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RESEARCH ARTICLE

Molecular Effects of Selected Copper Compounds on Novel Oncogenic Signaling Marker Expression in Cancer Cells

Salih Erat [1✉], Alparslan Kadir Devrim [1], Ali Şenol [2], Büşra Moran [3], Canan Çakır Çoban [4], Elisha Apatewen Akanbong [2]

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ABSTRACT

This study evaluated the cytotoxic, apoptotic, and gene expression–modulatory effects of $CuSO_4$, $CuCl_2$, and $CuCO_3$ on L929 fibroblasts, DLD-1 colorectal cancer cells, and Capan-1 pancreatic cancer cells. Cells were exposed to concentrations ranging from 7.8 to 500 µM for 24 h. Cell viability was assessed by MTT assay, apoptosis/necrosis by double staining, and gene expression (EZH2, PGK1, HER2, USP28, AURKA, HPRT1, ER-α) by qPCR. At 500 µM, $CuSO_4$, $CuCl_2$, and $CuCO_3$ showed no significant cytotoxicity in L929 cells, with viability values of 99.81±9.4%, 90.69±5.58%, and 104.66±1.48%, respectively. In DLD-1 cells, limited cytotoxicity was observed, with viability remaining above 58% at most concentrations. In contrast, a pronounced, concentration-dependent cytotoxic effect was detected in Capan-1 cells. At 500 µM, viability decreased to 37.28±1.61% ($CuSO_4$), 36.28±3.29% ($CuCl_2$), and 31.34±4.93% ($CuCO_3$). At 250 µM, viability ranged between 42.66±3.44% and 53.50±4.11%, indicating sustained cytotoxic activity. Double staining analysis demonstrated that copper-induced cell death occurred predominantly via apoptosis. In Capan-1 cells, the apoptotic index reached up to 74.2% for $CuCl_2$ and showed a dose-dependent increase across all compounds, while necrotic rates remained comparatively low. qPCR results revealed significant modulation of cancer-related genes in Capan-1 cells. EZH2 expression was consistently downregulated, particularly in $CuSO_4$ and $CuCO_3$ groups. PGK1 and AURKA expression exhibited concentration-dependent changes, with peak upregulation observed at intermediate doses (notably 125–250 µM). HER2 and ER-α expression levels increased across most treatment groups, whereas USP28 expression was generally decreased, with compound-specific variations. HPRT1 expression showed a marked reduction and was nearly undetectable in $CuCO_3$-treated groups. In conclusion, $CuSO_4$, $CuCl_2$, and $CuCO_3$ exhibit selective and dose-dependent cytotoxic and pro-apoptotic effects in pancreatic cancer cells, while sparing healthy fibroblasts. These compounds also induce significant alterations in gene expression profiles associated with proliferation, metabolism, and tumor progression, highlighting their potential as candidates for targeted anticancer strategies.

INTRODUCTION

Cancer is recognized as the predominant cause of death in developed nations and the second most common cause of mortality in developing countries (WHO, 2008; Jemal et al., 2011). Pancreatic cancer is a rapidly progressing malignant tumor with a poor prognosis and high mortality rate (Watanapa et al., 1992; Tempero et al., 2011). The 5-year survival rate for pancreatic cancer is less than 10% (Siegel et al., 2023). Pancreatic cancer, a leading cause of cancer-related deaths due to its aggressive nature, is predicted to become the second leading cause in Western countries within the next decade (Neoptolemos et al., 2018). Colorectal cancer, which arises due to various reasons such as dietary habits, smoking, genetic factors,

and aging, is the most observed cancer among digestive system cancers and is a significant cause of cancer-related deaths (Granados-Romero et al., 2017). In the United States, colorectal cancer constitutes the third major cause of cancer-associated mortality, accounting for nearly 9% of deaths among men and 8% among women. Pancreatic cancer ranks fourth, with an estimated 8% mortality rate in both sexes (Siegel et al., 2023).

Copper is an essential metal involved in various biological processes such as oxidative phosphorylation, iron transport, cross-linking in connective tissue, and pigment formation (Robinson and Winge, 2010). It is known that excess copper can cause various diseases in animals, and Cu(II) complexes have specific effects, such as inducing apoptosis in cancer cells, exhibiting cytotoxic selectivity, and copper compounds are antiproliferative anticancer agents due to the accumulation of copper in high concentrations in cancer cells (Krajčiová et al., 2014).

Cancer is a complex disease characterized by molecular alterations in fundamental biological processes such as epigenetic regulation, cellular metabolism, cell cycle control, and hormone signaling. The enhancer of zeste homolog 2 (EZH2), phosphoglycerate kinase 1 (PGK1), human epidermal growth factor receptor 2 (HER2), ubiquitin-specific protease 28 (USP28), aurora kinase A (AURKA), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and estrogen receptor alpha (ER-α) genes, which play a role in these processes, can be considered potential biomarkers in the molecular characterization of the disease, prognosis, and evaluation of treatment response (Wang et al., 2018; Wolff et al., 2018; Cui et al., 2024; Gao et al., 2024; Lu et al., 2024; Varshney et al., 2024; Xu et al., 2025). In this study, the genes EZH2, PGK1, HER2, USP28, AURKA, HPRT1, and ER-α were selected due to their established roles in these processes.

Enhancer of zeste homolog 2, which serves as a histone methyltransferase in the Polycomb repressor complex, is characterized by recurrent mutations in diverse cancers and by high expression levels in many additional tumor types (Kim and Roberts, 2016). PGK1 is a key enzyme involved in ATP synthesis in glycolysis and has been identified as a molecular regulator associated with tumor progression and patient prognosis in many cancer types, including breast cancer (Yang and Han, 2025). HER2 is a key growth factor receptor encoded by the ERBB2 gene and associated with proliferation, invasion, and treatment resistance in cancer cells (Raggi et al., 2025). USP28 is a critical deubiquitinase that regulates the stability of signaling proteins by removing their ubiquitin tags, thereby influencing key biological processes such as cell proliferation, stress response, and carcinogenesis (Wang et al., 2018). AURKA is a serine/threonine kinase involved in cell division by regulating mitosis and is considered an important therapeutic target due to its overexpression in many cancer types and its involvement of oncogenic signaling pathways (Du et al., 2021). HPRT1 is a key enzyme involved in the purine salvage pathway, regulating nucleotide synthesis and DNA repair, and has recently gained attention as a molecular regulator due to its increased expression in many cancer types and its association with tumor development (Lu et al., 2024). ER-α is a key transcription factor regulating cellular proliferation and hormone signaling, and maintaining its stability through intracellular protein complexes can affect treatment response (Jaiswal et al., 2025).

This study aimed to investigate the effects of various concentrations of three different copper compounds ($CuSO_4$, $CuCl_2$, and $CuCO_3$) on the healthy cell line L929, the colorectal cancer cell line DLD-1, and the pancreatic cancer cell line Capan-1. We hypothesized that copper compounds would exert selective cytotoxic and gene regulatory effects particularly in pancreatic cancer cells.

MATERIALS AND METHODS

Cell Culture

L929 fibroblast cells, Capan-1 pancreatic cancer cells, and DLD-1 colorectal cancer cells were used. 89% DMEM (Biological Industries), 10% Fetal Bovine Serum (FBS; Biological Industries), 1% L-glutamine (Biological Industries), and 1% Penicillin/Streptomycin (Biological Industries) antibiotics were used as complete culture media for all cells. 0.05% Trypsin-EDTA solution (Trypsin-Ethylenediaminetetraacetic acid) was used to remove cells from the culture flasks, and 1X concentration calcium/magnesium-free phosphate buffer (PBS) was used for washing and cleaning. Trypan blue was used for cell counting. Cell viability was determined using the tetrazolium salt MTT (Serva) in the cytotoxicity test. In double staining, apoptosis/necrosis ratios were determined using ribonuclease-A, propidium iodide (Serva), and hoechst 33342 (Serva). All cell culture studies were performed using serological pipettes, culture dishes, and multi-well plates (Corning, USA).

Frozen cells were thawed briefly at 37°C. Thawed cells were transferred to 15 mL Falcon tubes in a sterile Class II laminar flow cabinet. The Falcon tubes were centrifuged at 250 g for 5 minutes. 3 mL of complete culture medium was added to the Falcon tube and, after homogenization, plated into 25 cm² flasks. The flasks were incubated in a 37°C incubator with 5% CO2.

Sample Preparation

The prepared materials were sterilized under UV light for 15 minutes and mixed with ready-made culture medium at concentrations of 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.625 µM, and 7.8 µM. Cell application was performed without delay.

Cytotoxicity Test (MTT)

For this test method, the MTT method described in reference standard 10993-5 was applied according to the standard. A 96-well plate was used for the toxicity test. After cell counting, calculations were made to ensure there were 1.0 x 10^4 cells in each well according to the number of live cells. In each well of the 96-well plate, 100 µL of complete culture medium was placed in cells and incubated for 24 hours. After 24 hours, it was checked whether the cells adhered to the well plate surface. The medium in the wells was drained. The prepared samples were added to the cells at concentrations of 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.625 µM, and 7.8 µM. Only the medium was added to the negative control group. The cells were incubated with the applied materials for 24 hours. At the end of the incubation, the medium in the well plates was removed, and 50 µl of MTT (1 mg/mL) solution was added to the wells. After incubation at 37°C for 2-2.5 hours, the MTT solution in the wells was drained, and 100 µL of MTT solvent (isopropanol) was added. To determine cell viability, absorbance density values from a 96-well plate were read at 570 nm using an ELISA plate reader. The percentage of cell viability for each group was calculated using the equation “Cell viability (%) = Optical Density, OD570 (sample) / Optical Density, OD570 (control) x 100”, assuming 100% viability for the control cells.

Determination of Apoptosis and Necrosis by Double Staining Method

Cells were plated in 48-well plates, with 15 x 10^3 cells per well. The cells were incubated for 24 hours (5% CO_2, 37°C, >90% humidity). After incubation, the media in the plates were discarded, and 200 µL of samples at concentrations of 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.625 µM, and 7.8 µM were added. A negative control consisting only of cells and culture medium was also added. Incubation was allowed for 24 hours. After incubation, the media in the plates were discarded, 70 µL of double staining solution was added, the plates were covered to prevent light exposure and incubated for 15 minutes. After incubation, apoptotic cells and necrotic cells were evaluated using an inverted microscope (Leica, DM6000B, Germany) with a DAPI filter (Preparation of the double staining solution: ribonuclease A 10 mg/1 mL PBS, hoechst 33342 200 µg/1 mL PBS, propodium iodide 100 µg/1 mL PBS were prepared from stock solutions, and 500 µL hoechst 33342, 100 µL propodium iodide, and 100 µL ribonuclease A were added to 10 mL PBS).

Quantitative Real-Time PCR (qPCR) Analyses

Total RNA was obtained using cell culture samples as specified in the RNA isolation kit (Norgen, Canada) protocol. The concentration and purity of total RNA were assessed using a nanodrop spectrophotometer (MultiSkan GO, Thermo Scientific, USA). cDNA synthesis reactions, performed using 500 ng of template total RNA, were carried out in a thermal cycler (Bio-Rad, USA) with the ab185916 cDNA synthesis kit (Abcam, UK). Quantification of the resulting cDNA samples was performed using a nanodrop spectrophotometer (MultiSkan GO, Thermo Scientific, USA). Subsequently, expression analyses were performed on these samples in a qPCR system (Bio-Rad, USA) using the primers given in Table 1. The BrightGreen (ABM, Canada) protocol was applied for this stage, and enzyme activation was performed at 95 °C for 10 minutes. PCR reactions were run for 35 cycles: 15 seconds at 95 °C and 60 seconds at 57-60 °C. Relative quantifications of qPCR amplicons were determined using the ΔΔCt method. These values were calculated by subtracting the ΔCt values of the control group from the ΔCt values of the cancer cells. The relative expression of target genes was determined using the equation 2^−ΔΔCt, and the levels of EZH2, PGK1, HER2, USP28, AURKA, HPRT1, and ER-α genes were normalized using GAPDH as the primary housekeeping gene (Devrim et al., 2025). In addition, HPRT1 expression variability was also evaluated rather than being used as a stable reference gene.

Table 1. Primer sequences used in qPCR analyses.

Gene	Forward Primer	Reverse Primer
EZH2	GGCTCCTCTAACCATGTTTACAACT	AGCGGTTTTGACACTCTG AACTAC
PGK1	GGGAAAAGATGCTTCTGGGAA	TTGGAAAGTGAAGCTCGGAAA
HER2	CCAGGACCTGCTGAACTGGT	TGTACGAGCCGCACATCC
USP28	GCAAATGCTGGACACT	ACTTCCTGCTCAAACC
AURKA	GGAATATGCACCACTTGGAACA	TAAGACAGGGCATTTGCCAAT
HRPT1	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA
ER-α	GTGGGAATGATGAAAGGTGG	TCCAGAGACTTCAGGGTGCT
GAPDH	CAATGACCCCTTCATTGACC	GACAAGCTTCCCGTTCTCAG

RESULTS

Cytotoxicity results in the MTT test, the absorbance values of the cells were compared with control cells and the % viability values were calculated. For a material to have an anticancer effect in vitro, it should not be toxic to healthy cells (L929) but should be toxic to cancer cells (DLD-1, Capan-1). Furthermore, it is desirable that the dying cells choose the apoptotic pathway as their death pathway, meaning that necrotic death is not present. According to ISO 10993-5, a material is considered toxic if its viability is less than 70%. After drug design or invention, it is necessary to meet the requirements of the Food and Drug Administration (FDA), and according to the FDA, a viability of 50% lethality is considered acceptable for the drug to be effective in drug development studies. According to the MTT test results; $CuSO_4$ at a concentration of 500 µM showed 99.81±9.4% viability in L929 cells, while DLD-1 and Capan-1 cancer cells showed 96.02±3.96% and 37.28±1.61% viability, respectively. All concentrations of the prepared material did not show a toxic effect on the healthy cell group, L929 fibroblast cells. It was determined that there was no toxic effect on DLD-1 colorectal cancer cells at the applied concentration; although cell viability changed with decreasing concentration, no dose showed the desired toxic effect. However, the toxic effect on Capan-1 cells is noteworthy, and it was found that the toxic effect decreased and viability increased with decreasing concentration (250 µM and 125 µM, respectively) (50.38±3.89% and 63.18±4.07%, respectively).

At a concentration of 500 µM ($CuCl_2$), 90.69±5.58% viability was recorded in L929 cells, while viability was observed in DLD-1 and Capan-1 cancer cells at 58.79±1.67% and 36.28±3.29%, respectively. All concentrations of the prepared material showed no toxic effect on the healthy cell group, L929 fibroblast cells, and were almost at the same level as the control group cells. In DLD-1 cells, toxic effects were observed at concentrations of 250 µM and 62.5 µM, with viability rates of 62.61±4.17% and 68.63±5.74%, respectively, while no toxic effects were detected at other concentrations. In Capan-1 cells, toxic effects were detected at concentrations of 250 µM, 125 µM, and 62.5 µM, with viability rates of 42.66±3.44%, 46.97±0.66%, and 68.03±0.62%, respectively. The toxic effect decreased in Capan-1 cells as the material concentration decreased.

At a concentration of 500 µM, $CuCO_3$ showed viability of 104.66±1.48% in L929 cells, while it showed viability rates of 110.46±6.15% and 31.34±4.93% in DLD-1 and Capan-1 cancer cells, respectively. All concentrations of the prepared material did not show a toxic effect on the healthy cell group, L929 fibroblast cells, and were at almost the same level as the control group cells. Although a decrease in cell viability was detected in DLD-1 colorectal cancer cells as the concentration decreased, it did not reach a level that could be considered toxic. However, it was determined that the concentrations applied at 250 µM and 125 µM showed a toxic effect in Capan-1 cells, with viability rates of 53.50±4.11% and 55.88±1.51%, respectively. The toxic effect decreased and

viability increased in Capan-1 cells as the concentration decreased.

In addition to all these results, during microscopic imaging, $CuCO_3$, $CuCl_2$, and $CuSO_4$ induced morphological changes such as cell shrinkage and membrane bulging, especially in the Capan-1 cell line. To determine the relationship between this observed phenomenon and apoptosis, a double staining (apoptosis/necrosis) study was conducted.

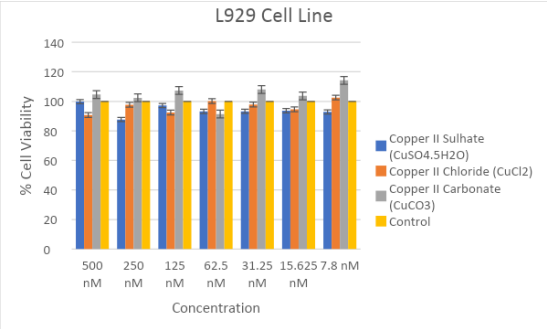


Figure 1. L929 cell line MTT test results.

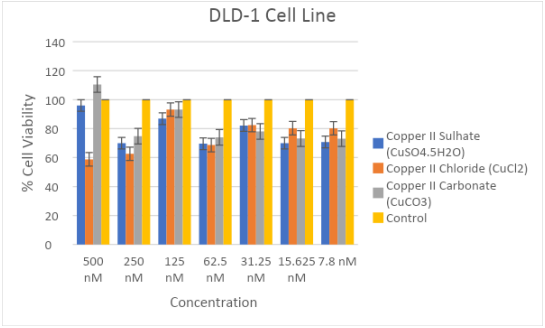


Figure 2. DLD-1 cell line MTT test results.

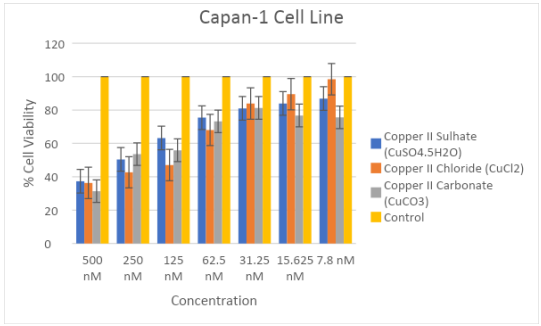


Figure 3. Capan-1 cell line MTT test results.

Double Staining (apoptosis/necrosis) Results

The percentages of apoptosis and necrosis were determined by counting the stained cells using DAPI and FITC filters. The results show that the apoptotic index of $CuCO_3$, $CuCl_2$, and $CuSO_4$ applied to DLD-1 and Capan-1 cells was higher than the necrotic index, indicating that the cells predominantly died by apoptosis. Furthermore, in the Capan-1 cell line, the apoptotic index of $CuCl_2$ increased at concentrations of 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.625 µM, and 7.8 µM, respectively, with percentages of 74.2%, 67.5%, 65.3%, 35.5%, 24.6%, 22.5%, 3.2%, and 0.5%, respectively, while the apoptotic index of $CuSO_4$ at the same concentrations was higher. The apoptosis rate was determined to increase with increasing concentrations of $CuCO_3$, $CuCl_2$, and $CuSO_4$, respectively, at the same concentrations of $CuCO_3$, $CuCl_2$, and $CuSO_4$.

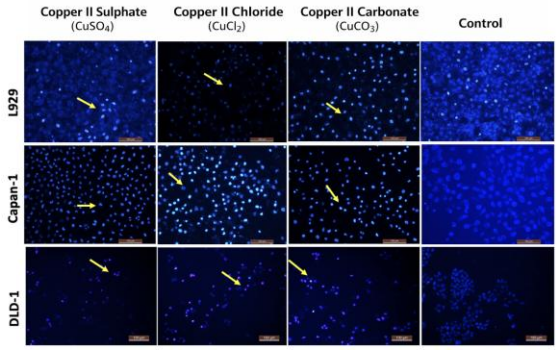


Figure 4. Images of L929, Capan-1, and DLD-1 cells under a DAPI filter after double staining. The material concentration applied in the photographs is 250 µM. Arrows indicate apoptotic cells.

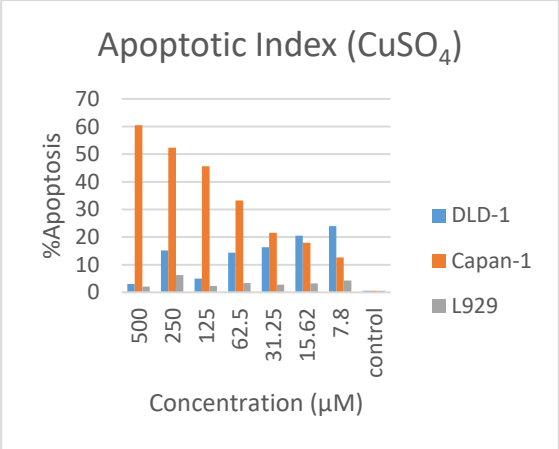


Figure 5. Change in apoptosis rate in DLD-1, Capan-1, and L929 cells depending on $CuSO_4$ concentration.

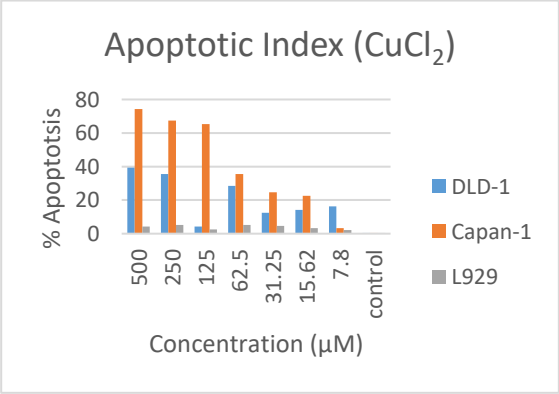


Figure 6. Variation of apoptosis rate in DLD-1, Capan-1, and L929 cells with $CuCl_2$ concentration.

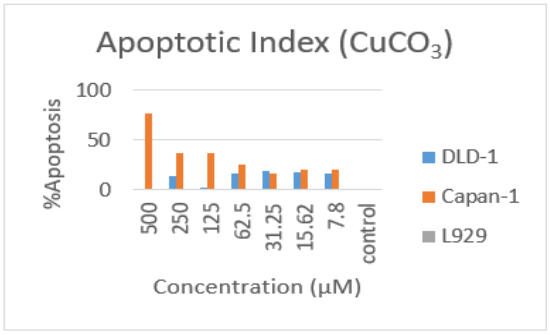


Figure 7. Variation of apoptosis rate in DLD-1, Capan-1, and L929 cells depending on $CuCO_3$ concentration.

Quantitative Real-Time PCR (qPCR) Analysis Results

When cytotoxicity and apoptosis necrosis findings obtained from different concentrations of $CuSO_4$, $CuCl_2$, and $CuCO_3$ compounds on DLD-1 and Capan-1 cell lines were evaluated, it was decided to use this cell line in the qPCR phase studies due to the clear effect on cell viability in Capan-1 cell lines and the high apoptotic index in Capan-1 cell lines.

