ORIGINAL ARTICLE

Myeloperoxidase as an Indicator of Oxidative Stress in Metabolic Syndrome

Mieloperoxidasa como indicador de estrés oxidativo en síndrome metabólico

MARÍA L. GARAGIOLA[1], MARIANA TARÁN[1], MARÍA P. SCRIBANO[1], ARIEL BALCEDA[1], EMILIA GARCÍA[1], ISMAEL FONSECA[3], MÓNICA MOYA[1, 2], MARÍA C BAEZ[1, 2]

ABSTRACT

Background: Increased myeloperoxidase (MPO) activity would be the link between the rise of the inflammatory response and oxidative stress (OS) in metabolic syndrome (MS).
Objective: The aim of this study was to determine the enzymatic activity of MPO associated with OS in animals with MS and establish their relationship with probable cardiovascular injury.
Methods: Male Wistar rats were divided into two groups: Group A, control (n=12) and Group B, induced MS (n=12). Metabolic syndrome was produced by 6-week administration of 10% fructose diluted in the drinking water. Insulin (μU/ml), glucose (mg/dl), lipid panel (mg/dl), HOMA (homeostatic model assessment), MPO (IU/ml) and superoxide dismutase (SOD) activity (U/ml) were measured. Light microscopy was used for the histological study of the heart and thoracic aorta.
Results: Group B showed significantly increased levels of plasma glucose (176±17.3 mg/dl), insulin (29.5±4.52 μU/ml), HOMA (11±1.3), total cholesterol (133±9.6 mg/dl) and triglycerides (75±12.9 mg/dl) compared with Group A: plasma glucose (115±1.1 mg/dl), insulin (4±0.82 μU/ml), HOMA (3±0.38), total cholesterol (69.7±1.6 mg/dl) and triglycerides (46.2±6 mg/dl), ($p<0.001$ for all variables). A significant decrease in HDL (28.3±1.14 mg/dl) in Group B vs. Group A (61±1.0 mg/dl) ($p<0.001$) validated the experimental MS model. Myeloperoxidase activity increased significantly in Group B (181.3±15.7 IU/ml) vs. Group A (116.07±4.2 IU/ml) ($p<0.001$). A similar behavior was seen with SOD antioxidant activity in Group B (181±6 U/ml) vs. Group A (138±3.6 U/ml) ($p<0.01$). Light microscopy of the heart and thoracic aorta revealed histopathological changes in animals with induced MS.
Conclusion: Increased MPO and SOD in Group B would indicate the presence of OS in MS, with consequences at the vascular level.

Key words: Myeloperoxidase - Oxidative Stress - Metabolic Syndrome - Insulin Resistance - Cardiovascular Risk

RESUMEN

Introducción: Los incrementos en la actividad de la mieloperoxidasa (MPO) serían el nexo entre el aumento de la respuesta inflamatoria y el estrés oxidativo (EO) en el síndrome metabólico (SM).
Objetivo: Determinar la actividad de la enzima MPO asociada con EO en animales con SM y establecer su relación con las probables lesiones cardiovasculares.
Material y métodos: Se utilizaron ratas macho de la cepa Wistar, que se dividieron en: Grupo A, control (n = 12) y Grupo B, inducción de SM (n = 12). El SM se indujo con la administración de fructosa al 10% diluida en agua de bebida durante 6 semanas. Se cuantificaron insulina (μU/ml), glucemia (mg/dl), perfil lipídico (mg/dl), HOMA (homeostatic model assessment), MPO (UI/ml) y actividad de la superóxido dismutasa (SOD) (U/ml). Se estudió la histología de la aorta torácica y el corazón por microscopia óptica.
Resultados: Se observaron niveles de glucemia (176 ± 17,3 mg/dl), insulina (29,5 ± 4,52 μU/ml), HOMA (11 ± 1,3), colesterol total (133 ± 9,6 mg/dl) y triglicéridos (75 ± 12,9 mg/dl) incrementados significativamente en el Grupo B en comparación con el Grupo A: glucemia (115 ± 1,1 mg/dl), insulina (4 ± 0,82 μU/ml), HOMA (3 ± 0,38), colesterol total (69,7 ± 1,6 mg/dl) y triglicéridos (46,2 ± 6 mg/dl) ($p < 0,001$ para todas las variables); se verificó disminución significativa de los valores de HDL (28,3 ± 1,14 mg/dl) en el Grupo B en comparación con el Grupo A (61 ± 1,01 mg/dl) ($p < 0,001$), validando así el modelo experimental de SM. La actividad de la MPO se incrementó significativamente en el Grupo B (181,3 ± 15,7 UI/ml) respecto del Grupo A (116,07 ± 4,2 UI/ml) ($p < 0,001$). Similar comportamiento presentó la actividad antioxidante de la SOD en el Grupo B (181 ± 6 U/ml) respecto del Grupo A (138 ± 3,6 U/ml) ($p < 0,01$). Las microscopias ópticas de corazón y de aorta torácica evidenciaron cambios histopatológicos en los animales con SM inducido.
Conclusión: Los incrementos de la MPO y la SOD en el Grupo B demostrarían la presencia de EO, con repercusión a nivel vascular en el SM.

