

Investigation of the Effects of Partial Estrogen Antagonist Tamoxifen and Aromatase Inhibitor Letrozole on the Endometrium in Rats and Determination of the Effects in Combination with Nonselective Cox Inhibitor Acetylsalicylic Acid (Aspirin)

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Abstract

Objective: This study investigates the effects of tamoxifen, a selective estrogen receptor modulator, and letrozole, an aromatase inhibitor, on the endometrium in a rat model. It further evaluates the combined impact of these drugs with acetylsalicylic acid (aspirin), a non-selective COX inhibitor, through immunohistochemical and biochemical analyses, aiming to compare endometrial alterations and the potential mitigating effects of aspirin. **Methods:** Seventy female rats were divided into seven groups receiving tamoxifen, letrozole, their combinations with aspirin, and control treatments. Endometrial tissues were analyzed for VEGF, COX-2, Ki-67, BCL-2, and PECAM-1 expression using immunohistochemistry, while serum VEGF-C levels were quantified via

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ELISA. Statistical comparisons were conducted across groups. **Results:** Tamoxifen significantly increased VEGF and COX-2 expression in the endometrium compared to controls, whereas aspirin co-administration significantly reduced these levels. No significant Ki-67 expression changes were observed in the glandular epithelium or stroma. Tamoxifen did not affect BCL-2 expression in the epithelium but decreased stromal expression. PECAM-1 expression remained unchanged across groups, and tamoxifen-induced serum VEGF-C elevation was not statistically significant. **Conclusions:** Tamoxifen increases VEGF and COX-2 expression, potentially contributing to endometrial pathologies, while aspirin co-administration effectively mitigates these effects, suggesting its protective role against uterine complications. Letrozole demonstrated minimal endometrial impact, reinforcing its favorable safety profile. The combination of tamoxifen and aspirin may represent a viable strategy to alleviate endometrial side effects in clinical applications.

Keywords

Selective Estrogen Receptor Modulator (SERM), Tamoxifen, Aspirin, Uterus, Endometrial Pathologies

1. Introduction

Breast cancer accounts for one-quarter of all cancer types in women and ranks third in cancer-related mortality, following lung and colorectal cancers [1]. Globally, over one million women are diagnosed with breast cancer annually, and approximately 650,000 women lose their lives to this disease. Until 2022, breast cancer was the leading cause of cancer-related deaths, but advancements in early-stage diagnosis and adjuvant therapy protocols after surgery have relegated it to the third position. Among these advancements, the introduction of tamoxifen for clinical use in 1973 is particularly notable [2] [3]. Tamoxifen, a synthetic non-steroidal antiestrogen agent, plays a pivotal role in the hormonal treatment of breast cancer and is recognized as the progenitor of selective estrogen receptor modulators (SERMs) [4] [5]. While tamoxifen exerts antiestrogenic effects on breast tissue, it exhibits estrogenic effects on other tissues such as serum lipids, bone, and endometrium, thereby increasing the risk of developing endometrial pathologies in patients using tamoxifen [6]. This paradoxical tissue-specific action is due to its partial estrogen agonist activity in the uterus, where it can stimulate endometrial proliferation and angiogenesis, potentially leading to hyperplasia and malignancy. Numerous studies have reported various endometrial pathologies associated with tamoxifen use. Interestingly, different regions of the endometrial cavity respond differently to tamoxifen. Ye *et al.* examined the effect of tamoxifen use on endometrial cancer risk in postmenopausal women with endometrial polyps.

Among 675 women undergoing hysteroscopic polypectomy, tamoxifen users ($n = 169$) had a cancer prevalence of 4.14%, while non-users ($n = 506$) had 8.1%, with no significant difference ($P = 0.083$). Multivariate analysis showed that tamoxifen was not an independent risk factor. However, abnormal uterine bleeding, age over 60, and nulliparity increased the risk in both groups. The study concluded that tamoxifen does not raise cancer risk but highlighted the need for careful monitoring of women with these risk factors [7]. However, the primary concern is the increased risk of endometrial cancer in women using tamoxifen. Studies have demonstrated that the risk of endometrial cancer is 2 - 3 times higher in patients who use tamoxifen for more than two years and up to five times higher in those using it for more than five years [8]. This risk is attributed to the estrogenic and proliferative effects of tamoxifen on the endometrium, highlighting the need for effective and well-tolerated alternative therapies to tamoxifen. Selective aromatase inhibitors have emerged as a approach in the endocrine treatment of advanced breast cancer [9]. Letrozole, a synthetic benzimidazole derivative, and a highly selective non-steroidal aromatase inhibitor is used as a first-line treatment option for advanced breast cancer as an alternative to tamoxifen [10]. Clinical studies have shown that letrozole surpasses tamoxifen in terms of clinical benefit, objective response rate, and progression-free survival [11]. Based on these findings, letrozole has been recommended as a preferred first-line treatment for metastatic breast cancer. Epidemiological studies have observed a reduction in breast cancer incidence among women using non-steroidal anti-inflammatory drugs (NSAIDs), suggesting that these effects may be related to the NSAIDs themselves. NSAIDs exert their effects by inhibiting cyclooxygenase (COX) enzymes in target tissues. COX-2 plays a significant role in cancer development and progression [12]-[14]. Elevated COX-2 levels are associated with poor prognostic characteristics in many cancer types, particularly solid tumors such as breast cancer. Studies have demonstrated that increased COX-2 levels are linked to tumor cell proliferation, angiogenesis, aromatase induction, and metastatic potential [15]. Consequently, research has increasingly focused on the therapeutic and preventive use of COX-2 inhibitors in breast cancer. Prolonged exposure to estrogen, especially in post-menopausal women, is a well-established risk factor for breast cancer. Some studies have shown that COX-2 enzyme activity in breast cancer enhances tissue aromatase enzyme activity through prostaglandins, leading to increased estrogen synthesis [16] [17]. Given the severe side effects of tamoxifen, the development of novel therapeutic models to mitigate these adverse effects has become increasingly important. Aromatase inhibitors, which do not cause endometrial side effects, have emerged as promising alternatives in breast cancer treatment. This study aimed to compare the histopathological changes induced by tamoxifen and letrozole in the rat uterus, including changes in angiogenesis, apoptosis, cell proliferation, COX-2, VEGF expression, and serum VEGF-C levels. Additionally, the study investigated the potential changes caused by combining these drugs with aspirin, a non-selective COX inhibitor.

2. Materials and Methods

2.1. Animal Model and *in Vivo* Experimental Conditions

This study utilized adult nulliparous female Sprague Dawley rats weighing 200 - 250 grams. The rats were sourced from the Medical and Surgical Experimental Research Center (TİCAM) of Eskişehir Osmangazi University Faculty of Medicine, Turkey, and were housed under controlled care at the center until the start of the experiment. The Ethics Committee of Eskişehir Osmangazi University Faculty of Medicine approved the experimental procedures under protocol number 89 and dated 17/12/2008. The rats were maintained under standard environmental conditions, including a 12-hour light/dark cycle, a temperature of $24 \pm 2^{\circ}\text{C}$, and a humidity level of $55 \pm 5\%$. They underwent a one-week acclimatization period before the experimental procedures. Daily feeding and hydration needs were met, and the health status of the animals was regularly monitored. The body weights of the rats were measured at the beginning and end of the experiment, while uterine weights were recorded after the study.

To simulate a postmenopausal hormonal environment and eliminate endogenous estrogen influence, all animals underwent bilateral oophorectomy before drug administration. This surgical approach is a well-established method for creating a postmenopausal model in experimental studies, ensuring that the observed endometrial effects result from the administered compounds rather than native hormonal fluctuations.

The animals were randomly assigned to seven groups of 10 rats. A total of 70 rats were included in the study; however, due to the death of one rat during the experiment, the study was completed with 69 subjects. This comprehensive design ensured robust data collection and adherence to ethical standards.

Table 1. Experimental groups and treatment protocols.

Group Name	Number of Animals (n)	Dose Administered	Administration Method	Duration of Treatment
Control (Saline) Group	10	1 ml/kg/day saline	Intraperitoneal (i.p.)	30 days, 07:00 - 09:00
Control (DMSO) Group	10	1 mg/200g rat weight/day DMSO	Intraperitoneal (i.p.)	30 days, 07:00 - 09:00
Tamoxifen Group	10	1 mg/200g rat weight/day Tamoxifen	Intraperitoneal (i.p.)	30 days, 07:00 - 09:00
Letrozole Group	10	0.1 mg/200g rat weight/day Letrozole	Intraperitoneal (i.p.)	30 days, 07:00 - 09:00
Aspirin Group	10	2 mg/200g rat weight/day Aspirin	Intraperitoneal (i.p.)	30 days, 07:00 - 09:00
Tamoxifen + Aspirin Group	10	1 mg/200g rat weight/day Tamoxifen + 2 mg/200g Aspirin	Intraperitoneal (i.p.)	30 days, 07:00 - 09:00
Letrozole + Aspirin Group	9	0.1 mg/200g rat weight/day Letrozole + 2 mg/200g Aspirin	Intraperitoneal (i.p.)	30 days, 07:00 - 09:00

Table 1 summarizes the experimental groups, the number of animals in each group, the administered doses, the administration method, and the duration of treatment. Each group received the designated intraperitoneal treatments consistently at the same time daily, between 07:00 and 09:00, for 30 days. During the experimental period, one animal was lost in the Letrozole + Aspirin Group. A pilot study was conducted to assess the feasibility of the experimental procedures before initiating the main study. Bilateral oophorectomy was performed under anesthesia, and postoperative care was provided for 30 days. In the final stage of the experiment, the rats were fasted for 18 hours, and water was withheld for 2 hours before the final procedures to standardize the study conditions.

