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Effect of Combined Oral Contraceptive on Flap Surgery in Female Rat Model

Efecto de los anticonceptivos orales combinados en la cirugía de colgajo en el modelo de rata hembra

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ABSTRACT

Combined oral contraceptives (COCs) are said to have a negative effect on flap surgery's outcomes due to their adverse effects on tissue perfusion, managing inflammation, and oxidative stress. This study aimed to investigate the effects of **COCs** use on flap survival in a female rat model. In this randomized controlled experimental study, a total of 20 female Wistar Albino rats were divided into control and COC groups (n=10/group). The COC group received Desolett® (Desogestrel 0.15 mg, Ethinyl estradiol 0.03 mg) orogastrically for three weeks. The McFarlane flap model was surgically implemented, and flap survival was evaluated using digital planimetry. Histopathological and biochemical analyses were performed during the postoperative period. The flap survival rate in the COC group was significantly lower compared to the control group (74.4 vs 83.7%, P=0.012). Furthermore, the COC group demonstrated a higher necrotic area ratio (25.7 vs 18.4%, P=0.015) and more severe inflammatory cell infiltration (2.4 ± 0.5 vs 1.8 ± 0.4, P=0.011). Biochemical analyses revealed higher malondialdehyde (MDA) levels (4.86 ± 0.72 vs 3.24 ± 0.58 nmol/mg, P=0.008) and lower superoxide dismutase (SOD) activity (14.2 ± 2.1 vs 18.5 ± 2.4 U/mg, P=0.012) in the COC group. Using COCs reduces the chances of a successful flap surgery and promotes the chances of complications arising owing to oxidative stress, inflammation and impairment of tissue perfusion. These observations merit caution while planning flap surgery for COCs users and a recommendation for a brief halt in the usage of COCs should be made. The results underscore the importance of understanding the potential implications of COCs use in surgical planning and postoperative care management.

Key words: Combined oral contraceptives; flap surgery; oxidative stress; inflammation; flap survival.

RESUMEN

Los efectos de los anticonceptivos orales combinados (AOC) sobre la perfusión tisular, la inflamación y el estrés oxidativo pueden afectar negativamente a los resultados de la cirugía de colgajo. El objetivo de este estudio fue investigar los efectos del uso de AOC sobre la supervivencia del colgajo en un modelo de rata hembra. En este estudio experimental aleatorizado y controlado, se dividió a un total de 20 ratas hembra Wistar Albino en grupos de control y de AOC (n=10/grupo). Se administró Desolett® (desogestrel 0,15 mg, etinilestradiol 0,03 mg) por vía orogástrica al grupo AOC durante 3 semanas. Se aplicó quirúrgicamente el modelo de colgajo de McFarlane y se evaluó la supervivencia del colgajo mediante planimetría digital. Se realizaron análisis histopatológicos y bioquímicos postoperatorios. La tasa de supervivencia del colgajo fue significativamente inferior en el grupo AOC en comparación con el grupo control (74,4 frente a 83,7%, P=0,012). Además, la tasa de zonas necróticas fue mayor en el grupo AOC (25,7 frente a 18,4%, P=0,015) y la infiltración de células inflamatorias fue más grave (2,4 ± 0,5 frente a 1,8 ± 0,4, P=0,011). En los análisis bioquímicos, los niveles de malondialdehído (MDA) fueron superiores (4,86 ± 0,72 frente a 3,24 ± 0,58 nmol/mg, P=0,008) y la actividad de la SOD fue inferior (14,2 ± 2,1 frente a 18,5 ± 2,4 U/mg, P=0,012) en el grupo de AOC. El uso de AOC afecta negativamente a la supervivencia del colgajo y aumenta el riesgo de complicaciones a través de mecanismos como el estrés oxidativo, la inflamación y el deterioro de la perfusión tisular. Estos hallazgos sugieren que debe tenerse precaución al planificar la cirugía de colgajo en pacientes que utilizan AOC y que el cese temporal del uso de AOC puede ser beneficioso.

Palabras clave: Anticonceptivos orales combinados; cirugía de colgajo; estrés oxidativo; inflamación; supervivencia del colgajo

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INTRODUCTION

Studies in the field of tissue engineering and flap surgery emphasise that sex differences and tissue oxygenation play an important role in healing processes. Brandenburg et al. demonstrated that female mice showed a higher efficacy in distal flap revascularisation compared to male mice and that this was related to sex-specific biological mechanisms [1]. In addition, in a study titled 'Unconventional Perfusion Flaps', it was stated that perfusion and reoxygenation mechanisms are critical in determining the success of flap surgery [2]. In addition, the study by Diaz-Raval [3] examined the effect of oral contraceptives on metabolic processes and stated that hormonal regulation plays a key role in wound healing. Based on this literature, our study aims to further investigate the effects of oral contraceptives on flap survival and healing processes.

In recent time, a critical area of study is tissue engineering and flap surgery which examines various causes that affect the healing process of wounds and the preservation of tissues. However, the effects of oral contraceptive use on tissue perfusion, oxidative stress and flap survival have not been adequately investigated. According to literature, not much is known about the possible adverse effects that can result from oral contraceptives on healing of tissues. For example, the study by Diaz-Raval [3] drew attention to the potential consequences of hormonal interventions by demonstrating the negative effects of oral contraceptives on metabolic processes. Similarly, in a study conducted by Brandenburg *et al.* [1] in mouse models, it was emphasised that sex differences in ischaemia/reperfusion processes and hormone levels play an important role in tissue healing. However, studies evaluating the direct effects of oral contraceptives in flap surgery are limited. In this context, our study aims to fill this important research gap by examining the effects of oral contraceptive use on flap survival and complication rates.

