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Research Article

Detection of central nervous system tissues in minced meat by ELISA and histological examination

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ABSTRACT

The aim of this study was to compare two histological staining methods and an ELISA for the detection of central nervous system (CNS) tissue in raw minced meat obtained from tissues surrounding the spinal cord of a bovine carcass split using a band saw, as well as in experimentally prepared fresh minced meat samples containing different proportions of brain or spinal cord tissue. Minced meat samples containing CNS tissue at concentrations ranging from 0.1% to 10% were analyzed using Crossmon's modified triple staining, Kluver–Barrera staining, and ELISA. No statistically significant differences were detected between the staining methods or between histological examination and ELISA. However, ELISA detected CNS tissue at a lower concentration (0.1%) than histological examination (0.2%), indicating greater analytical sensitivity. In contrast, histological examination enabled direct visualization of tissue morphology, including ganglion tissue that was not detected by ELISA. Thus, the two techniques provided complementary information. These findings may facilitate the identification of adulteration in meat and meat products and help prevent the consumption of CNS tissues that may pose a public health risk associated with bovine spongiform encephalopathy (BSE).

Keywords: Adulteration, BSE, Central nervous system, ELISA, Histological examination

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Introduction

Meat is consumed either unprocessed or processed into various products. Ready-to-eat meat products such as sucuk, salami, and sausages are among the most commonly consumed. Due to the methods used in their preparation, these products are highly susceptible to adulteration (2). Adulteration is the term given to the deliberate act of reducing the quality of any substance offered for sale by adding another substance, substituting it with lower-quality raw materials, or by failing to use, or by using only a small amount of, an expensive raw material that should be present in the product. Adulteration is carried out for economic gain. It is encountered across all subsectors of the food industry. However, incidence is higher in high-value products. Consequently, activities that could be classified as adulteration may be observed in the meat and meat products sector (34).

According to the Ministry of Agriculture and Forestry's Turkish Food Codex Regulation No. 2018/52 on Meat, Prepared Meat Mixtures and Meat Products, the addition of offal to meat products is prohibited (4). Tissues of lower commercial value (e.g., pieces of tendon and fascia, genital organs, eyes, ears, skin, stomach, intestines, spleen, lungs, liver, mammary tissue, cartilage, and bones) may be added to meat and meat products (14, 17). This not only creates unfair competition for businesses producing high-quality products, but also misleads consumers and poses a threat to public health (5, 37). Contamination of meat and meat products with central nervous system (CNS) tissues, such as the brain and spinal cord, whether through deliberate adulteration or inadvertent contamination during production, is significant with respect to bovine spongiform encephalopathy (BSE). Transmissible spongiform encephalopathies (TSEs), which occur in both animals and humans, are chronic,

degenerative and fatal diseases affecting the central nervous system. These diseases are also known as “prion diseases”. Because the immune system does not recognize prions as foreign, affected individuals do not develop an immune response against the agent (15, 16, 35).

BSE is characterized by the accumulation of an abnormal isoform of the cellular prion protein in the central nervous system (28). Transmission of the agent from animals to humans is thought to occur through the consumption of contaminated meat products derived from cattle infected with BSE. Research has demonstrated an association between exposure to the BSE agent and the development of variant Creutzfeldt-Jakob disease (vCJD) in humans (1, 7).

In cattle aged 30 months or older, the brain, spinal cord, tonsils, distal ileum and dorsal root ganglia are defined as specified risk materials (SRMs) (10). CNS tissues, such as the brain and spinal cord, are considered the primary sources of infection and account for 95% of total infectivity (33). Legislation enacted in European countries and Türkiye prohibits the inclusion of CNS tissue in meat and meat products (4, 8), and the Turkish Standards Institute (TSE) has stated that animals suspected of showing clinical signs must be destroyed rather than made available for human consumption (3).

During slaughter, there is a potential for meat to become contaminated with CNS tissue through the stunning of the animal, carcass splitting, and the washing of the carcass (29). Due to this risk, methods such as histological examination, immunohistochemistry (IHC), ELISA, Western blotting, cholesterol analysis, RT-PCR and GC-MS are used to detect the presence of CNS tissue in meat and meat products (6). The aim of this study was to compare two histological staining methods with ELISA for the detection of CNS tissue in raw minced meat obtained from tissues surrounding the spinal cord of a bovine carcass that had been split using a band saw and in experimentally prepared fresh minced meat samples containing different proportions of brain or spinal cord tissue.

