Study of Dynamics of Immunobiochemical Parameters and Pharmacological Interferences in the Metabolic Syndrome

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The metabolic syndrome (MetS) represents a frequent disorder of the present age, with increased global prevalence, with complex etiopathogenesis and physiopathology, with great impact upon the patients, society and economy and which often is difficult to treat. The main aim of the study was the evaluation of the pharmacodynamic effects of associated angiotensin-converting enzyme (ACE) inhibitors with non-steroidal anti-inflammatory drugs (NSAIDs) on blood pressure values and markers of oxidative stress in rats with MetS. Material and methods: 9 groups of 6 Wistar rats weighing between 150-200g with uniform gender distribution were subjected to cholesterol diet. During 4 weeks, their exercise capacities were analyzed over a 10-minute interval after administration of the test substances. Results: All groups who received cholesterol showed significant increases in serum cortisol. The group receiving Enalapril noted the highest levels of superoxide dismutase (SOD). Conclusions: Comparing the results obtained in this study with the literature, it was noted that the administration of ACE and / or NSAIDs significantly improves the process of chronic inflammation.

Keywords: metabolic syndrome, immuno-biochemical markers, cortisol, interleukins

Metabolic syndrome (MetS) is a progressive disorder, which comprises a large variety of disturbances with specific metabolic anomalies, it being defined by the presence of three out of five criteria: increased waist circumference, systolic blood pressure (BP) 1 exceeding 30 mmHg and/or a diastolic BP above 85 mmHg, hyperglycemia, a low value of HDL-cholesterol and increased values of the triglycerides [1, 2].

Literature data shows that the global prelevance of MetS of 5% in the normal-weight subjects, 22% in those with excess weight and 60% in the obese and it increases with age (10% in those aged between 20 and 29, 20% in those with ages comprised between 40-49 and 45% in those aged 60-69). The prevalence varies between 8% and 43% in men and from 7% to 56% in women [3]. In Romania, there is a national prevalence of excess weight of 33.1% and an obesity prevalence of 8.6% [4].

Hypertension treatment involves the use of various classical associations of antihypertensive drugs, in many cases, depending on the response, requiring a complex therapeutic regimen. Usually, the hypertensive patient as an associated, inflammatory pathology or pain-related disease requiring treatment with NSAIDs, medication known to increase BP. Additionally, dysregulated inflammatory response in MetS raises serious concerns related with its long term metabolic impact on the liver causing chronic liver disease, liver fibrosis and organic failure [5-8].

Experimental part

Material and methods

The purpose of the study is to investigate the pharmacodynamic effects of associated ACE-NSAIDs administration on pressure values and markers of oxidative stress in rats with MetS. For experiments, Wistar white rats were used (weighing between 185-200g), with a uniform gender distribution.

For induction of dyslipidemia, all animals have been subjected to cholesterol diet (0.2 g/kg body weight/day, 4 weeks). The animals were distributed in 9 groups (6 rats/group) and received the following substances, single intraperitoneally injection, following protocol: Group M1 (control1): physiological saline - 0.5mL/100g body; Group M2 (control2): cholesterol diet; Group ENP: Enalapril -1 mg/kg body weight/day; Group IND: Indometacin -1 mg/kg body weight/day; Group KET: Ketoprofen- 3 mg/kg body weight/day; Group NMS: Nimesulid - 1.5 mg/kg body weight/day; Group ENP+IND: Enalapril-1 mg/kg body weight/day + Indometacin - 1 mg/kg body weight/day; Group ENP+KET: Enalapril -1 mg/kg body weight/day + Ketoprofen - 3 mg/kg body weight/day; Group ENP+NMS: Enalapril-1 mg/kg body weight/day + Nimesulid -1.5 mg/kg body weight/day.

The current research was carried out using the instructions provided by the *Guide for the Care and Use of laboratory animals* [9]. The study complied with the national and international ethical regulation [10, 11]. During this in vivo animal experimentation the guidelines of the Ethics Committee of Gr. T. Popa, Iasi was taken in account in strict accordance with the International Ethical Regulations, some published models and guidelines related to the handling of laboratory animals (20840/ 03.10.2017) [12].

