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RESEARCH ARTICLE

Evaluation of Some Spermatological Parameters Following *Escherichia coli* Contamination in Bull Semen

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ABSTRACT

This study aimed to evaluate the effects of extended-spectrum beta-lactamase (ESBL) producing and non-producing Escherichia coli concentrations on bull semen motility parameters and spermatozoa viability. A total of 50 frozen semen straws from the same Simmental bull were used. All semen straws were thawed in a water bath at 37 °C and divided into 5 groups of 10 semen samples. Group 1 (G1) (n=10) was the uncontaminated control group. Group 2 (G2) (n=10) was contaminated with 100.000 cfu/ml and Group 3 (G3) (n=10) was contaminated with E. coli ATCC 25922 at a concentration of 1.000.000 cfu/ml. Group 4 (G4) (n=10) was contaminated with 100.000 cfu/ml and Group 5 (G5) (n=10) was contaminated with ESBL producing E. coli BAA-196 at a concentration of 1.000.000 cfu/ml. In the study, progressive motility, motility and percentages of dead-live spermatozoa in semen samples of these groups were analyzed over time. Significant decreases in spermatozoa motility and viability were observed and the most significant effects were seen in groups (G4 and G5) contaminated with high concentrations of ESBL-producing E. coli ($p<0.05$). It was determined that the effect of bacterial contamination on spermatological parameters was dose-dependent, with higher concentrations causing more rapid and severe deterioration in semen quality. These findings highlight the role of bacterial contamination, especially with resistant strains, in reducing semen quality and draw attention to the importance of microbial contamination in artificial insemination practices. Further research is needed to explore alternative methods to control contamination in reproductive technologies and combat antibiotic resistance.

Keywords: Bull, Contamination, *Escherichia coli*, Sperm parameters

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Boğa Spermasında Escherichia coli Kontaminasyonu Sonrası Bazı Spermatolojik Parametrelerin Değerlendirilmesi

ÖZ

Bu çalışma, genişlemiş spektrumlu beta laktamaz (ESBL) üreten ve üretmeyen Escherichia coli konsantrasyonlarının boğa sperma motilite parametreleri ve spermatozoa canlılığı üzerindeki etkilerini değerlendirmeyi amaçlamıştır. Aynı Simental boğasından toplam 50 adet donmuş sperma payeti kullanıldı. Tüm sperma payetleri 37 °C'de su banyosunda çözdürülerek, her biri 10 sperma örneğinden oluşan 5 gruba ayrıldı. Grup 1 (G1) (n=10), kontamine edilmeyen kontrol grubunu oluşturdu. Grup 2 (G2) (n=10), 100,000 cfu/ml ve Grup 3 (G3) (n=10), 1,000,000 cfu/ml konsantrasyonunda E. coli ATCC 25922 ile, Grup 4 (G4) (n=10), 100,000 cfu/ml konsantrasyonda ve Grup 5 (G5) (n=10), 1,000,000 cfu/ml konsantrasyonda ESBL üreten E. coli BAA-196 ile kontamine edilen grupları oluşturuldu. Çalışmada bu gruplara ait sperma örneklerinde progresif motilite, motilite ve ölü-canlı spermatozoa yüzdelerinin zaman içinde değişimi analiz edildi. Spermatozoa motilitesi ve canlılığında önemli azalmalar gözlemlendi ve en belirgin etkiler, yüksek ESBL üreten E. coli konsantrasyonlarıyla kontamine olan gruplarda (G4 ve G5) görüldü ($p<0.05$). Bakteriyel kontaminasyonun sperma parametreleri üzerindeki etkisinin doza bağlı olduğu ve daha yüksek konsantrasyonların sperma kalitesinde daha hızlı ve ciddi bozulmaya neden olduğu belirlendi. Bu bulgular, özellikle dirençli suşlarla bakteriyel kontaminasyonun sperma kalitesini azaltmadaki rolünü vurgulamakta ve suni tohumlama uygulamalarında mikrobiyal kontaminasyonun önemine dikkat çekmektedir. Reprodüktif teknolojilerde kontaminasyonu kontrol etmede ve antibiyotik direnciyle mücadele etmeyi sağlamada alternatif yöntemleri keşfetmek için daha fazla araştırmaya ihtiyaç vardır.

