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# Modulatory effects of concomitant quercetin/sitagliptin administration on the ovarian histological and biochemical alterations provoked by doxorubicin in a streptozotocin-induced diabetic rat model

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## ABSTRACT

Limited literature was available on the effects of sitagliptin or quercetin treatments on doxorubicin induced ovarian dysfunction in diabetic animals. The study aim was test the efficacy and suggested mechanisms of quercetin/sitagliptin combined treatment on the doxorubicin-induced ovarian toxicity in rat model with streptozotocin-induced diabetes. Forty eight female Wistar rats were divided into six groups: 1) Control; 2) Streptozotocin induced diabetes; 3) Streptozotocin-induced diabetes + doxorubicin ovarian damage; 4) Streptozotocin-induced diabetes + doxorubicin ovarian damage with; 5) Streptozotocin-induced diabetes + doxorubicin ovarian damage with sitagliptin treatment and 6) Streptozotocin-induced diabetes + doxorubicin ovarian damage with concomitant quercetin/sitagliptin treatment. Biochemical tests for serum estrogen, progesterone, insulin, blood glucose, and ovarian levels of malondialdehyde, nitric oxide, and superoxide dismutase and qRT-PCR for NOBOX, FSHr, and iNOS genes were performed. Histological evaluation was done on ovary sections with hematoxylin and eosin and immunohistochemistry for 8-OHdG and iNOS followed by morphometric analysis. The streptozotocin-induced diabetic group showed varying degrees of follicle atresia and altered biochemical parameters, both were marked in the streptozotocin-induced diabetic + doxorubicin group. The mRNA of NOBOX, FSHr, and iNOS genes were disturbed with increased immunoeexpression of iNOS and 8-OHdG. Quercetin and/or sitagliptin administration improved all altered histological and biochemical parameters and was more effective as a combined treatment. The study suggested equal efficacy of both quercetin and sitagliptin in mitigating the doxorubicin-induced ovarian toxicity in the streptozotocin diabetic rat model, and the combined therapy showed anti-inflammatory, anti-antioxidant, and anti-DNA damage mechanisms.

## KEYWORDS

Doxorubicin; sitagliptin; quercetin; streptozotocin-induced diabetes; immunohistochemistry; follicular atresia; inflammation

## Introduction

Diabetes Mellitus (DM) is a chronic systemic disorder of carbohydrate metabolism that was estimated to affect more than 460 million people worldwide in 2019 and was expected to rise to 578 million by 2030 [1]. DM has been linked with an increase in both cancer incidence and cancer mortality according to Ling et al. [2]. Both are frequently co-existing fatal diseases so that the diversity of the clinical conditions of diabetic patients suffering from cancer might encourage the oncologists to decrease the dose and/or the duration of chemotherapy, even at the expense of its effectiveness [3]. In reality, both patients and physicians may neglect the control of blood glucose being overwhelmed by cancer management. However, poor diabetes control has been

associated with poor cancer prognosis. Patients suffering from both DM and cancer might present a unique challenge to oncologists regarding the decision of their management plan [4]. Therefore, the innovation of dual-action drugs that could treat diabetes, reduce the side effects of chemotherapy, and/or potentiate anti-tumor activity of chemotherapeutic agents might be beneficial for those patients.

Cancer chemotherapy has targeted rapidly dividing cells by inhibiting their proliferation and growth [5] so that mitotically active non-tumor cells such as germ cells are at risk of these toxic effects. Doxorubicin (DOX) is a chemotherapeutic agent used to treat a wide spectrum of malignancies in the clinical field, but its detrimental

effects on nontargeted tissues, particularly gonads, minimize its use [6]. Since diabetic patients are more vulnerable to cancer than normal individuals as reported by Ling et al. [2], DOX therapy becomes inevitable, with a warning to cause severe ovarian damage to the already ailing gonads of diabetic female patients.

Oogenesis, follicular development, and maturation are regulated by several controlling factors [7]. Newborn ovary homeobox (*NOBOX*) gene is an essential transcriptional regulator of oocyte-specific genes, and *NOBOX*-deficient humans were associated with downregulation of approximately 28 oocyte-specific genes with subsequent follicular atresia and failure of oocyte differentiation as demonstrated by Batiha et al. [8]. As reported by Chen et al. [9] and Li et al. [10], *NOBOX* deficiency and mutations were associated with mammalian premature ovarian failure, postnatal oocyte loss, and follicular atresia during the early stages of ovarian folliculogenesis. Other studies have shown that, follicle-stimulating hormone receptors (FSHr) and their ovarian expression are involved in the production of estrogen and have a crucial role in follicular development and ovulation [11,12]. A recent study conducted by Casarini et al. [13] reported the importance of estrogen receptor/FSHr complex in the survival of human ovarian follicles. Reactive oxygen species (ROS)-rich ovarian micro milieu could affect the ovarian reserve. DNA damage mediated by ROS generation and oxidation can affect oogenesis by interfering with the ovarian follicular reserve [14]. The biomarker, 8-hydroxy-2'-deoxyguanosine (8-OHdG), was used for detection of oxidative DNA damage as demonstrated by Qing et al. [15].

Quercetin (QCT), a natural flavonoid normally found in daily consumed diets such as onions, was reported to have various anti-inflammatory, antioxidant, and anti-tumor activities [16]. Of interest, the Minaei group reported that QCT enhanced the antineoplastic activity of DOX in a breast cancer cell line and reduced its toxic effects on nontargeted organs [17]. Sitagliptin is an oral hypoglycemic medication classified as dipeptidyl peptidase-4 (DPP-4) enzyme inhibitor. Sitagliptin has been reported to have antioxidant and anti-inflammatory activities, in a diabetes mellitus study, with proven efficacy and safety as demonstrated by Shawky group [18]. Moreover, El-Agamy et al. suggested sitagliptin provided cardioprotection in rats with doxorubicin-induced cardiotoxicity via antioxidant, anti-inflammatory, and anti-apoptotic effects [19].

The current study was designed to mimic diabetes and investigate the role of QCT, and/or sitagliptin in treating the concurrent DOX-induced ovarian toxicity in female rats with preexisting diabetes induced by STZ. The role of *NOBOX*, FSHr, and iNOS expressions, in addition to the 8-OHdG-mediated DNA damage, was

investigated as possible mechanisms for this ovarian toxicity, using histological, immunohistochemical, biochemical, and gene studies.

## Material and methods

### Animals

In this study, 48 adult female Wistar rats (age 7 wk, 180–200 g) were obtained from the animal house of Moshtohor Faculty of Veterinary Medicine, Benha University, Egypt. The rats were housed 4 per cage at ambient temperature of  $24 \pm 2^\circ\text{C}$ , a 12 h/12 h light/dark cycle and supplied standard food and water *ad libitum*. To acclimatize to the laboratory environment, the rats were left without manipulation for 7 days before the start of the experiment. All ethical issues regarding animal husbandry and experimentation were following the guidelines of the Faculty of Medicine, Benha University, and were consistent with recommendations of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health [20].

