Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

Biochemistry Letters

Journal home page:

Anti-inflammatory response of Metformin and/or low doses of γ-Irradiation on Acute Pancreatitis induced by L-arginine in Rats

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ARTICLE INFO

Received : 19/6/2025
Accepted : 20/7/2025
Accepted to Online publish: 20/7/2025

Keywords:
Acute pancreatitis,
Metformin,
Gamma Irradiation,
Cytokines.

ABSTRACT

Background: Pancreatitis is an inflammatory disorder of the pancreas. **Aim:** The purpose of the current study was to assess the anti-inflammatory properties of metformin (Met) and/or in combined with low doses of γ-irradiation (IR) against acute pancreatitis (AP) caused by L-arginine in an animal model. **Methods:** From among thirty adult Wister rats, five groups (n=6) were randomly selected. Normal control; AP rats; AP rats exposed to IR; AP rats treated with Met, and AP rats treated with Met and exposed to IR. One week following the end of experimental treatments, microscopic examination on pancreatic tissue and biochemical parameters were measured. **Results:** AP was evidenced by elevating the serum pancreatic enzymes (amylase & lipase) levels. a significant increase in pro-inflammatory markers (IL-6, IL-8, TNF-α, RBS, and CRP), and a significant decrease in anti-inflammatory marker (IL-10), insulin levels, and Insulin growth factor 1 (IGF1) were detected. Microscopic examination revealed that L-arg. treated animals showed a cluster of immune cells, lymphoid aggregates, hemorrhage, necrotic acini and detachment of lobules. All the serological parameters and the histopathological observations were markedly improved by Met and/or low doses of γ-irradiation treatment either alone or combined. **Conclusion:** Met and/or low doses of IR could have a therapeutic effect on the acute pancreatitis model induced in rats.

Introduction:

Acute pancreatitis (AP) is an inflammatory condition of the pancreas and one of the most prevalent and serious gastrointestinal diseases. It frequently results in multiple organ dysfunction syndrome or systemic inflammatory response syndrome, which has a high death rate (~39%) [1]. Metformin, the biguanide antidiabetic, reduces hepatic glucose production and insulin resistance and is recommended as the primary oral

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treatment for type 2 diabetes. [2]. Besides its anti-diabetic benefits, metformin has notable characteristics, including antioxidant, anticancer, anti-inflammatory, and anti-fibrotic effects, making it a compelling candidate for adjunctive usage in oncology [3]. Metformin has been in clinical use for more than 50 years and has a good safety record with limited toxicity. it has been reported that metformin has no effect on glucose levels in nondiabetic individuals and this represents the idea of using metformin as adjuvant therapy [4]. Lower cancer incidence and cancer-specific deaths have been reported among diabetics on metformin compared to diabetics on other anti-diabetic medications [5].

Pre-treatment with non-lethal LDR has been shown to have a protective effect against oxidative injury in animal tissues. At low doses, radiation is generally regarded as safe and its effect, if any, is considered to be negligible. Induction of hormesis and adaptive response by low-dose radiation has been extensively indicated [6]. LDR was reported to stimulate some biological activities in vitro as well as in vivo, including DNA repair, increase in cellular antioxidant capacity, prolongation of life span and activation of immune functions by induction of immune responses and apoptosis in certain cancer cell types. In addition, LDR has been shown to enhance the efficacy of chemotherapy and immunotherapy [7]. The aim of this study was to investigate the possible anti-inflammatory role of Met and/or low doses of γ- irradiation against acute pancreatitis in Rats model.

Material and Methods:

1. Materials:

1.1 Drugs and chemicals:

Metformin; L-Arginine; were purchased from Sigma Chemicals Co., U.S.A.

1.2 Irradiation Process:

Gamma irradiations were carried out in Gamma cell units at the National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority. Rats were exposed to whole-body gamma radiation (0.25 Gy twice weekly for two weeks using Canadian Cs-137 γ-cell-40, at a dose rate of 0.423 Gy/min which was calculated according to the dosimetry department guidelines at the NCRRT at the time of the experiment.