At the end of the experiments, the animals were euthanized by provoking a quick death without physical

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and mental suffering [13, 14]. The method was painless with rapid unconsciousness, cessation of heartbeat, respiration and death. This is a standard procedure and is carried out in a separate area away from the place where animals are housed. Euthanasia was performed by inhalation of a volatile anesthetic (isoflurane), which induces a state of unconsciousness in a few seconds. The absence of vital signs (heartbeats, respiratory movements, reflexes) was followed over 5 min. After death confirmation, the animals were decapitated [15].

Absolute BP values were determined with the HAMEG sphygmomanometer.

The physical exercise capacity analysis of rats after administration of the test substances was performed using forced treadmill exercise over a 10 min interval. This experimental model is used to evaluate the motor function and effort resistance of laboratory animals.

To determine serum cortisol levels, blood was harvested in vacutainer without anticoagulant, with or without a separating gel. The vacutainer was shaken very easily by overturning and was immediately placed on ice. Within 30 minutes of harvesting, the plasma was separated by centrifugation (in a cool centrifuge with at 2-8°C), 15 minutes 1500 x g [g = 0.00001118 x radius in cm x RPM^2]. For plasma separation, a plastic pipette was used.

The serum level of the hormone was determined by the immunochemical method with electrochemi-luminescence detection (ECLIA).

Interleukin (IL)-1 acts on both T lymphocytes (with IL-2 production stimulation) as well as B lymphocytes (stimulating B lymphocyte proliferation and immuno-globulin production), IL-6 provides growth and differentiation of B cells, stimulates immunoglobulin production, promotes activation, growth and differentiation of T-cell; tumor necrosis factor (TNF-α), proinflammatory cytokine, is involved in cellular apoptosis [16-18].

A venous blood sample (at least 0.5 mL of serum) was harvested in vacutainer without anticoagulant. The serum was separated by centrifugation as soon the clot formation was complete, and the sample was immediately frozen at -20°C. IL levels were determined by an immunochemical method with chemiluminescence detection.

Evaluation of oxidative stress

SOD, antioxidant metalloenzyme occurs in the conversion of superoxide radicals into water and hydrogen peroxide, subsequently decomposed to oxygen and water by the intervention of glutathione peroxidase and catalase [19]. SOD determination was performed by spectro-photometric monitoring (at 505 nm) of superoxide anion generation by the participation of xanthine and xanthine oxidase.

After the interaction with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride, a Formazan-type substance was formed which showed a red reaction.

It is believed that SOD activity is proportional to the degree of inhibition of the color reaction, in that a SOD unit represents that enzymatic activity for which the 50% inhibition of the color reaction is inhibited.

Glutathione peroxidase (GPX) is an enzyme mediating the protection of proteins, lipids and nucleic acids from the action of oxidizing molecules, using electron donor glutathione, or in some cases, thioredoxin or glutaredoxin [20].

Determination of the level of glutathione peroxidase was performed spectrophotometrically. One reaction mixture contains: 1 mL of 0.4M phosphate buffer (at *p*H 7.0) with 0.4 mM EDTA, 1 mL of 5 mM NaN_3, 1 mL of 4 mM GSH, and 0.2 mL of supernatant.

It was pre-incubated at 37°C for 5 min. Then 1mL of 4mM H_2O_2 was added and incubated at 37°C for a further 5 min. The excess amount of GPX was quantified using the DTNB method.

Malondialdehyde (MDA) is a highly reactive enzyme mediating the biosynthesis of prostaglandins and thromboxane [21].

Determination of its values was done by spectro-photometric measurement using the thiobarbituric acid method. 200μL of supernatant was added and vigorously mixed with 1 mL of 50% trichloroacetic acid in 0.1 M HCl and 1 mL of 26 mM thiobarbituric acid.

The samples were maintained at 95°C for 20 min. Then, they were centrifuged at 3000 rpm for 10 min. The supernatant was read at 532 nm in a spectrophotometer. The standard calibration curve was constructed using MDA as a control.