### Induction of diabetes

Streptozotocin ( $\geq 98\%$  purity, powder, # S0130, Sigma-Aldrich, USA) was freshly prepared in a sterile aq. solution of 0.1 M sodium citrate, pH 4.5, and a single dose was injected intravenously (i.v.) through the tail vein of overnight fasted rats. STZ was given one time immediately after drug preparation at a dose of 60 mg/kg as described by previous work [21]. After 3 days, the rats were checked for diabetes induction via blood drawn from tail veins to determine fasting blood glucose level using an Accu-Check Active glucometer (#07444141200, Roche Diagnostics, Germany). After 3 weeks (day 21), overnight fasting animals were checked for blood glucose, and rats with a fasting blood glucose level of  $\geq 250$  mg/dl were considered diabetic and were included in the study [21].

### Study design

#### *Doxorubicin, sitagliptin, and quercetin preparations*

The preparations used in the experiment were doxorubicin (provided as Adriblastina® vials, 50 mg/25 ml, Pfizer, Egypt) and sitagliptin (JANUVIA® 100 tablet, Merck Sharp & Dohme Ltd., Italy). Sitagliptin tablet was ground and dissolved in 40 ml 0.5% carboxymethylcellulose (CMC), # C5678, Sigma-Aldrich) for final concentration of 2.5 mg/ml and was given in a dose of 10 mg/kg body weight (b.w.). Quercetin (95 HPLC,

solid  $\geq 95\%$  purity, #Q4951, Sigma-Aldrich) was prepared as follows: 40 mg was dissolved in 10 ml corn oil (purchased from a local market, Benha, Egypt) for a final concentration of 4 mg/ml and was given in a dose of 20 mg/kg b.w.

Forty-eight animals were equally divided into 6 groups,  $n = 8$  rats per group as follows:

- **Control:** Normal saline/quercetin/sitagliptin treatment. Saline (0.9% w/v sodium chloride, Nasrpharma for Pharmaceutical Chemicals, Egypt) was injected twice. The first dose was 5 ml/kg i.v. and the second dose was 7.5 ml/kg intraperitoneal (i.p.) on day 25. Quercetin in corn oil at 20 mg/kg b.w. (5 ml/kg b.w.) and sitagliptin in CMC at 10 mg/kg b.w. (4 ml/kg b.w.) were given by oral gavage for 21 consecutive days starting 3 days before the second i.p. saline dose.
- **Diabetes group:** Rats ( $n = 8$ ) received a single dose of STZ (5 ml/kg, equivalent to 60 mg/kg) i.v. and then received the corresponding vehicles (corn oil, 5 ml/kg and CMC, 4 ml/kg) on days 22 and for 21 consecutive days.
- **Diabetes/DOX group:** Diabetic rats ( $n = 8$ ) were divided equally into subgroups ( $n = 4$  rats per subgroup). Both subgroups received DOX (15 mg/kg b.w., single dose, i.p.) [22] on day 25 after STZ diabetes induction. Three days before DOX administration, one subgroup received 5 ml/kg corn oil and the other subgroup received 4 ml/kg CMC by oral gavage for 21 consecutive days.
- **Diabetes+DOX+QCT:** diabetic rats ( $n = 8$ ) were given quercetin (20 mg/kg b.w. by oral gavage) on day 22 after STZ diabetes induction (3 days before DOX administration) for 21 consecutive days [22].
- **Diabetes+DOX+SIT:** diabetic rats ( $n = 8$ ) were given sitagliptin (10 mg/kg b.w. by oral gavage) on day 22 after STZ diabetes induction (3 days before DOX administration) for 21 consecutive days [19].
- **Diabetes+DOX+SIT+QCT:** diabetic rats ( $n = 8$ ) were given both quercetin and sitagliptin dosages as described (3 days before DOX administration) for 21 consecutive days.

### **Blood sampling, blood glucose, and hormonal profile assay**

Twenty-four hours after the last oral gavage, the rats were anesthetized (ketamine/xylazine; 80/10 mg/kg b.w.). Ketamine (Ketam 50 mg/ml) was purchased from EIPICO, Egypt, and Xylazine (Xylaject 20 mg/ml) was

purchased from ADWIA Pharmaceuticals, Egypt. Blood sampling was done via cardiac puncture using 5 ml syringe and was collected into red top vacuum blood collection tubes (#367812, Becton Dickinson, USA). Centrifugation was carried out to separate sera and were stored at  $-20^{\circ}\text{C}$  (Frimed Laboratory Freezer, Italy) for later assays of estrogen, progesterone, blood glucose, and serum insulin. After blood collection, the rats were humanely euthanized by cervical decapitation, abdomens were incised longitudinally, both ovaries removed, and washed with ice cooled saline. The left ovaries were stored at  $-20^{\circ}\text{C}$  (Frimed Laboratory Freezer, Italy) for biochemical study.

### **Biochemical assay of oxidative markers**

The ovarian tissues were minced and homogenized in potassium phosphate buffer (10 ml, 50 mM,  $4^{\circ}\text{C}$ , pH 7.5, #P5244, Sigma Aldrich) using an Ultra-Turrax tissue homogenizer (IKA, Germany). Centrifugation (4000 rpm, for 15 min, at  $4^{\circ}\text{C}$ ) was done, and homogenate supernatant was collected for estimation of the ovarian levels of nitric oxide (NO) (Catalog #NO 2533), malondialdehyde (MDA) (Catalog #MD 2528), and superoxide dismutase (SOD) (Catalog #SD 2520). All kits were manufactured by Biodiagnostic, Egypt, and assays performed using manufacturers' kit instructions. Thiobarbituric acid reactive substances (TBARS) assay [23] was done for MDA measurement (nmol/mg protein). Superoxide dismutase SOD (U/mg protein) was measured according to Sun et al [24]. NO level (nmol/mg protein) was estimated indirectly by measuring nitrite concentrations [25].

### **Quantitative RT-PCR assay for mRNA expression of NOBOX oogenesis, FSHr, and iNOS**

Total RNA was obtained from the ovarian samples of all experimental groups utilizing TRIzol (#15596026, Invitrogen, USA) according to the manufacturer guidelines. The purity of isolated RNA was calculated by the Nano-Drop 2000C spectrophotometer (Thermo Scientific, USA). RNA purity from all samples was determined using 2% agarose gel electrophoresis image with  $>1.9$  at absorbance ratio A260/A280 with Gel Doc, Biorad, USA, according to El-Attrouny et al. [26]. Complementary DNA (cDNA) was manufactured for the genes with SensiFast cDNA synthesis kits (# BIO-65053, Sigma Bioline, UK) according to kit instructions. NCode VILO miRNA cDNA Synthesis Kit (#A48570, Invitrogen) was used for cDNA synthesis from miRNAs following the manufacturer's instructions using a T100 Thermal Cycler (#1861096, Bio-Rad). Quantitative PCR was done using

Maxima SYBR Green/ROX qPCR master mix (2x) (#K0221, Thermo Scientific) according to Abdelatty et al. [27]. Primer pairs for target and selected genes (*NOBOX*) oogenesis, follicle-stimulating hormone receptor (*FSHR*), inducible nitric oxide synthase (*iNOS*), and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes were obtained from GENEWIZ, USA (Table 1). An individual PCR reaction contained 500 ng per reaction of cDNA, 12.5 µl Maxima SYBR Green qPCR Master Mix (Maxima SYBR Green qPCR, #K0221, Thermo Fisher Scientific), 0.3 µmol of each forward and reverse primer, 10 nmol/100 Nm ROX Solution, and Nuclease- Free (not DEPC- treated) (#4387936, Invitrogen) to reach a total volume of PCR reaction equal to 25 µl. The Primer-BLAST program was used to check the specificity of the primer sets [<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>]. Relative gene expression ratios (RQ) between treated and control groups were calculated using  $\Delta\Delta Ct$  method [28].