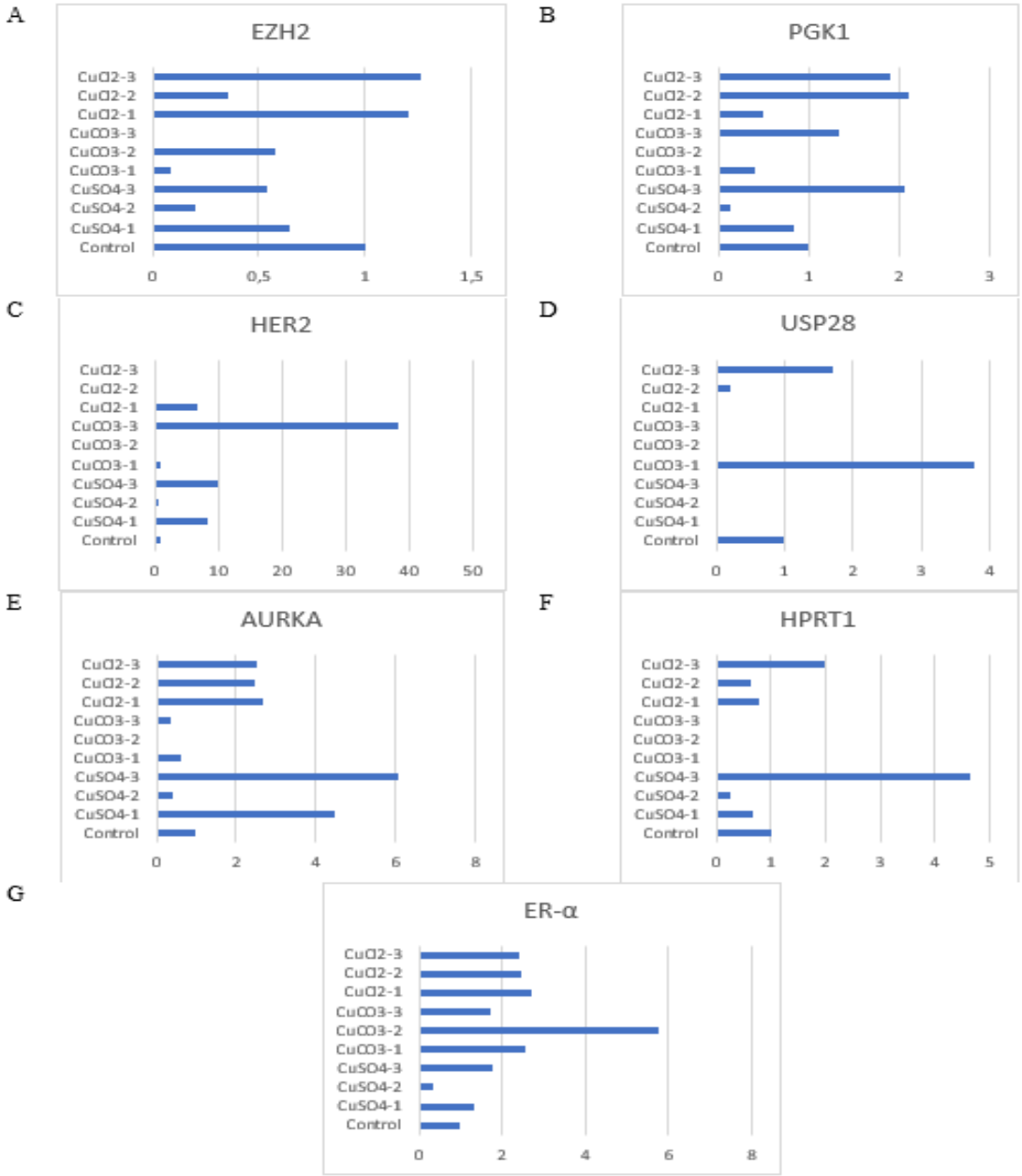


Figure 8. Quantitative real-time PCR (qRT-PCR) analyses of EZH2, PGK1, HER2, USP28, AURKA, HPRT1 and ER-α expression in Capan-1 pancreatic cancer cells treated with $_{Cu}SO_4$, $CuCl_2$ and $CuCO_3$. A: EZH2, B:PGK1, C: HER2, D: USP28, E: AURKA, F: HPRT-1, G: ER-α. $CuSO_4$-1: 500 µM, $CuSO_4$-2: 250 µM, $CuSO_4$-3: 125 µM, $CuCO_3$-1: 500 µM, $CuCO_3$-2: 250 µM, $CuCO_3$-3: 125 µM, $CuCl_2$-1:500 µM, $CuCl_2$-2: 250 µM, $CuCl_2$-3: 125 µM. Data are presented as the fold changes.

Compared to the control group, EZH2 gene expression was generally reduced in the groups treated with copper compounds. This reduction was more pronounced with $CuSO_4$ and $CuCO_3$, while suppression was more limited in the $CuCl_2$ group.

When examining the data for the PGK1 gene, the groups treated with copper compounds showed changes in expression levels compared to the control group. Increases in PGK1 gene expression were detected in all three copper compounds, particularly at a concentration of 125 µM. On the other hand, at a concentration of 500 µM, decreases were detected in all three compounds compared to the control. The highest PGK1 RNA expression level occurred at a concentration of 250 µM $CuCl_2$.

When evaluating HER2 gene RNA expression, it was noted that expression increased significantly in the groups treated with copper compounds at 125 and 500 µM concentrations with $CuSO_4$. The greatest increase was observed with 125 µM $CuCl_2$.

The results for the USP28 gene showed a general decrease in expression levels in the groups treated with copper compounds, with this decrease being particularly pronounced in the $CuSO_4$ group. Increases were observed with 500 µM $CuCO_3$ and 125 µM $CuCl_2$ applications.

When evaluating AURKA gene expression, a general upward trend was evident in the groups treated with copper compounds. Although limited decreases were seen in some groups, the dominant response was an increase in expression compared to the control group. This suggests that copper compounds may enhance cell cycle progression and mitotic activity. On the other hand, a gene silencing effect was detected in the AURKA gene at a concentration of 250 µM $CuCO_3$.

When examining the expression levels of the HPRT1 gene, a general decrease in HPRT1 was detected in the groups treated with copper compounds compared to the control group. Furthermore, it was noted that HPRT1 expression ceased at all three concentrations of copper compounds treated with $CuCO_3$. In the other two genes, the changes detected at a concentration of 125 µM were noteworthy biological responses.

When RNA expression related to the ER-α gene was evaluated compared to the control group, a consistent increase was observed in the groups treated with copper compounds. This increase was particularly pronounced in the 250 µM $CuCO_3$ group, while only the $CuSO_4$ group at the same concentration showed a decrease.

DISCUSSION AND CONCLUSION

Cancer is a complex disease closely related to the mechanism of programmed cell death caused by various factors. Apoptosis is one of the main mechanisms of programmed cell death (Mishra et al., 2018). Copper and copper-based biomaterials currently have various applications in cancer imaging and cancer treatment (Zhong et al., 2022). Because copper activates apoptosis, it can be used in the elimination of cancer cells (da Silva et al., 2022).

$CuCl_2$ and $CuSO_4$ are novel compounds exhibiting interesting cytotoxic activity against cancer cell lines and are considered to have potential to be selective for cancer cells compared to non-cancerous cells. $CuCO_3$, $CuCl_2$, and $CuSO_4$ have yielded remarkable results in terms of selectivity against metastatic Capan-1 pancreatic cancer cells. Characterizing the antitumor activity against this cancer type and determining the cellular pathways is considered important for the effective use of the drug.

When we examined the effects of $CuSO_4$, $CuCl_2$, and $CuCO_3$ compounds at various concentrations, we found that they did not show a significant toxic effect in the healthy cell line L929 and the colorectal cancer cell line DLD-1. However, when we examined the effects of these compounds in the pancreatic cancer cell line Capan-1, a toxic effect was found. Subsequently, double staining was performed to determine whether the cell death pathway was apoptosis or necrosis. When the cells were examined under a fluorescence microscope after double staining, it was found that their apoptotic index was higher than their necrotic index.

In this study, the molecular effects of $CuSO_4$, $CuCl_2$, and $CuCO_3$ compounds on the pancreatic cancer cell line Capan-1 were evaluated through the expression levels of genes associated with proliferation, metabolism, and tumor progression. qPCR findings show that copper compounds produce selective and multidimensional gene expression modulation in cancer cells compared to the control group. Since previous studies have reported that EZH2 is overexpressed in pancreatic cancer cells in association with proliferation, tumor progression, and treatment resistance, and that suppression of EZH2 can limit tumor growth, it is thought that the decrease in EZH2 gene expression observed in Capan-1 cells treated with copper compounds in this study may indicate a potential antitumor effect through epigenetic mechanisms (Ougolkov et al., 2008).

Liang et al. (2020) reported that PGK1 can modulate the balance between proliferation and metastasis in pancreatic cancer depending on its cellular localization and metabolic phenotype. In our study, copper compounds were observed to modulate PGK1 expression in cancer cells; this is likely related to cellular energy balance and stress response mechanisms. However, the increases or decreases observed at different concentrations and in different cell lines suggest that PGK1 regulation varies depending on cellular binding and compound properties. These findings indicate that the functional effects of changes in PGK1 expression, such as glycolysis rate, ATP production, and cell proliferation, should be investigated in more detail in future studies.

The literature reports that transition metals, particularly copper, can modulate cell proliferation and growth responses by influencing intracellular signaling pathways (Turski and Thiele, 2009). In our study, copper compounds were observed to increase HER2 expression. This finding suggests that copper may influence oncogenic gene expression by modulating intracellular signaling pathways. This may provide preliminary insights into the potential pro-tumor effects of copper in cancer cells.

The role of the USP28 gene in cancers has been reported in the literature as both tumor suppressor and oncogenic (Prieto-Garcia et al., 2021). In our study, a general decrease in USP28 expression was observed with the application of copper compounds, but increases were also detected in some groups. These results suggest that the role of USP28 may vary depending on the cell type and the dose of the applied compounds.

When AURKA gene expression was evaluated, a general upward trend was observed in the groups treated with copper compounds. Although limited decreases were seen in some groups, the dominant response was an increase in expression compared to the control group (Barr ve Gergely, 2007). This may indicate a potential association with enhanced cell cycle progression and mitotic activity, considering that Aurora kinase A is a key

regulator in the cell cycle, particularly in the G2/M transition, centrosome maturation, and mitotic spindle formation (Barr and Gergely, 2007).

Although HPRT1 is frequently used as a reference "housekeeping gene," it has been previously reported that its expression can vary under different cellular stress conditions and experimental applications (Suzuki et al., 2000; Vandesompele et al., 2002). This suggests that HPRT1 may not always be a stable reference gene under experimental conditions and should be carefully evaluated, especially in studies involving metal ions or metabolic stress-inducing agents. In this study, it is unclear whether the decrease in HPRT1 expression is transcriptional or a secondary effect to decreased cell viability. Future studies examining cell proliferation, apoptosis markers, and other purine metabolism genes (e.g., IMPDH, GMPS) together will provide a clearer understanding of the mechanism of copper compounds.

It is well-established in the literature that increased ER-α expression can support hormone-dependent tumor growth by activating estrogen-mediated proliferative and anti-apoptotic signaling pathways (Ali and Coombes, 2002; Nilsson and Gustafsson, 2011). Increased ER-α expression in the study groups suggests that hormone-dependent growth may be supported in cancerous cells. This raises the possibility that copper compounds may have the potential to increase proliferation in hormone-sensitive tumors and influence treatment resistance or tumor progression via ER-α. Therefore, the use of copper-based compounds as anticancer agents should be carefully evaluated in terms of their effects on hormone receptor expression.

In conclusion, copper-based compounds can modulate molecular pathways associated with proliferation, metabolism, and tumor progression in pancreatic cancer cells. The more pronounced antitumor potential of $CuSO_4$ and $CuCl_2$, in particular, necessitates detailed investigation of their mechanisms of action through further molecular and functional studies. On the other hand, while this study evaluated RNA expression of genes, verification of protein levels, investigation of functional activity, and examination of different dose-time effects are important for future research. These findings are limited to in vitro conditions and require further validation.

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The authors declare that there are no acknowledgements.

Ethical Declaration
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Conflict of Interest
The authors declare that there is no conflict of interest regarding the publication of this article.

Authorship Contributions
Concept: S.E., A.K.D., Design: S.E., A.K.D., E.A.A., Data Collection or Processing: A.Ş., B.M., C.Ç.Ç., Analysis or Interpretation: A.K.D., E.A.A., B.M., C.Ç.Ç., Literature Search: S.E., A.K.D., B.M., Writing: S.E.

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REFERENCES
Ali S, Coombes RC. 2002. Endocrine-responsive breast cancer and strategies for combating resistance. Nature Reviews Cancer, 2(2): 101–112.

Barr AR, Gergely F. 2007. Aurora-A: the maker and breaker of spindle poles. Journal of Cell Science, 120(17): 2987–2996.

Cui J, Chai S, Liu R, Shen G. 2024. Targeting PGK1: A new frontier in breast cancer therapy under hypoxic conditions. Current Issues in Molecular Biology, 46(11): 12214–12229.

da Silva DA, De Luca A, Squitti R, Rongioletti M, Rossi L, Machado CM, Cerchiaro G. 2022. Copper in tumors and the use of copper-based compounds in cancer treatment. Journal of Inorganic Biochemistry, 226: 111634.

Devrim AK, Devrim T, Ekici H, Sözmen M, Şenol A, Kuruca N, Sudağıdan M. 2025. Impact of melamine exposure on apoptotic proteins and oxidative stress markers in mouse hepatic tissue. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 72(2): 211–217.

Du R, Huang C, Liu K, Li X, Dong Z. 2021. Targeting AURKA in cancer: molecular mechanisms and opportunities for cancer therapy. Molecular Cancer, 20(1): 15.

Gao Y, Yu Y, Zhang M, Yu W, Kang L. 2024. Mechanisms of endocrine resistance in hormone receptor-positive breast cancer. Frontiers in Oncology, 14: 1448687.

Granados-Romero JJ, Valderrama-Treviño AI, Contreras-Flores EH, Barrera-Mera B, Herrera Enríquez M, Uriarte-Ruíz K, Ceballos-Villalba JC, Estrada-Mata AG, Alvarado Rodríguez C, Arauz-Peña G. 2017. Colorectal cancer: a review. International Journal of Research in Medical Sciences, 5(11): 4667–4676.

International Organization for Standardization (ISO). 1992. ISO 10993-5: Biological evaluation of medical devices—Part 5: Tests for cytotoxicity: in vitro methods. ISO, Geneva.

Jaiswal SK, Fedkenheuer K, Khamar R, Tan H, Gotea V, Raj S, Fedkenheuer M, Elkahloun A, Zhao M, Jenkins LM, Annunziata CM, Elnitski L. 2025. The megacomplex protects ER-alpha from degradation by fulvestrant in epithelial ovarian cancer. Cancer Letters, 608: 217129.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. CA: A Cancer Journal for Clinicians, 61: 69–90.

Kim KH, Roberts CW. 2016. Targeting EZH2 in cancer. Nature Medicine, 22(2): 128–134.

Krajčiová D, Melník M, Havránek E, Forgácsová A, Mikuš P. 2014. Copper compounds in nuclear medicine and oncology. Journal of Coordination Chemistry, 67(9): 1493–1519.

Liang C, Shi S, Qin Y, Meng Q, Hua J, Hu Q, Ji S, Zhang B, Xu J, Yu XJ. 2020. Localisation of PGK1 determines

metabolic phenotype to balance metastasis and proliferation in patients with SMAD4-negative pancreatic cancer. Gut, 69(5): 888–900.

Lu Y, Chen R, Zhang H, Sun X, Li X, Yang M, Zhang X. 2024. Prognostic significance and immunological role of HPRT1 in human cancers. Biomolecules and Biomedicine, 24(2): 262.

Mishra AP, Salehi B, Sharifi-Rad M, Pezzani R, Kobarfard F, Sharifi-Rad J, Nigam M. 2018. Programmed cell death, from a cancer perspective: an overview. Molecular Diagnosis & Therapy, 22: 281–295.

Neoptolemos JP, Kleeff J, Michl P, Costello E, Greenhalf W, Palmer DH. 2018. Therapeutic developments in pancreatic cancer: current and future perspectives. Nature Reviews Gastroenterology & Hepatology, 15(6): 333–348.

Nilsson S, Gustafsson JA. 2011. Estrogen receptors: therapies targeted to receptor subtypes. Clinical Pharmacology & Therapeutics, 89(1): 44–55.

Ougolkov AV, Bilim VN, Billadeau DD. 2008. Regulation of pancreatic tumor cell proliferation and chemoresistance by the histone methyltransferase enhancer of zeste homologue 2. Clinical Cancer Research, 14(21): 6790–6796.

Prieto-Garcia C, Tomašković I, Shah VJ, Dikic I, Diefenbacher ME. 2021. USP28: oncogene or tumor suppressor? A unifying paradigm for squamous cell carcinoma. Cells, 10(10): 2652.

Raggi D, Crupi E, Pederzoli F, Martini A, Briganti A, Alhalabi O, O'Donnell PH, Ross J, Gupta S, Kamat AM, Faltas BM, Black PC, Spiess PE, Grivas P, Gao J, Apolo AB, Huddart RA, Necchi A, Galsky MD. 2025. HER2 and urothelial carcinoma: current understanding and future directions. Nature Reviews Urology, 22: 1–23.

Robinson NJ, Winge DR. 2010. Copper metallochaperones. Annual Review of Biochemistry, 79: 537–562.

Siegel RL, Miller KD, Wagle NS, Jemal A. 2023. Cancer statistics, 2023. CA: A Cancer Journal for Clinicians, 73(1): 17–48.

Suzuki T, Higgins PJ, Crawford DR. 2000. Control selection for RNA quantitation. BioTechniques, 29(2): 332–337.

Tempero MA, Berlin J, Ducreux M, Haller D, Harper P, Khayat D, Van Cutsem E. 2011. Pancreatic cancer treatment and research: an international expert panel discussion. Annals of Oncology, 22(7): 1500–1506.

Turski ML, Thiele DJ. 2009. New roles for copper metabolism in cell proliferation, signaling, and disease. Journal of Biological Chemistry, 284(2): 717–721.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 3(7): research0034-1.

Varshney N, Pandey RK, Mishra A, Kumar S, Jha HC. 2024. Aurora kinase A: integrating insights into cancer, inflammation, and infectious diseases. Gut Microbes Reports, 1(1): 2419069.

Wang X, Liu Z, Zhang L, Yang Z, Chen X, Luo J, Zhou Z, Mei X, Yu X, Shao Z, Feng Y, Fu S, Zhang Z, Wei D, Jia L, Ma J, Guo X. 2018. Targeting deubiquitinase USP28 for cancer therapy. Cell Death & Disease, 9(2): 186.

Watanapa P, Williamson RC. 1992. Surgical palliation for pancreatic cancer: developments during the past two decades. British Journal of Surgery, 79(1): 8–20.

Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, Bilous M, Ellis IO, Fitzgibbons P, Hanna W, Jenkins RB, Press MF, Spears PA, Vance GH, Viale G, McShane LM, Dowsett M. 2018. Human epidermal growth factor receptor 2 testing in breast cancer: ASCO/CAP clinical practice guideline focused update. Archives of Pathology & Laboratory Medicine, 142(11): 1364–1382.

World Health Organization. 2008. World health statistics 2008. World Health Organization, Geneva.

Xu M, Xu C, Wang R, Tang Q, Zhou Q, Wu W, Wan X, Mo H, Pan J, Wang S. 2025. Treating human cancer by targeting EZH2. Genes & Diseases, 12(3): 101313.

Yang Z, Han Y. 2025. PGK1 is a prognostic molecule and silencing PGK1 inhibits growth in breast cancer cells. Gene Reports, 102396.

Zhong X, Dai X, Wang Y, Wang H, Qian H, Wang X. 2022. Copper-based nanomaterials for cancer theranostics. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 14(4): e1797.

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RESEARCH ARTICLE

Evaluation of Transcutaneous Electrical Stimulation (TENS), Therapeutic Ultrasound (TU), and Low-Level Laser Therapy (LLLT) Applications for Physical Therapy in Neurological and Orthopedic Disorders in Cats and Dogs; 20 Cases

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ABSTRACT

This study aimed to evaluate the applications of transcutaneous electrical stimulation (TENS), therapeutic ultrasound (TU), and low-level laser therapy (LLLT) for physical therapy and rehabilitation (PTR) in cats and dogs with neurological and orthopedic disorders, and the results obtained from these applications. The study material consisted of 11 cats and 9 dogs (20 cases). Transcutaneous electrical stimulation (TENS), therapeutic ultrasound (TU), and low-level laser therapy (LLLT) were used for PTR in these cases. In the study, 4 (20%) cases had muscular dystrophy, 9 (45%) cases had patellar dislocation or hip joint-related orthopedic problems (1 case of Leg Calve Perthes (5%), 8 cases of hip dislocation and patellar luxation (40%), while the remaining 7 cases (35%) were neurogenic in origin (6 spinal traumas (30%), 1 radial paralysis (5%). Only 6 (30%) of the cases included in the study underwent physical therapy alone without any surgical planning. In the other 14 cases (70%), physical therapy was applied after a surgical procedure. While physical therapy resulted in significant improvement in orthopedic problems, complete recovery was not achieved in some neurological cases. Based on the findings from this study regarding the dosages and durations used, the results of this combination are quite satisfactory. We recommend using physical therapy applications consisting of a combination of TENS, TU, and LLLT alone or as a complementary treatment for orthopedic and neurological disorders in cats and dogs.

INTRODUCTION

Physical therapy and rehabilitation (PTR) applications in animals are becoming more widespread every day (Mcgowan and Goff, 2016). Postoperative rehabilitation, which was previously overlooked, has recently started to receive more attention in the veterinary field. PTR applications are becoming even more valuable thanks to the increasing demands due to their ability to accelerate the healing process of patients, contribute to pain management and early mobilization (Marcellin-Little et al., 2015; Sarıerler and Ulutürk 2022).

Muscles, joints, tendons, ligaments, and bones that make up the musculoskeletal system are often treated with medical and operative treatments, as well as conservative supportive treatments, in both acute and chronic diseases. These may include heat, light, electricity, and exercise. Physical therapy is a treatment method used to treat diseases or pains that negatively affect the body's motor functions and to provide rehabilitation for patients (Chuet al., 2022; Ünsaldı, 2024).

Among the treatment methods applied for physical therapy, there are many different or combined applications that show different mechanisms of action and whose effects have also been defined, such as massage, therapeutic exercises, thermal applications, aquatherapy, transcutaneous electrical nerve stimulation (TENS), therapeutic ultrasound, extracorporeal shock wave therapy, low-level laser therapy (LLLT) (Özdinçerler et al., 2013; Vance et al., 2014; Johnsonet al., 2022; Sarıerler and Ulutürk, 2022).