A major hypothesis exists for this study according to which the use of COCs in female rats may obstruct the success of flap surgery. In particular, it was predicted that COCs use may decrease flap survival by decreasing tissue perfusion, altering the inflammatory response and increasing oxidative stress levels. It has also been suggested that COCs may increase the risk of necrosis in flap tissue by affecting microvascular circulation and slow the wound healing process. Given hypotheses are based on the previous research that has shown that COCs use increases clot formation chances and disrupts endothelial function. We also hypothesize that COCs use will lead to higher oxidative stress markers within flap tissue, and hence undermine its antioxidant defense systems as well. To test these hypotheses, a randomised controlled experiment was designed in a female rat model and flap survival rates, tissue perfusion, histopathological changes and biochemical parameters were evaluated [4, 5, 6].

The combined oral contraceptives used in this study contain desogestrel (0.15 mg) and ethinyl estradiol (0.03 mg), which may have specific effects on the mechanisms relevant to flap survival. Desogestrel, a third-generation progestin, has been shown to affect vascular tone and endothelial function through its interaction with progesterone receptors, potentially altering microvascular perfusion in flap tissue. Ethinyl estradiol, on the other hand, may influence clotting cascade activation, inflammatory mediator expression, and nitric oxide production, all of which are critical factors in tissue healing and perfusion. These hormonal components may synergistically impair angiogenesis and microvascular function in flap tissues through multiple pathways including increased oxidative stress, altered inflammatory response, and compromised endothelial integrity.

The major aim of this study was to assess the impact of combined oral contraceptives on survival and viability of flap surgical outcomes using a rat model. We wanted specifically to examine the influence that COCs have on rates of survival, tissue blood flow, wound healing, complications as well as inflammatory response, oxidative stress markers and antioxidant defense systems in the flap tissue. Furthermore, our objectives included assessing how COCs would impact histopathological alterations as well as changes in the vascular network within the flap tissues. In line with these aims, a multidimensional evaluation was planned by using scintigraphic imaging, biochemical analyses and histopathological examinations.

MATERIALS AND METHODS

Experimental Animals and Ethical Approval

This experimental study was carried out with the approval of Dicle University Animal Experiments Local Ethics Committee (DÜHADEK) dated 18.12.2015. In the study, 20 female Wistar Albino rats aged 12-16 weeks with an average weight of 250±10 g (measured using Precisa XB 220A precision balance, Precisa Instruments AG, Switzerland) were used. The experimental animals were housed in an environment with a temperature of 22±2°C and a humidity of 50-60% (monitored using Testo 608-H1 thermo-hygrometer, Testo SE & Co. KGaA, Germany) and a 12-hour light/dark cycle. All rats were fed ad libitum with standard laboratory feed and water (FIG. 1).

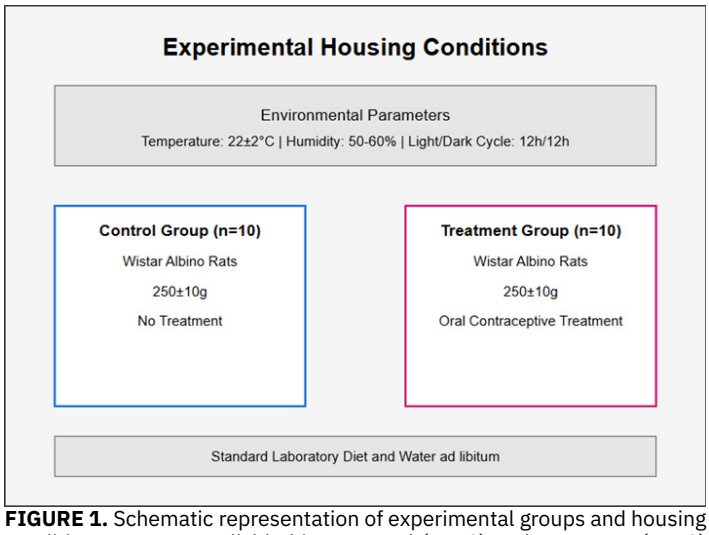

FIGURE 1. Schematic representation of experimental groups and housing conditions. Rats were divided into control (n=10) and treatment (n=10) groups and all animals were housed under standardised environmental conditions (22±2°C temperature, 50-60% humidity, 12 h light/dark cycle). Rats in both groups were fed ad libitum with standard laboratory feed and water

Study Groups and Drug Administration Rats were randomly divided into two groups (n=10/group):

- Control Group: No medical treatment was administered
- Experimental Group: Combined oral contraceptive (Desolett®; Desogestrel 0.15 mg, Ethinyl estradiol 0.03 mg) was administered with a daily orogastric catheter for 3 weeks. The COCs dosage corresponded to approximately 0.6 mg/kg desogestrel and 0.12 mg/kg ethinyl estradiol based on the average rat weight of 250 g, which was selected to proportionally simulate human therapeutic dosing.

Drug administration was performed at the same time every day (between 09:00-10:00). While orogastric administration was chosen to ensure complete dosing, we acknowledge this method may introduce some variability in drug absorption compared to oral self-administration. To minimize this potential variability, administration was performed by the same researcher using standardized technique and timing to ensure consistency in drug delivery and absorption patterns across all experimental animals.

Surgical Procedure

After three weeks of drug administration, all rats underwent a standardised surgical protocol. The surgical procedure was performed in a five-stage systematic approach (FIG. 2). In the first stage, Ketamine Hydrochloride (50 mg/kg) and Xylazine (5 mg/kg) were administered intraperitoneally for induction of anaesthesia. In the second stage, the dorsal region was shaved and antisepsis was provided with povidone iodine (FIG. 3a). In the third stage, a 3x9 cm flap area was marked with a surgical pen in accordance with the McFarlane flap technique. In the fourth stage, the subcutaneous tissue was carefully prepared by incision along the marked area and subfascial dissection was completed (FIG. 3b). In the final stage, the flap was eluted (FIG. 3c) and closed primerally with 4/0 silk suture (FIG. 3d).

