

Innovative Dual Therapy: Magnesium Oxide Nanoparticles and Royal Jelly for Parotid Gland Protection in Diabetic Male Rats

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Purpose: Nanoparticles and natural products have gained attention for their beneficial properties, but limited studies exist on the co-administration of these two therapeutic agents. In this study, both magnesium oxide nanoparticles (MgO NPs) and royal jelly (RJ) were evaluated for their protective potential against parotid gland damage in induced diabetic rats through the investigation of their antidiabetic, antioxidative, and anti-inflammatory effects.

Material and Methods: 64 male rats were divided into 8 groups: control group, MgO NPs treated group (300mg/kg/day), RJ treated group (100mg/kg/day), streptozotocin-induced diabetic group, diabetic group and concomitant MgO NPs, diabetic group and concomitant RJ, diabetic group and co-administration of both MgO NPs and RJ, and diabetic group and concomitant metformin (100mg/kg/day). After 8 weeks, the rats of all groups were anaesthetized, and blood was drawn to estimate different diabetic parameters. Saliva was collected to determine the salivary parameters. The parotid glands were excised and cut into pieces, where half was frozen for further biochemical investigations (oxidative stress and inflammatory markers) and the other assessed for histological and immunohistochemical changes.

Results: The administration of either MgO NPs or RJ to diabetic rats significantly improved the diabetic parameters and nearly restored the salivary and oxidative stress parameters to control values. There was a significant improvement in the histological and immunohistochemical alterations in the parotid gland.

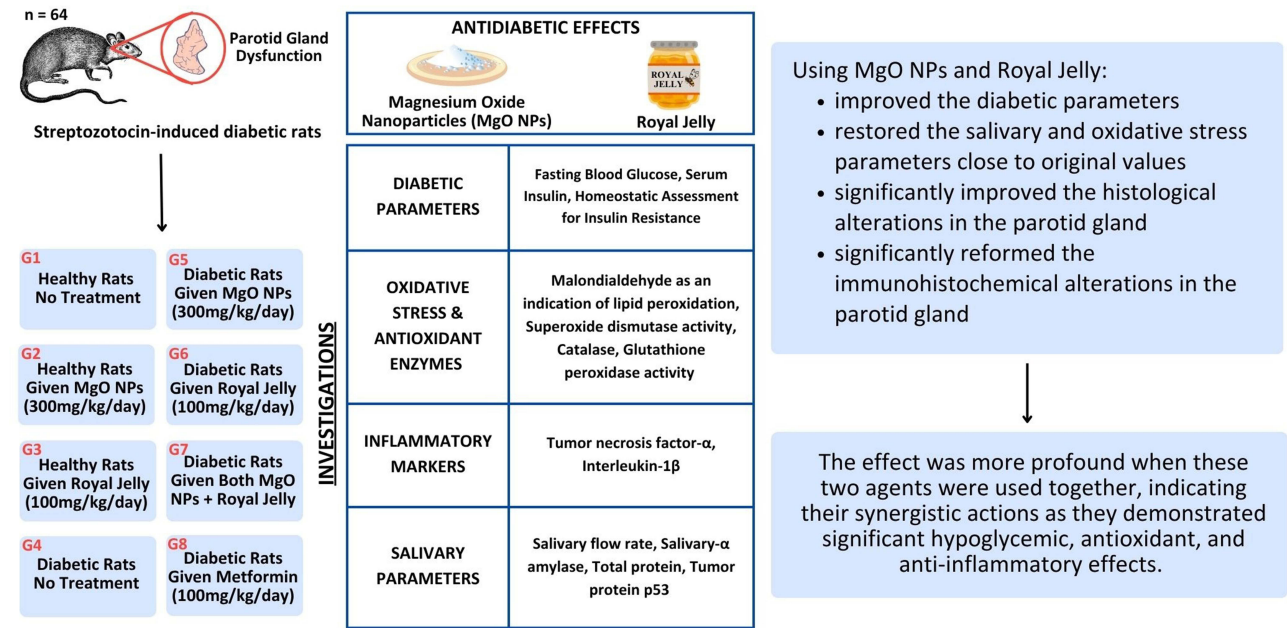
Conclusion: Both MgO NPs and RJ were effective in protecting the parotid gland from damage in the diabetic rats. These effects were even more profound when the two agents were administered together.

Keywords: magnesium oxide nanoparticles, royal jelly, diabetes mellitus, parotid gland, hyposalivation, oxidative stress

Introduction

Diabetes mellitus (DM) is characterized by chronic hyperglycemia, resulting from defects in insulin secretion, insulin action, or both. This persistent elevation in blood glucose leads to a range of metabolic disturbances in carbohydrate, lipid, and protein metabolism. Insulin-dependent tissues experience a reduction in glucose uptake, which, in turn, stimulates increased glucose production by the liver and the breakdown of glycogen. Additionally, lipid metabolism is disrupted, leading to higher levels of free fatty acids and triglycerides, thereby exacerbating insulin resistance and lipotoxicity. These metabolic disorders collectively lead to both acute and chronic complications, which can include vascular damage, oxidative stress, and inflammation.^{1,2}

Graphical Abstract



Many components of the body are affected by DM, and the oral cavity is no exception. The major oral manifestations can present as xerostomia (dry mouth), soft tissue inflammation, periodontal disease, dental caries, oral candidiasis, burning mouth, altered taste, heightened infection susceptibility, and impaired wound healing.³ The literature has recently demonstrated that DM modifies the tissue structure and mechanism of saliva secretion, thereby precipitating xerostomia.⁴ Many diabetic patients experience hypofunction of the salivary glands, which can worsen and result in hyposalivation.⁵

Parotid gland alteration is commonly observed in diabetic individuals. Approximately 80% of diabetic patients exhibit sialadenosis, a non-inflammatory and asymptomatic enlargement of the parotid glands. Ultrasonographic studies further support these findings, revealing increased glandular volume in diabetic individuals compared to healthy controls, with such changes closely associated with the duration of diabetes and the level of glycemic control.^{6,7}

Beyond the well-documented impact on the parotid glands, DM also significantly impairs the function and structure of the submandibular, sublingual, and minor salivary glands.⁸ Collectively, these alterations contribute to the pathogenesis of xerostomia and other oral health issues frequently observed in diabetic individuals.^{4,9,10}

The pathogenesis of DM-related salivary gland hypofunction occurs as a result of the persistent hyperglycemia generating oxidative and cellular damage, which in turn hinders the structure of the tissues.³ Chronic oxidative stress induces the excessive production of reactive oxygen species, thereby exacerbating the complications associated with DM.¹¹ Reports have mentioned that DM-associated neuropathy, microvascular abnormalities, endothelial dysfunction, and hindered microcirculation could lead to degenerative changes in salivary glands.¹²

Currently, the most commonly used drug for the treatment of DM is metformin, with other available treatments such as sulfonylureas, thiazolidinedione, α -glucosidase inhibitors, repaglinide, and insulin therapies. However, while these treatments can reverse hyperglycemia temporarily, they possess numerous side effects in the form of hyponatremia (abnormally low sodium in the blood), obstructive jaundice, nausea, headaches, vomiting, and weight gain. Long-term insulin administration from an external source can gradually diminish insulin formation and secretion in β -cells.¹³ The suggested medical treatments for diabetes-associated hyposalivation and xerostomia include artificial saliva spray, chemical reagents such as malic acid and locarpine, and systemic sialagogues. Nonetheless, these provide only a temporary alleviation of the salivary flow rate and can trigger adverse effects like excessive sweating, tremors,

nervousness, and diarrhea.⁵ Therefore, there is a pressing need to develop more effective and safe therapeutic approaches for the prevention of salivary gland damage that occurs as a complication of DM.¹⁴

In recent years, nanotechnology has played a crucial role in the diagnosis and treatment of DM and its complications.¹⁵ Nanoparticles possess several advantages over their larger-sized counterparts and can demonstrate superior physicochemical properties. The nano-delivery systems improve the solubility of poorly soluble drugs, enhance bioavailability, enhance the rate of absorption, and exhibit targeted delivery to a desired site of action.^{16,17} In this regard, magnesium oxide nanoparticles (MgO NPs) have great potential as therapeutic agents for managing diabetes and combating oxidative stress.^{18,19} Magnesium, a fundamental component in insulin receptor signaling, can influence the activity of insulin receptor tyrosine kinase and glucose transporters, thereby improving insulin sensitivity.^{20,21} Moreover, MgO NPs can inhibit the levels of insulin-inhibiting pro-inflammatory cytokines such as TNF- α and IL-6, and can efficiently scavenge free radicals, ultimately reducing cellular damage.²²

Additionally, implementing natural compounds and supplements into therapeutic agents has become progressively prevalent. Many natural agents are capable of combating oxidative stress and inflammation, which in turn aids in preventing DM or reducing DM-associated complications.²³ Recent research indicates that natural products offer various advantages. Besides enhancing insulin sensitivity, they also possess anti-inflammatory, antioxidant, and cholesterol-lowering properties.²⁴ Royal Jelly (RJ), also known as Queen Bee Jelly, is a secretion from honeybees that has been popularized for use in alternative medicine.²⁵ The composition of RJ includes a wide array of proteins, lipids, carbohydrates, polyphenols, vitamins, and hormones, with the most important bioactive compounds involving 10-hydroxy-2-decenoic acid, a crucial source of RJ's beneficial properties.^{26,27} RJ's pharmacological characteristics involve antioxidant,²⁵ anti-inflammatory,²⁸ anti-aging,²⁹ neuroprotective,³⁰ anticancer,³¹ and antidiabetic behavior.³²

The need for a novel and improved treatment for the parotid gland damage as a result of DM-associated complications propelled the necessity of the current study. This study was designed to evaluate the protective role of both MgO NPs and RJ against the parotid gland damage in experimentally induced diabetic rats through the investigation of their antidiabetic, antioxidative, and anti-inflammatory effects.

Materials and Methods

Chemicals

The following agents were procured from Sigma-Aldrich (St. Louis, USA): streptozotocin [CAS: 18883–66-4], metformin [CAS: 1115–70-4], magnesium oxide nanoparticles (MgO NPs) [< 50 nm, 99.9%, CAS: 1309–48-4], ELISA kits for tumor necrosis factor- α (RAB0480), and interleukin-1 β (RAB0311). Assay kits for malondialdehyde (MDA - ab118970), superoxide dismutase (SOD – ab65354), catalase (CAT - ab83464), and glutathione peroxidase (GPx - ab102530) were purchased from Abcam Inc. (Massachusetts, USA).