Materials and Methods

The study material consisted of four main groups of minced meat samples with different compositions, all prepared from tissues obtained from a single bovine carcass. Group 1 consisted of minced meat samples obtained solely from the leg section of this carcass. As these samples did not contain CNS tissue, this group was used as a control for comparison with the other groups. Group 2 consisted of minced meat samples prepared from meat obtained from the area surrounding the spinal cord of the same carcass, which had been split into two using a band saw. This group was used to determine whether the bandsaw caused contamination of the surrounding muscle tissue by spinal cord tissue. Group 3 consisted of minced meat samples (subgroups 3.1–3.6) prepared by adding six different proportions of bovine brain tissue obtained from the same carcass to minced meat from the same region used for Group 1. Group 4 consisted of minced meat samples (subgroups 4.1–4.6) prepared by adding six different proportions of bovine spinal cord tissue, obtained from the same carcass, to minced meat obtained from the same region as that used for Group 1. Groups 1 and 2 each consisted a single composition and were not subdivided; the four main groups therefore corresponded to 14 experimental groups in total.

Each experimental group and each subgroup was prepared as a 500-g sample. Experimental groups containing brain or spinal cord tissue were prepared in six subgroups at concentrations of 0.1%, 0.2%, 0.4%, 1%, 5% and 10% (w/w), taking into account the detection limits of ELISA and histological techniques. Each of the 14 experimental groups was analyzed using Crossmon’s modified triple staining, Kluver-Barrera staining, and ELISA. The experimental groups and their contents are presented in Table 1.

The fresh red meat, bovine brain and bovine spinal cord tissues used to prepare the experimental groups were obtained from a single carcass supplied by a local butcher in Bursa and transported under cold-chain conditions to the Histology and Embryology Laboratory at the Faculty of Veterinary Medicine, Uludağ University. The tissues were stored at 4 °C. Samples from the experimental groups were minced once using the medium-sized mincing disc of a meat mincer (Vestel, product code: 20242454). To prevent contamination, all parts of the mincer were washed before processing each new sample. The experimental groups specified in Table 1 were prepared using an analytical balance and stored at 4 °C.

Table 1. Experimental groups

Group	Brain tissue content (%)	Spinal cord tissue content (%)
Group-1	0	0
Group-2	0	0
Group-3.1	0.1	0
Group-3.2	0.2	0
Group-3.3	0.4	0
Group-3.4	1	0
Group-3.5	5	0
Group-3.6	10	0
Group-4.1	0	0.1
Group-4.2	0	0.2
Group-4.3	0	0.4
Group-4.4	0	1
Group-4.5	0	5
Group-4.6	0	10

ELISA Technique: Samples were analyzed using a fully automated ELISA analyzer (ChemWell 2900) in accordance with the manufacturer's instructions for the R-Biopharm Ridascreen Risk Material 10/5 (R-6703) ELISA kit for the detection of glial fibrillary acidic protein (GFAP). Absorbance values were measured at a wavelength of 450 nm. Samples with absorbance values greater than that of the 0.1% CNS standard (0.326) were classified as CNS-positive (+). The absorbance values were calculated using Ridasoft Win software. To assess the reliability of the analysis, the correlation coefficient (R) of the standard curve was verified against the value reported on the kit certificate. The R value was found to be 0.9989, and the results were deemed reliable. In accordance with the kit method, one swab sample was collected from each experimental group, a total of 14 swabs were analyzed in duplicate.