Anahtar kelimeler: Boğa, *Escherichia coli*, Kontaminasyon, Sperm parametreleri

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INTRODUCTION

In cattle breeding, various genetic diseases or environmental factors are associated with infertility. In addition, bacterial contamination of cattle semen significantly contributes to reduced birth rates and increased prevalence of reproductive disorders in cattle (Thibier and Guerin 2000). The known mechanisms by which infection causes infertility include (a) bacterial adhesion to spermatozoa, (b) immobilizing factors produced by certain bacteria, (c) enhanced activation of the immune system, and (d) disruption of glandular function (Cottell et al. 1996).

The presence of bacteria in ejaculates may originate from intrinsic bacterioses within the male urogenital system, extending from the testes to the penis and prepuce (Marcus et al. 1994). Commonplace bacteria can also be introduced through the artificial vagina, lab equipment, semen extenders, or even the lab environment, even in the face of stringent biosecurity regulations during semen collection. (Rana et al. 2012). Additionally, the presence of bacterial pathogens such as *Staphylococcus* spp*., Micrococcus* spp*., Escherichia coli (*E. *coli), Pseudomonas* spp*., Corynebacterium* spp*., Proteus* spp*., Klebsiella* spp*.,* and *Bacillus* spp. has been reported in frozen semen (Mitra et al. 2016). Bacteria such as *E. coli* significantly reduce sperm motility and increase sperm agglutination. *E. coli* has been shown to adhere to the sperm membrane/surface through mannose-binding sites (Monga and Roberts 1994; Wolff et al. 1993). Class A β-lactamases, also known as extended-spectrum β-lactamases (ESBLs), are a fast developing category of β-lactamases that may hydrolyze and produce resistance to monobactams (aztreonam) and oxy-imino cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefuroxime, and cefepime) (Peirano and Pitout 2010). Calves exhibited the highest prevalence of ESBL-producing *E. coli* on mixed farms, with %56.2 of fecal samples testing positive for this pathogen. This was followed by cows, with %41.1 of fecal samples, and beef cattle, which showed a prevalence of %21.4 in their fecal samples (Schmid et al., 2013). Semen samples containing these bacteria have been associated with reproductive failure following insemination. Pathogenic bacterial contamination of ejaculates can impair spermatological parameters by reducing sperm motility and viability, causing morphological abnormalities, and decreasing sperm concentration (Folliero et al. 2022). Moreover, antibiotic-resistant *E. coli* strains can exert more rapid and severe adverse effects on sperm function. These resistant bacteria produce toxins and metabolites that disrupt sperm motility, viability, and morphological integrity. Such bacterial infections can damage sperm membrane structure and compromise DNA integrity, reducing viability and motility. Additionally, they may increase oxidative stress, resulting in cellular damage (Oghbaei et al. 2020). These spermatological parameters play a crucial role in fertility (Khalili et al. 2000). Although bacterial contamination of ejaculates contributes to infertility in cattle, standardized protocols for the routine microbiological analysis of bovine semen remain lacking. Additionally, the microbial flora of semen can also induce infertility in females, increase embryonic mortality rates, reduce pregnancy success, and, on a larger scale, lead to abortion and other reproductive complications. In addition, failure to control contamination in reproductive biotechnologies can lead to negative outcomes such as infectious infertility, endometritis and abortion in the cows, and can seriously reduce fertilization success rates by reducing the quality of the semen used (Stringfellow et al. 2000). Given the widespread use of artificial insemination in livestock production and genetic improvement, addressing bacterial contamination in semen is essential (Garba et al. 2023). Therefore, the identification of opportunistic bacteria and the prevention of potential transmission between bulls and from bulls to cows is crucial, as it may help prevent economic losses in cattle production. Furthermore, future bacteriospermia therapy and prevention may benefit from an understanding of the mechanisms behind bacterial damage in spermatozoa (Cojkic et al. 2021; Ďuračka, et al. 2021).

This study aims to investigate the effects of *E. coli*, the most common bacterium found in feces and associated with contamination risk during semen collection, on various spermatological parameters in commercially available semen used routinely by veterinarians in artificial insemination practices.