Preparation and Characterization of MgO NPs Solution

A fresh solution of MgO NPs (white powder form) was prepared every two days by dissolving 20mg of the MgO nanopowder in 100mL of deionized water. The solution was stirred overnight, and then ultra-sonicated using an ultrasonic bath (30.8 W; Elma Schmidbauer GmbH, Germany) for 30min at room temperature to create a homogeneous suspension. The characterization of the solution was performed in the Department of Pharmacology, Faculty of Pharmacy, King Abdulaziz University, Saudi Arabia, using the following techniques: X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and Zeta Potential analysis (ZP).

XRD

The crystalline nature of the inorganic nanoparticles was analyzed using a Shimadzu XRD-6000 X-ray diffractometer (Shimadzu Corporation, Kyoto, Japan) set with Cu K α radiation (0.15406nm at 15kV and 30mA for the X-ray tube). For every measurement, the phase type and content were analyzed at 5° min⁻¹. The XRD spectra were scanned in the 2 θ range of 10–80° with an accelerating voltage of 40kV.³³

FTIR

The functional groups and chemical residues on the surface of the inorganic nanoparticles were detected using FTIR (Perkin Elmer Spectrum 1000, Perkin Elmer, Waltham, Massachusetts, USA). It was used to obtain an infrared spectrum of absorption of a sample at a wave range of $600\text{--}4000\text{cm}^{-1}$. This measured how well a sample can absorb light at different wavelengths.

ZP

The stability of the inorganic nanoparticles was assessed by the zeta potential analyzer (Malvern Zetasizer Nano-ZS90, Malvern Panalytical, Worcestershire, United Kingdom). The investigation of ZP involves analyzing the electrostatic charge on the surface of nanoparticles in colloidal dispersion.³⁴

Animal and Experimental Design

64 adult male Sprague-Dawley rats (weighing 200–220 grams) were used in this study. They were kept in separate metallic cages with free access to a normal diet formed of Purina Rat Chow (Purina, St. Louis, MO, USA) and water ad libitum and were maintained under controlled conditions of a 12h light–dark cycle, room temperature of $22\text{--}25^{\circ}\text{C}$, and relative humidity of 40–50%. Care and management of the animals were conducted in compliance with the National Institutes of Health's 'Guide for the Care and Use of Laboratory Animals', which outlines standards for housing, environmental enrichment, pain management, and humane endpoints. Special attention was given to reducing stress and discomfort during the experimental period, in line with current best practices in laboratory animal science.^{35,36} Approval was also granted by the Institutional Animal Care and Use Committee of the Faculty of Medicine at King Abdulaziz University.³⁷ After acclimatization for one week, the rats were randomly divided into 8 equal groups, with 8 rats per group:

- Group 1 (control group): the rats received a normal diet and water throughout the experimental period
- Group 2 (MgO NPs group): the rats received MgO NPs at a dose of 300mg/kg by weight per day. This dose was given according to previous studies in rats.³⁸
- Group 3 (RJ group): the rats received RJ at a dose of 100mg/kg by weight per day. This dose was given according to a previous study in rats.³²
- Group 4 (diabetic group): the rats underwent streptozotocin-induced DM. They were left untreated throughout the experiment.
- Group 5 (diabetic and concomitant MgO NPs group): the rats underwent induced DM and received 300mg/kg by weight per day of MgO NPs.
- Group 6 (diabetic and concomitant RJ group): the rats underwent induced DM and received 100mg/kg by weight per day of RJ.
- Group 7 (diabetic and concomitant MgO NPs and RJ group): the rats underwent induced DM and received 300mg/kg by weight per day of MgO NPs and 100 mg/kg by weight per day of RJ.
- Group 8 (diabetic and metformin group): the rats underwent induced DM and received 500mg/kg by weight per day of metformin, which is employed as a standard antidiabetic agent and is commonly utilized to mitigate and treat DM.³⁹

All treatments administered to the diabetic rats started after the establishment of the diabetic state on the 3rd day of streptozotocin injection. The agents were administered through a gastric tube at 10am. After 8 weeks, the rats of different groups were anaesthetized by intraperitoneal injection with 30mg/kg of pentobarbital sodium, and their limbs were fixed. The blood samples were immediately withdrawn from the retro-orbital venous plexus by capillary tubes and centrifuged at 3000rpm (CLS6758 Corning® LSE™ Compact Centrifuges, Glendale, AR, USA) to separate the sera, which were then stored at -80°C . Afterwards, the saliva was collected according to the described method of Bagavant et al.⁴⁰

Prior to stimulation, the basal saliva of rats was collected for 3min with a micro-sampler. Then, the stimulated saliva was achieved by an intraperitoneal injection of pilocarpine nitrate (5mg/kg of body weight). After 5min of stimulation, saliva was collected using a micro-sampler. The collected saliva samples were centrifuged at 3000rpm for 5min, and the supernatant was stored at -80°C for assessment of salivary parameters. Moreover, a skin incision was done ventral to the

external ear, where the parotid glands were dissected carefully on both sides and excised, then cut transversely. Half of the cut pieces were fixed in 10% neutral buffer formalin for subsequent histological and immunohistochemical studies, while the remaining pieces were immediately wrapped in aluminum foil and frozen in liquid nitrogen to be stored at -80°C to evaluate biochemical parameters (oxidative and inflammatory markers).

Induction of DM

To induce DM, overnight fasted rats (12hr) received a single intraperitoneal injection of newly prepared streptozotocin at a dose of 50 mg/kg by weight, dissolved in 0.1M citrate buffer (pH 4.5).⁴¹ To avoid the animal mortality related to hypoglycemia and hyperinsulinemia induced by streptozotocin, 10% glucose was given in drinking water for 24hr post-injection. On the 3rd day after the streptozotocin injection, blood glucose levels were detected by obtaining blood samples through pricking the tail vein using a blood glucose testing kit. The level of blood glucose measuring 250mg/dL or more was diagnostic and confirmed the onset of the diabetic state. The rats were kept on a high-carbohydrate diet to keep them diabetic.

Assessment of the Diabetic Parameters

The fasting blood glucose (FBG) level was determined in rats of different groups using an Accu-Chek glucometer (Roche, Basel, Switzerland). The glycated hemoglobin (HbA1C) was determined using high-performance liquid chromatography and commercial kits (Recipe Chemicals – Instruments GmbH, Munich, Germany) according to the manufacturer's instructions. The serum insulin (SI) concentration was determined by enzyme-linked immunosorbent assay (ELISA) using an insulin detection kit (CBS-E05070R, CUSABIO, Boehringer Mannheim, Germany) according to the manufacturer's protocol. The insulin level in serum was expressed in $\mu\text{U/mL}$. The homeostatic assessment for insulin resistance (HOMA-IR) formula was used to estimate insulin resistance as follows (1):

$$\text{HOMA} - \text{IR} = \frac{\text{insulin} \times \text{glucose}}{405} \quad (1)$$

Where the insulin was expressed in ($\mu\text{U/mL}$) and the glucose in (mg/dl).

Measurement of Oxidative and Antioxidative Parameters

The frozen parotid gland pieces were defrosted before being mixed with 2mL of ice-cold Tris-HCl (pH 7.4) with 1% protease inhibitor and homogenized using a Teflon homogenizer (Heidolph Silent Crusher M Homogenizer, Heidolph, Schwabach, Germany) at 4000rpm. The buffer was then added to adjust the final volume to be 10-fold of the tissue weight. Using a spectrophotometer (Shimadzu UV-1700 Series, Shimadzu Corporation, Kyoto, Japan), the supernatant was utilized to determine the activities of the lipid peroxidation and antioxidative enzymes using specific kits and according to the manufacturer's instructions at specific absorbances. The level of malondialdehyde (MDA) as an indication of lipid peroxidation was expressed as nmol/gm tissue, while the antioxidative enzyme superoxide dismutase (SOD) activity was expressed as Unit/gm of tissue. Catalase (CAT) activity was expressed as Unit/min/gm of tissue. Glutathione peroxidase (GPx) activity was expressed as Unit/gm of tissue.⁴²

Examination of Salivary Parameters

Both salivary alpha amylase (sAA) and total protein were measured as done by Chen et al.⁴ The sAA concentration (U/mL) was determined using the kinetic reaction assay with a commercially available kit (142817005, Mindray, Shenzhen, China) on the BS-180 Automatic Biochemical Analyzer (Mindray, Shenzhen, China). The total protein concentration (mg/mL) was determined using the Bicinchoninic Acid Protein Assay Kit (P0011, Beyotime Biotech Inc, Haimen, China). All procedures were carried out exactly according to the reagent's manual and instructions.

Histopathological and Immunohistochemical Studies

The neutral buffer formalin-fixed parotid gland specimens were dehydrated in ascending degrees of alcohol, cleared in xylol, and infiltrated with molten paraffin wax to build up a block. Serial tissue sections with a thickness of 5 μm were

mounted on a glass slide to be stained with Hematoxylin and Eosin (H&E) for routine histological examination, and Masson's trichrome for the collagen fibers.

For immunohistochemical staining, the streptavidin–biotin–peroxidase technique was performed to the anti- α -smooth muscle actin (α -SMA) rat monoclonal antibody (Dako Cytomation, Heverlee, Belgium) with dilution 1:1000 for identification of myoepithelial cells. Anti-p53 monoclonal mouse antibody (Clone Do-7, M7001; Dako, California, USA) was assessed at a dilution of 1:50 for detection of tumor protein p53 (TP53).

Paraffin sections were mounted on poly-L-lysine-coated glass slides, dried, dewaxed, and rehydrated with water. Microwave heat-mediated antigen retrieval was performed at 110°C for 2min in 1mM EDTA buffer with pH 8.0. Then, the slides were treated with 0.3% hydrogen peroxide in methanol for 10min to reduce endogenous peroxidase. Afterwards, they were incubated in a moist chamber at 4°C for 12–14hr with the primary antibody. After 5min of rinsing, the sections were incubated for 1hr with anti-mouse immunoglobulin (secondary antibody) conjugated to a peroxidase-labeled dextran. The sections were dehydrated, left to dry, mounted, and then covered with glass covers. Finally, all the slides were examined, and representative photos were taken using an Olympus BX41 Optical Microscope equipped with an Olympus DP25 digital camera (Olympus Corporation, Tokyo, Japan).