Histological Examination: Samples weighing between 1.00 and 1.50 g were taken from five regions of each experimental group and fixed in 10% neutral-buffered formalin for 24 hours. Following routine histological processing, the specimens were embedded in paraffin. After trimming, serial sections 5–6 µm thick were prepared from the blocks using a microtome (Leica RM 2135). Two suitable sections from this series were placed on separate slides. Subsequently, 10 series of sections were discarded, and a new series of 5–6 µm-thick sections was prepared. The serial sections obtained were added to the slides as a second set of sections. The first slide was stained using Crossmon's modified triple staining (9), whilst the second slide was stained using the Kluver-Barrera staining technique (25). In this way, the ability of the two staining techniques to stain CNS tissue was compared using corresponding serial sections. Consequently, 20 sections were analyzed on 10 slides for each experimental group. A total of 280 sections were analyzed across all experimental groups. For evaluation, the entire surface of each slide was examined. Samples in which substantia grisea and/or substantia alba, (characteristic of brain and spinal cord tissue) or ganglion cells (characteristic of ganglion tissue) were detected were interpreted as positive (+) for CNS tissue, while samples in which neither of these structures was observed were interpreted as negative (−). Substantia grisea was identified by the presence of neuronal and glial cell nuclei, and substantia alba by the detection of myelinated and unmyelinated nerve fibers. Ganglion tissue was identified by the presence of large ganglion cells with a vesicular nucleus and a prominent nucleolus, surrounded by satellite cells. All of these structures were clearly distinguishable from the striated muscle fibers and connective tissue. Examinations and photographic recordings were performed using a Nikon Eclipse 80i light microscope.

Statistical Analysis: Statistical analysis was carried out using IBM SPSS 20.0. The Pearson chi-square test was used to determine whether there was any difference between the histological and ELISA methods in the detection of CNS tissue. The Pearson chi-square test was used to assess whether there was any difference in the detection of CNS tissue between Crossmon's modified triple staining and the Kluver-Barrera staining methods. P-values below 0.05 were considered statistically significant.

Results

The results of CNS tissue detection in the experimental groups are presented in Table 2. The absorbance values of the kit standards, as determined by ELISA analysis, are presented in Table 3, whilst those obtained for the experimental groups are presented in Table 4. For Groups 1 and 2, the absorbance values were 0.040 and 0.067, respectively. Because these values were lower than the absorbance value of the 0.1% CNS standard (0.326), both groups were classified as negative (–) for CNS tissue. For Groups 3.1–3.6 and 4.1–4.6, the absorbance values were greater than 0.326. Therefore, these groups were classified as positive (+) for CNS tissue.

Table 2. Histological examination and ELISA results.

Group	Crossmon's modified triple staining	Kluver-Barrera staining	ELISA
Group-1	-	-	-
Group-2	+	+	-
Group-3.1	-	-	+
Group-3.2	+	+	+
Group-3.3	+	+	+
Group-3.4	+	+	+
Group-3.5	+	+	+
Group-3.6	+	+	+
Group-4.1	-	-	+
Group-4.2	+	+	+
Group-4.3	+	+	+
Group-4.4	+	+	+
Group-4.5	+	+	+
Group-4.6	+	+	+

(+) : Detected. (-) : Not detected.

Table 3. Absorbance values for ELISA kit standards.

Kit standards	Absorbance value
Standard-1 (0.0%)	0.109
Standard-2 (0.1%)	0.326
Standard-3 (0.2%)	0.558
Standard-4 (0.4%)	1.059

Table 4. Absorbance values for the experimental groups.

Group	Absorbance value
Group-1	0.040
Group-2	0.067
Group-3.1	2.300
Group-3.2	0.517
Group-3.3	0.937
Group-3.4	1.750
Group-3.5	2.654
Group-3.6	2.543
Group-4.1	0.983
Group-4.2	1.295
Group-4.3	1.619
Group-4.4	2.077
Group-4.5	2.205
Group-4.6	1.069

No CNS tissue was detected in Group 1 (Figure 1). Ganglion tissue was detected in Group 2 using both staining methods (Figure 2). No CNS tissue was detected in Groups 3.1 and 4.1 using either staining method. In Groups 3.2–3.6 and 4.2–4.6, CNS tissue was detected using both staining methods in samples containing 0.2%, 0.4%, 1%, 5%, and 10% brain or spinal cord tissue (Figures 3 and 4).

A chi-squared test was performed to assess whether a statistically significant difference existed between histological examination and the ELISA method in the detection of CNS tissue. The calculation yielded a P-value of 1.00. Because the calculated P value exceeded 0.05, no statistically significant difference was detected between histological examination and the ELISA method for detecting CNS tissue. Subsequently, the two staining methods used for histological examination were compared in terms of their ability to detect CNS tissue. The P-values for the detection of brain and spinal cord tissues were 0.436 and 0.438, respectively. As both P values were greater than 0.05, no statistically significant difference was detected between Crossmon's modified triple staining and Kluver–Barrera staining in the detection of brain or spinal cord tissue.