2.2. Chemical Substances and Administration

The experimental design included the administration of specific chemical substances; each selected for its relevance to the study's objectives. Tamoxifen was administered intraperitoneally at a dose of 1 mg per 200 g rat body weight daily (Sigma Chemical Co., St. Louis, USA), based on previous studies demonstrating this dose effectively induces uterine and systemic estrogenic responses in ovariectomized rat models [18] [19]. Letrozole was administered intraperitoneally at a dose of 0.1 mg per 200 g rat body weight daily (Sigma Chemical Co., St. Louis, USA), a dose validated in prior studies for achieving significant aromatase inhibition and endocrine effects in rodents [20] [21]. Aspirin was administered intraperitoneally at 2 mg per 200 g rat body weight daily (Sigma Chemical Co., St. Louis, USA), consistent with previous *in vivo* studies that used low-dose aspirin to evaluate anti-inflammatory and COX-inhibitory effects in rats [22] [23].

To ensure solubility and standardization, Dimethyl Sulfoxide (DMSO) was employed as a solvent across all experimental groups at a consistent dose of 0.02 ml per animal (Sigma Chemical Co., St. Louis, USA). All injections were conducted using sterile, separate syringes for each group and administered at the same time each day to minimize variability (07:00 - 09:00).

Anesthesia for pre-euthanasia procedures was induced via an intraperitoneal injection of a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg), ensuring a humane and standardized process. The doses and administration routes were determined based on prior pilot studies and standardized protocols to ensure reproducibility and reliability of the experimental outcomes.

2.3. Intraoperative Macroscopic Evaluation and Uterus Removal

At the final stage of the experiment, full anesthesia was achieved by administering a high dose of ether. Following anesthesia, intracardiac blood samples were collected from each subject and transferred into appropriate tubes for biochemical analysis. During the blood sampling procedure, sterility was meticulously maintained, and all steps were performed according to standard biosafety protocols. After blood collection, euthanasia was performed using the cervical dislocation

method. This technique was selected as a rapid and humane method to ensure animal welfare.

Subsequently, the animals underwent laparotomy. During this surgical procedure, abdominal incisions were made using sterile surgical instruments, and the layers of the abdominal wall were carefully separated to access the uterine tissues. After removing the uterine tissues, macroscopic observations were made, and findings were recorded for each group. These observations included assessments of uterine discoloration, abnormal growths, cysts, or polyps as pathological indicators. The uterine tissues were then carefully excised, and the surrounding fat tissues were meticulously cleaned to prepare the samples. The prepared uterine tissues were weighed using a precision scale, and the weights were recorded. Uterine weights were considered an important parameter for evaluating differences among experimental groups. All samples were maintained under sterile conditions during the weighing process to preserve tissue integrity. After recording the weights, the excised tissues were immediately fixed in 10% formalin to preserve their structural integrity for subsequent biochemical and histological analyses. This fixation step ensured optimal conditions for detailed histological evaluations.

2.4. Tissue Processing and Immunohistochemical Staining

The extracted uterine tissues were fixed in 10% formalin to preserve their structural integrity and prepare them for subsequent histological analyses. Samples containing all tissue layers (endometrium, myometrium, and serosa) were collected from each uterine horn. Following fixation, the tissues were sectioned into 4-micron-thick slices using a microtome. These sections were stained with hematoxylin-eosin (H&E) and evaluated under a light microscope for histological assessment. After the initial histological examination, new sections were prepared for immunohistochemical analysis using the antibodies listed in **Table 2**. The Avidin-Biotin Complex (ABC) method was used to perform the immunohistochemical staining procedure. The sections were first immersed in distilled water for 1 minute and then treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Following two washes with PBS (pH 7.4, 2×3 minutes), the sections were incubated in a blocking solution for 5 minutes. Primary antibodies were applied for 60 minutes, followed by PBS washes, and treated with a link solution and streptavidin consecutively. The reaction was developed with AEC chromogen, and the sections were counterstained with hematoxylin, rinsed in distilled water, air-dried, and mounted using an aqueous mounting medium. The prepared slides were examined under a light microscope by three independent observers, and results were classified based on staining intensity as negative (0), weak (1), moderate (2), or intense (3). Evaluations were performed in specific regions, including luminal and glandular epithelium, stromal tissue, vascular endothelium, and myometrium. Findings were recorded for comparative analysis among the experimental groups.

Table 2. Antibodies used for immunohistochemical staining.

ANTIBODY	CLONE	DILUTION RATIO	SUPPLIER	TARGET TISSUE AND LAYERS
COX-2	SP21	OCA.50	NEOMARKERS, USA	EPITHELIUM, STROMA, VASCULAR TISSUE
Ki-67	SP6	1/150	SPRINGBIOSCIENCE, USA	NUCLEAR STAINING
BCL-2	C21	OCA.50	SANTACRUZ, USA	EPITHELIUM AND STROMA
PECAM-1	M-20	1/100	SANTACRUZ, USA	VASCULAR ENDOTHELIUM
VEGF	POLYCLONAL	1/150	NEOMARKERS, USA	VASCULAR AND STROMAL REGIONS

2.5. Evaluation of Immunoreactivity

The immunohistochemical evaluation was conducted to assess the expression levels of VEGF, COX-2, BCL-2, Ki-67, and PECAM-1 in uterine tissues. The analysis focused on specific regions, including the luminal and glandular epithelium, stromal tissue, vascular endothelium, and myometrium. Staining intensity was classified into four categories based on visual scoring under a light microscope: negative (0), weak (1), moderate (2), and intense (3). Three observers independently evaluated each sample, and any discrepancies in scoring were resolved through consensus to ensure reliability and minimize observer bias. For Ki-67, sections were scanned at low magnification ($\times 100$) to identify areas with the highest intensity of nuclear staining. These regions were analyzed at $\times 200$ magnification, and the percentage of cells with positively stained nuclei was calculated. The final score represented the average percentage of positive cells from the three regions with the most intense staining (hot spots). For PECAM-1, vascularization in the stromal regions was assessed by identifying three hot spots with the most prominent vascular structures under low magnification ($\times 100$). The number of positively stained blood vessels was counted at $\times 400$ magnification, and the highest value among the three regions was recorded as the representative score.

The results were systematically documented for comparative analysis among the experimental groups and to investigate potential correlations between staining intensities and treatment protocols.

2.6. Biochemical Analysis

Serum VEGF-C levels were quantified using the RAT VEGF-C Immunoassay Kit (Catalog No: KRG0111, Invitrogen) following the manufacturer's instructions. The biochemical analyses were performed at the ESOGÜTF Biochemistry Laboratory. Blood samples collected during the experimental procedures were centrifuged at 3000 rpm for 10 minutes to isolate the serum, which was aliquoted into sterile tubes and stored at -80°C for future analysis. Prior to the assay, serum samples were thawed at room temperature for 24 hours to ensure consistency. Reagent preparation and assay setup were conducted following the manufacturer's instructions to ensure consistency and accuracy in the measurements. A wash buffer was prepared by diluting 50 ml of the concentrate with 950 ml of distilled water. VEGF-C standards were prepared through serial dilution to gen-

erate a calibration curve for quantifying the serum levels. Biotin conjugate was diluted at a 1:100 ratio using the assay buffer, while Streptavidin-HRP was prepared at a 1:400 dilution with the same buffer. These steps were performed under standardized laboratory conditions to maintain the integrity of the reagents and ensure reliable assay performance.