Statistical Analysis

The acquired measurements of this study were expressed as means $\pm$ SD and were analyzed using the Statistical Package for Social Sciences program, Version 23 (SPSS Inc., Chicago, Illinois, USA). The significance of the differences between groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values were considered statistically significant at $p < 0.05$.

Results

Characterization of MgO NPs

XRD Analysis

The XRD spectra demonstrated 5 intense peaks of MgO NPs at 2θ values at 36.9°, 42.6°, 62.2°, 74.7°, and 78.8°, which corresponded to (111), (200), (220), (311), and (222), respectively. These peaks represent the reflection lines of the cubic inorganic nanoparticles. No significant characteristic peaks of any impurities were detected in the pattern, which confirmed the highly crystalline nature of the metal oxide nanoparticles [Figure 1A].

FTIR Analysis

The FTIR spectra of the nanoparticles exhibited a major absorption peak at 675cm⁻¹, confirming the presence of the MgO NPs vibrations. The broad absorption bands at 1660cm⁻¹ and 2760cm⁻¹ corresponded to the characteristic asymmetric stretching mode of the C=C and CH₂ groups, respectively [Figure 1B].

ZP Analysis

The ZP of the inorganic nanoparticles suspended in water showed a negative potential value around -12.5 mV, which indicates the physical stability of the inorganic nanoparticles in suspension [Figure 1C].

Diabetic Parameters

For the non-diabetic rats, the levels of FBG and SI were closely similar in the control (Group 1), MgO NPs (Group 2), and RJ (Group 3) treated groups with non-significant differences. In the diabetic group (Group 4), the level of FBG was significantly higher ($p=0.0009$) as compared to the control and treated groups [Figure 2A]. Moreover, treatment with either MgO NPs (Group 5) or RJ (Group 6) or metformin (Group 8) to the diabetic rats was found to significantly ($p=0.00016$) reduce FBG in comparison to the non-treated diabetic rats. The co-treatment with both agents to the diabetic rats (Group 7) resulted in normalization of FBG with a highly significant difference as compared to the diabetic group ($p=0.00012$) and was lower than that in the diabetic rats treated with metformin (Group 8).

Regarding the SI, the level was significantly lower ($p=0.0007$) in the diabetic rats (Group 4) as compared to the control rats (Group 1) [Figure 2B]. The treatment with either MgO NPs (Group 5), RJ (Group 6), or metformin (Group 8)

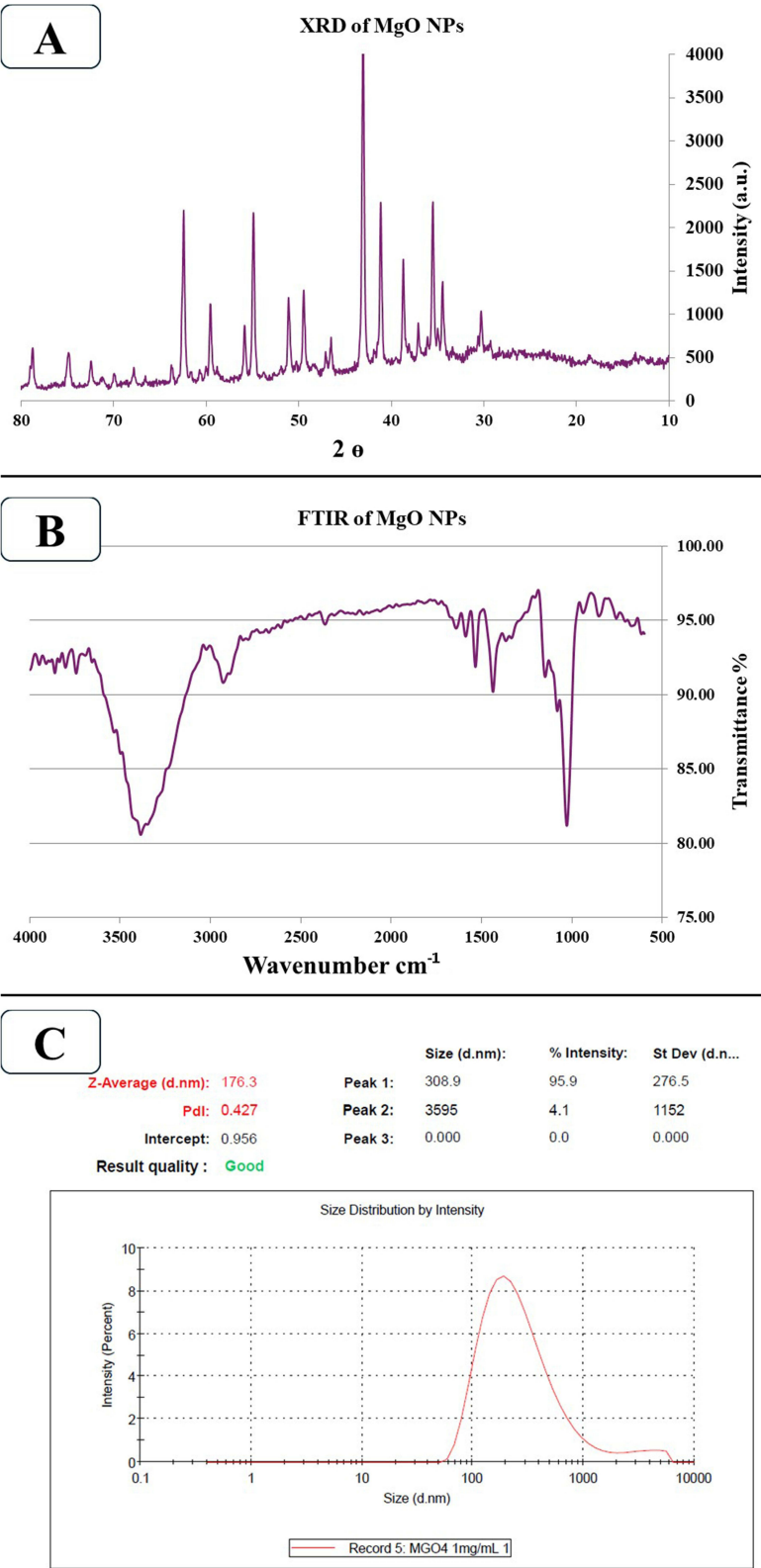


Figure 1 Graphs demonstrating the characterization of the MgO NPs through **(A)** X-ray diffraction; **(B)** Fourier transform infrared spectroscopy; **(C)** Zeta potential analysis.

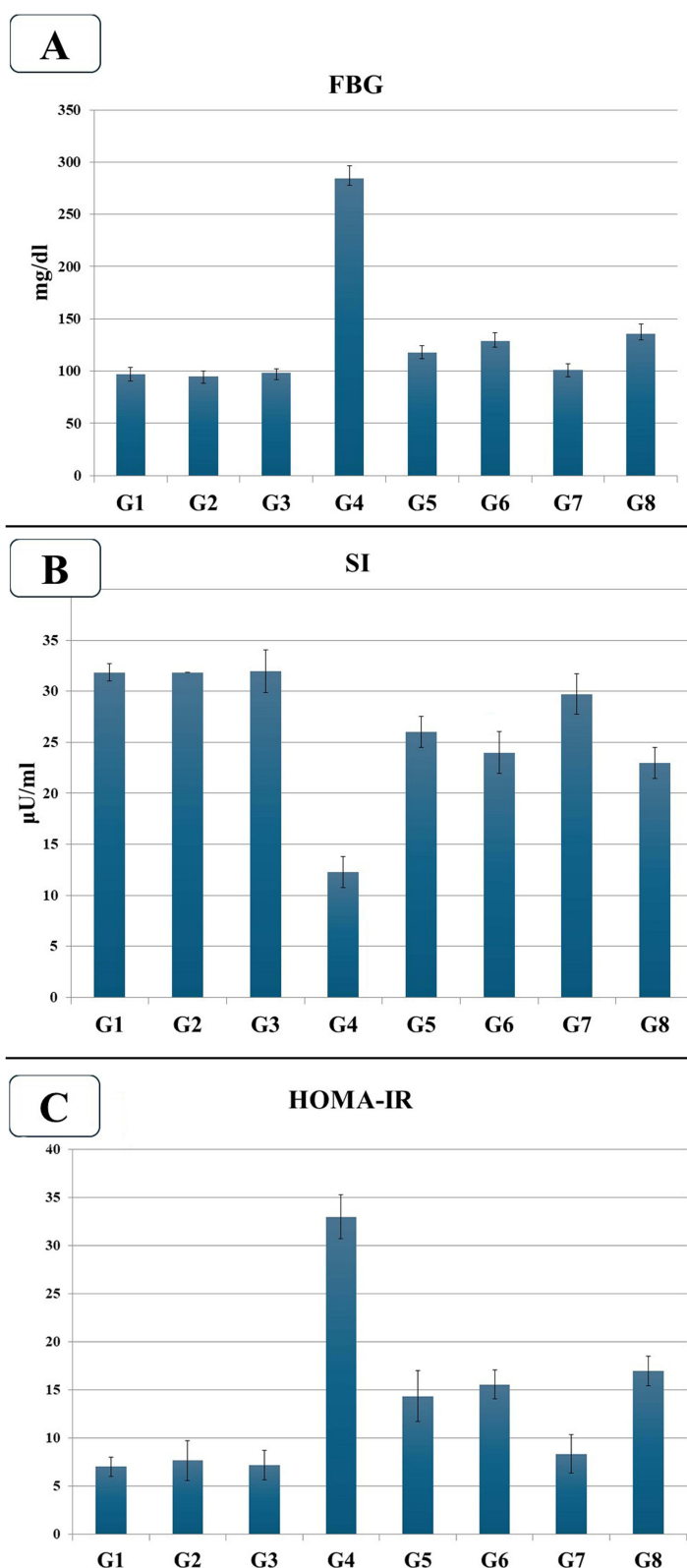


Figure 2 Charts of the statistical analysis of the diabetic parameters in different groups: **(A)** Fasting Blood Sugar; **(B)** Serum Insulin; **(C)** Homa-IR[G1=Control group, G2=MgO NPs group, G3=RJ group, G4=untreated diabetic group, G5=Diabetic group treated with MgO NPs, G6=Diabetic group treated with RJ, G7=Diabetic group treated with MgO NPs and RJ, G8=Diabetic group treated with metformin].

to the diabetic rats was found to increase the SI in comparison to the untreated diabetic rats ($p=0.03$). Furthermore, the co-treatment of both MgO NPs and RJ to the diabetic rats (Group 7) resulted in a significant elevation ($p=0.0008$) of SI, close to the SI of the control group (Group 1), and was higher than that of the diabetic rats treated with metformin (Group 8).