TENS refers to the stimulation of nerves near the skin's surface with electrical signals (Marcellin-Little et al., 2015; Sarıerler and Ulutürk, 2022). These signals use small currents to activate sensory axons without affecting muscle contraction (Özdinçerler et al., 2013; Chuet al., 2022). TENS is an inexpensive and easy self-administered

technique (Vance et al., 2014). The commonly used frequency for TENS in clinical treatment is 1-150 Hz (Johnsonet al., 2022). These frequencies have high and low modes. Low-frequency modes provide pulsed currents of 10 Hz or less, while high-frequency TENS is used to describe frequencies greater than 10 Hz (up to 215 Hz at the maximum setting). TENS is generally used in the treatment of pain control, urinary incontinence, and muscle atrophy (Mirza et al., 2019; Johnsonet al., 2022).

The effects of therapeutic ultrasound are considered non-thermal and typically a combination of cavitation, acoustic flow, and micromassage. Ultrasound waves interacting with tissue cause a local increase in blood flow and a temporary increase in the elongation of collagenous structures such as tendons, ligaments, and joint capsules. It creates a mild inflammatory reaction, reducing pain and muscle spasm in joint contractions (Cotler et al., 2015; Mirza et al., 2019; Gölgeli and Yanmaz, 2023; Ünsaldı, 2024).

LLLT, commonly referred to as photobiology or biostimulation, is a non-invasive light source therapy that does not emit heat, sound, or vibration. It is believed to accelerate connective tissue repair by affecting the function of connective tissue cells (fibroblasts) and to act as an anti-inflammatory agent. Lasers with different wavelengths ranging from 632 to 904 nm are commonly used in the treatment of musculoskeletal disorders. Wavelengths between 660 nm and 905 nm have the ability to penetrate the skin and soft/hard tissues. This light has a good effect on pain, inflammation, and tissue repair. The effect of LLLT is photochemical, not thermal, and its positive effects on pain, inflammation, and tissue repair are known (Bockstahler, 2006; Anders et al., 2015; Yayla et al., 2026).

This study aimed to evaluate transcutaneous electrical stimulation (TENS), therapeutic ultrasound (TU), and low-level laser therapy (LLLT) applications for physical therapy in cats and dogs for neurological and orthopedic disorders.

MATERIALS AND METHODS

This study was conducted on 11 cats and 9 dogs (20 cases) with physiotherapy indications at the Surgical Clinics of the Animal Hospital, Faculty of Veterinary Medicine, Dicle University. The owners of the animals included in the study were informed about the study and their written consent was obtained through patient consent forms. Furthermore, ethical approval for the study was obtained from the Dicle University Local Ethics Committee for Animal Experiments, with protocol number 2024/05 dated 26/12/2024.

In this study, TENS, TU, and LLLT applications were performed using the “M1890 CHATTANOOGA Intelect VET Combo Physical Therapy Unit.” This unit contains TENS, TU, and LLLT modules and can be easily controlled from a single panel.

TENS application: All patients received TENS once daily for 1200 seconds for the first three days, using a premode waveform, continuous cycle, 80/150 Hz frequency, and 1.3 mA. On subsequent days, the treatment was continued at 1.5 mA and increased to 1.8 mA until the patient responded. Additionally, TENS electrodes were placed at the beginning and end points of the affected muscle. In patients with spinal cord trauma, they were placed on the right and left sides of the affected area.

TU therapy was administered once daily using the TU probe located on the device (Intelect Vet; 1 MHz, continuous mode, SATA intensity 1.5 W, exposure time: 900 seconds; Chattanooga Group Inc., Chattanooga, TN, USA). The intensity was increased to 1.8 W during the second week and then to 2.0 W for 3 or 4 weeks (in patients requiring continued treatment).

For LLLT applications, a GaAs (gallium arsenide) laser device was set to continuous mode with λ=905 µm, 10,000 Hz, 25 mW, and a peak power of 25 W. The monodiode laser probe (MLA1/25) was held perpendicular to the skin and applied at close range once daily. Laser treatment was calculated according to the formula (power/beam area) X time = J/cm² and applied transcutaneously for 180 seconds daily for 3 or 4 weeks with regional scanning movements at 9 J/cm². In addition, all patients were advised to perform passive exercises, massage, and hot hydrotherapy. In addition to physical therapy lasting 3 or 4 weeks, none of the patients in the study received additional supplements such as muscle relaxants or analgesics.

Generally, when working on a specific muscle group (such as cases 1, 2, and 5), TENS electrodes were placed at the origin and insertion points of the affected muscle groups, while the TU and LLLT probes were applied to the area with circular movements. In spinal diseases or after spinal surgery (cases 3, 4, 6, 8, 10, and 20), TENS electrodes were placed bi-directionally, while the TU and LLLT probes were held perpendicular to the skin and applied to the area with circular movements. If a metal implant was present, the application was performed starting more caudally, without applying to the implant itself. In cases involving the hip joint (cases 12, 15, 16, 17, 19) or cases with local inflammation (case 14), application was performed only to the affected area (Figure 1 and 2).

The study consisted of clinical cases, predominantly orthopedic and neurological. There was no control group. Within the scope of the study, criteria such as treatment duration, faster recovery, and a more comfortable postoperative period were interpreted solely based on observation and previous experiences. On the other hand, in conditions such as muscular dystrophy, recovery was assessed by observing the increase in muscle mass and the clinical use of the affected extremity. Similarly, a more comfortable postoperative period was interpreted based on the patient's general condition and indicators such as appetite, willingness to walk or cheerfulness, liveliness, response to environmental stimuli, and communication. All these observations were made by the same person (first author).

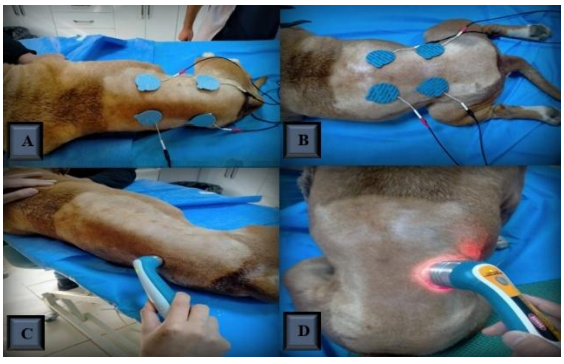


Figure 1. Case 4: Male dog with spondylitis (15-day-old case). *After determining the treatment protocol, A, B: bidirectional TENS (20 min 1.3 MA), C: TU (20 min 2 w/cm2), and D: LLLT (3 doses at 50 sec intervals) were applied.*

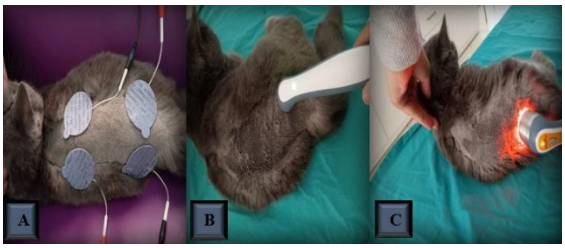


Figure 2. Case 6; improvement was observed in a cat with spinal trauma after 10 sessions of physical therapy protocol: TENS (A), TU (B), LLLT (C).

RESULTS

The distribution of cases evaluated in this study was 55% cats and 45% dogs. When the cases were evaluated among themselves, the average age of the cats was calculated as 18.90 months, and that of the dogs as 31.77 months. Six of the cats were male and five were female, and all of the dogs were male (Table 1). Of these cases, 4 cases (20%) had muscular dystrophy, 9 cases (45%) had patellar dislocation or hip joint-related orthopedic problems (1 case Leg Calve Perthes (5%), 8 cases hip dislocation (40%), while the remaining 7 cases (35%) were determined to be of neurogenic origin (6 spinal trauma (30%), 1 radial paralysis (5%).

It was determined that only 6 of the cases included in the study (30%) received physiotherapy alone without surgical planning. In the other 14 cases (70%), physiotherapy was applied after a surgical procedure for therapeutic purposes.

During the physical therapy applications, patients expressed their satisfaction, stating that they allowed the applications without resistance from the second session onwards, and that they were more comfortable at home with their owners. Other findings from the study are summarized in Table 2.

Table 1. Patient data including type, race, gender, age, weight

Case No	Type	Race	Gender	Age	Body weight (kg)
1	Cat	Tabby	Male	6 month	3
2	Dog	Anatolian shepherd	Male	7 month	35
3	Cat	Tabby	Female	1 year	4.5
4	Dog	Crossbreed	Male	1.5 year	26
5	Dog	Belgian Shepherd	Male	2 year	25
6	Cat	Scottish	Female	6 month	1.5
7	Dog	German Shepherd	Male	3 year	25
8	Cat	British Shorthair	Male	3 year	5
9	Dog	Japanese spits	Male	4 year	27
10	Cat	British Shorthair	Male	1 year	4
11	Dog	German Shepherd	Male	2 year	35
12	Dog	Poodle	Male	9 month	15
13	Cat	Tabby	Female	4 month	1.5
14	Cat	Tabby	Male	3 year	4.5
15	Cat	Tabby	Male	2 year	3.5
16	Cat	Turkish Van	Male	3 year	4
17	Cat	Tabby	Male	1 year	2
18	Dog	Malinois	Male	4 year	25
19	Dog	German Shepherd	Male	6 year	35
20	Cat	British Shorthair	Female	2 year	4

Table 2. Clinical status of the cases

Case No	Diagnosis	Clinical status	Developments or clinical findings related to the case
1	Muscular atrophy	Due to surgery on the femur, the cat was unable to use its leg, resulting in muscle atrophy and lameness. No surgery other than physical therapy was performed for treatment.	Regarding lameness, the leg was functionally usable. There was no lameness, but it could not be said that a complete recovery had occurred. This improvement was achieved after 3 weeks of TENS, TU, and LLLT treatment.
2	Delayed movement after femoral osteosynthesis	The dog was unable to use its leg due to a femur operation. No surgery other than physical therapy was performed for treatment.	The patient's treatment was not completed because they were not brought in regularly for TENS, TU, and LLLT treatments for 3 days.
3	Spinal trauma	Prior to physical therapy, dorsal laminectomy and vertebral stabilization were performed. Physical therapy applications were performed regularly starting 24 hours after the operation.	The patient started weight-bearing after 4 weeks of TENS, TU, and LLLT therapy.
4	Discospondylitis	No surgery other than physical therapy was performed for the treatment.	The patient, who was completely paraplegic on the first day of arrival at the clinic, started walking by the end of the second session. The treatment consisted of 2 weeks of TENS, TU and LLLT applications.

Table 2. (continued)

Case No	Diagnosis	Clinical status	Developments or clinical findings related to the case
5	Muscular atrophy	No surgery other than physical therapy was performed for the treatment.	Three weeks of TENS, TU, and LLLT therapy significantly prevent muscle atrophy, as the developmental gap between the two arms was observed to have decreased compared to baseline.
6	Spinal trauma	Dorsal laminectomy was performed prior to physical therapy. Physical therapy applications were performed regularly 24 hours after the operation.	Despite the challenges of home care following the application of TENS, TU, and LLLT treatments, the patient weight-bearing on the 45th day.
7	Patellar luxation	A combination of tibial tuberosity transposition, extracapsular stabilization, and trochlear sulcoplasty was performed, and physical therapy was applied in the postoperative period.	Within a short period of time, approximately one week, the patient functionally recovered as a result of TU and LLLT treatment, and no relapse was observed during the following 6 months.
8	Spinal trauma	Physical therapy was performed after hemilaminectomy.	The patient could not be contacted after 1 week of TENS, TU, and LLLT treatment.
9	Patellar luxation and subluxation of the coxofemoral joint	A combination of tibial tuberosity transposition, extracapsular stabilization, and trochlear sulcoplasty was performed, and physical therapy was applied in the postoperative period. Physical therapy was applied without any surgery on the hip joint.	Functional recovery occurred within a short time, approximately one week, following TU and LLLT application, and no recurrence was observed during the 6-month follow-up period. The hip joint became tighter, and the subluxation corrected.
10	Spinal trauma	Physical therapy was performed after laminectomy and vertebral stabilization.	After 3 weeks of TENS, TU, and LLLT treatment, the patient showed functional improvement, and no relapse was observed during the 6-month follow-up period.
11	Patellar luxation and aseptic arthritis	A combination of extracapsular stabilization and trochlear sulcoplastywas performed, and physical therapy was applied in the postoperative period.	Within a short period of time, approximately one week, the patient functionally recovered as a result of TU and LLLT treatment, and no relapse was observed during the following 6 months.
12	Legg calve perthes (LCP)	Physical therapy was applied after excision arthroplasty.	No complications were observed in the surgical area, and the patient was able to walk functionally from the third day onwards.
13	Radial paralysis	Physical therapy was planned without any surgical procedure.	Complete recovery was not achieved after 3 weeks of TU, LLLT treatment. Even though it started using its paw, it couldn't fully put weight on it.
14	Pain control after multiple fracture surgery	After stabilizing multiple fractures with intramedullary nailing and plate osteosynthesis, physical therapy was applied.	Three weeks of TU and LLLT treatment prevented the development of local edema, increased limb mobility, and contributed to both muscle growth and bone healing.
15	Hip dislocation	Physical therapy was applied after excision arthroplasty.	With TU and LLLT application, he walked functionally without limping on the 3rd postoperative day. Since the time interval between trauma and surgical intervention is not precisely known in these cases, rapid recovery may be attributed to the pain reduction achieved through surgery. However, in such situations requiring surgery, physical therapy can be considered as an additional supplementary support to the surgical plan.
16	Hip dislocation	Physical therapy was applied after excision arthroplasty.	With TU and LLLT application, he walked functionally without limping on the 3rd postoperative day. Since the time interval between trauma and surgical intervention is not precisely known in these cases, rapid recovery may be attributed to the pain reduction achieved through surgery. However, in such situations requiring surgery, physical therapy can be considered as an additional supplementary support to the surgical plan.

Table 2. (continued)

Case No	Diagnosis	Clinical status	Developments or clinical findings related to the case
17	Hip dislocation	Physical therapy was applied after excision arthroplasty.	With TU and LLLT application, he walked functionally without limping on the 3rd postoperative day. Since the time interval between trauma and surgical intervention is not precisely known in these cases, rapid recovery may be attributed to the pain reduction achieved through surgery. However, in such situations requiring surgery, physical therapy can be considered as an additional supplementary support to the surgical plan.
18	Regional atrophy due to lameness originating from the elbow.	Physical therapy was planned without any surgical procedure.	After three weeks of TENS, TU, and LLLT treatment, atrophy in the treated area stopped, and muscular development was achieved. Information was obtained indicating an increase in the dog's capacity during exercise and training.
19	Hip dislocation	Physical therapy was applied after excision arthroplasty.	With TU and LLLT application, he walked functionally without limping on the 3rd postoperative day.
20	Spinal trauma	Physical therapy was performed after laminectomy.	After 4 weeks of TENS, TU, and LLLT therapy, the child started walking on day 25.

DISCUSSION AND CONCLUSION

In veterinary practice, FTR applications have become quite popular recently and are sometimes used as a treatment alone, and sometimes as a secondary application supporting surgical treatment (Bockstahler, 2006). In our study, cats or dogs with indications for FTR applications were included in the study and the results were evaluated. It can be used in many different conditions, from acute and chronic injuries to various musculoskeletal or nervous system diseases (Martorell et al., 2006; Sarıerler and Ulutürk, 2022). In our study, the use of physical therapy for orthopedic and neurological disorders was discussed. Among these, muscle atrophy developing in animals that have undergone faulty orthopedic interventions or been kept immobile for a long time was noteworthy. In addition, patellar dislocation and hip-related orthopedic diseases also occupied a significant place. Almost all of our patients with neurological disorders had spinal trauma, and the main cause was trauma. Physical therapy applications can be used alone for therapeutic purposes or to support a treatment (Wiputhanuphongs et al., 2015; Henea et al., 2023). In our cases, physical therapy and rehabilitation (PTR) applications (TENS, TU, LLLT, respectively) were used alone for muscle atrophy, while in spinal trauma patients, PTR was applied following laminectomy and vertebral stabilization surgery. In cases of patellar dislocation and hip dislocation, PTR was continued to support treatment while orthopedic surgery for the dislocation was planned. This approach aims to shorten treatment time, accelerate recovery, make the postoperative period more comfortable, and contribute to pain control. All of these are expected effects of TENS, TU, and LLLT. Due to the limited number of cases for each disease or the lack of a control group in our study, it is extremely difficult to explain the specific mechanism of action of PTR for each disease; however, it is possible to interpret these effects based on the known effects of TENS, TU, and LLLT. Therefore, whether PTR is planned alone or in conjunction with surgery depends on many factors, primarily the disease, the patient's condition, and the physician's preference.

Physical rehabilitation should be started as soon as possible (Sarıerler and Ulutürk, 2022). In this study, if surgery was not planned when the cases were brought in, physical rehabilitation applications were started immediately. If surgery was planned, physical rehabilitation applications were started the day after the surgery to accelerate recovery. Physical rehabilitation applications should be considered important for early mobilization and muscle development, especially since mobilization is important for the health of regional muscles and tendons after many orthopedic surgeries and complete immobilization is not always a desirable situation.

Although physical therapy applications were not widely known in veterinary practice initially, they have found new application areas in veterinary medicine thanks to the successful results obtained in humans. Postoperative rehabilitation, in particular, has gained importance recently due to its contribution to accelerating the healing process, early mobilization, and pain management (Wright, 2021; Sarıerler and Ulutürk, 2022). In our study, the aim of postoperative rehabilitation was to accelerate the healing process in patients, enable early mobilization, and support pain management, and in this sense, most of the cases included in the study expressed great satisfaction by the owners by the end of the second day. The absence of a control group in this study and the unstandardized distribution of patients from different disease groups were limitations in providing a complete picture of the results. However, it can be said that the authors' experience was somewhat better than expected.

In recent years, many different physical therapy methods have become an integral part of postoperative rehabilitation, in addition to being conservative treatment for animals with orthopedic and neurological disorders. In spinal cord injuries and neurological injuries, physical therapy can be used to help improve and protect muscle elasticity, joint mobility, and the nervous system (Grecu et al., 2018; Hyytiäinen et al., 2022; Sarıerler and Ulutürk, 2022). Our cases consisted of animals with orthopedic and neurological disorders, and the application of physical

therapy, mostly in the form of conservative treatment, was among the important findings in postoperative rehabilitation. Furthermore, physical therapy and rehabilitation (FTR) can be beneficial in spinal cord injuries or neurological disorders, helping to improve muscle elasticity, joint mobility, and the nervous system.

In veterinary practice, electrotherapy methods generally use electrical current to affect nerves, muscles, bones, or other tissues. TENS is one type of electrotherapy and can be used as part of the rehabilitation process or as a standalone treatment (Okur and Okumuş, 2022). TENS can be applied to many different muscles and can be easily applied to the medial or lateral side of a joint or inflamed area. When it needs to be applied to the spine, electrodes can be placed to the right and left of the affected area, and also cranially and caudally to the affected area (Sarıerler and Ulutürk, 2022). In the cases included in our study, the placement of TENS electrodes was based on previously reported author recommendations. The effectiveness of this approach on treatment or its relationship to dose was not evaluated, but it is considered to offer an advantage based on the reference of previous studies.

One of the FTR applications is TU, which has two main features. The first is its anti-inflammatory property. This property consists of high-frequency vibrations that create cavitation, increase cell permeability, and thus support cell communication between joint membranes (Arioli et al., 2008; Cheng et al., 2014). The second important feature of TU is that it reduces pain by decreasing the nerve conduction velocity of C fibers through stable cavitation. In addition, when used in continuous mode, TU can increase intratissue temperature. Therefore, the important benefits of the thermal effects of ultrasound are the reduction of muscle fiber activity and consequently the reduction of muscle spasms and pain (Arioli et al., 2008; Yanmaz et al., 2021). We believe that TU is largely effective in cases, especially in terms of its effect on muscles and its ability to provide pain control. However, of course, the regional effects of TU could not be specifically determined in this study due to many limiting factors. Nevertheless, clinically, TU may be beneficial in situations where it is indicated due to the lack of defined effects and side effects.

LLLT has been used in many clinical applications since the 1980s. The main therapeutic advantages of LLLT are to enhance tissue repair, reduce inflammation, edema, and pain (Pallotta et al., 2012; Okur and Okumuş, 2022). It can be used to stimulate cell function. Laser photons are thought to be absorbed by chromophores in cells, such as cytochrome C oxidase located in mitochondria. Changes in cytochrome C oxidase activity lead to increased production of adenosine triphosphate, the main source of cellular energy, and consequently to normalization of metabolism (Aimbire et al., 2006; Pallotta et al., 2012; Alves et al., 2013; Hentschke et al., 2013; Cheng et al., 2014). In our study, LLLT was the final stage of the treatment plan. Energy metabolism is undoubtedly very important in a healthy muscle or joint structure or tissue. We also believe that if inflammation occurs in a muscle, this condition should quickly return to normal and be sustainable. Therefore, the application of LLLT aimed to alleviate local inflammation, edema, and pain, and to regulate energy metabolism in muscle and joint structures. We planned the LLLT application as a complement to TENS and TU applications.

The results obtained at the doses and durations used in this study were quite satisfactory, and it was observed that physical therapy applications consisting of a combination of TENS, TU, and LLLT can be used alone or as a complementary treatment for orthopedic and neurological disorders in cats and dogs. Furthermore, we believe that further studies using different doses and durations will lead to the widespread use of physical therapy in veterinary clinical practice.

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Ethical Declaration

The owners of the animals included in the study were informed about the study and their written consent was obtained through patient consent forms. Furthermore, ethical approval for the study was obtained from the Dicle University Local Ethics Committee for Animal Experiments, with protocol number 2024/05 dated 26/12/2024.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authorship Contributions

Concept: A.D., S.Y., Design: A.D., S.Y., Data Collection or Processing: A.D., S.Y., Analysis or Interpretation: A.D., S.Y., Literature Search: A.D., S.Y., Writing: SY.

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REFERENCES

Aimbire F, Albertini R, Pacheco MTT, Leonardo PSLM, Iversen VV, Bjordal JM. 2006. Low-level laser therapy induces dose-dependent reduction of TNF alpha levels in acute inflammation. Photomedlaser Surg, 24(1): 33-37.

Alves ACA, de Carvalho PT, Parente M, Xavier M, Frigo L, Ambire F, Junior ECPL, Albertini R. 2013. Low-level laser therapy in different stages of rheumatoid arthritis: a histological study. Lasersmed Sci, 8(2): 529-536.

Anders JJ, Lanzafame RJ, Arany PR. 2015. Low-level light/laser therapy versus photobiomodulation therapy. Photomedlaser Surg, 33(1): 183–184.

Arioli G, Maddali Bongi S, Pappone N. 2008. L'approccio riabilitativo nell'artrite reumatoide [The rehabilitative approach in rheumatoid arthritis]. Reumatismo, 60(4): 242-248.

Bockstahler B. 2006. The orthopedic patient: conservative treatment, physiotherapy and rehabilitation. In Iams clinical nutrition symposium. Vienna. Austria, p:25-30.

