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Orlistat mitigates nitrosative stress and enhances paraoxonase-1 activity in serum and tissues of induced obese rats

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This study examined the effects of orlistat, an anti-obesity medication, on nitrosative stress and paraoxonase-1 (PON-1) enzyme activity in various tissues of rats with experimentally induced obesity. A total of 24 Wistar albino rats were divided into three groups: control, obese, and obese treated with orlistat (ORL). The ORL group received oral doses of 10 mg/kg/day for six weeks following an eight-week high-fat diet. During the study, body weights were monitored weekly, and various biochemical markers, including TNF-alpha, IL-1 beta, nitric oxide, nitrotyrosine, PON-1 enzyme activity, urea, creatinine, lactate dehydrogenase, and creatine kinase, were measured after the rats were sacrificed. The ORL group had significantly lower body weights compared to the obese group ($P < 0.05$). Obesity increased levels of inflammatory markers (TNF-alpha and nitric oxide) in serum, heart, and kidney tissues, as well as nitrotyrosine levels in serum, kidney, and testis tissues, compared to the control group ($P < 0.05$). Orlistat treatment reduced levels of TNF-alpha, IL-1 beta, nitric oxide, and nitrotyrosine in various tissues, as well as urea, creatinine, lactate dehydrogenase, and creatine kinase levels, compared to the obese group ($P < 0.05$). Moreover, orlistat significantly increased PON-1 enzyme activity in the heart, kidney, and testis tissues compared to the obese group ($P < 0.05$). These findings suggest that orlistat reduces inflammation and nitrosative stress and enhances antioxidant activity by increasing PON-1 enzyme levels. While orlistat shows promise as a therapeutic option for obesity, its systemic effects should be carefully considered.

Keywords: Orlistat, Obesity, Nitrosative stress, Paraoxonase enzyme activity

Obesity stands as a crucial risk factor for cardiovascular diseases, musculoskeletal disorders, diabetes mellitus, and certain types of cancer[1]. In addition to the array of health complications it engenders, obesity exerts a significant economic burden on healthcare systems. It is estimated that the medical costs for obese individuals are 30% higher compared to those with normal weight, accounting for approximately 0.7-2.8% of a country's total healthcare expenditures[2]. Obesity triggers chronic inflammation, endothelial dysfunction, and oxidative stress, leading to increased nitric oxide (NO) production. This excess NO, driven by the proinflammatory state, promotes the formation of reactive nitrogen species (RNS), which can cause DNA damage[1]. In comparison to superoxide anions, reactive nitrogen species exhibit a threefold higher reactivity with other molecules and possess a prolonged average half-life. Moreover, they impose irreversible damage and perturb the functionalities of cellular membranes, proteins, mitochondria, endoplasmic reticulum, nucleic acids, and enzymes, ultimately culminating in necrotic processes and cell death. The current understanding recognizes nitrosative stress as a contributing factor in the pathogenesis of various diseases[3]

Orlistat is a potent inhibitor of pancreatic and gastric lipases, preventing the breakdown of dietary triglycerides into absorbable fats. This results in undigested triglycerides being excreted, reducing fat absorption and promoting a calorie deficit[4]. Orlistat, approved by the FDA in 1999 for obesity treatment, not only reduces weight but also improves insulin sensitivity, lowers blood glucose, and has positive effects on blood pressure, cholesterol, and LDL levels[5]. It is known that obesity causes oxidant damage in heart, kidney, and testicular tissues[6–8]. In some studies, it has been shown that the application of orlistat reduces the damage[9]. Paraoxonase-1 enzyme (PON-1), an indispensable endogenous antioxidant enzyme, plays a crucial role in preventing lipid peroxidation and is intricately linked to the regulation of nitrosative stress[10].

This study aimed to investigate the effects of orlistat on nitrosative stress markers, including nitrotyrosine levels and the antioxidant enzyme PON-

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1 in the serum, heart, kidney, and testicular tissues of obese rats. The research also assesses the impact of orlistat on renal function, cardiac health, and testicular response through serum urea, creatinine, lactate dehydrogenase, and testosterone levels. The findings will address a gap in the literature concerning orlistat's role in modulating nitrosative stress in obesity.