The effectiveness of both MgO NPs and RJ in the improvement of insulin sensitivity was further tested with the HOMA-IR index [Figure 2C], which presented a marked rise ($p=0.0007$) in the diabetic rats (Group 4) as compared to the control rats (Group 1). The treatment with either MgO NPs (Group 5), RJ (Group 6), or metformin (Group 8) to the diabetic rats was found to reduce the elevated HOMA-IR in comparison to the untreated diabetic rats ($p=0.03$), with no significant difference between the three groups. Additionally, the co-treatment with both MgO NPs and RJ (Group 7) to the diabetic rats resulted in a significant reduction ($p=0.0006$), which was lower than that of the diabetic rats treated with metformin (Group 8).

Oxidative Stress Markers

As seen in Figure 3, there were no statistically significant differences ($p=0.08$) in mean values of antioxidant enzymes SOD, GPx, CAT, and MDA level (marker of lipid peroxidation) in the parotid gland homogenate between the control group, MgO NPs, and RJ treated groups (Group 1, 2, and 3). In the untreated diabetic group (Group 4), there was a marked diminution in the activity of SOD, GPx, and CAT, as well as a marked increase in the MDA level, as compared to those of the control groups ($p=0.0002$). However, the treatment with either MgO NPs (Group 5) or RJ (Group 6) or metformin (Group 8) alone to the diabetic rats was found to increase the levels of SOD, GPx, CAT, and reduce the MDA level in comparison to the untreated diabetic rats ($p=0.01$) with no significant difference between the three groups. Furthermore, the co-treatment with both MgO NPs and RJ (Group 7) to the diabetic rats resulted in significant improvement ($p=0.0007$) of the parameters, with the resultant values close to those of the control groups.

Inflammatory Markers

As shown in Figure 4, there were no statistically significant differences in mean values of serum tissue TNF- α and IL-1 β levels in the control (Group 1), MgO NPs (Group 2), and RJ (Group 3) treated groups ($p>0.05$). In contrast, the levels of these markers were significantly elevated ($p=0.0001$) in the diabetic group (Group 4) compared to the control group. However, the treatment with either MgO NPs (Group 5), RJ (Group 6), or metformin (Group 8) to the diabetic rats was found to decrease the levels of both TNF- α and IL-1 β in comparison to the untreated diabetic rats ($p=0.02$), with no significant difference between the three groups. Moreover, the co-treatment with both MgO NPs and RJ (Group 7) to the diabetic rats resulted in a significant reduction ($p=0.0008$) of both markers, with the values close to the control values.

Salivary Parameters

To evaluate the function of the parotid gland, the salivary flow rate, sAA, and total protein content of the saliva were determined. There were no statistically significant differences in mean values of the three parameters between the control (Group 1), MgO NPs (Group 2), and RJ (Group 3) treated groups ($p>0.05$) [Figure 5]. When compared with the control group, the diabetic group (Group 4) had significantly lower sAA and total protein content as well as salivary flow rate when compared to the control group ($p=0.0005$). These parameters displayed a significant increase in the diabetic groups treated with either MgO NPs (Group 5), RJ (Group 6), or metformin (Group 8) ($p=0.009$). Moreover, the administration of both MgO NPs and RJ to the diabetic rats (Group 7) resulted in a significant increase of these parameters ($p=0.0007$).

Histopathological Results

H&E Staining

Examination of the parotid gland sections from the control (Group 1), MgO NPs (Group 2), and RJ (Group 3) treated groups presented nearly similar histological results. The normal structure of stromal and parenchymal elements was composed of several lobules of different sizes and separated by a fine network of interlobular connective tissue [Figure 6A]. Each lobule contained regular, densely packed serous acini and striated ducts, separated by thin fibrous connective tissue septa. The serous acini with well-defined, rounded boundaries were lined by one row of long,

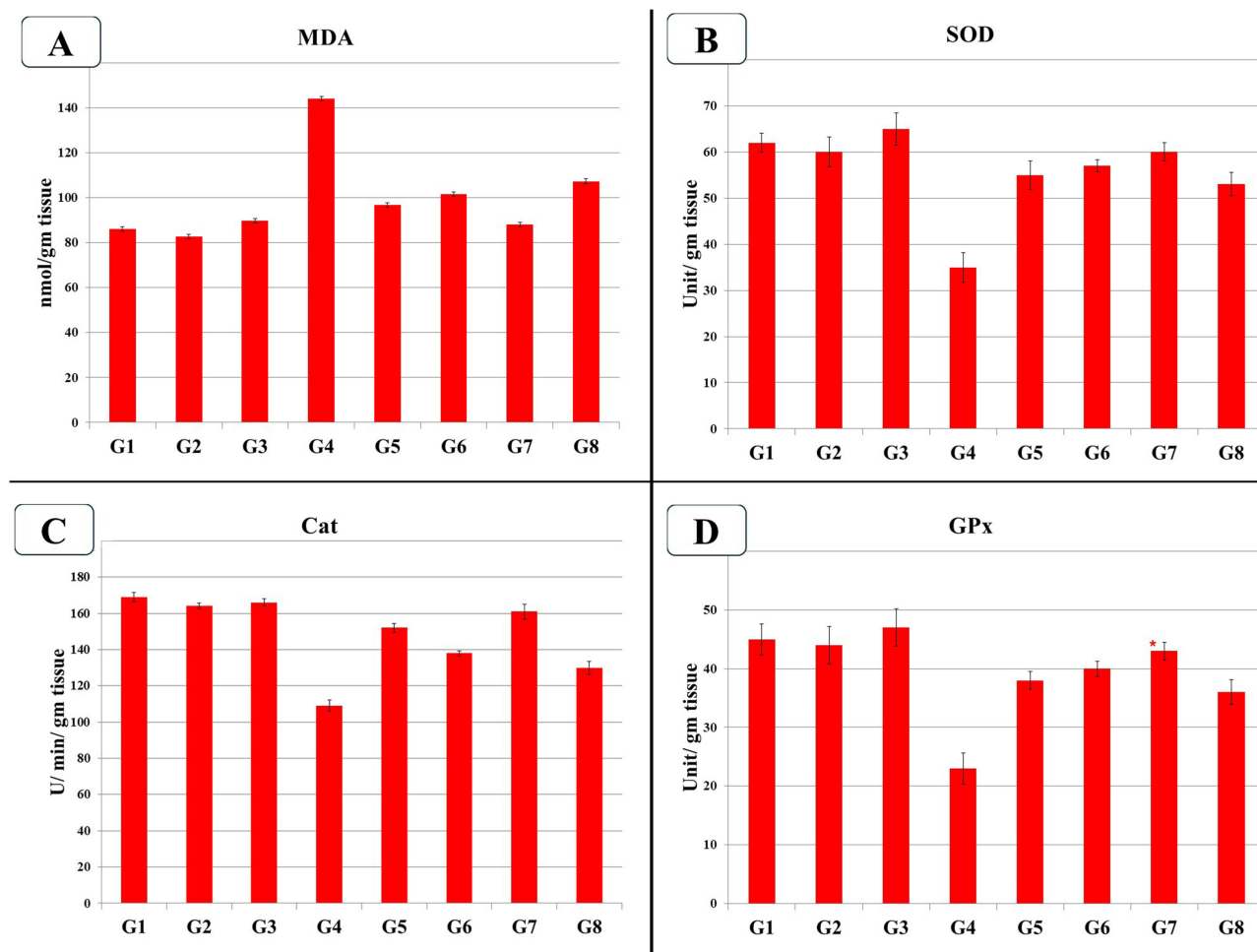


Figure 3 Charts representing the levels of oxidative stress and antioxidant enzymes in different groups: **(A)** Malondialdehyde; **(B)** Superoxide dismutase; **(C)** Catalase; **(D)** Glutathione peroxidase [G1=Control group, G2=MgO NPs group, G3=RJ group, G4=untreated diabetic group, G5=Diabetic group treated with MgO NPs, G6=Diabetic group treated with RJ, G7=Diabetic group treated with MgO NPs and RJ, G8=Diabetic group treated with metformin].

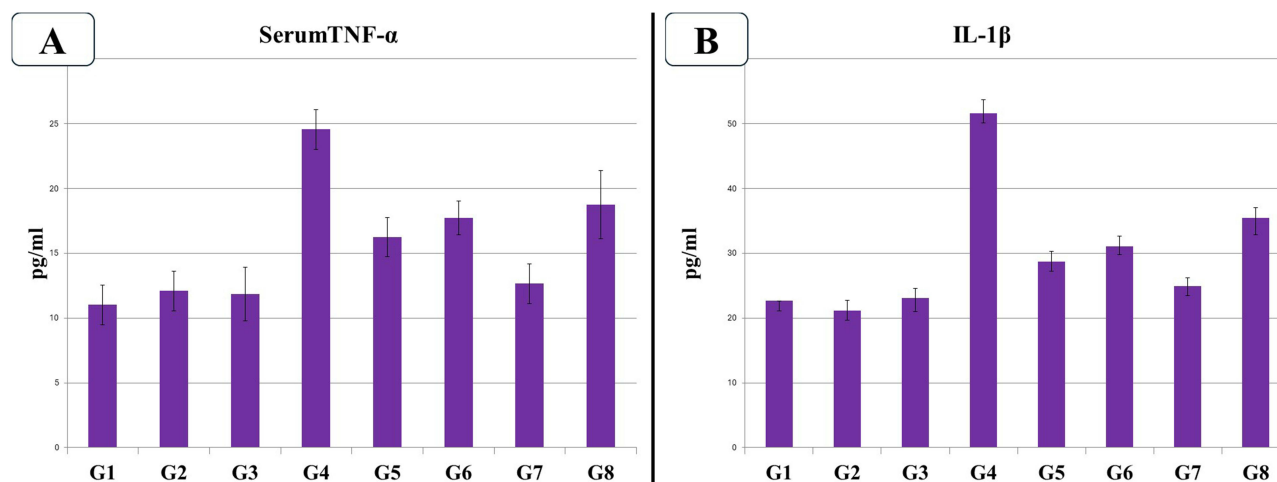


Figure 4 Analysis of the levels of inflammatory markers in different groups: **(A)** Tumor necrosis factor-alpha; **(B)** Interleukin-1 β [G1=Control group, G2=MgO NPs group, G3=RJ group, G4=untreated diabetic group, G5=Diabetic group treated with MgO NPs, G6=Diabetic group treated with RJ, G7=Diabetic group treated with MgO NPs and RJ, G8=Diabetic group treated with metformin].

