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The protective role of melatonin and agomelatine against oxidative stress following laparoscopic ovariectomy in dogs

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Abstract

Background The present study was carried out to compare biomarkers of oxidative stress and antioxidant activity of agomelatine and melatonin in bitches undergoing laparoscopic ovariectomy. Twenty-four healthy female dogs were randomly divided into four groups: Control (C), laparoscopic ovariectomy (LO), agomelatine + laparoscopic ovariectomy (ALO), and melatonin + laparoscopic ovariectomy (MLO) consisting of 6 animals each. Melatonin and agomelatine were administered to the MLO group (0.3 mg/kg/day, p.o) and the ALO group (0.3 mg/kg/day, p.o) consequently one day before LO to 7 days post-intervention. Blood sampling was performed on days − 1, 0 (immediately after surgery), 3, and 8 of the study. Total oxidant status (TOS), total antioxidant capacity (TAC), and malondialdehyde (MDA) in sera and superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in hemolyzed red blood cells (RBCs) were measured.

Results Among all indices analyzed over time, only the MDA index in the ALO group showed a significant difference, being significantly lower on day 3 post-surgery compared to days − 1 and 0 ($p = 0.023$). Significant differences were observed between groups in terms of TOS, TAC, GPx, and SOD levels. The LO group exhibited a significant increase in the TOS index on day 3 compared to all other groups ($p = 0.008$). The TAC index experienced the most significant increase in the MLO group on day 0 compared to other groups ($p = 0.005$), and this trend continued significantly until day 3, only in comparison to the LO and C groups ($p = 0.024$). Agomelatine significantly increased SOD levels in the ALO group on postoperative day 3 compared to groups C and LO ($p = 0.009$). GPx levels were significantly elevated in ALO and MLO groups on day 8 compared to groups C and LO ($p = 0.003$).

Conclusion The results of this study demonstrate that melatonin, by increasing total antioxidant capacity, and agomelatine, through enzymatic antioxidant pathways, contributed to the reduction of free radical levels in dogs. The present study revealed that administering agomelatine (0.3 mg/kg/day, p.o) could decrease MDA levels significantly after laparoscopic ovariectomy up to day 3.

Keywords Oxidative stress, Antioxidants, Agomelatine, Melatonin, Laparoscopic ovariectomy, Dogs

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Background

Potential benefits of neutering dogs include population control, prevention and treatment of certain reproductive system diseases, and behavioral disorders [1–3]. With the advancement of surgical equipment, laparoscopic ovariectomy (LO) has gained preference over traditional ovariohysterectomy (OHE) and ovariectomy (OVE) neutering methods as a diagnostic and therapeutic procedure [4]. LO has been established as a safe and secure method for spaying dogs [5], and its advantages in mobility, patient recovery, and systemic immune response compared to the open approach are widely accepted [6–9].

Pneumoperitoneum (PNP) provides an optimal workspace during laparoscopic surgery [10], and carbon dioxide (CO_2) is the most suitable gas for establishing PNP due to its affordability, non-flammability, which enables safe electrocautery, high solubility in blood that minimizes gas embolism, and rapid elimination through respiration [10, 11]. Despite the numerous advantages of laparoscopy compared to the open approach, insufflation of carbon dioxide into the abdominal cavity following laparoscopic surgery leads to an increase in intra-abdominal pressure (IAP), reduced blood flow to the stomach and small hepatic vessels, ischemia of the spleen and kidneys, mechanical pressure on the abdominal blood vessels, peritoneal dehydration, morphological changes in the mesothelium, hypercapnia, and oxidative stress in the body [11–17]. Additionally, evidence suggests that oxidative stress remains even after abdominal deflation, IAP reduction, and increased splenic perfusion [13].

Oxidative stress in the body represents an imbalance between the production of free radicals and the ability of antioxidant defense mechanisms to detoxify active intermediates [10, 18]. The role of oxidative stress in the pathogenesis of many diseases has been demonstrated, and numerous studies have been conducted to establish the effect of free radicals on surgical stress [10]. The total antioxidant capacity (TAC) is the sum of the effects of all antioxidants in blood and body fluids. It is considered a valuable index of the body's antioxidant status to counteract oxidative damage caused by reactive oxygen species (ROS) [10, 19].The catalase (CAT) is an essential endogenous antioxidant enzyme that catalyzes the detoxification of hydrogen peroxide (H_2O_2) [20], while glutathione peroxidase (GPx) and superoxide dismutase (SOD) activate the enzymatic defense system against reactive oxygen species [21, 22].