Cheng K, Xia P, Lin Q, Shen S, Gao M, Ren S, Li X. 2014. Effects of low-intensity pulsed ultrasound on integrin-FAK-PI3K/Akt mechanochemical transduction in rabbit osteoarthritis chondrocytes. Ultrasound Med Biol, 40 (7): 1609-1618.

Chu XL, Song XZ, Li Q, Li YR, He F, Gu XS, Ming D. 2022. Basic mechanisms of peripheral nerve injury and treatment via electrical stimulation. Neural Regen Res, 17(10): 2185-2193.

Cotler HB, Chow RT, Hamblin MR, Carroll J. 2015. The use of low level laser therapy (LLLT) for musculoskeletal pain. MOJ Orthoprheumatol, 2(5): 00068.

Gölgeli BA, Yanmaz LE. 2023. The effects of cream-based Triticum vulgare with and without therapeutic ultrasound on excisional wound healing in diabetic rats. Cutaneous and Ocular Toxicology, 42(2): 61-67. DOI: 10.1080/15569527.2023.2201833

Grecu M, Rîmbu CM, Mareș M, Năstasă V, Rusu OR. 2018. Alternative methods of therapy in articular disorders in dogs and cats. Series Vet Med, 66(4): 1-275.

Henea ME, Șindilar EV, Burtan LC, Mihai I, Grecu M, Anton A, Solcan G. 2023. Recovery of spinal walking in paraplegic dogs using physiotherapy and supportive devices to maintain the standing position. Animals (Basel), 13(8), 1398.

Hentschke VS, Jaenisch RB, Schmeing LA, Cavinato PR, Xavier LL, Dal Lago P. 2013. Low-level laser therapy improves the inflammatory profile of rats with heart failure. Lasersmed Sci, 28(3): 1007-1016.

Hyytiäinen HK, Boström A, Asplund K, Bergh A. 2022. A systematic review of complementary and alternative veterinary medicine in sport and companion animals: Electrotherapy. Animals (Basel), 13(1): 64.

Johnson MI, Paley CA, Jones G, Mulvey MR, Wittkopf PG. 2022. Efficacy and safety of transcutaneous electrical nerve stimulation (TENS) for acute and chronic pain in adults: a systematic review and meta-analysis of 381 studies (the meta-TENS study). BMJ Open, 12(2): 1-12.

Marcellin-Little DJ, Levine D, Millis DL. 2015. Veterinary rehabilitation and physical therapy. Preface. Vet Clin North Am Small Anim Pract, 45(1): 9-10.

Martorell J, Espada Y, Ramis A. 2006. Bilateral adrenalectomy in a ferret (Mustela putorius furo) with hyperadrenocorticism. Comp Anim Pract, 16(3): 191-194.

Mcgowan C, Goff L. 2016. Animal physiotherapy: assessment, treatment and rehabilitation of animals. İngiltere. p: 384-417.

Mirza U, Makhdoomi DM, Anjum S, Baba WN. 2019. Phonophoresis in animals-A review. Agricultural Reviews, 40(1): 37-40.

Okur S, Okumuş Z. 2022. Effects of low-level laser therapy and therapeutic ultrasound on Freund's complete adjuvant-induced knee arthritis model in rats. Arch Rheumatol, 38(1): 32-43.

Özdinçerler AR, Tarakçı E, Hüseyinsinoğlu BE, Alpözgen AZ, Baydoğan SN. 2013. Isı İle İlgili Genel Bilgiler, "Fiziksel Modaliteler ve Elektroterapi. İstanbul Tıp Kitap Evi. İstanbul. s: 65-70.

Pallotta RC, Bjordal JM, Frigo L, Leal JEC, Teixeira S, Marcos RL, Ramos L, Messias FM, Lopes-Martins RAB. 2012. Infrared (810-nm) low-level laser therapy on rat experimental knee inflammation. Lasersmed Sci, 27(1): 71-78.

Sarıerler M, Ulutürk SE. 2022. Fizik tedavi ve rehabilitasyon, Özaydın İ (Ed), Veteriner Genel Cerrahi, Nobel Tıp Kitabevleri. Ankara. s: 266-279.

Ünsaldı E. 2024. Kedi ve köpeklerde fizik tedavi ve rehabilitasyon, Nobel Tıp Kitabevleri. Ankara. s: 1-5.

Vance CG, Dailey DL, Rakel BA, Sluka KA. 2014. Using TENS for pain control: the state of the evidence. Pain Management, 4(3): 197-209.

Wiputhanuphongs A, Soontornvipart K, Janwantanakul P. 2015. Effect of physical therapy program after surgical correction of medial patellar luxation in small breed dogs. The Thai J. Vet. Med, 45(4): 573-580.

Wright BD. 2021. Acupuncture for the treatment of neuromuscular conditions in dogs and cats, with emphasis on intervertebral disc disease. J Am Holist Vet Med Assoc, 63(1): 23-33.

Yanmaz LE, Ersoz U, Okur S, Yildirim BA, Comakli S, Sağlam YS, Dogan E, Karatas O, Turgut F, Okumuş Z. 2021. The effect of low-level laser therapy on rat unicortical femoral defect. The Thai J of Vet Med, 51(2): 277-284.

Yayla S, Altan S, Çatalkaya E, Ersöz-Kanay B, Saylak N, Altan F, Sağsöz H, Topaloğlu U. 2026. Effectiveness of low-level laser therapy for treatment of cats with thoracic trauma. Pak Vet J, 46(3): 701-709. http://dx.doi.org/10.29261/pakvetj/2026.059

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RESEARCH ARTICLE

A New Surgical Approach in Bitches with Vaginal Fold Prolapse

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ABSTRACT

The aims of present study were to investigate the efficiency of new surgical approach by clinical (complete blood count, operation time) and to determine the response of oxidative stress, immun system and acute phase proteins with total antioxidant status, total oxidant status, oxidative stress index, nitric oxide, interleukin 1, 6, 8, 10, tumour necrosis factor alpha, haptoglobin, C-reactive protein and serum amyloid A in bitches with vaginal fold prolapse. The seven bitches with vaginal fold prolapse were conducted as a study group and operated vaginal amputation or submucosal resection with a new technique by Reverdin needle. The six bitches with vaginal fold prolapse were planned as a control group and treated by routine vaginal amputation or submucosal resection. The blood samples were collected to just before operation and after operation two hours, seven days, fourteen days in two groups. The mean weight of the excised prolapsed fold in the study group was 157.31 ± 44.46 g with an operative time of 39.28 ± 6.74 min. In comparison, the removed vaginal tissue in the control group weighed 165.17 ± 36.82 g and took 45.67 ± 7.87 min (P>0.05). In the groups, the above parameters were evaluated preoperatively and postoperatively (after 2 hours, 7 and 14 days) and were not find difference except for few blood findings in both groups and interleukin 1 in the control group. In the intergroup comparison, the study group showed statistically higher interleukin 1, 8, haptoglobin, and C-reactive protein levels on postoperative day 14 compared to the control group, while its total oxidant status value was found to be lower. The result of this study, the new technique used did not demonstrate a clear superiority over the routine operative procedure. However, it can be chosen to shorten the operation time and press of all tissue.

INTRODUCTION

In the bitches, masses protruding from the vulva may occur due to many reasons such as vaginal fold prolapse (VFP), true vaginal prolapse, vaginal neoplasia, clitoral enlargement or uterine prolapse (Sontas et al., 2010). VFP occurs often at the peak of estrogen concentration in intact female dogs which are usually young and during between first and third estrous cycle (Schutte, 1967; Post et al., 1991; Johnston et al., 2001; Nak and Kasikci, 2013; Singh et al., 2022; Antonov and Ivanova, 2023). VFP is a result of the vaginal mucosa overreacting to estrogens during prooestrus or early oestrus. On the other hand, true vaginal prolapse is the protrusion of the entire vaginal mucosa through the vulva. This rarely occurs after copulation, during pregnancy and before or after parturition due to increased oestrogen levels, or as a result of increased intra-abdominal pressure, abdominal trauma in a pregnant bitch, vaginal tumours (transmissible venereal tumour), or constipation. In the past, VFP is also named vaginal hyperplasia, vaginal prolapse, vaginal hypertrophy, estrual hypertrophy, vaginal eversion and vaginal protrusion. Today, the more common term VFP is used to describe this condition, which is different from other species (Sontas et al., 2010; Ozenc et al., 2016 and 2017).

There are three degrees of VFP in bitches. Slight to moderate eversion of vaginal floor with no observation this tissue through the vulvar lips is classified as Type I. In Type II; prolapse tissue of the cranial floor and lateral walls of the vagina is observed through the vulvar lips like a tongue or pear. Type III, the vaginal wall is completely prolapsed as a doughnut shaped (Johnston et al., 2001; Kurt et al., 2019; Anya et al., 2020; Bucci et al., 2022; Antonov et al., 2023; Methai et al., 2023).

Slight cases can be spontaneously resolved during diestrus stage without any treatment (Semacan, 2007; Singh et al., 2022; Antonov et al., 2023). However, in the majority of spontaneous regressing cases will be recurrence at the subsequent estrous cycle (66-100%) (Johnston et al., 2001; Antonov et al., 2023). Ovulation may be induced by GnRH or hCG in affected bitches that are not yet during preovulatory stage with serum progesterone level below 2 ng/ml (Johnston et al., 2001; Nak and Kasikci, 2013).

Hysteropexy can be tried, if the prolapsed vagina tissue is not returned or stabilized its normal position. Ovariohysterectomy can be applied in chronic cases (Semacan, 2007). If the prolapsed vagina tissue prevents urination, there is a possibility of infection or impair of vagina and uterine circulation, serious necrosis and it becomes persistent, it must be excision by operation (Nak and Kasikci, 2013; Bucci et al., 2022). Submucosal resection of vagina and vaginal amputation can be performed in Type II and III, respectively (Nak and Kasikci, 2013).

Wound healing processes consist of cellular and biochemical events that begin with trauma and result in new tissue formation. Many factors affect this recovery (Gonzalez et al., 2016). Antioxidants play a role in wound healing by removing reactive oxygen species that damage wounds due to their effects on cells and tissues (Kumar et al., 2007). Cellular immunity also plays an important role in the regulation of wound healing. Proinflammatory cytokines such as interleukin 1 (IL-1), IL-6 and tumour necrosis factor alpha (TNF-α) help protect against infections, prepare injured tissue for repair and increase the migration and activation phagocytes (Lowry, 1993). It had been reported that IL-8 modulates the functions of many inflammatory cells during wound healing (Konstantinova et al., 1996). Acute phase proteins are used to assess the immune system against infection, inflammation or trauma. These blood proteins are synthesized in the liver and their secretion is regulated by proinflammatory cytokines, especially IL-6 (Tiftik, 1996; Murata et al., 2004; Petersen et al., 2004). They play a role in limiting microbial growth and reorganizing hemostasis in animals exposed to infection, inflammation, surgical trauma or stress (Eckersall, 2004; Eckersall and Bell, 2010).

The aims of present study were to investigate the efficiency of new surgical approach by clinical (complete blood count, operation time) and to determine the response of oxidative stress, immune system and acute phase proteins with total antioxidant status (TAS), total oxidant status (TOS), nitric oxide (NO), oxidative stress index (OSI), IL-1, IL-6, IL-8, IL-10, TNF-α, haptoglobin (Hp), C-reactive protein (CRP) and serum amyloid A (SAA) in bitches with VFP.

MATERIALS AND METHODS

Groups

The thirteen female Turkish-Anatolian Shepherd Dogs with VFP, ranging in age from 8-36 months, were used in the present study. Seven of these animals were in the study group (applied new surgical technique) (Figure 1) and six bitches were in the control group (applied routine surgical technique). The bitches with VFP were taken groups sequentially. Operations of all animals were performed by the same operators.

Study design

Amoxicillin (Vilamoks-LA®) (15 mg/kg, SC) was administrated before xylazine-ketamine anesthesia. Anesthesia was induced with xylazine (Basilazin®) (2 mg/kg, IM), followed by ketamine (Ketasol®) (10 mg/kg, IM) ten minutes later. The bladder was catheterized following anesthesia. Operation time was recorded from the placement of surgical drape to end of the operation. In addition, the weight of the excised tissue was measured.

Operation in the study group (n=7). Figure 1 was showed operations of study group in Type III (n=5).

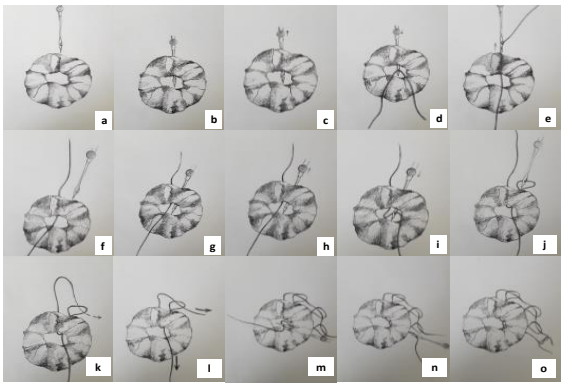


Figure 1. Operation technique applied to bitches with Type III vaginal fold prolapse in the study group (up to 4 o'clock).
a-b: Direction and application of Reverdin needle (without suture material). c: Opening the eye of Reverdin needle. d: Threading the suture material into the eye of Reverdin needle and closing the needle's eye. e: Pulling out the half of the suture material. f-g-h: Re-application of Reverdin needle at another angle. i: Threading the suture material (part of inside) into the eye of Reverdin needle and closing the needle's eye. j: Forming a loop by pulling out the Reverdin needle. k: Passing the suture material (part of outside) through this loop. l: Tightening the inner and outer ends of the suture. m: Repeating of this technique. n-o: Ligation of the suture.

All of prolapse tissue was surrounded using this suture technique (ligated at 4, 8 and 12 o'clock) in Type III.

This suture technique was also applied in Type II (n= 2) without interruption. The Reverdin needle was passed from right to left through the base of the prolapsed tissue without a suture material. The eye of the Reverdin needle was then opened, threaded with the suture material, and closed. Half of the suture material was pulled through from left to right. Next, the Reverdin needle was passed through the tissue again, directly underneath, without the suture material. The left part of the suture material was threaded into the needle's eye, and the eye was closed. A loop was formed by drawing the Reverdin needle back to the right, and the right end of the suture was passed through this loop. Both ends of the suture were then tightened. This technique was repeated along the entire length of the prolapsed tissue. Upon reaching the end of the tissue, the left end of the suture was pulled to the right using the Reverdin needle, and the suture was ligated.

In both types, the prolapsed tissue was removed just in front of the suture line.

Operation in the control group (n=6). Submucosal resection and vaginal amputation were applied to the bitches with Type II (n=1) and Type III (n=5) in the control group, respectively (Nak and Kasikci, 2013).

Sample collection and preparing for analysis

The blood samples were collected just before induction of anesthesia and 2 hours, 1 week and 2 weeks after operation in two groups. Blood samples were collected into serum, EDTA and heparinized test tubes by *V. cephalia antebrachium*. The sera and plasma samples were obtained in the blood sample collected into the sera (3000 rpm, 10 min) and heparinized tubes (3000 rpm, 10 min, at 4°C) by centrifugation (Hettich, Germany), respectively. The sera and plasma samples were stored at -80°C until biochemical analysis.

Hematological parameters

Into the EDTA tubes, blood samples were used to determine white blood cell (WBC), erythrocyte (RBC), lymphocyte (LYM), monocyte (MON), neutrophil (NEU),

eosinophil (EOS), basophil (BAS), hemoglobin (HGB), hematocrite (HCT) and platelet (PLT) count by using commercial test kits using blood counter (Abacus Junior Vet 5, Austria). All samples were evaluated on the same day.

Cytokines and acute phase proteins, nitric oxide levels

Blood samples were individually collected into serum test tubes for cytokines, acute phase proteins and NO. IL-1 (ECA0030), IL-6 (ECA0013), IL-8 (ECA0014), IL-10 (ECA0012), TNF-α (ECA0020), CRP (ECA0006), HP (ECA0008), SAA (ECA0029) and NO (EH4229) levels were determined by an ELISA reader (Biotek, USA) with Canine ELISA diagnostic kits (Fine Test, China).

Oxidative stress parameters

Blood samples were individually collected into heparinized test tubes for TOS and TAS were determined by an ELISA reader (SIRIO S, Indonesia) with test kits (Rel Assay Diagnostics, Türkiye). The ratio of TOS to TAS is defined as OSI. The units of TOS and TAS were equated to calculate the index. Results were expressed in Arbitrary Units (AU). OSI (arbitrary unit) = TOS (µmol H2O2 Eq/L)/TAS (µmol Trolox Eq/L)x100 (Kosecik et al., 2005).

Statistical analysis

Data processing was performed with the SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). The normality of all data was assessed by the Shapiro-Wilk test. In the blood samples taken before the operation, on the 2nd hour, 7th day, and 14th day after the operation, repeated measurements and comparisons between groups, variance analysis (General Linear Model) was used for parameters with normal distribution, and Friedman's test was used for non-normally distributed parameters. In the statistical analysis of whole blood findings, TAS, TOS, OSI, acute phase proteins, and cytokine levels of dogs in the study and control groups, one-way ANOVA test was performed in repeated measurements. In the test, $p<0.05$ was accepted as important. RBC, PLT (study group) and RBC, LYM, NEU (control group) counts, IL-1, IL-6, IL-10, TNF-α, HP, SAA, NO (study group) and IL-6, SAA (control group) levels were tested using the Friedman test and $p<0.0083$ in the Wilcoxon test with a Bonferroni adjustment to determine which of the 4 groups differed from the others. When comparing the control and study groups; Normality assessments for intergroup comparisons (Shapiro-Wilk test) revealed that IL-6 and SAA levels (preoperative, postoperative hour 2, days 7 and 14), TNF-α levels (preoperative and postoperative days 7 and 14), HP levels (preoperative and postoperative hour 2), and NO levels (postoperative days 7 and 14) exhibited a non-parametric distribution. Accordingly, the Mann-Whitney U test was used to compare the data at these specific time points, and statistical significance was evaluated at a Bonferroni-adjusted $p<0.025$. Independent Samples T Test was performed on the other parameters. In the test, $p<0.05$ was accepted as important.

RESULTS

The bitches were 17±3.66 and 11.67±2.54 months old in the study and control groups, respectively. There was no statistically difference between groups ($p>0.05$) (Table 1). According to the owners, eight bitches (study group=3, control group=5) were at first estrous period, four bitches (study group=3, control group=1) were at second estrous period and one bitch (study group) was at third estrous period. However, it was learned that all of the bitches, which were at second and third estrous periods, were also observed at the previous estrous periods by anamnesis. The owners stated that vaginal fold prolapsed were lasting for 17.28±7.25 and 13.67±3.67 days in the study and control groups, respectively (Table 1). There was no statistically difference between groups ($p>0.05$).

Table 1. Age and lasting of vaginal fold prolapse of the bitches in the study and control groups.

Groups	Study (n=7)			Control (n=6)			
Parameters	**Mean±SE**	**Median**	**Mean Rank**	**Mean±SE**	**Median**	**Mean Rank**	**p**
Age (month)	17±3.66	12	8.79	11.67±2.54	9	4.92	NS
Lasting of vaginal fold prolapse (day)	17.28±7.25	10	7	13.67±3.67	12.50	7	NS

NS: not significant and n: number of animals.

The weight of the extracted vagina tissue from the bitches was 157.31±44.46 and 165.17±36.82 g in the study and control groups, respectively. There was no statistically difference between groups ($p>0.05$). The mean operation time of the bitches with VFP was 39.28±6.74 and 45.67±7.87 min in the study and control groups, respectively. There was also no statistically significant difference between the groups ($p>0.05$).

The results of complete blood count of preoperative and postoperative (hour 2, days 7 and 14) in the study group are shown in Table 2.

In the study group, the count of WBC ($p<0.01$), MON ($p<0.05$) and NEU ($p\leq0.001$) were significantly increased 2nd hour after the operation compared to the 14th day after the operation. Other hematological parameters did not differ statistically among the preoperative and postoperative (hour 2, days 7 and 14) ($p>0.05$). The results of complete blood count of preoperative and postoperative (hour 2, days 7 and 14) in the control group are shown in Table 3.

In the control group, the count of WBC ($p<0.05$) and NEU ($p<0.01$) were significantly increased 2nd hour after the operation compared to the 14th day after the operation. Other hematological parameters did not differ statistically among the preoperative and postoperative (hour 2, days 7 and 14) ($p>0.05$).

No statistically significant difference was found when complete blood count findings were compared between the groups (Table 4).

The levels of IL-1, IL-6, IL-8, IL-10, TNF-α, Hp, CRP and SAA of preoperative and postoperative (hour 2, days 7 and 14) in the study group are shown in Table 5.

Table 2. Preoperative and postoperative (hour 2, days 7 and 14) hematological parameters in the study group (n=7).

Parameters	Preoperative	Postoperative (hour 2)	Postoperative (day 7)	Postoperative (day 14)	p
WBC (10^9/l)	17.59±2.79^{ab}	21.52±2.89^{a}	15.07±1.47^{ab}	10.57±1.49^{b}	<0.01
LYM (10^9/l)	1.03±0.19	0.92±0.23	1.80±0.35	1.80±0.34	NS
MON (10^9/l)	0.78±0.14^{ab}	0.94±0.08^{a}	0.57±0.09^{ab}	0.41±0.05^{b}	<0.05
NEU (10^9/l)	14.46±2.39^{ab}	18.68±2.65^{a}	11.86±1.37^{ab}	7.31±1.41^{b}	≤0.001
EOS (10^9/l)	1.10±0.51	0.78±0.36	0.59±0.08	1.00±0.18	NS
BAS (10^9/l)	0.22±0.05	0.20±0.06	0.21±0.02	0.15±0.02	NS
RBC (10^{12}/l)	7.13±0.44 6.83	6.06±0.40 5.87	5.91±0.60 6.41	6.18±0.48 6.13	NS
HGB (g/dl)	14.41±0.67	12.51±0.44	12.16±0.96	12.40±0.71	NS
HCT (%)	40.19±1.59	33.75±0.90	33.23±2.47	34.87±1.78	NS
PLT (10^9/l)	495.29±44.71 449	407.57±69.52 478	556.57±46.37 533	567.71±73.03 532	NS

Mean values within the same row with different superscripts letter a, b are significantly different (p<0.05; p<0.01; p≤0.001). Parametric data were expressed as mean ± SE. Nonparametric values were expressed as mean ± SE and median. NS: not significant and n: number of animals.

Table 3. Preoperative and postoperative (hour 2, days 7 and 14) hematological parameters in the control group (n=6).