Palabras clave: Mieloperoxidasa - Estrés oxidativo - Síndrome metabólico - Insulinorresistencia - Riesgo cardiovascular

Rev Argent Cardiol 2016;84:514-518. http://dx.doi.org/10.7775/rac.v84.i6.9946

Received: 10/27/2016 – *Accepted:* 11/18/2016

Address for reprints: Garagiola María Luz - Cátedra de Física Biomédica. Facultad de Ciencias Médicas, Universidad Nacional de Córdoba - e-mail: garagiolamarialuz@hotmail.com

[1] Chair of Biomedical Physics. School of Medicine, Universidad Nacional de Córdoba.
[2] Biomedical Physics. School of Medicine, Universidad Nacional de La Rioja.
[3] II Chair of Anatomy Pathology, Facultad de Ciencias médicas. Universidad Nacional de Córdoba.
FINANCIAL SUPPORT:
- Secretariat of Science and Technology (SECYT) of Universidad Nacional de Córdoba
- Science and Technology (CYCYT) of Universidad Nacional de La Rioja
- PID 2010, MINCYT, Ministry of Industry of the Province of Córdoba

Abbreviations

CVD	Cardiovascular disease	**MS**	Metabolic syndrome
HDL	High density lipoproteins	**OS**	Oxidative stress
HOMA	Homeostatic model assessment	**RNS**	Reactive nitrogen species
LDL	Low density lipoproteins	**ROS**	Reactive oxygen species
MPO	Myeloperoxidase	**SOD**	Superoxide dismutase

INTRODUCTION

Oxidative stress (OS) is currently considered a potential cause of inflammation, implicated in the development of chronic systemic diseases, as metabolic syndrome (MS). The presence of OS implies an imbalance in oxidation-reduction metabolism generated by the uncontrolled production of reactive oxygen (ROS) and nitrogen species (RNS). (1, 2) It is known that overproduction of ROS and RNS in the vascular wall generates endothelial dysfunction, a condition that increases the risk of developing cardiovascular diseases (CVD).

The prooxidative state triggered by OS may induce insulin resistance through the phosphorylation of insulin receptors and increase in proinflammatory cytokine levels, both conditions expressed in MS. (3) Moreover, it has been postulated that excessive formation of reactive species would directly impact on insulin effect, modifying antioxidant enzymatic mechanisms such as superoxide dismutase (SOD). Superoxide dismutase catalyzes the breakdown of harmful oxidants, neutralizing their toxicity and avoiding their pathological concentrations; (4) thus, OS would condition endothelial dysfunction. (5)

In the continuous search of new prooxidant markers, myeloperoxidase (MPO) acquires relevance to identify the factors determining MS. (6) Excessive MPO activity may lead to tissue injury through the production of oxidants, thus forming lipid and protein reactive species. Recent studies have shown that exposure of activated leukocytes to low density lipoproteins (LDL) generates, through MPO, nitrogen and halogenated species facilitating lipid peroxidation, protein nitration and conversion to proatherogenic forms of LDL in the vascular wall. (7) In addition, MPO participates in the generation of dysfunctional high-density lipoproteins (HDL) transforming their anti-inflammatory properties to proinflammatory ones. (8)