Materials and Methods

Animals and diet

This study was approved by the Local Ethics Committee for Animal Experiments of Baskent University (Approval Number: DA 22/25). In the study, 24 male Wistar albino rats weighing 200-250 g were utilized. The animals were obtained from the Animal Production and Research Center of Baskent University, and all research activities were conducted at the same facility. Throughout the study period, the animals were housed under standard atmospheric conditions, maintaining a temperature of 21±2°C and a relative humidity ranging from 30% to 70%. A 12-hour light/dark cycle was maintained, and the rats had *ad libitum* access to food and water. The obesity groups were provided with a high-fat diet (Altromin, High-Fat Diet, C1090-60) containing 35% fat, while the control group received a standard diet for a duration of 14 weeks. The high-fat diets were stored at -20°C and brought to room temperature before being administered to the animals. Orlistat (Thincal®), which is used in the experimental group, was dissolved in sunflower oil and administered via gavage at a dosage of 10 mg/kg/day. There are studies in literature that apply this dose (10 mg/kg/day)[8,9,11,12]. Since ORL has been shown to be effective in these literatures, we decided to use this dose.

Experimental design of animals

The rats were randomly divided into three groups by utilizing a computer software program GraphPad by Dotmatics, with each group consisting of 8 animals: Control group (C) received a normal diet for 14 weeks and was administered 0.5 mL of sunflower oil via gavage during the last 6 weeks; The experimental obesity group (E) was fed a high-fat diet for 14 weeks and received 0.5 mL of sunflower oil* via gavage during the last 6** weeks; The obesity + orlistat (ORL) group was fed a high-fat diet for 14 weeks and received a daily gavage of 10 mg/kg/day of orlistat in addition to the diet during the last 6 weeks. The animals were randomly divided into groups in a blind manner by the veterinarian in charge of the experimental animals. *In the studies on orlistat administration, although there are those who dissolve orlistat in solvents such as water[13] and oil[14], we preferred to dissolve it in oil since the diet was also HFD. ** The fact that the effect was seen in this period in the articles in the literature[7–9,15], which preferred a 6-week period for orlistat administration, caused us to choose 6 weeks for Orlistat administration in the study. Weight measurements were recorded weekly before and throughout the experiment. At the end of the 14th week, the rats were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (7 mg/kg). Subsequently, cardiac blood samples were collected, and the rats were sacrificed.

Identification of obesity

The development of obesity in the groups receiving a high-fat diet was determined by calculating the Lee obesity index and body mass index (BMI). The indices were calculated using the following formula. Rats with a Lee obesity index greater than 315 and a BMI value of 0.68 g cm^{-2} were classified as obese[8,16]. Once the rats were identified as obese, the administration of orlistat was initiated in the obesity + orlistat group.

$$Lee\ obesity\ index = \frac{\sqrt[3]{\text{body weight}}\ (g)}{\text{nasoanal length (cm)}}$$

$$\text{Body mass index} = \text{body weight (g)}/\text{length}^2\ (\text{cm}^2)$$

Sample collection

Blood samples were collected in serum tubes and centrifuged at 3000 rpm for 10 min. The sera obtained were stored at -20°C for subsequent hormone and enzyme activity measurements. Heart, kidney, and testis tissues were preserved at -80°C until the analysis day. Prior to analysis, the tissues were homogenized in a 1/9 ratio (0.1 g tissue: 0.9 mL 140 mmol KCl) potassium chloride buffer and centrifuged at 7000 rpm for 5 min at +4°C. The analysis was performed using the supernatants.

Measurement of TNF-alpha and IL-1 beta levels in the serum

To assess the impact of obesity and orlistat administration on inflammatory pathways, the concentrations of TNF-alpha (E0764Ra) and IL-1 beta (E0119Ra) in the serum were measured using the ELISA method with commercial kits from BT-Lab.

Determination of nitric oxide, nitrotyrosine level and paraoxonase-1 enzyme activity

The NO level in both serum and tissue homogenates (heart, kidney, and testis) was quantified

using the Elabscience (E-BC-K035-S) commercial kit based on a colourimetric method. Due to the high reactivity and rapid oxidation of nitric oxide in aerobic environments, its direct determination in biological samples is challenging. Nitrite and nitrate, the end products of nitric oxide oxidation, serve as indicators of the amount of NO produced. The level of nitrotyrosine was measured using the Elabscience (E-EL-0040) commercial kit, and the activity of paraoxonase-1 enzyme was measured using the Rel-Assay (RL0031) commercial kit.