MATERIALS and METHODS

The study was conducted in the laboratories of the Department of Reproduction and Artificial Insemination and the Department of Microbiology at the Faculty of Veterinary Medicine, X University. The material used in the study consisted of 50 randomly selected semen straws, each with a volume of 0.25 ml, from imported bull semen. These straws were all from a single bull, collected on the same date, and all analyses throughout the study were performed on these samples.

Experimental Design and Group Formation

Before the start of the study, three Simmental semen straws were randomly selected from the nitrogen tank and thawed in a 37 °C water bath to assess initial sperm motility and concentration, which were determined to be 70–80% and 10–20 × 10^6 spermatozoa per straw, respectively. The aim was to check for any significant differences between the straws. All materials used were subjected to sterilization procedures, and the temperature was maintained at 37 °C throughout the study.

All straws (n=50) were thawed at 37 °C for 30 seconds and then divided into five experimental groups (n=5x10). Subsequently, the semen samples were contaminated with *E. coli* at varying concentrations and prepared in the microbiology laboratory. Spermatological parameters (motility and live-dead sperm ratio) were evaluated at 20-minute intervals during the first two hours and subsequently at hourly intervals.

Microbiological Process

E. coli ATCC 25922 and ESBL producing *E. coli* BAA-196 were thawed at room temperature from -20 °C and then passaged into 5 ml of Brain Heart Infusion Broth (1.10498, Merck, KGaA, Darmstadt, Germany), where it were incubated at 37 °C for 18 hours. The resulting bacterial culture was serially diluted 10-fold in sterile physiological saline (FTS, pH=7.2). Two aliquots were plated from each dilution onto MacConkey Agar (1.05465, Merck, KGaA, Darmstadt, Germany) and incubated at 37 °C for 18 hours. Colonies were counted, and the bacterial concentrations in the dilutions were determined, considering McFarland standards. The bacterial suspensions, stored at +4 °C in the refrigerator, were added to the semen samples as soon as the bacterial concentrations (cfu/ml) were determined.

In this context, Group 1 (G1) (n=10) consisted of 10 semen samples of 0.25 ml each, thawed at 37 °C in a water bath, forming the control group. The semen samples were incubated in a 37 °C incubator with %5 CO_2 to evaluate spermatological parameters. Group 2 (G2) (n=10) consisted of semen samples thawed using the same method and contaminated with *E. coli* ATCC 25922 at a concentration of 100.000 cfu/ml and were incubated at 37 °C with %5 CO_2. Group 3 (G3) (n=10) involved semen samples thawed using the same method, contaminated with *E. coli* ATCC 25922 at a concentration of 1.000.000 cfu/ml and incubated under the same conditions (37 °C, %5 CO_2). Group 4 (G4) (n=10) involved semen samples thawed using the same method and contaminated with ESBL producing *E. coli* BAA-196 at a concentration of 100.000 cfu/ml and were incubated under the same conditions (37 °C, %5 CO_2). Group 5 (G5) (n=10) consisted of semen samples thawed using the same method, contaminated with ESBL producing *E. coli* BAA-196 at a concentration of 1.000.000 cfu/ml, and incubated under the same conditions (37 °C, %5 CO_2). Spermatological parameters were evaluated for all groups.

Spermatological Analysis

Sperm motility was evaluated using a Computer-Assisted Sperm Analyzer (CASA) system (SCA, Sperm Class Analyzer, Version 6.5.0.91; Microptic, Barcelona, Spain). The samples were examined under a phase-contrast microscope (Nikon, Eclipse, Tokyo, Japan) equipped with a 10× objective lens and a high-speed camera (60 frames/sec) at 37 °C. At least 200 randomly selected spermatozoa were analyzed for each sample from at least five microscopic fields.

Sperm viability was assessed using eosin-nigrosin staining. For this, 15 µL of eosin-nigrosin stain and 10 µL of semen were placed side by side on a glass slide and gently mixed with a pipette tip. A cover slip spread the sample across the slide at a 30° to 40° angle. The slide was placed on a heating plate until dry, then evaluated under a light microscope at 200x magnification (with immersion oil). Spermatozoa that appeared partially or fully pink or red were considered dead, while spermatozoa that did not absorb the stain were considered alive.

