# *SAFETY OF CHRONIC USE OF AGOMELATINE AND COMBINATIONS: IMPACT ON HEMATOLOGICAL, BIOCHEMICAL, AND HISTOPATHOLOGICAL PARAMETERS IN GENDER-SPECIFIED RATS*

# SEGURANÇA DO USO CRÔNICO DA AGOMELATINA E ASSOCIAÇÕES: IMPACTO EM PARÂMETROS HEMATOLÓGICOS, BIOQUÍMICOS E HISTOPATOLÓGICOS EM RATOS ESPECIFICADAS POR GÊNERO

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*Abstract*

***Introduction:*** *Polypharmacy may lead to changes in the action profile and toxicity of medicines.* ***Objective:*** *This study evaluated the effect of the association of the antidepressant agomelatine (AGO) with losartan (LOS), simvastatin (SIM), and metformin (MET) after subchronic treatment on hematological, biochemical, and histopathological parameters in Wistar rats.* ***Methods:*** *The animals were randomly divided into ten groups for each sex and treated for 90 days, orally, with saline, AGO, LOS, SIM, MET, or combinations (AGO+LOS, AGO+SIM, AGO+MET, LOS+SIM+MET, and AGO+LOS+SIM+MET). We evaluated hematological, biochemical, and histopathological parameters.* ***Results and Discussion:*** *The main hematological alterations occurred in the erythrogram, and females subjected to polypharmacy were more prone to develop hematological and biochemical alterations. Hepatic and renal functions were the most affected among biochemical parameters. Agomelatine in combination increased the risk of histopathological changes in both males and females, especially in the liver, kidneys, and spleen. Females presented more intense hepatic histopathological changes when AGO was associated with SIM and MET. Females using MET or AGO+MET were more prone to develop histopathological changes than males. The spleen was significantly affected in females, corroborating erythrogram alterations.* ***Conclusion:*** *Agomelatine in combination*

*altered the safety profile of drugs, especially in females, indicating caution in their use in polypharmacy with commonly prescribed drugs.*

***Keywords:*** Polypharmacy; Agomelatine; Losartan; Simvastatin; Metformin.

## Resumo

**Introdução:** A polifarmácia pode levar a alterações no perfil de ação e toxicidade dos medicamentos. **Objetivo:** Este estudo avaliou o efeito da associação do antidepressivo agomelatina (AGO) com losartana (LOS), sinvastatina (SIM) e metformina (MET) após tratamento subcrônico sobre parâmetros hematológicos, bioquímicos e histopatológicos em ratos Wistar. **Metodologia:** Os animais foram divididos aleatoriamente em dez grupos para cada sexo e tratados por 90 dias por via oral com solução salina, AGO, LOS, SIM, MET ou combinações (AGO+LOS, AGO+SIM, AGO+MET, LOS+SIM+MET e AGO+LOS+SIM+MET). Avaliamos parâmetros hematológicos, bioquímicos e histopatológicos. **Resultados e Discussão:** As principais alterações hematológicas ocorreram no eritrograma, e as fêmeas submetidas à polifarmácia apresentaram maior propensão a alterações hematológicas e bioquímicas. As funções hepática e renal foram as mais afetadas entre os parâmetros bioquímicos. A agomelatina em associação aumentou o risco de alterações histopatológicas em ambos os sexos, especialmente no fígado, rins e baço. As fêmeas apresentaram alterações histopatológicas hepáticas mais intensas quando a AGO foi associada ao SIM e ao MET. Fêmeas em uso de MET ou AGO+MET tiveram maior propensão a desenvolver alterações histopatológicas que os machos. O baço foi significativamente afetado no sexo feminino, corroborando alterações eritrográficas. **Conclusão:** A agomelatina associada alterou o perfil de segurança dos medicamentos, principalmente no sexo feminino, indicando cautela no seu uso na polifarmácia com medicamentos comumente prescritos.

**Palavras-chave:** Polifarmácia; Agomelatina; Losartana; Sinvastatina; Metformina.

Recebido em: 21-11-2023 Publicado em: 04-12-2024

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## 1. Introduction

Polypharmacy, defined as the regular use of five or more medications at the same time, is common in older adults and at-risk younger individuals. as aging individuals often contend with multiple chronic health conditions, the use of multiple medications becomes common, posing risks of adverse outcomes such as falls, frailty, disability, and mortality. Conventionally, polypharmacy is

considered something to be avoided, but recent research has identified that appropriately managing patients with targeted medications can prevent unplanned hospital admissions[1]. The use of polytherapy is justified when there is a necessity to increase the effectiveness of treatment with synergic effects, or when the patient has more than one disease[2]. Moreover, polypharmacy was found to be more prevalent among patients with depression versus non-depressed comparisons, especially among older patients with early-onset depression. in these cases, polypharmacy needs to be identified and managed appropriately, as it is an independent risk factor for chronic depression[3].

Agomelatine is a recent therapeutic option for treating depression. It is a melatonin receptor agonist (mt1/mt2) and a serotonergic receptor antagonist ($5\text{-}HT_{2c}$)[4]. This mechanism induces the release of dopamine and norepinephrine without significantly altering serotonin concentrations in the synaptic cleft. However, agomelatine has been associated with increased hepatotoxic potential, especially in older adults, women, and patients under polypharmacy regimens[4]. Hepatotoxic reactions, such as acute liver injury (ali), are an identified risk in the european risk management plan for agomelatine. Population studies suggest that antidepressants are widely prescribed and often used by patients with risk factors for ali, such as metabolic syndrome[5]. Therefore, understanding the safety profile of agomelatine in combination with other drugs commonly used in chronic conditions, such as losartan, metformin, and simvastatin, is essential.

Polypharmacy, in many instances, cannot be avoided, as many patients suffering from multiple chronic conditions will require drugs from different classes. This phenomenon has led to the concept of "adequate polypharmacy," which defines the use of a sufficient number of drugs to treat the existing pathologies and comorbidities of a patient. Certain classes of drugs, including anticoagulants, antibiotics, psychiatric medications, antidiabetics, and antihypertensive agents, are associated with an increased risk of drug interactions[6]. Considering this context, it is critical to evaluate the impact of drug interactions involving agomelatine, metformin, losartan, and simvastatin, all of which are frequently used in the clinical management of chronic diseases.

The objective of this study was to evaluate the effect of subchronic treatment with the association of agomelatine plus losartan, simvastatin, or metformin on

hematological, biochemical, and histopathological parameters in male and female rats. This study provides novel insights into the safety profile of agomelatine in polypharmacy settings, highlighting the different responses between male and female subjects, which is crucial for understanding gender-specific risks in clinical practice.

## 2. Materials and methods

### Animals and Ethical considerations

We used 120 adult Wistar rats of both sexes (60 males and 60 females). At the beginning of the experiments, the animals were 50-60 days old, weighing between 200-250 grams. Rats, obtained from the Animal House of the Federal University of Ceara (UFC), were maintained at a controlled temperature (22 ± 2°C) with a 12-h dark/light cycle, food (FRI-LAB Rat II, FRI-Ribe), and water ad libitum. The animals were housed in groups of same-sex 4 animals in 42 × 25 × 20 cm polypropylene wire-bottom cages. Different sex rats were maintained in separate rooms.

The animals were manipulated according to the NIH Guide for the Care and Use of Laboratory Animals[7], the Guide for the Care and Use of Laboratory Animals (NRC, 2011), and the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The Ethics Committee on Animal Research of the Federal University of Ceara approved the study protocol (57/2016). All efforts were made to reduce or minimize the number and the suffering of experimental animals.

### Drugs and treatment protocol

We used the drugs agomelatine (AGO, Valdoxan®, Servier laboratory, Brazil), potassium losartan (LOS, Cozaar® Merck Sharp & Dohme laboratory, Brazil), simvastatin (SIM, Zocor® Merck Sharp & Dohme laboratory, Brazil), and metformin hydrochloride (MET, Glifage® Merck Sharp & Dohme Laboratory, Brazil). The pills were dissolved in sterile water in sufficient volume to obtain the desired dose of each drug. Drug doses were selected based on clinical equivalence between humans and rats, using the interspecies dose conversion guide[8]. This conversion is widely accepted in preclinical studies to ensure that dosages used in animals reflect therapeutic doses in humans, minimizing risks and maximizing translational relevance. Accordingly, based on this calculation, the following doses were used: AGO 2.3 mg/kg, LOS 4.6 mg/kg, SIM 1.8 mg/kg and MET 7.9 mg/kg. The drugs were made up freshly within 1–2 h of dosing and were administered orally by gavage ina volume of 1 mL/100 g body weight.

The experimental protocol was based on the international guidelines for testing drug safety in a subchronic treatment, namely the *OECD Guideline for the Testing of Chemicals - Test 408*[14]. To do this, the rats received daily consecutive oral administrations of vehicle (VEH) or the drugs alone or in combination for 90 days. In the combination groups, the time interval between each drug administration was 30 min. All experimental manipulations were conducted between 8:00 - 12:00 AM. On the first day of drug administration, the animals were randomly divided into 10 groups of 12 animals according to sex. Additionally, we divided each group of 12 animals into 3 cages to allow the housing of 4 rats per cage.

The following experimental groups were conducted: Group 1 – G1/VEH, rats that received four consecutive daily administrations of the VEH; Group 2 – G2/AGO, rats that received a daily administration of AGO; Group 3 – G3/LOS, rats that received one daily administration of LOS; Group 4 – G4/SIM, rats that received one daily administration of SIM; Group 5 – G5/MET, rats that received one daily administration of MET. The groups G1 to G5 received daily doses of the drugs followed by three administrations of the VEH. Group 6 – G6/LOS+SIM+MET, rats received LOS, SIM, MET daily consecutive oral administrations of the drugs, in this order; Group 7 – G7/AGO+LOS, rats received AGO and LOS daily consecutive oral administrations, in this order; Group 8 – G8/AGO+SIM, rats received AGO and SIM daily consecutive oral administrations, in this order; Group 9 – G9/AGO+MET, rats received AGO and MET daily consecutive oral administrations, in this order. G7 to G9 received AGO followed by LOS or SIM or MET and two daily administrations of VEH. Group 10 – G10/AGO+LOS+SIM+MET, rats received daily administrations of AGO, followed by LOS, SIM, and MET administration (FIGURE. 1).