Determination of urea, creatinine, lactate dehydrogenase, and creatine kinase activities in the serum

To assess the impact of a high-fat diet and orlistat on kidney, heart, and testis tissues, the renal function markers urea (OttoBC157) and creatinine (OttoBC139), cardiac damage markers LDH (OttoBC129) and CK (OttoBC136) were measured by colourimetric.

Determination of testosterone level

The serum testosterone level was assessed employing ELISA technique, utilizing the commercially available BT-Lab kit (EA0023Ra).

Statistical analysis

The collected experimental data were analyzed utilizing the JAMOVI statistical program (2.3.18., Australia). The outcomes were expressed as mean ± standard error. A P value < 0.05 was considered statistically significant. The Kruskal-Wallis test was employed for the assessment of the obtained results, and subsequently, the post-hoc Dwass-Steel-Critchlow-Fligner pairwise test was applied to ascertain the significance between the groups.

Results

Changes in body weight

At the commencement of the study, the weights of animals in all groups (C, E, ORL) were approximately equivalent, and no significant disparities were detected among the groups (P=0.361). Both the rats consuming the normal diet and those consuming the high-fat diet exhibited a consistent increase in weight. A comparison of weight changes between the control and obesity groups suggested that the group subjected to the high-fat diet would exhibit a significantly greater weight gain in comparison to the normal diet group (P=0.007).

Following the administration of orlistat, it was observed that only the group receiving the high-fat diet exhibited continued weight gain, whereas the group receiving the high-fat diet in conjunction with orlistat commenced weight loss. At the end of the study, the body weight of the experimental obesity (E) group was significantly greater than that of the ORL group (P=0.017) (Fig. 1).

Biochemical analyses

The obese group exhibited a significantly elevated serum TNF-alpha level in comparison to the control group (P=0.017). However, in the orlistat-treated group, both TNF-alpha and IL-1 beta levels exhibited a significant decrease compared to the obese group (P=0.017 and P=0.007, respectively), as depicted in Fig. 2. The impact of orlistat on nitrosative stress in experimentally induced obese rats was elucidated by evaluating the levels of nitric oxide (Fig. 3) and nitrotyrosine (Fig. 4) in serum, heart, kidney, and testis tissues. A comparative analysis between the

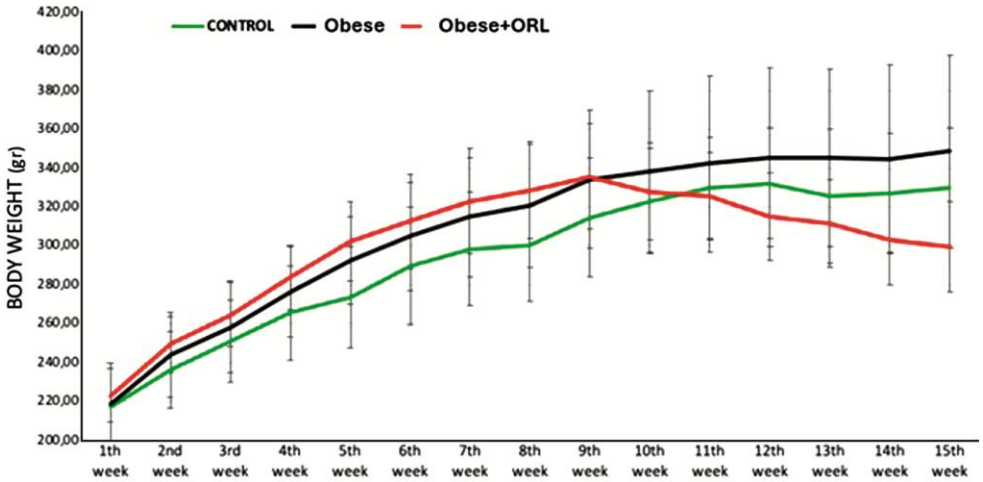

Fig. 1 — 15 weeks weight change in all groups. At the beginning, all groups were approximately equivalent (P=0.361). At the end of the 15th week, a significant difference was obtained between the obese group and the obese+orlistat group (P =0.017).