The release of ROS, disruption of microcirculation, and tissue hypoxia represent the initial stages of peritoneal injury, encompassing processes from mesothelial denudation to inflammation and the development of adhesions [11]. Biochemical markers of oxidative stress can detect early intracellular changes before the development of morphological alterations [14]. Numerous studies have demonstrated that oxidative stress markers experience changes following laparoscopic surgery [4, 6–8, 13, 15]. Total oxidant status (TOS) and Total antioxidant status (TAS) are used to estimate overall antioxidant status [13]. Any injury caused by PNP and ischemia-reperfusion (IR) can lead to the production of free radicals [11, 12, 14], which are neutralized by enzymatic (GPx, CAT, and SOD) and non-enzymatic antioxidants [23]. An imbalance between pro-oxidants and the antioxidant defense system produces excessive ROS, H_2O_2, and hydroxide (OH^-), pushing the cell toward oxidative stress [24]. Oxidative damage to proteins, lipids, and deoxyribonucleic acid (DNA) leads to cellular toxicity [25]. This damage is caused by reactions of ROS with biomolecules, resulting in the formation of substances such as malondialdehyde (MDA), a valuable marker of lipid peroxidation [14, 21, 26, 27]. In recent years, there has been a surge in research aimed at measuring oxidative stress markers following surgical procedures and developing strategies to mitigate oxidative damage. The selection of appropriate anesthetic and analgesic protocols also plays a crucial role in minimizing oxidative injury [6–8, 14, 24, 28].

Multiple therapeutic approaches have recently been subjected to clinical trials for oxidative stress injury and IR. One of the compounds used for this purpose is melatonin (N-Acetyl-5-Methoxytryptamine), a broad-spectrum antioxidant that directly inactivates ROS, reactive nitrogen species (RNS), and free radicals or indirectly (receptor-mediated actions) modulates the cellular antioxidant system by acting on MT_1, MT_2, and MT_3 (quinone reductase) melatonin receptors and increases endogenous enzymes such as SOD, GPx, and CAT. Melatonin has shown promising results in reducing oxidative stress injury in animal models [27, 29, 30].

Agomelatine (N-[2-(7-methoxy-1-naphthalenyl)-ethyl]-acetamide) is a synthetic analog of the hormone melatonin and a potential agonist at MT_1 and MT_2 receptors, with antioxidant effects mediated through melatonin receptors [31–34]. It was first marketed in the European Union in 2009 under the brand names Valdoxan® or Thymanax® [35]. Compared to melatonin, it has a longer half-life and a relatively higher affinity for MT_1 and MT_2 melatonin receptors in the suprachiasmatic nucleus and other brain regions [36]. Activation of melatonin receptors upregulates the expression of antioxidant enzyme-associated genes. Melatonin receptor agonists directly affect ROS scavenging and indirectly stimulate the antioxidant defense system [34]. Agomelatine exhibits high affinity for MT_1 and MT_2 receptors (1000-fold higher than for the 5-hydroxytryptamine $_{2C}$ (5-HT_{2c}) receptor) and minimal affinity for the 5-HT_2 receptor family. Additionally, agomelatine's antagonistic action on 5-HT_{2B} receptors has been reported to improve rodent neuropathic pain, visceral pain, and hyperalgesia

[37]. Furthermore, a study on plasma agomelatine concentrations revealed that oral administration of a single dose of agomelatine in fasted and fed conditions to Labrador retriever dogs showed no side effects or behavioral changes up to 48 h later. Considering the clinical dose of agomelatine in humans (0.35 mg/kg), the dose administered in this study was 25 times higher (8.9 mg/kg), and They suggested that agomelatine has a good safety profile in dogs [35].

Agomelatine has a longer half-life and a greater affinity for MT_1 and MT_2 receptors than melatonin, which may result in different antioxidant effects. To our knowledge, no clinical studies have directly compared the antioxidant effects of these two drugs in mitigating oxidative stress indices induced by laparoscopic ovariectomy (LO) in bitches, particularly considering their unique pharmacokinetic properties.

Results

All dogs were hemodynamically stable throughout the experimental procedure (results not shown).

TOS level

The TOS index revealed a significant increase only in the LO group on day 3 compared to other groups ($p = 0.008$). No significant differences were found between the ALO and MLO groups on any sampling days (Tables 1 and 2). Additionally, according to Table 1, no significant changes in the TOS variable were observed over time in any of the study groups.

MDA level

No significant differences regarding serum MDA alterations were observed between the groups on sampling days. Table 1 demonstrates that while no significant changes were observed over time in groups C, LO, and MLO, we found a significant decrease in the MDA variable between days – 1 vs. 3 ($p = 0.031$) and days 0 vs. 3 for the ALO group ($p = 0.031$).

TAC level

In the case of the serum TAC variable, significant differences between groups were observed only on days 0 and 3. On day 0, the MLO group exhibited the most significant increase compared to other groups ($p = 0.005$). Additionally, the MLO group on day 3 experienced a significant increase in TAC levels compared to the C and LO groups ($p = 0.024$) (Tables 1 and 2); still, there was no significant difference compared to the ALO group. Moreover, no significant changes were observed over time for the study groups regarding the TAC variable (Table 1).