Also, proinflammatory and prooxidative states have been implicated in myocardial damage and endothelial impairment, (9) which emphasizes the importance of implementing oxidative biomarkers, as MPO, in asymptomatic stages of MS. This enzyme could define cardiovascular risk and would be an indicative test for this pathology. (10)

The purpose of this study was to determine MPO activity associated with OS in animals with MS and establish its relationship with probable cardiovascular injury.

METHODS

Animals

Male Wistar rats, weighing 280±20 g, inbred in the Institute of Physiology of the School of Medicine at Universidad Nacional de Córdoba were used to perform the experimental and analytical study. Animals were fed a balanced rat diet rat containing at least 17% protein.

Study groups

Twenty-four male rats were divided into two groups and consecutively studied in either of two different experimental situations:

- Group A: Control, animals without intervention (n=12)
- Group B: MS, induced by hydric diet supplemented with 10% fructose in the drinking water for 6 weeks (n=12).

Induced metabolic syndrome

Metabolic syndrome was induced by the administration of 10% fructose diluted in the drinking water for 6 weeks. (11) To confirm the presence of MS, fasting blood glucose, insulin, triglycerides, total cholesterol and HDL cholesterol were determined and HOMA (homeostatic model assessment) was calculated.

Biochemical assays

Plasma samples

Blood was obtained from decapitated animals previously anesthetized with ketamine (Ketal™) (16.25 mg/kg) and 2% xylazine (Alfasan™)(2 mg/kg), 42 days after fructose administration or tap water and following 24-hour fasting. Blood was collected in Petri dishes with an anticoagulant combination of ammonium/potassium oxalate in a 2:1 ratio. EDTA-anticoagulated blood samples were used for SOD and MPO assays. Then, blood was centrifuged at 3000 rpm for 15 minutes to obtain plasma and lysed red blood cells, respectively.

The following variables were quantified

- Glucose: Plasma glucose levels were determined by spectrophotometry, using commercial kits (Wiener, Buenos Aires, Argentina) according to the enzymatic method. Results were expressed in mg/dl. (12)
- Insulinemia: Insulin levels were quantified by radioimmunoassay and the results were expressed in μU/ml. (13)
- The homeostasis assessment model (HOMA) was used as index to measure the degree of insulin resistance and was calculated using the formula: [insulin (μU/ml) × plasma glucose (mmol/L) 22.5]. (14)
- Lipid panel: The lipid panel was assessed by enzymatic method (15) and the results were expressed in mg/dl.
- Myeloperoxidase: ELISA was used to quantify MPO enzymatic activity, and the results were expressed in IU/ml. (16)

- Superoxide dismutase: SOD enzymatic activity was assessed by spectrophotometry in lysed red blood cell using the Randox kit. Results were expressed in U/ml. (17)

Tissue samples for pathological anatomy by light microscopy

In all animals, 16 heart samples were collected, performing 4 μm longitudinal sections, and 30 thoracic aortic samples were collected from its origin to the last thoracic portion performing 4 μm sections. Samples for pathological anatomy were fixed in 10% buffered formalin and stained with hematoxylin-eosin. Light microscopy analysis was performed at 40×, 60× and 400× amplification

Statistical analysis

Multivariate ANOVA followed by the Hotelling post-hoc test was used to compare all possible combinations of pairs of means. In all cases, the significance level was set at $p<0.05$.

Ethical considerations

The procedures were performed following the guidelines and protocols approved by the Institutional Committee for the Care and Use of Laboratory Animals of the School of Medicine at Universidad Nacional de Córdoba, based on the American Physiological Society "Principles for the Care and Use of Laboratory Animals" (NIH Publication 85-23, 1996).

RESULTS

Biochemical characteristics to validate the MS model in the study groups are shown in Table 1.