Statistical data analysis

The data was centralized and statistically processed using the SPSS version 22.0 for Windows 10 and the ANOVA method. In the statistical analysis, both descriptive and analytical methods were used at 95% significance (CI 95%). In calculating the significant difference between two media, the t-Student test or F (ANOVA) test was used to compare the average values in three or more groups with normal distributions. P-value values of less than 0.05 were considered to be statistically significant.

Results and discussions

Analysis of metabolic syndrome components

Exploration of lipid metabolism was performed by evaluating serum titres of total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol.

Indomethacin caused the highest cholesterol levels, which can also be observed with triglycerides.

A significant difference of LDL-cholesterol was observed between the ENP + IND vs. IND, where it appears that ACE administration had a role its decrease.

Regarding the values obtained from the control group + cholesterol diet, they were significantly higher compared to the rest of the groups except for groups 4 and 7 (fig. 1). Following analysis of the distribution of lipid metabolism components, we can objectively claim that Enalapril has a beneficial effect on the decrease of total cholesterol, LDL-cholesterol, and triglycerides.

Except for Indomethacin, the remaining NSAIDs used in the experiment did not significantly affect the values outlined in figure 1.

Analysis of the components of oxidative stress and inflammatory syndrome in rats subjected to physical stress

Glucocorticoids increase the activity of enzymes involved in the synthesis of fatty acids and contribute to lipoprotein secretion, inducing the hepatic gluconeogenic pathway [22].

The most prominent increase in serum cortisol values under experimentally induced forced effort conditions was found in the control group with cholesterol diet.

NSAIDs treatment reduced plasma cortisol levels but was statistically insignificant compared to the control group receiving cholesterol diet under the stress test.

The association of Enalapril with the studied NSAIDs decreased serum cortisol values, but was found statistically insignificant compared to both control groups, the cholesterol free group and the cholesterol diet control group, under stress conditions. The most pronounced effect

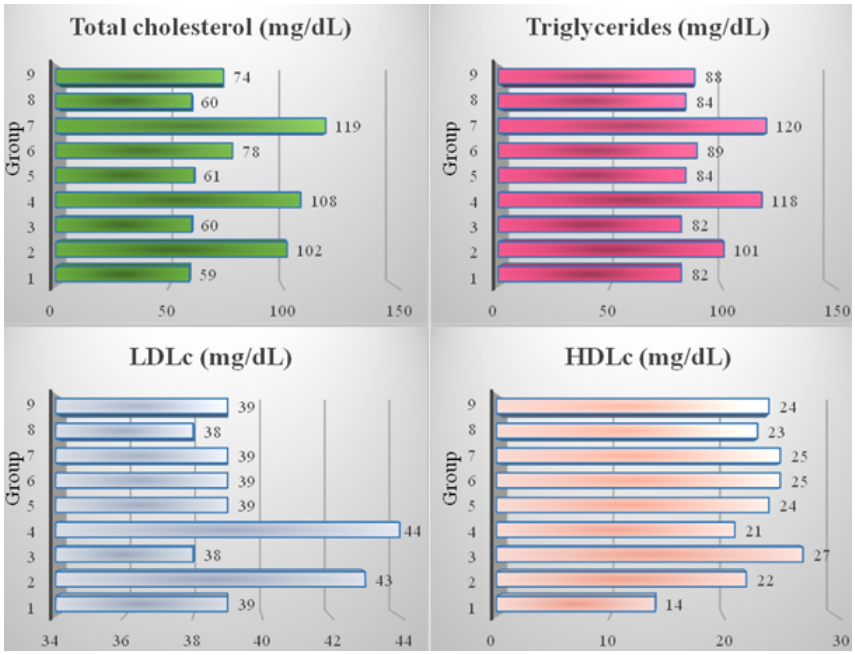

Fig. 1. Distribution of average values of lipid metabolism components in rats

was found for the combination of Enalapril + Ketoprofen (fig. 2).