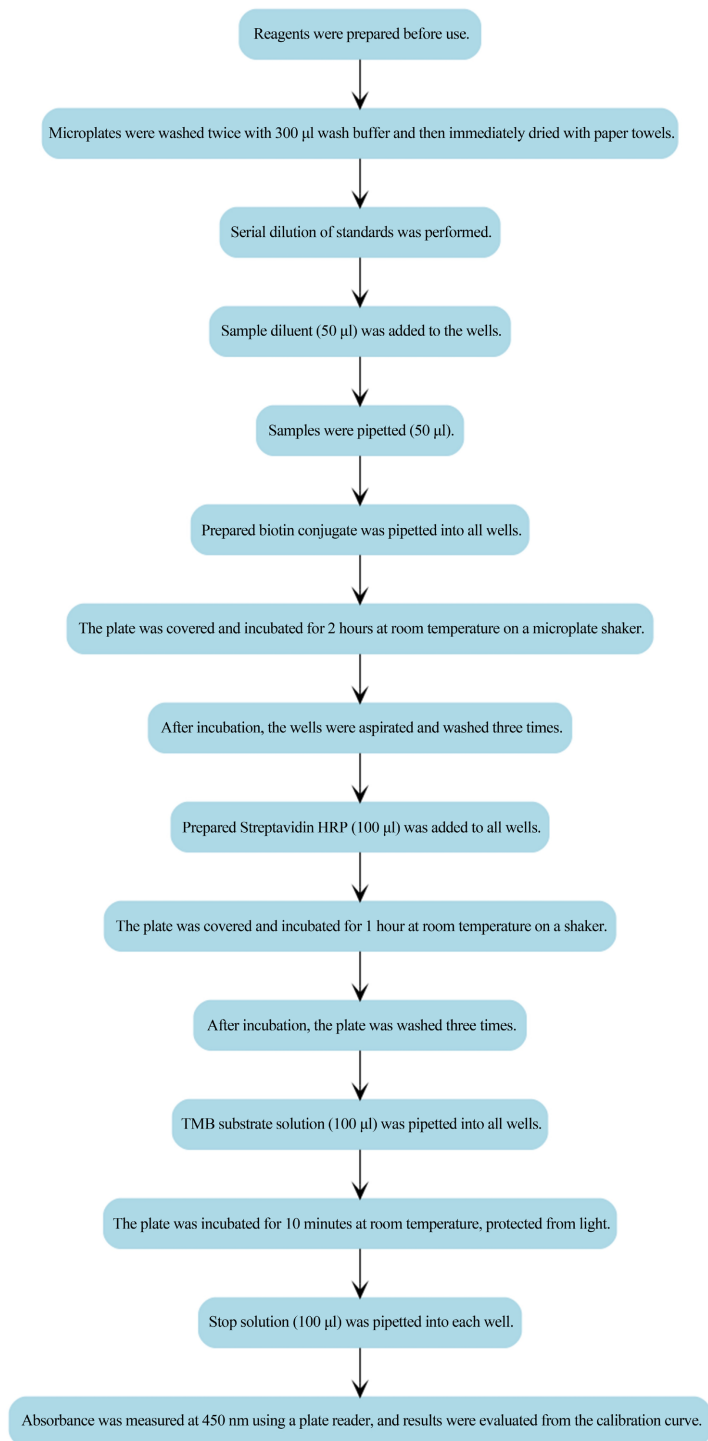


Figure 1. Workflow of the RAT VEGF-C immunoassay procedure.

The immunoassay workflow involved multiple steps to ensure precision and accuracy in VEGF-C quantification, as summarized in **Figure 1**. Each step, from reagent preparation to the final absorbance reading, was executed under controlled conditions to maintain the consistency and reliability of the assay results.

2.7. Statistical Analysis

All statistical analyses were conducted using SPSS (Statistical Package for Social Sciences, version 13.0). The data were first tested for normality using the Kolmogorov-Smirnov test to determine whether parametric or non-parametric methods would be appropriate. Descriptive statistics were presented as median with interquartile range (25th and 75th percentiles) and mean $\pm$ standard error, depending on the distribution of the data. For comparisons of body weights and uterine weights before and after treatments within the same groups, a paired t-test was employed for normally distributed data, while the Wilcoxon signed-rank test was used for non-normally distributed data. One-way analysis of variance (ANOVA) was performed to evaluate differences among experimental groups, followed by Tukey's multiple comparison test for post hoc analysis to identify specific group differences. The Kruskal-Wallis test was applied to assess differences among groups for immunohistochemical scoring and non-parametric data, and the Mann-Whitney U test was used for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant for all analyses. The results of statistical evaluations were summarized in appropriate tables and figures, with annotations to highlight significant differences between groups.

3. Results

Body Weight Changes

At the beginning of the experiment, the subjects' body weights were recorded. Following a thirty-day postoperative care and monitoring period, daily injections were administered for 30 days. At the end of the injection period, the subjects were weighed again, and the uterine tissues were collected via laparotomy. The uterine weights were measured with a precision scale. Macroscopic intraoperative findings revealed significant variations among the experimental groups. In the SF group, the uteri appeared pale and atrophic, with thin cornua, normal vascularization, minimal intra-abdominal adhesions, and widespread fat planes surrounding the uterus. The DMSO group exhibited normal uterine morphology, normal vascularization, and an absence of intra-abdominal adhesions. The Tamoxifen group displayed hypertrophic uteri with shortened cornua, increased vascularization of surrounding tissues, and prominent intra-abdominal adhesions. In the Letrozole group, the uteri appeared atrophic with thinned and elongated cornua, and two subjects presented bladder stones completely filling the bladder, a finding absent in other groups, with minimal intra-abdominal adhesions. The Aspirin group exhibited hypertrophic uteri with increased vascularization in the surrounding fat planes, no intra-abdominal adhesions, and easy access to pelvic or-

gans. In the tamoxifen + Aspirin group, severe intra-abdominal adhesions (frozen pelvis) were observed, along with atrophic uteri and elongated cornua. The Letrozole + aspirin group displayed moderate intra-abdominal adhesions and atrophic uteri with thinned cornua. These findings highlight the differential effects of treatments on the macroscopic characteristics of the uterine tissues and surrounding structures.

Table 3. Comparison of pre-oophorectomy (0 months) and post-injection (2 months) weights of subjects by groups (mean $\pm$ standard error).

Experimental Groups	Weights Before Oophorectomy (g)	Post-Injection Weights (g)	T Statistic, p
SF	221.6 $\pm$ 4.3	239.9 $\pm$ 9.1	T = -2.137, p > 0.05
DMSO	222.6 $\pm$ 4.6	236.7 $\pm$ 4.1	T = -3.154, p < 0.05
Tamoxifen	230.0 $\pm$ 4.2	215.3 $\pm$ 6.6	T = 1.842, p > 0.05
Letrozole	241.5 $\pm$ 7.1	250.1 $\pm$ 4.6	T = -1.130, p > 0.05
Aspirin	240.2 $\pm$ 7.3	244.3 $\pm$ 6.6	T = -0.485, p > 0.05
Tamoxifen + Aspirin	245.4 $\pm$ 7.0	227.8 $\pm$ 5.9	T = 2.342, p < 0.05
Letrozole + Aspirin	235.0 $\pm$ 6.9	238.6 $\pm$ 9.3	T = -0.318, p > 0.05

[Weights Before Oophorectomy (0 Months): F = 2.39, p > 0.05; Post-Injection Weights (2 Months): F = 2.89, p > 0.05. F = One-way analysis of variance statistics, T = Paired t-test].

The body weights recorded before oophorectomy (0 months) and after injections (2 months) are presented in **Table 3**. Significant weight changes were observed within the DMSO and the Tamoxifen + Aspirin groups, showing statistically significant differences between pre- and post-injection weights (p < 0.05). However, no statistically significant differences in body weight were noted among the groups at either 0 or 2 months when evaluated by one-way ANOVA (p > 0.05). At 2 months post-injection, the Tamoxifen group exhibited lower body weights compared to the Letrozole and Aspirin groups, although these differences were not statistically significant. Among all groups, the most pronounced weight loss was observed in the tamoxifen + Aspirin group, which demonstrated a statistically significant reduction in body weight compared to its baseline and to the other experimental groups at 2 months (p < 0.05).

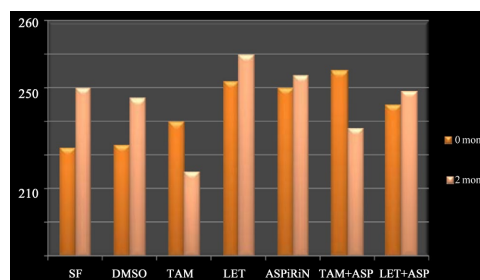


Figure 2. Comparison of pre-oophorectomy (0 months) and post-injection (2 months) body weights across experimental groups.

Figure 2 illustrates the body weights of experimental groups before oophorectomy (0 months) and after 30 days of injections (2 months). The bar chart demonstrates weight changes in each experimental group, highlighting intergroup differences and within-group variations over time. The DMSO group and the tamoxifen + Aspirin group showed significant differences in body weight between 0 and 2 months, with the tamoxifen + aspirin group exhibiting the most pronounced weight loss post-injection. The Tamoxifen group, despite showing a reduction in body weight compared to the Letrozole and Aspirin groups, did not reach statistical significance. These trends indicate that the combined treatment of tamoxifen and aspirin had a notable effect on reducing body weight, likely reflecting the physiological impact of these interventions on metabolism and tissue response during the experimental period. The chart effectively visualizes the comparative impact of different treatments, supporting the statistical findings outlined in **Table 3**.

Table 4 presents the uterine dry weights (g) of subjects in each experimental group. The results indicate no statistically significant differences among the groups ($F = 1.58$, $p > 0.05$), suggesting that the interventions did not produce a measurable effect on the uterine dry weight. The SF group and tamoxifen group showed slightly higher mean uterine weights (1.25 ± 0.07 g and 1.25 ± 0.03 g, respectively), whereas the DMSO group and Letrozole + Aspirin group exhibited lower values (1.06 ± 0.08 g and 1.08 ± 0.07 g, respectively). Despite these differences in mean values, the lack of statistical significance implies that the treatments had no substantial impact on the uterine dry weight across the experimental groups. This uniformity in uterine weights across groups aligns with the absence of treatment-specific effects observed in the statistical analysis.

