesoporous silica nanoparticles (MSNs) have recently gained significance as nanomaterials due to their stability, inert properties, thermal durability, low density, minimal toxicity, and biocompatibility. These attributes will make mesoporous silica nanoparticles instrumental in drug delivery systems, cellular imaging, and therapeutic interventions. In this study, aimed at assessing the potential of MSNs as effective carriers for various materials to spermatozoa (sptz), experimental groups were established as control (C), MSN10 (10 µg/10^7 sptz), MSN20 (20 µg/10^7 sptz), and MSN40 (40 µg/10^7 sptz). To investigate this hypothesis, semen was collected from five healthy rams (2-3 years of age), pooled, diluted, and divided into experimental groups. Following this study, analyses were conducted on motility, dead-live spermatozoa ratio, functional integrity of the sperm's plasma membrane (HOST), and abnormal spermatozoa ratios across five replicates. It was observed that MSNs did not have a negative effect on the freezability of ram semen in terms of dead-live spermatozoa ratio and HOST data, but caused an increase in motility (MSN40) and head and tail abnormality levels compared to the control group, though still within acceptable ranges. In the light of the data obtained, it is suggested that MSNs can be used as biocarriers for ram semen in terms of basic spermatological parameters, but it is necessary to expand the scope of the research and conduct more detailed analyzes.
Keywords: Mesoporous slica nanoparticles, nanotechnology, ram, sperm

Mezogözenekli Silika Nanopartiküllerinin Merinos Koçlarının Spermatolojik Parametreleri Üzerindeki Etkileri

Öz: Nanoteknoloji, son yıllarda oldukça gelişim gösteren ve kullanım alanı bulan bir teknolojidir. Malzeme ve imalat sektörü, elektronik ve bilgisayar teknolojileri, tıp ve sağlık sektörü, havacılık ve uzay araştırmaları, çevre ve enerji, savunma sanayii, biyoteknoloji, tarım ve gıda gibi pek çok alanda kullanılmaya başlanmıştır. Son dönemde kullanım alanı bulan nanomalzemelerden bir tanesi de mezoporoz slika nanoparçacıklardır (MSN). Kararlılıkları, inert yapıları, termal dayanıklıkları, düşük yoğunlukları, düşük toksisiteleri ve biyouyumlulukları sayesinde mezoporoz silika nanopartiküller ilaç taşıma sistemlerinde, hücre görüntülemede ve tedavi yöntemlerinde önemli bir rol oynamaktadır. Bu noktada MSN'lerin çeşitli materyalleri spermatozoaya (sptz) taşıma noktasında etkili bir ajan olup olmayacaklarının test edilmesi amacıyla yapılan bu çalışmada kontrol (C), 10 µg/10^7 sptz (MSN10), 20 µg/10^7 sptz (MSN20) ve 40 µg/10^7 sptz (MSN40) µg olacak şekilde gruplar oluşturulmuştur. Sağlıklı ve 2-3 yaşlarında 5 adet koçtan spermalar alınarak pooling yapılmış ve sulandırılarak deney gruplarına bölünmüştür. Toplamda 5 tekrar halinde yapılan bu çalışma sonucunda motilite, ölü-canlı spermatozoa oranı, membran dayanım oranı (HOST) ve anormal spermatozoa oranları hesaplanmıştır. Proje sonucunda MSN lerin koç spermasının dondurulabilirliği üzerine ölü-canlı spermatozoa oranı ve HOST verileri bakımından olumsuz bir etkisinin olmadığı, ancak kabul edilebilir sınırlar içerisinde olsa da, kontrol grubuna kıyasla, motilite ve anormalite düzeylerinde artışa neden olduğu görülmüştür. Elde edilen veriler ışığında MSN lerin koç sperması için biyo taşıyıcı olarak kullanılabileceği temel spermatolojik parametreler bakımından ön görülse de, araştırma kapsamının genişletilerek daha detaylı analizlerin yapılmasına ihtiyaç duyulmaktadır.
Anahtar Kelimeler: Koç, mezogözenekli silika nanoparçacıklar, nanoteknoloji, sperma

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Introduction

The term "nano" in Nanotechnology originates from the Greek word for "dwarf" and signifies one billionth of a meter (1x10^{-9} m). Nanotechnology facilitates the

practical utilization of structures at the nanometer scale (1-100 nm) and generally materials smaller than 100 nm are categorized as nanomaterials, whereas in the medical field, particles measuring 200 nm in size are also classified as nanomaterials (Bayda et al., 2019; Contera et al., 2020).