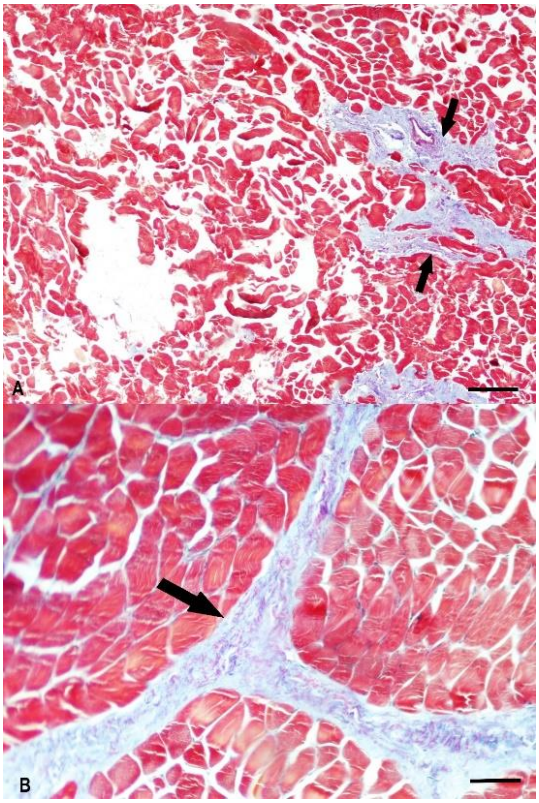


Figure 1. (A) Striated muscle tissue and connective tissue, Crossmon's modified triple stain x40 (scale: 200 µm), (B) Striated muscle tissue and connective tissue, Crossmon's modified triple stain x100 (arrow: connective tissue, scale: 80 µm).

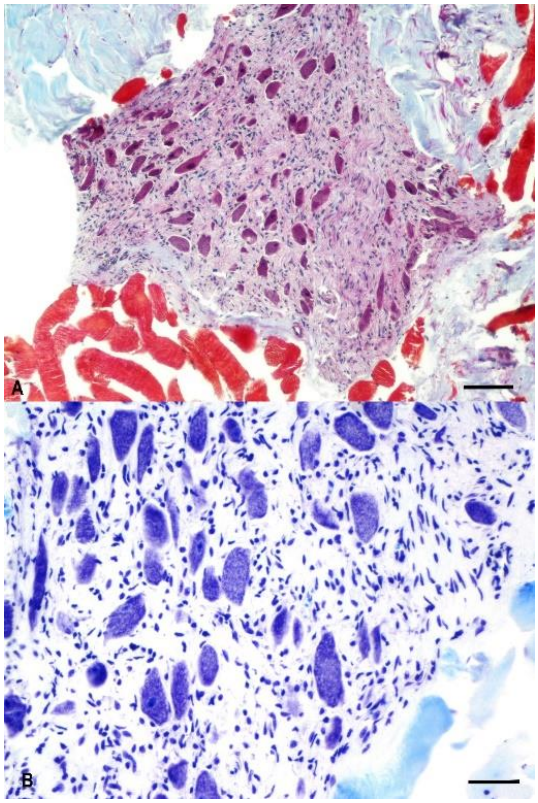


Figure 2. (A) Ganglion tissue, Crossmon's modified triple stain x100 (scale: 80 µm), (B) Ganglion tissue, Kluver-Barrera stain x200 (scale: 40 µm).