1.3 Experimental animals:

Male Wistar rats weighing (100 ± 20) g, purchased from the Egyptian National Authority for Drug Research and Control, Ministry of Health, Cairo, Egypt, were used in this study. Under standard laboratory conditions of 12-h light/dark cycle, appropriate temperature, good ventilation and humidity level, the animals were housed in specially built plastic cages, six per each. The animals received a pellet-concentrated diet containing all the nutrients and tap water required. Throughout the study, drinking water and food were supplied ad libitum. Handling, treatments, and scarification of rats were performed as per the guidelines of the ethics by Public Health Guide for Care and Use of Laboratory Animals [8], and in accordance with the recommendation for the proper care and use of laboratory animals approved by animal care committee of the NCRRT, EAEA, Cairo, Egypt.

1.4 Animal classification (induction of acute pancreatitis and drug treatment):

The Wistar rats were equally classified into five groups of six rats each, as follows:

1. Control group [G1]: rats in this group were kept as controls.

2. Acute pancreatitis (AP) group [G2]: rats were injected intraperitoneally (i.p.) with L-arginine (250 mg/100g b.wt., twice at 1-hour intervals) day over day for 14 days to induce acute pancreatitis [9].

3. Gamma irradiated (IR) group [G3]: rats were injected with L-arginine like group (2) and after that, they were exposed to whole-body γ-radiation at a dose level of 0.25 Gy twice weekly for 2 weeks [10].

4. Metformin-treated group [G4]: rats were injected with L-arginine like group (2) and after that, they were treated orally with Metformin at a dose of 150 mg/kg body weight, daily for 14 days [11].

5. Metformin/IR treated group [G5]: rats were injected with L-arginine like group (2) and after that, they were exposed to whole-body γ-radiation like group (3) and were treated with Metformin similar to group (4).

After one week of all treatments, all rats were IP injected with urethane (1.3-1.5 g/kg in a ~1.5 g/5 ml solution) for euthanasia before being sacrificed [12], and then the blood was collected via cardiac puncture by using disposable plastic syringes. The coagulated blood samples were centrifuged at 3000 rpm for 15 minutes, and the serum was collected for different estimates of biochemical parameters. Portions of pancreatic tissue were rinsed and set for histopathological examination in 10% neutralized formalin.

2. Methods:

2.1 Measurement of biochemical parameters:

In serum, using a diagnostic kit purchased from TRUE chemie Company, India., Activities of amylase and lipase expressed as U/I were evaluated. Serum glucose was measured by the kinetic method of **Kaplan** [13] using a commercial kit obtained from (SPINREACT Company, Spain). **IL-6** was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) using a pre-coated rat kit (R&D Systems, Inc., Minneapolis, USA, Catalog No. CSB-E04640r). While, **TNF-α, IL-8, and IGF1** levels were determined by the ELISA pre-coated rat kit (CUSABIO, USA, Catalog No. CSB-E11987r, CSB-E07273r, CSB-E04582r respectively). On the other hand, **IL-10** and **CRP** levels were determined by the ELISA pre-coated rat kit (My BioSource, USA, Catalog No. MBS355232 and MBS2508830 respectively). Serum **Insulin** was determined by the ELISA pre-coated rat kit (CELL BIOLABS, INC. Company, USA, Catalog No. MET-5063). According to the manufacturers' instructions, the ELISA microplate was read using an ELISA reader with an absorbance maximum at 450 nm wavelength. The parameters levels were calculated after plotting the standard curves and expressed as pg/mL.

2.2 Histopathological examination:

Samples of pancreas tissue were fixed in 10% formaldehyde solution and inserted in paraffin using standard methods. Sectioned tissues at 5 μm thickness were treated with hematoxylin-eosin (H&E) stain for routine examination using light microscopy according to the method of **Bancroft and Stevens** [14].