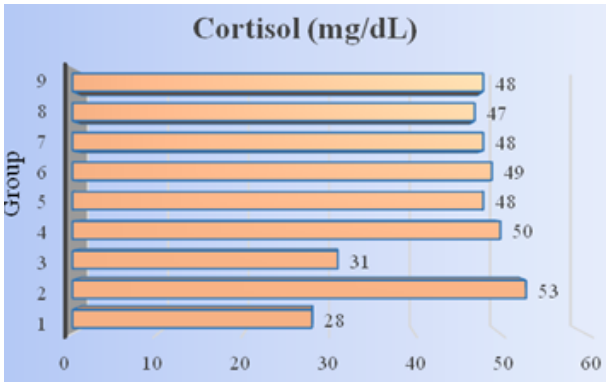

Fig. 2. Batch distribution based on serum cortisol value

Compared with cortisol results, the group receiving Enalapril noted the highest values of SOD, compared with data from the control groups (fig. 3).

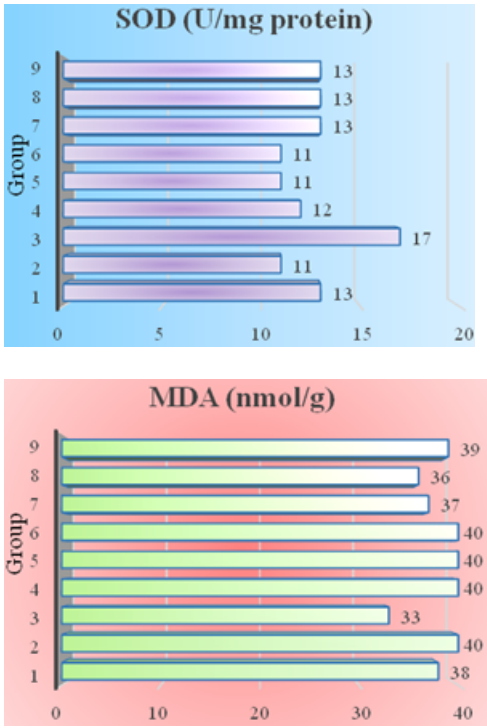

Fig. 3. Batch distribution based on superoxide dismutase and malondialdehyde value

Enalapril treatment resulted in an increase in SOD activity, statistically significant from the cholesterol-treated group and underwent the conveyance test. Administration of NSAIDs did not conclusively alter the activity of SOD in the serum and cholesterol diet, under strenuous conditions for the rat. The use of the combination between Enalapril and the NSAIDs studied did not result in significant variations in the determined levels of SOD, as compared to the control cholesterol test group, in the rat exercise test (fig. 3).

In the test animals receiving cholesterol diet, there was a slight, though increase in MDA, compared to the group with saline, but without cholesterol diet. Enalapril treatment resulted in an increase in MDA activity, statistically significant over the group receiving the cholesterol diet that underwent the treadmill test. Administration of NSAIDs did not conclusively alter MDA activity against the serum with saline and cholesterol diet under forced rat exertion. The use of the combination between Enalapril and the NSAIDs studied did not result in significant variations in the determined levels of MDA, as compared to the control cholesterol test group, in the rat exercise test (fig. 3).

IL-1β represents the key cytokine in triggering visceral destructive phenomena (pancreas, liver, kidney) in chronic inflammatory diseases.

The use of Enalapril in exercise-induced cholesterol diet rats was accompanied by a decrease in serum IL-1β but statistically insignificant from the control group with cholesterol diet (fig. 4).

The administration of tested NSAIDs weakly reduced IL-1β values, statistically insignificant compared to the group receiving saline and cholesterol diet under stress conditions. The combination of Enalapril with Indomethacin resulted in a decrease in the statistically significant IL-1β level over the physiological serum lot and the cholesterol diet one. It can be appreciated that in the context of experimentally induced MetS rats, administering Enalapril in combination with Indomethacin significantly improves the process of chronic inflammation (fig. 4).

The statistical analysis revealed the establishment of weak positive relationships between the control group and the NSAIDs lots, this means that an increase in IL-6 value for one of the two compared lots will associate an increase in the value for IL-6 for the other batch. The only lots with a high IL-6 value were the control + cholesterol diet and Enalapril (fig. 4).