Over the past few years, nanotechnology has been widely incorporated in biomedical uses, notably influencing biological sensing, medication transport, and diagnostic imaging. This is particularly important in the emerging field of reproductive health, which has become an important area of research (Acharya et al., 2024). Infertility, endometriosis, ectopic pregnancy, erectile dysfunction, benign prostatic hyperplasia, sexually transmitted infections and reproductive cancers have become worldwide concerns. Nanotechnology presents hopeful remedies to tackle these issues through novel methods and tools, enabling early detection, precise drug delivery, and enhanced imaging Technologies (Ammar et al., 2019; Bansal et al., 2020; Chakraborty et al., 2023; Zhao et al., 2023). Nanotechnology holds immense promise in the field of reproductive medicine, paving the way for advancements in diagnosis, personalized treatments, and fertility preservation. Utilizing nanotechnology-based drug delivery systems is anticipated to enhance treatment outcomes, reduce side effects, and offer patients treatments that are not only more accurate but also more efficient (Acharya et al., 2024; Friend, 2017).

Nanomaterials, tiny forms of various materials measured in nanometers, originate diversely. Among these, mesoporous silica nanoparticles (MSNs) are notable examples. Due to their expansive surface area, stability, and biocompatibility, mesoporous silica nanoparticles (MSNs) find applications in biomedical, pharmaceutical, and biochemistry fields. Their mechanical stability and resistance to microbial degradation can be attributed to the siloxane bonds (Si-O -Si) present in their structure. The cost-effective and convenient synthesis of MSNs makes them favorable for various industries. Their distinctive features, including azoles-containing structures, make them valuable in biomedical, chemical, and food-related sectors (Usul et al., 2022).

Mesoporous silica nanoparticles (MSNs) are a favorable choice for cellular transport investigations due to their exceptional chemical properties, minimal toxicity, and structural resilience. Their compatibility with diverse materials and ease of functionalization make them versatile. These nanoparticles are valuable for detailed cell imaging and advancing reproductive health and genetic studies. MSNs serve as efficient carriers for various substances into sperm cells, ensuring safe and effective use without adverse effects on the reproductive system (Barkalina et al., 2014; 2015). Consequently, mesoporous silica nanoparticles hold promise in reproductive biology research. With these advantages in mind, the aim was to explore the impact of mesoporous silica nanomaterials on ram semen.

Material and Method

Chemicals and Reagents

All the necessary chemicals for the Tris-based egg yolk extender used in the research were sourced from Sigma-Aldrich (Citric Acid C0706, Glycerol G2025, Fructose F2543, Trisma Base T6066). Local services for producing mesoporous silica nanoparticles (MSNs) were provided by Nano Enzyme Hybrid Technology (Kayseri, Türkiye).

Characterisation of MSN with Electron Microscopic Imaging

The characterization (size and shape) of the produced nanomaterials was carried out using STEM images. The necessary preparations for STEM images and acquisition of the images were carried out with the services of Erciyes University Technology Research and Application Center (ERUTAUM).