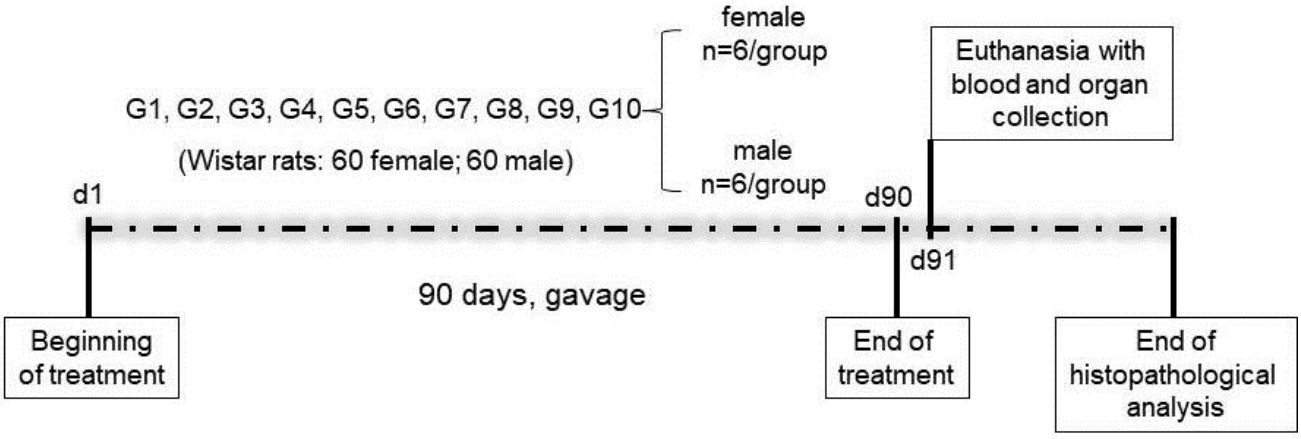


FIGURE 1 - Experimental design. Rats were treated daily with oral administration of the

drugs alone or combined. At the end of the protocol, the animals were sacrificed, and their organs were dissected for histopathological analysis. Abbreviations: G: group; G1: VEH/control; G2: AGO/agomelatine; G3: LOS/losartan; G4: SIM/simvastatin; G5: MET/metformin; G6: LOS+SIM+MET/losartan + simvastatin + metformin; G7: AGO+LOS/agomelatine + losartan; G8: AGO+SIM/agomelatine + simvastatin; G9: agomelatine + metformin; G10: AGO+LOS+SIM+MET/agomelatine + losartan + simvastatin + metformin. Source: Prepared by the authors (2024).

The division of the groups was performed to evaluate both the monotherapeutic and combined effects of the drugs in question, reflecting real clinical situations of polypharmacy in the management of chronic diseases. The combination groups (G6 to G10) were assigned to examine potential interactions between drugs that are often prescribed together in clinical practice, while the monotherapy groups (G2 to G5) allowed for the evaluation of individual effects of each drug.

After 90 days of daily administration of the medications, two hours after the last administration, the animals were anesthetized with intraperitoneal administration of xylazine 10 mg/kg and ketamine 90 mg/kg. Afterward, samples were collected by abdominal vein venipuncture (3 to 5 mL). Lastly, the animals were decapitated, and the organs, liver, kidneys, stomach, intestine, spleen, pancreas, and heart were quickly removed.

Histopathological analysis

The organs were analyzed macroscopically and subsequently submitted to histological processing through the automated system PT05 LupTec® undergoing dehydration in increasing alcohol series (70, 90, 95, and 100%), xylol diaphanization, paraffin impregnation, casting at 60°C, and inclusion of specimens forming paraffin blocks at room temperature. The fragments were sectioned 5 µm thick and stained using routine staining for hematoxylin-eosin histology. The slides were analyzed by optical microscope (100 and 400x magnification) by two experienced blinded pathologists. Part of the tissues from six different rats were taken to compose each group. At least five aleatory fields were imaged in each animal sample.

The choice of histopathological methods, such as the use of the hematoxylin-eosin staining technique and the evaluation of light microscopy, was made to detect microscopic alterations in tissues of target organs, such as the liver, kidneys and heart. These techniques are international standards in preclinical toxicology studies and are fundamental to evaluate the morphological effects of pharmacological treatments in animal models, offering detailed and quantifiable information on

the degree of tissue injury.

The following parameters were carefully evaluated in each organ: liver (hepatocyte cell swelling, hydropic degeneration, Kupffer cell hyperplasia, inflammatory foci, microvesicular steatosis, and focal hepatocyte necrosis); kidneys (tubular epithelial swelling, vacuuming of the tubular epithelium, tubular epithelium necrosis or scaling, hyaline cylinders and inflammatory foci); gut (villus flattening, crypt loss, inflammatory foci, vacuuming or edema of the enterocytes, lymphoid follicles enlargement, and ectatic vessels); spleen (presence of hemosiderin and/or lipofuscin, presence of megakaryocytes); pancreas (acinar vacuolization, acinar necrosis, inflammation, and lymph node hyperplasia); stomach and heart (inflammatory foci) (TABLE 1). Blinded experimenters qualitatively described the findings from the histopathological analysis. They described major observed changes and the number of animals affected in each group for each organ.

TABLE 1 – Histopathological criteria evaluated in each organ for experienced and blinded histopathologists.

| Organ | Histopathological criteria evaluated |
|---|---|
| Liver | Hepatocyte cell swelling; Hydropic degeneration; Kupffer cell; Hyperplasia; Inflammatory foci; Macro or microvesicular steatosis; Focal hepatocyte necrosis |
| Kidney | Tubular epithelial swelling; Tubular epithelium vacuuming, necrosis, or scaling; Hyaline cylinders; Inflammatory foci |
| Gut | Villus flattening; Crypt loss; Inflammatory foci; Vacuuming or edema of the enterocytes; Lymphoid follicles; Ectatic vessels |
| Spleen | Presence of hemosiderin and/or lipofuscin Presence of megakaryocytes |
| Pancreas | Acinar vacuolization; Acinar necrosis Inflammation and lymph node hyperplasia |
| Stomach | Inflammatory foci |
| Heart | Inflammatory foci |

Source: Adapted from Alves et al., 2019[15]; Paz et al., 2017[16].

Hematological analyses

The animal's blood was collected in tubes containing 10% sodium EDTA, where the SDH-3-VET® device (Labtest, Brazil) was used to perform the blood count. Thus, the hematological evaluation consisted of the performance of an automated complete blood count, including the following parameters: total leukocytes, lymphocytes (absolutes and %), monocytes/eosinophils/basophils (absolute and %), granulocytes (absolute and %), erythrocytes, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MVC), mean corpuscular hemoglobin (HCM), mean corpuscular hemoglobin concentration (CHCM), anisocytosis index (RDW) and platelet count (PLT).

Biochemical analyses

The blood was collected in tubes containing a coagulation accelerator and centrifuged at 3500 rpm for 10 minutes to obtain the serum, separated into Eppendorf tubes for subsequent biochemical analysis in the Labmax-Plenno Automatic Analyzer®, standard model (Labtest, Brazil). The tests were performed according to the manufacturer's protocols of the equipment and reagent kits for each parameter to be evaluated. The following parameters were analyzed: total cholesterol, triglycerides, total bilirubin, direct bilirubin, gamma-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, urea, and creatinine.

Hematological and biochemical analyses, such as blood cell counts and biochemical parameters, were essential to monitor the systemic response to treatment. These parameters are critical indicators of liver, kidney, metabolic, and immune health and are widely used in pharmacological safety studies to identify subclinical changes.

The data from hematological and biochemical analyses were submitted to variance analysis (two-way ANOVA), followed by significance tests of Tukey for comparison between groups and Dunnett for comparison of groups with saline control. Organ weight was represented as means ± S.E.M. and analyzed by regular one-way ANOVA followed by Dunnett's multiple comparison test using the VEH group as the control group. The significance level was set at $p < 0.05$. Statistical analysis was performed with GraphPad Prism for Windows (version 7.0, San Diego, USA).

## 3. Results

Hematological analyses

The mean ± Standard Error of the Mean (SEM) of the hematological parameters analyzed compared between sexes are shown in Tables 2 and 3.

The comparisons with significant differences between females and males are bold and marked with an asterisk. Thus, it is possible to verify that most of the hematological parameters analyzed (nine out of fifteen) presented a significant difference between genders in at least one of the treatment groups (without considering saline).

TABLE 2 - Hematological parameters analyzed by sex in the groups treated in monotherapy (G1 to G5).

| Parameter (unit) | G1/VEH | | G2/AGO | | G3/LOS | | G4/SIM | | G5/MET | |
|---|---|---|---|---|---|---|---|---|---|---|
| | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 |
| Leukocytes (109/L) | 9.78±1.29 | 9.17±1.78 | 12.78±1.39 | 8.09±1.33 | 7.04±0.31 | 8.07±0.37 | 13.88±2.49* | 7.89±1.47* | 9.70±0.62 | 11.3±1.38 |
| Lymphocytes (109/L) | 8.83±1.32 | 7.72±1.59 | 11.30±1.19* | 6.24±1.09* | 6.20±0.22 | 6.27±0.27 | 13.25±2.32* | 6.18±1.16* | 8.75±0.64 | 9.00±1.34 |
| Monoc/Eosin /Bas (109/L) | 0.36±0.11 | 0.16±0.12 | 0.51±0.24 | 0.70±0.14 | 0.21±0.05 | 0.73±0.06 | 0.20±0.07 | 0.57±0.16 | 0.28±0.03 | 0.61±0.06 |
| Granulocytes (109/L) | 0.59±0.08 | 1.28±0.09 | 0.96±0.45 | 1.16±0.17 | 0.56±0.14 | 1.07±0.14 | 0.57±0.17 | 1.14±0.18 | 0.68±0.15 | 1.70±0.18 |
| Lymphocytes (%) | 89.60±2.95 | 84.20±0.90 | 89.10±3.35 | 76.57±1.63 | 88.27±1.49 | 77.87±1.52 | 94.60±1.16 | 78.18±1.53 | 90.17±1.77 | 78.78±2.91 |
| Monoc/Eosin /Bas (%) | 3.98±1.62 | 1.55±0.95 | 3.35±1.33* | 8.78±1.40* | 2.97±0.64* | 6.85±0.75* | 1.35±0.41* | 6.85±0.75* | 2.80±0.20 | 5.75±0.96 |
| Granulocytes (%) | 6.42±1.35 | 1.28±0.09 | 7.50±3.67 | 1.16±0.17 | 8.75±1.57 | 1.07±0.14 | 4.05±1.18 | 1.14±0.18 | 7.12±1.65 | 1.69±0.18 |
| Erythrocytes (1012/L) | 10.64±0.48 | 9.17±0.21 | 10.21±0.12 | 9.38±0.10 | 9.99±0.15 | 9.05±0.11 | 10.25±0.10 | 9.42±0.23 | 9.92±0.28 | 9.46±0.23 |
| Hemoglobin (g/dL) | 16.18±2.81 | 15.20±0.00 | 18.33±0.30* | 9.38±0.10* | 18.18±0.16* | 9.05±0.11* | 16.38±1.76 | 15.68±0.36 | 17.78±0.60* | 9.46±0.23* |
| Hematocrit (%) | 72.59±2.64 | 55.80±0.17 | 72.49±9.94* | 56.94±0.37* | 71.36±0.78* | 56.13±0.55* | 71.11±0.42* | 55.96±1.15* | 70.94±2.31 | 58.12±0.64 |
| MCV (fL) | 68.80±1.20* | 61.00±1.00* | 70.83±0.91* | 60.67±0.49* | 71.50±0.42* | 61.83±0.87* | 69.67±0.84* | 59.50±0.42* | 71.50±0.56* | 61.50±1.56* |
| MCH (pg) | 17.06±0.14 | 16.6±0.40 | 17.63±0.42 | 16.60±0.17 | 18.07±0.28 | 17.05±0.17 | 17.63±0.30 | 16.67±0.22 | 17.90±0.14 | 16.80±0.41 |
| MCHC (g/dL) | 24.56±0.23 | 27.20±0.10 | 25.30±0.24 | 27.35±0.21 | 25.52±0.18 | 27.45±0.24 | 23.73±1.70* | 28.02±0.25* | 25.07±0.17 | 27.13±0.20 |
| RDW (%) | 14.14±0.27* | 8.60±5.00* | 14.00±0.13 | 13.53±0.14 | 13.55±0.18 | 13.45±0.16 | 13.68±0.17 | 13.80±0.14 | 13.98±0.19 | 14.08±0.19 |
| Platelets (109/L) | 1475±543.1 | 1060±9.5 | 1702±239.8 | 920.2±32.63 | 1368±80.22 | 868.8±51.21 | 2701±508* | 1067±75.3* | 2117±568* | 845.3±104* |