### Histopathological examination

Right ovaries were fixed in 10% neutralbuffered formalin for 48 h at room temperature (RT) for histological and immunohistochemical studies. The specimens were manually processed through ascending concentration grades of ethyl alcohol (Sigma-Aldrich, USA), 70%, 90%, and 100% (3 changes, 15 min per change), cleared in xylene (#RXSOL-19- 1489-210RX, Marine International, India) 3 changes, 20 min each, infiltrated with paraffin (Histoplast PE, Thermo Scientific, #8330) 3 changes, 30 min each, at 60°C, and embedded in paraffin. Serial sections 5 µm thick were cut using a manual rotary microtome (HistoCore Biocut, 149BIO000C1, Leica Biosystems, USA) and one section from 10 serial sections was selected for the H&E stain. Sections were deparaffinized in xylene, rehydrated through descending concentration grades of ethanol (100%, 95%, 70%, 50%) 2 changes, 5 min each,

**Table 1.** PCR primer sequences and accession numbers of the studied genes.

Gene	Accession	
	Numbers <sup>a</sup>	Primer sequence (5' → 3')
NOBOX oogenesis ( <i>NOBOX</i> )	NM_001192013.1	F: AGGGTGTGAGAGGGTGGCAG R: GGCGATACTAGTCCCCAGGAC
Follicle stimulating hormone receptor ( <i>FSHR</i> )	XM_021218040.1	F: TGGAGGCGGCAAACTCTGAAC R: TCTGGCTTTGGCGAGCAGGTC
Nitric oxide synthase 3 ( <i>NOS3</i> )	NG_011992.1	F: TCTTGCTAACTCTGGCGCAC R: AAGAAGTGGCATCAAGCGGA
Glyceraldehyde 3-phosphate dehydrogenase ( <i>GAPDH</i> )	AB047300.1	F: GATGCTGGTCTGAGTATGTCG R: GTGGTGCGGATGCATTGCTGA

F forward, R Reverse, <sup>a</sup>NIH National Library of Medicine

and manually stained with Harris Hematoxylin (H9627, Sigma-Aldrich) and Eosin (E4009, Sigma Aldrich) [29]. Stained sections were cover slipped with permanent mounting media, Canada balsam (#101691, Merck, Darmstadt, Germany).

### Immunohistochemistry

Immunohistochemical (IHC) assay was done according to the method described by Kiernan [30] and manufacturer's kit protocol for the detection of expression of the DNA oxidative damage biomarker, 8-hydroxy 2 deoxyguanosine (8-OHdG), and the inflammation marker, inducible nitric oxide synthase (iNOS). The primary antibodies were mouse monoclonal anti-8-OHdG antibody [N45.1], IgG1, 50 µg size (#ab48508, Abcam UK), and rabbit recombinant multiclonal anti-iNOS antibody [RM1017] (IgG, 100 µg at 1.025 mg/ml #ab283668, Abcam). Formalin-fixed/paraffin-embedded sections were deparaffinized and rehydrated as previously described. Buffer washing was done between each step using PBS, pH7.4 (#97062-730, VWR). Antigen retrieval was done for 10 min in citrate buffer solution pH 6 (#C9999, Sigma-Aldrich) at 90 C. Peroxidase Blocking Reagent (S2023, Agilent, USA) was used for 60 min to remove endogenous peroxidase activity. The sections were incubated with the primary antibodies at dilutions 1:20 for 8-OHdG, and 1:2000 for iNOS, at RT for 1 h followed by the biotinylated goat anti-rabbit IgG secondary antibody (#ab64261, Abcam,) for 30 min. The primary antibodies were diluted using antibody diluent (#ab64211, Abcam). The IHC technique was performed manually inside a humidified chamber. After that, 3,3-diaminobenzidine (DAB) chromogen (GV825, Agilent) was applied to sections until brown color developed, rinsed, and counterstained with Mayer's hematoxylin (#26043-05; VWR). Positive expression for 8-OHdG was nuclear, and iNOS expression was cytoplasmic. The positive controls were mouse skin for 8-OHdG and human lung for iNOS and the negative control was replacing the primary antibody with phosphate buffer solution (PBS, pH7.4, #97062-730, VWR). Slide examination and image acquisition were done by light microscope (Leica DM3000) with a fixed camera (Leica MC 190 HD), and Microscope Software Platform

LAS X Life Science, all from Leica Microsystems, Germany

### Morphometric analysis

For morphometric analysis, 10 non-overlapping fields in three non-serial H&E and IHC sections from each right ovary (n = 8 rats per group) were evaluated. Sections were evaluated by an expert in the field blinded from the study groups. The analysis was performed at the Faculty of

Medicine, Cairo University, Egypt, using Leica Qwin

500MC image analyzer with Leica Qwin 500MC software, Windows version, (Leica Microsystems, Germany) installed on an IBM personal computer system, connected to a light microscope supported by a Panasonic video camera. This was used for counting the ovarian follicles in H & E sections at 100× magnification and calculation of the area percentage of 8-OHdG and iNOS positive expression in the IHC assayed sections at 200× magnification. Initial automatic calibration of the image analyzer was done to convert the pixels into measurement units (micrometer). Atretic follicles were identified if they contained ≥20 apoptotic granulosa cells, disarrayed granulosa cells, degenerated oocyte, and/or fragmented oocyte nucleus according to Nishi et al. [31]. The number of follicles in a particular stage of development represented their number per ovary. The results were presented as the mean number of follicles, in a certain stage, from eight ovaries per group.

Percentage (%) of follicular atresia = number of atretic follicles ÷ total number of follicles × 100.

## Statistical analysis

The results were presented as a mean ± SD. One-way ANOVA was used to detect significance and followed by the Tukey test for comparison between the means of the study groups. GraphPad Prism Version 8.0 for Windows 10, GraphPad Software, USA) was used to carry out the data analysis. Initial check of normality was done depending on the result of the Kolmogorov–Smirnov test. Statistical significance was considered at  $p < 0.05$ .

## Results

### Effect of quercetin and or sitagliptin on blood glucose and hormonal profile

The hormonal profile and blood glucose profile are presented in Table 2 and show a decrease ( $p < 0.001$ ) in the serum insulin, estrogen, and progesterone in the STZ-diabetic-rat-only group as compared to control

animals. Injection of DOX in the diabetic rats resulted in a larger decrease in the measured hormones when compared to the diabetes-only group ( $P < 0.001$ ) except insulin that was non-significant. Treatment by QCT and/or sitagliptin led to an increase in all hormones when compared to DOX-treated diabetic rats ( $P < 0.05$ ) and the combination of both showed a greater improvement ( $P < 0.001$ ) with complete restoration of the hormonal profile when compared to the control ( $P > 0.05$ ). However, the blood glucose level showed an increase in the diabetes-only group when compared to control ( $P < 0.001$ ) and a further increase was noted after DOX treatment when compared to diabetic-only rats ( $P < 0.001$ ). Both QCT and sitagliptin significantly decreased ( $P < 0.05$ ) blood glucose and normalization of blood glucose values was obtained in the QCT/SIT combination therapy when compared to the control ( $P > 0.05$ ).