Table 4. Comparison of uterine dry weights across experimental groups (mean $\pm$ standard error).

Experimental Groups	Uterine Dry Weight (g)
SF	1.25 ± 0.07
DMSO	1.06 ± 0.08
Tamoxifen	1.25 ± 0.03
Letrozole	1.13 ± 0.09
Aspirin	1.24 ± 0.08
Tamoxifen + Aspirin	1.24 ± 0.05
Letrozole + Aspirin	1.08 ± 0.07

[F (ANOVA): 1.58; p-value: $p > 0.05$; F = One-way analysis of variance statistics; T = Paired t-test].

Figure 3 depicts histopathological findings from the SF group, highlighting distinct structural and inflammatory changes. In **Figure 3(A)**, the luminal and glandular epithelium appears low columnar, with pigment-laden histiocytes sparsely

distributed in the stromal tissue, suggesting localized alterations. In **Figure 3(B)**, neutrophil clusters are evident within the glandular lumens, indicative of an acute inflammatory response.

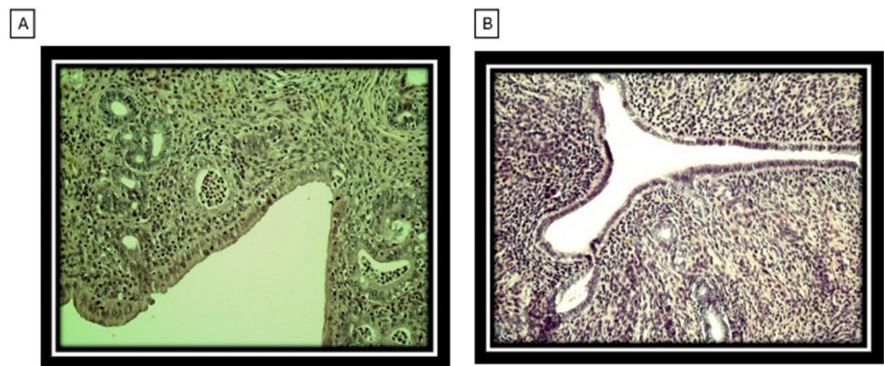


Figure 3. Histopathological findings in the SF Group. (A) Low columnar luminal and glandular epithelium with pigment-laden histiocytes in the stroma (H&E $\times 200$). (B) Neutrophil clusters within glandular lumens indicate acute inflammation (H&E $\times 200$).

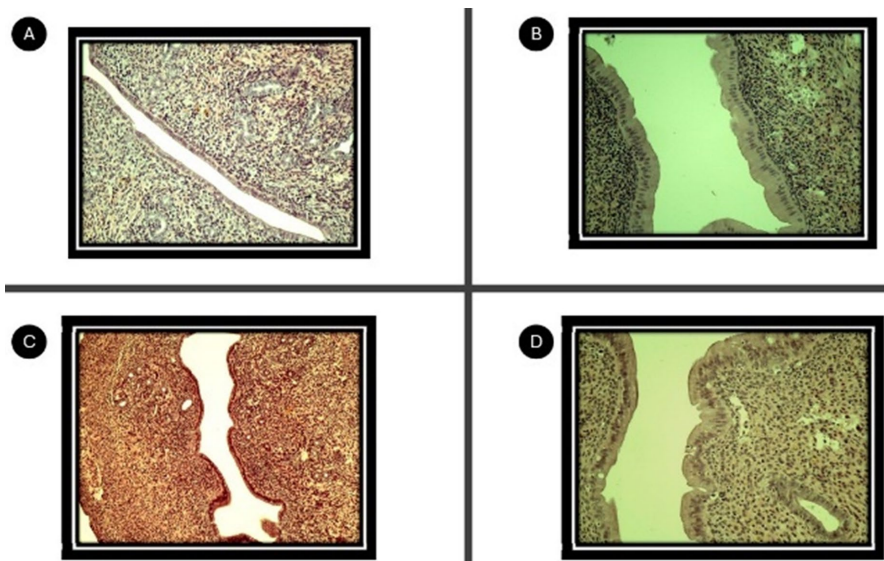


Figure 4. Comparative histopathological analysis of endometrial tissues across experimental groups. (A) DMSO Group: Low columnar luminal epithelium with fibrotic stroma and mild inflammatory infiltration. (B) Tamoxifen Group: High columnar luminal epithelium with eosinophilic cytoplasm and prominent inflammatory changes. (C) Letrozole Group: High columnar luminal epithelium with tubular glands and predominantly fibrotic stroma. (D) Aspirin Group: Luminal epithelium with variable columnar characteristics and pigment-laden histiocytes in the stroma.

The histopathological examination of the endometrial tissues across the experimental groups revealed distinct structural and inflammatory changes, as illustrated in **Figures 4(A)-(D)**. In the DMSO group (**Figure 4(A)**), the luminal epithelium was predominantly low columnar, and the stroma alternated between fibrotic and cellular characteristics, with occasional eosinophils and neutrophils ob-

served, indicating mild inflammation. In the Tamoxifen group (**Figure 4(B)**), the luminal epithelium was high columnar with abundant eosinophilic cytoplasm, accompanied by squamous metaplasia in some cases and neutrophil infiltration in the stroma and glands, reflecting significant epithelial and inflammatory activity. The Letrozole group (**Figure 4(C)**) displayed high columnar luminal epithelium, tubular glands lined with cuboidal epithelium, and predominantly fibrotic stroma, with neutrophil clusters observed in a single case, suggesting localized inflammation. In the Aspirin group (**Figure 4(D)**), the luminal epithelium varied between low and high columnar, with eosinophilic cytoplasm, tubular glands lined by low columnar epithelium, and pigment-laden histiocytes and eosinophils in the fibrotic stroma, indicating subtle inflammatory changes.

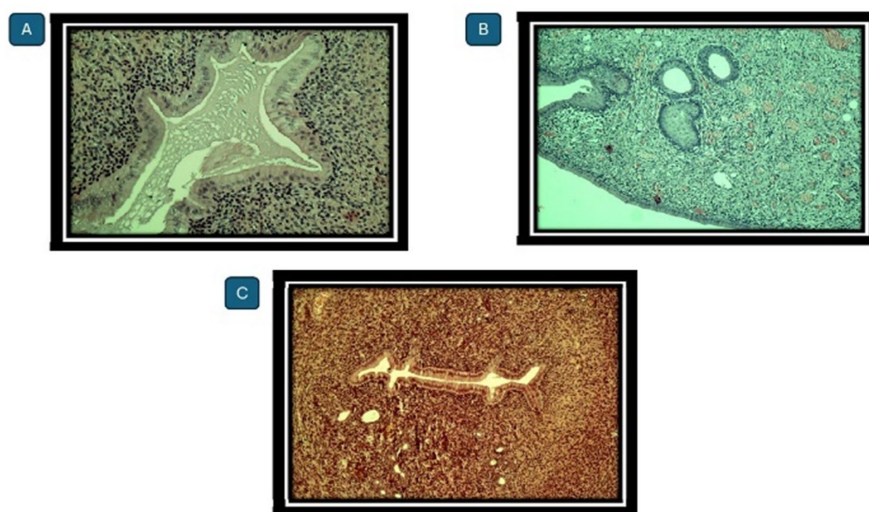


Figure 5. Histopathological features of Tamoxifen + Aspirin and Letrozole + Aspirin Groups. (A) Tamoxifen + Aspirin Group: The endometrial luminal epithelium is high columnar with eosinophilic cytoplasm. Tubal metaplasia is observed, and the stroma contains increased connective tissue, eosinophils, and pigments. Endometrial polyps are visible with vascular-rich regions (H&E $\times 100$). (B) Tamoxifen + Aspirin Group: Squamous metaplasia of the glandular epithelium is observed in vascular-rich endometrial polyps (H&E $\times 100$). (C) Letrozole + Aspirin Group: The luminal epithelium is high columnar, and the glandular epithelium is cuboidal with tubular organization. The stroma is mildly fibrotic in some cases, cellular in others, with a small number of neutrophils and occasional squamous metaplasia (H&E $\times 200$).