G1/VEH: saline control group; G2/AGO: agomelatine group; G3/LOS: losartan group; G4/SIM: simvastatin group; G5/MET: metformin group; MVC: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration. RDW: amplitude of red blood pressure distribution. The results are expressed in mean ± SEM. *Significant $p<0.05$ values according to the ANOVA followed by the Tukey test. Source: Research data (2023).

TABLE 3 - Hematological parameters analyzed by sex in rats treated with the drug combinations (G6 to G10).

| Parameter (unit) | G6/LSM | | G7/AL | | G8/AS | | G9/AM | | G10/ALSM | |
|---|---|---|---|---|---|---|---|---|---|---|
| | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 |
| Leukocytes ($10^9$/L) | 8.09±0.70 | 8.19±1.09 | 6.87±0.79 | 9.62±0.98 | 5.82±0.82 | 5.98±1.64 | 7.35±0.33 | 9.53±0.71 | 7.10±0.68 | 9.60±0.76 |
| Lymphocytes ($10^9$/L) | 7.31±0.68 | 6.41±0.81 | 6.03±0.72 | 7.97±0.80 | 5.02±0.99 | 4.88±1.29 | 6.46±0.37 | 7.78±0.52 | 5.64±0.62 | 7.54±0.42 |
| Monoc/Eosin/Bas (109/L) | 0.21±0.06 | 0.41±0.13 | 0.19±0.02 | 0.60±0.19 | 0.15±0.06 | 0.32±0.13 | 0.18±0.38 | 0.39±0.12 | 0.19±0.03 | 0.64±0.18 |
| Granulocytes ($10^9$/L) | 0.58±0.14 | 1.39±0.19 | 0.64±0.18 | 1.05±0.15 | 0.65±0.30 | 0.78±0.23 | 0.70±0.14 | 1.36±0.17 | 1.28±0.68 | 1.41±0.21 |
| Lymphocytes (%) | 91.12±2.23 | 78.70±1.15 | 87.87±2.36 | 83.08±2.47 | 82.65±8.4 | 83.38±2.13 | 87.17±2.4 | 81.88±1.80 | 80.92±7.12 | 77.72±2.50 |
| Monoc/Eosin/Bas (%) | 2.42±0.56 | 4.68±1.18 | 2.98±0.37 | 5.88±1.42 | 2.92±1.33 | 4.48±1.08 | 2.53±0.53 | 3.93±0.94 | 2.62±0.42 | 6.33±1.41 |
| Granulocytes (%) | 7.47±1.69 | 1.39±1.19 | 9.38±2.22 | 1.05±0.15 | 14.42±8.73 | 0.78±0.23 | 9.61±1.90 | 1.36±0.17 | 16.47±7.39 | 1.41±0.21 |
| Erythrocytes ($10^{12}$/L) | 7.94±0.21 | 8.85±0.86 | 7.91±0.18 | 8.10±0.59 | 7.84±0.17 | 6.65±1.42 | 7.79±0.19 | 8.93±0.11 | 7.56±0.19 | 8.91±0.20 |
| Hemoglobin (g/dL) | 13.83±0.37* | 8.85±0.86* | 13.95±0.26* | 8.10±0.59* | 13.65±0.32 | 11.48±2.05 | 13.53±0.18 | 14.4±0.14 | 13.38±0.31 | 14.23±0.32 |
| Hematocrit (%) | 54.77±1.09 | 54.07±5.09 | 55.05±1.28 | 50.73±3.50 | 54.63±1.29* | 40.15±8.47* | 54.03±0.96 | 53.99±0.55 | 52.53±1.28 | 54.47±0.80 |
| MCV (fL) | 69.00±0.51* | 61.50±0.67* | 69.67±0.33* | 62.67±0.56* | 69.83±0.87* | 60.60±0.60* | 69.33±0.76* | 60.33±0.67* | 69.67±0.33* | 61.17±0.75* |
| MCH (pg) | 17.43±0.10* | 15.28±0.49* | 17.70±0.17 | 16.60±0.24 | 17.43±0.14 | 16.18±0.57 | 17.38±0.20 | 16.10±0.21 | 17.70±0.13* | 15.97±0.29* |
| MCHC (g/dL) | 25.15±0.29 | 24.93±0.85 | 25.38±0.23 | 26.47±0.46 | 25.00±0.21 | 26.60±0.80 | 25.03±0.16 | 26.67±0.30 | 25.48±0.18 | 26.12±0.27 |
| RDW (%) | 12.87±0.14 | 14.13±0.26 | 12.90±0.14 | 12.12±1.67 | 13.05±0.11 | 13.80±0.17 | 12.62±0.18 | 13.95±0.13 | 12.78±0.19 | 13.88±0.11 |
| Platelets ($10^9$/L) | 662.3±25.31 | 1386±311.1 | 618.2±13.07 | 1083±150.7 | 646.2±49.04 | 1280±146.1 | 541.5±24.93 | 1383±65.13 | 576.8±39.38 | 1541±205.8 |

G6/LSM: losartan group + simvastatin + metformin; G7/AL: agomelatine + losartan group; G8/AS: agomelatine group + simvastatin; G9/AM: agomelatine group + metformin; G10/ALSM: agomelatine group + losartan + simvastatin + metformin. MVC: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration. RDW: amplitude of red blood pressure distribution. The results are expressed in mean ± SEM. *Significant $p<0.05$ values according to the ANOVA followed by the Tukey test. Source: Research data (2023).

Biochemical analyses

The mean ± Standard Error of the Mean (SEM) of the biochemical parameters analyzed compared between sexes are shown in Tables 4 and 5. The comparisons with significant differences between females and males are bold and marked with an asterisk. Thus, it is possible to verify that most of the biochemical parameters analyzed (seven out of eleven) presented a significant difference between sexes in at least one of the treatment groups (without considering saline).

Histopathological analysis

The results of the histopathological analysis were documented descriptively and. The most relevant ones, illustrated by photomicrographs. The comparisons between organs weight are shown in Table 6 for males and in Table 7 for females.

TABLE 4 - Biochemical parameters analyzed by sex in the groups treated with the drugs alone (G1 to G5).

| Parameter (unit) | G1/VEH | | G2/AGO | | G3/LOS | | G4/SIM | | G5/MET | |
|---|---|---|---|---|---|---|---|---|---|---|
| | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 |
| Cholesterol (mg/dL) | 52.0±6.8 | 61.7±6.2 | 39.5±5.0* | 77.3±7.0* | 43.2±5.4 | 57.8±7.8 | 42.5±2.5 | 57.0±6.7 | 130.0±12.2* | 66.3±6.8* |
| Triglycerides (mg/dL) | 78.0±10.4 | 119.5±18.4 | 50.5±6.5* | 110.7±10.2* | 58.5±4.1 | 79.5±9.0 | 44.8±4.9 | 58.7±10.4 | 194.8±19.0* | 94.0±7.9* |
| Glucose (mg/dL) | 135.7±5.0* | 48.2±5.5* | 74.2±5.7 | 72.3±5.8 | 99.0±6.9 | 74.3±5.1 | 100.0±6.5 | 71.2±6.4 | 157.7±5.5* | 57.0±4.7* |
| AST (U/L) | 151.5±9.6 | 91.0±7.1 | 142.7±30.0 | 83.7±8.1 | 150.0±16.3 | 127.3±45.0 | 145±18.0 | 80.3±5.3 | 178.8±6.1 | 77.7±7.7 |
| ALT (U/L) | 50.5±3.2 | 49.3±4.8 | 40.4±6.5 | 66.3±7.8 | 37.0±2.7 | 53.8±9.8 | 34.3±4.0 | 37.0±3.7 | 70.7±4.4* | 52.0±6.3* |
| Creatinine (mg/dL) | 0.46±0.02 | 0.42±0.03 | 0.30±0.03* | 0.51±0.04* | 0.43±0.02 | 0.51±0.04 | 0.43±0.06 | 0.38±0.03 | 0.53±0.04 | 0.44±0.04 |
| Urea (mg/dL) | 32.7±1.2 | 31.7±1.8 | 36.0±2.0 | 41.7±1.7 | 39.3±2.0 | 38.17±2.0 | 42.2±1.8 | 33.5±3.8 | 41.8±1.9 | 35.5±2.6 |
| Gamma GT (U/L) | 2.5±0.3 | 3.5±0.7 | 2.3±0.5 | 3.0±0.3 | 4.0±0.9 | 3.7±0.5 | 3.7±0.6 | 4.2±0.6 | # | 3.1±0.5 |
| Total Bilirubin (mg/dL) | 0.05±0.01 | 0.05±0.01 | 0.21±0.09 | 0.56±0.36 | 0.25±0.06 | 0.18±0.05 | 0.16±0.04 | 0.10±0.03 | 0.04±0.01 | 0.14±0.09 |
| Direct Bilirubin (mg/dL) | 0.08±0.00 | 0.18±0.03 | 0.06±0.01 | 0.17±0.06 | 0.09±0.01 | 0.08±0.02 | 0.06±0.02 | 0.07±0.02 | 0.09±0.00 | 0.13±0.03 |

# date not available. G1/SAL: saline control group; G2/AGO: agomelatine group; G3/LOS: Losartan group; G4/SIM: simvastatin group; G5/MET: metformin group. AST: aspartate aminotrasferase. ALT: alanine aminotrasferase. Gamma GT: gamma-glutamyl transferase. The results are expressed in mean ±SEM. *Significant $p<0.05$ values according to the ANOVA followed by the Tukey test. Source: Research data (2023).