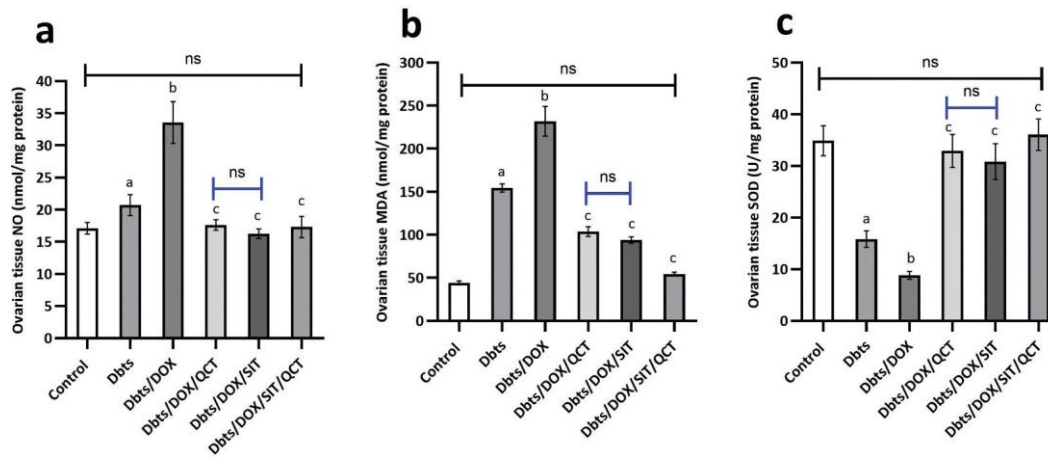
### Effect of quercetin (QCT) and or sitagliptin (SIT) on ovarian NO, MDA, and SOD activities

Results for NO, MDA, and SOD activity are seen in Figure 1. There was a large increase noted in the ovarian tissue levels of NO ( $P = 0.03$ ) and MDA ( $P < 0.001$ ) in the diabetic-only group when compared to the control. However, DOX injection in the STZ diabetic rats showed a bigger increase in both NO and MDA levels when compared to the STZ diabetic-only group ( $P < 0.001$ ). Treatment by quercetin and/or sitagliptin reduced NO and MDA levels when compared to DOX-treated STZ-diabetic rats ( $P < 0.001$ ) with no statistical significance between individual QCT and sitagliptin treatments ( $P > 0.05$ ). Complete normalization of both NO and MDA was noted in the QCT/SIT combination group as compared to control ( $P > 0.05$ ). However, the opposite was observed with a large decrease in SOD level in STZ diabetic-only animals in relation to the control ( $P < 0.001$ ), became even larger with a further decrease on DOX injection ( $p < 0.001$ ) as when compared to the

**Table 2.** Effect of quercetin and/or sitagliptin treatment on the serum estrogen, progesterone, insulin, and blood glucose levels of doxorubicin-treated streptozotocin-induced diabetic rats.

Experimental Groups	Estrogen (pg/ml)	Progesterone (ng/ml)	Insulin (μU/ml)	Glucose (mg/dl)
Control	23.71 ± 1.2	37.60 ± 1.97	1.86 ± 0.17	89.32 ± 3.42
Dbts	18.50 ± 1.05 <sup>a</sup>	28.82 ± 2.68 <sup>a</sup>	0.8 ± 0.25 <sup>a</sup>	271.9 ± 12.32 <sup>a</sup>
Dbts/DOX	12.62 ± 1.08 <sup>b</sup>	17.93 ± 0.94 <sup>b</sup>	0.63 ± 0.13	293.5 ± 9.95 <sup>b</sup>
Dbts/DOX/QCT	19.52 ± 1.43 <sup>c</sup>	35.12 ± 2.44 <sup>c</sup>	1.51 ± 0.2 <sup>c</sup>	107.8 ± 7.28 <sup>c</sup>
Dbts/DOX/SIT	20.06 ± 1.43 <sup>c</sup>	36.55 ± 1.57 <sup>c</sup>	1.27 ± 0.27 <sup>c</sup>	102.8 ± 10.05 <sup>c</sup>
Dbts/DOX/SIT/QCT	22.80 ± 1.16 <sup>cd</sup>	40.21 ± 2.19 <sup>cd</sup>	2.04 ± 0.24 <sup>cd</sup>	85.64 ± 3.73 <sup>cd</sup>

The results were presented as a mean of 8 rats ± SD, using one-way ANOVA followed by post hoc Tukey's test at  $p < 0.05$ . <sup>a</sup>Significant versus control. <sup>b</sup>Significant versus Dbts. <sup>c</sup>Significant versus Dbts/DOX. <sup>d</sup>Significant versus Dbts/DOX/QCT and Dbts/DOX/SIT. Dbts: streptozotocin-induced diabetes, DOX: doxorubicin, QCT: quercetin, SIT: sitagliptin.



**Figure 1.** Changes in the ovarian tissue concentrations of NO, MDA, and SOD after treatment by quercetin and or sitagliptin in the studied groups. The results were presented as a mean  $\pm$  SD,  $n = 8$  animals, using one-way ANOVA followed by post hoc Tukey's test. a: significant versus control, b: significant versus diabetes (Dbts) group, c: significant versus doxorubicin-treated diabetic (Dbts/DOX) group, ns: non-significant when comparing between quercetin (QCT) and sitagliptin (SIT) effect, at  $P < 0.05$ .

diabetic-only rats, and then the SOD level was completely restored on combined treatment QCT and SIT ( $P > 0.05$ ), in relation to the control.

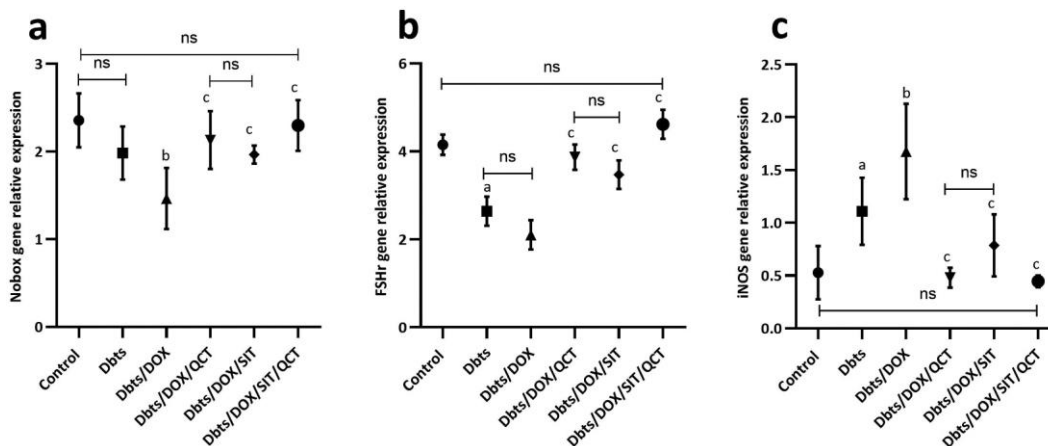
### Effect of quercetin and or sitagliptin on *NOBOX*, *FSHr*, and *iNOS* gene expression

The *NOBOX*, *FSHr*, and *iNOS* gene expression profiles are shown in Figure 2. A decrease in the mean values of the *NOBOX* and *FSHr* genes was shown in the STZ-induced diabetic-only group that was only statistically significant in *FSHr* gene expression as compared to the control ( $P < 0.05$ ) and an increase was noted in *iNOS* gene expression when compared to the control ( $P < 0.05$ ). However, further DOX administration in diabetic rats resulted in a marked

decrease in both *NOBOX* and *FSHr* as compared to the control ( $P < 0.05$ ). However, *iNOS* gene expression showed a marked increase in comparison to the STZ diabetic-only group ( $P < 0.05$ ). Treatments with quercetin, sitagliptin, or both combined resulted in an obvious reversal in expression of all three genes when compared to the DOX-treated diabetic rats ( $P < 0.05$ ), with normalization of the gene values in the combined QCT/SIT therapy group.