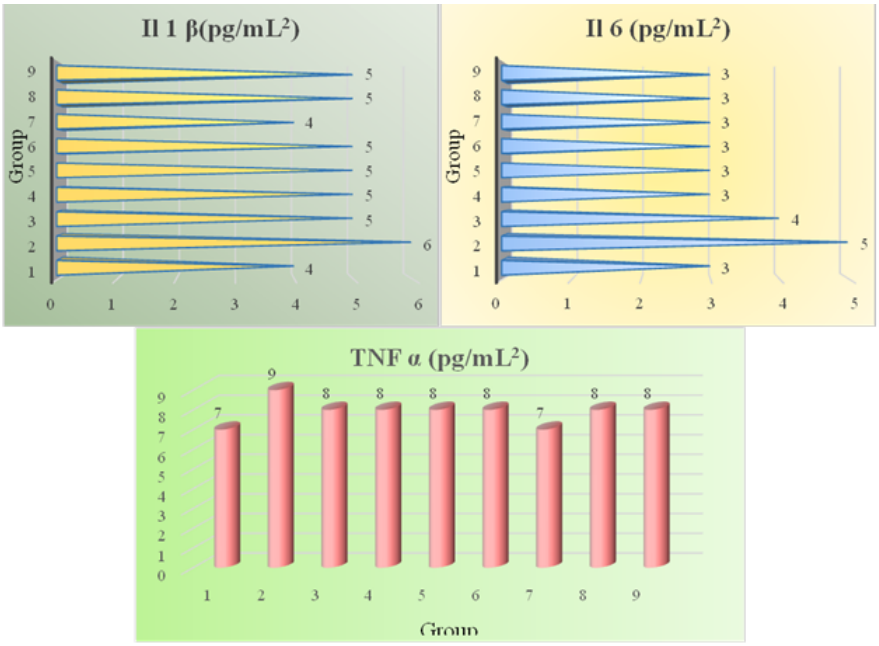

Fig. 4. Batch distribution based on interleukin-1β, interleukin 6 and TNF-α

Administration of Enalapril in cholesterol diet rats slightly diminished the IL-6 though it was statistically insignificant in comparison to the group receiving saline and cholesterol a diet under physical strain conditions (fig. 4). The administration of tested NSAIDs and their association with Enalapril in cholesterol-lowering rats reduced serum levels of IL-6, statistically significant compared to the control group with a cholesterol diet, subjected to forced exercise (fig. 4).

In test animals with saline and cholesterol diet there was an increase in serum TNF-α, statistically insignificant to the control group without cholesterol diet and effort-free. Enalapril treatment in rats with cholesterol diet lowered the TNF-á slightly, statistically insignificant from the physiological saline group and cholesterol diet, subjected to forced exercise. The use of tested NSAIDs in cholesterol-lowering diet in test animals lowered serum levels of IL-6, but statistically insignificant compared to the control group with cholesterol diet under physical exercise.

The combination of Enalapril with Indomathacin in rats with cholesterol diet produced the most significant reduction in TNF-α compared to the cholesterol-treated group and was tested in the treadmill exercise test.

Cortisol and BP are two related variables; their correlation field is not uniform (it consists of three disjoint surfaces, at the cortisol values 30 and 50), which is why the link between them occurs after the stratification of the values according to the group they are part of (fig. 5).

The variable effects of NSAIDs used in this study are influenced by the selective inhibitory action on either COX-1 or COX-2 (fig. 5).

The complex interactions of ACE and NSAIDs, both from a pharmacodynamic point of view and in terms of producing changes in the body at different levels of apparatus and systems under stress, offer the possibility of complex experimental investigations with modern laboratory equipment, using standardized experimental models from the literature.

Despite therapeutic advances, hypertension maintains its position as a *silent killer*, impacting cardiovascular morbidity and mortality. A sustained increase of 3 mmHg in systolic BP could elevate by 10-20% the risk of congestive heart failure and 12% in angina pectoris [23]. Another study has shown that an increase in systolic BP of 5 mmHg has a 25% higher risk of cardiovascular events [24].