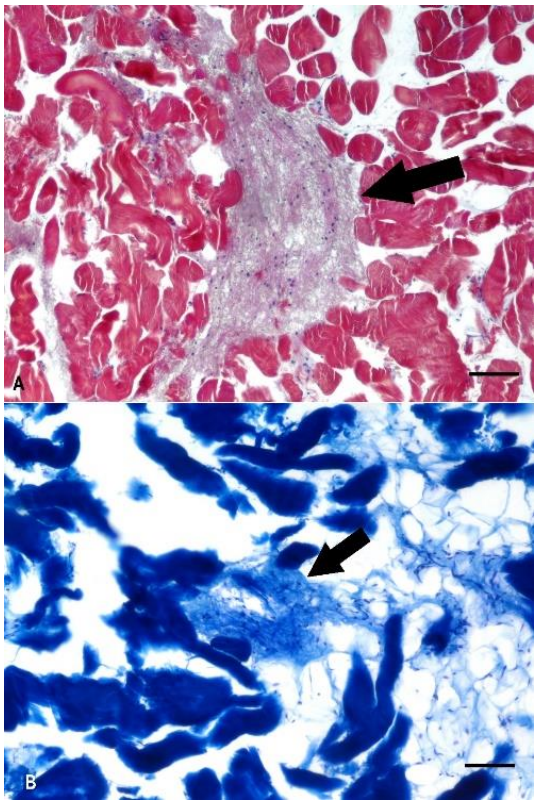


Figure 3. (A) 0.2% Brain tissue, Crossmon's modified triple stain x100 (scale: 80 µm), (B) 0.2% Brain tissue, Kluver-Barrera stain x100 (arrow: substantia grisea, scale: 80 µm).

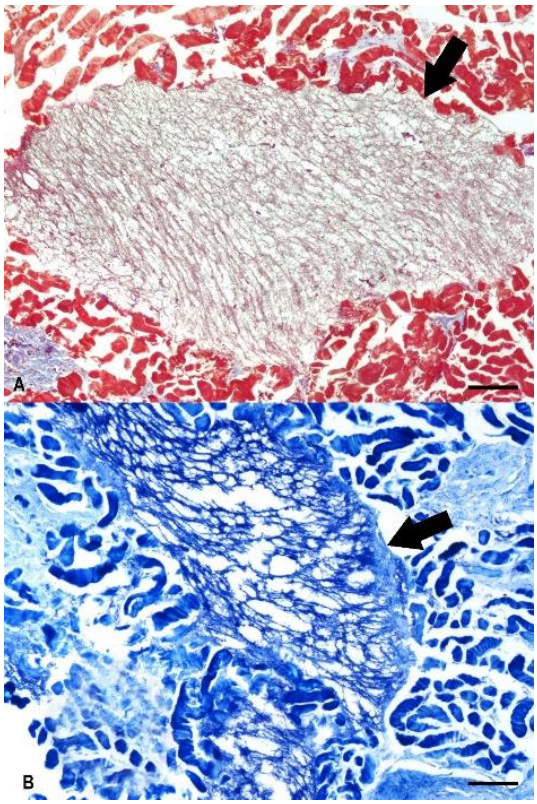


Figure 4. (A) 0.2% Spinal cord tissue, Crossmon's modified triple stain x40 (scale: 200 µm), (B) 0.2% Spinal cord tissue, Kluver-Barrera stain x40 (arrow: substantia alba, scale: 200 µm).

Discussion and Conclusion

Previous studies have shown that the presence of CNS tissue in beef and beef products is of significant concern, as it poses a public health risk in relation to variant Creutzfeldt-Jakob disease (vCJD), which is associated with exposure to the bovine spongiform encephalopathy (BSE) agent. Studies using ELISA to detect CNS tissue in meat and meat products have indicated that GFAP is a major protein component of glial intermediate filaments in differentiated fibrous and protoplasmic astrocytes of the CNS (12, 13). Analyses of GFAP concentrations in various tissues revealed levels of 55.000–220.000 ng/mg in the spinal cord, 9.000–55.000 ng/mg in the whole brain, and 13.5–51 ng/mg in the sciatic nerve. ELISA has been reported to be a simple, cost-effective, and reliable method for the detection of CNS tissue (30). Studies conducted in Türkiye to detect CNS tissue using ELISA reported positivity rates of 9.4% in fresh raw minced meat, 18.8% in raw meatballs, 0.0% in pastrami (24), 14.48% in raw meatballs (11), 21.51% in raw minced meat samples, and 15% in processed meat products such as salami and sausages (23).

Our ELISA results were consistent with the minimum detection limits reported in previous studies (11, 23). CNS tissue was correctly detected in all experimental groups containing 0.1–10% brain or spinal cord tissue. In Group 2, ganglion tissue could not be detected, and the ELISA result was therefore negative. This may be due to the GFAP concentration in ganglion tissue (1.4 ng/mg) (31) being significantly lower than that in brain and spinal cord tissues, and therefore falling below the limit of detection (LOD) of the kit. A previous study also noted that the detection of ganglion tissue using ELISA is particularly difficult (20).