### Effect of quercetin and or sitagliptin on the ovarian histopathological changes

Light microscopic examination of H & E-stained ovarian sections in the control group showed normal ovarian microstructure. The ovarian surface was covered by



**Figure 2.** Changes in the *NOBOX*, *FSHr*, and *iNOS* genes expression profile after treatment by quercetin and or sitagliptin in the studied groups. The results were presented as a mean of 8 rats  $\pm$  SD, using one-way ANOVA followed by post hoc Tukey's test. a: significant versus control, b: significant versus diabetes (Dbts) group, c: significant versus doxorubicin-treated diabetic (Dbts/DOX) group, ns: non-significant comparison between the study groups, at  $P < 0.05$ .

a single-layered germinal epithelium separated from an underlying connective tissue tunica albuginea. The cortical stroma showed normal ovarian follicles at different stages of maturation. Each follicle was formed by an oocyte surrounded by a wall of follicular cells. The inner medulla appeared normal with regular vascularity separated by fibroblast and interstitial cells (Figure 3a).

The STZ diabetic-only group showed degenerative changes in all sections. Atretic follicles had loose detached granulosa cells with oocyte degenerative changes. There was a reduction in the number of healthy follicles with interfollicular degenerative changes (Figure 3b).

The DOX-treated diabetic group showed drastic histoarchitectural changes in the form of atrophic and degenerative ovarian changes. Cortical stromal edema denoted by widely separated spaces was evident (Figure 3c). Numerous follicular atresia (primordial, primary, secondary, and mature stages) was observed with dilated congested blood vessels (Figure 3c, d). The atretic follicles appeared to have degenerated, absent oocytes, and loose, detached granulosa cells with apoptotic nuclei. The corpus luteum was irregular with focal areas of degeneration and vacuolated granulosa lutein cells (Figure 3d). Completely degenerated corpora lutea were seen and appeared as ghosts of vacuolated granulosa cells with pyknotic nuclei (Figure 3c).

The quercetin (Figure 3e) and sitagliptin (Figure 3f) treatments caused a marked improvement of the histological structure of the ovary in all specimens. In the diabetic+DOX+sitagliptin-treated group, there were few atretic follicles, and numerous intact follicles were observed at different developmental stages with few interstitial vacuolar changes (Figure 3f).

In QCT/SIT combined therapy group (Figure 3g), there was almost complete restoration of the ovarian morphology that was very similar to the control section. Different stages of ovarian follicles were clearly evident with regular numbers and structure. Normal corpora lutea were seen, meanwhile, almost absence of atretic follicles was noticed.

### **Effect of quercetin and or sitagliptin on the 8-OHdG and iNOS expression**

#### **8-OHdG Immunohistochemical Assay**

The results for 8-OHdG showed diffuse negative nuclear expression in all the control group sections (Figure 4a). In STZ diabetic-only group, an obvious 8-OHdG positive nuclear expression of granulosa cells and interstitial cells was observed (Figure 4b, c). The DOX-treated STZ diabetic group has heavy positive 8-OHdG expression in the nuclei (Figure 4d,e). The DOX/SIT-treated diabetic group had a few nuclei with positive expression (Figure 4e). The

DOX/QCT-treated diabetic and DOX/SIT/QCT-treated diabetic groups had negative 8-OHdG expression (Figure f-h).

#### **iNOS Immunohistochemical Assay**

The IHC assay in the control group found that iNOS had few cytoplasmic expression limited to the germinal epithelium and follicular fluid (Figure 5a). However, in both the diabetes-only (Figure 5b) and DOX-treated diabetic groups (Figure 5c), there was a marked diffuse expression evident in the ovarian follicles and interfollicular stroma. The DOX/QCT-treated, DOX/SIT-treated diabetic and DOX/SIT/QCT-treated diabetic groups (Figure 5d-f) showed a little positive iNOS expression in follicular and stromal cells.

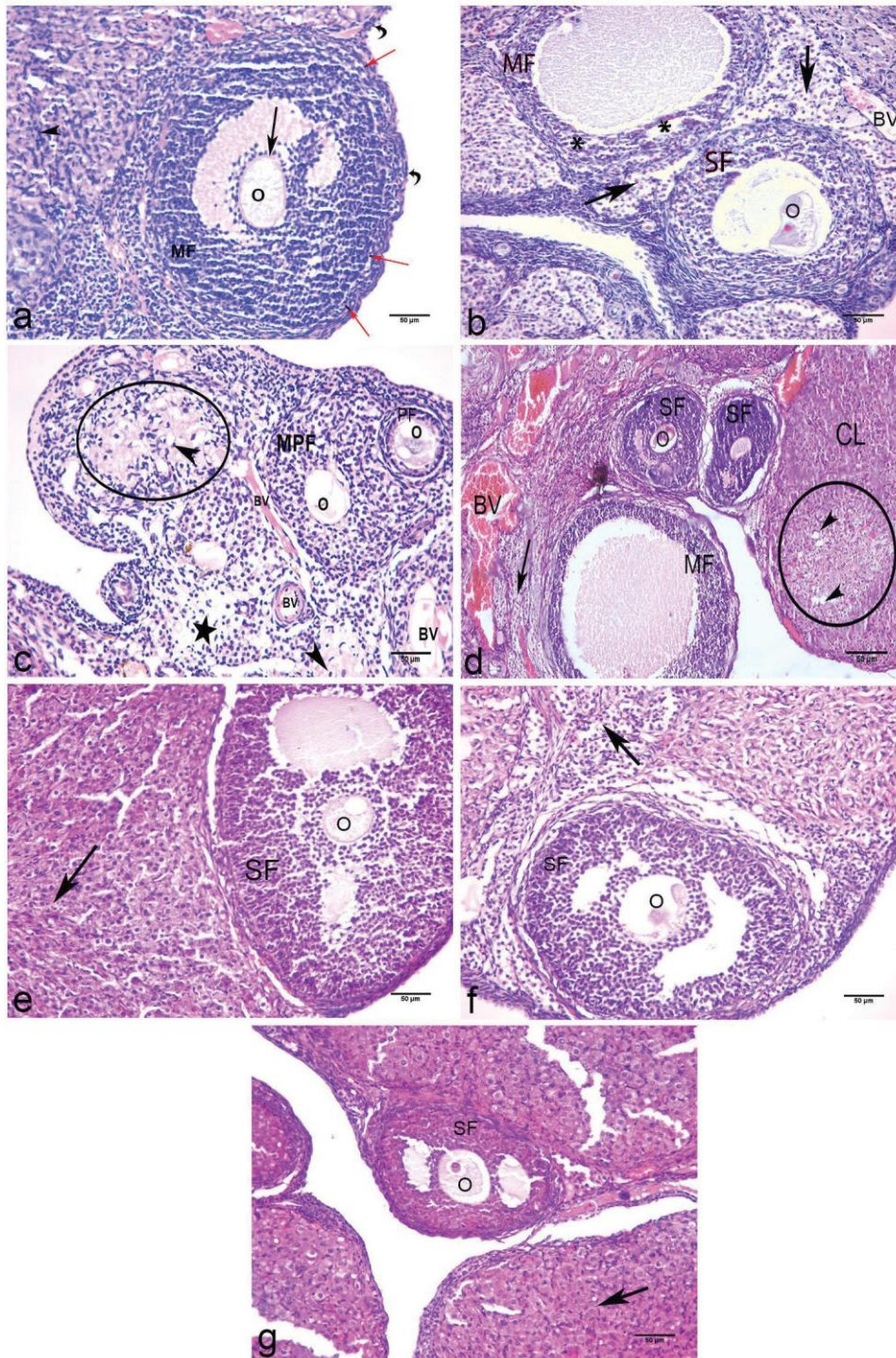
### **Effect of quercetin and or sitagliptin on the ovarian morphometric measurements**