2.3 Statistical analyses:

Data were analysed using one ways analysis of variance (ANOVA) for testing the significance between various treated groups followed by the Least Significant Difference (LSD) test for multiple comparisons. Statistical significance was set at $p < 0.05$ or $p < 0.01$, and with high significance considered at $p < 0.001$. Results were expressed as mean ± standard error of the mean (SEM) and analysed using SPSS software (version 20, SPSS Inc., Chicago, IL, USA).

Results:

1. Effect of metformin and low doses γ radiation on AP in rat's model.

1.1 Histological findings

In the pancreatic sections of the normal control group stained with H&E, the typical histological architecture was

observed. The islets of Langerhans appeared as pale-staining, scattered spherical clusters of polygonal cells containing fine secretory granules (endocrine portion) amidst the darker-staining, well-organized, densely packed pyramidal-shaped acinar cells (exocrine portion), all interspersed with connective tissue and blood vessels **(Figure 1, A&B).** In contrast, the pancreatic tissues from rats treated with L-arginine showed marked histopathological changes one week after treatment, including clusters of immune cells, lymphoid aggregates, hemorrhage, and necrotic acinar cells, in addition to detachment of lobules **(Figure 1, C & D)**.

Furthermore, pancreatic sections from rats with AP exposed to low doses of γ-radiation exhibited near-normal histological architecture, including the preservation of islets of Langerhans and dark-staining acini, following one-week post treatment **(Figure 1, E)**. On the other hand, pancreatic sections from rats with AP treated with Metformin, revealed a marked improvement in the tissue architecture including normal appearance islets of Langerhans, normal acini, as well as well-defined intralobular and interlobular ducts, except for some cluster of immune cells (lymphoid aggregate) and some hemorrhages were observed **(Figure 1, F).**

Meanwhile, pancreatic sections from rats with AP treated with Metformin and low-dose γ-radiation, recorded normal pancreatic histology, with clear islets of Langerhans, well-preserved acini, and intact intralobular and interlobular ducts, except for some clusters of immune cells (lymphoid aggregates) were observed **(Figure 1, J).**

1.2 Serological findings:

1.2.1 Pancreatic enzymes activities

Amylasemia and lipasemia, the high amylase and lipase activity, are among the key clinical criteria for the diagnosis of acute pancreatitis. In this study the activities of serum amylase and lipase in the L-arginine treated group were elevated when compared to control at the end of the experiment, suggesting a successful induction of AP. However, treating the animals suffering from acute pancreatitis with metformin (Met), exposure to γ-radiation, or their combination, produced substantial suppression of amylase and lipase activity **(Table 1).**

1.2.2 Glycaemic parameters

One of the complications of AP is endocrine dysfunction, particularly impaired glucose metabolism. Moreover, blood glucose levels are closely correlated with the inflammatory responses in AP, influencing disease progression. The data of the current study represented in **table (2)** revealed that the induction of AP in rats was associated with a significant elevation in the glucose levels accompanied with low levels of insulin, when compared with the control group. On the other hand, treatment with fractionated low doses of γ-irradiation (γ-IR), metformin (Met), or their combination effectively restored glucose and insulin levels toward normal. These findings suggest that hyperglycemia may serve as a useful indicator for assessing the severity of AP.

1.2.3 Inflammatory markers

Pancreatitis is identified by the destruction of acinar cells besides the activation of inflammatory cells including macrophages and neutrophils. thus, a significant change in the levels of many inflammatory mediators was observed. Systemic manifestations of the AP are mediated by various pro- and anti-inflammatory mediators released from the injured pancreas. Local recruitment and activation of inflammatory cells in AP lead to the production of inflammatory markers, such as interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), and C-reactive

protein (CRP), which are important in predicting the severity of the disease.