Furthermore, the absorbance values obtained using ELISA did not increase proportionally with the CNS tissue concentrations in the experimental groups (Table 4). This was attributed to the use of the swab sampling method because the amounts of CNS tissue in randomly collected swabs may have varied. Studies employing the same method have also reported that swab sampling may lead to variable results (20, 27, 32).

Our histological examination results differed from those of previous studies. Previous studies on fermented and heat-treated sucuk reported that CNS tissue could be detected in limited areas (21) or could not be detected in sausages subjected to heat treatment at 80–100 °C (36). In our study, CNS tissue was detected in all experimental samples containing 0.2% or more brain or spinal cord tissue using both Crossmon's modified triple staining and Kluver–Barrera staining. Although ganglion tissue could not be detected by ELISA in minced meat samples

obtained from muscle tissue near the spinal cord in Group 2, it was detected using both histological staining methods. This may be attributed to the fact that the samples had not been subjected to heat treatment or fermentation. The detection of ganglion tissue was consistent with previous findings indicating a risk of neural tissue spreading to surrounding tissues during carcass splitting (18) and even to subsequent carcasses (19). The difference in results between the two techniques in Group 2 stems from their differing analytical objectives. ELISA measures GFAP, which is present in ganglion tissue at a concentration of approximately 1.4 ng/mg (31); this is lower than the concentration in the spinal cord and brain, and therefore remains below the kit's detection limit even if ganglion tissue is present in the sample. In contrast, histological examination relies on the direct visualization of tissue morphology rather than the concentration of a single marker protein; consequently, ganglion cells can be identified independently of their GFAP content. This may have practical implications for meat inspection and food safety. A positive ELISA result indicates the presence of CNS tissue, but histological examination reveals the identity and distribution of the detected tissue. The presence of ganglion tissue alone in muscles adjacent to the spinal cord, in the absence of substantia grisea or substantia alba, is consistent with accidental contamination occurring during carcass dissection. However, the presence of brain or spinal cord fragments distributed throughout the product is more consistent with the deliberate addition of central nervous system tissue. Therefore, histological examination can assist food safety control mechanisms in distinguishing between hygiene breaches requiring corrective action at the slaughterhouse and adulteration requiring legal sanctions.

Studies conducted in Türkiye have reported that no BSE-related prion proteins were detected in the samples examined (22, 26). However, there is always a risk that BSE-positive animals may enter the country through animal imports or other routes. Therefore, specified risk materials should be removed during slaughter, and post-mortem BSE testing should be performed (11, 23).

The study was carried out on fresh samples prepared experimentally using muscle, brain and spinal cord tissues obtained from the same carcass. For the ELISA analysis, a kit suitable for detecting GFAP in raw samples was selected based on a literature review. In all experimental groups, the amount of brain and/or spinal cord tissue was prepared taking into account the detection limit of this kit, and the ELISA results were compared with the results of histological examination. Based on the results of this study, further research could be conducted on the detectability of CNS tissue using fresh, heat-treated and/or fermented meat and meat products obtained from multiple carcasses. In future studies, these materials could be analyzed using various methods such as immunohistochemistry, Western blot, real-time PCR and gas chromatography-mass spectrometry (GC-MS).

In conclusion, the analyses carried out demonstrated that both histological examination and ELISA methods can be used to detect central nervous system (CNS) tissue in raw minced meat samples, and no statistically significant difference was found between the methods. ELISA detected experimentally added brain and/or spinal cord tissue at a concentration of 0.1%, whereas histological examination detected CNS tissue at concentrations of 0.2% and above. Consequently, ELISA provided higher analytical sensitivity. The main advantage of histological examination is not a lower detection limit, but the ability to visualize tissue morphology directly and identify ganglion tissue that ELISA cannot detect. Therefore, both techniques can be used with confidence to detect CNS tissue in raw meat and meat products, and the results provide complementary information.

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Ethical Statement

No live animals were used in experimental procedures in this study. Therefore, ethical approval was not required.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

OBN and NÖ proposed the idea, planned the article, provided scientific support and contributed to the interpretation of the results.

Data Availability Statement

Data supporting the findings of this study may be obtained from the relevant author upon reasonable request.

Declaration of Generative Artificial Intelligence (AI)

The authors declare that no generative artificial intelligence (AI) or AI-assisted technologies were used in the writing of this article or the creation of the figures.

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