Figure 5 illustrates the histopathological changes observed in the tamoxifen + Aspirin and Letrozole + Aspirin groups, highlighting distinct structural and inflammatory alterations. In the Tamoxifen + Aspirin group, the luminal epithelium is high columnar with abundant eosinophilic cytoplasm, and the glandular epithelium demonstrates squamous metaplasia, as seen in vascular-rich endometrial polyps (**Figure 5(A)**). Tubal metaplasia is observed in one case, and the stroma shows a mild increase in connective tissue with scattered eosinophils and pigment deposition, indicating notable structural remodeling. An endometrial polyp with rich vascularity is also evident (**Figure 5(B)**), further reflecting significant epithelial

lial and stromal modifications induced by the combined treatment. In the Letrozole + Aspirin group, the luminal epithelium is high columnar, and the glandular epithelium retains a cuboidal, tubular architecture, with the stroma displaying mild fibrosis in four cases and cellular characteristics in others (**Figure 5(C)**). Minimal inflammatory activity is noted, with rare neutrophils observed in two cases and squamous metaplasia present in one case, indicating a comparatively less pronounced but distinct response to the treatment. The immunohistochemical findings of BCL-2 expression across different experimental groups are summarized in **Table 4** and visualized in **Figures 6(A) and 6(B)**. Significant differences in stromal cell staining intensity were noted between specific groups. The Aspirin group exhibited significantly higher stromal cell staining compared to the Tamoxifen-Aspirin (A, $p < 0.05$) and Tamoxifen groups (B, $p < 0.05$). Similarly, significant differences were observed between the DMSO and Tamoxifen-Aspirin groups (C, $p < 0.05$), as well as the DMSO and Tamoxifen groups (D, $p < 0.05$). In contrast, no significant differences in BCL-2 expression were detected in the luminal and glandular epithelium across the groups ($p > 0.05$), and no staining was observed in the vascular endothelium or myometrium in any group. Notably, tamoxifen significantly reduced BCL-2 expression in stromal cells. Letrozole and aspirin, however, did not show alterations in BCL-2 expression compared to the control group. Furthermore, the combination of tamoxifen or letrozole with aspirin did not result in changes in BCL-2 expression compared to the use of these agents alone. **Figure 6(A)** represents the Tamoxifen group, where no staining was observed in the luminal and glandular epithelium. In contrast, **Figure 6(B)** shows the Tamoxifen-Aspirin group, demonstrating weak positivity for BCL-2 in the luminal and glandular epithelium. These findings suggest that while tamoxifen modulates stromal BCL-2 expression, its combination with aspirin does not yield additive or synergistic effects.

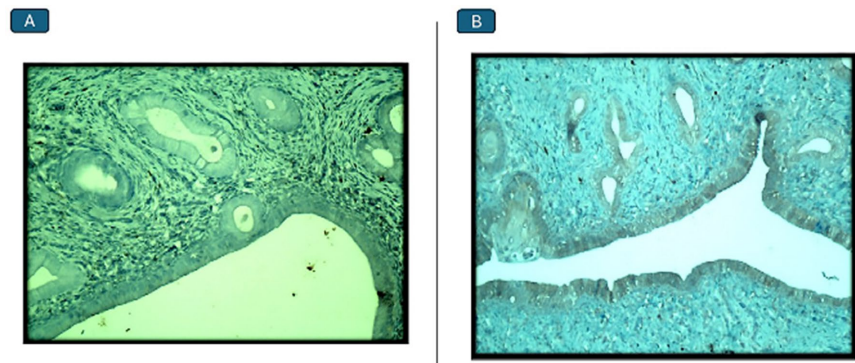


Figure 6. BCL-2 immunohistochemical staining in experimental groups. (A) Tamoxifen Group: No BCL-2 staining observed in the luminal and glandular epithelium (H&E $\times 200$). (B) Tamoxifen-Aspirin Group: Weak BCL-2 positivity observed in the luminal and glandular epithelium (H&E $\times 200$).

Table 5 summarizes VEGF immunoreactivity scores across the experimental groups, highlighting differences in angiogenic response within uterine tissues. In

the luminal epithelium, the Tamoxifen group displayed the highest VEGF expression (Median = 3), significantly exceeding other groups ($H = 12.96$, $P < 0.05$). The letrozole + aspirin group exhibited the lowest staining intensity (Median = 1). This trend suggests a strong angiogenic stimulus associated with tamoxifen. In the glandular epithelium, VEGF expression was significantly elevated in the Tamoxifen group (Median = 2) compared to the control (SF) and Aspirin-only groups ($P < 0.01$). This indicates a tamoxifen-driven proliferative effect, particularly in glandular structures. Regarding the stroma, tamoxifen induced a markedly higher VEGF expression (Median = 2) than the DMSO, Letrozole + Aspirin, and control groups ($H = 25.06$, $P < 0.001$). Aspirin-treated groups also demonstrated moderate stromal VEGF expression, reflecting its potential modulatory effect on angiogenesis. Interestingly, no VEGF immunoreactivity was observed in vascular endothelium or myometrium across any experimental group, suggesting that VEGF activity in this study was primarily confined to epithelial and stromal compartments. Tamoxifen elicited the most robust VEGF expression across multiple uterine layers, emphasizing its strong pro-angiogenic role. While aspirin modulated this response when combined with Tamoxifen or Letrozole, the changes were not as pronounced as with tamoxifen alone (**Figure 7**).

Table 5. VEGF immunohistochemical staining scores across experimental groups.

Experimental Groups	Luminal Epithelium	Glandular Epithelium	Stromal Cells	Vascular Endothelium	Myometrium
SF	1 (1 - 2)	1 (1 - 1.25)	0 (0 - 1.25) C	0 (0 - 0)	0 (0 - 0)
DMSO	1.5 (1 - 2.25)	1 (1 - 1.25)	0 (0 - 1) B	0 (0 - 0)	0 (0 - 0)
TAM	3 (2 - 3)	2 (1.75 - 3) A	2 (1 - 2) B, C, D	0 (0 - 0)	0 (0 - 0)
LET	2 (1 - 2.25)	1 (0.75 - 2)	1 (0 - 1)	0 (0 - 0)	0 (0 - 0)
ASPIRIN	2 (1 - 2.25)	1 (0.75 - 1) A	1 (0.75 - 1)	0 (0 - 0)	0 (0 - 0)
TAM + ASP	2 (2 - 3)	2 (1.75 - 2)	1 (1 - 1.25)	0 (0 - 0)	0 (0 - 0)
LET + ASP	1 (1 - 2.5)	1 (0 - 2.5)	0 (0 - 1) D	0 (0 - 0)	0 (0 - 0)
Statistics (P)	$H = 12.96$, $P < 0.05$	$H = 18.35$, $P < 0.01$	$H = 25.06$, $P < 0.001$	$H = 0$, $P > 0.05$	$H = 0$, $P > 0.05$

[A: Significant difference in glandular epithelium staining between TAM and other groups ($P < 0.05$). B, C, D: Significant differences in stromal staining between respective groups ($P < 0.05$).]

Table 6 demonstrates significant differences in COX-2 immunostaining among the experimental groups in the glandular epithelium, stromal cells, and vascular endothelium. For the glandular epithelium, the DMSO group showed significantly reduced staining compared to the Tamoxifen (A) and Letrozole-Aspirin (C) groups, with significant differences also observed between the Tamoxifen and Aspirin (B) and Aspirin and Letrozole-Aspirin (D) groups. Stromal cell staining with COX-2 was markedly reduced in the DMSO group compared to the Tamoxifen (E) and Aspirin groups (F), while the Tamoxifen group also differed significantly from the Letrozole-Aspirin group (G). In the vascular endothelium, significant

differences were observed between the SF group and the Letrozole (U), Aspirin (I), and Letrozole-Aspirin (K) groups. The Tamoxifen group exhibited greater vascular endothelial staining compared to the Aspirin (M) and Letrozole-Aspirin (N) groups, while the Letrozole group differed significantly from the Tamoxifen-Aspirin group (O). Notably, the combination groups (Tamoxifen-Aspirin and Letrozole-Aspirin) showed differences in vascular staining (R). No significant differences were found in luminal epithelial staining across groups ($P > 0.05$), and no staining was observed in the myometrium for any group (Figure 8).

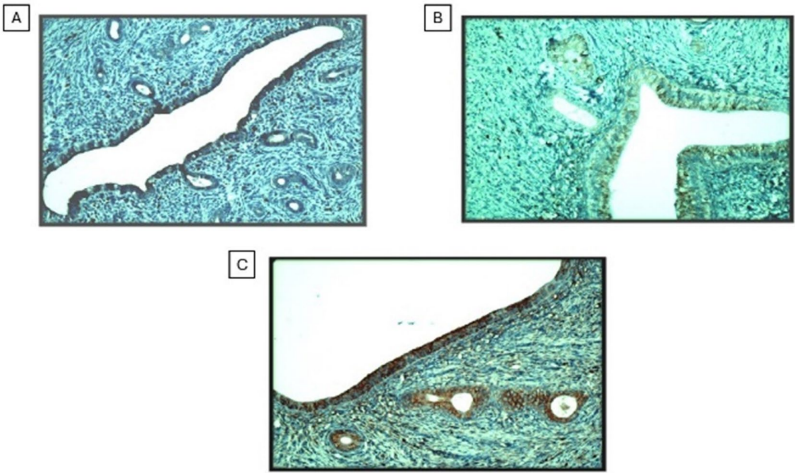


Figure 7. VEGF immunohistochemical staining across experimental groups. (A) VEGF expression in the Letrozole Group demonstrated moderate positivity in the luminal epithelium, while weak positivity was noted in the glandular epithelium and stromal cells (×200). (B) VEGF staining in the Tamoxifen-Aspirin Group displayed poor positivity in the luminal and glandular epithelium and stromal cells (×200). (C) VEGF expression in the Tamoxifen Group revealed strong cytoplasmic staining of the luminal and glandular epithelium, indicating a pronounced pro-angiogenic effect (×200).