TABLE 5 - Biochemical parameters analyzed by sex in the groups treated with the drug combinations (G6 to G10).

| Parameter (unit) | G6/LSM | | G7/AL | | G8/AS | | G9/AM | | G10/ALSM | |
|---|---|---|---|---|---|---|---|---|---|---|
| | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 | female n=6 | male n=6 |
| Cholesterol (mg/dL) | 47.3±6.1 | 70.2±3.0 | 47.5±4.6 | 57.7±4.0 | 26.6±2.5 | 53.0±3.5 | 48.8±3.4 | 58.7±4.1 | 63.2±5.3 | 71.8±3.9 |
| Triglycerides (mg/dL) | 42.3±7.4* | 114.3±4.8* | 52.3±7.0 | 97±9.6 | 42.8±6.4 | 84.8±8.1 | 54.2±4.6 | 88.3±12.3 | 65.7±3.7 | 84.3±6.9 |
| Glucose (mg/dL) | 50.0±5.9* | 112.0±10.3* | 54.8±5.9* | 116.2±9.3* | 47.8±4.4* | 117.0±6.0* | 55.0±2.1* | 109.3±6.6* | 68.5±7.6 | 87.5±10.9 |
| AST (U/L) | 165.5±10.0 | 104.7±5.6 | 198.8±10.4 | 105.7±5.6 | 180.5±17.3 | 95.0±7.1 | 186.2±16.7 | 102.5±4.8 | 218.8±39.8 | 130.5±28.8 |
| ALT (U/L) | 41.1±5.1 | 52.7±7.6 | 39.0±2.3 | 42.0±4.6 | 36.0±2.6 | 39.5±4.6 | 34.0±1.6 | 47.3±6.2 | 64.6±8.0 | 56.3±13.7 |
| Creatinine (mg/dL) | 0.35±0.01 | 0.42±0.03 | 0.12±0.01* | 0.38±0.03* | 0.12±0.01* | 0.36±0.03* | 0.20±0.02* | 0.39±0.02* | 0.48±0.04 | 0.41±0.03 |
| Urea (mg/dL) | 34.5±2.5 | 36.0±1.3 | 30.8±3.4 | 36.0±1.4 | 25.3±1.6 | 37.2±1.3 | 34.8±3.3 | 34.3±1.8 | 38.7±4.2 | 36.7±3.2 |
| Gamma GT (U/L) | 2.8±0.4 | 2.8±0.3 | 5.6±1.2 | 2.8±0.6 | 4.7±0.9 | 3.3±0.6 | 4.2±1.0 | 2.8±0.3 | 2.5±0.3 | 2.8±0.3 |
| Total Bilirubin (mg/dL) | 0.13±0.03 | 0.08±0.02 | 0.52±0.03* | 0.05±0.01* | 0.49±0.03* | 0.05±0.01* | 0.44±0.01 | 0.06±0.01 | 0.20±0.03 | 0.07±0.01 |
| Direct Bilirubin (mg/dL) | 0.06±0.01 | 0.15±0.01 | 0.06±0.01 | 0.13±0.01 | 0.06±0.02 | 0.12±0.01 | 0.09±0.01 | 0.14±0.02 | 0.16±0.10 | 0.11±0.01 |

G6/LSM: losartan group + simvastatin + metformin; G7/AL: agomelatine + losartan group; G8/AS: agomelatine group + simvastatin; G9/AM: agomelatine group + metformin; G10/ALSM: agomelatine group + losartan + simvastatin + metformin. AST: aspartate aminotrasferase. ALT: alanine aminotrasferase. Gamma GT: gamma-glutamyl transferase. The results are expressed in mean ±SEM. *Significant $p<0.05$ values according to the ANOVA followed by the Tukey test. Source: Research data (2023)

TABLE 6 – Absolute organ weight of male animals relative to the experimental group.

| Organ/Group Male | Vehicl | AGO | LOS | SIM | MET | LOS+SIM+MET | AGO+LOS | AGO+SIM | AGO+MET | AGO+LOS+ SIM+MET |
|---|---|---|---|---|---|---|---|---|---|---|
| Liver | 13.8 ± 0.6 | 11 ± 0.5* | 10.2 ± 0.35* | 10.9 ± 0.6* | 12 ± 0.47 | 13.4 ± 0.5 | 12.6 ± 0.38 | 11.6 ± 0.52* | 2.5 ± 0.52 | 12.5 ± 0.28 |
| Right Kidney | 1.2 ± 0.2 | 1.1 ± 0.03 | 1 ± 0.02* | 1.1 ± 0.05 | 1.2 ± 0.03 | 1.2 ± 0.06 | 1.2 ± 0.03 | 1.1 ± 0.04 | 1.2 ± 0.04 | 1.2 ± 0.04 |
| Left Kidney | 1.3 ± 0.06 | 1.1 ± 0.03* | 1 ± 0.04* | 1.1 ± 0.06 | 1.2 ± 0.05 | 1.2 ± 0.04 | 1.2 ± 0.04 | 1.1 ± 0.02* | 1.2 ± 0.04 | 1.2 ± 0.05 |
| Spleen | 0.7 ± 0.02 | 0.06 ± 0.03 | 0.6 ± 0.03 | 0.7 ± 0.05 | 0.7 ± 0.06 | 0.7 ± 0.01 | 0.7 ± 0.03 | 0.6 ± 0.01 | 0.7 ± 0.02 | 0.07 ± 0.02 |
| Pancreas | 1.5 ± 0.12 | 1.3 ± 0.1 | 1.4 ± 0.12 | 1.4 ± 0.16 | 1.3 ± 0.07 | 1.4 ± 0.17 | 1.5 ± 0.15 | 1.4 ± 0.11 | 1.3 ± 0.10 | 1.7 ± 0.12 |
| Stomach | 2 ± 0.08 | 1.9 ± 0.07 | 1.8 ± 0.08 | 1.9 ± 0.06 | 2 ± 0.07 | 2.3 ± 0.16 | 2.1 ± 0.07 | 2 ± 0.11 | 2.1 ± 0.12 | 2.1 ± 0.06 |
| Gut | 8.5 ± 0.33 | 7.9 ± 0.4 | 7.7 ± 0.20 | 7.5 ± 0.49 | 8.7 ± 0.44 | 8.4 ± 0.41 | 7.6 ± 0.58 | 7.4 ± 0.34 | 8.7 ± 0.73 | 8.4 ± 0.37 |
| Heart | 1.3 ± 0.05 | 1.1 ± 0.05* | 1.1 ± 0.06* | 1.1 ± 0.04 | 1.2 ± 0.05 | 1.1 ± 0.02 | 1.1 ± 0.02* | 1 ± 0.02* | 1.1 ± 0.05 | 1.1 ± 0.04 |

The results are expressed in mean ± SEM. *Significant $p<0.05$ values according to the ANOVA followed by the Tukey test. Source: Research data (2023).

TABLE 7 – Absolute organ weight of female animals from each experimental group.

| Organ/Group Female | Vehicl | AGO | LOS | SIM | MET | LOS+SIM+MET | AGO+LOS | AGO+SIM | AGO+MET | AGO+LOS+ SIM+MET |
|---|---|---|---|---|---|---|---|---|---|---|
| Liver | 8.3 ± 0.3 | 7.3 ±0.3 | 7.9 ± 0.6 | 7.4 ± 0.2 | 8.3 ± 0.96 | 8 ± 0.4 | 7.6 ±0.22 | 6.7 ±0.2** | 7.5 ±0.2 | 8.3 ± 0.4 |
| Right Kidney | 0.9 ± 0.02 | 0.8 ±0.03 | 0.8 ±0.03 | 0.8 ± 0.01 | 0.9 ± 0.02 | 0.95±0.04 | 0.9 ± 0.02 | 0.8 ±0.01 | 0.9 ±0.03 | 0.9 ± 0.04 |
| Left Kidney | 0.9 ± 0.03 | 0.8 ± 0.02 | 0.9 ± 0.02 | 0.8 ± 0.01 | 0.8 ± 0.02* | 0.9 ± 0.02 | 0.9 ± 0.01 | 0.8 ± 0.02* | 0.8 ± 0.02* | 0.9 ± 0.02 |
| Spleen | 0.6 ± 0.04* | 0.5 ± 0.02** | 0.5 ± 0.01** | 0.5 ± 0.01** | 0.5 ± 0.03** | 0.5 ± 0.01** | 0.5 ± 0.01* | 0.5 ± 0.02*** | 0.5 ± 0.02** | 0.6± 0.01* |
| Pancreas | 1 ± 0.09 | 1 ± 0.15 | 1.2 ± 0.1 | 1.1 ± 0.09 | 1.1 ± 0.1 | 0.9 ± 0.06 | 1.1 ± 0.09 | 1 ± 0.05 | 0.9 ± 0.05 | 0.8 ± 0.08 |
| Stomach | 1.7 ± 0.1 | 1.5 ± 0.06 | 1.5 ± 0.08 | 1.6 ± 0.1 | 1.5 ± 0.07 | 1.8 ± 0.08 | 1.6 ± 0.06 | 1.5 ± 0.1* | 1.7 ± 0.04 | 1.8 ± 0.08 |
| Gut | 6.8 ± 0.6 | 5.9 ± 0.4 | 6.4 ± 0.6 | 6.1 ± 0.4 | 7 ± 0.4 | 6.1 ± 0.3 | 5.8 ± 0.23 | 5.6 ± 0.24 | 5.8 ± 0.2 | 5.9 ± 0.24 |
| Heart | 0.8 ± 0.01 | 0.8 ±0.04 | 0.8 ± 0.02 | 0.8 ± 0.01 | 0.9 ± 0.03 | 0.8 ± 0.02 | 0.8 ± 0.02 | 0.7 ± 0.01 | 0.8 ± 0.02 | 0.8 ± 0.05 |

The results are expressed in mean ± SEM. *Significant p<0.05 values according to the ANOVA followed by the Tukey test.

Liver

In all experimental groups, hepatocyte swelling was observed. However, it was more discrete among the VEH group in both males and females. The presence of hepatocyte swellingwas more intense in male animals in AGO and LOS groups. This finding was more pronounced in females in the group treated with the four drugs combined (AGO+LOS+SIM+MET).

Hydropic degeneration was only observed in males, occurring in 100% (6/6) of animals treated with the three drugs combined (LOS+SIM+MET group), either focally or dispersed. This alteration was also observed in one male animal from the AGO+MET group and two animals from the SIM group. Foam cells were found just in the organ's periphery (Fig. 2).

All-female rats had Kupffer cell hyperplasia without any critical difference between the experimental and control groups. Among males, this hyperplasia occurred in all groups at monotherapy, namely AGO, LOS, SIM, and MET groups. However, there was a decrease or even complete absence of hyperplasia in the groups of drug association. In this regard, one animal in the LOS+SIM+MET group, three animals in the AGO+LOS group, and none in AGO+SIM, AGO+MET, and AGO+LOS+SIM+MET groups.