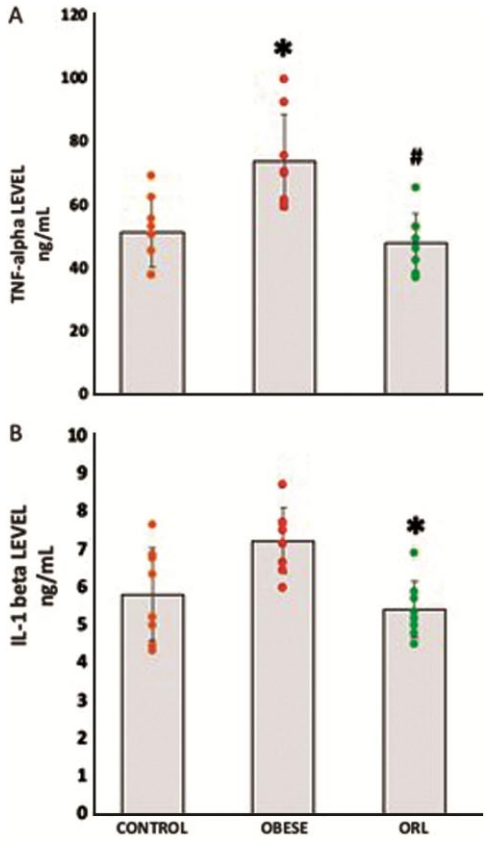

Fig. 2 — The levels of TNF-alpha and IL-1 beta in the serum. (A), *control *vs* obese (P =0.017), # obese *vs* ORL (P =0.017). (B) *obese *vs* ORL (P =0.007)[TNF-alpha: Tumor Necrosis Factor Alpha; IL-1beta: Interleukin-1 Beta; ORL: obese+orlistat group]

control and obese groups revealed noteworthy elevations in nitric oxide levels in the serum (P=0.009), heart (P=0.005), and kidney (P=0.005) tissues of the obese group in contrast to the control group. Conversely, these elevations in the orlistat group exhibited a significant decrease in serum, heart and kidney compared to the obese group (P=0.017, P=0.023, P=0.024, respectively). Notably, no substantial difference was observed in testis tissue concerning nitric oxide levels. As depicted in Fig. 4, the levels of nitrotyrosine in the serum, kidney, and testis tissues of the obese group exhibited a substantial increase in comparison to the control group (P=0.008; P=0.013; P=0.006, respectively). Conversely, in the ORL group, this elevation was significantly mitigated (P=0.007; P=0.015; P=0.002, respectively). Regarding the heart tissue, no significant disparity was observed between the C and E groups. However, when juxtaposed with the E group, the ORL group displayed notably lower levels of nitrotyrosine (P=0.017).