Blood glucose, insulin, increased total cholesterol and triglyceride levels together with elevated HOMA and decreased HDL values in group B (MS) validate the experimental model (see Table 1).

Assessment of MPO activity evidenced a statistically significant increase in the group with induced MS (181.3±15.7 I U/ml) vs. the control group (116.07±4.2 I U/ml) ($p<0.001$). This increased MPO activity would indicate the proinflammatory state of the animals with MS, as illustrated in Figure 1.

Results of SOD enzymatic activity in lysed red blood cells of rats with MS and control rats are shown in Figure 2.

The persistence of the proinflammatory and prooxidative stimulus in group B (MS) (181±6 U/ml) generated a significant increase in the enzymatic activity of SOD when compared with group A (control) (138±3.6 U/ml) ($p<0.01$).

The increase in MPO and SOD in animals with experimentally induced MS compared with the control group would indicate the persistence of the proinflammatory and prooxidative stimulus in the group with MS.

Figures 3 and 4 show the vascular and cardiac impact in rats with experimentally induced MS.

DISCUSSION

The experimental model of MS showed that rats chronically receiving fructose in the drinking water provide a useful model for the diagnosis of factors constituting MS, which is induced by changes in food intake and expresses numerous alterations similarly to humans with MS. (18) The administration of 10% fructose in group B (MS) generated hyperglycemia, hypertriglyceridemia, reduced HDL levels and increased total cholesterol levels, together with hyperinsulinemia, confirming that the experimental model presents characteristic manifestations of MS. In addition, the assessment of blood glucose/insulin homeostasis, HOMA parameter, constitutes a useful model

Table 1. Plasma levels of glucose, insulin and lipid panel in group A (control) and group B (metabolic syndrome)

	Control (A)	MS (B)
Blood glucose (mg/dl)	115±1.1	176±17.3*
Insulinemia (μU/ml)	4±0.82	29.5±4.52*
HDL (mg/dl)	61±0.01	28.3±1.14*
Total cholesterol (mg/dl)	69.7±1.6	133±9.6*
Triglycerides (mg/dl)	46.2±6	75±12.9*
HOMA	3±0.38	11±1.3*

Results are expressed as mean±standard error of the mean. n = 12. * p <0.001 vs. control rats (A). MS: Metabolic syndrome. HDL: High density lipoproteins. HOMA: Homoeostatic model assessment.

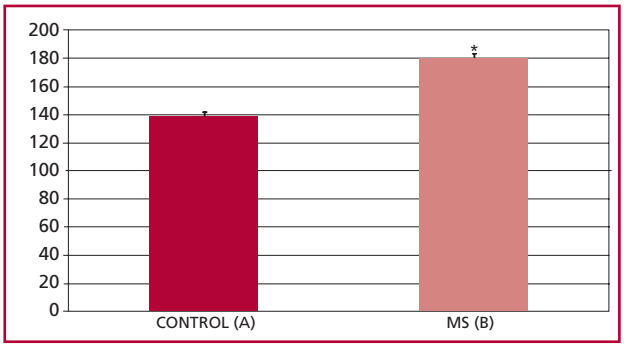

Fig. 1. Quantification of myeloperoxidase (MPO) activity in control (A) and with metabolic syndrome (MS) [B] rats. Values are expressed as mean±standard error of the mean. n = 12. * p <0.001 vs. control rats (A).

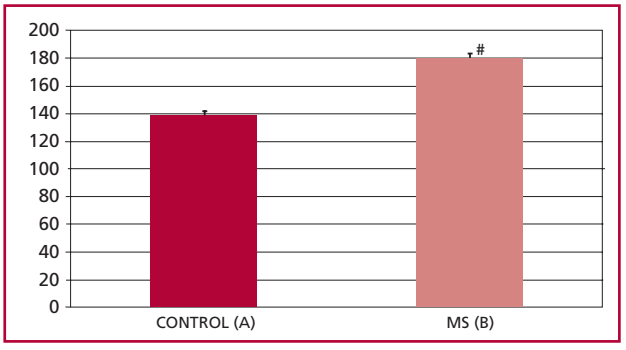

Fig. 2. Analysis of superoxide dismutase (SOD) enzymatic activity in control and with metabolic syndrome (MS) [B] rats. Values are expressed as mean±standard error of the mean. n = 12. # p <0.001 vs. control rats (A).

to quantify insulin resistance, reflecting beta cell function, only needing a fasting serum sample. Our results showed an increased HOMA value in the MS group, indicating the presence of insulin resistance.