Statistical Analysis

Statistical analyses were performed using RStudio (version 2023.06.0+421, "Mountain Hydrangea") (R Core Team, 2023). Data normality was assessed using the Shapiro-Wilk test, and none of the parameters followed a normal distribution ($p<0.05$). Therefore, non-parametric tests were employed for statistical comparisons.

For each of the three general evaluation parameters, differences between the five groups were assessed at each of the ten-time points using the Kruskal-Wallis test. When significant differences were detected, pairwise comparisons were conducted using Dunn's test, with Holm's method applied for p-value adjustment. The significance thresholds were set as follows: $p<0.05$ (significant), $p<0.01$ (highly significant), and $p<0.001$ (very highly significant).

RESULT

This study aimed to evaluate the effects of *E. coli* contamination on the spermatological parameters of bull semen. The data demonstrate that *E. coli* contamination harmed sperm progressive motility, live sperm and motility rates (Figure 1).

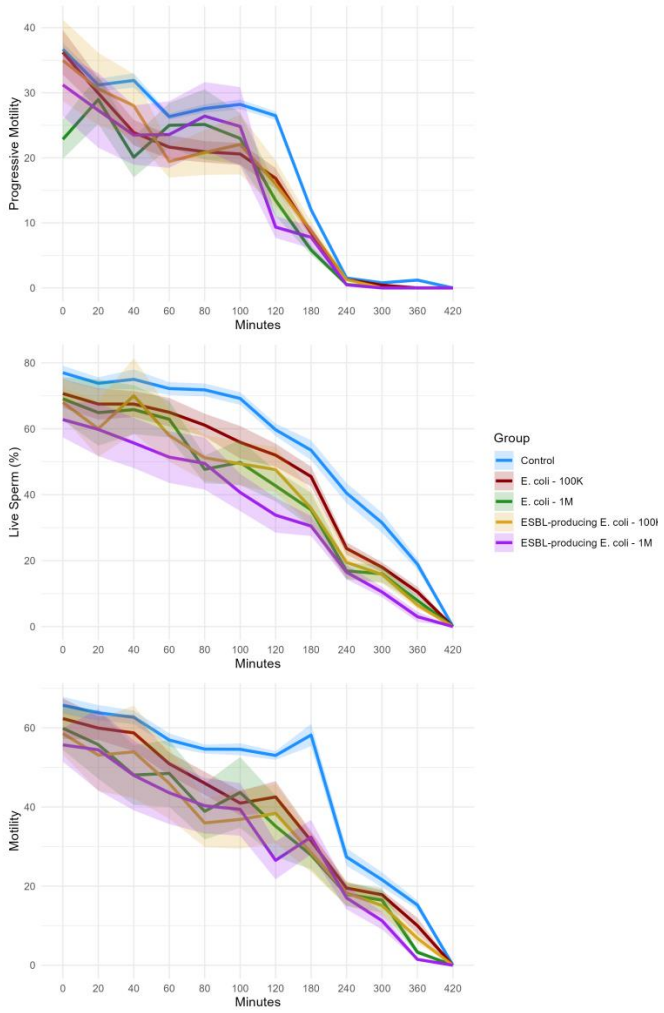

Figure 1. Changes in progressive motility (%), live sperm (%) and motility (%) parameters over time as a result of *E. coli* contamination, respectively. [blue line, control group (G1); pink line, 100.000 cfu/ml *E. coli* contaminated group (G2); green line, 1.000.000 cfu/ml *E. coli* contaminated group (G3); yellow line, 100.000 cfu/ml *E. coli* contaminated group (G4); purple line, 1.000.000 cfu/ml *E. coli* contaminated group (G5)]

Progressive Motility: Group 1 (G1) significantly differed from Groups 2 (G2) and 4 (G4) starting from the 60th minute ($p<0.05$). At the 120th minute, a notable decrease in Group 5 (G5) was observed ($p<0.001$). At the 180th minute, Group 1 (G1) was significantly different from all other groups ($p<0.001$). At the 300th and 360th minutes, differences between Groups 3-5 (G3-G5) disappeared, with the effects of the high-density ESBL-producing groups becoming more pronounced in the later hours ($p<0.01$).