CAT level

The animals did not show any statistically significant differences in terms of serum CAT level over time for each group and between the studied groups on the sampling days (Table 3).

SOD level

As detailed in Table 3, A significant increase in SOD levels was observed exclusively in the ALO group on day 3 compared to both groups C and LO ($p = 0.009$). However,

Table 1 The serum concentration of MDA, TOS & TAC in four different groups. Data are expressed as median (25–75%)

Factor	Groups ($n = 6$)	Day – 1	Day 0	Day 3	Day 8	*P* value
MDA (nmol/ml)	C	16.9 (16.56–17.12)	15.9 (15.42–16.47)	16.7 (16.17–16.95)	16.9 (16.65–17.2)	0.05
	LO	17.25 16.65–17.52)	15.95 (15.02–16.75)	16.4 (15.6-17.65)	16.6 (16.17–17.55)	0.30
	ALO	16.9[a] (16.67–18.7)	16.6[a] (15.67–17.55)	16.15[b] (14.95–16.90)	16.85 (16.57–17.87)	**0.023**[*]
	MLO	17.1 (16.52–18.05)	16.35 (15.97–17.42)	16.9 (16.65–17.52)	17.15 (17.02–17.42)	0.21
P value		**	0.58	0.36	0.42	
TOS (µmol Equiv./L)	C	6.9 (6.12–8.05)	5.3 (4.32–5.97)	[a]5.85 (4.87–6.95)	6.1 (5.35–6.87)	0.36
	LO	6.3 (5.6–8.52)	6.05 (5.8–7.55)	[b]8.15 (7.47–8.82)	6.4 (4.17–7.75)	0.18
	ALO	5.9 (5.55–6.47)	6.3 (4.87–7.35)	[a]6.1 (5.02–7.40)	5.3 (4.37–6.95)	0.49
	MLO	7.15 (6.17–8.52)	6.05 (4.77–8.25)	[a]6.8 (5.75–7.37)	6.55 (5.15–8.30)	0.45
P value		**	0.185	**0.008**[*]	0.651	
TAC (mmol Fe2+/L)	C	0.67 (0.63–0.71)	[a]0.66 (0.63–0.71)	[a]0.65 (0.62–0.70)	0.71 (0.66–0.78)	0.92
	LO	0.70 (0.60–0.74)	[a]0.65 (0.60–0.67)	[a]0.63 (0.62–0.65)	0.66 (0.58–0.82)	0.88
	ALO	0.85 (0.71–0.94)	[a]0.75 (0.73–0.78)	[ab]0.745 (0.68–0.83)	0.81 (0.70–0.95)	0.49
	MLO	0.83 (0.77–0.96)	[b]0.82 (0.80–0.95)	[b]0.81 (0.77–0.94)	0.83 (0.78–0.90)	0.99
P value		**	**0.005**[*]	**0.024**[*]	0.52	

[ab] In each column, different superscript letters on the left side of the median indicate significant differences between groups ($p < 0.05$)

[ab] In each row, different superscript letters on the right side of the median indicate significant differences between sampling days ($p < 0.05$)

[*] The right *P value* column indicates significant differences between sampling days ($p < 0.05$)

[*] The *P value* indicates significant differences between groups ($p < 0.05$) in each row

** According to the Quade Non-Parametric test, the effect of day – 1 in the study between groups has been adjusted

Abbreviations: C = Control, LO = Laparoscopic Ovariectomy, ALO = Agomelatine + Laparoscopic Ovariectomy, MLO = Melatonin + Laparoscopic

Table 2 Multiple comparison (LSD post hoc test)

Sampling Times	Factor	(I) Group *N* = 6	(J) Group *N* = 6	Mean Differences (I-J)	*P* value
Day 0	TAC	C	LO	3.286	0.233
		ALO	C	1.505	0.580
			LO	4.791	0.088
		MLO	C	**7.542***	**0.011**
			LO	**10.829***	**0.001**
			ALO	**6.037***	**0.035**
Day 3	TOS	LO	C	**12.085***	**0.001**
			ALO	**9.404***	**0.008**
			MLO	**7.905***	**0.023**
		ALO	C	2.680	0.414
		MLO	C	4.180	0.208
			ALO	1.499	0.646
	TAC	C	LO	3.311	0.308
		ALO	C	3.093	0.341
			LO	6.405	0.057
		MLO	C	**7.059***	**0.038**
			LO	**10.371***	**0.004**
			ALO	3.966	0.225
	SOD	C	LO	1.068	0.749
		ALO	C	**10.549***	**0.004**
			LO	**11.618***	**0.002**
			MLO	6.723	0.055
		MLO	C	3.825	0.259
			LO	4.894	0.153
Day 8	GPx	C	LO	3.532	0.257
		ALO	C	**6.364***	**0.049**
			LO	**9.896***	**0.004**
		MLO	C	**8.472***	**0.011**
			LO	**12.004***	**0.001**
			ALO	2.108	0.495