Myeloperoxidase is an important cardiovascular risk factor, producing an increase in the inflammatory response capable of enhancing the oxidative effects of its cosubstrate hydrogen peroxide (H_2O), with a main role in endothelial damage and inflammation. (19)

Several investigations describe the association of chronic inflammatory states and insulin resistance with increased MPO activity, (20) increased OS, endothelial dysfunction and their influence on cardiovascular risk. (21) In our work, the significant positive relationship between fasting glucose/insulin (HOMA) and MPO activity, confirms the probable participation of dysglycemia in the altered redox metabolism of animals with MS.

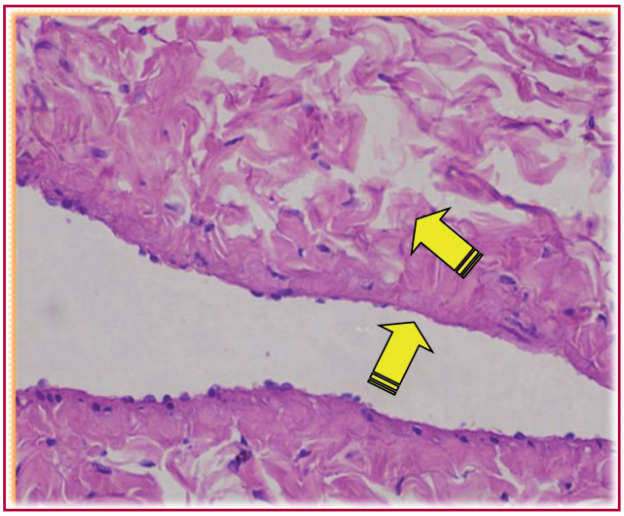

Fig. 3. Light microscopy of the thoracic aorta in rats with induced metabolic syndrome showing endothelial denudation with red blood cell adhesion, intimal thickening, myxoid changes in the extracellular matrix and internal muscular lamina disorganization in most sections (*arrow head*) (HE 60×)

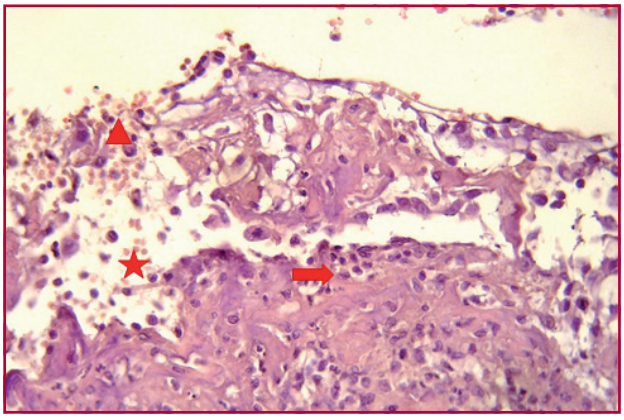

Fig. 4. Cardiac light microscopy of rats with induced metabolic syndrome showing fibrolipomatous pericardium (*star*), with presence of fibrinous leukocyte exudate with cell detritus (*triangle*) and mononuclear and polymorphonuclear neutrophil inflammatory infiltrate (*arrow*). Thick-walled vessels are also observed with intense mononuclear and polymorphonuclear neutrophil inflammatory infiltrate (vasculitis phenomena) (HE 60×)

Increase SOD activity associated with modifications in fasting blood glucose levels, insulin resistance, dyslipidemia due to decreased HDL and hypertriglyceridemia, and increased proinflammatory and prooxidative components are indicators of OS present in the experimental model of MS. This situation reflects the importance of SOD as endogenous antioxidant mechanism attempting to compensate the increase in free radicals, though it is not always enough, either by its low productivity or because it is overcome by the enzymatic production rate. (22-24) Concurrent with the biochemical parameters analyzed in this experimental model, thoracic aorta histopathological results with light microscopy show the vascular impact of this disease. (25)