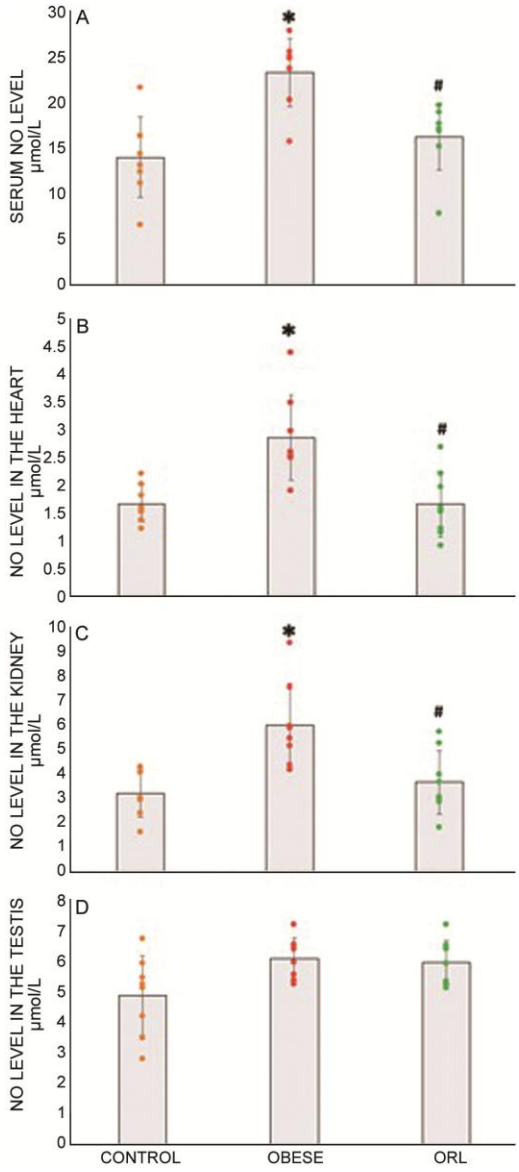

Fig. 3 — Changes in NO levels in serum, heart, kidney, and testis tissues were assessed. (A) *control *vs* obese (P =0.009), # obese *vs* ORL (P =0.017). (B) *control *vs* obese (P =0.005), # obese *vs* ORL (P =0.023). (C) *control *vs* obese (P =0.005), # obese *vs* ORL (P=0.024) [NO: nitric oxide; ORL: obese+orlistat group]

As illustrated in Fig. 5, the assessment of PON-1 enzyme activity, an antioxidant enzyme, exhibited no significant alterations in the serum, heart, kidney, and testis tissues when comparing the C group with the E group. Nevertheless, a noteworthy increase in PON-1 enzyme activity was observed in the heart, kidney, and testis tissues of the ORL group relative to the E group (P=0.009; P=0.013; P=0.002, respectively). Although a similar increase was observed in serum PON-1 levels of the same groups, statistical significance was not attained.

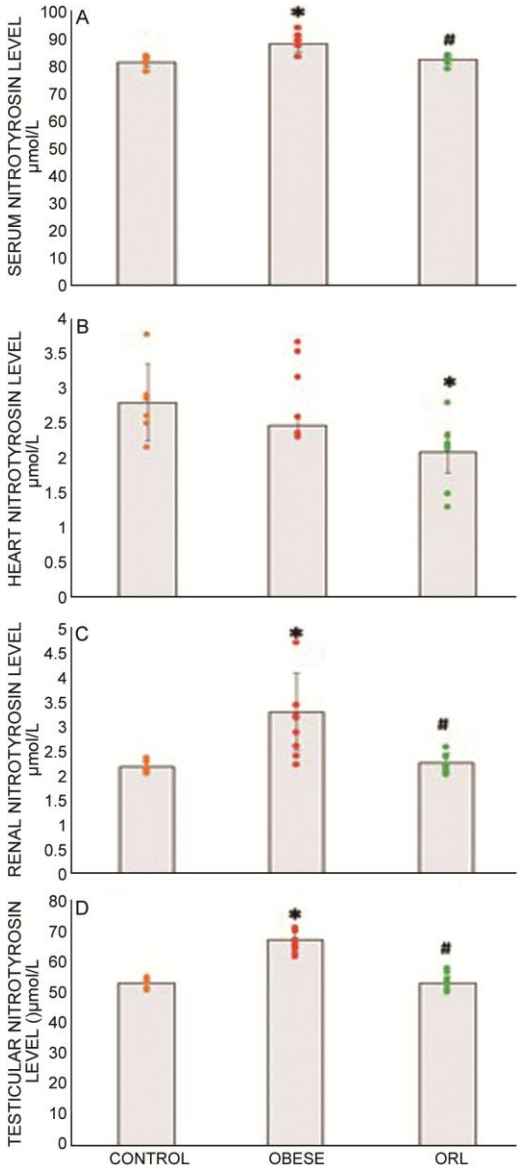

Fig. 4 — Changes in nitrotyrosine levels in serum, heart, kidney, and testis tissues. (A) *control *vs* obese (P =0.008), # obese *vs* ORL (P =0.007). (B) *obese *vs* ORL (P =0.0017). (C) *control *vs* obese (P =0.013), # obese *vs* ORL (P =0.015(D) *control *vs* obese (P =0.006), # obese *vs* ORL (P =0.002) [ORL: obese+orlistat group]