In **table (3)** the obtained data showed a high significant increase ($P \leq 0.001$) in the mean values of serum pro-inflammatory markers (IL-6, IL-8, TNF-α, and CRP) in AP group relative to their corresponding levels in the negative control group. In contrast, treatment of AP-induced rats with low doses of γ-radiation, metformin (Met), or their combination for two weeks resulted in a significant reduction in these pro-inflammatory markers relative to the untreated AP group. As shown in **table (4)**, the serum levels of anti-inflammatory cytokine IL-10 and insulin-like growth factor 1 (IGF-1) were markedly reduced in the AP group compared to the control group ($P < 0.001$), While, exposure of Ap rats to γ-radiation, treatment with Met, or their combination significantly enhanced and increased IL-10 and IGF-1 levels compared to the AP group.

Discussion:

Acute pancreatitis (AP) is a common severe critical illness associated with a high death rate that may resulting from systemic inflammatory response syndrome or multiple organ dysfunction syndrome. Sometimes repeated attacks of AP lead chronically to loss of pancreatic function and fibrosis [15, 16]. Hallmarks of AP include inflammation, apoptosis of acinar cells, and elevated levels of digestive enzymes such as amylase and lipase [17].

Metformin is the most widely prescribed oral anti-diabetic medicine in the world. It has been clinically used for over 50 years and has a strong safety record with low toxicity. Epidemiological studies have shown that Metformin-treated diabetics had a lower cancer incidence and cancer-specific death rate than diabetics taking other anti-diabetic drugs [18]. Based on these findings, the present study was designed to evaluate the anti-inflammatory and protective effect of the Met on L-arginine-induced acute pancreatitis and its complications on body tissues in Wistar Wister rats.

Handling with L-arginine was characterized by significant elevations of pancreatic enzymes activities both lipase and amylase levels as compared to negative controls. This observation is consistent with numerous previous studies that have employed L-arginine as a model to induce acute pancreatitis. **Salem, Lokman** [19] reported that the elevated levels of the pancreatic enzymes, mainly amylase and lipase, likely due to the production of hydrolytic enzymes that hydrolyse phospholipids to liberate arachidonic acid and lysophospholipids and the latter has a cytotoxic function, causing acinar cells necrosis. Furthermore, **Wang, Zhang** [20] reported that L-arginine selectively destroys pancreatic acinar cells by inducing amino acid imbalance, decreasing the synthesis of polyamine, nucleic acid and proteinase and resulting in excessive activation of the zymogen. **Yang, Tang** [21] indicated that pancreatic lipase leakage initiates adipose tissue lipolysis and elevates unsaturated fatty acid levels, further stimulating the release of inflammatory mediators that can drive disease progression with eventual multi-organ failure.

In the present study, L-arginine injection effectively induced acute pancreatitis in rats, markedly through histological changes, and higher pathological scores in the pancreas. Pancreatitis was further evidenced by hyperamylasemia and hyperlipasemia, which is consistent with the earlier results of **Kononczuk, Lukaszuk** [22].

On the other hand, our results revealed a remarkable increase in the levels of IL-6, IL-8, TNF-α and CRP accompanied with a significant reduction in the levels of IL-10 in AP group. These results were similar to that of **Al-Hashem** [23] who found that toxic doses of L-

arginine induced pancreatic tissue injury and increased the pro-inflammatory mediators such as TNF-α coupled with a reduction in the anti-inflammatory cytokine IL-10.

Acute pancreatitis is an inflammatory disease of the pancreas with the involvement of both local tissues and distant organs [24]. **Rehman, Rashid** [25] indicated that the prevalence of AP may lead to a systemic illness that may progress to multiple organ dysfunction and even death. Inflammatory cytokines (IL-6, IL-8) and TNF-α generated during the pathogenesis of AP are considered responsible for the development of multiple organ failure.