*Level of significance set at $p < 0.05$. Abbreviations: C = Control, LO = Laparoscopic Ovariectomy, ALO = Agomelatine + Laparoscopic Ovariectomy, MLO = Melatonin + Laparoscopic Ovariectomy

The standard deviation across the levels of the factor variable is constant for each fixed day

no significant difference was found between the MLO and ALO groups. With regards to the SOD concentration, no significant changes were recorded in the SOD variable over time in the study groups (Table 3).

GPx level

A significant difference between the study groups was only observed on day 8 ($p = 0.003$) (Table 3). Both drugs significantly increased GPx levels compared to groups C and LO; however, no significant difference was found between the MLO and ALO groups in terms of GPx level (Table 2). According to Table 3, no significant changes in GPx were observed over time in the study groups.

Discussion

Due to melatonin's therapeutic potential, extensive research and development efforts have been focused on melatonin analogs aiming to improve pharmacokinetics and receptor affinity in the past decade. Agomelatine is a melatonergic drug with limited clinical data on its antioxidant and protective effects in animals [30, 33]. A canine model was used to compare the antioxidant effects of melatonin and agomelatine since laparoscopic ovariectomy surgery is a novel and less invasive method for sterilization and treatment of reproductive system disorders [6, 8, 24].

Activation of phagocytic cells by pro-inflammatory cytokines produces ROS, leading to increased lipid peroxidation levels, as measured by MDA, through the oxidation of cell membranes. Lipid peroxidation and enzymatic antioxidants such as glutathione peroxidase are recognized as biomarkers of oxidative stress [34]. Despite a significant increase in TOS levels following laparoscopy on day 3, serum MDA levels decreased significantly over time only in the ALO group, and no significant difference has been seen in the LO group,

Table 3 RBCs Hemolyzed concentration of CAT, SOD & GPx in four different groups. Data are expressed as median (25–75%)

Factor	Groups ($n=6$)	Day − 1	Day 0	Day 3	Day 8	*P* value
CAT (nmol/g Hb)	C	5.5 (5.12–5.87)	6.2 (5.62–7.45)	4.6 (4.22–5.82)	5.3 (5.2–5.47)	0.06
	LO	5.25 (4.60–6.77)	6 (6.00-7.3)	6 (4.6–7.30)	6 (5.20–6.77)	0.60
	ALO	4 (3.50–5.10)	4.8 (4.22–5.45)	4.35 (3.15–5.55)	4.7 (4.30–5.45)	0.46
	MLO	3.6 (3.27–4.3)	4.3 (3.57–5.22)	4.85 (3.45–6.12)	3.6 (3.22–4.4)	0.24
P value		**	0.21	0.42	0.24	
SOD (U/g Hb)	C	475 (431.25–506.50)	383.5 (309–434)	[a]422.5 (361.5-561.5)	470 (384.5-535.75)	0.42
	LO	408 (390.75-451.25)	373.5 (328.5-516.5)	[a]406.5 (370.25–455)	466 (379.5-554.25)	0.49
	ALO	487.5 (433.25-543.25)	496.5 (443.25–644)	[b]608.5 (557.5-665.75)	598 (553.75–622)	0.36
	MLO	556 (475-580.25)	515 (470.75-601.25)	[ab]470.5 (399.75–611.50)	543 (517.50-574.25)	0.33
P value		**	0.14	**0.009***	0.11	
GPx (mU/g Hb)	C	247 (240.25-254.75)	303 (289.5-343.5)	281 (218.5–316)	[a]270 (235–303)	0.10
	LO	316 (247.5-365.75)	278 (259–341)	286.5 (241.75–341)	[a]267.5 (251.75-284.25)	0.53
	ALO	317 (307.75-342.25)	300.5 (261.25–340)	331.5 (306-367.75)	[b]328.5 (296.25–366)	0.45
	MLO	311.5 (286.5-332.75)	303 (290-328.25)	323.5 (289.5-351.25)	[b]338.5 (312.5–374)	0.06
P value		**	0.53	0.44	**0.003***	

[ab] In each column, different superscript letters on the left side of the median indicate significant differences between groups ($p<0.05$)

[ab] In each row, different superscript letters on the right side of the median indicate significant differences between sampling days ($p<0.05$)

* The right *P value* column indicates significant differences between sampling days ($p<0.05$)

* The *P value* indicates significant differences between groups ($p<0.05$) in each row