The same finding was observed regarding the intensity of inflammatory foci. Females, therefore, were revealed to be more sensitive to liver inflammation, especially when exposed to drug combinations in which AGO is present. In males, drug combination, especially with SIM, seems to attenuate AGO-induced liver inflammation. In accordance, the AGO group in male subjects showed the most intense inflammation compared to the other male experimental groups, affecting 5/6 animals. Microvesicular steatosis was observed in (4/6) female subjects of the following groups: LOS, SIM, AGO+SIM, AGO+LOS+SIM+MET, highlighting the presence of SIM in three of these groups. Only one (1/6) animal presented microvesicular steatosis among male groups, which happened in the SIM group. Thus, a higher occurrence of this finding was observed among female animals and in the SIM-treated ones alone or combined with other drugs, pointing to the possible influence of SIM in the development of steatosis (Fig. 2).

Focal hepatocyte necrosis was detected in two (2/6) females of AGO+SIM and AGO+MET groups. Among the males, one (1/6) subject presented focal necrosis in the SIM group (Fig. 2). Thus, AGO was present in two of the three cases of focal necrosis detected, just as SIM was also present in two of the three cases, including in association with AGO. Overall,

all proposed treatments (except VEH) caused signs of toxicity in hepatocytes, especially in females, and more pronounced in AGO-treated ones.

Regarding liver weight, there was a significant reduction ($p < 0.05$) in the AGO, LOS, SIM groups and in the AGO+SIM combination compared with the VEH control group in male subjects (Table 6). In female ones (Table 7), it was observed a significant reduction in liver weight in the AGO+SIM group ($p < 0.05$) compared with VEH controls. No additional significance was observed.

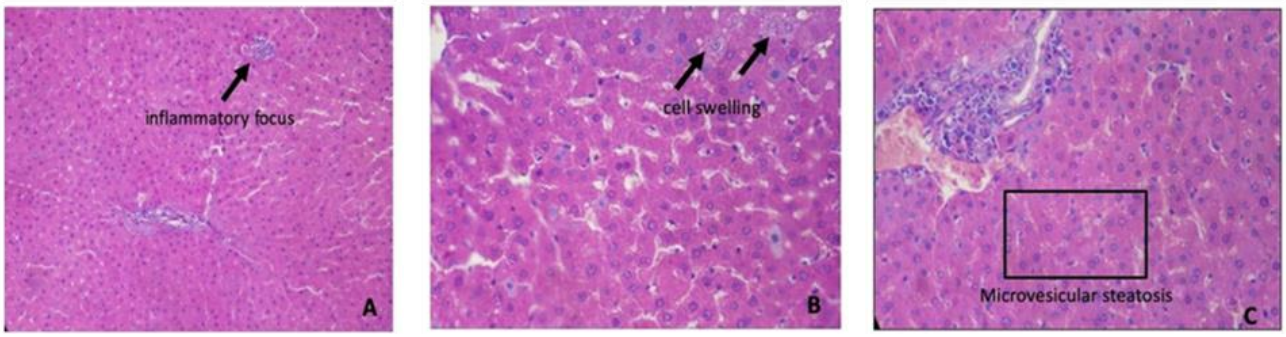


FIGURE 2 - Liver photomicrographs of a male animal (code 3M) from the simvastatin group. The figure shows hepatocyte strands with cell swelling, scattered inflammatory foci, and microvesicular steatosis. Hematoxylin-eosin staining 200X (A) and 400x (B and C). Source: Research data (2024).

Kidney

Cellular swelling of the tubular epithelium was observed in 100% of females, ranging from mild (in the VEH group) to marked in the MET group. Additionally, in two (2/6) animals of the MET group, marked tubular swelling led to the collapse of the lumen of the proximal tubules, suggestive of interstitial edema, with evident scaling of the tubular epithelium at the cortical-medullary junction suggesting acute tubular necrosis (ATN). In males, the number of animals with tubular epithelial swelling was smaller. In the groups, AGO+SIM, AGO+MET, and AGO+LOS+SIM+MET tubular swelling were not observed in any subject. Even in affected males, swelling prevailed as mild, except in males of the MET group, where the observed swelling was severe, similarly to the respective female group. Also, in this male group, marked swelling of the cortical tubular epithelium resulted in areas of tubular lumen collapse, vacuolar degeneration, and the focal regions of proximal tubular epithelium desquamation in one (1/6) animal, suggestive of ATN (Figs. 3 and 4).

Also, vacuolization of the tubular epithelium occurred in 100% of females,

ranging from discrete in the VEH group to severe in three AGO-combined groups: AGO+LOS, AGO+SIM, and AGO+MET. Among males, vacuolization was not detected in the groups LOS, AGO+MET, and AGO+LOS+SIM+MET. Thus, MET showed more significant nephrotoxic potential with the development of ATN when used alone than when associated with other drugs in the study, affecting 33.3% of treated animals (4 animals/12),

Hyaline cylinders were found in 100% of females, ranging from rare in the VEH group to frequent in all other treated groups. Among males, hyaline cylinders were not detected in the combined drug groups LOS+SIM+MET and AGO+LOS+SIM+MET and the group LOS. In the other groups, hyaline cylinders were scarce, affecting a small number of animals per group, as observed in AGO, SIM, and MET groups. Therefore, the occurrence of hyaline cylinders was notably higher in females.

Inflammatory cells were found only in females and males treated with MET alone, affecting all-female subjects in this group (6/6) and four (4/6) males. Thus, 10 out of 12 animals treated with MET (83.33%) presented considerable inflammatory focus in the kidneys, the most affected females (Figs. 3, 4, and 5).

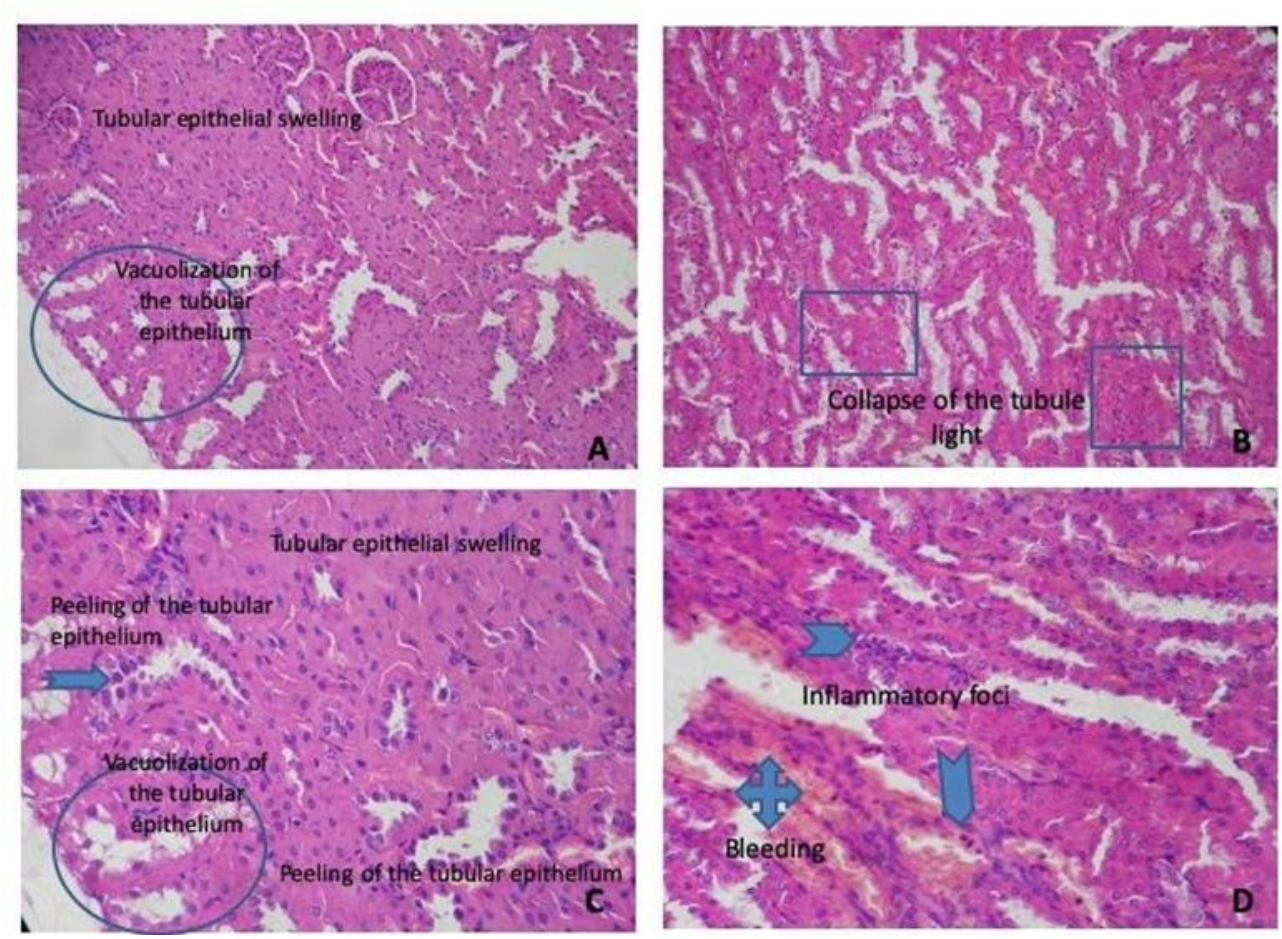

FIGURE 3 - Kidney photomicrographs of one male animal (code 3M) of the metformin group.

The figure shows a marked swelling of the tubular epithelium, sometimes with the collapse of the tubule lumen, vacuolar degeneration, scaling of the proximal tubular epithelium, cortical vascular congestion, and foci of lymphocyte infiltrate. Hematoxylin-eosin staining 200X (A and B) and 400x (C and D). Source: Research data (2024).

In males, both right and left kidneys had a significant reduction in mean weight in the AGO and LOS groups ($p < 0.05$), as well as in the AGO+SIM one ($p < 0,05$), compared with the VEH control group (Table 6). Regarding females, a significant reduction in left kidney weight was observed in the groups MET, AGO+MET, and AGO+SIM compared with VEH controls ($p < 0.05$) (Table 7).

Also, vacuolization of the tubular epithelium occurred in 100% of females, ranging from discrete in the VEH group to severe in three AGO-combined groups: AGO+LOS, AGO+SIM, and AGO+MET. Among males, vacuolization was not detected in the groups LOS. AGO+MET, and AGO+LOS+SIM+MET. Thus, MET showed more significant nephrotoxic potential with ATN development when used alone than when associated with other drugs in the study, affecting 33.3% of treated animals (4 animals/12).

Hyaline cylinders were found in 100% of females, ranging from rare in the VEH group to frequent in all other treated groups. Among males, hyaline cylinders were not detected in the combined drug groups LOS+SIM+MET and AGO+LOS+SIM+MET and the group LOS. In the other groups, hyaline cylinders were scarce, affecting a small number of animals per group, as observed in AGO, SIM, and MET groups. Therefore, the occurrence of hyaline cylinders was notably higher in females.