It was reported that high-level glucose can be used as one of the reference indicators for evaluating the severity of AP in clinical practice [26]. The obtained results revealed a remarkable increase in the levels of glucose coupled with a significant decrease in the insulin levels in the group of acute pancreatitis induced by L-arginine compared to the control group. this is in accordance with **Shoman and Nafeh** [27] who reported that AP affects not only exocrine pancreatic function, manifested by significantly higher serum amylase and lipase levels, but also affects pancreatic endocrine function as manifested by decreased fasting plasma insulin (FPI) levels in association with hyperglycemia.

Metformin acts not only as a glucose-lowering drug but exhibits additional benefits, including moderate anti-inflammatory and anti-oxidative effects [28]. **Sena, Matafome** [29] reported that the anti-inflammatory actions of metformin were by suppressing the main components of inflammation (endothelial cells and smooth muscle cells, monocytes, macrophages, and other cell types) and restoring cell functions. Furthermore, metformin has been shown to reduce levels of common pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in a mouse model of olanzapine-induced insulin resistance [30, 31].

It has been suggested that metformin improves metabolic parameters such as hyperglycemia, and insulin resistance thereby reducing chronic inflammatory responses [32]. Metformin reduces blood glucose levels primarily by decreasing hepatic glucose production through suppression of gluconeogenesis, ameliorating insulin signaling leading to reduction the intestinal glucose absorption, and improving glucose uptake by peripheral tissues, such as skeletal muscle and adipose tissue [33, 34]. Interestingly, it was reported that metformin could have direct protective effects on β-cells under metabolic stress, including non-diabetic and T2D human islet cells via alleviating the oxidative stress and endoplasmic reticulum stress which are responsible for pancreatic β-cells destruction [35].

While several mechanisms have been proposed to explain the effects of γ-irradiation, the mechanisms underlying the biological effects of γ-irradiation remain largely speculative due to limited direct empirical evidence, variability in biological responses across different systems, and challenges in isolating γ-irradiation-specific effects from other environmental or experimental factors [36]. Traditionally, targeted DNA damage induced by radiation has been considered the primary cause of its biological effects, with the prevailing view that any amount of radiation is potentially harmful to the organism. However, despite multiple proposed mechanisms, a comprehensive understanding of γ-irradiation's effects is still lacking [37]. Despite these risks, low-dose radiation therapy (LDRT) has been investigated for certain inflammatory conditions (like arthritis or degenerative joint disease), showing some efficacy. However, it's typically reserved for cases

where: Other therapies have failed, the potential benefits outweigh the risks or it's applied in highly controlled settings with careful dose planning [38].

It was reported that a low dose of IR is essential to life, acknowledging that the natural production of ROS that is adequate to stimulate the protective systems and provoke a beneficial health effect which is known as radiation hormesis [39]. In the present study, low-dose radiation (LDR) was associated with decreased circulating levels of inflammatory markers, such as C-reactive protein (CRP), alongside increased levels of the anti-inflammatory cytokine IL-10. Interestingly, LDR at doses of 0.5–1.5 Gy modulates the activity of cells involved in the inflammatory response- including endothelial cells, polymorphonuclear leukocytes, and macrophages- eliciting anti-inflammatory effects and promoting IL-10 production by both endothelial and immune cells. Presumably, it is in this phase could be effective by acting as a powerful anti-inflammatory agent against the cascade of proinflammatory cytokines [40].

Conclusions:

In conclusion, metformin attenuates the severity of acute pancreatitis by modulating inflammatory responses. Therefore, metformin may represent a promising therapeutic agent for the treatment of acute pancreatitis. However, further studies are warranted to elucidate the precise underlying mechanisms.

Declaration of competing interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Funding and Author Contribution:

Funding*:* The authors declare that they did not receive any financial support from any organization for the research, authorship, and/or publication of this article.

Author Contribution:

- ***Mohamed Mosleh:*** Validation, Resources, Methodology, Writing Original Draft, Formal Analysis, Writing Review & Editing;
- ***Neamat H. Ahmed:*** Conceptualization, Methodology, Investigation, Writing Review & Editing;
- ***Faten Zahran:*** Conceptualization, Investigation, Writing Review & Editing.