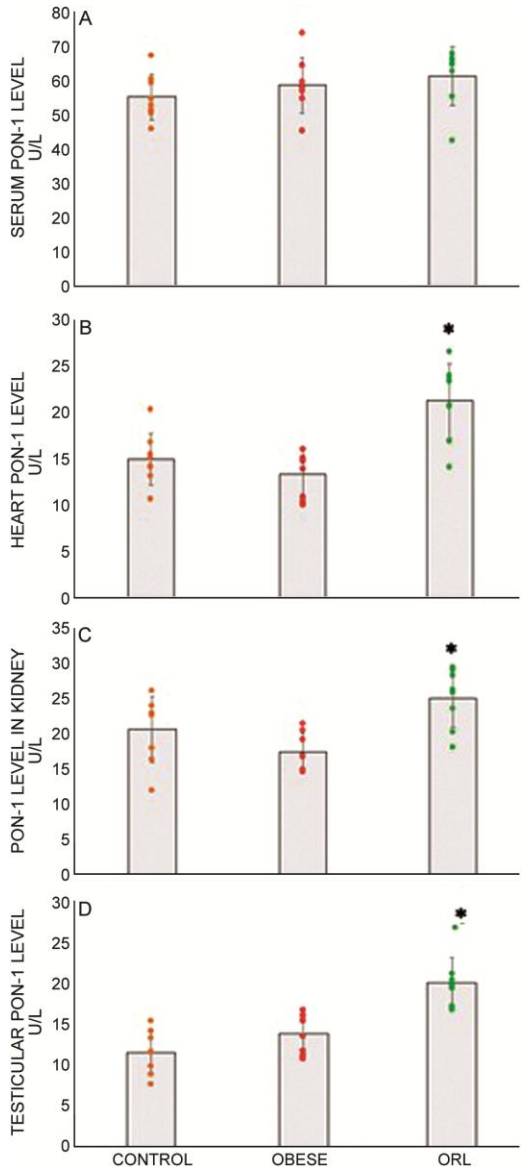

Fig. 5 — Changes in PON-1 levels in serum, heart, kidney, and testis tissues. (B) *obese *vs* ORL (P =0.009). (C) *obese *vs* ORL (P =0.013). (D) *obese *vs* ORL (P =0.002) [PON-1: paraoxonase-1; ORL: obese+orlistat group]

In terms of urea, creatinine, LDH, and creatine kinase, no statistically significant difference was observed between the C and E groups (P>0.05). However, significant reductions in urea, creatinine, LDH, and creatine kinase were detected in all orlistat-treated groups compared to group O (respectively, P= 0.007; P=0.002; P=0.003; P=0.024) (Fig. 6). At the same time, significant decreases in urea, creatinine, LDH and creatine kinase were detected in all orlistat-treated groups compared with control (respectively, P= 0.002; P=0.002; P=0.002; P=0.013) (Fig. 6). Regarding testosterone levels, no significant difference was observed among the groups.

Discussion

Given the substantial risk associated with obesity-related diseases, there is an increasing emphasis on the use of anti-obesity medications, such as orlistat. Scientific literature has provided evidence not only for the anti-obesity effects of orlistat but also for its diverse pharmacological properties, such as anti-inflammatory[17] and anti-tumor activities[18] as well as