Experimental Design

The research involved 5 Merino rams aged between 2 and 3 years, previously used in breeding. These animals were housed and nourished at Selçuk University Prof. Dr. Humeyra Özgen Research and Application Farm. Semen from the selected rams, healthy and suitable for breeding, was collected twice a week for a total of 5 replications using an electroejaculator. Samples with optimal mass movement score (3 or above), at least 80% motility, and a density of $2.5x10^9$ were pooled after collection to reduce individual variations in a 37°C water bath. Subsequently, the spermatozoa were divided into experimental groups comprising a control group and three groups treated with 10, 20, and 40 µg MSNs (Table 1). After grouping, the samples were equilibrated at +4°C for 2 hours, frozen using liquid nitrogen vapor, and stored until thawing (Figure 1) (Bodu et al., 2024). The ethical approval required for the study was obtained through decision number 22/266 from the Erciyes University Local Animal Experiments Ethics Committee.

Figure 1. Illustration of the experimental design.

Determination of Live/Dead Spermatozoa

Eosin-Nigrosin staining method was used to determine the ratio of dead to live semen. Smears were prepared by mixing 1 drop of semen with 2 drops of Eosin-Nigrosin stain on the slide. The prepared frotis were examined under phase contrast microscope at 400X magnification. At least 300 spermatozoa were counted and those with red staining on the head were considered dead and those without red staining (white) were considered alive and the result was noted in percentages (Björndahl et al., 2003).

Table 1. Experimental groups.

Groups	Extender Content
Control	Tris-Egg Yolk Extender
MSN10	Tris + 10 µg MSNs / 10^7 sptz
MSN20	Tris + 20 µg MSNs / 10^7 sptz
MSN40	Tris + 40 µg MSNs / 10^7 sptz

Sperm Motility Assessment

Frozen semen was thawed after 2 weeks in 38°C water for 25 seconds, 10 micro liters was dropped onto the slide, covered with a cover slip and examined at 400x magnification under a phase contrast microscope by looking at 5 different areas. The motility of semen was subjectively determined as a percentage value by taking the average of 5 different areas (Evans and Maxwell, 1987).

Hypoosmotic Swelling Test

Test solution was prepared with 0.735 g sodium citrate + 1 g fructose + 100 ml distilled water. Then, 300µl of the prepared solution was mixed with 30µl semen and incubated at 37°C for 30 minutes. 20µl of the mixture was taken and a total of 300 spermatozoa were counted at 400x magnification under phase contrast microscope. The number of spermatozoa with swollen and curled tails was determined and the membrane resistance ratio was determined by calculating the percentage of these spermatozoa (Revell and Mrode, 1994).

Determination of Abnormal Spermatozoa

Ten micro liters of the dissolved sperm was taken and then completed to 200µl with Hancock's solution. A drop of 10 µl was placed on a slide, covered with a cover slip. At least 200 spermatozoa were counted at 100x magnification under a phase contrast microscope, and anomalies in the acrosome, head, midpiece, and tail were identified (Evans and Maxwell, 1987).

Statistical Analysis

The IBM SPSS Statistics 25 program was used for the statistical evaluation of the data obtained in the study. The averages of the results emerging in the spermatological parameters and imaging were determined for each experimental group using variance analysis (One-Way ANOVA). The comparison of groups showing significant differences within the same parameters was conducted using the post-hoc multiple comparison test Duncan. When evaluating the results, the presence of differences at a significance level of $P<0.05$ was considered statistically important.

Results

Electron Microscopic Imaging of MSN

The STEM image of the produced MSNs is given in Figure 2. When the image is examined, it is seen that the produced MSNs are round-shaped and planar, and their sizes are between approximately 20-50 nm.

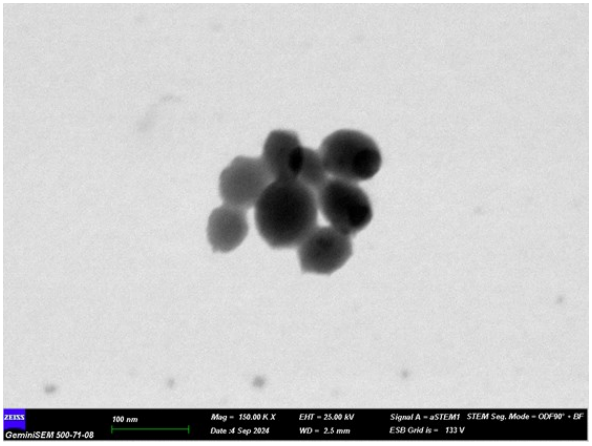

Figure 2. STEM images of synthesized MSNs.