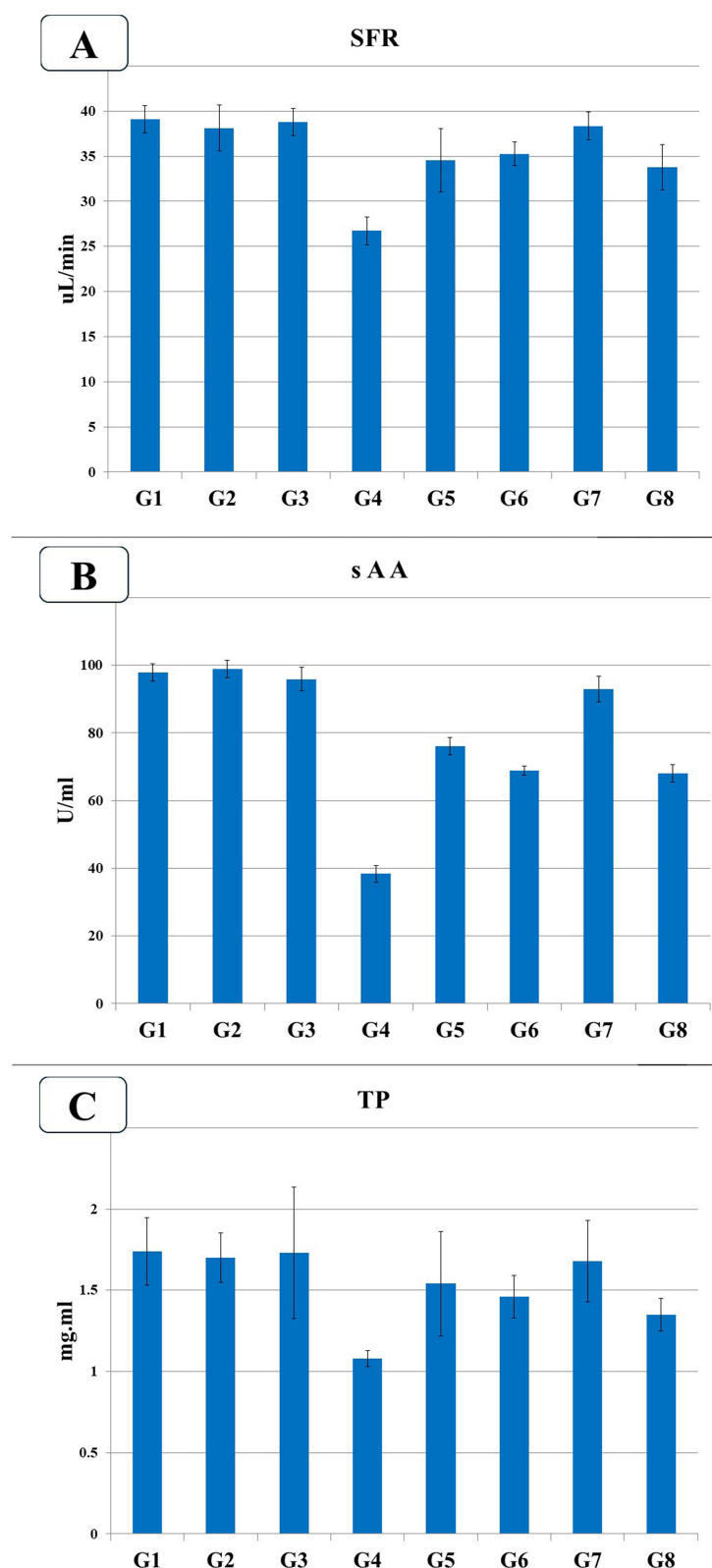


Figure 5 Evaluation of the salivary parameters in different groups: (A) Salivary flow rate; (B) Salivary alpha amylase; (C) Total protein [G1=Control group, G2=MgO NPs group, G3=RJ group, G4=Diabetic group, G5=Diabetic group treated with MgO NPs, G6=Diabetic group treated with RJ, G7=Diabetic group treated with MgO NPs and RJ, G8=Diabetic group treated with metformin].

pyramidal epithelial cells surrounding a central lumen and demonstrated dark basophilic cytoplasm and large spherical basally located nuclei with numerous apical acidophilic secretory granules. The intralobular ducts were formed mainly of large, striated ducts, which had a wide lumen and were lined by columnar cells with central nuclei and eosinophilic cytoplasm.

In the diabetic group (Group 4), the examination revealed variable degrees of histopathological alterations in the parotid gland in the form of disorganized and widely separated lobules. Most of the acini were shrunken, widely separated, fewer in number, and irregular in shape [Figure 6B]. Many acinar cells lost their normal arrangement and appeared condensed with cytoplasmic vacuolations and pyknotic nuclei. Furthermore, some cellular infiltration and congested blood vessels around the ducts were detected. Many of these ducts appeared irregular and dilated, with their lining cells showing pyknotic nuclei.

For the diabetic group treated with either MgO NPs (Group 5) or RJ (Group 6), the parotid gland displayed a more preserved structure when compared to the untreated diabetic group. Most of the acini presented a normal configuration; some acini were atrophic and had an irregular outline, with their acinar cells still containing vacuolated cytoplasm and pyknotic nuclei [Figure 6C]. Also, most ducts appeared regular with normal epithelial lining, with the exception of a few, which displayed some dilatation and degenerated lining epithelium. However, when the diabetic group was treated with both MgO NPs and RJ (Group 7), the parotid gland showed a preservation of the normal appearance of the lobules [Figure 6D]. Most of the serous acini and striated ducts appeared intact, with their epithelial lining presenting a regular arrangement of the cells, similar to the control group.

In the diabetic group treated with metformin (Group 8), the parotid gland appeared relatively well-organized compared to the untreated diabetic group. Although many acini showed normal appearance, some others appeared irregular and degenerated with condensed acinar cells [Figure 6E]. Moreover, most of the striated ducts exhibited a normal cell lining, with the exception of some ducts that displayed degenerated cells. Additionally, congested blood vessels were observed in the connective tissue stroma surrounding the ducts.

Masson Trichrome Staining

Examination of the Masson Trichrome-stained sections showed a low quantity of collagen fibers around the ducts and blood vessels and in between the serous acini in the control (Group 1), MgO NPs (Group 2), and RJ (Group 3) groups [Figure 7]. Meanwhile, there were excessive amounts of collagen fibers around the dilated ducts and blood vessels and between the deformed acini in the diabetic group (Group 4). In contrast, mild to moderate amounts of collagen fibers were detected in the diabetic groups treated with either MgO NPs (Group 5), RJ (Group 6), or metformin (Group 8). However, in the diabetic group treated with both MgO NPs and RJ (Group 7), there was a minimal amount of collagen fibers around the ducts and serous acini.

Immunohistochemical Staining for α -SMA

As seen in Figure 8, the immunohistochemical stained sections of the control (Group 1), MgO NPs (Group 2), and RJ (Group 3) treated groups displayed positive brown cytoplasmic immunostaining for α -SMA of the myoepithelial cells surrounding the acini and ducts, as well as in the lining wall of the blood vessels. In the diabetic group (Group 4), there was weak immunoreactivity for α -SMA at the periphery of both the serous acini and ducts as compared to the control group. Furthermore, some abnormal staining was observed in the interlobular ducts and in the lining of blood vessels. The diabetic groups treated with either MgO NPs (Group 5) or RJ (Group 6) displayed increased positive cytoplasmic immunoreactivity for α -SMA compared to the diabetic untreated group.

Immunohistochemical Staining for P53

As seen in Figure 9, the immunostaining of the p53 antibodies showed negative nuclear and cytoplasmic immunoreaction in the control (Group 1), MgO NPs (Group 2), and RJ (Group 3) treated groups in both acinar and ductal cells. In the diabetic group (Group 4), there was strong p53 cytoplasmic immuno-expression in the acinar and ductal cells; however, there was regression of immunostaining in the diabetic groups treated with MgO NPs (Group 5) or RJ (Group 6). Furthermore, in the diabetic group treated with both MgO NPs and RJ (Group 7), a negative immunostaining was observed.