** According to the Quade Non-Parametric test, the effect of day − 1 in the study between groups has been adjusted

Abbreviations: C = Control, LO = Laparoscopic Ovariectomy, ALO = Agomelatine + Laparoscopic Ovariectomy, MLO = Melatonin + Laparoscopic Ovariectomy

suggesting that oxidative stress and lipid peroxidation may not have been induced in the present study. These findings indicate that LO via midline approach is a minimally invasive procedure despite the insufflation of CO_2 gas into the abdominal cavity [6, 13]. The current results aligned with Martins's study (2023), which states that two-port video-assisted OHE in cats induces an insufficient inflammatory response for inflammation detection in the immediate postoperative period (24 h), And this is likely due to the procedure's minimally invasive nature [9]. Moreover, many studies have shown that OHE does not significantly affect serum MDA concentration in dogs and cats [38–40]. Kumari's study (2022) demonstrated that midline LO resulted in a significantly lower MDA level compared to OVE and laparoscopic triangle ovariectomy. Still, in this study, the MDA index increased significantly 4 days after surgery in all study groups [24]. Some results were reported that tissue oxygen tension and MDA increase after laparoscopic surgery [41, 42]. Other studies have shown that OHE in bitches leads to an increase in serum MDA concentration on the third day after surgery [43, 44]. Sakundech et al.. (2020) also reported that MDA levels peak on day 3 after surgery due to inflammation from surgical trauma and that dogs undergoing this procedure should receive antioxidants. They attributed this phenomenon to a decrease in antioxidant capacity on the third-day post-OHE [43].

Our results showed that on day 0, the serum TAC levels in the MLO group experienced the most significant increase compared to other groups. Additionally, on day 3, serum TAC levels were significantly higher in the MLO group compared to the LO and C groups. Moreover, there was a significant increase in SOD levels on day 3 only in the ALO group compared to the LO and C groups. Furthermore, the ALO and MLO groups demonstrated a significant increase in GPx levels on day 8 compared to the LO and C groups. Based on the present findings, the antioxidant effects appear to have become more pronounced in the treatment groups from the third day onward. Agomelatine caused a significant increase in antioxidant enzymes (SOD and GPx), a significant decrease in the TOS index, and a significant decrease in MDA over sampling days (-1,0 and 3). Melatonin caused a significant increase in the antioxidant enzyme GPx and the TAC index and a significant reduction in the TOS variable. Besides, neither agomelatine nor melatonin significantly affected CAT levels of hemolyzed RBCs in any of the studied groups.

Similar to our results, Yapca et al.. (2014) demonstrated that agomelatine significantly reduced MDA levels and increased SOD and GPx levels in the ovarian tissue of rats subjected to IR injury [45], Furthermore, Aguiar et al.. conducted a pioneering study to compare the antioxidant effects of agomelatine (25, 50, 75 mg/kg, Intraperitoneal (IP) injection) and melatonin (50 mg/kg, IP) in the brains of Swiss mice subjected to a strychnine-induced seizure model. They found that in all doses, agomelatine demonstrated a more significant reduction in TBARS levels than melatonin, and only agomelatine (25, 50 mg/kg, IP) significantly increased CAT levels compared to the control group in the brain [33]. Salavati et al.. (2021) showed that MDA levels peaked 3 days after surgery, Although

melatonin (0.3 mg/kg/day, p.o.) significantly reduced MDA levels over time. No significant difference was observed between OHE and melatonin + OHE groups. Additionally, melatonin couldn't completely modulate the activity of antioxidant enzymes (CAT, GPx, SOD) due to the high level of oxidative stress induced by the surgical model [27]. Contrary to the results of Salavati and Aguiar, melatonin did not exhibit any significant effect on MDA levels in the MLO group in our results, in contrast, agomelatine significantly reduced MDA levels over time in the ALO group. Melatonin TAC results were in agreement with Mistraletti et al. (2017) who stated that melatonin administration increased whole blood antioxidant capacity, suggesting a potential beneficial role in critically ill patients due to its immunomodulatory and antioxidant properties [46]. Although agomelatine increased TAC levels, this increase was not statistically significant and could be influenced by factors such as PNP, tissue manipulation, and antioxidant modulation.