The proinflammatory and prooxidative signals triggered by the biomarkers present in the experimental model of MS probably activate the endothelium using the superoxide radical to generate the free radical chain reaction initiating lipid peroxidation, causing loss of cellular structure and function as observed in the histopathology of the heart and thoracic aorta. (26) Several authors have established the combination of proinflammatory state, insulin resistance and obesity with the progression of endothelial dysfunction, (27-29) which is a common factor for the development of MS and CVD.

The results of this study demonstrate that insulin resistance evidenced by hyperinsulinemia, modified HOMA, decreased HDL levels and hypertriglyceridemia are associated with the proinflammatory properties and endothelial dysfunction demonstrated in the experimental model of MS.

CONCLUSION

Increased MPO and SOD enzymatic activity in the group with induced MS, together with the anatomopathological findings would demonstrate increased OS and would justify the usefulness of these markers for the evaluation of cardiovascular risk in the early stages of MS.

Conflicts of interest

None declared. (See authors' conflicts of interest forms in the website/Supplementary material).

REFERENCES

1. Scribano MP, Baez MC, Becerra F, Tarán MD, Signorini F, Balceda AG, et al. Effects of atorvastatin on oxidative stress biomarkers and mitochondrial morphofunctionality in hyperfibrinogenemia-induced atherogenesis. Adv Med 2014, Article ID 947258.

2. Pastori D, Carnevale R, Pignatelli P. Is there a clinical role for oxidative stress biomarkers in atherosclerotic diseases? Intern Emerg Med 2014;9:123-31. http://doi.org/bv7c