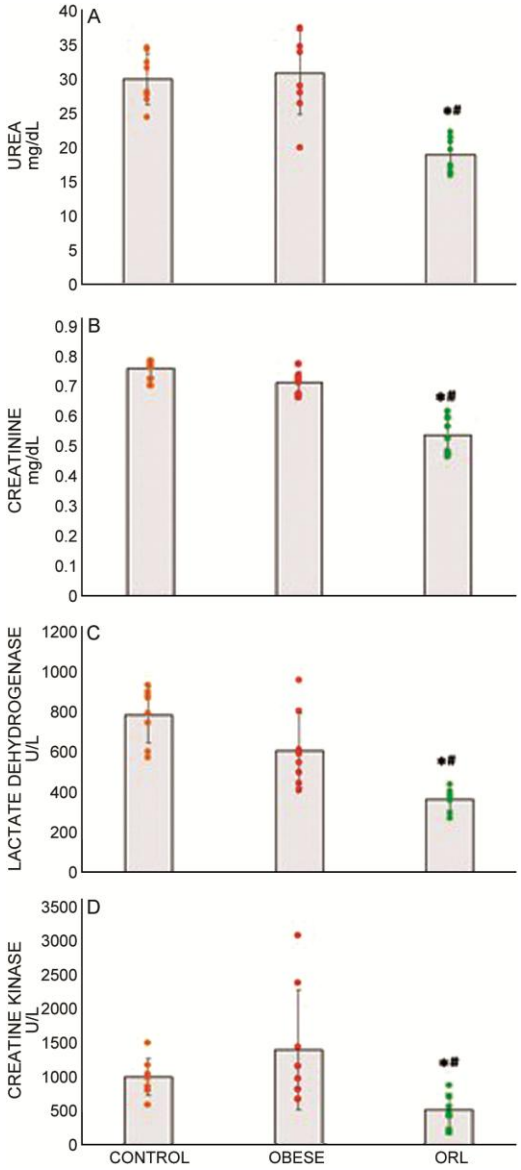

Fig. 6 — The levels of serum urea, creatinine, lactate dehydrogenase, and creatine kinase. (A) *control *vs* ORL (P =0.002), # obese *vs* ORL (P =0.007). (B) *control *vs* ORL (P =0.002), # obese *vs* ORL (P =0.002). (C) *control *vs* ORL (P =0.002), # obese *vs* ORL (P =0.003). (D) *control *vs* ORL (P =0.013), # obese *vs* ORL (P =0.024) [ORL: obese+orlistat group]

its potential to alleviate oxidative stress in different tissues[9,19]. However, it is important to consider that orlistat has been associated with adverse effects in various tissues, including the liver[20], kidney[21], bone[22–24], and nervous system[25,26]. In our study, we aimed to investigate the effects of orlistat at a dosage of 10 mg/kg/day, administered for a duration of 6 weeks in experimentally induced obese rats, on nitrosative stress and paraoxonase enzyme activity in the heart, kidney, and testis tissues. Additionally, we aimed to assess any potential tissue damage caused by orlistat.

The increase in body weight observed in the high-fat diet group compared to the control group, as well as the reduction in weight gain observed after orlistat treatment in obese mice, is consistent with previous scientific studies that have consistently reported the weight-reducing effects of orlistat[8,16].

It is well-established that excessive expansion of adipose tissue in obesity induces a state of hypoxia, thereby triggering the release of adipokines and acute-phase proteins from adipocytes, consequently leading to systemic inflammation[27]. Proinflammatory cytokines, which are prominently elevated in obesity, have been identified to promote oxidative stress[28,29] and augment NO synthesis via the upregulation of iNOS and superoxide production through the activation of NADPH oxidase enzyme[30]. Consequently, this cascade of events contributes to the escalation of oxidative and nitrosative stress, thereby characterizing obesity as a chronic inflammatory disorder[22]. The relationship between adipose tissue expansion in obesity and the subsequent increase in inflammatory cytokines is widely recognized. It is hypothesized that orlistat, acting as a lipase inhibitor, reduces fat absorption, thereby reducing adipose tissue mass and subsequently attenuating inflammatory cytokines levels. This study was parallel with this assumption. In the context of physiological conditions, NO exerts numerous beneficial effects within the body when maintained at normal levels. However, in inflammatory circumstances such as obesity, the overproduction of NO gives rise to the generation of reactive nitric oxide species (RNOS)[30]. It is important to note that since the detrimental effects of nitric oxide predominantly originate from its oxidized species, nitrosative stress is indeed a constituent of oxidative stress, contributing to the overall pathophysiology. In our study, we assessed the levels of NO and nitrotyrosine as markers of nitrosative stress in both serum and tissues including the heart, kidney, and testis. We observed elevated levels of NO in the serum, heart, and kidney tissues, as well as increased levels of nitrotyrosine in the serum, kidney, and testis tissues in the obese group. However, following a 6-week treatment with orlistat, the levels of NO and nitrotyrosine in these samples were found to be reduced. Consistent with our findings, previous research conducted in humans has also reported higher NO levels in overweight and obese individuals compared to those with normal weight[1]. Given the chronic inflammatory nature of obesity, it is

reasonable that increased NO production occurs in this context[30]. One possible explanation for the elevated NO levels in obesity is the upregulation of iNOS synthesis induced by elevated inflammatory cytokines[31]. Notably, iNOS, which exhibits the highest capacity for NO production, is expressed in various cells in response to inflammatory signals. Therefore, the increased NO levels associated with iNOS activity in morbidly obese individuals may contribute to the inflammatory effects observed[32]. Nevertheless, it is worth mentioning that studies in literature have reported decreased NO levels in the long term in the presence of obesity[33,34]. One potential explanation for the divergent outcomes regarding NO in obese individuals could be the presence of a negative feedback mechanism, whereby the initial acute-phase increase in NO may lead to a reduction in NOS synthesis[31]. In our study, the decrease in NO and nitrotyrosine levels following Orlistat administration may be attributed to its negative impact on the inflammatory process, stemming from decreased fat absorption. Consequently, this reduction in nitrosative stress could be mediated by the modulation of NO production.