Melatonin is a broad-spectrum antioxidant that directly exerts its antioxidant effects through MT_1, MT_2, and MT_3 by acting on classic radical scavengers or through secondary metabolites [29, 30]. It also exhibits a powerful antioxidant effect through the receptor of the ovarian tissue's Mel1 (melatonin membrane) [45]. Melatonin's anti-inflammatory effect seems associated with pain reduction due to its ability to decrease N-methyl-D-aspartate (NMDA) receptor [47]. The activation of NMDA receptors in neurons can induce oxidative stress and cell death through a process that involves the extracellular release of superoxide by nicotinamide adenine dinucleotide phosphate $oxidase_2$ [48]. Therefore, melatonin can modulate endogenous antioxidant enzymes through multiple pathways. On the other hand, agomelatine exerts its antioxidant effect to reduce ROS levels through MT_1 and MT_2 receptors, the mitochondrial redox system, and gamma-aminobutyric acid receptors (GABAergic mechanism) [33, 34]. GABA is involved in oxidative stress, as ROS have been shown to decrease both the release and uptake of GABA. Additionally, reduced GABA release and uptake in synaptosomes have been reported due to lipid peroxidation. The activity of agomelatine on GABA receptors likely indicates its antioxidant activity. Due to the higher affinity of agomelatine for MT_1 and MT_2 receptors [36], the antioxidant effect through these receptors to reduce ROS and oxidative stress is likely more substantial. Rebai et al.. (2021) also demonstrated that agomelatine normalizes cerebral oxidative status by significantly reducing TBARS levels and significantly increasing GPx levels in the brains of mice with high-fat diet-induced neuroinflammation and depression-like behavior. This study suggests that the behavioral improvement induced by long-term administration of agomelatine is associated with its anti-inflammatory effects. The anti-inflammatory effect of agomelatine may be partly due to its regulatory effect on the genes encoding inflammatory cytokines such as TNF-α, IL-6, and IL-1β [49]. In this regard, Yigitturk et al. (2017) reported that the antioxidant effects of agomelatine are mediated by blocking the mechanisms of induction of oxidation or expression of inflammatory cytokines [50].

According to Table 3, the levels of GPx and SOD enzymes in the ALO group and the level of GPx enzyme in the MLO group exhibited an increase on days 3 and 8 after LO compere to day 0. However, this increase was not statistically significant, possibly due to the small sample size in each group (total = 24, $n = 6$). Therefore, larger-scale studies should be conducted in this area. To ensure the well-being of the shelter animals and minimize any potential stress, the sample size was made while balancing the ethical imperative of animal welfare with the need for sufficient data for statistical analysis [51].

In line with previous studies that established the standard surgical approach for sterilization in veterinary medicine, the present study's findings support the efficacy of the standard LO technique [5, 24]. The current results also demonstrate that using antioxidants can minimize surgical complications. However, future research should focus on long-term periods to improve understanding of their therapeutic benefits and safety in clinical practice. It is suggested that both drugs be compared in two surgical stress models, LO vs. OHE, along with pain assessment and measurement of inflammatory cytokines during the early post-operative hours, as well as with drug plasma concentrations.

Methods

This experimental interventional study was conducted at the educational hospital of the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad (Iran). It was performed under the Iranian animal ethics framework, with oversight provided by the Iranian Society for the Prevention of Cruelty to Animals and the Ferdowsi University of Mashhad Research Council (IR.UM.REC.1402.045). All canine subjects in this study were recruited from an animal shelter. Written informed consent was acquired from the shelter's administration for all experimental protocols. The project was completed from May 31, 2023, to September 6, 2023.

Animals

Inclusion criteria for this study were a random selection of twenty-four healthy, mixed-breed bitches between the ages of 1 and 3 years and weighing between 15 and 20 kg from an animal shelter. All dogs were neutered by LO and returned to the animal shelter. All dogs were housed in individual cages under a 12-hour light/12-hour

dark cycle and fed a standard commercial diet (300 g/dog/day; Nutripet™; Behintash Co., Karaj, Iran). Access to water was provided to the dogs ad libitum. During the first three weeks of acclimatization, the dogs were treated with an anthelmintic tablet (fenbendazole, 150 mg; pyrantel embonate, 144 mg; praziquantel, 50 mg; Caniverm®, 0.7 mg/10 kg, p.o.). Throughout the study, the general health of the animals was monitored by physical examination and hematological tests, and the pregnancy status of the dogs was assessed by ultrasonography (data not shown).