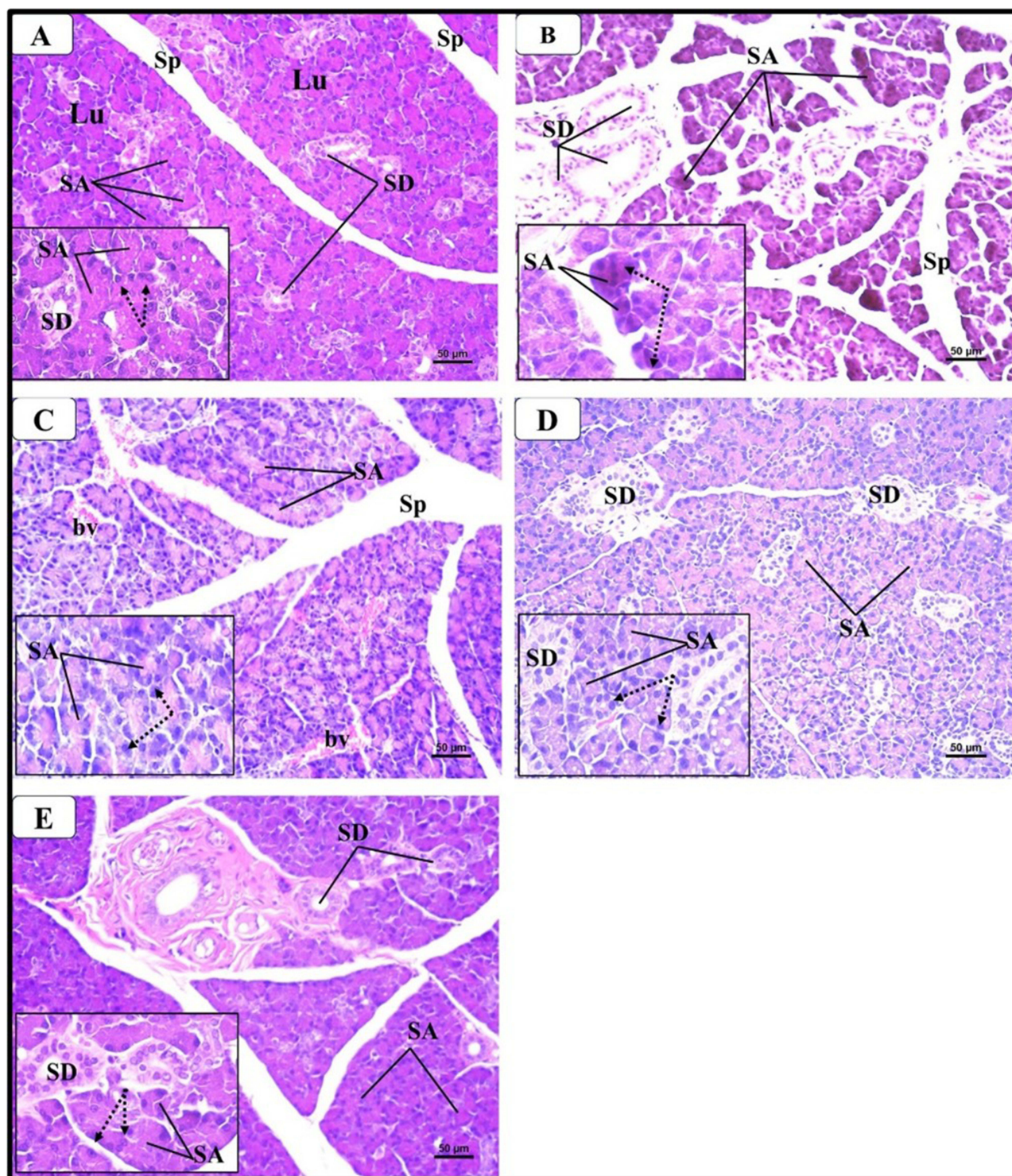


Figure 6 Representative photomicrographs of parotid gland sections stained with H&E from different groups: **(A)** The control group showed normal structure in the form of multiple lobules (Lu) separated by thin connective tissue septa (Sp). Each lobule was formed of uniform, closely packed serous acini (SA). The acini were composed of pyramidal acinar cells with spherical basal nuclei and basophilic cytoplasm (dotted arrow) [Inset]. Among the acini, the striated ducts (SD) had wide lumen and were lined by simple columnar cells with rounded nuclei and eosinophilic cytoplasm. **(B)** The diabetic group showed widely separated and irregular lobules. Most of the serous acini (SA) appeared atrophic and widely separated with an ill-defined outline. Many acinar cells (AC) exhibited irregularly shaped, darkly-stained pyknotic nuclei (dotted arrow) [Inset]. A lot of the striated ducts (SD) appeared dilated with disorganized epithelial lining and darkly-stained nuclei. Additionally, dilated, congested blood vessels (bv) were clearly seen. **(C)** The diabetic group treated with MgO NPs or RJ demonstrated nearly preserved histological features; however, there were some atrophic acini (SA) with shrunken acinar cells and condensed nuclei (dotted arrow) [Inset] in addition to mild blood vessels congestion (bv). **(D)** The diabetic group treated with both MgO NPs, and RJ presented with the normal histological picture, similar to that of the control group. The serous acini (SA) were regular and had normal pyramidal cells (dotted arrow) [Inset]. The striated ducts (SD) showed round nuclei (arrow). **(E)** The diabetic group treated with metformin showed nearly preserved histological features, but there were some atrophic acini (SA) with shrunken acinar cells and condensed nuclei (dotted arrow) [Inset] in addition to dilated striated ducts (SD). (H&Ex200 - Insetx400).

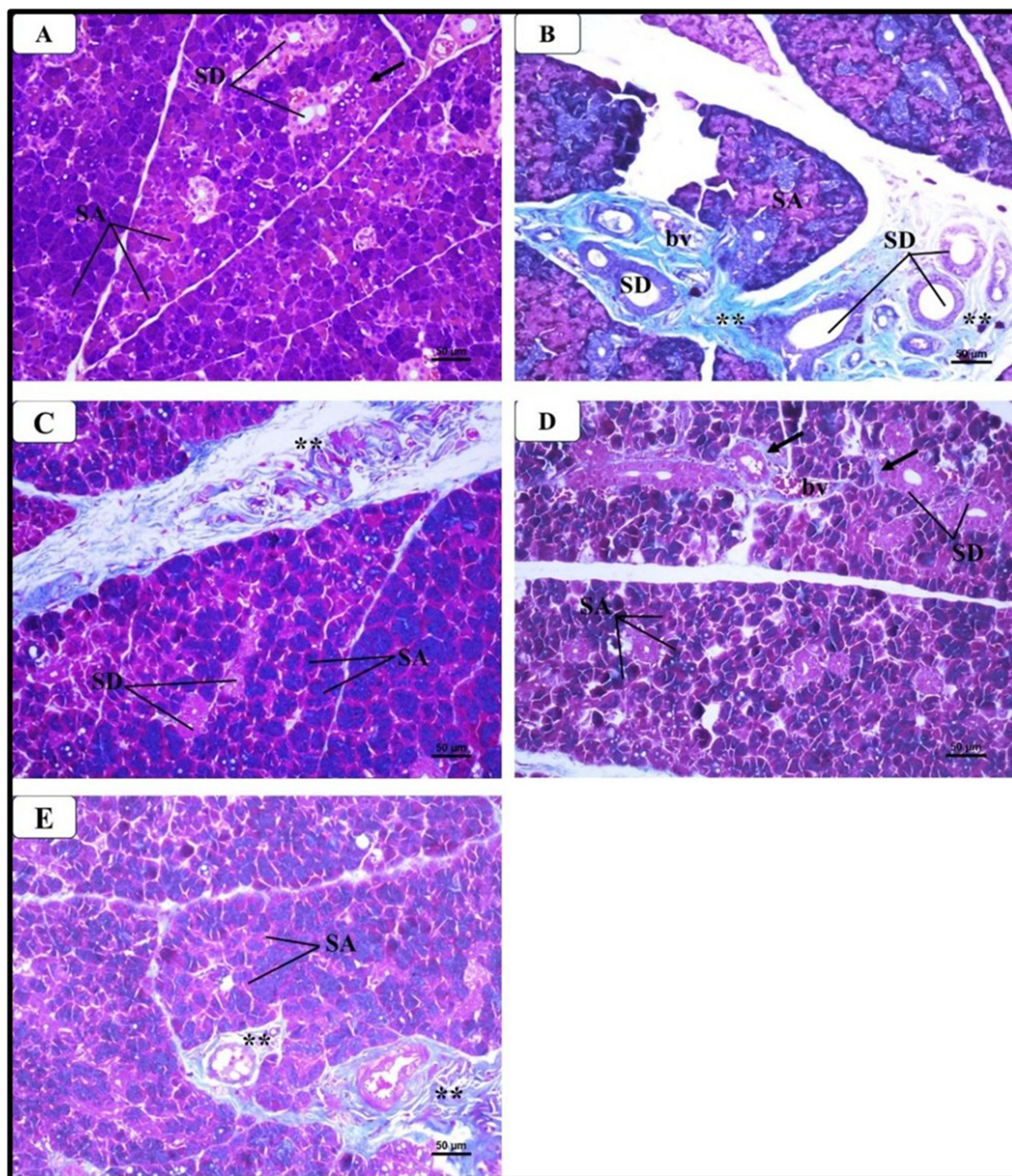


Figure 7 Representative photomicrographs of parotid gland sections stained with Masson's trichrome from different groups: **(A)** The control group showed very little amounts of collagen fibers around the ducts and blood vessels and in between the acini. **(B)** The diabetic group demonstrated an apparent increase in the collagen fibers deposited around the striated ducts and blood vessels (**). **(C)** The groups of diabetic rats treated with either MgO NPs or RJ showed mild to moderate amounts of collagen fibers around the ducts and acini (**). **(D)** The diabetic group treated with both MgO NPs and RJ exhibited a minimal amount of collagen fibers around ducts and acini (arrow). **(E)** The diabetic group treated with metformin displayed mild to moderate amounts of collagen fibers around ducts and acini (**). (MTx200).