Table 6. COX-2 immunohistochemical staining in various groups.

Experimental Groups	Luminal Epithelium	Glandular Epithelium	Stromal Cells	Vascular Endothelium	Myometrium
SF	2 (1 - 3)	1 (0 - 1.25)	1 (1 - 2)	3 (3 - 3) U, I, K	0 (0 - 0)
DMSO	1 (0.75 - 2)	0 (0 - 0) A, C	0 (0 - 1) E	2 (1.75 - 2)	0 (0 - 0)
TAM	2 (2 - 3)	2 (2 - 2.25) A, B	2 (1.75 - 2) E, F, G	3 (3 - 3) L, M, N	0 (0 - 0)
LET	2 (1.75 - 3)	1 (0.75 - 2)	1 (1 - 1.25)	1 (1 - 2) U, L, O	0 (0 - 0)
ASPIRIN	1 (0.75 - 2.25)	0 (0 - 0.25) B, D	0 (0 - 1) F	2 (0.75 - 2) I, M, P	0 (0 - 0)
TAM + ASP	1 (1 - 2.25)	1 (0.75 - 1)	1 (1 - 1)	3 (3 - 3) O, P, R	0 (0 - 0)
LET + ASP	2 (1.5 - 3)	1 (1 - 3) C, D	0 (0 - 1) G	1 (1 - 2) K, N, R	0 (0 - 0)
Statistics	H = 10.67, $P > 0.05$	H = 33.03, $P < 0.001$	H = 34.81, $P < 0.001$	H = 48.62, $P < 0.001$	H = 0, $P > 0.05$

[A, B, C, D: Significant differences in glandular epithelial staining intensity ($P < 0.05$). E, F, G: Significant differences in stromal cell staining intensity ($P < 0.05$). U, I, K, L, M, N, O, P, R: Significant differences in vascular endothelium staining intensity ($P < 0.05$). H = Kruskal-Wallis test statistic.]

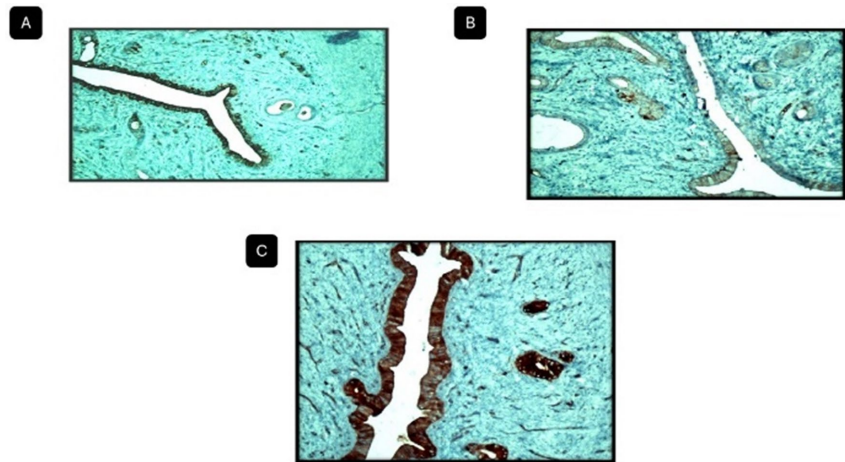


Figure 8. Comparative COX-2 immunoreactivity across experimental groups. (A) Letrozole Group: COX-2 showed strong positivity in the luminal epithelium and weak positivity in the glandular epithelium (H&E ×200). (B) Tamoxifen-Aspirin Group: Weak cytoplasmic positivity was observed in the luminal and glandular epithelium with COX-2 (H&E ×200). (C) Tamoxifen Group: Strong cytoplasmic positivity was observed in the luminal and glandular epithelium with COX-2 (H&E ×200).

Table 7. KI-67 immunohistochemical marker in experimental groups.

Experimental Groups	Luminal Epithelium	Glandular Epithelium	Stromal Cells	Vascular Endothelium
SF	15 (7.75 - 36.25) U	48.5 (23 - 83.5)	3 (1 - 9.25) V, Y	1 (0 - 2.5)
DMSO	25 (15.5 - 40.5) S	19 (10.75 - 41.75)	0 (0 - 1.25)	0 (0 - 0)
Tamoxifen	4.5 (1 - 7.25) S, T, U	18 (4.75 - 27)	0.5 (0 - 2.25)	0 (0 - 0)
Letrozole	13.5 (6.5 - 30.75)	6.5 (2 - 48)	0 (0 - 1) V, Z	0 (0 - 0)
Aspirin	16.5 (11.5 - 40.75) T	33.5 (42.75 - 59.4)	2.5 (0 - 3.25)	0 (0 - 0)
Tamoxifen + Aspirin	6.5 (4.5 - 12.7)	21.5 (2.5 - 33.25)	2 (1.75 - 3.25) Z, X	0 (0 - 0)
Letrozole + Aspirin	9 (1.5 - 14)	22 (7.5 - 32.5)	0 (0 - 0) Y, X	0 (0 - 0)

[Luminal Epithelium: H = 25.72, P < 0.001 (S, T, U: P < 0.05). Glandular Epithelium: H = 10.89, P > 0.05. Stromal Cells: H = 29.99, P < 0.001 (V, Y, Z, X: P < 0.05). Vascular Endothelium: H = 32.70, P < 0.001.]

Table 7 highlights the immunoreactivity percentages with KI-67 across the experimental groups. A significant difference in luminal epithelial staining was observed between the DMSO and Tamoxifen groups (S, P < 0.05), the Tamoxifen and Aspirin groups (T, P < 0.05), and the SF and Tamoxifen groups (U, P < 0.05). For stromal cell staining, significant differences were noted between the SF and Letrozole-Aspirin groups (Y, P < 0.05), the SF and Letrozole groups (V, P < 0.05), and the Tamoxifen-Aspirin and Letrozole groups (Z, P < 0.05). The Tamoxifen-Aspirin and Letrozole-Aspirin groups exhibited significant differences (X, P < 0.05). No statistically significant differences were observed among the groups for glandular epithelium (P > 0.05). KI-67 positivity in vascular endothelium was not detected across all experimental groups except for a single subject in the SF group (**Figure 9**).

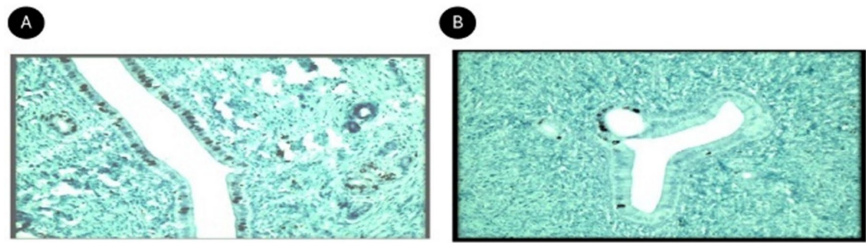


Figure 9. KI-67 immunohistochemical staining in experimental groups. (A) Letrozole Group: Prominent nuclear positivity in the luminal and glandular epithelium. (B) Tamoxifen Group: Limited nuclear positivity in the luminal and glandular epithelium.

Table 8. Microvessel density evaluated with PECAM-1 (CD-31) immunohistochemical marker.

Experimental Groups	Number of Veins
SF Group	13 (10 - 16)
DMSO Group	20 (16 - 24)
Tamoxifen Group	21.5 (20 - 26)
Letrozole Group	25 (16 - 35)
Aspirin Group	16.5 (14 - 22)
Tamoxifen + Aspirin	20.5 (15 - 22)
Letrozole + Aspirin	29 (19 - 34.5)

[Median (25th Percentile - 75th Percentile). $H = 13.091$, $P > 0.05$ (Kruskal-Wallis statistic)].

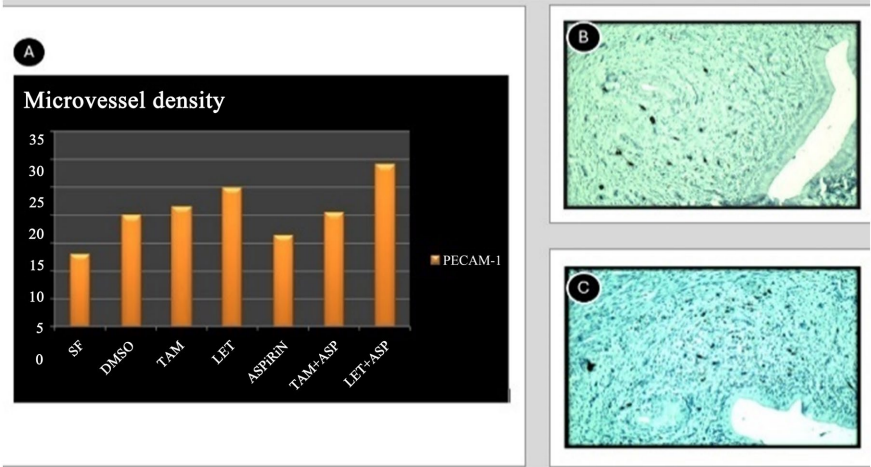


Figure 10. PECAM-1 immunohistochemical analysis across experimental groups. (A) Microvessel densities in the experimental groups were visualized through a PECAM-1 immunohistochemical marker. (B) Tamoxifen Group: PECAM-1 positivity is observed in many vascular structures within the endometrial stroma (H&E $\times 200$). (C) Tamoxifen-Aspirin Group: PECAM-1 demonstrates increased vascularization in the endometrial stroma, indicating enhanced microvessel formation (H&E $\times 200$).