Ethics approval:

The present study was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, Zagazig University, Egypt (Approval No. ZU-IACUC/1/F/28/2019).

Availability of data and materials:

All data obtained from this study are included in the current manuscript.

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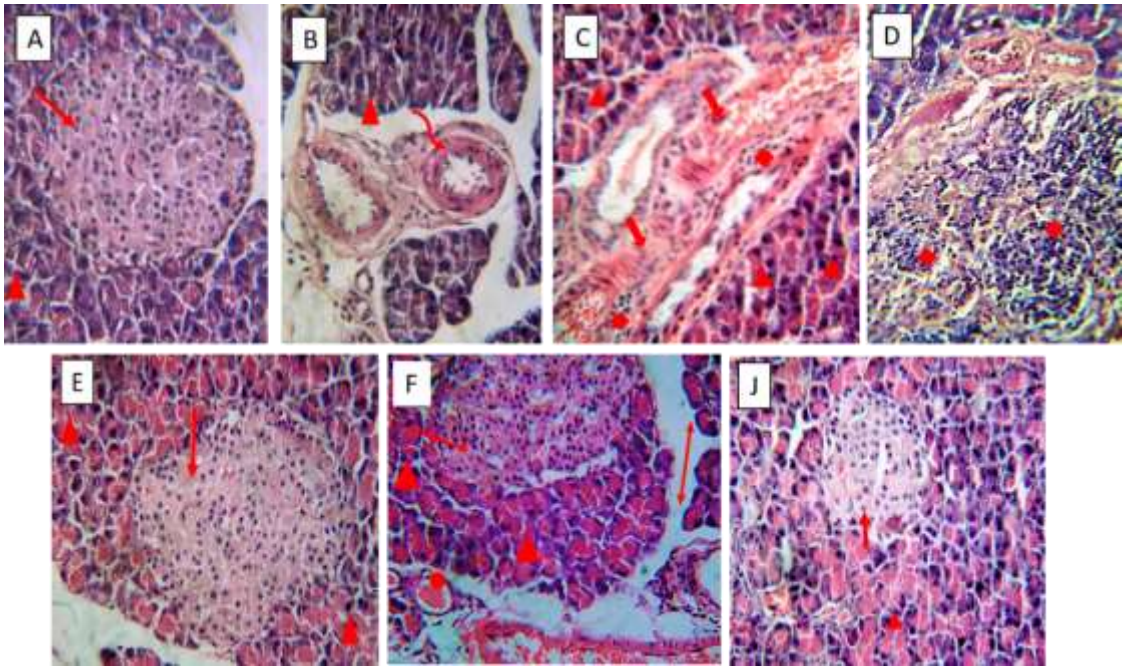

Figure (1): ***Photomicrographs of sections in pancreas of Wister rats. [A, B]: pancreas of control Wister rat*** *showing normal appearance of tissue structure (islets of Langerhans* (↓)*, acini* (▲) *and pancreatic arteries ( ).* ***[C, D]: section in the pancreas of Wister rat treated with ARG (pancreatitis model)*** shows a cluster of lymphoid cells aggregate (♦), Hemorrhage (Bold arrow) and necrotic acini (↓). ***[E]: section in pancreas of AP Wister rat exposed to low doses of γ- radiation*** showing normal islets of Langerhans (↓) and normal acini (▲). ***[F]: section in pancreas of AP Wister rat treated with Metformin,*** showing normal appearance islets of Langerhans (↓), normal acini (▲), intralobular duct (↕), interlobular duct (●), except for some cluster of immune cells and hemorrhage were observed. ***[J]: section in pancreas of AP Wister rat treated by Metformin and exposed to low doses of γ- radiation,*** recording normal appearance islets of Langerhans (↓), normal acini (▲). *(H & E stain x400).*

Table (1): Pancreatic enzymes activities (serum amylase and lipase) along all studied groups.