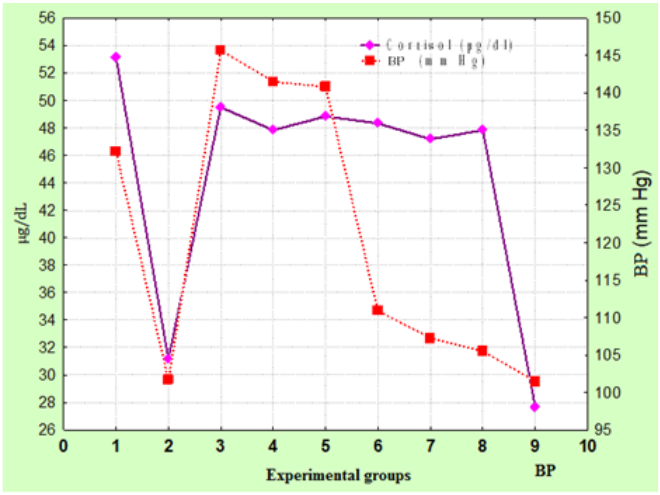

Fig. 5. Batch distribution according to BP and cortisol

NSAIDs have different effects depending on the dose administered. Reports on the effect of NSAIDs on the cardiovascular system are controversial [25]. NSAIDs increase BP by blocking the synthesis of prostaglandins that regulate vascular tone and sodium excretion. Small doses of aspirin and selective COX-2 inhibitors may improve or aggravate endothelial dysfunction of hyper-cholesterolaemia, atherosclerosis and hypertension, according to some authors [26].

By comparing the results obtained in this study with the literature, a significant increase in IL-1β secretion in the control group with cholesterol diet was indeed noted compared to the control group with saline.

In animals with physiological serum and cholesterol diet subjected to forced exercise test, the decrease in SOD values was noted compared to the control cholesterol control group, which is in line with the data in the literature on activity variations SOD in forced locomotor activity [27].

In line with previous studies, the increase in TNF-α concentration in the Indometacin group was statistically insignificant [28].

Similar to the study on stressed rats, IL-1β and TNF-α values in the NSAIDs treated group were increased compared to the control group [29]. Nevertheless, the metabolic syndrome and associated diseases is characterized by an overall poor prognosis due to the borderline adhesion and compliance of patients to treatment [30-33].

Conclusions

Cortisol secretion attests the activity of the hypothalamic-pituitary-adrenocortical axis, activity dependent on the diurn and metabolic rhythm, but also on the stress response. Cortisol is an important factor with role in atherosclerotic pathophysiological processes (metabolic imbalances and adiposopathy, insulin resistance, vascular and prothrombotic inflammatory response). After the treadmill stress test of Winstar rats, significant increase in serum cortisol was observed in all groups receiving cholesterol, but a value close to that of the control group was obtained in rats given Enalapril.

The highest values of SOD were noted in the group receiving Enalapril, compared even to the control groups. Antioxidant systems are an important link in controlling the destructive oxidative process associated with metabolic syndrome, marking the complex pharmacological relationship between the ACE inhibitors and reactive oxygen species.

Comparing the results obtained in this study with the literature, a significant increase in IL-1β secretion in the control group with cholesterol was indeed noted compared to the control group with saline and an improvement in IL-1β secretion in animals test lots, which allows us to conclude that in the context of induced metabolic syndrome rats, ACE and/or NSAIDs administration significantly improves the process of chronic inflammation.

At the same time, we have found a significant increase in serum cortisol levels along with elevated IL-1β levels, so we can anticipate new research directions on the cytokine study and correlations with the symptoms and values of classical markers.

List of abbreviations

ACE = angiotensin-converting-enzyme
NSAIDs = Non-steroidal anti-inflammatory drugs
BP = Blood pressure
IL = Interleukin
TNF = Tumor necrosis factor
SOD = Superoxide dismutase
GPx = Glutathione peroxidase
MDA = Malondialdehyde

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