Parameters	Preoperative	Postoperative (hour 2)	Postoperative (day 7)	Postoperative (day 14)	p
WBC (10^9/l)	22.70±2.82^{ab}	25.30±2.90^{a}	16.74±1.54^{ab}	13.73±1.05^{b}	<0.05
LYM (10^9/l)	1.69±0.31 1.7	1.43±0.39 1.23	2.39±0.74 2.00	2.63±1.03 1.88	NS
MON (10^9/l)	0.62±0.11	0.84±0.11	0.56±0.07	0.55±0.10	NS
NEU (10^9/l)	19.71±3.01 17.47^{ab}	22.50±3.20 20.19^{a}	12.66±1.75 11.07^{ab}	8.24±0.58 8.29^{b}	<0.01
EOS (10^9/l)	0.76±0.23	0.37±0.10	0.96±0.31	2.14±0.83	NS
BAS (10^9/l)	0.25±0.07	0.15±0.04	0.17±0.03	0.17±0.02	NS
RBC (10^{12}/l)	7.09±0.54 7.01	6.43±0.46 6.83	5.99±0.40 6.31	6.75±0.45 6.98	NS
HGB (g/dl)	14.70±1.36	13.58±1.31	12.60±1.13	13.75±1.00	NS
HCT (%)	41.87±4.11	38.18±4.06	35.75±3.83	39.67±3.65	NS
PLT (10^9/l)	504.50±79.62	508.50±58.32	531.33±92.29	438.67±66.52	NS

Mean values within the same row with different superscripts letter a, b are significantly different (p<0.05; p<0.01). Parametric data were expressed as mean ± SE. Nonparametric values were expressed as mean ± SE and median. NS: not significant and n: number of animals.

Table 4. Comparison of hematological parameters between the control (n=6) and study (n=7) groups at preoperative and postoperative (hour 2, days 7 and 14).

	Preoperative		Postoperative (hour 2)		Postoperative (day 7)		Postoperative (day 14)	
	control	study	control	study	control	study	control	study
WBC (10^9/l)	22.70±2.82	17.59±2.79	25.30±2.90	21.52±2.89	16.74±1.54	15.07±1.47	13.73±1.05	10.57±1.49
LYM (10^9/l)	1.69±0.31	1.03±0.19	1.43±0.39	0.92±0.23	2.39±0.74	1.80±0.35	2.63±1.03	1.80±0.34
MON (10^9/l)	0.62±0.11	0.78±0.14	0.84±0.11	0.94±0.08	0.56±0.07	0.57±0.09	0.55±0.10	0.41±0.05
NEU (10^9/l)	19.71±3.01	14.46±2.39	22.50±3.20	18.68±2.65	12.66±1.75	11.86±1.37	8.24±0.58	7.31±1.41
EOS (10^9/l)	0.76±0.23	1.10±0.51	0.37±0.10	0.78±0.36	0.96±0.31	0.59±0.08	2.14±0.83	1.00±0.18
BAS (10^9/l)	0.25±0.07	0.22±0.05	0.15±0.04	0.20±0.06	0.17±0.03	0.21±0.02	0.17±0.02	0.15±0.02
RBC (10^{12}/l)	7.09±0.54	7.13±0.44	6.43±0.46	6.06±0.40	5.99±0.40	5.91±0.60	6.75±0.45	6.18±0.48
HGB (g/dl)	14.70±1.36	14.41±0.67	13.58±1.31	12.51±0.44	12.60±1.13	12.16±0.96	13.75±1.00	12.40±0.71
HCT (%)	41.87±4.11	40.19±1.59	38.18±4.06	33.75±0.90	35.75±3.83	33.23±2.47	39.67±3.65	34.87±1.78
PLT (10^9/l)	504.50±79.62	495.29±44.71	508.50±58.32	407.57±69.52	531.33±92.29	556.57±46.37	438.67±66.52	567.71±73.03

Parametric data were expressed as mean ± SE. n: number of animals.

Table 5. Preoperative and postoperative (hour 2, days 7 and 14) cytokine and acute-phase protein levels in the study group (n=7).

Parameters	Preoperative	Postoperative (hour 2)	Postoperative (day 7)	Postoperative (day 14)	p
IL-1 (pg/ml)	13.31±0.77 13.00	14.09±1.34 12.50	14.33±1.03 14.33	13.41±0.57 12.96	NS
IL-6 (pg/ml)	5123.72	5151.08	5238.20	5382.23	NS
IL-8 (pg/ml)	84.28±0.62	83.82±0.49	83.38±0.54	83.20±0.59	NS
IL-10 (pg/ml)	152.91±18.82 129.08	322.96±83.59 212.65	179.17±25.67 197.99	304.72±108.75 183.22	NS
TNF-α (pg/ml)	12.38±2.48 10.93	13.99±2.77 13.99	7.99±2.56 6.07	17.89±5.60 12.58	NS
Hp (ng/ml)	1.64±1.22 0.22	0.98±0.66 0.22	0.35±0.09 0.23	0.24±0.02 0.24	NS
CRP (ng/ml)	3.35±0.94	3.49±0.79	2.88±0.62	3.88±0.36	NS
SAA (ng/ml)	0.14	0.24	0.19	0.21	NS

Parametric data were expressed as mean ± SE. Nonparametric values were expressed as mean ± SE and median. NS: not significant and n: number of animals.

The levels of IL-1, IL-6, IL-8, IL-10, TNF-α, Hp, CRP and SAA did not differ statistically significant between the preoperative and postoperative (hour 2, days 7 and 14) in the study group (p> 0.05). The levels of IL-1, IL-6, IL-8, IL-10, TNF-α, Hp, CRP and SAA of preoperative and postoperative (hour 2, days 7 and 14) in the control group are shown in Table 6.

Table 6. Preoperative and postoperative (hour 2, days 7 and 14) cytokine and acute-phase protein levels in the control group (n=6).

Parameters	Preoperative	Postoperative (hour 2)	Postoperative (day 7)	Postoperative (day 14)	p
IL-1 (pg/ml)	11.93±0.15^{ab}	12.46±0.30^{a}	12.16±0.25^{ab}	11.62±0.10^{b}	<0.05
IL-6 (pg/ml)	5411.10	5385.09	5571.57	5319.39	NS
IL-8 (pg/ml)	82.78±0.62	82.17±0.55	82.28±0.32	81.57±0.19	NS
IL-10 (pg/ml)	158.43±22.59	166.52±28.48	175.05±22.63	114.62±24.93	NS
TNF-α (pg/ml)	23.42±1.52	13.36±1.00	18.32±2.52	24.97±2.01	NS
Hp (ng/ml)	0.16±0.00	0.16±0.00	0.16±0.00	0.17±0.00	NS
CRP (ng/ml)	1.99±0.62	2.18±0.63	2.44±0.45	2.61±0.39	NS
SAA (ng/ml)	0.14	0.14	0.14	0.14	NS

Mean values within the same row with different superscripts letter a, b are significantly different (p<0.05). Parametric data were expressed as mean ± SE. Nonparametric values were expressed as median. NS: not significant and n: number of animals.

The levels of IL-1, IL-6, IL-8, IL-10, TNF-α, Hp, CRP, and SAA were compared between the groups (Table 7). In the preoperative period, the control group exhibited significantly higher TNF-α levels than the study group (p<0.05). This statistically significant difference was observed on postoperative day 7. At postoperative hour 2 and day 14, IL-8 levels were significantly higher in the study group than in the control group (p<0.05). Furthermore, IL-1, Hp, and CRP levels were also found to be higher in the study group compared to the control group on postoperative day 14 (p<0.05).

In the control group, the levels of IL-1 were significantly increased 2nd hour after the operation compared to the 14th day after the operation (p<0.05). The levels of NO, TOS, TAS and OSI of preoperative and postoperative (hour 2, days 7 and 14) in the study group are presented in Table 8.

There were no statistically significant changes in terms of NO level, TOS, TAS and OSI between preoperative and postoperative 2nd hour and 7th and 14th days in the study group (p>0.05). The levels of NO, TOS, TAS and OSI of preoperative and postoperative (hour 2, days 7 and 14) in the control group were given in Table 9.

When comparing the oxidative stress parameters of the control and study groups, only the TOS value on postoperative day 14 in the study group was found to be statistically significantly lower than that of the control group (p<0.05). The comparison of oxidative stress parameters between the groups is summarized in Table 10.

Recurrence was not observed any bitches. Gestation information was obtained from five bitches in the study group and three bitches in the control group and all were found to have delivered.

Table 7. Comparison of preoperative and postoperative (hour 2, days 7 and 14) cytokine and acute-phase protein levels between the control (n=6) and study (n=7) groups.

	Preoperative		Postoperative (hour 2)		Postoperative (day 7)		Postoperative (day 14)	
	control	**study**	**control**	**study**	**control**	**study**	**control**	**study**
IL-1 (pg/ml)	11.93±0.15	13.31±0.77	12.46±0.30	14.09±1.34	12.16±0.25	14.33±1.03	11.62±0.10	13.41±0.57*
IL-6 (pg/ml)	5411.10	5123.72	5385.09	5151.08	5571.57	5238.20	5319.39	5382.23
IL-8 (pg/ml)	82.78±0.62	84.28±0.62	82.17±0.55	83.82±0.49*	82.28±0.32	83.38±0.54	81.57±0.19	83.20±0.59*
IL-10 (pg/ml)	158.43±22.59	129.08	166.52±28.48	212.65	175.05±22.63	197.99	114.62±24.93	183.22
TNF-α (pg/ml)	23.42±1.52* 24.48	12.38±2.48 10.93	13.36±1.00 12.51	13.99±2.77 13.99	18.32±2.52* 19.18	7.99±2.56 6.07	24.97±2.01 27.13	17.89±5.60 12.58
Hp (ng/ml)	0.16±0.00 0.16	1.64±1.12 0.22	0.16±0.00 0.16	0.98±0.66 0.22	0.16±0.00	0.35±0.09	0.17±0.00	0.24±0.02*
CRP (ng/ml)	1.99±0.62	3.35±0.94	2.18±0.63	3.49±0.79	2.44±0.45	2.88±0.62	2.61±0.39	3.88±0.36*
SAA (ng/ml)	0.14	0.19	0.14	0.24	0.14	0.19	0.14	0.21

Parametric data were expressed as mean ± SE. Nonparametric values were expressed as mean ± SE and median. n: number of animals. *p<0.05

Table 8. Preoperative and postoperative (hour 2, days 7 and 14) oxidative stress parameters in the study group (n=7).

Parameters	Preoperative	Postoperative (hour 2)	Postoperative (day 7)	Postoperative (day 14)	p
NO (ng/ml)	0.27±0.02 0.25	0.27±0.03 0.22	0.29±0.04 0.26	0.33±0.04 0.30	NS
TOS (μmolH_2O_2/l)	5.32±1.28	5.35±0.99	6.21±0.86	4.18±0.75	NS
TAS (mmol Trolox Equiv/l)	0.92±0.22	0.58±0.08	0.51±0.10	0.83±0.28	NS
OSI (arbitrary unit)	0.83±0.22	0.98±0.16	1.52±0.30	1.13±0.42	NS

Parametric data were expressed as mean ± SE. Nonparametric values were expressed as mean ± SE and median. NS: not significant and n: number of animals.

Table 9. Preoperative and postoperative (hour 2, days 7 and 14) oxidative stress parameters in the control group (n=6).

Parameters	Preoperative	Postoperative (hour 2)	Postoperative (day 7)	Postoperative (day 14)	P
NO (ng/ml)	0.26±0.04	0.25±0.03	0.28±0.02	0.30±0.02	NS
TOS (µmolH_2O_2/l)	7.12±0.53	4.72±1.31	6.56±0.84	6.93±0.65	NS
TAS (mmol Trolox Equiv/l)	0.45±0.13	0.41±0.13	0.26±0.07	0.49±0.13	NS
OSI (arbitrary unit)	2.26±0.55	1.92±0.79	3.21±0.76	1.91±0.43	NS

NS: not significant and n: number of animals.

Table 10. Comparison of preoperative and postoperative (hour 2, days 7 and 14) oxidative stress parameters between the control (n=6) and study (n=7) groups.

	Preoperative		Postoperative (hour 2)		Postoperative (day 7)		Postoperative (day 14)	
	control	study	control	study	control	study	control	study
NO (ng/ml)	0.26±0.04 0.23	0.27±0.02 0.25	0.25±0.03 0.24	0.27±0.03 0.22	0.28±0.02 0.27	0.29±0.04 0.26	0.30±0.02 0.30	0.33±0.04 0.30
TOS (µmolH_2O_2/l)	7.12±0.53	5.32±1.28	4.72±1.31	5.35±0.99	6.56±0.84	6.21±0.86	6.93±0.65	4.18±0.75*
TAS (mmol Trolox Equiv/l)	0.45±0.13	0.92±0.22	0.41±0.13	0.58±0.08	0.26±0.07	0.51±0.10	0.49±0.13	0.83±0.28
OSI (arbitrary unit)	2.26±0.55	0.83±0.22	1.92±0.79	0.98±0.16	3.21±0.76	1.52±0.30	1.91±0.43	1.13±0.42

Parametric data were expressed as mean ± SE. Nonparametric values were expressed as mean ± SE and median. n: number of animals. *p<0.05

DISCUSSION AND CONCLUSION

Vaginal fold prolapse is observed in young bitches which were not neutered and usually occurred during between first and third estrous cycle (Schutte, 1967; Post et al., 1991; Johnston et al., 2001). Schutte (1967) reported that 22 bitches with vaginal prolapse were between 9 and 40 months (approximately 22 months) and they were during between first and forth estrous cycle. Trafer (1970) found this average as 18 months (Post et al., 1991). In the present study, it was determined that the bitches were young and average age was 14.53 months (8-36 months). It was also determined that eight of these bitches were at the first estrus period, four bitches were at second estrous period and one bitch was at third estrous period. However, it was learned that VFP was observed at the previous estrous periods in five bitches, which were at second and third estrous periods. These findings were similar to the results of the researchers.

Mild VFP cases can be spontaneously resolved during diestrus stage without treatment (Semacan, 2007; Singh et al., 2022). GnRH or hCG may be used for hormonal treatment (Nak and Kasikci, 2013). Surgical treatment can be choose including purse-string sutures, circumferential excision of prolapsed vaginal tissue, hysteropexy (Johnston et al., 2001), cervicopexy (Ozturkler et al., 2001) and ovariohysterectomy (Alan et al., 2007). While submucosal resection of vagina and vaginal amputation, which are based on horizontal mattress suture, are used as the standard methods (Wykes and Olson, 2002; Kalkan and Alacam, 2007; Nak and Kasikci, 2013; Anya et al., 2020), modified new techniques have been tried (Post et al., 1991; Antonov et al., 2009; Bucci et al., 2022; Singh et al., 2022). Canooglu (2013) reported that a new technique, which was modified by Antonov et al. (2009) and based on passing through each other ligature, was started to be used by researchers. Bühner vulvar suture, which was modified for the bitch, has been performed by Bucci et al. (2022) and Singh et al. (2022). Anovulvar cleft and vaginal prolapse-hyperplasia in a bitch was treated with perineoplasty (Mostachio et al., 2007). A new technique was tried in the present study. This technique is original and it is not a modification of any suture or methods. It was examined for alternative of other methods. This technique is a continuous suture performed by Reverdin needle and it can pressure the whole prolapsed vaginal tissue.

In the present study, submucosal resection of vagina and vaginal amputation, which are standard methods and based on horizontal mattress suture (Wykes and Olson, 2002; Kalkan and Alacam, 2007; Nak and Kasikci, 2013), were compared the new technique. There was no statistically significant difference between the groups in parameters that could affect results such as age (study group: 17±3.66 months; control group: 11.67±2.54 months), prolapse duration (study group: 17.28±7.25 days; control group: 13.67±3.67 days) and weight of prolapsed tissue (study group: 157.31±44.46 g; control group: 165.17±36.82 g).

The complete blood count is used for determination of inflammatory, prognosis and recovery (Turgut, 2000). In this study WBC, monocyte and neutrophil counts were significantly higher in postoperative 2 hours of complete blood count as compared to postoperative day 14 of complete blood count in the study group ($p<0.05$). WBC and neutrophil counts were also significantly higher in postoperative 2 hours of complete blood count as compared to postoperative day 14 of complete blood count in the control group ($p<0.05$). The neutrophil count increases of postoperative hour 2 in both groups were might be due to induced endogenous corticosteroids by stress. The monocyte count increase of postoperative 2 hours was considered due to its role in tissue repair and regeneration (Turgut, 2000). The increase in WBC count was result of the increase in neutrophil and monocyte in the study group and the increase in neutrophil in the control group. Decreasing of these values on the postoperative day 14 was considered as treatment of infection. On the other hand, the lack of a statistically significant difference in the comparison between the groups suggests that the new technique does not offer an advantage over the standard method in terms of infection.

Trauma and wound healing cause pro and anti-inflammatory cytokines to rise significantly (Uluisik and Keskin, 2012; Hismiogulları et al., 2013). In particular, cytokines such as IL-1, IL-6, IL-10, TNF-α and CRP as an acute phase protein are markers used to monitor healing and traumatic inflammation in both human and veterinary medicine (Schijns and Horzinek, 1997; Reikeras et al., 2014; Ok et al., 2015). Ozcinar et al. (2014) found that IL-1 and TNF-α levels increased after thyroid operation in humans. It has been reported that pre-operative IL-6 and IL-10 concentrations decrease after 3 days and 10 days after operation in bitches with pyometra (Dabrowski et al., 2015). It was stated that there were change in acute phase response and biosynthesis of pro and anti inflammation cytokines during inflammation (Fransson et al., 2007; Kjelgaard-Hansen et al., 2007; Karpinski et al., 2009). Kandir and Keskin (2016) determined that serum IL-1, IL-6, IL-10 levels of the rats which had thyroid operation increased slightly compared to control group, but only the increase in TNF-α level was statistically significant. In this presented study, it was determined that in both control (excluding IL-1) and study groups pro and anti inflammation cytokines and acute phase proteins levels difference were not statistically significant. Despite there was statistically significant difference in IL-1 levels of control group, it was noted that values were close to each other. In our study, the significantly higher levels of IL-1, IL-8, Hp, and CRP on postoperative day 14 in the study group (Table 7) may suggest a prolonged inflammatory response. This extended elevation could be attributed to a higher degree of tissue manipulation or a subclinical tissue reaction associated with the procedures of the new technique. However, these increases in values were not reflected in hematological parameters (Table 4).

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and antioxidant defense system (Sies, 1997). While TAS reflects the defense system of metabolism against oxidative stress, TOS is a marker of oxidative stress. The OSI indicates the tolerance level of the oxidant compounds by the antioxidant compounds (Erel, 2004). Nitric oxide is responsible for providing hemostasis in physiological conditions and in many pathological conditions. In cases of tissue damage, excessive amounts of NO are produced (Kuyumcu et al., 2004). Lee and Kim (2014) performed laparoscopic and laparotomic ovariectomy in dogs. After the surgery, there was an increase in TOS value in both techniques while a decrease in TAS value was detected. In the presented study, it was determined that the preoperative TAS, TOS, NO, and OSI values of the animals in both groups showed no significant intra-group differences across the postoperative time points. On the other hand, in the inter-group analysis, while oxidative stress markers were similar between the groups preoperatively, at postoperative hour 2, and on day 7; a significant decrease in TOS levels was observed on postoperative day 14 in the study group compared to the control group. This delayed divergence suggests that the new technique does not alter the initial, unavoidable surgical inflammatory response, but accelerates the resolution of oxidative stress during the late healing phase.

In conclusion, the new technique used did not demonstrate a clear superiority over the routine operative procedure, except for the lower TOS value on day 14. However, it is believed that it can be preferred due to the shorter operation time, easy application to thick tissues and providing pressure to the entire tissues.

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Ethical Declaration

This study was approved by the Local Ethics Committee of Laboratory Animals (14/50).

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authorship Contributions

Concept: H.C.M., Design: H.C.M., H.K., Data Collection or Processing: H.C.M.., H.K., Analysis or Interpretation: M.Ç., Literature Search: H.C.M., H.K., M.Ç. Writing: H.C.M., H.K., M.Ç.

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REFERENCES

Alan M, Cetin Y, Sendag S, Eski F. 2007. True vaginal prolapse in a bitch. Anim. Reprod. Sci., 100, 411-414. doi:10.1016/j.anireprosci.2006.10.022

Antonov AL, Atanassov AS, Georgic PI. 2009. A modified technique for prolapsed fold excision in a bitch with vaginal hyperplasia. BJVM, 12, 260-264.

Antonov A, Ivanova B. 2023. True Vaginal Prolapse Complicated with Uterine Horn Intussusception and Urethral Obstruction in a Postpartum Bitch. Veterinarija ir Zootechnika, 81(1): 10-13.

Antonov A, Atanasov A, Fasulkov I, Karadaev M. 2023. Clinical retrospective study of vaginal hyperplasia in the bitch (2012–2022). Reproduction in Domestic Animals, 58(10): 1352-1358.

Anya KO, Oguejiofor CF, Nnaji TO, Udeani IJ. 2020. Vaginal hyperplasia and progressive vaginal fold prolapse in a bullmastiff bitch. Open Journal of Veterinary Medicine, 10, 55-63. doi: 10.4236/ojvm.2020.105005

Bucci R, Fusi J, Robbe D, Veronesi MC, Carluccio A. 2022. Management of vaginal hyperplasia in bitches by Bühner suture. Animals, 12, 3505. doi: 10.3390/ani12243505

Canooglu E. 2013. Vaginal hyperplasia in the bitch: Diagnosis and treatment options. Erciyes Univ. Vet. Fak. Derg., 10, 177-183.

Dabrowski RJ, Pastor J, Szczubial M, Piech T, Bochniarz M, Wawron W, Tvarijonaviciute A. 2015. Serum IL-6 and IL-10 concentrations in bitches with pyometra undergoing ovariohysterectomy. Acta. Vet. Scand., 57, 61. doi: 10.1186/s13028-015-0153-8

Eckersall PD. 2004. The time is right for acute phase protein assay. Vet. J., 168, 3-5. doi: 10.1016/j.tvjl.2003.09.003.

Eckersall PD, Bell R. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet J., 2010, 185: 23–27. doi: 10.1016/j.tvjl.2010.04.009.

Erel O. 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin. Biochem., 37: 277-285. doi: 10.1016/j.clinbiochem.2003.11.015

Fransson BA, Lagerstedt A, Bergstrom A, Hagman R, Park JS, Chew BP, Evans MA, Ragle CA. 2007. C-reactive protein, tumor necrosis factor α, and interleukin-6 in dogs with pyometra and SIRS. J. Vet. Emerg. Crit. Car., 17, 373-381. doi:10.1111/j.1476-4431.2006.00203.x.

Gonzalez AC, Costa TF, Andrade ZA, Medrado ARAP. 2016. Wound healing - A literature review. An. Bras., Dermatol. 91: 614-620. doi:https://doi.org/10.1590/abd1806-4841.20164741

Hismiogulları SE, Hismiogulları AA, Yavuz MT, Yavuz O, Yaman I, Seyrek K, Hayirli A, Rahman K. 2013. The protective effect of resveratrol in experimentally induced non-sterile clean wound inflammation in rats. Kafkas Univ. Vet. Fak. Derg., 19 (Suppl.-A): A1-A5. doi: 10.9775/kvfd.2012.6989

Johnston SD, Kustritz MVR, Olson PNS. 2001. Disorders of the canine vagina, vestibula, and vulva. In: Canine and Feline Theriogenology. (Johnston, S. D., M.V.R. Kustritz, P.N.S. Olson, Eds.), W.B., Saunders Company, Philadelphia, pp. 225-242.