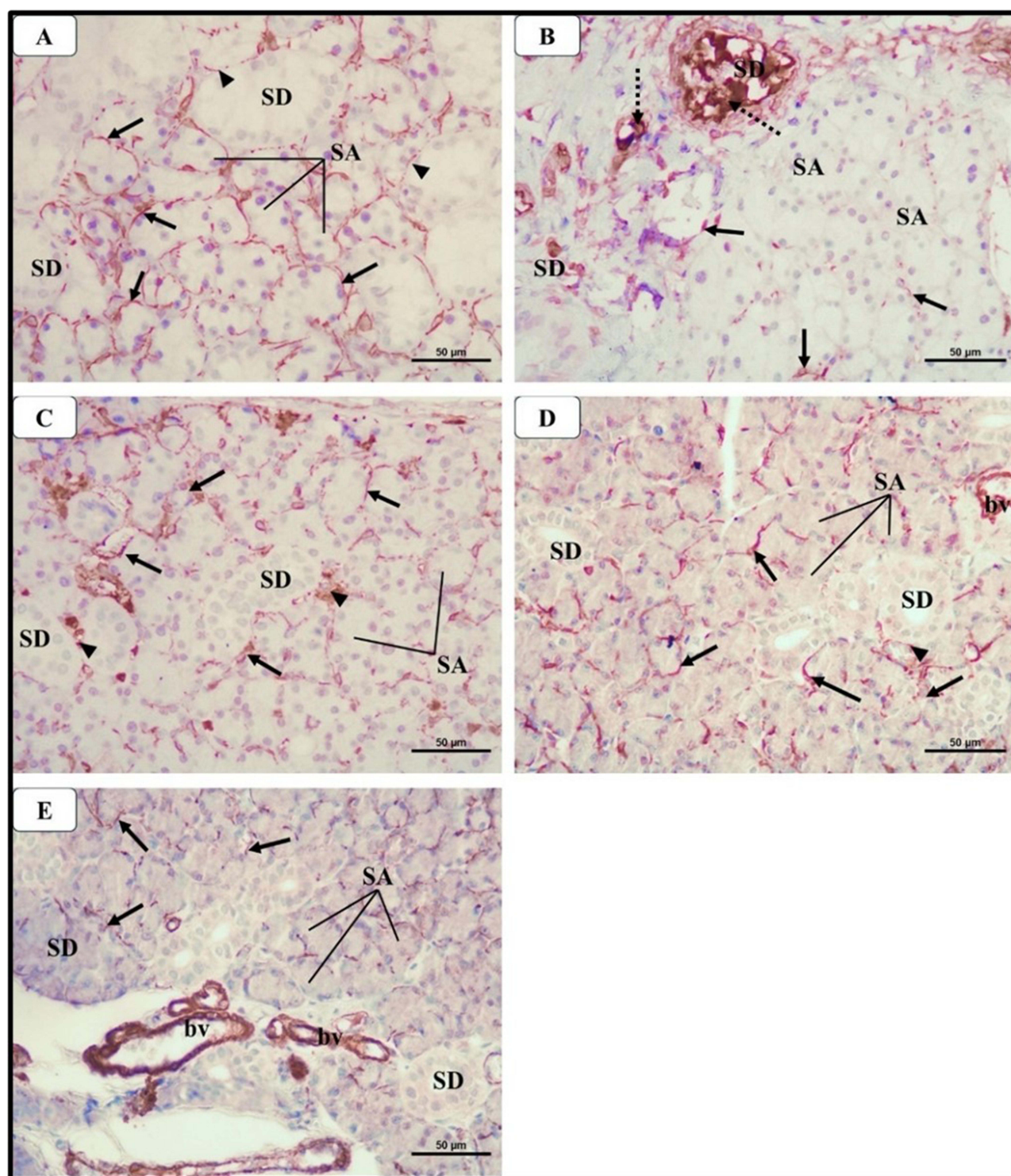


Figure 8 Representative photomicrographs of immunohistochemical staining of α -SMA in the parotid gland in different groups: **(A)** The control group showed positive brown immunostaining of myoepithelial cells at the periphery of the acini (→) and around the striated ducts (SD). **(B)** The diabetic group presented with decreased immunostaining of myoepithelial cells at the periphery of many acini and ducts. However, abnormal staining was observed around the blood vessels (dotted arrow). **(C)** The diabetic rats treated with either MgO NPs or RJ demonstrated moderate immunostaining of myoepithelial cells at the periphery of some acini (→) and around some striated ducts (SD). **(D)** The diabetic rats treated with both MgO NPs, and RJ displayed increased immunostaining of myoepithelial cells at the periphery of many acini (→) and around the striated ducts (SD). **(E)** The diabetic rats treated with metformin showed mild immunostaining of myoepithelial cells at the periphery of many acini (→). Notice the presence of positive staining around the blood vessels (bv). (α -SMA immunostaining $\times 400$).

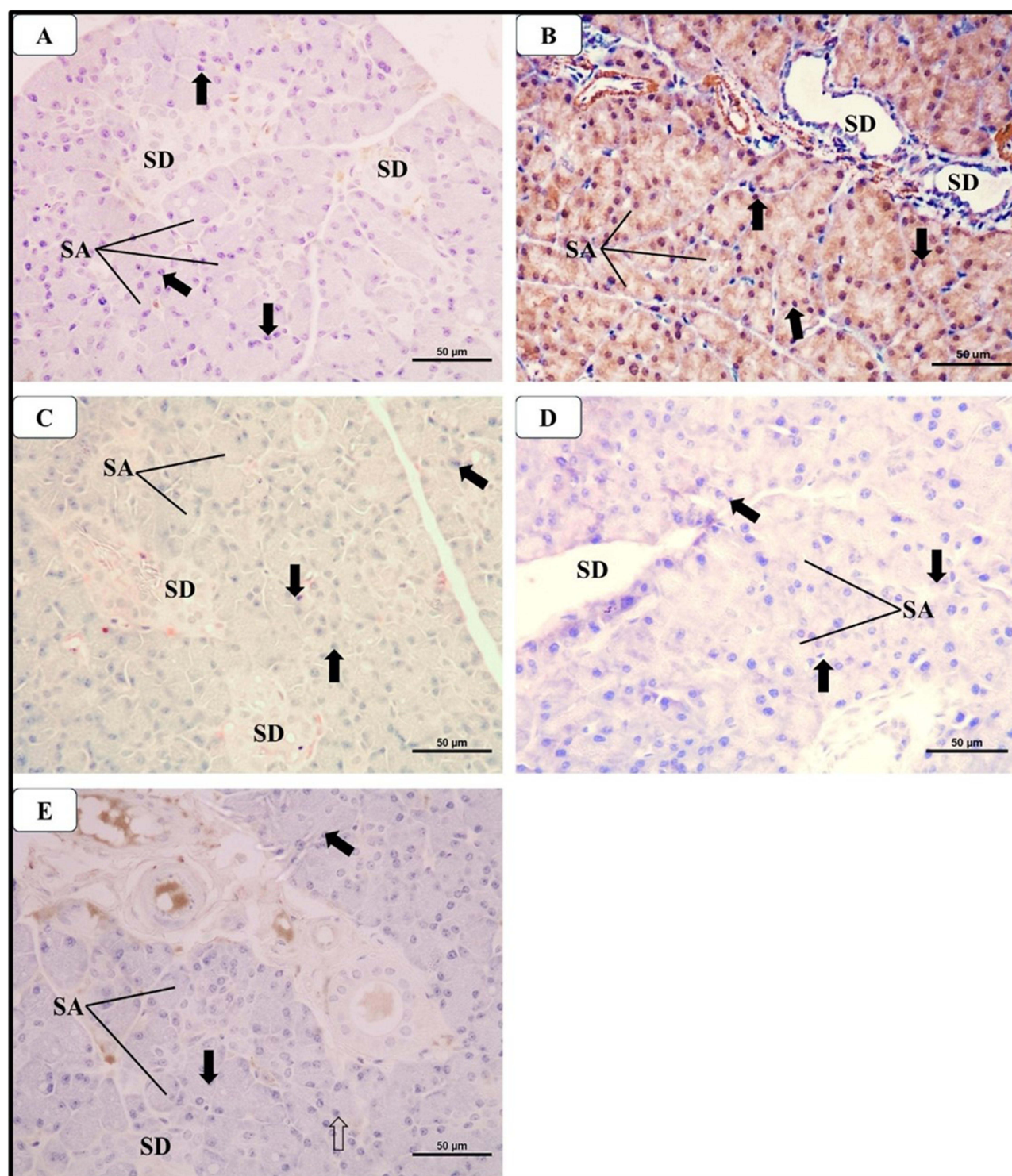


Figure 9 Representative photomicrographs of immunohistochemical staining of p53 in the parotid gland in different groups: **(A)** The control group exhibited negative p53 nuclear and cytoplasmic immunostaining in the acinar and ductal cells (thick arrow). **(B)** The diabetic group showed strong p53 immunostaining in the acinar and ductal cells (thick arrow). **(C)** The groups of diabetic rats treated with either MgO NPs or RJ demonstrated mild p53 immunostaining in the acinar and ductal cells (thick arrow). **(D)** The diabetic rats treated with both MgO NPs and RJ showed negative p53 immunostaining in the acinar and ductal cells (thick arrow). **(E)** The diabetic rats treated with metformin group presented with moderate p53 immunostaining in the acinar and ductal cells (thick arrow). (p53 immunostaining×400).

Discussion

The pathogenesis of parotid gland damage in DM is multifactorial, primarily driven by chronic hyperglycemia, which induces metabolic disturbances that impair glandular function. Increased glucose levels lead to the creation of advanced glycation end-products, which interfere with normal cellular functions and cause tissue damage. Moreover, DM is marked by increased oxidative stress arising from an imbalance between reactive oxygen species (ROS) and the body's antioxidant defenses, resulting in damage to the structure and function of salivary glands. Microangiopathy further compromises blood supply to these glands, resulting in hypoxia and nutrient deficiencies that contribute to atrophy and reduced secretory capacity. Moreover, diabetic neuropathy can impair autonomic control of salivary secretion, exacerbating xerostomia and worsening oral health outcomes.^{43–45}

Given the individual therapeutic approaches of MgO NPs and RJ, this study was devised as a novel approach to utilize the benefits of both to manage parotid gland dysfunction that occurs as a complication of DM, which can considerably worsen the patient's quality of life and increase the possibility of periodontitis and other oral infections.

The quality and yield of the MgO NPs were confirmed through a qualitative assessment using XRD, FTIR, and ZP. These analytical techniques are suitable and effective in providing information on the composition and interactions of nanomaterials; however, some previous investigations have reported that the results depend on various factors such as particle size, distribution, shape, aggregation, and surface charge of the tested nanoparticles.⁴⁶ The MgO NPs' nature was highly crystalline and exhibited physical stability when suspended in water. Our findings agreed with previously published works that reported similar results.^{38,47}

Metformin (dimethyl biguanide) was used in this study as a standard oral antidiabetic drug, as it is commonly prescribed to lower blood glucose levels in patients with non-insulin-dependent DM.⁴⁸ Investigations have demonstrated its efficacy in blood sugar control with low risk of hypoglycemia, although common side-effects can include nausea, diarrhea, abdominal discomfort, and vitamin B12 deficiency.^{49,50} Throughout the various investigations in our study, our results expressed that the administration of both MgO NPs and RJ to diabetic rats was comparable or superior in promoting good results of the diabetic and oxidative stress parameters in comparison to metformin.