Inflammatory cells were found only in females and males treated with MET alone, affecting all-female subjects in this group (6/6) and four (4/6) males. Thus, 10 out of 12 animals treated with MET (83.33%) presented considerable inflammatory focus in the kidneys, the most affected females (Figs. 3, 4, and 5).

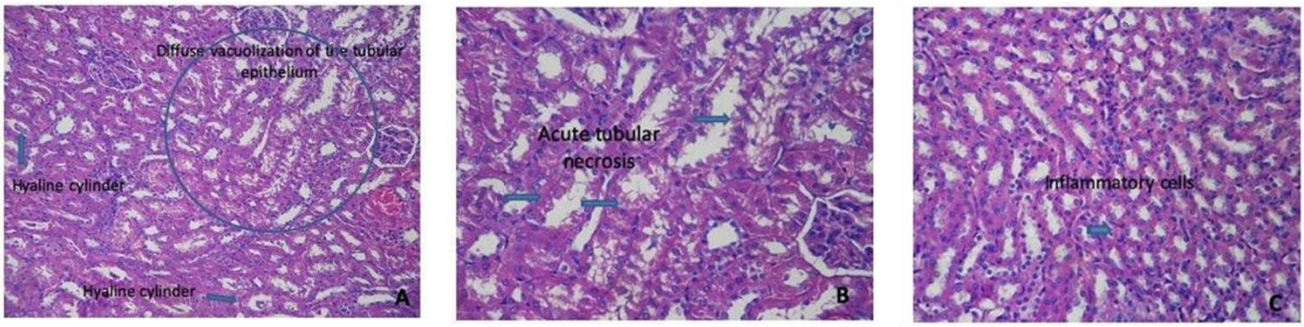


FIGURE 4 - Kidney photomicrographs of a female animal (code 4F) from the metformin group.

The figure shows a diffuse vacuolization of the tubular epithelium, cortical medullary hyaline cylinders, dispersed inflammatory cells, and foci of desquamation of the tubular epithelium at the cortico-medullary junction, suggestive of acute tubular necrosis. Hematoxylin-eosin staining 200X (A) and 400x (B and C). Source: Research data (2024).

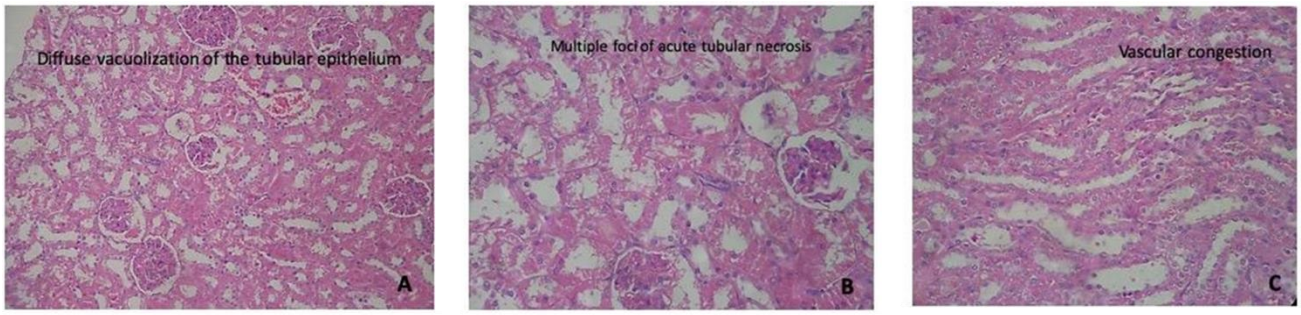


FIGURE 5 - Kidney photomicrographs of a female animal (code 5F) of the metformin group. It was found a diffuse vacuolization of the tubular epithelium. Cortical vascular congestion and multiple flaking foci of the tubular epithelium at the cortico-medullary junction suggest acute tubular necrosis. Hematoxylin-eosin staining 200X (A) and 400x (B and C). Source: Research data (2024).

In males, both right and left kidneys had a significant reduction in mean weight in the AGO and LOS groups ($p < 0.05$), as well as in the AGO+SIM one ($p < 0.05$), compared with the VEH control group (Table 6). Regarding females, a significant reduction in left kidney weight was observed in the groups MET, AGO+MET, and AGO+SIM compared with VEH ($p < 0.05$) (Table 7).

Stomach

All-female treated groups had inflammatory foci in the stomach, occurring in 100% (6/6) of the animals in the groups: SIM, AGO+LOS, and LOS+SIM+MET. Only one (1/6) female from the VEH group had inflammatory cells in this organ. Among males, inflammatory cells were not observed in the groups treated with MET or AGO+SIMcombination and in the VEH group. In the other male groups, that is, AGO, LOS, SIM, LOS+SIM+MET, AGO+LOS. AGO+MET and AGO+LOS+SIM+MET inflammatory foci were found in similar proportions (6/6) found in females.

Regarding this organ weight, we found a reduction in the AGO+SIM group compared with VEH controls in females ($p < 0.05$) (Table 7). In male animals, no significant weight difference was observed.

Gut

Flattening of villi and loss of crypts were not observed in any female animal. However, these alterations were detected in males of the LOS group (2/6 animals), SIM group (5/6 animals), and AGO+LOS+SIM+MET combination group (1/6 animals). In the latter, crypt loss was observed in two animals. Therefore, females did not suffer alterations in the architecture of the intestinal epithelium, being these alterations were restricted to the males' groups mentioned above. However, the presence of inflammatory cells was observed in all-female groups except for the VEH group affecting 24 of the 60 females (40%) using one or more drugs, AGO to AGO+LOS+SIM+MET group. In males, all groups presented inflammatory cells, except the animals of the LOS+SIM+MET group, in which no animal showed inflammation in the intestine. Thus, inflammatory cells were detected in 31 of 57 male animals whose gut was analyzed (about 54%).

Vacuolization or edema of intestinal epithelial cells was uncommon, occurring in only one female (1/6) of the SIM group and one male (1/6) of the AGO+LOS+SIM+MET combination group.

The submucosal and muscular layers were generally preserved, with no changes occurring in any group of females. The submucosa lymphoid follicle was observed in one male animal from each of the following groups, LOS, LOS+SIM+MET, and AGO+LOS+SIM+MET groups. Also, only in males was the presence of ecstatic vessels in fiveof the six (5/6) males in the SIM group.

In general, males were more susceptible to intestinal alterations than females, especially animals from LOS, SIM, LOS+SIM+MET, and AGO+LOS+SIM+MET. However, no significant difference was observed between the experimental groups in males or females regarding weight changes.

Spleen

The accentuated presence of hemosiderophages was verified in all females using one or more drugs, corresponding to 80% of the analyzed samples. In the group using AGO+SIM, similarly to the VEH control group, there was a moderatepresence of hemosiderophages. These findings were confirmed by blood count, which showed a significant reduction in the number of erythrocytes in females, especially in the combination drug groups, LOS+SIM+MET, AGO+LOS+AGO+SIM, AGO+MET, and AGO+LOS+SIM+MET, all with $p < 0.0001$ compared with the VEH control group. Also, the hemoglobin rate of females was lower than the VEH group, especially in the AGO combination groups, AGO+SIM ($p = 0.0371$). AGO+MET ($p = 0.0296$) and AGO+LOS+SIM+MET ($p = 0.0219$) (Table 3).

Among males, the total number of animals in which the significant presence of hemosiderophages was verified was lower than the number of affected females, occurring in 32 of the 54 male animals (approximately 59%) that had the spleen analyzed. Male blood count showed a significant reduction of

hemoglobin in some groups compared with the VEH control group, but not to the number of erythrocytes. Significantly lower hemoglobin rates than control ($p < 0.05$) occurred in groups whose presence of hemosiderophages in histopathology was marked, of note, LOS, MET, LOS+SIM+MET, and AGO+LOS-treated groups. However, this association was not observed in the AGO+LOS+SIM+MET group, in which there was a substantial presence of hemosiderophages, but the hemoglobin rate was like VEH controls.

The presence of megakaryocytes was observed in 100% of females, but rarely in the VEH group, mild in AGO+SIM and AGO+MET, moderate in LOS+SIM+MET, AGO+LOS, and AGO+LOS+SIM+MET groups, and marked in all groups using drugs alone, AGO, LOS, SIM, and MET. Among males, there was a smaller number of animals presenting megakaryocytes in all experimental groups, and this presence occurred more discreetly with females. Exception for the groups using LOS and SIM alone, in which the presence of megakaryocytes was pronounced, occurring in 100% (6/6) and 50% (3/6) of the animals of these groups, respectively.

Regarding spleen weight, no significant difference was observed in the male groups. However, in females, all-treated groups showed a significant reduction in this organ weight compared with VEH controls, mainly in the groups AGO, LOS, SIM, and MET ($p < 0.01$).

No alterations were observed in the groups using the drugs alone, either in females or males. However, in one of the six females in the group using three drugs combined, LOS+SIM+MET, acinar vacuolization was observed. This was also observed in two of the five males in the four-drugs combination AGO+LOS+SIM+MET group, suggesting a possible tendency for increased pancreatic toxicity in polypharmacy conditions. In both sexes,no difference was observed related to this organ weight compared to controls.

Heart

We found mononuclear inflammatory infiltrate in the cardiac tissue of a female of the SIM group and animals treated with drug combinations, LOS+SIM+MET (3/6) and AGO+LOS+SIM+MET (2/6). Furthermore, using SIM alone or in combination in all females induced an inflammatory infiltrate. The same occurred in 6 of the 18 females using SIM alone or in combination (about 33%) and 10% of the total female sample (6 of 60).

In males, the presence of mononuclear inflammatory infiltrate was more significant than in females, affecting 12 of 60 animals (20%), distributed in groups LOS (2/6 animals), SIM (2/6), MET (3/6), AGO+LOS (2/6), AGO+SIM (1/6) and AGO+LOS+SIM+MET (2/6) groups.

Furthermore, the groups AGO, LOS, AGO+LOS, and AGO+SIM had a significant reduction in mean heart weight relative to the VEH control group ($p < 0.05$) (Table 6).

## 4. Discussion

Evidence points to sex as a contributing factor for the effect of drugs. For example, pharmacokinetic

and toxicokinetic profiles are distinct in males and females, affecting, therefore, plasma levels of the drugs, their efficacy, pharmacological and toxicological profile, besides sensitivity to adverse effects[9]. Also, in animal studies, the influence of sex in drugs response has been widely described[10,11].

In the present study, we observed that hematological, biochemical, and histopathological parameters were significantly influenced by sex. Notably, AGO presented an increased risk for deleterious alterations when used alone or a protective profile when combined with the other drugs. Furthermore, hematological analyses revealed that, in females, the groups using the drugs in combination showed relevant hematological alterations in relation to the administration alone. However, this effect was not observed in males. Hence, we observed that females were more prone to hematological alterations induced by multiple drugs administration than males.