Parameters / Groups	Lipase Mean± SEM	Amylase Mean± SEM
Cont.	37.0 ± 1.3 b∗	2133 ± 16 b‡
AP	42.3 ± 0.6 a∗	3771 ± 104 a‡
AP + IR	45.1 ± 2.4 a‡	2454 ± 57 a† b‡
AP + Met	43.0 ± 2.0 a∗	2401 ± 66 a∗ b‡
AP + IR + Met	43.8 ± 1.9 a‡	2262 ± 99 b‡

Data are presented as means ± SEM. Superscript letters (a, b) indicate significant differences compared to the control and AP groups respectively. Symbols denote levels of statistical significance: ∗p<0.05, †p<0.01, and ‡p<0.001.

Table (2): Glycaemic parameters (RBS and Insulin) among studied groups.

Parameters / Groups	RBS Mean± SEM	Insulin Mean± SEM
Cont.	180.6 ± 6.9 b‡	1.4 ± 0.03 b‡
AP	229.2 ± 10.1 a‡	1.03 ± 0.08 a‡
AP + IR	197.5 ± 3.3 a∗ b‡	1.32 ± 0.02 b†
AP + Met	175.8 ± 3.0 b‡	1.60 ± 0.04 a∗ b‡
AP + IR + Met	184.1 ± 3.9 b‡	1.39 ± 0.08 b‡

Data are presented as means ± SEM. Superscript letters (a, b) indicate significant differences compared to the control and AP groups respectively. Symbols denote levels of statistical significance: ∗p<0.05, †p<0.01, and ‡p<0.001.

Table (3): Pro-inflammatory markers levels among studied groups.

Parameter / Groups	IL-6 (pg/mL) Mean± SEM	IL-8 (pg/mL) Mean± SEM	TNF-α (pg/mL) Mean± SEM	CRP (pg/mL) Mean± SEM
Cont.	78.4 ± 2.3 b‡	33.0 ± 1.2 b‡	13.7 ± 0.7 b‡	12.8 ± 0.5 b‡
AP	276.8 ± 5.1 a‡	142.3 ± 1.4 a‡	120.2 ± 2.0 a‡	96.1 ± 2.5 a‡
AP + IR	156.7 ± 1.9 a‡ b‡	75.3 ± 2.9 a‡ b‡	41.6 ± 2.2 a‡ b‡	43.4 ± 0.9 a‡ b‡
AP + Met	131.5 ± 2.0 a‡ b‡	68.7 ± 1.9 a‡ b‡	58.1 ± 1.9 a‡ b‡	38.8 ± 1.2 a‡ b‡
AP + IR + Met	141.4 ± 2.9 a‡ b‡	52.2 ± 2.5 a‡ b‡	57.2 ± 1.7 a‡ b‡	34.7 ± 1.6 a‡ b‡

Data are presented as means ± SEM. Superscript letters (a, b) indicate significant differences compared to the control and AP groups respectively. Symbols denote levels of statistical significance: ∗p<0.05, and ‡p<0.001.

Table (4): Anti-inflammatory markers levels among studied groups.

Parameter / Groups	IL-10 (pg/mL) Mean± SEM	IGF1(pg/mL) Mean± SEM
Cont.	183.0 ± 1.4 b‡	8.4 ± 0.18 b‡
AP	101.9 ± 1.3 a‡	3.4 ± 0.18 a‡
AP + IR	163.9 ± 1.4 a‡ b‡	7.9 ± 0.25 b‡
AP + Met	151.7 ± 2.3 a‡ b‡	6.0 ± 0.15 a‡ b‡
AP + IR + Met	175.1 ± 1.4 a∗ b‡	7.9 ± 0.08 b‡

Data are presented as means ± SEM. Superscript letters (a, b) indicate significant differences compared to the control and AP groups respectively. Symbols denote levels of statistical significance: ∗p<0.05, and ‡p<0.001.