As presented in **Table 8**, no statistically significant differences in microvessel density were identified among the experimental groups based on PECAM-1 stain-

ing results ($P > 0.05$). However, median values indicate relatively higher microvessel density in the Letrozole and Letrozole + Aspirin groups compared to other groups, suggesting potential trends that were not statistically significant. **Figure 10** shows representative PECAM-1 staining images across the experimental groups. Specifically, **Figure 10(B)** illustrates PECAM-1 positivity in numerous vascular structures within the endometrial stroma of the Tamoxifen group, while **Figure 10(C)** depicts increased vascularization in the stroma of the tamoxifen + Aspirin group. These findings qualitatively support observations of microvascular density variations among the groups, despite the absence of statistically significant differences.

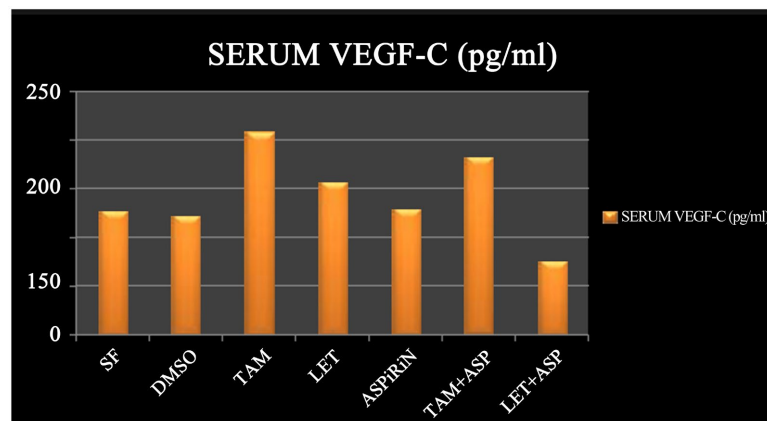


Figure 11. Comparison of serum VEGF-C levels among experimental groups.

Table 9. Comparison of serum VEGF-C levels among experimental groups.

Experimental Groups	Serum VEGF-C Levels (pg/ml)
SF	127.00 (81.02 - 161.09)
DMSO	121.47 (76.10 - 184.62)
TAM	207.10 (38.39 - 232.38)
LET	154.91 (84.31 - 204.45)
ASPIRIN	128.78 (58.19 - 198.30)
TAM + ASP	183.46 (151.69 - 304.57)
LET + ASP	75.90 (31.98 - 121.74)

The serum VEGF-C levels across the experimental groups are detailed in **Table 9** and visualized in **Figure 11**. The findings reveal variations in VEGF-C levels influenced by different treatment regimens. Pairwise comparisons using the Mann-Whitney U test showed significant differences among groups. VEGF-C levels in the tamoxifen + Aspirin group were significantly higher than in the SF group ($z = -2.12$; $p < 0.05$) and the Aspirin group ($z = -1.97$; $p < 0.05$). Similarly, the Letrozole group had higher VEGF-C levels than the letrozole + aspirin group ($z = -2.221$; $p < 0.05$). A significant difference was also observed between the Tamoxifen + Aspirin and Letrozole + Aspirin groups ($z = -2.86$; $p < 0.01$). **Figure 11**

illustrates these findings, showing that the Tamoxifen + aspirin group had the highest serum VEGF-C levels, while the Letrozole + aspirin group had the lowest. These results suggest that tamoxifen combined with aspirin significantly enhances VEGF-C levels compared to other combinations or individual treatments.

4. Discussion

The main purpose of this study was to compare the endometrial effects of tamoxifen used in the treatment of breast cancer with Letrozole, and to evaluate the histopathological changes, cell proliferation, apoptosis, angiogenesis, COX-2 inhibition and serum VEGF-C levels caused by these drugs in the uterine tissue. In addition, the potential effects of the combination of these drugs with a non-selective COX inhibitor such as aspirin on uterine pathologies were investigated. The main findings of our study revealed that while tamoxifen caused proliferative and estrogenic effects in the uterine tissue, Letrozole showed an atrophic and anti-estrogenic effect. These findings are also supported by Emons *et al.*, who stated that the estrogenic effects of tamoxifen on the uterus were associated with the increased expression of VEGF and COX-2 in the tissue [24]. This study emphasized the success of tamoxifen as a selective estrogen receptor modulator (SERM) in breast cancer treatment, while drawing attention to the fact that its proliferative effect on uterine tissue, especially with long-term use, increases the risk of endometrial cancer. The researchers stated that this effect of tamoxifen could be explained by differences in G-protein coupled estrogen receptor 1 (GPER-1) and co-activator/co-repressor expression profiles. In addition, it was stated that these effects of tamoxifen do not only pose a risk of cancer but may also pave the way for uterine hyperplasia and other endometrial pathologies. Letrozole, on the other hand, was shown to result in endometrial atrophy and suppression of proliferative effect in a study conducted on patients with disordered proliferative endometrium or simple hyperplasia by Mostafa Gharabaghi *et al.* [25]. In this randomized controlled trial, it was reported that 58.7% of patients receiving letrozole treatment developed endometrial atrophy and 34.78% developed poorly proliferative endometrium. The researchers stated that letrozole suppresses the proliferative process in uterine tissue by reducing estrogen levels through aromatase inhibition and therefore can be used as an effective alternative in the treatment of endometrial pathologies. In addition, a prospective study by McGonigle *et al.* reported that tamoxifen has mild estrogenic effects on uterine tissue in postmenopausal women, leading to an increase in endometrial thickness and uterine volume [26]. The study showed that endometrial thickness increased from 2.6 mm to 5.8 mm in the first year of tamoxifen treatment and uterine volume increased significantly. In addition, it was stated that proliferative endometrium was detected in 26% of women using tamoxifen, but no serious pathologies such as hyperplasia or adenocarcinoma were observed. These findings revealed that tamoxifen can show limited estrogenic activity in uterine tissue and that this effect may contribute to proliferative processes in the long term.

Tamoxifen did not increase the expression of Ki-67, an indicator of cell proliferation, while it caused a significant increase in VEGF and COX-2 expressions. In contrast, VEGF and COX-2 expressions were suppressed in the Letrozole group, and an increase in BCL-2 expression was observed in stromal tissue. The combination of aspirin and tamoxifen provided a potential benefit in preventing endometrial side effects by reducing VEGF and COX-2 expressions. However, no significant difference was observed in the effects of the combination with aspirin on the uterine stroma and glandular epithelium. The combination of letrozole and aspirin exhibited less proliferative effects on uterine weight and histopathological findings compared to the Tamoxifen group. Nynca *et al.* reported that tamoxifen suppressed apoptosis in ovarian tissue and reduced chemotherapy-induced ovarian toxicity by supporting DNA repair pathways. It was also emphasized that tamoxifen provided these protective effects without reducing the effectiveness of the treatment [27]. Chlebowski *et al.* [28], reported that aromatase inhibitors significantly reduced the risk of endometrial cancer in women using tamoxifen and suggested that tamoxifen-related endometrial side effects could be reduced with aromatase inhibitors. In addition, clinical findings regarding the antiproliferative effects of aromatase inhibitors on endometrial hyperplasia and early-stage endometrial cancer were supported. This suggests that aromatase inhibitors may be a safer option in terms of uterine side effects, which is consistent with the findings of our study. It was concluded that tamoxifen did not increase the expression of Ki-67, an indicator of cell proliferation, but caused a significant increase in VEGF and COX-2 expressions, and these findings are consistent with studies in literature. In a study conducted by Ferrandina *et al.*, it was reported that high-dose short-term tamoxifen administration caused changes in biological parameters in cervical cancer tissue, a significant decrease in the percentage of Ki-67 positive cells and an increase in apoptotic activity [29]. In the same study, a significant decrease in microvessel density was observed and it was stated that these changes may differ with estrogen receptor status. Another study by Morena *et al.* reported that tamoxifen treatment caused nuclear changes such as decreased cellular volume, chromatin condensation and increased nuclear borders in breast cancer tissues within two days, but there was no significant change in VEGF, ILK and Ki-67 expression [30]. However, it was stated that TGF-beta1 protein expression increased and Erb-B2 protein expression decreased. Du *et al.* showed that the combined use of tamoxifen and COX-2 silencing suppressed VEGF expression, reduced angiogenesis and induced apoptosis in breast cancer cells both *in vitro* and *in vivo* models [31]. This combination has also been reported to limit tumor growth and reduce metastatic potential. These findings support the idea that the VEGF and COX-2 expression increases observed in our study are related to the estrogenic effects of tamoxifen and that COX-2 inhibitors or other complementary treatments can reduce these effects. The finding that the combination with aspirin reduced VEGF and COX-2 expression in our study is interpreted in accordance with this literature.