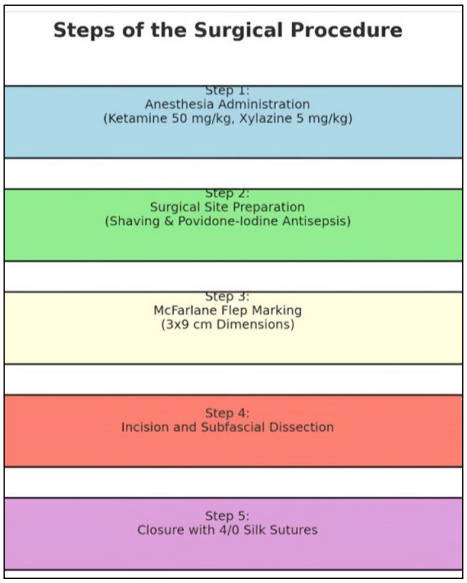

FIGURE 2. Schematic representation of the surgical procedure. The procedure was performed in five main steps: (1) induction of anaesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg), (2) surgical site preparation (shaving and povidone-iodine antisepsis), (3) McFarlane flap marking (3x9 cm), (4) incision and subfascial dissection, (5) closure with 4/0 silk suture

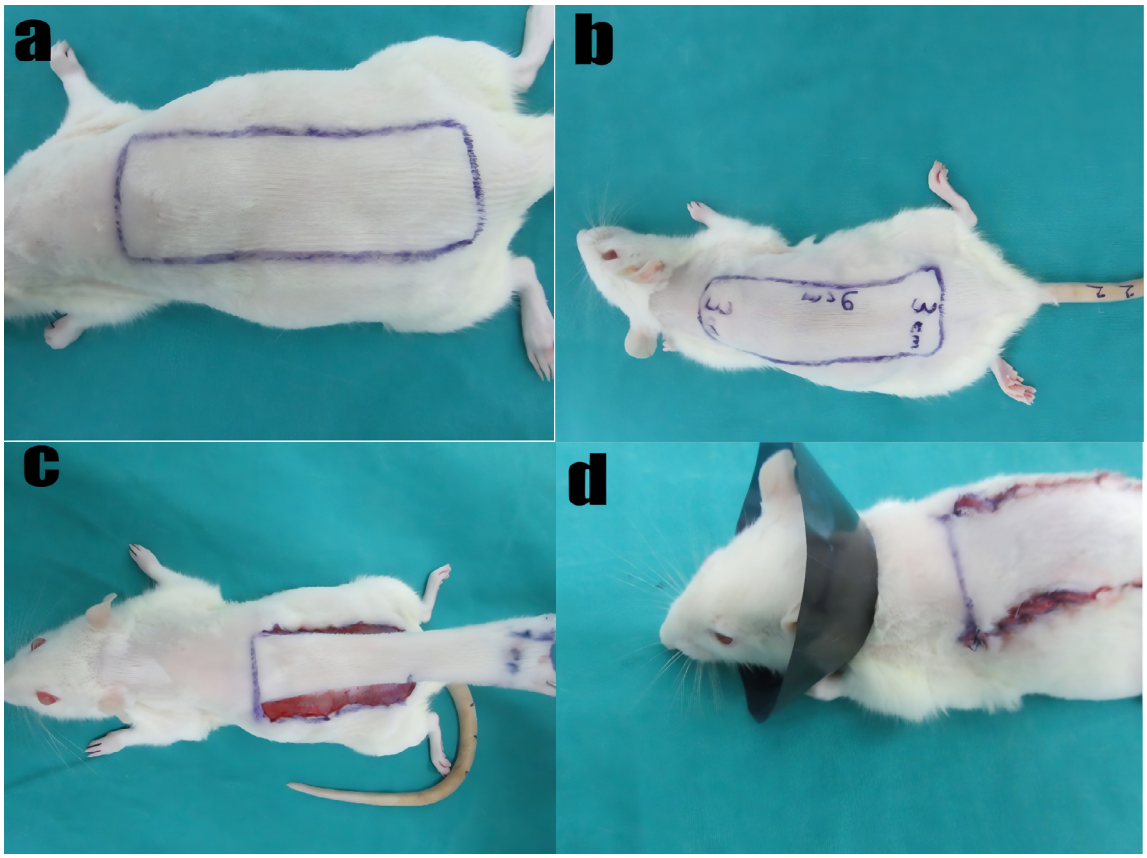

FIGURE 3. Photographic representation of the surgical procedure steps. (a) Marking of the flap area after site preparation and antisepsis, (b) Preparation and exposure of subcutaneous tissue, (c) Elevation of the subfascial flap, (d) Completion of closure with primary suturing

Postoperative follow-up and evaluation

In the early postoperative period (first 24 h), the flap area was evaluated every 6 h and tissue integrity, capillary filling and colour changes were recorded (FIG. 4a). During the following 48 h, tissue perfusion in the proximal and distal regions of the flap was checked twice daily (FIG. 4b). In the mid postoperative period (d 3-5), the viability of the flap tissue was monitored and hyperemic and ischaemic areas were identified and mapped (FIG. 4c).