There was a detrimental effect of STZ-induced diabetes on ovarian folliculogenesis that resulted in a significant increase ( $P < 0.05$ ) in the % of follicular atresia of the primordial, primary, secondary, and tertiary follicles as compared to the corresponding control follicles (Table 3). DOX injection in the STZ diabetic rats led to significant toxicity with marked follicular atresia. There was an increase ( $P < 0.05$ ) in the percentage of atresia of these follicles as compared to the diabetes-only group. Quercetin and/or sitagliptin treatment reversed ( $P < 0.05$ ) the percentage of atresia in all follicular stages (primordial, primary, secondary, and tertiary follicles) and became the least in the QCT/SIT combination therapy group.

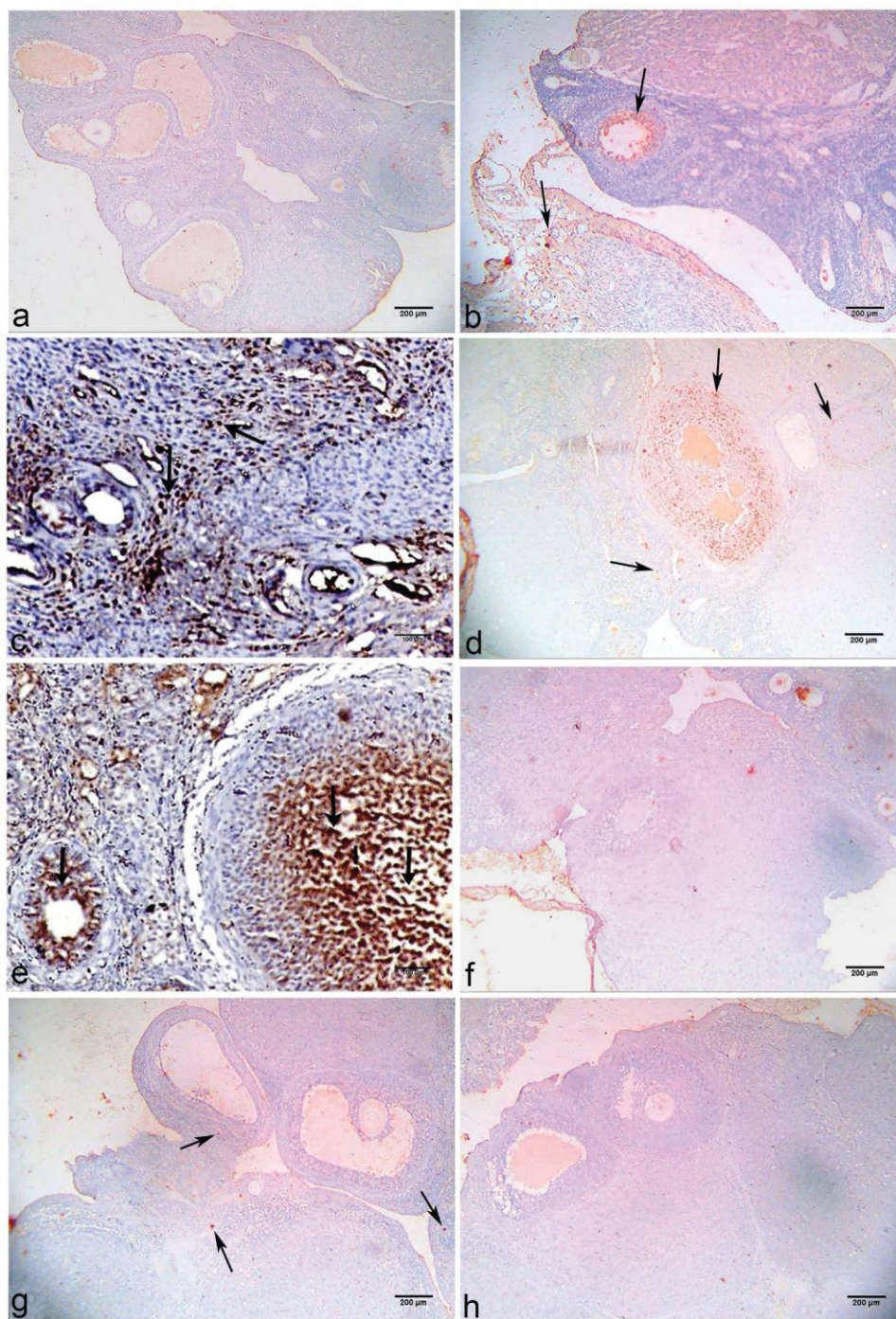
### **Area percentage comparisons for 8-OHdG and iNOS Immunoassays**

Anti-8-OHdG (Figure 6a) and anti-iNOS (Figure 6b) immunoassays, there was an increase ( $P < 0.05$ ) in the mean area percentage of both reactions in the diabetic-only group as compared to the control. Doxorubicin administration in diabetic rats resulted in an increase ( $P < 0.001$ ) in the measured parameters when compared to the diabetic-only group. Quercetin, sitagliptin, and their combined treatments showed a greater decrease ( $P < 0.001$ ) when compared to the DOX-treated diabetic group, but there was better restoration of the immunoreaction in the QCT/SIT combined therapy group when compared to the control ( $P > 0.05$ ). When comparing QCT and SIT treatments, there was no statistical significance in the mean area percentage for iNOS expression.