Spermatologic Parameters

When sperm motility data were analyzed, it was observed that MSN 10 and MSN 20 groups gave similar results with the control group, while MSN 40 group gave statistically more successful results than all other groups (P<0.05) (Figure 3).

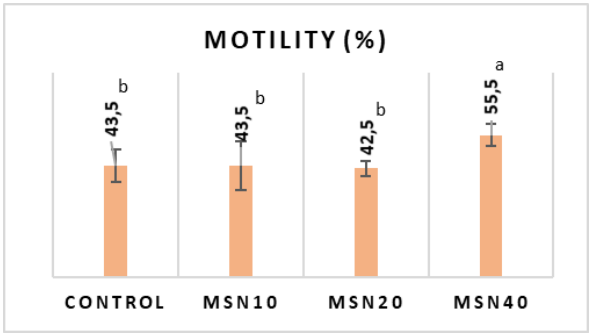

Figure 3. Motility results of the study groups. Control, MSN10 and MSN20 groups showed similar motility data, while MSN40 group had a higher motility value than all other groups (P<0.05). *a,b: Shows the statistical difference between experimental groups.

No significant differences were observed among the experimental groups in terms of the ratio of live/dead spermatozoa and the Hypo-Osmotic Swelling Test (HOST). The live/dead ratio reflects the membrane integrity of semen under optimal conditions, whereas HOST assesses the membrane resistance of semen in a low osmotic pressure environment. The viability percentage in the control group was 69.75%, and the functional integrity of the sperm's plasma membrane (HOST+) was 18.95%. Similar declines were noted in all groups, suggesting that mesoporous silica nanoparticles (MSNs) did not have a discernible impact on membrane resilience (Figure 4).

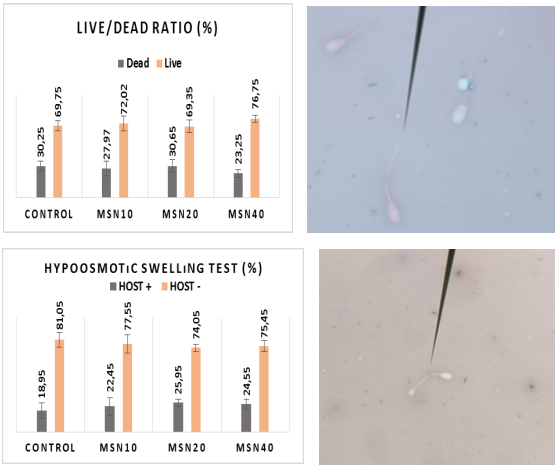

Figure 4. A. Graphic of Live/Dead ratio. B. Counting of Live/Dead spermatozoa. Dead spermatozoa with red arrow and live spermatozoa with white arrow. C. Graphic of Hypoosmotic Swelling Test. D. HOST positive spermatozoa with round head (blue arrow) and curved tail (green arrow) (P>0.05).

When the abnormal spermatozoa data were analyzed, MSNs significantly increased both head- and tail-related abnormality rates (Figure 5). However, since the abnormality data obtained are within acceptable limits, they do not pose any danger in terms of using MSNs as biocarriers.

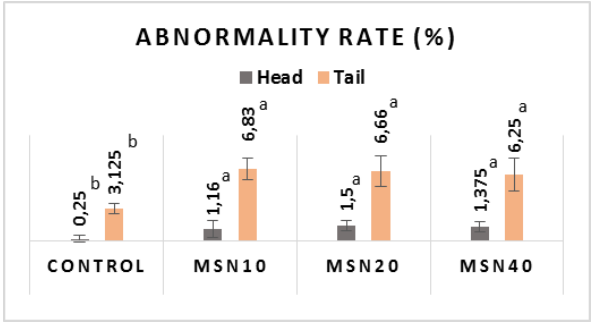

Figure 5. Head and Tail anormality rates. All groups containing with MSN increased abnormality rates (P<0.05). *a,b: Shows the statistical difference between experimental groups

Discussion and Conclusion

MSNs are one of the nanomaterials that have come to the fore in drug delivery in recent years. They are frequently used especially in cancer therapy and bioimaging systems. They can modify their surfaces to be suitable for the target tissue and thus become an advantageous nanomaterial at the point of cellular

uptake (Argyo et al., 2014; Baek et al., 2015).