Flap survival analysis

A standardised digital photographic analysis method was used to evaluate flap survival (Figure 4d). Flaps were photographed using Canon EOS 750D digital camera (Canon Inc., Japan) at a standard distance (30 cm) on postoperative day 7, and total flap area, viable tissue area, and necrosis area were measured pixel-wise using ImageJ software (version 1.53k, National Institutes of Health, USA). The flap survival rate was calculated by dividing the living tissue area by the total flap area.

Complication assessment

Complications were evaluated according to standardised criteria. Partial necrosis was defined as the presence of a necrotic area larger than 1 cm^2 in the distal part of the flap. Superficial infection was diagnosed according to the presence of erythema, oedema and purulent discharge. Seroma evaluation was performed by physical examination and ultrasonographic confirmation when necessary. All complications were recorded and photographed daily.

Scintigraphic evaluation

At 72 h after the surgical procedure, 1 mCi Tc-99m pertechnetate was administered intravenously to each rat and images were taken with a gamma camera. Imaging was performed 10 min after injection as 5-mine static images with a matrix size of 128x128. Standard regions of interest (ROIs) were determined by dividing the proximal, middle and distal regions of the flap into three areas of equal size. Mean count values for each region were calculated in arbitrary units (AU). The percentages of hyperaemic area (>25 AU), normally perfused area (5-25 AU) and hypoperfused/necrotic area (<5 AU) were determined. The proximal/distal activity ratio was obtained by dividing the mean activity value of the proximal region by the distal region.

Histopathological examination

Samples taken from the flap tissue were fixed in 10% formaldehyde solution for 48 h. After routine tissue follow-up, 5 µm thick sections were taken from the tissues embedded in paraffin blocks and stained with Haematoxylin-Eosin (H&E). FIGURE 5, shows the scintigraphic perfusion analysis of flap tissue, not the histological sections. The histological evaluation methodology is described here, while representative histology

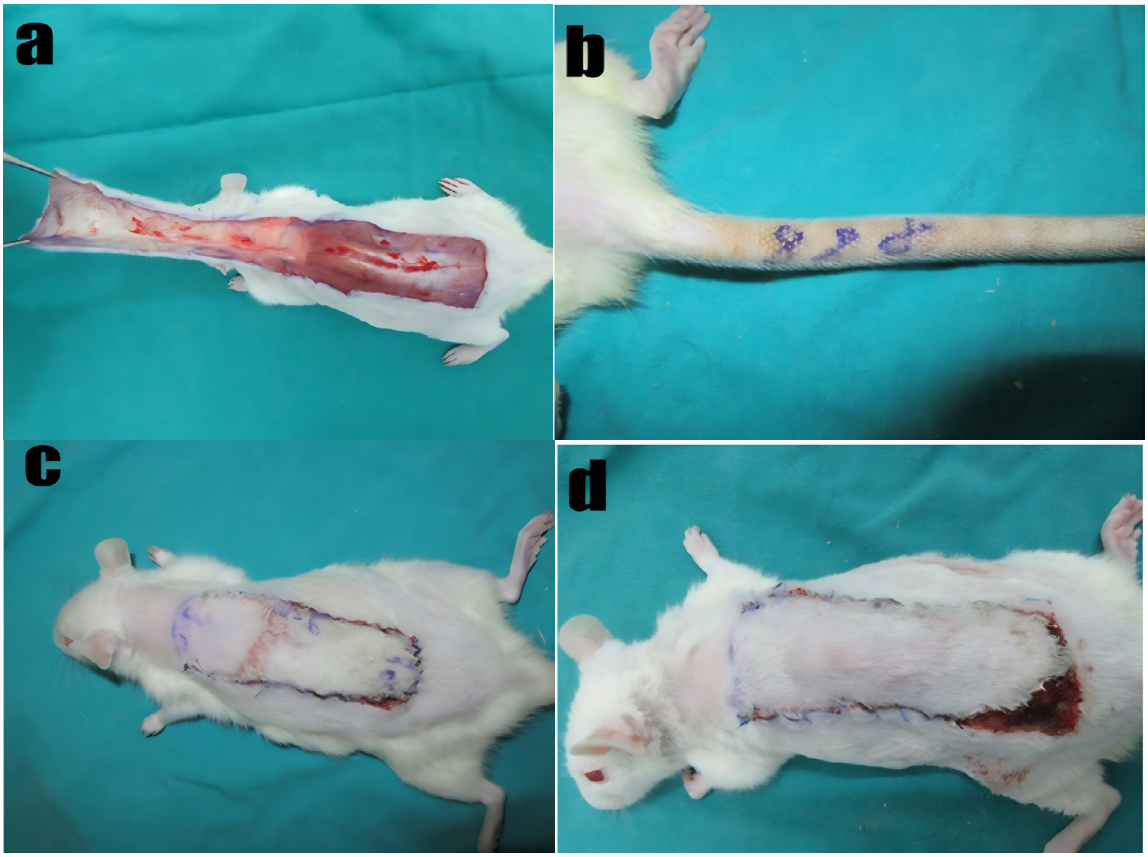

FIGURE 4. Photographic documentation of postoperative flap follow-up. (a) Macroscopic appearance of the flap showing areas of viable tissue (pink color) and early signs of compromised perfusion (pale areas), (b) Close-up view of the flap demonstrating perfusion assessment technique with visible suture lines, (c) Day 5 postoperative assessment showing clear demarcation between viable and non-viable tissue regions, (d) Standardized photographic documentation for planimetric analysis with ruler scale for accurate necrosis area measurement

images are not shown in this manuscript. The sections were examined blindly by an experienced pathologist. The degree of inflammatory cell infiltration, fibroblastic proliferation, collagen formation, neovascularisation and epithelialisation was scored between 0-3 (0=absent, 1=mild, 2=moderate, 3=severe). At least 5 different fields for each preparation were evaluated and the mean score was calculated.