Kalkan C, Alacam E. 2007. Jinekolojik operasyonlar. In: Evcil Hayvanlarda Doğum ve İnfertilite. (Alacam, E., Ed.), Medisan, Ankara, pp. 383-400. (in Turkish)

Kandir S, Keskin E. 2016. Serum IL-1β, IL-6, IL-10 and TNF-α levels in thyroidectomized rats. Kafkas Univ. Vet. Fak. Derg., 22(2): 297-300. doi: 10.9775/kvfd.2015.14371

Karpinski L, Plaksej R, Derzhko R, Orda A, Witkowska M. 2009. Serum levels of interleukin-6, interleukin-10 and C-reactive protein in patients with myocardial infarction treated with primary angioplasty during a 6-month follow-up. Pol. Arch. Med. Wewn., 119: 115-121.

Kjelgaard-Hansen M, Luntang-Jensen M, Willesen J, Jensen AL. 2007. Measurement of serum interleukin-10 in the dog. Vet. J., 173: 361–365. doi: 10.1016/j.tvjl.2005.11.016

Konstantinova NV, Duong DM, Remenyik E, Hazarika P, Chuang, A, Duvic M. 1996. Interleukin-8 is induced in skin equivalents and is highest in those derived from psoriatic fibroblasts. J. Invest. Derm. 107: 615–621., doi: 10.1111/1523-1747.ep12584215.

Kosecik M, Erel O, Sevinc E, Selek S. 2005. Increased oxidative stress in children exposed to passive smoking. Int. J. Cardiol. 100: 61-64., doi: 10.1016/j.ijcard.2004.05.069

Kumar B, Vijayakumar M, Govindarajan R, Pushpangadan P. 2007. Ethnopharmacological approaches to wound healing—Exploring medicinal plants of India. Journal of Ethnopharmacology, 114: 103-113. doi:10.1016/j.jep.2007.08.010

Kurt S, Salar S, Baştan A. 2019. Effect of vaginal fold prolapse occurrence in a pregnant bitch on parturition process. Journal of Turkish Veterinary Medical Society, 90(1): 50-54. doi: 10.33188/vetheder.496890

Kuyumcu A, Duzgun AP, Ozmen MM, Besler HT. 2004. The role of nitric oxide in trauma and infection. Ulus. Travma Derg., 10: 149-159.

Lee JY, Kim MC. 2014. Comparison of oxidative stress status in dogs undergoing laparoscopic and open ovariectomy. J. Vet. Med. Sci., 76: 273-276. doi: 10.1292 /jvm s.13 -0062

Lowry SF. 1993. Cytokine mediators of immunity and inflammation. Arch. Surg., 128: 1235-1241. doi: 10.1001/archsurg.1993.01420230063010.

Methai A. Shiju Simon M, Gowri B, Arun Prasad A. 2023. Type III recurrent vaginal fold prolapse (VFP) in a Labrador bitch and its management. Pharma Innovation., 12(6): 3874-3875.

Mostachio G, Vicente WRR, Cardilli D, Pires EA, Toniollo GH. 2007. Anovulvar cleft and vaginal prolapse-hyperplasia in a bitch. J. Small Anim. Pract., 48: 713-715. doi: 10.1111/j.1748-5827.2007.00365.x

Murata H, Shimada N, Yoshioka M. 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet. J., 168: 28-40. doi: 10.1016/S1090-0233(03)00119-9.

Nak D, Kasikci G. 2013. Infertilite. In: Köpek ve Kedilerde Doğum ve Jinekoloji. (Kaymaz, M., M. Findik, A. Risvanli, A. Koker, Eds.), Medipres, Malatya, pp. 223-273.

Ok M, Er C, Yildiz R, Col R, Aydogdu U, Sen I, Guzelbektas H. 2015. Evaluation of acute phase proteins, some cytokines and hemostatic parameters in dogs with sepsis. Kafkas Univ. Vet. Fak. Derg., 21(5): 761-766. doi: 10.9775/kvfd.2015.13418

Ozcinar B, Aksakal N, Yanar F, Agcaoglu O, Peker KD, Turkoglu U, Mercan S, Ozmargan S, Erbil F. 2014. Increased interleukin 1 and tumor necrosis factor alpha levels after thyroid surgery. Ulusal Cer. Derg., 30: 80-84. doi: 10.5152/UCD.2014.2484

Ozenc E, Baki Acar D, Birdane MK, Bozkurt MF. 2016. Vaginal prolapse and Transmissible Venereal Tumor in a bitch. Kocatepe Vet. J., 9: 255-258. doi: 10.5578/kvj.27798

Ozenc E, Birdane MK, Yaprakci MV, Demirkan I. 2017. Bladder retroflexion in a pointer bitch during pregnancy. Kafkas Üniv. Vet. Fak. Derg., 23: 513-514. doi: 10.9775/kvfd.2017.17521

Ozturkler Y, Ucar O, Colak A, Ozaydin I, Ozba B, Kamiloglu A. 2001. Vaginal hyperplasia and its treatment with cervicopexie in a Kangal (Anatolian shepherd) bitch. Kafkas Üniv. Vet. Fak. Derg., 7(1): 101-103.

Petersen HH, Nielsen JP, Heegaard PMH. 2004. Application of acute phase protein measurement in veterinary clinical chemistry. Vet. Res., 35: 163–187. doi: 10.1051/vetres:2004002.

Post K, Haaften BV, Okkens AC. 1991. Vaginal hyperplasia in the bitch: Literature review and commentary. Can. Vet. J., 32: 35-37.

Reikeras O, Borgen P, Reseland JE, Lyngstadaas SP. 2014. Changes in serum cytokines in response to

musculoskeletal surgical trauma. BMC Research Notes, 7, 128. doi:10.1186/1756-0500-7-128

Schijns VECJ, Horzinek MC. 1997. Cytokines in veterinary medicine. 1st ed., CAB International, Wallingford, pp. 1-352.

Schutte AP. 1967. Vaginal prolapse in the bitch. J. S. Afr. Vet. Med. Ass., 38: 197-203.

Semacan A. 2007. Prolapsus vagina. In: Evcil Hayvanlarda Doğum ve İnfertilite. (Alacam, E., Ed.), Medisan, Ankara, pp. 137-142.

Sies H. 1997. Oxidative stress: oxidants and antioxidants. Exp. Physiol., 82: 291-295. doi: 10.1113/expphysiol.1997.sp004024

Singh GD, Yadav D, Kumar A, Shekhar P, Singh VK, Prasad JK. 2022. Management of recurrent vaginal fold prolapse in a bitch: A case report. Journal of Farm, Companion and Aquatic Animals, 1: 54-56.

Sontas HB, Ekici H, Romagnoli S. 2010. Canine vaginal fold prolapse: a comprehensive literature review. The European Journal of Companion Animal Practice, 20(2): 127-135.

Tiftik AM. 1996. Kan Proteinleri, Klinik Biyokimya, Mimoza, Konya, pp. 279-299. (in Turkish)

Turgut K. 2000. Lökosit Bozuklukları ve Testleri, Veteriner Klinik Laboratuvar Teşhis, Bahçıvanlar Basımevi, Konya, pp. 80-119. (in Turkish)

Uluisik D, Keskin E. 2012. The effects of ginseng and echinacea on some plasma cytokine levels in rats. Kafkas Univ. Vet. Fak. Derg., 18(1): 65-68. doi: 10.9775/kvfd.2011.4814

Wykes PM, Olson PN. 2002. Vagina, vestibule, and vulva. In: Textbook of Small Animal Surgery. (Slatter, D., Ed.), Saunders, Philadelphia, pp. 1502-1510.

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RESEARCH ARTICLE

Dose- and Time-Dependent Inhibitory Effects of Mancozeb on Viability and Steroid Synthesis of Bovine Granulosa Cells *In Vitro*

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ABSTRACT

Mancozeb (MAN) is a widely used fungicide considered an endocrine disrupting compound with potential reproductive toxicity. This study aimed to evaluate the cytotoxic and endocrine-disrupting effects of MAN on bovine granulosa cells (bGCs) by assessing cell viability and steroid hormone secretion in vitro. Bovine granulosa cells were isolated from ovarian follicles of slaughtered healthy cows at a local abattoir. Cells were then cultured in DMEM/F12 medium and exposed to increasing concentrations of MAN (0 - 100 µM) for 12, 24, and 48 h. At the end of the experiment, cell viability was assessed using the MTT assay while estradiol (E2) and progesterone (P4) concentrations in culture media were measured using electrochemiluminescence immunoassay. Our results revealed that low concentrations of MAN (0.001–1 µM) did not significantly affect cell viability at any incubation period ($p>0.05$) while a transient increase in MTT activity was observed at 10 µM after 24 h exposure ($p<0.05$). In contrast, exposure to 100 µM MAN significantly reduced cell viability at all time points ($p<0.001$), accompanied by morphological deterioration and reduced cell confluency. Hormone analyses showed that 10 µM MAN did not significantly alter E2 or P4 secretion. However, 100 µM MAN significantly decreased progesterone production at both 24 and 48 h ($p<0.001$) and significantly reduced estradiol secretion after 48 h exposure ($p<0.001$). In conclusion, we showed for the first time that MAN exerts dose- and time-dependent inhibition of cellular metabolic activity and steroid secretion of bGC, indicating potential disruption of ovarian function.

INTRODUCTION

Endocrine disrupting compounds (EDCs) are defined as exogenous chemicals that disrupt homeostasis, reproduction, and developmental processes by interfering with processes such as the synthesis, secretion, transport, or binding of hormones in the body (Gore et al., 2015). These chemicals can cause adverse effects on numerous physiological systems, including both male and female reproductive functions (Kabakci and Yigit, 2020; Kabakci et al., 2021). Exposure to EDCs generally occurs through dermal contact, contaminated water, air, or soil and food products where agricultural chemicals are used. Livestock exposed to pesticides, particularly through grasses, crop residues, and ground water sources, are at a higher risk compared to other species (Diamanti-Kandarakis et al., 2009; Arikan et al., 2017; Atuhaire et al., 2017).

Mancozeb (MAN) is a broad-spectrum ethylene-bis-dithiocarbamate fungicide widely used throughout the world in agricultural areas to protect fruits, vegetables, and field crops against fungal diseases. Although it is reported to have low acute toxicity in mammals, it is established that chronic exposure of MAN poses serious health risks due to the persistence of it and its metabolite in the environment (WHO, 1988; Černohlávková et al., 2009; Runkle et al., 2017). It has been demonstrated in various animal models that MAN acts as an EDC and causes reproductive toxicity by disrupting the hypothalamic-pituitary-gonadal axis. Studies conducted on rodents have reported that MAN exposure disrupts the estrous cycle, inhibits the embryonic implantation, decreases the number of healthy follicles, and increases the number of atretic follicles (Nordby et al., 2005; Mohanty et al., 2017). Furthermore, direct gamete toxicity effects, such as the disruption of the meiotic process in mouse oocytes and reduced fertilization competence, have also been documented (Pocar et al., 2003; Rossi et al., 2006).

Granulosa cells play critical role in regulation of follicular development, oocyte maturation, and steroid hormone production. Ovarian folliculogenesis and oogenesis are strictly dependent on the bidirectional communication between the oocyte and its surrounding granulosa cells. Through this dynamic interaction, granulosa cells support meiotic progression, cumulus expansion, and steroidogenesis, thereby it is required a providing the specialized microenvironment for optimal oocyte growth and maturation. Consequently, any

disruption in granulosa cell viability or functional integrity may directly compromise oocyte quality and ultimately impair fertility potential (Hutt and Albertini, 2007; Palmerini et al., 2018). *In vivo* and *in vitro* studies have shown that MAN disrupts the cytoskeleton structure in mouse and human granulosa cells, reduces p53 expression mitochondrial dysfunction and ATP production, and increases oxidative stress (Paro et al., 2012; Iorio et al., 2015). Ultrastructural analyses have revealed that this fungicide triggers cell degeneration consistent with apoptosis by causing mitochondrial damage, extensive vacuolization, and loss of intercellular contacts in mouse granulosa cells (Palmerini et al., 2018; Dinisri et al., 2021).

A recent *in vitro* study conducted on caprine models confirmed that MAN exposure significantly reduced granulosa cell viability and impaired oocyte cytoplasmic and nuclear maturation. However, it has also reported a dose-dependent increase in progesterone secretion despite reduced cell viability in this study (Dinisri et al., 2021). In contrast, previous studies on mouse theca cells suggested a decrease in progesterone production (Palmerini et al., 2018). These contradictory findings and variations observed across different species suggest that the effect of MAN on steroidogenesis may vary via species-specific mechanisms.

Although the effects of MAN on laboratory animals and human cell lines have been extensively studied (Cecconi et al., 2007; Paro et al., 2012; Iorio et al., 2015; Runkle et al., 2017; Atmaca et al., 2018; Palmerini et al., 2018), its effects on the granulosa cells of bovine species which are at risk of direct and intensive exposure to this chemical through grazing in agricultural areas are still limited. Cattle farming is one of the crucial issues of the global livestock economy, and losses in reproductive performance in this species result in serious economic consequences. In this context, the specific effects of MAN on bovine granulosa cell (bGC) viability and hormone production capacity need to be elucidated. On the other hand, bGCs serve not only as a valuable model for understanding follicular physiology in domestic ruminants but also as a translational model for reproductive toxicology (Mohammed and Donadeu, 2018).

In view of the findings these reported previous studies, we hypothesized that MAN exposure would reduce cell viability and disrupt steroid hormone production in bGCs *in vitro*. Considering that cattle raised in agricultural regions may be chronically exposed to pesticide residues through contaminated forage and water sources, understanding the reproductive toxicology of widely used fungicides such as MAN is essential for sustainable livestock production. To date, the direct effects of MAN on bovine granulosa cells and their steroidogenic activity remain largely unexplored. Therefore, the present study aimed to evaluate the cytotoxic effects of different concentrations of MAN on the viability and in vitro estradiol (E2) and progesterone (P4) secretion of bovine granulosa cells.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all reagents and chemicals used in the present study were purchased from Sigma-Aldrich (Munich, Germany). The study was conducted in accordance with the national guidelines Regulation on Working Procedures and Principles for Ethical Committees of Animal Experimentations of Türkiye. The ethics committee approval was not required since the tissue samples used in this study were collected from slaughtered animals.

Isolation of Bovine Granulosa Cells

Bovine ovaries were collected from clinically healthy Simental cows (between 2-5 years of age) slaughtered at a local abattoir and transported to the laboratory within 2 h in sterile phosphate-buffered saline (PBS) supplemented with 1% antibiotic–antimycotic solution (AB/AM) at 4°C. Upon arrival, ovaries were washed with 70% ethanol followed by sterile PBS to minimize contamination. Granulosa cells were isolated from the antral follicles as previously described by Kabakci et al. (2023). Briefly, follicular fluid of follicles in diameter approximately 2–5 mm containing granulosa cells were aspirated using a 19-gauge needle attached to a sterile syringe and transferred into tubes containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 1% AB/AM. Afterwards, follicular walls were gently scraped to collect more cells which were added to the same tubes. The obtained cell suspension was filtered through a 100-µm cell strainer to remove tissue debris and centrifuged at 300 g for 10 min at 4°C. The resulting pellet was resuspended in 1 mL fresh culture medium (DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% AB/AM). Cell viability was determined using the trypan blue exclusion method. Equal volumes (10 µL) of cell suspension and 0.4% trypan blue were mixed and counted using an automated cell counter (Countess II, Thermo Fisher Scientific, USA). Cells with viability ≥80% were used for subsequent experiments.

Cell Culture and Mancozeb Treatment

Bovine granulosa cells (bGC) were seeded into 96-well and 12-well culture plates at a density of 1×10^4 viable cells per well in 100 µL and at a density of 2×10^5 cells per well in 1 mL culture medium consist of DMEM/F12 + 10% FBS + 1% AB/AM for cytotoxicity and hormone analyses, respectively. During the first 24-h period, cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in order to allow attachment. Following this attachment period, the culture medium was replaced with serum-free DMEM/F12 medium supplemented with 1% ITS, 1% AB/AM and increasing concentrations of mancozeb (0, 0.001, 0.01, 0.1, 1, 10, and 100 µM). These concentrations were selected based on previously published in vitro ovarian toxicity studies (Atmaca et al., 2018; Cao et al., 2019). Before the treatment period MAN was dissolved in dimethyl sulfoxide (DMSO) and final concentration of it in culture medium was 0.01%. Cells were exposed to MAN for 12, 24, and 48 h. Each experimental group was set out in triplicate wells within each plate, and the entire experiment was repeated three times.

Assessment of Cell Viability (MTT Assay)

Cell viability and cytotoxicity were evaluated using a commercial colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay kit according to the manufacturer's instructions. At the end of each incubation period (12, 24, and 48 h), 10 µL of MTT reagent at the concentration of 5 $mg.mL^{-1}$ was added to each well of the 96-well plates and incubated for an additional 2 h at 37°C to allow for formazan crystal formation. After incubation, the culture medium was removed, and formazan crystals were dissolved in DMSO. The absorbance of wells was measured at 450 nm using a

microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA). Cell viability was expressed as a percentage relative to the untreated control group.

Measurement of Hormone Secretion

At the end of each incubation period, culture media from 12-well plates were collected and stored at −20°C until analysis. Estradiol (E2) and progesterone (P4) concentrations were measured using commercial electrochemiluminescence immunoassay (ECLIA) kits according to the manufacturer's protocols. Hormone levels were expressed as pg.mL^{-1} for E2 and ng.mL^{-1} for P4.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, USA) version 9. Data distribution was assessed using the Shapiro–Wilk normality test. The differences observed among the groups with respect to the dose and time effects were analyzed by two-way analysis of variance (ANOVA), followed by post-hoc Tukey's multiple comparison test. Data are presented as mean ± standard error of the mean (SEM). A p-value < 0.05 was considered statistically significant.

RESULTS

Effects of Mancozeb on Bovine Granulosa Cell Viability

The effects of MAN on bovine granulosa cell viability were evaluated by MTT assay following 12, 24, and 48 h of exposure. As shown in Figure 1, exposure to low and moderate concentrations of mancozeb (0.001–1 µM) did not significantly affect cell viability compared to the control group ($p>0.05$) at any incubation periods. Interestingly, 10 µM MAN significantly increased MTT absorbance values compared to control ($p<0.05$) at only after 24 h, contrary to the results after 12 and 48 h. However, exposure to the highest concentration of mancozeb (100 µM) led to a dramatic decrease in cell viability ($p<0.0001$).

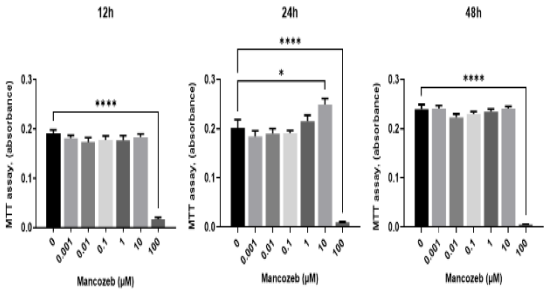


Figure 1. Effects of increasing concentrations of mancozeb exposure for 12, 24, and 48 h on the viability/metabolic activity of bovine granulosa cells assessed by MTT assay. Data were presented as mean ± SEM. Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple-comparison test. Only statistically significant comparisons with the corresponding control are indicated. *p<0.05; ****p<0.0001.

Microscopic examination of bGCs collected from control (0 µM) and the highest doses of MAN (100 µM) treated groups at 12, 24, and 48 h supported the MTT findings (Figure 2). While control cells exhibited normal morphology and confluency over time, cells exposed to 100 µM MAN showed progressive morphological deterioration, reduced confluency, and increased cellular detachment, particularly at 24 and 48 h.

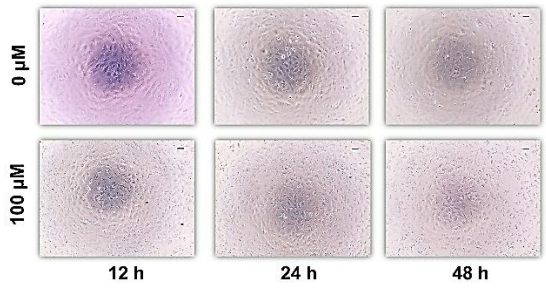


Figure 2. Representative microscopic images of bovine granulosa cells exposed to 0 µM (control) or 100 µM mancozeb for 12, 24, and 48 h. Images were acquired at 100× magnification. Scale bar = 50 µm.

Effects of Mancozeb on Estradiol and Progesterone Secretion

Due to the absence of significant cytotoxicity at low doses, hormone analyses were performed in 0, 10, and 100 µM treatment groups. Although both concentrations of MAN (10 and 100 µM) slightly decreased estradiol secretion compared to control (0 µM), this difference was not statistically significant ($p>0.05$) at 24 h. Following 48-h exposure period, 100 µM MAN significantly reduced estradiol secretion compared to control ($p<0.0001$), while 10 µM MAN did still not produce a statistically significant ($p>0.05$) change in E2 level (Figure 3).

Similarly, as demonstrated in Figure 3, progesterone secretion was not significantly affected by lower concentration of MAN (10 µM) at both exposure time. However, 100 µM MAN dramatically reduced in vitro progesterone production of granulosa cells compared to untreated cells at 24 and 48 h ($p<0.0001$).

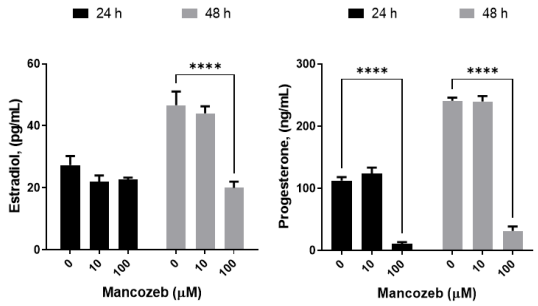


Figure 3. Effects of 0, 10, or 100 µM mancozeb exposure for 24 and 48 h on estradiol (E2) and progesterone (P4) production by bovine granulosa cells. Data are presented as mean ± SEM. Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple-comparison test. Only statistically significant comparisons with the corresponding control are indicated. ****p<0.0001.

DISCUSSION AND CONCLUSION

The present study demonstrates that MAN exerts dose- and time-dependent effects on bGCs viability and steroid hormone production. While low concentrations (≤1 µM) did not significantly affect cell viability, 10 µM MAN did not significantly alter hormone secretion, whereas exposure to 100 µM MAN resulted in a remarkable reduction in cell viability and a marked suppression of estradiol and progesterone secretion, particularly after prolonged incubation period.