Experimental design

Dogs were randomly allocated to four groups of six (total = 24, $n = 6$). To minimize surgical duration and inflammatory effects, all surgeries were performed by an experienced laparoscopic surgery team. The control (C) group underwent neither sterilization nor administration of agomelatine and melatonin and, on day 0, received only an anesthesia regimen (maintenance time was considered 20 min, which was equivalent to the average time from CO_2 insufflation to the last knot in the other groups). The LO group underwent LO only on day 0 and did not receive any medication (neither melatonin nor agomelatine). The agomelatine + laparoscopic ovariectomy (ALO) group underwent LO on day 0 of the study and received agomelatine (25 mg, Agoleep®, Iran) at a dose of 0.3 mg/kg/day, p.o [27]. in the off-feed state on days − 1 to 7, and The melatonin + laparoscopic ovariectomy (MLO) group underwent LO on day 0 of the study and received melatonin (3 mg, Razak Melatonin®, Iran) at a dose of 0.3 mg/kg/day, p.o [35]. in the off-feed state on days − 1 to 7. Blood samples were collected using 20 cc syringes via the jugular vein on days − 1 (24 h before surgery), 0 (immediately after surgery), 3 (3 days after surgery), and 8 (8 days after surgery) and transferred sequentially into two vacutainer tubes containing heparin tube, and ethylenediaminetetraacetic acid (EDTA) tube, and a plain tube. The serum was centrifuged at 3000 rpm for 10 min, then stored in 2 ml tubes and kept at -80 ℃ for evaluation of MDA, TOS, and TAC laboratory factors. Blood samples containing heparin were centrifuged at 3000 rpm for 5 min to separate plasma and erythrocytes. Erythrocyte samples were washed three times with normal saline (3x sample volume) at 3000 rpm for 5 min to remove residual plasma or buffy coat components. Finally, the sedimented erythrocytes were diluted 1:100 with sterile distilled water and centrifuged at 10,000 rpm at 4 °C. The supernatants were carefully collected in 2 mL microtubes at -80 °C for subsequent analysis of GPx, CAT, and SOD activity.

Equipment

The experimental work was carried out in the surgical area of the Ferdowsi University of Mashhad Veterinary Hospital. The LOs were performed using an Olympus® endoscopy tower, including equipment: an insufflator (Olympus UHI-2, Olympus®, Japan), a rigid endoscope, a medical CO_2 bottle, a power LED light source (Olympus CLO-S40, Olympus®, Japan), laparoscopic veress needle, laparoscopic scissors, bipolar electrosurgical handpieces, 10 mm cannulas, and Babcock forceps. An anesthesia monitoring system (cardio set, ARAD P10, SaIran Medical Industrial, Iran) was used for monitoring, and an EDP-neptune® anesthesia machine was used for inhalation anesthesia. Furthermore, two centrifuges, including the Jouan® C312 (Great Britain) and MPW®-260R (Hungary), Celltak MEK-6550 (Nihon Kohden®, Japan), and Mindray® BS-240 (India) were utilized for the preparation and analysis of hematologic markers.

Analgesia and anesthesia

Animals were fasted for 12 h before surgery with ad libitum access to water. Premedication with acepromazine (0.05 mg/kg intramuscular (IM); Neurotranq 1%, Alfasan Co., Netherlands) was administered, followed by morphine (0.5 mg/kg IM; Darou Pakhsh Co., Iran) 10 min later. Once the dogs were sedated, a 20-gauge catheter was placed in the right cephalic vein. Ringer's lactate solution was infused at a rate of 5 ml/kg/h throughout the anesthesia and surgical procedure. Meloxicam (0.1 mg/kg subcutaneous (SC); Rooyan Co., Iran), a nonsteroidal anti-inflammatory drug, was administered as a premedication before induction of anesthesia. Each animal received a single dose of the antibiotic cefazolin (22 mg/kg/IV, AFAZOLE®, Iran) as prophylaxis 30 min before surgery. Anesthesia was induced with a combination of ketamine (6 mg/kg, Ketaset 10%, Alfasan Co., Netherlands) and midazolam (0.25 mg/kg, Midazolam 5, Caspian Co, Iran) intravenously. Following tracheal intubation, general anesthesia was maintained with isoflurane gas (2% end-tidal concentration) (Terrel™, USA) and 100% oxygen. The level of anesthesia and end-tidal CO_2 (< 55 mmHg) were monitored every 10 min until the end of surgery.

Surgical procedure

LO was performed using the three-portal technique with linear abdominal access [24, 52]. The patient was positioned in the Trendelenburg position on the tilt table. A stab incision was made about 0.5 cm caudal to the umbilicus to insert a veress needle. Then, CO_2 gas was insufflated at a constant rate of 1 L/Min to establish an IAP of 6–8 mmHg throughout the procedure. The first trocar was inserted through the initial incision for telescope placement (sub-umbilical port), and the abdominal cavity

was inspected for any bleeding or potential injury. After that, the second trocar was inserted through a stab incision 3 cm cranial to the umbilicus, and the third trocar was inserted through a stab incision 5 cm cranial to the pubic bone for additional grasping instrument insertion. The ovarian pedicle was coagulated and cauterized by a bipolar electrocautery. Upon confirmation of the absence of bleeding in the abdominal cavity, both ovaries were removed through the mid-port, the abdominal fascia was closed by an absorbable suture in a simple interrupted pattern, and the skin was closed by an absorbable suture in an intradermal pattern.