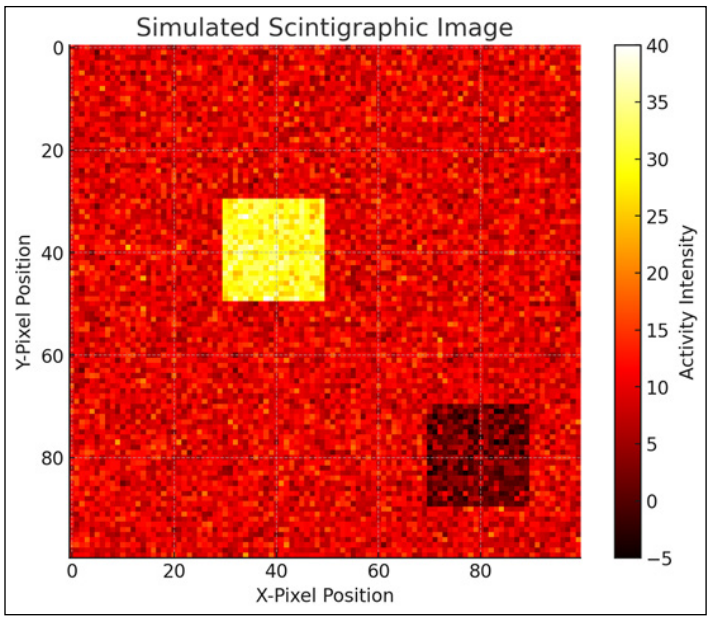

FIGURE 5. Scintigraphic perfusion analysis of flap tissue. Gamma camera image obtained using Tc-99m pertechnetate shows the activity distribution. Yellow area (activity intensity between 30-40) represents hyperaemic region, red areas (between 5-15) represent normally perfused tissue, dark/black areas (activity below 0) represent hypoperfused/necrotic regions. The Y-axis and X-axis show pixel positions and the colour scale on the right shows the activity intensity (arbitrary units)

Biochemical analysis

Tissue samples were homogenised in phosphate buffer using Ultra-Turrax T25 homogenizer (IKA-Werke GmbH & Co., Germany). MDA levels were measured spectrophotometrically using Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Japan) by thiobarbituric acid reactive substance (TBARS) method. NO levels were determined by Griess reaction. SOD activity was calculated by measuring the degree of inhibition of dismutation of superoxide radicals. Total protein content was determined by Bradford method. All measurements were performed in duplicate using Thermo Scientific Multiskan GO microplate reader (Thermo Fisher Scientific Inc., USA) and mean values were used.

Statistical analysis

Data analysis was performed using IBM SPSS Statistics 21.0 programme. The conformity of the data to normal distribution was evaluated by Shapiro-Wilk test. Student's t-test was used for continuous variables showing normal distribution and Mann-Whitney U test was used for continuous variables not showing normal distribution. Chi-square test was used to compare categorical variables. The relationships between variables were evaluated by Pearson or Spearman correlation analysis according to the distribution of the data. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

There was no significant difference between the experimental and control groups in terms of age, body weight and operation time. Both groups received standard sized flaps (3x9 cm). However, the area of viable tissue was significantly decreased (20.1 ± 1.9 cm² vs 22.6 ± 1.8 cm²) and the area of necrosis was increased (6.9 ± 1.2 cm² vs 4.4 ± 0.9 cm²) in the COCs group. Similarly, flap survival rates were lower in the COCs group (74.4% vs 83.7%). When complications were evaluated, the rate of partial necrosis was twice as high in the COCs group (40% vs 20%) and the overall complication rate was significantly higher (70% vs 40%) (TABLE I).

TABLE I
Demographic Characteristics, Baseline Parameters, Flap Survival, and Complication Analysis of Study Groups

Variable	Control Group (n=10)	COCs Group (n=10)	p-value
Number of Rats (n)	10	10	1.000[a]
Age (weeks)	13.8 ± 1.2	14.1 ± 1.3	0.582[b]
Body Weight (grams)	248.5 ± 8.4	251.2 ± 7.9	0.463[b]
Operation Time (minutes)	42.3 ± 5.6	43.1 ± 4.9	0.735
Flap Dimensions (cm)	3.0 x 9.0	3.0 x 9.0	1.000[a]
Total Flap Area (cm²)	27.0 ± 0.0	27.0 ± 0.0	1.000[a]
Viable Tissue Area (cm²)	22.6 ± 1.8	20.1 ± 1.9	0.009[a]
Necrotic Area (cm²)	4.4 ± 0.9	6.9 ± 1.2	0.007[a]
Flap Survival Rate (%)	83.7 ± 4.2	74.4 ± 4.8	0.012[b]
Partial Necrosis	2 (%20)	4 (%40)	0.018
Superficial Infection	1 (%10)	2 (%20)	0.342
Seroma	1 (%10)	1 (%10)	1.000
Overall Complication Rate	4 (%40)	7 (%70)	0.024

Abbreviations: COCs: Combined Oral Contraceptives, referring to the group treated with oral contraceptive methods. P-value: A statistical measure indicating the probability that the observed differences occurred by chance. [a]: Denotes results obtained using the Chi-squared test. [b]: Denotes results obtained using the Student's t-test.