Our viability data are consistent with previous in vitro studies reporting cytotoxic effects of MAN in reproductive cell types. Dinisri et al. (2021) demonstrated that 3 and 30 $\mu g.mL^{-1}$ MAN significantly reduced caprine granulosa cell viability. Similarly, Wang et al. (2021) reported decreased viability in human endometrial and placental cell lines at concentrations of 10, 50, and 100 $\mu g.mL^{-1}$. In vivo evidence also supports the reproductive toxicity of MAN. For instance, Liu et al. (2016) showed reduced oocyte numbers in mice following 4-week oral exposure of MAN, while Sakr et al. (2009) reported degenerative changes in rat spermatogenic germ cells and Leydig cells. These findings indicate that MAN adversely affects both male and female reproductive cells across species. Interestingly, in our study, 10 µM MAN transiently increased MTT absorbance at 24 h without affecting viability at 12 or 48 h. This temporary elevation may reflect a compensatory increase in mitochondrial metabolic activity rather than real proliferative stimulation. It is well established that the MTT assay primarily measures mitochondrial dehydrogenase activity and therefore reflects metabolic activity in addition to cell numbers. The marked reduction (approximately 97.5%) in MTT activity observed at 100 µM MAN was not accompanied by an equivalent decrease in cell density under microscopic examination as seen in Figure 2. This discrepancy may indicate an early impairment of cellular metabolic activity before overt cell detachment or death, although specific mitochondrial dysfunction was not directly assessed in the present study. In consistent with our findings, several previous studies have shown that MAN disrupted mitochondrial integrity, reduced ATP production, and increased oxidative stress in mammalian and rodent granulosa cells (Paro et al., 2012; Iorio et al., 2015). As these reports supported our suggestion, the inhibited MTT activity in bGCs may have resulted from suppressed or disrupted mitochondrial activity.

Regarding steroidogenesis, our results demonstrated that 100 µM MAN significantly reduced progesterone secretion at both 24 and 48 h, whereas estradiol secretion was significantly decreased only after 48 h exposure. These findings suggest that progesterone synthesis may be more rapidly or more sensitively affected by MAN exposure. Atmaca et al. (2018) similarly reported that 1 µM MAN significantly decreased progesterone production in bovine luteal cells. Moreover, Tsagué Manfo et al. (2011) observed reduced basal pregnenolone and testosterone production in rat Leydig cells following MAN exposure. In vivo studies further indicate that MAN decreased circulating testosterone, estradiol, and progesterone levels Zago et al. (2022). These reports are consistent with our findings, which can be associated with the endocrine disrupting properties of mancozeb. On the other hand, Dinisri et al. (2021) reported a significant increase in progesterone production in caprine granulosa cells exposed to 3 and 30 $\mu g.mL^{-1}$ MAN, in contrast to reduced cell viability. Although these findings are inconsistent with our results, such discrepancies may be attributed to species-specific responses, differences in exposure concentrations, serum conditions, or experimental design. These different results indicated the necessity of further research to understand the molecular mechanism of time- and dose-dependent effects of MAN on ovarian steroidogenesis.

In addition, as we observed in our study, the reduction of progesterone at 24 h and the suppression of estradiol at 48 h may reflect inhibition at multiple steps of the steroidogenic biosynthesis pathway. Although it was not included in the experimental design of the present study, mancozeb may interfere with key steroidogenic proteins/enzymes such as steroidogenic acute regulatory protein (StAR), cytochrome p450 (CYP11A1), and 3β-hydroxysteroid dehydrogenase (3β-HSD), as well as aromatase (CYP19A1) activity. Girish and Reddy (2018) suggested that reduced testosterone synthesis following MAN exposure may be associated with inhibition of 3β- and 17β-HSD activities. If similar mechanisms occur in granulosa cells, early impairment of progesterone synthesis could result from disruption of cholesterol transport or pregnenolone conversion, whereas delayed estradiol suppression may reflect secondary effects on aromatase-mediated conversion pathways.

The present study has several limitations that should be acknowledged. One of them, E2 and P4 secretion, was evaluated only in the 0, 10, and 100 µM treatment groups. Therefore, it is possible that low-dose or non-monotonic endocrine responses at sub-cytotoxic concentrations (0.001–1 µM) remain to be excluded. Secondly, the MTT assay reflects cellular metabolic activity rather than providing a direct assessment of mitochondrial function or cell death, and specific mitochondrial parameters such as membrane potential and ATP production were not evaluated. Furthermore, oxidative stress, apoptosis-related parameters, and the expression or activity of key steroidogenic genes and proteins were not examined, which limits the mechanistic interpretation of the observed changes in cell viability and hormone secretion. Further studies incorporating these endpoints and a broader range of concentrations are required to better characterize the endocrine-disrupting mechanisms of mancozeb in bovine granulosa cells.

In conclusion, the present study demonstrates that mancozeb exerts dose- and time-dependent toxic effects on bovine granulosa cells. While low concentrations did not significantly affect cell viability or steroid hormone secretion, high-dose exposure (100 µM) markedly reduced cellular metabolic activity and significantly decreased progesterone and estradiol production, particularly following prolonged incubation. These findings suggest that mancozeb may disrupt ovarian function through both cytotoxic and steroidogenesis-related mechanisms. Given the widespread agricultural use of MAN and the potential exposure of livestock, further comprehensive in vivo and in vitro studies are required to elucidate the underlying molecular mechanism of its toxicity and to better clarify its impact on bovine fertility and reproductive health.

Acknowledgement

Part of the findings of this study was previously presented as an abstract at the 24th International Veterinary Students Scientific Research Congress (December 2024) under the title “The Effect of Mancozeb Fungicide on Bovine Granulosa Cells and In Vitro Hormone Production.” We would like to thank our undergraduate students, Ömer Faruk EROL and Sıla ÖZAY, for their valuable contributions to the experimental process.

Ethical Declaration

Ovaries used in this study were obtained from animals slaughtered for commercial purposes at a local abattoir. According to national regulations governing the ethical use of animal-derived materials in research in Türkiye, studies using tissues collected from slaughtered animals do not require approval from an institutional animal ethics committee.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authorship Contributions

RK: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – Original draft. TA: Investigation, Data curation, Methodology. RK, TA: Writing – Review & Editing, Interpretation of data, Validation. All authors have read and approved the final version of the manuscript.

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Artificial Intelligence Usage Declaration Statement

During the preparation of this work the authors used ChatGPT 4.0 Plus / Open AI solely to assist with English grammar checking, and spelling, and shortened some paragraphs. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

REFERENCES

Arikan S, Simsek O, Kalender H, Essiz D, Atmaca N, Kabakci R, Bilmen FS. 2017. Effects of acetamiprid, methomyl, and deltamethrin on steroidogenesis in cultured bovine luteal cells. Fresenius Environ. Bull., 26(5): 3280-3284.

Atmaca N, Arikan S, Essiz D, Kalender H, Simsek O, Bilmen FS, Kabakci R. 2018. Effects of mancozeb, metalaxyl and tebuconazole on steroid production by bovine luteal cells in vitro. Environ. Toxicol. Pharmacol., 59(2018): 114-118. https://doi.org/10.1016/j.etap.2018.03.009

Atuhaire A, Kaye E, Mutambuze IL, Matthews G, Friedrich T, Jørs E. 2017. Assessment of dithiocarbamate residues on tomatoes conventionally grown in Uganda and the effect of simple washing to reduce exposure risk to consumers. Environ. Health Insights, 11117863021771218. https://doi.org/10.1177/1178630217712218

Cao F, Souders II CL, Li P, Pang S, Liang X, Qiu L, Martyniuk CJ. 2019. Developmental neurotoxicity of maneb: Notochord defects, mitochondrial dysfunction and hypoactivity in zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicol. Environ. Saf., 170: 227-237. https://doi.org/10.1016/j.ecoenv.2018.11.110

Cecconi S, Paro R, Rossi G, Macchiarelli G. 2007. The effects of the endocrine disruptors dithiocarbamates on the mammalian ovary with particular regard to mancozeb. Curr. Pharm. Des., 13(29): 2989-3004. https://doi.org/10.2174/138161207782110516

Černohlávková J, Jarkovský J, Hofman J. 2009. Effects of fungicides mancozeb and dinocap on carbon and nitrogen mineralization in soils. Ecotoxicol. Environ. Saf., 72(1): 80-85. https://doi.org/10.1016/j.ecoenv.2008.07.001

Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev., 30(4): 293-342. https://doi.org/10.1210/er.2009-0002

Dinisri I, Kodikara S, Prasadani M, Pathirana I, Rathnayake C, Alexander B, Lee K-F, Kodithuwakku SP. 2021. Impairment of caprine oocyte maturation in vitro and alteration of granulosa cells functions by widely used fungicide mancozeb. Trop. Anim. Health Prod., 53(3): 406. https://doi:10.1007/s11250-021-02854-5

Girish B, Reddy PS. 2018. Forskolin ameliorates mancozeb-induced testicular and epididymal toxicity in Wistar rats by reducing oxidative toxicity and by stimulating steroidogenesis. J. Biochem. Mol. Toxicol., 32(2): e22026. https://doi.org/10.1002/jbt.22026

Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, Toppari J, Zoeller RT. 2015. EDC-2: the endocrine society's second scientific statement on endocrine-disrupting chemicals. Endocr. Rev., 36(6): E1-E150. https://doi.org/10.1210/er.2015-1010

Hutt KJ, Albertini DF. 2007. An oocentric view of folliculogenesis and embryogenesis. Reprod. Biomed. Online, 14(6): 758-764. https://doi.org/10.1016/S1472-6483(10)60679-7

Iorio R, Castellucci A, Rossi G, Cinque B, Cifone MG, Macchiarelli G, Cecconi S. 2015. Mancozeb affects mitochondrial activity, redox status and ATP production in mouse granulosa cells. Toxicol. In Vitro, 30(1): 438-445. https://doi.org/10.1016/j.tiv.2015.09.018

Kabakci R, Clark KL, Plewes MR, Monaco CF, Davis JS. 2023. Perfluorooctanoic acid (PFOA) inhibits steroidogenesis and mitochondrial function in bovine granulosa cells in vitro. Environ. Pollut., 338122698. https://doi.org/10.1016/j.envpol.2023.122698

Kabakci R, Kaya A, Yigit AA, Varisli O. 2021. Assessment of tebuconazole exposure on bovine testicular cells and epididymal spermatozoa. Acta Vet. Hung., 69(2): 180-188. https://doi.org/10.1556/004.2021.00023

Kabakci R, Yigit AA. 2020. Effects of bisphenol A, diethylhexyl phthalate, and pentabrominated diphenyl ether 99 on steroid synthesis in cultured bovine luteal cells. Reprod. Domest. Anim., 55(6): 683-690. https://doi.org/10.1111/rda.13665

Liu Y, Wang Y-L, He S-w, Chen M-H, Zhang Z, Fu X-P, Fu B-B, Liao B-Q, Lin Y-H, Qi Z-Q. 2016. Protective effects of resveratrol against mancozeb induced apoptosis damage in mouse oocytes. Oncotarget, 8(4): 6233. https://doi.org/10.18632/oncotarget.14056

Mohammed TB, Donadeu FX. 2018. Bovine granulosa cell culture. in Mario Baratta (Ed.), Epithelial Cell Culture: Methods and Protocols. Humana Press, New York, USA, pp. 79-87.

Mohanty B, Pandey SP, Tsutsui K. 2017. Thyroid disrupting pesticides impair the hypothalamic-pituitary-testicular axis of a wildlife bird, Amandava amandava. Reprod. Toxicol., 7132-7141. https://doi.org/10.1016/j.reprotox.2017.04.006

Nordby K-C, Andersen A, Irgens LM, Kristensen P. 2005. Indicators of mancozeb exposure in relation to thyroid cancer and neural tube defects in farmers' families. Scand. J. Work. Environ. Health, 31(2): 89-96. https://doi.org/10.5271/sjweh.855

Palmerini MG, Belli M, Nottola SA, Miglietta S, Bianchi S, Bernardi S, Antonouli S, Cecconi S, Familiari G, Macchiarelli G. 2018. Mancozeb impairs the ultrastructure of mouse granulosa cells in a dose-dependent manner. J. Reprod. Dev., 64(1): 75-82. https://doi.org/10.1262/jrd.2017-143

Paro R, Tiboni GM, Buccione R, Rossi G, Cellini V, Canipari R, Cecconi S. 2012. The fungicide mancozeb induces toxic effects on mammalian granulosa cells. Toxicol. Appl. Pharmacol., 260(2): 155-161. https://doi.org/10.1016/j.taap.2012.02.005

Pocar P, Brevini T, Fischer B, Gandolfi F. 2003. The impact of endocrine disruptors on oocyte competence. Reproduction, 125(3): 313-325. https://doi.org/10.1530/rep.0.1250313

Rossi G, Palmerini MG, Macchiarelli G, Buccione R, Cecconi S. 2006. Mancozeb adversely affects meiotic spindle organization and fertilization in mouse oocytes. Reprod. Toxicol., 22(1): 51-55. https://doi.org/10.1016/j.reprotox.2005.11.005

Runkle J, Flocks J, Economos J, Dunlop AL. 2017. A systematic review of Mancozeb as a reproductive and developmental hazard. Environ. Int., 9929-9942. https://doi.org/10.1016/j.envint.2016.11.006

Sakr S, Okdah Y, El-Adly E. 2009. Effect of ginger (Zingiber officinale) on mancozeb fungicide induced testicular damage in albino rats. Aust. J. Basic Appl. Sci., 3(2): 1328-1333.

Tsagué Manfo FP, Chao W-F, Moundipa PF, Pugeat M, Wang PS. 2011. Effects of maneb on testosterone release in male rats. Drug Chem. Toxicol., 34(2): 120-128. https://doi.org/10.3109/01480545.2010.482589

Wang Z, Kottawatta KS, Kodithuwakku SP, Fernando TS, Lee Y-L, Ng EH, Yeung WS, Lee K-F. 2021. The fungicide Mancozeb reduces spheroid attachment onto endometrial epithelial cells through downregulation of estrogen receptor β and integrin β3 in Ishikawa cells. Ecotoxicol. Environ. Saf., 208111606. https://doi.org/10.1016/j.ecoenv.2020.111606

WHO. 1988. Dithiocarbamate pesticides, ethylenethiourea, and propylenethiourea: a general introduction. World Health Organization, Environ Health Criteria 78 Geneva.

Zago AM, Faria NM, Favero JL, Meucci RD, Woskie S, Fassa AG. 2022. Pesticide exposure and risk of cardiovascular disease: a systematic review. Glob. Public Health, 17(12): 3944-3966. https://doi.org/10.1080/17441692.2020.1808693

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REVIEW ARTICLE

Establishment of an Experimental Aquatic Vertebrate Animal Laboratory within the Framework of Current Legislation: The Case of the Ankara University Faculty of Veterinary Medicine, Türkiye

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ABSTRACT

The necessity to develop alternative methods to reduce experiments on mammals in line with the 4R principle has brought aquatic animals to the fore due to their advantages. Vertebrate and invertebrate aquatic animals are now widely used in experimental studies covering many disciplines, including drug screening and development, preclinical trials, and research into toxicology, oncology, metabolism, the cardiovascular system, the kidneys, the immune system, dermatology, endocrinology, ophthalmology, genetics, infectious diseases and aging. As their use has increased, aquatic experimental animal units have been established around the world, including in Türkiye, and their number is growing daily. In this context, international guidelines containing basic information, recommendations and policies on environmental control for aquatic animals used in experimental studies for scientific purposes have been published. This review aims to provide information on the establishment steps, objectives and basic processes of the zebrafish unit at Ankara University Faculty of Veterinary Medicine's Aquatic Experimental Animal Centre (ADHAM), in line with current national and international guidelines, recommendations, policies and legislation.

INTRODUCTION

Driven by advancing ethical imperatives and aligned with the principles of the 4Rs (Replacement, Reduction, Refinement, and Responsibility), aquatic species have increasingly been recognized as valuable alternatives to traditional mammalian models in biomedical research. Their numerous advantages, including external fertilization, transparent embryonic development, and high genetic and physiological relevance, have positioned both vertebrate and invertebrate aquatic organisms, particularly fish, as powerful tools across a wide range of experimental domains. These include drug discovery and high-throughput screening, preclinical safety and efficacy testing, as well as research in toxicology, oncology, metabolism, cardiovascular and renal function, immunology, dermatology, endocrinology, ophthalmology, genetics, infectious disease, and aging biology (Kiani et al., 2022).

In Türkiye, the use of aquatic vertebrates in scientific research is governed by a comprehensive legislative framework established by the Ministry of Agriculture and Forestry. This framework includes the 2019 Regulation on the Welfare and Protection of Aquatic Vertebrates Used for Scientific Purposes and its 2022 amendment. These regulations articulate detailed standards concerning the infrastructure and operation of housing and care facilities, the authorization process for research institutions, procedural and ethical use of aquatic vertebrates, qualifications and responsibilities of personnel, systematic record-keeping, and overarching animal welfare and bioethical considerations. Within the scope of this legislation, aquatic vertebrates are defined as vertebrate species such as fish (*Pisces*), amphibians (*Amphibia*), and aquatic or semi-aquatic reptiles that must inhabit aquatic environments during all or part of their life cycle, excluding cetaceans (*Cetacea*). In addition to national regulations, internationally recognized guidelines provide essential directives on environmental parameters including water source and quality, temperature regulation, photoperiod control, noise and vibration levels, and the

operation of life-support and alarm systems. They also offer comprehensive recommendations on animal husbandry practices such as health monitoring, housing design, nutrition, sanitation, transportation, humane euthanasia, individual identification, and detailed documentation. The growing recognition of aquatic models as ethically and scientifically advantageous has led to a marked increase in the establishment of specialized research facilities, particularly zebrafish units, across universities, governmental institutions, and private research centers both in Türkiye and worldwide (OECD, 2002; CCAC, 2005; EC, 2007; FELASA, 2007; OECD, 2009; EC, 2010; NRC, 2011; OECD, 2015; The Ministry of Agriculture and Forestry of the Republic of Türkiye, 2019; CCAC, 2020; CCAC, 2021; The Ministry of Agriculture and Forestry of the Republic of Türkiye, 2022; OECD, 2023; Yipel and Altinok-Yipel, 2025).

Aquatic experimental animal facilities are specialized research infrastructures established and managed in strict accordance with national legislation and internationally recognized guidelines. These units operate within comprehensive regulatory frameworks that encompass animal welfare, scientific rigor, research ethics, and occupational health and safety standards. All procedures involving aquatic vertebrates, including reproductive management, controlled husbandry, environmental monitoring, and their utilization in ecological, physiological, and translational biomedical studies, are conducted by highly trained professionals with advanced expertise in veterinary science and laboratory animal care. The implementation of evidence-based practices ensures both the ethical treatment of research animals and the generation of scientifically valid and reproducible data (Yipel and Altinok-Yipel, 2025).

Zebrafish (*Danio rerio*) have emerged as a widely adopted vertebrate model in numerous stages of the drug discovery and development pipeline, including disease modeling, target identification and validation, pharmacological safety assessment, toxicological profiling, and high-throughput compound screening. Their increasing utilization in biomedical research is largely attributed to their significant genetic, molecular, morphological, and pathological homology with humans. From a veterinary and laboratory animal science perspective, zebrafish offer multiple logistical and scientific advantages. These include their small body size, ease of husbandry, low maintenance costs, expansive and well-characterized genomic resources, absence of ethical constraints in early embryonic experimentation, optical transparency during embryonic and larval stages, and prolific reproductive capacity. Moreover, the growing integration of zebrafish into research protocols aligns with the principles of replacement, reduction, and refinement, which are increasingly mandated by both national and international regulatory frameworks. Compared to traditional rodent models, zebrafish provide a more time-efficient and cost-effective alternative, without compromising translational relevance in preclinical investigations (Yipel, 2020; Yipel and İlhan, 2021; Habjan et al., 2024; Hughes and Hessel, 2024).

This review outlines the conceptualization, establishment, and strategic development of an Aquatic Experimental Animal Facility, using the Aquatic Experimental Animal Center (ADHAM) of Ankara University Faculty of Veterinary Medicine as a case model. The discussion is grounded in up-to-date guidelines, policy documents, and regulatory frameworks issued by both national and international competent authorities and reference institutions. The critical phases of facility establishment are examined in detail, encompassing physical infrastructure design, comprehensive budget planning, formation of a multidisciplinary research team, acquisition and installation of specialized equipment, navigation of formal application procedures and timelines, procurement of institutional and legal authorizations, transfer and quarantine protocols for initial aquatic specimens, implementation of breeding programs, standardization of husbandry and nutritional regimens, and the development and validation of relevant experimental models. The review further delineates clearly defined institutional goals across temporal categories, including short-term (6 months to 1 year), medium-term (1 to 3 years), and long-term (more than 3 years) objectives. Core procedural workflows specific to zebrafish research units are also described, highlighting key operational standards that align with contemporary veterinary research practices and ethical governance.

Establishment and Operational Licensing Application and Institutional Recognition of the Laboratory

In alignment with applicable regulatory frameworks, the laboratory's foundational infrastructure and aquatic rearing systems were established as illustrated in Figures 1A through 1D. Following these preparatory steps, an official application for operational authorization was submitted to the Directorate of Fisheries and Aquatic Resources under the Ministry of Agriculture and Forestry of the Republic of Türkiye. This submission was made through the "Preliminary Permit Application Form for the Use of Aquatic Vertebrate Animals for Scientific Purposes," as mandated by national legislation. As part of the procedural sequence, a Preliminary Permit was first secured. Subsequently, all requisite documentation for the issuance of a full operational license was compiled in accordance with Article 14, titled "Work Permit," of the relevant regulation.



Figure 1. Infrastructure and compartments of the laboratory.
A: Quarantine room, B: Care room, C: Breeding room, D: Examination and operation room.

The documentation included certified copies of academic diplomas for public officials and technical personnel designated to work within the facility, approved by the relevant institutional authority. In addition, an Emergency Action Plan was submitted, detailing the precautionary measures implemented against potential fire

and explosion risks. A comprehensive, authorized inventory was also provided, listing the technical specifications and capacities of all equipment intended for use in ensuring the health and welfare of aquatic vertebrate species, along with all chemical and biological agents to be employed within the facility.

IN THE COMPILED APPLICATION FILE FOR THE OPERATIONAL LICENSE, THE FOLLOWING COMPONENTS WERE INCLUDED IN ACCORDANCE WITH ARTICLE 14 OF THE RELEVANT REGULATION

Under Section 1, Clause (a)

The institutional director officially appointed the personnel designated to work within the facility. Additionally, in compliance with the regulation, a Welfare Unit was formally established within the laboratory. Written notifications of these assignments were issued to the respective personnel, and an official notification addressed to the Ankara Provincial Directorate of Animal Health was appended to the application dossier.