It was observed that VEGF and COX-2 expressions were suppressed and BCL-2 expression increased in stromal tissue in the letrozole group. These findings suggest that letrozole may support anti-angiogenic effects and apoptotic resistance mechanisms in stromal cells. These results are consistent with studies in the literature. In a study conducted by Alabiad *et al.*, it was shown that high-dose letrozole treatment significantly reduced VEGF expression in trophoblastic tissues, and as a result, vascular support signals were eliminated, leading to destruction of the vascular network and increased apoptosis [32]. However, a study by Xu and St. Croix suggested that inhibition of the COX-2/PGE2 axis may increase the efficacy of VEGF-targeted therapies and that this axis may contribute to the antiangiogenic effects of letrozole [33]. Tilghm *et al.* reported that in breast cancer cells with letrozole resistance, the estrogen signaling pathway was suppressed and cell motility and invasion increased despite aromatase inhibition [34]. This suggests that letrozole may increase BCL-2 expression through estrogen-independent mechanisms and may develop apoptotic resistance. These data support the results of our study, suggesting that letrozole creates different effects in stromal tissue by regulating both anti-angiogenic and apoptotic resistance mechanisms. The combination of aspirin and tamoxifen provided a significant decrease in VEGF and COX-2 expressions, offering a potential benefit in preventing endometrial side effects. This effect may be associated with the cellular mechanisms of aspirin and similar COX-2 inhibitors. In a study conducted by Li *et al.*, aspirin was shown to inhibit COX-2 activity and reduce bcl-2 expression in esophageal cancer cells [35]. This mechanism prevented tumor cell proliferation by promoting cellular apoptosis. The study also stated that COX-2-mediated tumor development could be prevented by suppressing prostaglandin E2 (PGE2) production. These findings may explain the protective effect of aspirin against endometrial pathologies. Similarly, in the study conducted by Wood *et al.*, aspirin and the COX-2 inhibitor were reported to suppress proliferation and increase apoptosis in endometrial cancer cells associated with hereditary nonpolyposis colorectal cancer. In this study, it was stated that the drugs were effective on cell cycle regulations and stopped cellular proliferation. It was suggested that COX-2 inhibition, especially with aspirin, could prevent endometrial cell growth through cell cycle modulation [36]. Olivarres *et al.* showed that selective COX-2 inhibitor celecoxib significantly suppressed proliferation, decreased VEGF secretion and increased apoptosis in endometrial epithelial cells. Celecoxib was reported to inhibit vasculogenesis and angiogenesis by suppressing PGE2 production. This mechanism emphasizes the therapeutic potential of selective COX-2 inhibitors in the treatment of endometrial pathologies [37]. Considering these studies, our study showed that the combined use of aspirin and tamoxifen was effective in preventing endometrial pathologies by reducing COX-2 and VEGF expressions. The effect of aspirin on COX-2-mediated mechanisms contributes to the suppression of estrogenic side effects of tamoxifen, suggesting that this combination may be a promising approach for endometrial pathologies. The combination of letrozole and aspirin has demonstrated less pro-

liferative effects on uterine weight and histopathological findings compared to tamoxifen. These findings are supported by several studies emphasizing the unique effects of letrozole on the endometrium and uterine tissue. In a study by Núñez *et al.*, the effects of tamoxifen and letrozole were compared in a preclinical breast cancer model [38]. Letrozole treatment resulted in significantly smaller uteri compared to tamoxifen-treated mice, where uterine hypertrophy was observed. This highlights letrozole's advantage in avoiding uterine proliferation, often associated with tamoxifen therapy. Similarly, research by Long *et al.* evaluated letrozole's effectiveness in delaying tumor progression compared to tamoxifen in breast cancer models [39]. The study also reported that tumors treated with letrozole exhibited reduced proliferation without the uterine hypertrophy characteristic of tamoxifen use. This further supports the notion that letrozole has a safer profile concerning uterine side effects. Furthermore, Pourmatroud *et al.* examined the co-administration of tamoxifen with letrozole in intrauterine insemination cycles [40]. While tamoxifen showed increased endometrial thickness, letrozole alone presented with less proliferative effects on the endometrium, demonstrating its potential as a safer alternative when combined with other treatments. These studies align with our findings, suggesting that the letrozole and aspirin combination could provide a therapeutic strategy with reduced endometrial side effects compared to tamoxifen. The combination's ability to mitigate uterine proliferation highlights its potential for safer and more effective therapeutic applications in hormone receptor-positive breast cancer treatment strategies.

While our findings offer valuable insight into the differential endometrial effects of tamoxifen and letrozole and the potential modulating role of aspirin, several limitations should be considered when translating these results into clinical settings. Notably, although ovariectomized rats serve as a widely accepted model for postmenopausal conditions, interspecies differences in drug metabolism, tissue sensitivity, and endocrine regulation may limit direct applicability to human physiology. For example, tamoxifen metabolites differ significantly between rodents and humans in terms of both potency and half-life, which may influence observed tissue responses. Similarly, the hormonal milieu and endometrial receptor distribution in rats do not fully replicate human conditions.

From a mechanistic standpoint, the differential expression of BCL-2 in response to tamoxifen and letrozole warrants further exploration. BCL-2, an anti-apoptotic protein, is known to be estrogen-regulated and plays a critical role in maintaining endometrial homeostasis. In our study, tamoxifen was associated with a reduction in stromal BCL-2 expression, potentially reflecting increased apoptotic turnover despite its overall proliferative action. Conversely, letrozole markedly upregulated stromal BCL-2, consistent with aromatase inhibition and reduced estrogen-driven apoptosis. These opposing effects suggest distinct regulatory pathways and tissue-specific interactions that merit further molecular investigation. Previous studies have also reported that estrogen deprivation can paradoxically upregulate anti-apoptotic markers in stromal compartments, possibly

as a compensatory survival mechanism [41] [42].

Clinically, these findings may help refine risk-benefit assessments in hormone therapy for breast cancer patients, particularly regarding uterine safety. The apparent protective effects of letrozole, and the potential of aspirin to counteract tamoxifen-induced angiogenesis and inflammation, could inform safer long-term treatment strategies. However, future studies involving human tissue models or clinical trials are necessary to validate these implications and to fully assess the translational value of combining COX inhibitors with hormonal agents.

5. Conclusions

This study investigated the endometrial effects of tamoxifen and letrozole in a postmenopausal rat model, with and without aspirin co-administration, by assessing histopathological, immunohistochemical, and biochemical parameters. Tamoxifen promoted estrogenic and proliferative changes in the endometrium, including increased VEGF and COX-2 expression, aligning with its known risk of endometrial hyperplasia and cancer. Letrozole, by contrast, exhibited anti-estrogenic effects, leading to endometrial atrophy and reduced angiogenic markers.

Notably, while tamoxifen reduced stromal BCL-2 expression compared to controls—suggesting increased apoptosis—letrozole significantly increased BCL-2 expression, indicating a protective anti-apoptotic effect on stromal cells. The combination of tamoxifen with aspirin mitigated angiogenic and inflammatory responses, while letrozole plus aspirin maintained minimal uterine stimulation.

These findings suggest that combining tamoxifen or letrozole with aspirin may help reduce endometrial side effects without compromising therapeutic action. However, due to limitations such as the use of an animal model, short treatment duration, and a limited biomarker panel, further long-term clinical studies are needed to confirm these outcomes and explore broader molecular mechanisms. Overall, the results support the potential of aspirin co-treatment strategies in improving the gynecological safety of hormonal therapies in breast cancer management.

CRedit Authorship Contribution Statement

Hande Nalan Töre: Writing - review & editing, Writing - original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization; **Rovshan Jabbarov:** Writing - review & editing, Visualization, Methodology, Formal analysis, Data curation, Conceptualization; **Hasan Bulut:** Formal analysis, Investigation, Data curation, Writing - review & editing; **Semra Can Mamur:** Writing - review & editing, Methodology, Formal analysis, Data curation; **Özben Özden Işıklar:** Writing - review & editing, Visualization, Formal analysis, Data curation; **Sabit Sinan Özalp:** Formal analysis, Data curation, Writing - review & editing; **Ömer Çolak:** Writing - review & editing, Formal analysis, Data curation; **Evrin Çiftçi:** Writing - review & editing, Formal analysis; **Engin Yıldırım:** Writing - review & editing, Formal analysis, Data curation; **Kubilay Uz-**

uner: Writing - review & editing, Visualization, Data curation; **Yasemin Aydın:** Writing - review & editing, Formal analysis, Data curation; **Canan Baydemir:** Formal analysis, Data curation, Writing - review & editing; **Mustafa Fuat Açıklan:** Writing - review & editing, Visualization, Supervision, Methodology, Investigation, Formal analysis.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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