In the scintigraphic evaluation of tissue perfusion, the percentage of hyperemic area was increased (25.7% vs 18.4%), normally perfused area was decreased (52.1 vs 65.3%) and hypoperfused/necrotic area was increased (22.2 vs 16.3%) in the COCs group. Mean activity values were lower in the COCs group (19.4 vs 22.6 AU). The proximal/distal activity ratio was significantly higher in the COCs group (2.4 vs 1.8). When the activity values in different regions of the flap were analysed, a significant decrease in perfusion was noted especially in the distal region (11.3 vs 16.1). Survival rates gradually decreased during the 7-day follow-up period, and this decrease was more pronounced in the COCs group. (TABLE II)

TABLE II
Scintigraphic Evaluation and Survival Analysis

Variable	Control Group (n=10)	COCs Group (n=10)	p-value
Hyperemic Area (%)	18.4 ± 3.2	25.7 ± 4.1	0.012[a]
Normally Perfused Area (%)	65.3 ± 5.8	52.1 ± 6.2	0.008[a]
Hypoperfused/Necrotic Area (%)	16.3 ± 3.5	22.2 ± 4.3	0.015[a]
Mean Activity Values (AU)	22.6 ± 3.8	19.4 ± 3.2	0.041[a]
Proximal/Distal Activity Ratio	1.8 ± 0.3	2.4 ± 0.4	0.009[a]
Proximal	28.5 ± 4.2	26.8 ± 3.9	0.024[b]
Mid	23.2 ± 3.6	19.1 ± 3.4	0.018[b]
Distal	16.1 ± 2.8	11.3 ± 2.5	0.007[b]
Survival Rate (Day 1, %)	100	100	-
Survival Rate (Day 3, %)	95.2	92.4	-
Survival Rate (Day 5, %)	89.6	83.5	-
Survival Rate (Day 7, %)	83.7	74.4	-
Log-rank Test p-value	-	-	0.016

Abbreviations: COCs: Combined Oral Contraceptives, referring to the group treated with oral contraceptive methods. AU: Arbitrary Units, used to represent relative measurements. p-value: A statistical measure indicating the probability that the observed differences occurred by chance. [a]: Denotes results obtained using the Mann-Whitney U test. [b]: Denotes results obtained using the Kruskal-Wallis test

In the histopathological scoring results, inflammatory cell infiltration (2.4 vs 1.8), fibroblastic proliferation (2.2 vs 1.6), collagen formation (2.1 vs 1.7) and neovascularisation (2.3 vs 1.5) values were significantly higher in the COCs group. There was no significant difference between the groups in terms of the degree of epithelialisation. When the score distributions were examined, it was noteworthy that especially severe (score 3) histopathological changes were observed more frequently in the COCs group (TABLE III).

In the biochemical evaluation, malondialdehyde (MDA) (4.86 vs 3.24 nmol/mg) and nitric oxide (NO) (58.3 vs 42.6 µmol/L) levels, which are markers of oxidative stress, were found to be significantly higher in the COCs group, while superoxide dismutase (SOD) activity (14.2 vs 18.5 U/mg), an important component of the antioxidant defence system, was found to be significantly lower. No significant difference was found between the groups in terms of total protein content (TABLE IV).

Correlation analyses revealed significant correlations between tissue perfusion and histopathological findings. Strong positive correlations were found especially between malondialdehyde (MDA) levels and necrosis area (r=0.712) and nitric oxide (NO) levels and neovascularisation score (r=0.695). A positive correlation was found between superoxide dismutase (SOD) activity and flap survival rate (r=0.642), while a negative correlation was observed between MDA levels and flap survival (r=-0.678) (TABLE V).

TABLE IV
Biochemical Analysis and Correlation Results

Variable	Control Group (n=10)	COCs Group (n=10)	p-value	Correlation Coefficient (r)
MDA Levels (nmol/mg protein)	3.24 ± 0.58	4.86 ± 0.72	0.008[a]	-
NO Levels (µmol/L)	42.6 ± 5.8	58.3 ± 6.4	0.006[a]	-
SOD Activity (U/mg protein)	18.5 ± 2.4	14.2 ± 2.1	0.012[a]	-
Total Protein Content (mg/ml)	6.8 ± 0.9	6.5 ± 0.8	0.428[b]	-
MDA - NO	-	-	0.003	0.642
MDA - SOD	-	-	0.007	-0.584
NO - SOD	-	-	0.021	-0.512
MDA - Total Protein	-	-	0.478	-0.168
NO - Total Protein	-	-	0.542	-0.145
SOD - Total Protein	-	-	0.417	0.192

Abbreviations: COCs: Combined Oral Contraceptives, referring to the group treated with oral contraceptive methods. MDA: Malondialdehyde, an indicator of lipid peroxidation and oxidative stress. NO: Nitric Oxide, a marker associated with vascular function and oxidative stress. SOD: Superoxide Dismutase, an enzyme that protects against oxidative damage by neutralizing superoxide radicals. P-value: A statistical measure indicating the probability that the observed differences occurred by chance. [a]: Denotes results obtained using the Mann-Whitney U test. [b]: Denotes results obtained using the Student's t-test

TABLE V
Correlation analysis results

Parameter Relationships	Correlation Coefficient (r)	p-value
Scintigraphic Findings - Histopathological Scores		
Hyperemic Area (%) - Inflammatory Cell Infiltration	0.584	0.007
Normally Perfused Area (%) - Neovascularization	0.632	0.003
Hypoperfused Area (%) - Collagen Formation	-0.548	0.012
Mean Activity - Fibroblastic Proliferation	0.512	0.021
Biochemical Parameters - Flap Survival		
MDA Levels - Flap Survival Rate	-0.678	0.001
NO Levels - Flap Survival Rate	-0.524	0.018
SOD Activity - Flap Survival Rate	0.642	0.002
Total Protein - Flap Survival Rate	0.186	0.432
NO - Neovascularization Relationship		
NO Levels - Neovascularization Score	0.695	<0.001
NO Levels - Proximal/Distal Activity Ratio	0.568	0.009
MDA - Necrosis Relationship		
MDA Levels - Necrotic Area	0.712	<0.001
MDA Levels - Hypoperfused Area Percentage	0.654	0.002