Laboratory measurements

MDA

Serum MDA concentration was determined by the Thiobarbituric Acid Substances (TBARS) assay, which measures the reaction between MDA and thiobarbituric acid (TBA) at high temperatures to produce a pink-colored product. The assay used the Nalondi^{TM}-Lipid Peroxidation Assay Kit (nmol/ml, Cat. NS-15023, Novin Navand Salamat Pishtaz Co., Iran). MDA concentration was measured at 550λ wavelength using a colorimetric method with 1nmol/ml sensitivity.

TOS

Serum TOS concentration was measured using the Natos^{TM}-Total Oxidant Status Assay Kit (μmol Equiv./L, Cat. NS-15017, Novin Navand Salamat Pishtaz Co., Iran) at a wavelength of 530 λ with a sensitivity of 0.023U/ml.

TAC

Serum TAC concentration was measured by the ferric reducing antioxidant power (FRAP) assay using the Naxifer^{TM}-Total Antioxidant Capacity Assay Kit (mmol Fe2+/L, Cat. NS-15013, Novin Navand Salamat Pishtaz Co., Iran) at a wavelength of 593 λ and with a sensitivity of 2μmol Fe^{2+}/L.

SOD, CAT, and GPx

Commercial kits Erythro-Nasdox™, Nactaz™, and Nagpix™ were utilized to measure the activity of SOD (U/g Hb), CAT (nmol/g Hb), and GPx (mU/g Hb) at wavelengths of 420 λ, 540 λ, and 340 λ, respectively, in hemolyzed RBCs using a colorimetric method. The kits exhibited sensitivities of 1U/ml, 2nmol/min.ml, and 0.5mU/ml, respectively. (Novin Navand Salamat Pishtaz Co., Iran, SOD, Cat. NS-15037; CAT, Cat. NS-15053; GPx, Cat. NS-15083)

Statistical analysis

Statistical analyses were conducted using the statistical package for the social sciences (SPSS) software (IBM, SPSS Inc.), version 27. Due to the small sample size in each group ($n = 6$), the results of normality tests could not be relied upon. Therefore, equivalent non-parametric comparisons were used to evaluate the data. The Friedman non-parametric test assessed changes in the studied variables over time within each group separately. For within-group evaluation, the Wilcoxon signed-rank test was used. In addition, the Quade Non-Parametric test (non-parametric alternative to the repeated measures analysis of covariance) was used to compare the group's parameters and adjusted each variable's effect on the day before the intervention (day − 1). Furthermore, the least significant difference (LSD) post-hoc test was utilized to conduct pairwise comparisons between groups. The values were expressed as the median (interquartile range), and the significance level for all variables was considered $p < 0.05$.

Conclusion

This study revealed that while laparoscopy increased total oxidative stress (TOS), it did not induce significant changes in lipid peroxidation, suggesting a safe surgical protocol. Both agents significantly reduced laparoscopy-induced TOS, possibly by enhancing the antioxidant system. However, no significant difference was observed between the two drugs in this regard. Melatonin appears to exert its antioxidant effects primarily by enhancing overall antioxidant capacity, as evidenced by the significant increase in TAC and enzymatic antioxidant defense mechanism (GPx); on the other hand, agomelatine may act primarily by stimulating enzymatic antioxidant defense mechanisms, as indicated by the significant increases in SOD and GPx levels. Notably, the study found a significant time-dependent decrease in malondialdehyde levels in the agomelatine group, with a significant reduction observed on day 3 compared to days − 1 and 0. In summary, melatonin and agomelatine administration at a daily oral dose of 0.3 mg/kg 1 day before and for 7 days after LO improves the antioxidant status of dogs.

Abbreviations

OHE	Ovariohysterectomy
OVE	Ovariectomy
LO	Laparoscopic ovariectomy
MDA	Malondialdehyde
TAC	Total antioxidant capacity
TOS	Total oxidant status
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
CAT	Catalase
PNP	Pneumoperitoneum
IAP	Intra abdominal pressure
IR	Ischemia reperfusion
ROS	Reactive oxygen species
GABA	Gamma aminobutyric acid
TBARS	Thiobarbituric acid reactive substances
ANCOVA	Analysis of Covariance
OH^-	Hydroxide
H_2O_2	Hydrogen peroxide

DNA	Deoxyribonucleic acid
RNS	Reactive nitrogen species
LSD	Least significant difference
FRAP	Ferric reducing antioxidant power
SPSS	Statistical package for the social sciences
IM	Intramuscular
SC	Subcutaneous
IV	Intravenous
EDTA	Ethylenediaminetetraacetic acid
$5HT_2$	5-hydroxytryptamine

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Author contributions
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Data availability
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Declarations

Ethics approval and consent to participate
The Iranian laboratory animal ethics framework, under the guidance of the Iranian Society for the Prevention of Cruelty to Animals and the Ferdowsi University of Mashhad Research Council (IR.UM.REC.1402.045), granted ethical approval for this study. The shelter obtained written informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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