Sex differences are evident in both the consequences of persistent drug use and the reasons for continuing drug use despite adverse outcomes. Notably, the negative consequences of substance use in humans (whether physical, psychological, or social/interpersonal) are often more severe in women compared to men. This heightened severity in women may, in part, be related to body composition and physiological differences, which increase their risk of developing substance-related health issues, including cardiovascular problems, cancer, and liver disease[12].

Among the most significant findings in the white blood count series, we observed a higher susceptibility of females to develop leukopenia and lymphopenia with AGO in combination with LOS, MET, and SIM, as well as the use of SIM in combination with AGO, LOS, and MET. Male animals, in turn, had a significant reduction in the number of leukocytes and absolute lymphocytes compared with females using SIM alone. This suggests that the physiological and metabolic differences between sexes could underlie the greater vulnerability of females to drug-induced toxic effects, supporting the notion that sex-specific factors need to be considered when evaluating drug safety profiles and therapeutic strategies. This effect can also be considered due to the fact that SIM has anti-inflammatory and immunomodulatory activity, with various effects on the differentiation and function of immune cells. This drug increases the number of Treg cells and inhibits Th17, Th1, Th2 and CTL cells, it also leads to the suppression of mast cells and basophils[13].

In our study, females from all groups treated with the combinations of two, three, or four drugs showed a significant reduction in the number of erythrocytes compared with the control group and the respective groups of drugs used in monotherapy. Among males, the decrease in erythrocytes was only observed in the group that received AGO+SIM in relation to the drugs alone. Therefore, females were much more affected than males concerning red blood cell count.

In females, the combination of AGO with the other three drugs resulted in lower hemoglobin rates

considering its use alone. In males, the reduction in hemoglobin level occurred when AGO was combined with SIM. This combination also impacted the number of erythrocytes and the reduction of hematocrit in these animals. In males, in the dual drug combination groups, and the polypharmacy group, a reduction in the hemoglobin rate was observed compared with the groups using each drug alone.

In our study, anemia was observed in both females and males. However, while females had a more significant impact on the number of erythrocytes and hematocrit, which were significantly reduced in all groups using drug combinations, males were more impacted concerning hemoglobin rates, especially in AGO+SIM group, and on anisocytosis index, which was increased in almost all groups treated with one or more drugs.

The interpretation for these results seems to be linked to the antithrombotic effects of statins, which seem to be accentuated when associated with other drugs and more pronounced in females. Statins reduce mean platelet volume, increase red blood cell deformability, appear to interfere with ATP release by red blood cells, and increase nitric oxide[13]. In a recent study, it was described that rosuvastatin causes a reduction in hemoglobin, MCHC and number of erythrocytes, and an increase in the volume of erythrocytes (MCV), suggesting complex effects of this statin on red blood cells.

These studies indicate that AGO may exert some influence on the coagulation profile. In the present study, AGO alone did not lead to thrombocytopenia, regardless of sex. However, the reduction in the number of platelets occurred in female animals using combinations of two (AGO+SIM; AGO+MET), three (LOS+SIM+MET), or four drugs (AGO+LOS+SIM+MET), when compared with the groups using SIM or MET in monotherapy. The comparison between sexes revealed that males had significantly lower platelet counts than females using SIM and MET. Thus, regarding the reduced platelet counts, male animals were impacted by the use of SIM or MET alone, while females used the drugs in combinations. Bourneau-Martin et al. (2017)[14] reported the occurrence of thrombocytopenia and thrombocytopenic purpura in one patient using AGO. Yang et al. (2019) demonstrated that AGO alone reduced platelet count in male Sprague-Dawley rats but caused no effect on female platelets.

Indeed, several biochemical parameters of the studied groups showed sex differences or were altered by the concomitant administration of AGO. Males had higher cholesterol and triglyceride rates than females when treated with AGO alone. Females, in turn, presented higher cholesterol and triglyceride rates than males when using MET alone. The lipid-lowering SIM did not reduce total cholesterol rates in males or females. We observed that the animals were in normal lipid profile conditions since hypercholesterolemia was detected. However, SIM reduced triglyceride rates in males compared with control animals, even under normal conditions of lipid profile.

The higher rate of triglycerides of males using three drugs (AGO+SIM+MET) compared with the group

treated with SIM alone may point to the possible interaction between these three drugs by altering the potential of SIM to act on triglycerides in male animals. As SIM and LOS compete for two CYP450 enzymes (CYP2C9 and CYP34A) in their biotransformation, the interaction between these two drugs is likely to have resulted in decreased action of SIM on triglycerides[15,16].

Total bilirubin was high in females of groups that used AGO in combination with both LOS, as well as SIM and MET when compared with each drug alone, as well as with the saline control group, demonstrating that AGO, when combined with these drugs, increased liver toxicity in females. This finding reinforces the hepatotoxic potential of AGO, already described in the literature, which seems to have been exacerbated in females treated with the drugs in combination[17-19].

Yang et al. (2019)[20], considering the elevation of total bilirubin as a demonstration of AGO hepatotoxicity, verified a total bilirubin increase in female rats using AGO alone, and noted the increase of this parameter in male animals. Contrarily, our study did not reveal any increase in total bilirubin in animals using AGO alone, but only in females using AGO combined with other drugs, while in males, the combination of AGO with LOS or with SIM resulted in lower rates of total bilirubin when compared with the drugs alone. In comparing sexes, the total bilirubin rates of females of the groups that used the combinations of AGO with LOS or with SIM were higher than the male rates of the same groups, demonstrating the higher susceptibility of females to hepatotoxicity through these combinations of drugs.

The rate of alanine aminotransferase (ALT) was significantly elevated in the group of females using four drugs in combination when compared with the rates of the groups using the drugs alone and with the double or triple combinations. Hence, we observed that in our experimental condition, the use of the drugs in combination resulted in an overload of liver function in females. Yang et al. (2019)[20] found that using AGO alone caused ALT elevation in female and male rats. The mechanism underlying AGO-induced liver injury appears idiosyncratic in humans[21]. According to Freiesleben and Furczyk (2015)[22], the mechanism of injury may be hepatocellular, with a predominant increase in alanine aminotransferase (ALT); cholestatic, with a predominance of alkaline phosphatase elevation; or mixed. However, in the present study, there was no elevation of ALT in animals using agomelatine, except in the group in which this drug was associated with the three others concomitantly, especially in females.

The animals used in the present study presented were normoglycemic. Nevertheless, in females, a reduction in glycemic level was observed in all groups compared to the untreated control group, revealing that the drugs in the study, except MET, in monotherapy or combination, influenced the glycemic profile. As an exception, no influence on blood glucose was observed in females treated with MET alone. Despite being a hypoglycemic drug, MET has not been associated with hypoglycemia when used by individuals under normoglycemia

conditions. Furthermore, this drug is used off label for certain pathological conditions, such as polycystic ovary syndrome, obesity, and weight control[23].

In female animals that used AGO alone, no change in blood glucose was observed compared with the groups of this drug combined with the other. However, there was significantly lower blood glucose in all groups using combinations compared to the groups that used LOS, SIM, or MET alone. Males showed increased blood glucose, significantly increased among animals from all groups that used some type of drug association compared with the untreated control group.

When comparing the sexes, it was observed that the glucose rate of females, both in the control group and in the drugs used in monotherapy, was higher than that of males; with the combination of drugs, all blood glucose levels in males were higher than in females, with a significant difference between sexes in the combined drug groups.

The association of agomelatine with simvastatin reduced the rate of urea compared to the simvastatin monotherapy group in female animals, suggesting agomelatine as a protective factor for urea elevation in patients using simvastatin. Among males however, finding was not verified, and there was also no difference between genders in urea rates in the different groups. Renal function tests performed by Yang et al. 2019[20] in rats treated with AGO alone showed that this drug increased the rate of urea in male and female animals. Differently, in the present study, the animals using AGO alone did not present elevation in this biochemical parameter.

Creatinine rates in females were lower in the groups in which AGO was used in combination paired with each of the other drugs in the study when compared with the drugs used alone, including with the control group and the group using only AGO. Agomelatine, in combination, seems to have a renal protective effect than drugs alone. However, the combination of AGO with the other three drugs concomitantly culminated in a significant increase in creatinine rate when compared with the use of AGO in monotherapy, revealing that, in polypharmacy conditions, the possible renal protection of AGO did not occur.

Cankara et al. (2022)[24] investigated the nephroprotective effect of AGO in a model of induction of nephrotoxicity by cisplatin in male rats, the findings of this study showed that ALT and AST levels were significantly reduced after treatment with AGO. Başol et al. (2016)[25] demonstrated that the use of AGO alone in animals submitted to the sepsis-induced renal injury model resulted in the reduction of urea and creatinine, in addition to better scores in the renal histopathological evaluation, signaling nephroprotective effect of the drug. Yang et al. (2019)[20] verified creatinine elevation in female animals using AGO alone, contradicting our findings.

In females, the combination of the four drugs significantly increased creatinine levels regarding the drugs used in pairs, suggesting that the multiple combinations of drugs may trigger greater

susceptibility to kidney damage in these animals. Comparatively, males presented higher creatinine rates than females in groups using agomelatine alone and in groups in which agomelatine was associated with each drug in pairs.

Although animal studies are reasonably good predictors of adverse renal effects in humans, important differences between species, such as the influence of sex hormones such as testosterone, on rodents, in which hormones play a role in regulating renal transport and metabolism of drugs other substances should be considered. Thus, male mice and rats are generally more sensitive to kidney injury than females to many chemicals[11].

In general, we found that the females presented significant alterations concerning the saline control group in six of the eleven biochemical parameters analyzed, demonstrating great susceptibility of liver and renal functions with the drugs used. On the other hand, males showed significant alterations compared with the saline group only in the triglyceride parameters, specifically in the SIM group, and glucose, which was altered in all groups in which the drugs were used in combination. Thus, males seem to suffer fewer impacts on biochemical parameters than females in the face of the use of AGO, LOS, SIM, and MET, corroborating what the literature has shown in both humans and animals[9-11,13].

The present study shows that chronic-use medications when used in combination (polypharmacy), induced important alterations mainly in the liver and kidneys. These organs presented irreversible cell damage, such as focal hepatocyte necrosis and NTA, respectively. Females were more susceptible to drug combination-induced toxic effects, suggesting more severe histopathological alterations in a more significant number of animals and organs such as the liver, kidneys, stomach, gut, and spleen. Heart alterations were more observed in males. Following previous studies, AGO was associated with substantial liver damage, mainly when combined with SIM and MET, with the animals presenting major kidney changes, such as NTA and weight atrophy. Our data bring new relevant evidence regarding the safety profile of the novel antidepressant AGO and its combinations with commonly prescribed chronic-use medications, which can help guide clinicians and future studies investigating the mechanisms related to these toxic effects.