Abbreviations: MDA: Malondialdehyde, an indicator of oxidative stress and lipid peroxidation. **NO: Nitric Oxide**, associated with vascular function and oxidative stress. **SOD: Superoxide Dismutase**, an enzyme involved in antioxidant defense mechanisms. **p-value:** Statistical measure indicating the probability of observed relationships occurring by chance

This study evaluated the safety implications of flap surgery in women using oral contraceptives. Our findings demonstrate that COCs negatively impact flap survival outcomes. Specifically, we observed that flap survival rates were significantly lower in the COCs group compared to the control group (74.4 vs 83.7%, P=0.012), with partial necrosis rates twice as high (40% vs 20%, P=0.018) and overall complication rates significantly increased (70% vs 40%, P=0.024). In addition, in our histopathological evaluations, we found that inflammatory cell infiltration was more prominent, fibroblastic

TABLE III
Histopathological Results and Score Distributions

Variable	Control Group (n=10)	COCs Group (n=10)	p-value	Score	Control Group	COCs Group
Inflammatory Cell Infiltration (0-3)	1.8±0.4	2.4±0.5	0.011[a]	Score Distribution	0: 0, 1: 3, 2: 6, 3: 1	0: 0, 1: 1, 2: 5, 3: 4
Fibroblastic Proliferation (0-3)	1.6±0.3	2.2±0.4	0.008[a]	Score Distribution	0: 0, 1: 4, 2: 6, 3: 0	0: 0, 1: 1, 2: 6, 3: 3
Collagen Formation (0-3)	1.7±0.4	2.1±0.3	0.022[a]	Score Distribution	0: 0, 1: 3, 2: 7, 3: 0	0: 0, 1: 1, 2: 7, 3: 2
Neovascularization (0-3)	1.5±0.3	2.3±0.4	0.007[a]	Score Distribution	0: 0, 1: 5, 2: 5, 3: 0	0: 0, 1: 1, 2: 5, 3: 4
Degree of Epithelialization (0-3)	1.9±0.4	2.0±0.5	0.645[a]	Score Distribution	0: 0, 1: 2, 2: 7, 3: 1	0: 0, 1: 2, 2: 6, 3: 2

Abbreviations: COCs: Combined Oral Contraceptives, referring to the group treated with oral contraceptive methods. P-value: A statistical measure indicating the probability that the observed differences occurred by chance. [a]: Denotes results obtained using the Mann-Whitney U test. [b]: Denotes results obtained using the Chi-squared test

proliferation was higher and neovascularisation was higher in the COC group. In our biochemical analyses, we found that oxidative stress markers were increased and antioxidant defence systems were weakened in the POP group. These findings suggest that POP use may adversely affect the results of flap surgery through various mechanisms.

In this study, flap survival rate was lower (74.4 vs 83.7%), partial necrosis rate was higher (40 vs 20%) and overall complication rate was higher (70 vs 40%) in the COC group. These findings are consistent with the data reported in the literature on flap surgery. The increase in partial necrosis rates may be explained by the findings that POP use increases the risks of hypercoagulability and microthrombosis. A meta-analysis by Kotamarti *et al.* [7], showed that hypercoagulability significantly affected flap survival rates and microthrombosis increased necrosis rates. This study supports our increased partial necrosis rates in the POP group. The decrease in flap survival rates may be related to the effects of oral contraceptives on tissue perfusion. In the study titled *Unconventional Perfusion Flaps in the Experimental Setting*, it was reported that hypoxia, inflammation and tissue perfusion disorders were the main factors affecting flap survival. These findings shed light on the potential mechanisms of our low flap survival rate in the POP group [2]. The increase in overall complication rates is consistent with studies reporting that oral contraceptives increase the inflammatory response and oxidative stress. In a study published in Scientific Reports, increased inflammatory markers and decreased antioxidant defence mechanisms were reported to increase complication rates in flap surgery. This supports the increase in overall complication rates in this study [8].

From COC group, the level of the hyperemic area was over 25.7 as compared to 18.4% of the control group whereas the necrotic/hypoperfused area existed within 22.2 of the POP group in comparison to 16.3% of control. The proximal to distal activity ratio was higher in the POP group at 2.4 than the control group at 1.8. Such results have been corroborated by multiple preceding analyzes that are documented in the literature. Studies on the role of estrogen in wound healing have shown that estrogen supplementation supports wound healing by reducing hypoxia and necrosis rates even at low doses [9]. In a study using silastic capsules with low doses of estrogen release, it was reported that the levels increased blood flow and decreased necrosis rate in skin flaps by maintaining physiological limits [9]. Similarly, the use of phytoestrogens such as genistein has been found effective in reducing hypoxia-induced ischaemia damage and has been reported to increase tissue viability.

In a study, it was reported that genistein treatment decreased the necrosis rate in skin flaps and contributed to the regulation of angiogenesis-related gene expressions [10]. Furthermore, in another research where the role of GPER deficiency on wound healing was analyzed, a substantial reduction in tissue viability and blood perfusion was noted in GPER knockout mice. This is vindicating the essential function of estrogen receptors in tissue repair [11]. The above findings support the higher percentage of hypoperfused/necrotic areas in the POP group observed in our study. The higher percentage of hyperaemic areas may be explained by the positive effect of estrogen on peripheral circulation. In addition, the differences in the proximal/distal activity ratio may be due to the effects of estrogen at the distal microvascular level. This seems to be consistent with previously reported studies [12].