3. Dąbrowski P, Majdan M. Insulin resistance and metabolic syndrome- a different image of disorders in rheumatoid arthritis and ankylosing spondylitis. Wiad Lek 2015;68:235-41.
4. Karbach S, Wenzel P, Waisman A, Münzel T, Daiber A. eNOS uncoupling in cardiovascular diseases- the role of oxidative stress and inflammation. Curr Pharm Des 2014;20:3579-94. http://doi.org/bv7d
5. Yubero-Serrano EM, Delgado-Lista J, Peña-Orihuela P, Perez-Martinez P, Fuentes F, Marin C, et al. Oxidative stress is associated with the number of components of metabolic syndrome: LIPGENE study. Exp Mol Med 2013;45:e28. http://doi.org/bv7f
6. Da Fonseca LJ, Nunes-Souza V, Guedes G da S, Schettino-Silva G, Mota-Gomes MA, Rabelo LA. Oxidative status imbalance in patients with metabolic syndrome: role of the myeloperoxidase/hydrogen peroxide axis. Oxid Med Cell Longev 2014;2014:898501. http://doi.org/bv7g
7. Sokolov AV, Kostevich VA, Runova OL, Gorudko IV, Vasilyev VB, Cherenkevich SN, et al. Proatherogenic modification of LDL by surface-bound myeloperoxidase. Sokolov AV, Chem Phys Lipids 2014;180:72-80. http://doi.org/bv7h
8. Yixin T, Qian X, Haiyang P, Zhaoya L, Tianlun Y, Zaixin Y, et al. The role of vascular peroxidase 1 in ox-LDL-induced vascular smooth muscle cell calcification. Atherosclerosis 2015;243:357-63. http://doi.org/bv7j
9. Zhang X, Dong L, Wang Q, Xie X. The relationship between fasting plasma glucose and MPO in patients with acute coronary syndrome. BMC Cardiovasc Disord 2015;25:15:93.
10. Shi P, Goodson JM, Hartman ML, Hasturk H, Yaskell T, Vargas J, et al. Continuous metabolic syndrome scores for children using salivary biomarkers. PLoS One 2015;10:e0138979. http://doi.org/bv7k
11. Renna N, Vázquez M, González S, Lama C, Cruzado M, Miatello R. Expresión vascular de factores de transcripción proinflamatorios en un modelo de síndrome metabólico. Rev Argent Cardiol 2007;75:36-41.
12. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, et al. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. Circulation 2002;106:2067-72. http://doi.org/fdwjv2
13. National Diabetes Data Group: Classification and diagnosis of diabetes and other categories of glucose intolerance. Diabetes 1979;28:1039-57. http://doi.org/bscx
14. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-9. http://doi.org/dbht47
15. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA 2001;285:2486-97. http://doi.org/d39ksv
16. Bergmeyer HU. Methods of enzymatic analysis. Vch Pub; 3 Sub edition, 1983.
17. Woolliams JA, Wiener G, Anderson PH, McMurray CH. Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. Res Vet Sci 1983;34:253-6.
18. Ajiboye TO, Raji HO, Adeleye AO, Adigun NS, Giwa OB, Ojewuyi OB, et al. Hibiscus sabdariff calyx palliates insulin resistance, hyperglycemia, dyslipidemia and oxidative rout in fructose-induced metabolic syndrome rats. J Sci Food Agric 2016;96:1522-31. http://doi.org/bv7m
19. Rovira-Llopis S, Rocha M, Falcon R, de Pablo C, Alvarez A, Jover A, et al. Is myeloperoxidase a key component in the ROS-induced vascular damage related to nephropathy in type 2 diabetes? Antioxid Redox Signal 2013;19:1452-8. http://doi.org/bv7n
20. Victor M, Rovira-Llopis S, Bañuls C, Diaz-Morales N, Martinez de Marañon A, Rios-Navarro C, et al. Insulin resistance in pcos patients enhances oxidative stress and leukocyte adhesion: Role of myeloperoxidase. PLoS One 2016;11:e0151960. http://doi.org/bv7p
21. Prieto D, Contreras C, Sánchez A. Endothelial dysfunction, obesity and insulin resistance. Curr Vasc Pharmacol 2014;12:412-26. http://doi.org/bv7q
22. Feoli AM, Macagnan FE, Piovesan CH, Bodanese LC, Siqueira IR. Xanthine oxidase activity is associated with risk factors for cardiovascular disease and inflammatory and oxidative status markers in metabolic syndrome: effects of a single exercise session. Oxid Med Cell Longev 2014;2014:587083. http://doi.org/bv7r
23. Vavrova L, Kodydkova J, Zeman M, Dusejovska M, Macasek J, Stankova B, et al. Altered activities of antioxidant enzymes in patients with metabolic syndrome. Obes Facts 2013;6:39-47. http://doi.org/bv7s
24. El Assar M, Ruiz de Adana JC, Angulo J, Pindado Martínez ML, Hernández MA, Rodríguez-Mañas L. Preserved endothelial function in human obesity in the absence of insulin resistance. J Transl Med 2013;11:263. http://doi.org/bv7t
25. Suman RK, Mohanty IR, Borde MK, Maheshwari U, Deshmukh YA. Development of an experimental model of diabetes co-existing with metabolic syndrome in rats. Advances in Pharmacological Sciences Article ID 9463476.
26. Bernabé García J, Zafrilla Rentero P, Mulero Cánovas J, Gómez Jara P, Leal Hernández M, Abellán Alemán J. Biochemical and nutritional markers and antioxidant activity in metabolic syndrome. Endocrinol Nutr 2014;61:302-8. http://doi.org/f2p5rz
27. Mahendra JV, Kumar SD, Anuradha TS, Prashanth T, Nagaraj RS, Vishali V. Plasma fibrinogen in type 2 diabetic patients with metabolic syndrome and its relation with ischemic heart disease (IHD) and retinopathy. J Clin Diagn Res 2015;9:BC18-BC21.
28. Sung KC, Ryu S, Lee JY, Lee SH, Cheong ES, BChir Wild SH, et al. Fatty liver, insulin resistance, and obesity: relationships with increase in coronary artery calcium over time. Clin Cardiol 2016;39:321-8. http://doi.org/bv7v
29. Bhatia LS, Curzen NP, Calder PC. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? Eur Heart J 2012;33:1190-200. http://doi.org/bv7w