PON-1, which is primarily associated with high-density lipoprotein (HDL), plays a crucial role in the antioxidant system by hydrolyzing lipid peroxides[10]. Given the strong correlation between obesity and lipid profiles, it is expected that PON-1 activity decreases in the presence of elevated lipid levels. Conversely, the administration of the lipase inhibitor orlistat is anticipated to result in increased PON-1 activity. In line with these expectations, our study demonstrated a significant increase in PON-1 activity in the heart, kidney, and testis tissues of the orlistat-treated group when compared to the obese group. This finding suggests that orlistat may positively modulate PON-1 activity in these tissues, potentially contributing to the regulation of oxidative stress and the prevention of cellular damage associated with obesity.

There are studies in the literature showing that heart enzymes, kidney function tests and testicular damage occur due to diet applied for a longer period (12 weeks or more)[7,35,36]. However, our study assessing the effects of an 8-week high-fat diet on cardiac, renal, and testicular parameters did not reveal a significant increase in LDH and CK levels as markers of cardiac damage, nor did it show significant alterations in urea and creatinine levels for kidney function evaluation or testosterone levels for testicular function evaluation. The absence of significant differences compared to the control group in our study may be attributed to the relatively shorter duration of the dietary intervention compared to previous literature. Although our study did not identify any adverse effects of the 8-week high-fat diet on the kidney, heart, or testis, it is evident that orlistat treatment resulted in decreased parameters associated with the heart and kidney in obese rats. A notable finding in our study was the even lower levels of urea, creatinine, LDH, and CK in the ORL group compared to the control group. The significant reduction in urea and creatinine levels could be attributed to the side effect of orlistat, namely increased intestinal motility leading to reduced protein absorption and subsequent decreased protein excretion. Despite reports suggesting low systemic effects of orlistat[37] our study results are consistent with reports indicating a decrease in CK[38] and LDH[39], which is more prevalent than CK, following orlistat administration. This decline could be associated with the inhibitory effect of orlistat on lipoprotein lipase in vascular endothelial cells, suggesting the possibility of systemic effects. Furthermore, there are studies reporting that chronic use of orlistat can lead to myopathy and reduced CK levels[40,41]. Although the duration of orlistat use in our study was shorter compared to those studies, the lower CK levels in the orlistat-treated group compared to the control group may be attributed to the potential cardiac muscle weakness caused by orlistat at the given dose and duration. The lack of differences in testosterone levels may be due to the lower dose and shorter duration of the orlistat intervention compared to the mentioned study.

Conclusion

In conclusion, our study using the high-fat diet-induced obesity model revealed an upregulation of proinflammatory activity and nitrosative stress in serum and the heart, kidney, and testis tissues. However, there was no significant alteration observed in the activity of PON-1 enzyme. Conversely, treatment with orlistat reducedduced inflammatory activity and enhanced antioxidant PON-1 enzyme activity, indicating a defensive response against the heightened nitrosative stress associated with obesity. Despite the observed increase in nitrosative stress levels in obese animals, it did not reach a threshold causing noteworthy tissue damage. Nonetheless, the beneficial effects of orlistat treatment on these tissues were still evident. Additionally, our findings indicated

that apart from its known effect on fat absorption inhibition, orlistat negatively affected protein absorption by diminishing the renal excretion of protein degradation byproducts. The ability of orlistat to lower urea, creatinine, LDH, and CK levels below those observed in the control groups suggests its efficacy not only in the gastrointestinal system but also in tissues where these enzymes are active, thereby suggesting systemic effects of orlistat. While our study successfully demonstrated the dual benefits of orlistat, namely its anti-obesity effects and its amelioration of nitrosative damage associated with obesity in rats subjected, it is crucial to consider its systemic effects when employing orlistat as a therapeutic intervention.

Ethical statement

This study was approved by the Local Ethics Committee for Animal Experiments of Baskent University (Approval Number: DA 22/25).

Funding statement

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Conflict of interest

The authors declare that there are no conflicts of interest related to this article.

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