Under Clause (e)

In order to obtain official recognition as an authorized institution to conduct research involving aquatic vertebrates or genetically modified aquatic vertebrates, a formal application was submitted to the General Directorate of Agricultural Research and Policies (TAGEM). Both the official petition and the written response from TAGEM were included in the application file.

Upon completion of the required Standard Operating Procedures (SOPs), laboratory forms, and the laboratory layout plan (Figure 2), the full application for authorization was submitted to the Ankara Provincial Directorate of Agriculture and Forestry, under the authority of the Ankara Governorate.

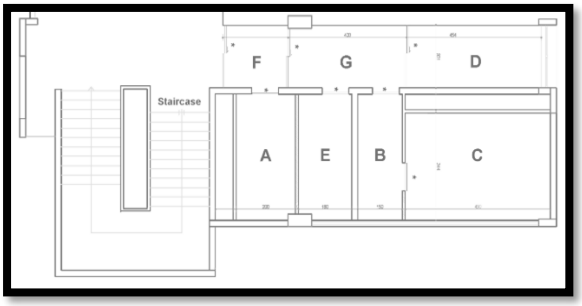


Figure 2. Schematic overview of the Laboratory infrastructure.
*A: Quarantine room, B: Care room, C: Breeding room, D: Examination and operation room, E: Feed storage room, F: Disinfection area, G: Corridor, *: Doors.*

Following the comprehensive evaluation of the submitted application dossier by the designated commission within the Directorate of Fisheries and Aquatic Products under the Ministry of Agriculture and Forestry of the Republic of Türkiye, an official on-site inspection of the facility was conducted in February 2023. As a result of this evaluation, the Aquatic Experimental Animal Research Center, established within Ankara University Faculty of Veterinary Medicine, was deemed compliant with regulatory standards and was formally approved for the issuance of an operational license.

Subsequently, the application fee mandated by the Directorate was transferred to the Ministry's revolving fund account, and the finalized species list for which authorization was requested was submitted to the relevant administrative body. The signing process for the official Work Permit was completed in April 2023, and the permit was formally issued to the institution on April 17, 2023 (Figure 3).

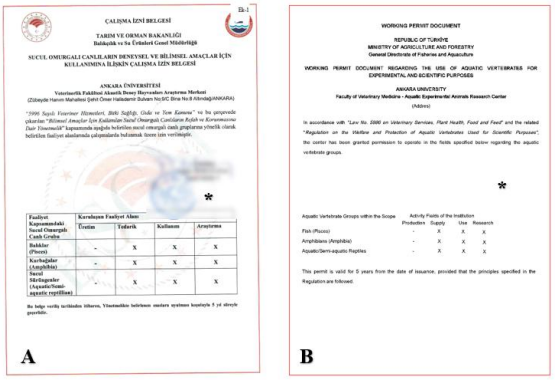


Figure 3. Approval Letter Issued by the General Directorate of Fisheries and Aquatic Products, Ministry of Agriculture and Forestry.
*A: original version, B: translated version, * Stamp, date, and signature.*

In March 2024, the Aquatic Experimental Animal Center underwent its first annual regulatory inspection, conducted by the Ankara Provincial Directorate of Animal Health. During the audit, a comprehensive overview of the Center's first-year activities was presented. All documentation required under the applicable legislation-including breeding records, health monitoring data, and routine quality control reports-was subjected to detailed examination. Based on the findings of this evaluation, it was determined that the institution remained in full compliance with regulatory expectations, and the continuation of its operational license was formally confirmed.

Zebrafish Reproductive and Embryological Research Unit

Equipment

The unit is comprehensively outfitted with specialized infrastructure and high-precision laboratory instrumentation to support zebrafish husbandry and experimental workflows. Core equipment includes a recirculating aquatic housing and breeding system (ZebTEC, Italy), a reverse osmosis water purification unit (Purelab Chorus 2), and ultrapure and distilled water systems (Millipore, Darmstadt, Germany), which together ensure optimal water quality and biosecurity. Microscopy capabilities are supported by a Euromex laboratory microscope, while incubation and sterilization needs are met by a laboratory-grade incubator (Lab Companion), an autoclave sterilizer (Panasonic MLS-3020U), and a programmable temperature-controlled incubator (Jeio Tech IB-11E, Korea).

Precision weighing and volumetric measurement are facilitated through a high-sensitivity analytical balance (Sartorius) and a complete set of adjustable-volume automatic micropipettes (Dlab, Omnipipette). Environmental control within the unit is maintained via a laboratory refrigerator (Panasonic), programmable lighting arrays (Philips), and an automated photoperiod timer (CATA), enabling regulation of circadian cues essential for zebrafish reproduction and embryogenesis. Additionally, a dedicated Artemia hatching system (CE-

PLAN Artemia Hatching Kit) is utilized for the production of live feed critical to larval rearing.

Chemicals

The unit is routinely supplied with high-purity, analytical-grade chemical reagents essential for zebrafish husbandry and experimental protocols. These include sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$, CAS No. 10039-32-4), potassium dihydrogen orthophosphate (KH_2PO_4, CAS No. 7778-77-0), calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, Product No. 12135), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, CAS No. 10034-99-8), and sodium bicarbonate ($NaHCO_3$, CAS No. 144-55-8), which are primarily used in the preparation and stabilization of aquatic media. To ensure accurate pH regulation and buffer formulation, a 1.0N sodium hydroxide solution (NaOH, Product Code: 145057, Reference: MB/26/616/M-552-05/6/16) is utilized. Additional reagents include sodium chloride (NaCl, CAS No. 7647-14-5) and potassium chloride (KCl, CAS No. 7447), both of which are essential for maintaining ionic balance in the aquatic environment. For toxicological and pharmacological studies, compounds such as 3,4-dichloroaniline (CAS No. 95-76-1) and dimethyl sulfoxide (DMSO, CAS No. 67-68-5) are employed as standard toxicants and solvent controls, respectively.

Procurement, Laboratory Transfer, and Quarantine of Adult Zebrafish Broodstock

Wild-type adult AB strain zebrafish, sourced from the Hacettepe University Laboratory Animal Research and Application Center (HÜDHAM), were transferred to the Aquatic Experimental Animal Research Center (ADHAM) of Ankara University Faculty of Veterinary Medicine, which had been established and officially licensed for scientific use under the project framework. In accordance with regulatory requirements, the animals were maintained under quarantine conditions for a period of 21 days. Upon successful completion of the quarantine, the broodstock were introduced into the automated recirculating zebrafish housing system (ZEBTEC, Italy) for acclimatization and continued use in experimental breeding protocols (Figure 4).



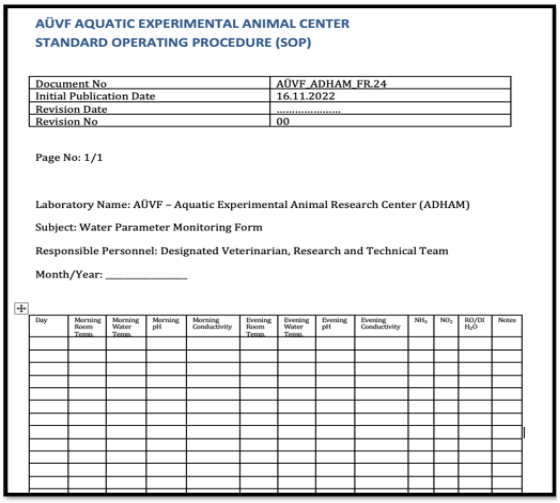
Figure 4. Automated zebrafish breeding system and parts (A: Breeding system, B: Control panel, C: Filtration system).

Zebrafish Facility Design, Tank Specifications, Environmental Conditions, Water Quality Monitoring, and Routine Husbandry Protocols

Adult zebrafish were housed in tanks with capacities ranging from 3.5 to 8 liters, depending on the number of individuals per group. Fish were grouped based on age to ensure uniform developmental stages within each tank. Photoperiod regulation essential for maintaining physiological rhythms was achieved through a lighting system set to 14 hours of light and 10 hours of darkness within the facility.

The ambient temperature was maintained at 28±1°C, with humidity stabilized at 61%, both controlled through an automated environmental system. The water pH was adjusted and stabilized within the range of 6.9 to 7.2. Water quality was preserved using an integrated filtration system comprising both mechanical and ultraviolet (UV) components.

In accordance with the manufacturer's recommended schedule automatic system sensor checks and tank cleaning were carried out routinely. Key environmental parameters, including pH, water temperature, conductivity, and ambient room temperature, were monitored daily and documented using a standardized recording form (Figure 5).



AÜVF AQUATIC EXPERIMENTAL ANIMAL CENTER
STANDARD OPERATING PROCEDURE (SOP)

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Revision No	00

Page No: 1/1

Laboratory Name: AÜVF – Aquatic Experimental Animal Research Center (ADHAM)

Subject: Water Parameter Monitoring Form

Responsible Personnel: Designated Veterinarian, Research and Technical Team

Month/Year: _____

Day	Morning Room Temp.	Morning Water Temp.	Morning pH	Morning Conductivity	Evening Room Temp.	Evening Water Temp.	Evening pH	Evening Conductivity	NH_3	NO_2	RO/DI H_2O	Notes

Figure 5. Water Parameter Monitoring Form.

Feeding Procedures

Feeding practices were implemented in accordance with the developmental stages of the zebrafish. Feed quantities were adjusted to ensure complete consumption within five minutes, thereby reducing waste and maintaining water quality. Adult fish were provided with a daily ration consisting of one feeding of dry feed and one feeding of live *Artemia nauplii* (brine shrimp). Juveniles, defined as individuals aged 15 days post-fertilization or older, were fed twice daily with dry feed and once daily with live food. Larvae received three feedings of dry feed and one feeding of *Artemia nauplii* per day (Figure 6).



Figure 6. Zebrafish nutrition by live and commercial feeds in the facility.

Embryos between 0 and 6 days post-fertilization were not externally fed, as they subsisted on endogenous yolk sac reserves. During this period, nutritional intake and development were monitored without exogenous feeding (Table 1).

Table 1. Weekly Feeding Schedule for Zebrafish

Day	Larval Feeding Time and Type				Juvenile Feeding Time and Type				Adult Feeding Time and Type			
Monday	10:00	-	Otohime	A1	10:00	-	Otohime	B1	10:00	-	Otohime	B2
	12:00	-	Otohime	A1	12:00	-	Otohime	B1	15:00	-	Artemia	
	14:00	-	Otohime	A1	15:00	-	Artemia					
	15:00	-	Artemia									
Tuesday	10:00	-	Otohime	A1	10:00	-	Otohime	B1	10:00	-	Otohime	B2
	12:00	-	Otohime	A1	12:00	-	Otohime	B1	15:00	-	Artemia	
	14:00	-	Otohime	A1	15:00	-	Artemia					
	15:00	-	Artemia									
Wednesday	10:00	-	Otohime	A1	10:00	-	Otohime	B1	10:00	-	Otohime	B2
	12:00	-	Otohime	A1	12:00	-	Otohime	B1	15:00	-	Artemia	
	14:00	-	Otohime	A1	15:00	-	Artemia					
	15:00	-	Artemia									
Thursday	10:00	-	Otohime	A1	10:00	-	Otohime	B1	10:00	-	Otohime	B2
	12:00	-	Otohime	A1	12:00	-	Otohime	B1	15:00	-	Artemia	
	14:00	-	Otohime	A1	15:00	-	Artemia					
	15:00	-	Artemia									
Friday	10:00	-	Otohime	A1	10:00	-	Otohime	B1	10:00	-	Otohime	B2
	12:00	-	Otohime	A1	12:00	-	Otohime	B1	15:00	-	Artemia	
	14:00	-	Otohime	A1	15:00	-	Artemia					
	15:00	-	Artemia									
Saturday	11:00	-	Otohime	A1	11:00	-	Otohime	B1	11:00	-	Otohime	B2
	14:00	-	Otohime	A1	14:00	-	Otohime	B1	14:00	-	Otohime	B2
Sunday	11:00	-	Otohime	A1	11:00	-	Otohime	B1	11:00	-	Otohime	B2
	14:00	-	Otohime	A1	14:00	-	Otohime	B1	14:00	-	Otohime	B2

Artemia Culture and Live Feed Preparation

Live feed in the form of *Artemia nauplii* was cultured daily by the research team to ensure optimal nutritional quality and bioavailability for zebrafish. Commercially sourced dormant *Artemia* cysts (Salt Lake Aquafeed Premium *Artemia* Cysts) were introduced into a conical hatching vessel containing saline water adjusted to a salinity of 14% (Figure 7A). The cysts were incubated under continuous aeration and constant illumination for 24 to 36 hours, in accordance with established hatching protocols (Figure 7B).

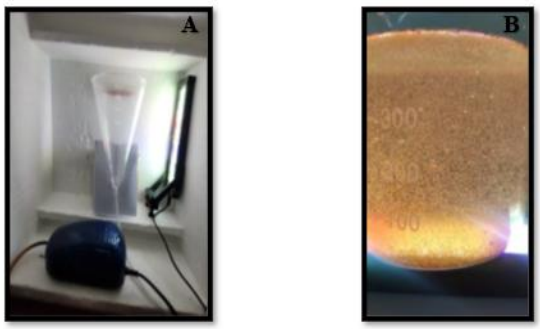


Figure 7. Cultivation of Artemia nauplii.
(A: Artemia separation funnel, B: Artemia larvae under illumination).

Upon completion of the incubation period, both unhatched cysts and newly hatched nauplii were collected through the bottom outlet of the separation funnel. The harvested material was passed through a fine mesh to remove excess salinity and debris. The nauplii were then resuspended in clean, oxygenated tank water to maintain viability and prevent osmotic stress. Hatched nauplii, which accumulated at the water surface, were gently siphoned and administered directly to the zebrafish as live feed.

Unhatched cysts were rinsed and returned to a freshly prepared hatching vessel for continued incubation. This procedure ensured a continuous supply of high-quality live feed. Feeding was performed daily in alignment with the stage-specific nutritional regimen outlined in the zebrafish feeding schedule.

Reproductive Classification Based on Morphological Traits

Phenotypic differentiation between male and female zebrafish (*Danio rerio*) was carried out by experienced personnel using established external morphological markers. Individuals with a slender, streamlined body shape and a yellow to golden hue were classified as males. In contrast, individuals exhibiting an enlarged abdominal profile-indicative of oocyte presence-and a prominent silvery-blue lateral stripe were classified as females. These visually distinguishable traits, representing clear sexual dimorphism, allowed for accurate non-invasive reproductive classification (Figure 8).

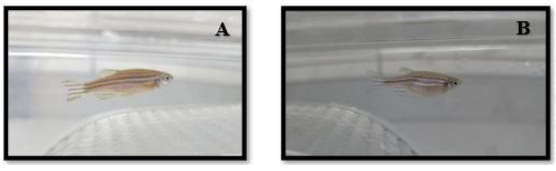


Figure 8. Adult Zebrafish.
A: Male, B: Female.

Breeding and Embryo Collection

Breeding was carried out using specially designed spawning tanks sourced from the manufacturer, optimized for egg collection and mating behavior. Adult males and females (6–12 months old) were placed in breeding tanks at a ratio of 1:2 or 5:8, depending on broodstock availability. The sexes were initially separated within the

tanks using dividers. Fish designated for breeding were fasted the day prior to spawning. In alignment with the circadian cycle, the broodstock were transferred into fresh, clean spawning tanks in the morning. The divider was removed to allow mating interactions, which were observed until sufficient egg deposition was achieved. Once adequate spawning had occurred, the broodstock were returned to their respective holding tanks. Fertilized eggs, which accumulated at the bottom of the spawning tank (Figure 9A), were carefully collected using a fine mesh strainer and transferred into sterile glass Petri dishes for further observation and incubation (Figure 9B).

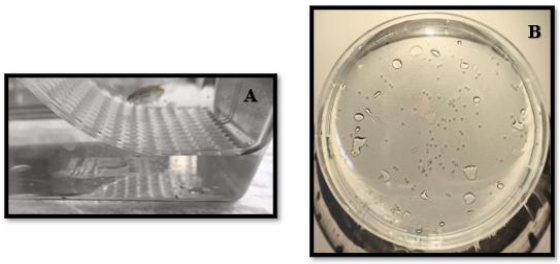


Figure 9. A: Embryos collected at the bottom of the spawning tank, B: Embryos transferred into petri dishes.

Embryo Collection and Handling

Eggs collected from the spawning tanks were rinsed with distilled water to remove debris and residual waste. Clean and intact embryos were transferred into embryo medium that had been freshly prepared the previous evening. Each embryo was individually assessed under a stereomicroscope to verify fertilization status and determine the developmental stage.

During microscopic examination, embryos exhibiting transparency were classified as viable (Figure 10A), while those with an opaque crescent-shaped appearance were considered non-viable or dead (Figure 10B). Non-viable embryos were separated and placed in a different Petri dish to avoid contamination.

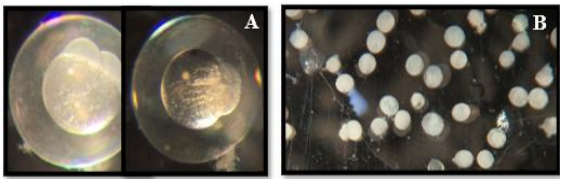


Figure 10. A: Fertilized zebrafish embryos, B: Coagulated (non-viable) zebrafish embryos.

Viable embryos were then transferred to an incubator pre-set to 28°C. Embryos and resulting larvae were maintained in the incubator for no more than seven days. Developmental progression was monitored daily under the microscope, and embryo media were refreshed three to five times per day to ensure optimal water quality and embryo viability.

CONCLUSION

As Türkiye's first faculty of veterinary medicine, the Faculty of Veterinary Medicine at Ankara University established the Aquatic Experimental Animal Research Center (ADHAM) with the dual aim of advancing academic training for both undergraduate and postgraduate students and providing a regulatory and procedural blueprint for the development of similar facilities across other institutions. Beyond serving as a zebrafish model laboratory, ADHAM was strategically conceptualized to address environmental toxicology within the scope of the One Health paradigm, offering a multidisciplinary research platform distinct from existing aquatic laboratories in the country.

In pursuit of this vision, a collaborative research team was formed, comprising experts from Physiology, Pharmacology-Toxicology, and Histology-Embryology. Together, they initiated work on embryotoxicity modeling, positioning the center at the intersection of veterinary biomedical science, environmental health, and regulatory toxicology. The establishment process, along with the outcomes achieved thus far, has laid a robust foundation that serves as both a national reference model and a scientific benchmark for faculties of veterinary medicine and related institutions across Türkiye.

The successful operationalization of the center is expected to contribute substantially to veterinary education, scholarly output, and interdisciplinary collaboration, while simultaneously providing a roadmap for future research in aquatic toxicology and animal welfare sciences. As veterinary surgeons and researchers, we recognize the critical importance of such infrastructure in generating translational knowledge and shaping evidence-based approaches to aquatic animal health.

In alignment with the short-term objectives (6 months to 1 year), the center has successfully developed zebrafish-based embryotoxicity models, submitted multiple institutional and inter-institutional research project applications, and initiated preparations for international scientific partnerships.

In the medium-term phase (1 to 3 years), the center has expanded its research capacity by incorporating alternative aquatic vertebrate models beyond zebrafish, launched scientific research services, and begun engaging with the private sector, particularly through educational offerings and initiatives aimed at establishing sustainable revenue streams via the university's revolving fund system.

Acknowledgement

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Ethical Declaration

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authorship contributions

Concept: E.Y., M.Y., F.G.A., S.S., S.N.A., A.G.B., H.Ö., Methodology: E.Y., M.Y., F.G.A., S.N.A., Writing of Original Draft: M.Y., F.G.A., Final Review and Editing: E.Y., M.Y., S.S., A.G.B., H.Ö.

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REFERENCES

CCAC. 2005. Guidelines on: the care and use of fish in research, teaching and testing. Canadian Council on Animal Care, Ottawa.

CCAC. 2020. CCAC guidelines: Zebrafish and other small, warm-water laboratory fish. Canadian Council on Animal Care, Ottawa.

CCAC. 2021. CCAC guidelines: Amphibians. Canadian Council on Animal Care, Ottawa.

EC. 2007. European Union Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (notified under document number C (2007) 2525).

EC. 2010. European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. Directive 2010/63/EU.

FELASA. 2007. Euroguide On the accommodation and care of animals used for experimental and other scientific purposes. Federation of European Laboratory Animal Science Associations, London, UK.

Habjan E, Schouten GK, Speer A, van Ulsen P, Bitter W. 2024. Diving into drug-screening: zebrafish embryos as an in vivo platform for antimicrobial drug discovery and assessment. FEMS Microbiology Reviews, 48(3), fuae011.

Hughes S, Hessel EV. 2024. Zebrafish and nematodes as whole organism models to measure developmental neurotoxicity. Critical Reviews in Toxicology, 54(5), 330-343.

Kiani AK, Pheby D, Henehan G, Brown R, Sieving P, Sykora P, Marks R, Falsini B, Capodicasa N, Miertus S, Lorusso L, Dondossola D, Tartaglia GM, Ergoren MC, Dundar M, Michelini S, Malacarne D, Bonetti G, Dautaj A, Donato K, Medori MC, Beccari T, Samaja M, Connelly ST, Martin D, Morresi A, Bacu A, Herbst KL, Kapustin M, Stuppia L, Lumer L, Farronato G, Bertelli M. 2022. Ethical considerations regarding animal experimentation. Journal of Preventive Medicine and Hygiene, 63(2 Suppl 3): E255.

NRC. 2011. Guide for The Care and Use of Laboratory Animals. 8th Ed. The National Academies Press, USA.

OECD. 2002. Environment, Health and Safety Publications Series on Testing and Assessment No. 23. Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD Publishing, Paris, France.

OECD. 2009. Test No. 231: The Amphibian Metamorphosis Assay. OECD Publishing, Paris, France.

OECD. 2015. Test No. 241: The Larval Amphibian Growth and Development Assay (LAGDA). OECD Publishing, Paris, France.

OECD. 2023. Test No. 248: Xenopus Eleutheroembryo Thyroid Assay (XETA). OECD Publishing, Paris, France.

The Ministry of Agriculture and Forestry of the Republic of Türkiye. 2019. Regulation Amending the Regulation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes.

The Ministry of Agriculture and Forestry of the Republic of Türkiye. 2022. Regulation Amending the Regulation on the Welfare and Protection of Aquatic Vertebrates Used for Scientific Purposes.

Yipel M. 2020. Zebrafish, In: Yarsan E. (Ed.), Fish Medicine (In Turkish), Güneş Tıp Kitapevi. Ankara, Türkiye, pp. 507-522.

Yipel M, İlhan, A. 2021. Zebrafish (*Danio Rerio*): Towardly Model Organisms for Toxicological Studies. Bulletin of Veterinary Pharmacology and Toxicology Association, 12(3): 161-167.

Yipel M, Altinok-Yipel F. 2025. Aquatic Animals in Experimental Studies, In: Yarsan E. (Ed.), Laboratory Animal Medicine (In Turkish). Güneş Tıp Kitapevi. Ankara, Türkiye, pp. 327-356.