The streptozotocin-induced diabetic rats showed a notable rise in blood glucose levels and a reduction in SI levels, together with an increase of HOMA-IR; these findings were consistent with former studies which stated that hyperglycemia can be considered a direct reflex to the marked hypo-insulinemia caused by the selective destructive cytotoxic effect of streptozotocin on the β -cells of the pancreas.^{51,52}

The administration of both MgO NPs and RJ to the diabetic rats notably improved these parameters, affirming their antidiabetic effect. In this regard, some recent studies have validated the efficacy of MgO NPs as antidiabetics, where they play a role in improving glucose metabolism and insulin sensitivity in diabetic rats.^{53–55} Through the regulation of blood sugar levels, MgO NPs can reduce the severity of DM-related complications.³⁸ MgO NPs have been found to improve insulin sensitivity in animal and cell levels, which in turn induces the receptivity of the body's cells to insulin and therefore facilitates the reduction of blood glucose and lowers the risk of developing Type 2 DM.^{56,57} These effects may be due to the physicochemical characteristics inherent to nanoparticles, allowing for increased uptake.¹⁶ Moreover, the small size allows for close interaction with biological tissue.¹⁷

Additionally, it was reported that magnesium is a necessary cofactor for enzymes shared in carbohydrate metabolism, particularly in the phosphorylation of tyrosine-kinase of the insulin receptor, along with all other protein kinases involved in insulin signaling.^{21,57,58} This is in accordance with previous studies, which reported that DM is associated with intracellular and extracellular magnesium depletion, and magnesium augmentation positively influences insulin receptor activity and reduces insulin resistance in a rat model.^{59,60}

Likewise, administration of RJ presented a significant decrease in the elevated glucose level and a significant increase in insulin concentration. Moreover, studies have illustrated that supplying RJ to diabetic rats lead to a significant alleviation of glucose, insulin, and insulin resistance.^{61,62} Ghanbari et al demonstrated that diabetic rats fed 100mg/kg of RJ for eight weeks had decreased fasting blood glucose and increased serum insulin levels.⁶³ Furthermore, RJ was found to regulate and reduce blood sugar levels through activity analogous to insulin, thereby improving insulin resistance in the rats.³² Additionally, Mousavi et al conducted a randomized controlled trial on 46 diabetic patients

treated with 1000mg/day of RJ for eight weeks. They reported a significant reduction in the mean FBS and HbA1C when compared to the patients on a placebo.⁶⁴

The level of MDA, a marker of oxidative stress, was significantly higher in the parotid tissue of the diabetic group, together with a marked reduction in the levels of antioxidant enzymes SOD, GPx, and CAT, suggesting oxidative damage in this gland. Some studies stated that the increased oxidative stress found with DM can lead to tissue damage of the parotid gland.^{65,66} The parotid gland is particularly susceptible to antioxidant imbalances and oxidative damage due to its predominantly aerobic metabolism and its inherent defense mechanisms against reactive oxygen species.^{4,67}

Studies have previously demonstrated that hyperglycemia significantly impacts the parotid gland, leading to increased lipid peroxidation, elevated MDA levels, and a reduction in antioxidant enzyme activity.^{4,68} Additionally, it was stated that in DM, the antioxidant enzymes are significantly reduced in many tissues, resulting in sustained reactive oxygen species formation, thereby heightening oxidative stress and leading to nuclear apoptosis and cellular degeneration in both the parotid and submandibular glands.^{69,70} Therefore, it can be concluded that the use of antioxidants is beneficial as an additional treatment for DM.^{71,72}

In this regard, the concurrent administration of both MgO NPs and RJ to the diabetic rats presented defensive characteristics through the significant reduction in MDA levels and significant elevation in catalase activity and GPx. One key mechanism through which MgO NPs exert their protective effects is by acting as antioxidants.^{38,53} MgO NPs can scavenge free radicals and reduce oxidative stress, thus aiding in the preservation of the structural and functional integrity of the parotid gland in diabetic conditions.¹⁸ Moreover, MgO NPs may modulate the inflammatory responses, which are often worsened in DM.⁷³ By reducing inflammation, these nanoparticles can aid in protecting the parotid gland against DM-induced damage. A study by Moeini-Nodeh et al illustrated MgO NPs' capacity to prompt anti-oxidative, anti-diabetic, and anti-apoptotic functions in the pancreatic islet.⁷⁴

Furthermore, a growing body of evidence suggests that RJ has the ability to diminish oxidative stress through its role as a free-radical scavenger, maintain an adequate antioxidant status, and protect against toxicity induced by reactive oxygen species.^{25,75} Maleki et al conducted a systematic review on the metabolic effects of RJ in diabetic patients and stated that RJ intake could improve glycemic status, HOMA-IR, lipid profiles, and oxidative stress.²³ It has also been reported to enhance levels and activity of the antioxidant enzymes CAT and SOD.^{20,76,77}

In this study, the diabetic rats had a marked decrease in salivary secretion parameters (salivary flow rate, sAA, and total protein content) compared to the control values. These findings coincided with previous human studies, which stated that most diabetic patients experience hypofunction of salivary glands, and further exacerbation can result in hyposalivation or xerostomia.^{5,78,79} Additionally, our results were in accordance with animal studies, which reported the deterioration of these salivary parameters resulting in the decline of secretory dysfunction in salivary glands, especially in the parotid gland.^{80,81}

Moreover, DM can lead to diabetic autonomic neuropathy, a complication that disrupts autonomic control of salivary glands, particularly the parotid glands. Since salivary secretion depends on a balance between parasympathetic and sympathetic nervous inputs, autonomic dysfunction in diabetes can impair salivary flow and composition. This may manifest as xerostomia and increased susceptibility to oral infections. Structural changes in the parotid glands, such as acinar atrophy and reduced expression of key proteins like aquaporin-5, further contribute to glandular dysfunction.⁸²

The conditions of DM result in the alteration of salivary gland tissue structure and the mechanism of saliva secretion.^{4,83} In the present study, the histopathology of the parotid gland of the diabetic rats displayed multiple lesions, including disorganization and atrophic changes in the serous acini, vacuolization and degeneration of both acinar and ductal cells, inflammatory cell infiltration, and accumulation of increased collagen fibers. These results were consistent with several previous studies conducted in rats in which DM induces morphological alterations in salivary glands, with changes seen in the density of secretory granules health.^{80,84,85}

The results also exhibited that many serous acini of the parotid gland of diabetic rats showed atrophic changes with cytoplasmic vacuolization and apoptotic nuclei of the acinar cells. Chen et al confirmed this, noting significant acinar cell deformation and atrophy, along with a reduction in secretory granules and vacuolation among the diabetic rats.⁴ Such findings were explained by the accumulation of degenerative substances and fatty degeneration in the cytoplasm, as well as the DM-induced oxidative stress in the parotid glands and the associated degenerative changes.^{69,85} Consistent with

our findings, a recent study found that these changes primarily result from damage caused by lipid peroxidation and oxygen radicals targeting chromatin, leading to DNA fragmentation, which ultimately causes cell death.⁸⁶

The administration of both MgO NPs and RJ to the diabetic rats established a beneficial role in improving the salivary secretion parameters towards the normal values. A recent study has shown that 12 weeks of RJ tablets (800 mg daily) were effective for patients who suffered from subjective dry mouth sensation with normal saliva function.⁸⁷ While many studies promote the benefits of oral consumption of magnesium, limited studies exist on the oral consumption of MgO NPs in humans, with most studies conducted in animals and focused on addressing cytotoxicity concerns.^{22,88,89} In consensus, most concluded that low doses of MgO NPs provided benefits with no cytotoxicity, while higher doses could produce significant tissue damage.^{74,90–92} Further toxicological profiling and biomedical investigations are imperative in determining safe exposure levels to utilize the multi-faceted benefits of MgO NPs.

In the present study, a significant increase in collagen deposition and dilated, congested blood vessels around the ducts was observed in the DM group. These findings coincide with what was detected by El Shahawy and El Deeb, who similarly found thick networks of collagen fiber bundles surrounding the blood vessels of the submandibular salivary gland in diabetic rats.⁹³ This could be attributed to the hyperglycemia triggering disordered remodeling activity of fibroblasts along with the inflammatory process associated with DM.^{69,94}

A marked improvement occurred in these histopathological alterations in the parotid gland of diabetic rats upon concurrent administration of MgO NPs and RJ. These results agreed with some recent studies that reported that MgO NPs possess antioxidant properties, which are essential for combating oxidative stress.^{22,95} Moreover, it was reported that RJ resulted in a decrease of oxidative stress indicators and an increase in antioxidant enzyme levels.^{25,77}

The immunohistochemical study of α -SMA expression, which is an important immunohistochemical marker for myoepithelial cells, showed a marked reduction in the parotid gland of the diabetic group as compared to the control group. It was reported that in the salivary glands, myoepithelial cells are stellate cells generally in the form of arcs surrounding the acini and appear along the long axes of the ducts. Their main function is to facilitate the expulsion of secretion from the acini.⁹⁶ This was similarly reported in diabetic mice by Nashida et al.⁹⁷ However, in another study by Morsy et al, the diabetic parotid glands showed a notably greater expression of α -SMA immunoreaction compared to the control group, suggesting a disproportionate proliferation or hypertrophy of myoepithelial cells.⁸⁶

The immunohistochemical study of TP53 displayed a higher expression in the parotid gland of diabetic rats compared to the control. TP53 is known to regulate cell survival and death, and animal studies have shown that it also responds to metabolic changes and affects metabolic pathways.⁹⁸ Several studies have established that chronic hyperglycemia causes cellular damage through oxidative injury to lipids, proteins, and DNA, which activates TP53. Subsequently, this activation controls the expression of apoptotic, pro-inflammatory, and metabolic genes.^{99,100} The administration of both MgO NPs and RJ to the diabetic rats showed mild p53 immuno-expression in the parotid gland, indicating stimulation of the antioxidant pathway. This illustrates that histological improvement could be a response to low levels of oxidative stress as a result of their antioxidant effect that stimulates p53.¹⁰¹

Several challenges were faced during this study. While combining STZ and a high carbohydrate diet is often used to mimic diabetes, these models may not accurately reproduce the complexities of β -cell dysfunction seen in humans. Furthermore, the effect of long-term complications could not be assessed within the eight-week time frame of the study. Alternative parameters and DM-associated complications should also be assessed.

Conclusion

The present study demonstrated that both MgO NPs and RJ exhibit promising effects in the defense against parotid gland damage in diabetic rats, which was more profound when these two agents were used together, as their synergetic actions were found to have significant hypoglycemic, antioxidant, and anti-inflammatory effects. However, further clinical trials are needed to cement the validity of the dual administration of MgO NPs and RJ to diabetic patients.

Abbreviations

DM, diabetes mellitus; MgO NPs, magnesium oxide nanoparticles; RJ, royal jelly; FTIR, Fourier transform infrared spectroscopy; XRD, X-ray diffraction; ZP, zeta potential analysis; FBG, fasting blood glucose; HbA1C, glycated

hemoglobin; SI, serum insulin; α -SMA, anti-alpha-smooth muscle actin; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; TP53, tumor protein p53.

Data Sharing Statement

Data is available from the corresponding author upon reasonable request.

Ethical Approval

This research was initiated after receiving the consent of the Medical Research Ethics Committee, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia (REF NO. [HA-02-J-008]). Care and management of the animals were conducted in compliance with the National Institutes of Health's 'Guide for the Care and Use of Laboratory Animals', which outlines standards for housing, environmental enrichment, pain management, and humane endpoints. Special attention was given to reducing stress and discomfort during the experimental period, in line with current best practices in laboratory animal science. Approval was also granted by the Institutional Animal Care and Use Committee of the Faculty of Medicine at King Abdulaziz University.

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Author Contributions

All authors made a significant contribution to the work reported, in the conception, study design, execution, acquisition of data, analysis and interpretation. Also, they took part in drafting, revising and critical reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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The authors have no competing interests to declare that are relevant to the content of this article.

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