Thanks to their drug delivery ability, MSNs have been tested in many therapeutic modalities. In one of these studies, Rathod et al. (2023) combined MSNs with Berberine, which is used as an anti-inflammatory in traditional Chinese medicine but can hardly be used in topical applications due to its low penetration ability. As a result of the study, Berberine used in combination with MSNs in rat paw edema was more successful than using it alone. In another study, Huang et al. (2022) utilized the ability of MSNs to transport 5 -Fluorororacil to reduce the proliferation ability and control the metastasis of oral squamous cancer cells.

In addition to these promising health studies, MSNs need some proof to gain toxicological credibility. Tacconi et al. (2022) stated that MSNs had a negative effect on the embryonic development of *Paracentrotus lividus*. They also emphasized that despite their promising potential, uncontrolled use of biomolecules may harm the environment and living organisms. In a similar study, Xu et al. (2014) revealed that silica nanoparticles negatively affected mouse spermatogenesis in a reversible manner, caused oxidative stress and damaged the structure of mitochondria. These and similar studies can be diversified. Rashidian et al. (2023) found that long-term exposure to silica nanoparticles decreased the reproductive performance of zebra fish.

Despite their high transport capacity, mesoporous silica nanoparticles have the potential to exhibit toxicity due to their size, surface properties, and pharmacokinetic aspects (Niroumand et al., 2023). Therefore, toxicity studies should be carried out considering dose and size. Considering the studies on semen, it was seen that there are very limited publications of MSNs in the literature.

In one of these studies, (Barkalina et al., 2014) incubated MSNs at 37 □C for 4 hours by adding 10, 15 and 30 µg/10^7 sptz doses in boar semen. When the data obtained were analyzed, it was stated that MSNs did not cause any negative effect on any of the basic parameters such as motility, viability, acrosomal integrity and DNA fragmentation. Again, Barkalina et al. (2015) reported that MSNs functionalized with cell penetrating peptide (C105Y) could bind to boar semen and did not cause any negative effect on motility data. Finally, in a study conducted in goat semen, Dev et al. (2020) reported that they did not observe any decrease in calculation data in the 3-hour incubation of 200 µg/ml MSNs.

In the current study, it was observed that 40 µg/10^7 sptz of mesoporous silica nanoparticles (MSNs) positively increased the motility data of ram sperm during the freezing and thawing process, while other groups showed similar results to the control groups. Regarding the live/dead analyses and Hypo-Osmotic Swelling Test (HOST), MSNs exhibited results similar to the control group, showing no positive or negative effects. Lastly, when abnormal spermatozoa data were examined, MSNs increased both head and tail abnormality rates, but as this increase remained within acceptable limits, it was not considered significant.

When the results were compared with the literature, both the studies by Barkalina et al. (2014; 2015), as well as the studies by Dev et al. (2020), yielded similar results. Just like in boar and goat sperm, no damage was observed on ram spermatozoa. Only a slight increase was noted in the head and tail abnormality rates. However, as other studies did not examine abnormality rates, comparisons cannot be made in this regard.

In conclusion, mesoporous silica nanoparticles (MSNs) can be utilized in any planned drug delivery study on ram sperm (within the current dosage limits). However, the present study only encompasses basic spermatological parameters. To reach conclusive results, detailed analyses including DNA damage, genetic analyses, and flow cytometric analyses are required. In this regard, the current study serves as a fundamental resource for future research endeavors.

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