Live Sperm Percentage: At the 100th minute, Group 1 (G1) showed a significant difference compared to Groups 3-5 (G3-G5) ($p<0.01$). At the 180th minute, the difference between Groups 2 and 5 (G2-G5) became statistically significant ($p<0.01$). At the 240th minute, Group 1 (G1) significantly differed from all other groups ($p<0.001$). At the 360th minute, a significant difference was observed between Groups 3 and 5 (G3-G5) ($p<0.05$), with the lowest viability recorded in Group 5 (G5).

Total Motility: Significant differences between Group 1 (G1) and Groups 3-5 (G3-G5) were observed starting from the 80th minute ($p<0.01$). At the 120th minute, Group 5 (G5) exhibited the lowest total motility ($p<0.001$). At the 240th minute, Group 1 (G1) significantly differed from all other groups ($p<0.05$), while at the 360th minute, a significant difference was observed between Groups 4 and 5 (G4-G5) ($p<0.01$).

In particular, sperm samples contaminated with ESBL producing *E. coli* strains showed a more rapid decline in motility over time, significantly reducing sperm viability rates. These findings indicate that resistant bacteria degrade sperm quality more quickly and distinctly, suggesting that such contamination could negatively affect spermatozoon functions. Additionally, it was found that contamination density (100.000 vs. 1.000.000 bacteria/ml) had an even more detrimental effect on spermatological parameters. These results highlight that microbiological contamination of bull semen, especially with resistant bacteria, poses a serious risk to sperm quality preservation and reproductive health.

DISCUSSION

This study evaluated the effects of different concentrations of ESBL-producing and non-producing *E. coli* bacteria on bull sperm motility and viability. Measurements taken at various time points during the study demonstrated the negative effects of bacterial contamination on sperm function. The results showed that *E. coli* had detrimental effects on sperm motility, progressive motility, and viability rates, with the impact being more rapid and pronounced in the presence of resistant bacteria.

Progressive motility data indicated a significant decrease in sperm cells exposed to higher bacterial concentrations. While no significant differences were found between groups at 20 minutes, significant differences were observed from 40 minutes onward, particularly between G1 (control group), G3 (1M *E. coli*), and G5 (10M ESBL-producing *E. coli*) groups. This suggests that contamination intensity negatively impacts sperm motility from the early stages. Notably, at 120 minutes, G2 was not statistically different from G3 and G4, but G2 and G4 significantly differed from G5 (which contained both high-density and ESBL-producing *E. coli*). This implies that contamination intensity has a more substantial effect in the early stages. In the mid-stage (120 minutes), ESBL-producing *E. coli* groups were more affected than non-producing strains, with only the group exposed to high-density and ESBL-producing contamination being statistically distinct from the others. By 300 minutes, the significant differences between G3, G4, and G5 had disappeared, but G2 remained significantly different from these three groups. The delayed negative effect on progressive motility in G2 compared to the other groups may be related to the lower contamination density and the influence of non-ESBL-producing *E. coli*, which was statistically significant. At

360 minutes, G1 was significantly different from all other groups, while no significant differences were observed between G3, G4, and G5, similar to what was observed at 300 minutes. Live sperm percentage data revealed the impact of bacterial exposure on sperm viability. From 60 minutes onward, significant differences between G1 and the G3, G4, and G5 groups became apparent, indicating that sperm viability is highly sensitive to bacterial contamination. Starting from 100 minutes, it was found that G5 had the lowest viability rate, suggesting that high bacterial concentrations may have a toxic effect on sperm cells. At 360 minutes, G1 significantly differed from all other groups, and the differences between G3, G4, and G5 became more pronounced. Total motility results followed a similar trend. Significant differences were observed between G1 and, particularly, G3, G4, and G5 groups at all time points. From 60 minutes onward, the impact of ESBL-producing *E. coli* on motility became more pronounced in the affected groups. From 100 minutes onward, especially at 360 minutes, it was observed that G1 differed from all other groups. This indicates that the effect of bacterial infection on sperm motility progressively increased and became more distinct over time.