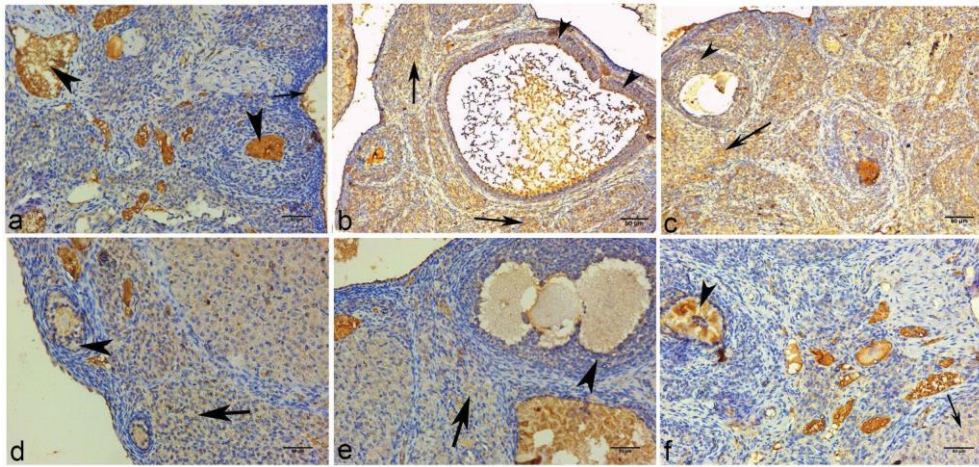




**Figure 3.** Photomicrographs from all experimental groups. H&E. (a) Control ovary shows normal germinal epithelium (curved arrows) with an underlying tunica albuginea (lines), mature follicle (MF) with intact oocyte (O), normal zona pellucida (arrow) and spindle-shaped interstitial cells (arrowhead). (b) STZ Diabetic-only group shows atretic secondary (SF) and mature follicles (MF) with degenerated oocyte (O) and lined by loose vacuolated granulosa cells (asterisks). There is a congested blood vessel (BV) and interstitial cells show vacuolar changes (arrow). (c-d) DOX-treated diabetic group shows a degenerated atretic primordial (PF), multilaminar primary follicles (MPF) with degenerated oocytes (O) and loose vacuolated granulosa cell lining. Corpus luteum (CL) shows focal degenerated areas (in circle). Some corpora appear completely degenerated leaving ghosts of vacuolated granulosa cells with pyknotic nuclei (arrowheads). Numerous congested blood vessels (BV), cortical stromal edema (star) with cellular infiltration is evident. (e) DOX/SIT-treated diabetic group (f) showing obvious improvement in the ovarian histomorphology with little interstitial cell vacuolar changes (arrow) in sitagliptin treated group (f). DOX/QCT/SIT-treated diabetic group (g) showing remarkable improvement of the ovarian cortical stroma with almost normal folliculogenesis. Scale bar = 50  $\mu$ m.



**Figure 4.** Immunohistochemical assay for 8-OHdG on ovarian sections from all experimental rat groups. (a) Control group shows 8-OHdG negative expression in nuclei. (b, c) Diabetic-only group shows highly positive expression in nuclei in granulosa and interstitial cells (arrows) and in (c) at high magnification in diabetic only group (white arrows). (d, e) DOX-treated diabetic group shows highly positive expression in nuclei in granulosa and interstitial cells (arrows) and in (e) high magnification. (f) DOX/QCT-treated diabetic group, the nuclei are 8-OHdG negative. (g) DOX/SIT-treated diabetic group has few nuclei with positive 8-OHdG expression (arrows). (h) DOX/QCT/SIT-treated diabetic group, the nuclei are 8-OHdG negative. DAB chromogen. Scale bar = 200 µm for a, b, d, f, g, h; Scale bar = 100 µm for c, e.



**Figure 5.** Immunohistochemical assay for iNOS in ovarian sections from all experimental rat groups. (a) Control group shows positive NOS expression limited to the germinal epithelium (arrow) and follicular fluid (arrowheads). (b) Diabetes-only group has marked iNOS expression in cytoplasm of granulosa (arrowheads) and interstitial cells (arrows). (c) DOX-treated diabetic rats shows diffuse extensive iNOS cytoplasmic expression in granulosa (arrowheads) and stromal (arrows) cells. (d) DOX/QCT-treated diabetic rats and (e) DOX/SIT-treated diabetic rats show a positive iNOS reaction restricted to follicular (arrowheads) and stromal (arrows) cells. (f) DOX/QCT/SIT-treated diabetic rats show iNOS expression limited to the cortical stroma, liquor folliculi (arrowhead) with weak expression in granulosa cells (arrow). DAB chromogen. Scale bar = 50 µm).

**Table 3.** The mean number of healthy (intact), atretic follicles, and percentage of ovarian follicular atresia in control and experimental groups after treatment with quercetin and/or sitagliptin.

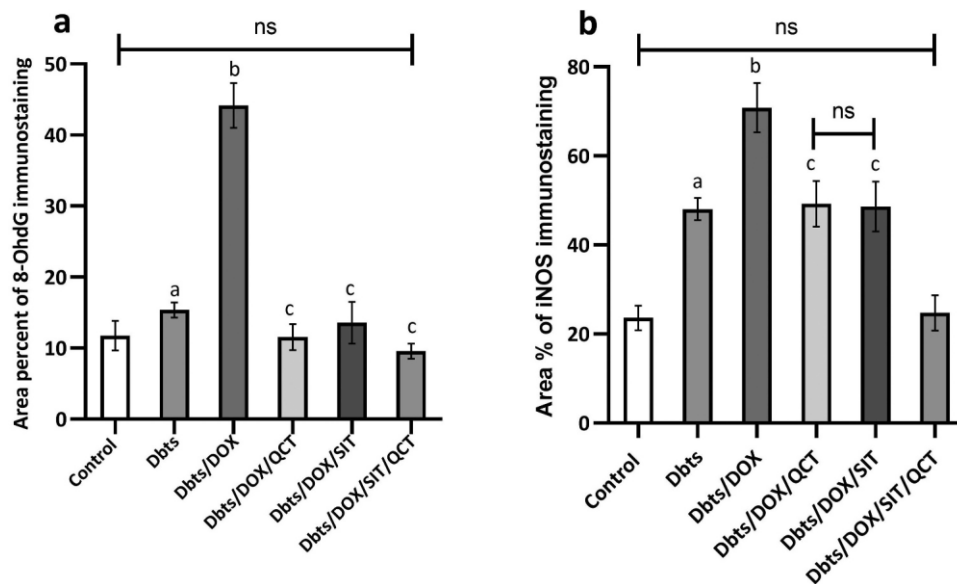
Follicles	Groups	Intact follicles (n)	Atretic follicles (n)	Atresia (%)
Primordial	Control	95.88 ± 3.46	11.69 ± 1.7	10.86 ± 1.52
	Dbts	63 ± 2.37 <sup>a</sup>	16.13 ± 1.7 <sup>a</sup>	20.37 ± 1.86 <sup>a</sup>
	Dbts/DOX	55.44 ± 3.18 <sup>ab</sup>	23.88 ± 2.31 <sup>ab</sup>	30.08 ± 2.18 <sup>ab</sup>
	Dbts/DOX/QCT	85 ± 2.99 <sup>abc</sup>	13.75 ± 1.98 <sup>abc</sup>	13.91 ± 1.89 <sup>abc</sup>
	Dbts/DOX/SIT	84.31 ± 2.68 <sup>abc</sup>	14.63 ± 1.5 <sup>ac</sup>	14.79 ± 1.53 <sup>abc</sup>
	Dbts/DOX/SIT/QCT	94 ± 3.98 <sup>bc</sup>	11.94 ± 1.29 <sup>bcd</sup>	11.26 ± 0.97 <sup>bce</sup>
Primary	Control	19.19 ± 1.76	4.69 ± 0.95	19.55 ± 3.05
	Dbts	14.19 ± 1.33 <sup>a</sup>	4.31 ± 1.45	24.59 ± 7.34 <sup>a</sup>
	Dbts/DOX	7.50 ± 1.21 <sup>ab</sup>	23.88 ± 2.31 <sup>ab</sup>	38.6 ± 4.78 <sup>ab</sup>
	Dbts/DOX/QCT	4.19 ± 0.83 <sup>c</sup>	13.75 ± 1.98 <sup>abc</sup>	20.83 ± 2.92 <sup>c</sup>
	Dbts/DOX/SIT	17 ± 2.94 <sup>abc</sup>	5.13 ± 0.89 <sup>c</sup>	23.55 ± 5.12 <sup>c</sup>
	Dbts/DOX/SIT/QCT	18.69 ± 2.15 <sup>bcd</sup>	3.75 ± 0.68 <sup>cd</sup>	16.74 ± 2.63 <sup>bcd</sup>
Secondary	Control	5.25 ± 1.06	1.37 ± 0.96	20.37 ± 13.01
	Dbts	3.19 ± 0.83 <sup>a</sup>	1.5 ± 0.82	33.8 ± 10.23 <sup>a</sup>
	Dbts/DOX	2.63 ± 0.89 <sup>ab</sup>	1.89 ± 0.81	41.67 ± 11.53 <sup>a</sup>
	Dbts/DOX/QCT	4.56 ± 0.89 <sup>bc</sup>	1.44 ± 0.81	22.57 ± 11.38 <sup>c</sup>
	Dbts/DOX/SIT	5.63 ± 0.81 <sup>bc</sup>	1.5 ± 0.97	19.71 ± 12.19 <sup>bc</sup>
	Dbts/DOX/SIT/QCT	5.13 ± 0.72 <sup>bc</sup>	1.44 ± 0.73	21.1 ± 8.63 <sup>bc</sup>
Tertiary	Control	4.06 ± 0.77	1.06 ± 0.68	20.22 ± 12.99
	Dbts	3 ± 1.7	1.38 ± 0.62	38.81 ± 27.95 <sup>a</sup>
	Dbts/DOX	1.06 ± 0.93 <sup>ab</sup>	2.38 ± 0.81 <sup>ab</sup>	71.88 ± 21.49 <sup>ab</sup>
	Dbts/DOX/QCT	3.06 ± 0.68 <sup>bc</sup>	1.5 ± 0.82 <sup>c</sup>	31.67 ± 15.76 <sup>c</sup>
	Dbts/DOX/SIT	2.25 ± 1.13 <sup>abc</sup>	1.19 ± 0.75 <sup>c</sup>	35.1 ± 20.38 <sup>c</sup>
	Dbts/DOX/SIT/QCT	5.25 ± 0.86 <sup>abce</sup>	1.38 ± 0.89 <sup>c</sup>	19.92 ± 10.72 <sup>c</sup>