In this study, inflammatory cell infiltration (2.4 vs 1.8), fibroblastic proliferation (2.2 vs 1.6) and neovascularisation (2.3 vs 1.5) were found to be increased in the POP group. However, these findings are considered as an indicator of pathological mechanisms rather than a positive process. Increased inflammation, neovascularisation and fibroblastic activity suggest that POP use has detrimental effects on wound healing. These effects are consistent with the low flap survival, high necrosis rate and increased complication rates in our study. McCracken *et al.* [13] demonstrated that estrogen regulates a controlled inflammatory process and accelerates wound healing by CD45+ cell infiltration. However, the chronic inflammatory state observed in the POP group is interpreted as an uncontrolled inflammation process rather than a regulated healing mechanism. El Mohtadi *et al.* [14] reported that estrogen enhances fibroblast proliferation, promotes TGF-β and VEGF expression, and supports connective tissue regeneration, thereby playing a critical role in improving wound healing and extracellular matrix formation. However, the increase in fibroblastic proliferation observed in the COC group indicates pathological fibrosis and delayed healing process instead of a regular regeneration. The study by Ebrahim *et al.* [15] showed that controlled estrogen therapies promoted vascular regeneration by increasing VEGF expression. However, the increase in neovascularisation observed in the COC group indicates an excessive and dysfunctional vascular formation, which is thought to disrupt normal vascular integrity and increase ischemic damage.

In this study, MDA levels were higher (4.86 vs 3.24 nmol/mg), NO levels increased (58.3 vs 42.6 µmol/L) and SOD activity decreased (14.2 vs 18.5 U/mg) in the POP group. These findings are consistent with various studies on oxidative stress and antioxidant system parameters. The increase in MDA levels indicates increased lipid peroxidation as an indicator of oxidative stress. One study reported that MDA levels were significantly higher in hormonal contraceptive users and this may be due to increased free radical formation [16]. Similarly, Abdelrazek *et al.* [17] reported that hormonal contraceptives increase oxidative stress and this was confirmed by an increase in MDA levels.

The increase in NO levels indicates an increase in oxidative stress and nitric oxide synthesis. Overproduction of nitric oxide may contribute to free radical formation in tissues. McCracken *et al.* [13], reported that oxidative stress levels increased with increased nitric oxide synthesis in oral contraceptive users. The decrease in SOD activity indicates that the antioxidant defence system is suppressed. SOD is an essential antioxidant enzyme that neutralises free radicals. Wang et al. reported that hormonal contraceptive use significantly decreased SOD activity, which may lead to increased oxidative stress levels [9]. In addition, similar findings were reported in the study of Akhiani et al. and it was shown that nano-selenium treatment may partially improve this situation [8].

In this study, we evaluated the effect of COCs on microvascular and angiogenic mechanisms. Examination

of markers such as VEGF and CD31, which are angiogenic factors, could be valuable for understanding the effect of COCs on flap tissue. In the literature, VEGF expression has been shown to play a critical role in tissue perfusion and new vessel formation; regulated by HIF-1α under hypoxic conditions, this factor initiates the process of angiogenesis [18]. Analyses of angiogenesis markers could better explain the perfusion defects in scintigraphic evaluations observed in our study. The potential effects of COCs use on endothelial function have been demonstrated in the literature in experimental animal models [19] and clinical studies [20]. The use of COCs may also affect the coagulation system. In the literature, estrogens have been reported to increase fibrinogen levels and decrease protein C activity [21]. More importantly, preparations containing 50 µg and 30 µg of ethinylestradiol have been shown to increase platelet activity in a dose-dependent manner [22], which may contribute to microthrombus formation in flap tissue. Bouck and colleagues [22] showed that ethinyl estradiol and drospirenone did not directly increase the procoagulant activity of endothelial cells, but this finding may contradict the perfusion disturbances observed in our in vivo study. The effect of combined oral contraceptives on angiogenic factors may affect the development of blood vessels in the flap by increasing the expression of VEGF, FGF1 and TF genes [23]. In future studies, more detailed evaluation of endothelial cell function and coagulation parameters may elucidate the mechanisms of the adverse effects of COCs use on flap tissue.

This study has some limitations. Firstly, the relatively small number of experimental animals and the short follow-up period limit the generalisability of the results. In addition, the use of a single dose of COC did not allow the evaluation of the dose-response relationship. However, our study also has strengths. In particular, our multifaceted evaluation approach (combined use of scintigraphic imaging, histopathological examination and biochemical analyses) allowed us to comprehensively evaluate the results of flap surgery. In addition, the application of a uniform surgical technique as well as the objective methods for assessment of the results, increased the reliability of this investigation. Future research should examine the influence of varying doses of COC, different timing of applications, and increase the follow-up period to gain understanding of the mechanisms at the molecular level. Besides, exploring potential treatment strategies (for example antioxidant therapies, vasodilator agents) which would mitigate the negative consequences of using POP would be also beneficial for the future investigations [24,25].

CONCLUSION

Analysis of our research findings reveals significant concerns regarding combined oral contraceptive use in flap surgery outcomes. The experimental data demonstrates markedly reduced flap survival alongside increased necrosis rates and complications. From the perusal of these images under a microscope, we identified some key factors that were the root cause: more oxidative stress markers, disrupted inflammatory pathways and less blood supply to tissues. These are important clinical observations especially when planning surgery for OC users. Thus, according to our observation it can be seen that there is need to reconsider perioperative OC management through either discontinuation or by using other contraceptive methods temporarily. Nevertheless, although our experimental findings provide vital insights into this matter, additional clinical investigations in humans about these findings are necessary to confirm them and design preventive strategies.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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Data availability

Data used in this study can be provided on reasonable request.

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