Overall, our study confirms the negative effects of ESBL-producing *E. coli* on sperm motility and viability. It was determined that groups containing high concentrations of ESBL-producing *E. coli* exhibited earlier and more pronounced effects than groups with lower concentrations. However, over time, all infected groups showed similar profiles in terms of sperm motility and viability. This suggests that the effects of bacterial contamination on spermatological parameters may reach a saturation point as time progresses.

These results are consistent with previous studies, confirming that bacterial contamination impairs sperm function and leads to motility loss due to metabolic disturbances in sperm cells (Yániz et al. 2010; Kuster and Althouse 2016). The deterioration of spermatological parameters, particularly sperm motility, due to bacterial contamination has also been reported in earlier research. In contaminated sperm samples, bacterial metabolites and toxins harm spermatozoa's cellular structure, leading to motility loss (Diemer et al. 2003; Ďuračka et al. 2021). There are different studies in various species. In a study conducted in boars, it was reported that progressive motility was significantly reduced and abnormal morphology rates increased in ejaculates contaminated with bacteria such as Escherichia coli (Gaczarzewicz et al., 2016). These deteriorations were explained by the deformation of the sperm membrane structure by bacterial endotoxins. Similarly, in a study conducted in boars, it was stated that total and progressive motility were significantly reduced in samples with bacteriospermia, and at the same time, an increase in reactive oxygen species (ROS) was observed (Kuster et al., 2016). This situation shows that bacteria impair sperm functions through oxidative stress. In a study conducted in dogs, a negatively significant correlation was found between sperm motility and colony forming unit (CFU) numbers in samples with high bacterial load in the ejaculate (Sorkytė et al., 2024). Researchers stated that in addition to direct cell membrane damage, bacteria also change environmental parameters such as pH and osmolarity, creating an unfavorable environment for sperm survival.

The mechanisms by which *E. coli* damages sperm cells occur through the endotoxins and various proteases produced by the bacteria. These toxins cause sperm cell membrane disruption, which raises oxidative stress and damages cells (Oghbaei et al. 2020). Additionally, type 1 adhesion molecules, which are present in both bacterial pili and spermatozoa, respectively, and which can be rendered inactive by preincubation with mannose, are used by *E. coli* to attach to the surface structures of spermatozoa. Spermatological parameters decline as a result of these adhesion processes, which alter and harm the plasma membrane and other surface features of spermatozoa (Mayer et al. 2000). A study investigating the prevalence of bacterial contamination of semen and whether contamination can reduce sperm quality has shown that contamination of sperm samples by certain species is more closely associated with infertility. The study showed that E. coli has a generally negative effect on sperm quality in men with infertility (Moretti et al. 2009). The findings of present study indicate that resistant bacteria accelerate these mechanisms, causing sperm quality to degrade more rapidly. This is an important finding, suggesting that the deterioration of spermatological parameters is not only related to the increase in bacterial count but also to the resistance characteristics of the bacteria.

The effect of contamination density on sperm motility was also noteworthy in our study. Samples contaminated with 1.000.000 *E. coli* cells/ml exhibited a more pronounced decrease in sperm motility, whereas samples with 100.000 *E. coli* cells/ml showed a more limited change. These results indicate that the effects of contamination density on sperm function are directly proportional, meaning that as bacterial density increases, sperm quality rapidly deteriorates.

CONCLUSION

The data from this study provide important insights for clinical applications. Bull semen is commonly used in artificial insemination procedures, and microbial contamination of semen can directly impact reproductive success. The presence of resistant bacteria indicates that such contaminations can become more challenging and that traditional treatment methods may be insufficient to control these infections. In this context, there is a need to develop alternative strategies to combat antibiotic resistance and prevent microbial contamination.

While our study contributes to the body of knowledge regarding microbial contamination in bull semen, it

highlights the need for more comprehensive research in this area. Further studies evaluating the effects of different bacterial strains and contamination durations on sperm quality will help improve the understanding of this issue.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: BE, MGSK and CK contributed to the project idea, design and execution of the study. BE and CK contributed to the acquisition of data. BE and VEE analysed the data. BE and CK drafted and wrote the manuscript. All authors reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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