Sex-related differences play an important role in drug toxicity in rodent models and humans[10,11]. In general, male rats are more prone to kidney damage caused by many drugs[11]. The study by Kadkhodaee et al. (2020)[26,] confirmed that male rats were more susceptible to kidney disease than female rats. This finding supports the hypothesis that sex difference plays a key role in the response of the rats' body to kidney injury. However, in our study, females were most affected, especially when using MET, manifesting renal toxicity demonstrated by the substantial presence of inflammatory foci and NTA occurrence. Quaile et al. (2010)[27], in a toxicokinetic study using a subchronic MET treatment in rats, had to euthanize femaleanimals earlier since they showed intense signs of toxicity,

mainly kidney failure, at the highest doses of MET tested 900 and 1200 mg/kg/day. In our study, no clinical signs of toxicity were observed, nor was there any mortality during the treatment, which can be explained by the lower dose of MET (79 mg /kg/day, which is much lower than those used by the previous authors. However, females' greater renal susceptibility to MET damage was demonstrated when histopathological analyzes were performed. We must highlight that the doses used in the present study were calculated based on human prescribed doses.

The spleen was significantly affected, and many megakaryocytes showed increased erythropoiesis rate and, at the same time, the intense hemolysis, particularly in females, was demonstrated by the substantial presence of hemosiderophages. These histopathological findings were corroborated by the blood count that revealed hemolytic anemia in females, with a reduction in the number of erythrocytes and hemoglobin and a decrease in hemoglobinrate in males (data not shown). Thus, the marked extramedullary erythropoiesis for erythrocyte replacement may result from intense hemolysis or direct spinal cord stimulation[28]. Interestingly, these considerable effects were observed in combined treatment groups, mainly in those that received more than three drugs and that AGO was present. This highlights the potential harmful risk of polypharmacy schemes using AGO and the potential ofthe interaction of this drug with commonly prescribed medication for hematologic health.

Simvastatin was the single drug most closely related to mild histopathological changes in the liver. According to Averbukh et al. (2022)[29] statins have been associated with mild liver dysfunction, but can rarely induce severe liver injury. Among statins, SIM overdose and atorvastatin have already been associated with liver injury with fatal outcomes. However, the author argues that if the patient has a good indication for statin therapy, even those with underlying liver disease such as nonalcoholic steatohepatitis should use the drug[30]. Patients with a higher risk of cardiovascular mortality can suffer from the previous liver disease and from other medical comorbidities and potentially use multiple drugs. Therefore, a better understanding of the safety profile, mainly regarding liver toxicity, is essential for improving the quality of the use of SIM in patients suffering from chronic diseases.

Here, AGO also partially contributed to the occurrence of liver alterations. This effect was predominant in males, with intense inflammation in almost all animals of AGO monotherapy group. Furthermore, when used in combination, AGO-treated groups showed the most relevant histopathological liver changes, such as intense inflammation, microvesicular steatosis, and focal hepatocyte necrosis. In a review by Souto et al. (2019)[17] on the use of AGO in humans, hepatotoxicity was the most cited and most relevant adverse effect associated with this drug. The findings of our animal study confirmed the hepatic toxicity associated with AGO that has been already demonstrated in the literature, including in humans, and bring attention to the potential increased risk when using itwith other potentially

hepatotoxic drugs such as SIM.

Regarding sex differences, Gochfeld (2007)[10] brings examples of studies that show greater hepatic susceptibility of female animals compared to males to some xenobiotics, such as ethanol. Still, this finding was not verified in relation to cocaine, suggesting a dependence of the type of substance to this susceptibility. However, the author focused on the limitation of rodent studies, which employ a small number of animals and are therefore insufficient to detect robust sex-related differences from experimental challenges, which is also a limitation of our study. Thus, further experimental studies with more significant numbers of animals would be needed to demonstrate the influence of sex on the toxicity of AGO, MET, SIM and LOS, alone or in combination in different organs and systems.

Metformin presented the highest renal impact, leading to NTA in females and males, although in a small number of animals in males. In an extensive study on MET toxicokinetic in rats, using oral doses ranging from 200 to 1200mg/kg/day. No significant adverse effect was evidenced at the dose of 200 mg/kg/day, twice the dose used in the present study. However, these authors detected adverse metabolic effects, clinical signs of toxicity, and increased mortality among MET-treated animals at the highest dose. They did not perform a histopathological examination on the kidneys to better characterize the cell damage induced by MET. Additionally, these authors verified an increase in the absolute kidney weight in females using MET dose of 1200 mg/kg/day, which was not found in males, corroborating our results of increased female susceptibility to MET renal effects[27]. However, instead of increased weight, we observed a decrease in absolute kidney weight in MET-treated female animals. This is compatible with the renal atrophy that follows chronic toxic conditions[31] and may help to explain the differences between our findings (more prolonged treatment - 90 days) and the previous studies.

Significantly, in patients with impaired renal function, the half-life of MET is increased, and renal clearance is significantly decreased. Therefore, renal function should be rigorously monitored, and MET dose should be adjusted to these patients to avoid the risk of MET-associated severe metabolic disturbances, such as lactic acidosis[32,33].

Simvastatin and LOS, when used alone, were the drugs associated with the major intestinal histopathological changes, especially in males. They were also associated with multiple inflammatory foci in the stomach when used alone or in combination. The effect of statins was evaluated in a model of indomethacin-induced gastric injury in rats. The results showed that while SIM 20 mg/kg inhibited mononuclear leukocyte infiltration, the 40 mg/kg dose induced hyperemia and inflammation. The authors even recommend that statin use in humans be done with caution in patients with gastric diseases[34]. In our study, SIM was used at the dose of 1.8 mg/kg, and the findings related to stomach inflammation were considered mild. Possible explanations for this mild effect may relate to the dose used (low) since it was calculated based on the clinically prescribed dose and the use of normal rats (not presenting gastric alterations).

In a pharmacovigilance study, Kim and colleagues (2017)[37] found that gastrointestinal disorders were the most common adverse effects among patients using statins, such as abdominal pain, constipation, gastritis, and nausea. Together with our data, this indicates the potential effect of SIM in the gut and the stomach, reinforcing the caution in using this drug in patients predisposed to the development of gastrointestinal disorders.

Data from the literature show that losartan has no relevant metabolic effects in humans, including renal function preserved even in patients with renal failure[35]. LOS had little impact on the kidneys in our study, and no inflammatory process or necrosis was observed in animals using LOS alone. When combined with AGO, LOS was associated with the mild occurrence of swelling and vacuolization of the tubular epithelium, highlighting the risk of combined treatments including AGO again.

As tested here, combinations of multiple medications, such as LOS+MET+SIM or AGO+LOS+MET+SIM, led to some pancreatic toxicity. Although our findings were not so pronounced, they draw attention to the presence of SIM in the groups that suffered major alterations in this organ. Statins, such as SIM, have been associated with pancreatic changes, decreased insulin production, and increased risk of developing diabetes mellitus, as well as the occurrence of acute pancreatitis[36,37].

Additionally, the groups that received multiple drugs combined with SIM presented inflammatory foci in the heart, counteracting this statin's possible anti-inflammatory and cardioprotective effect at monotherapy in previous studies[38,39]. Of note, this effect has been attributed to SIM-vasoprotective properties in conditions associated with acute systemic inflammation such as endotoxemia[40]. Hence, it is important to note that most studies demonstrated the beneficial effects of SIM against a pre-existing pathological condition, such as endotoxemia or ischemia, and not in sham conditions as adopted in our study, which. Thus, this observation difficult a direct comparison between our findings and previous ones.

In the heart, the combination AGO + MET reduced the number of maleanimals with inflammatory foci. Notably, in the MET group, inflammatory foci were detected in 50% of animals, but when AGO+MET was used, none of the animals presented inflammatory foci. The potential anti-inflammatory effect of AGO has been studied under different circumstances. Karaman et al. (2016)[41] demonstrated the antioxidant and anti-inflammatory effect of AGO against contrast-induced nephrotoxicity. Ozcan and colleagues (2019)[42] found that AGO reduced the progression of diabetic encephalopathy in rats and reversed glucose-induced neuronal injury. Thus, it is possible that the combination with AGO had an impact on decreasing heart inflammation compared with the animals treated with MET alone. Hence, based on our findings, we can suggest that AGO may improve the MET safety profile.

A major limitation of the study is the use of an animal model, which, while providing valuable insights into drug toxicity, may not fully represent

human responses due to physiological, metabolic, and hormonal differences. Furthermore, the study did not assess the impact of additional variables such as age and basal metabolic state, which can influence drug responses. Future studies could include these factors to provide a more comprehensive understanding of pharmacological interactions and their impacts on different populations.

Another limitation is the focus on a specific set of drug combinations. Although we have demonstrated that the combination of AGO with other chronic-use medications can significantly influence the toxicological profile, we did not evaluate the dose-response of each combination, which is essential for determining the clinical safety of these interactions. Future trials should include different dosages to assess the safety and efficacy of the combinations under various clinical conditions.

Given the observed impact of combinations of AGO with other medications, future research should explore the molecular mechanisms underlying these toxic effects, especially those related to sex differences. Mechanistic studies using techniques such as transcriptomics, proteomics, and metabolomics could provide more detailed insights into the biochemical pathways involved. Moreover, human studies, particularly clinical trials that take into account gender variability and the health status of patients, are essential to validate the clinical relevance of findings from animal models.

The findings of this study suggest that clinical practice should consider individualized therapy, particularly in the context of polypharmacy, where drug interactions and sex differences can play a significant role in patient outcomes. Given the susceptibility to hematological and biochemical alterations observed in females versus males, it is advisable for clinicians to closely monitor these changes in patients undergoing multiple pharmacological treatments, especially when combining antidepressants like AGO with other chronic-use medications.

## 5. Conclusion

This study demonstrates significant sex-specific differences in the toxicity of chronic medications, both when used alone and in combination. Our findings highlight the greater vulnerability of women to hematologic abnormalities, particularly when exposed to agomelatine combined with losartan, simvastatin, or metformin. Notably, polypharmacy involving these medications significantly affected erythrogram parameters in women, suggesting an increased risk of leukopenia and lymphopenia.

In addition, women exhibited a greater sensitivity to drug-induced hepatic and renal toxicity compared to men, as evidenced by significant changes in biochemical markers. This highlights the importance of considering sex as a crucial factor in clinical decision-making, especially when prescribing agomelatine in combination with other chronic medications.

To mitigate these risks, we recommend vigilant monitoring of hematologic, hepatic, and renal function parameters in female patients receiving multiple pharmacologic treatments. Furthermore, personalized medicine approaches that incorporate sex-specific responses are essential to optimize drug regimens. Future research should focus on elucidating the underlying molecular mechanisms responsible for these sex differences by investigating a broader range of drug combinations and conducting dose-response analyses. Integrating sex differences into clinical trial design and pharmacovigilance programs may enhance therapeutic strategies and improve the management of adverse effects in patients undergoing complex treatments.

## 6. Conflict of Interest

The authors have no conflicts of interest.

## 7. References


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