The data were presented as a mean of 8 rats ± SD <sup>a</sup>Significant versus control. <sup>b</sup>Significant versus Dbts. <sup>c</sup>Significant versus Dbts/DOX. <sup>d</sup>Significant versus Dbts/DOX/QCT. <sup>e</sup>Significant versus Dbts/DOX/QCT and Dbts/DOX/SIT. <sup>f</sup>Significant versus Dbts/DOX/SIT, using one-way ANOVA post hoc Tukey test,  $p < 0.05$ . n: number, Dbts: STZ-induced diabetes, DOX: doxorubicin, QCT: quercetin, SIT: sitagliptin.

## Discussion

The current study displayed there was a significant deterioration of ovarian microstructure and function in the DOX-treated diabetic group as compared to the control and diabetic-only rats. Therefore, the study investigated

a putative protective effect of both quercetin and sitagliptin treatments either alone or combined against the DOX-induced ovarian toxicity in STZ diabetic rats and the possible involved mechanisms. Both quercetin and sitagliptin showed a notable decrease in blood glucose, increased serum insulin, and were equally effective in



**Figure 6.** Representative figure of the histomorphometric evaluation of the mean area percentage of (a) 8-OHdG and (b) iNOS immunostainings showing a significant increase in both diabetic-only and DOX-treated diabetic groups with a significant improvement after treatment and complete restoration in the combined therapy group. The results are presented as mean  $\pm$  SD, using one-way ANOVA followed by post hoc Tukey's test. a: significant versus control, b: significant versus diabetes (Dbts) group, c: significant versus doxorubicin-treated diabetic (Dbts/DOX) group, ns: non-significant when comparing between study groups, at  $p < 0.001$ .

mitigating DOX-induced ovarian histopathological alterations in diabetic animals and with complete restoration by the QCT/SIT combination.

In the diabetic-only group, there was a decrease in serum insulin and an increase in the blood glucose level as compared to the control animals. DOX-treated diabetic animals showed a higher blood glucose levels. The hyperglycemic state is a key element in oxidative stress induction as reported by Olawale et al. [32]. This was consistent with our study for the increased MDA and NO levels and the decreased SOD level in ovaries from both diabetic-only and DOX-treated diabetic groups that indicated a prooxidant/antioxidant imbalance. Accordingly, the increase in the percentage of follicular atresia with cortical edema and disorganization noted in H&E stained sections was greater in DOX-treated diabetic rats in relation to diabetic-only rats. Consistent with the results of Olawale et al. [32] and Niringiyumukiza et al. [33], these histological observations could be explained by the inflammatory and oxidative status elicited in the ovary of both animal groups and might be an explanation for the decrease in serum estrogen and progesterone levels as compared to levels in the control.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) is an indicative biomarker for oxidative DNA damage as demonstrated by Qing et al. [15]. In diabetic-only and DOX-treated diabetic groups, the histomorphometric evaluation of

anti-8-OHdG and anti-iNOS IHC assays showed an increase in mean area percentage of both markers, indicating there were inflammatory and oxidative DNA damage-mediated mechanisms that was greater in DOX-treated diabetic rats. Similarly, Mahmoud et al. [34] reported an oxidative DNA damage in diabetic rats indicated by an increase in serum level of 8-OHdG. In contrast, Mis et al. [35] showed a non-significant increase in serum level of 8-OHdG in diabetic rats. This discrepancy might be related to the STZ dose used and/or the study design. Nevertheless, Poetsch [36] mentioned oxidative DNA damage and DNA strand breaks that are mediated by most cancer medications. Doxorubicin was reported to induce gonadal toxicity in both genders [37]. These findings were supported by Morsi et al. [38] and Kim et al. [39] who both confirmed an excellent antiproliferative activity for doxorubicin, and reported multiple mechanisms for spermatogenic toxicity and DOX-induced ovarian damage.

At the gene level, our finding of increased mRNA expression of *iNOS* in diabetic rats that was greater after DOX injection was consistent and confirmed by IHC for iNOS expression. Also, the *NOBOX* gene was decreased in diabetic-only and DOX-treated diabetic rats but was statistically non-significant in diabetic-only animals when compared to the control. *NOBOX* is an important transcriptional controller of oocyte-specific genes that regulates the process of follicular development and differentiation [8]. Xiao et al. [40] and Spears et al. [41]

reported a similar dose-dependent toxic effect of doxorubicin on oocyte maturation and development with early-stage follicular depletion. Inconsistent with current results, the H & E findings demonstrated a significant follicular atresia in diabetic-only rats when compared to the control. From the authors' point of view, diabetes might interfere with either folliculogenesis via downregulation of transcription factors other than *NOBOX* or act through a different signaling pathway. This could explain the atretic changes seen on H&E sections despite a insignificant decrease in *NOBOX* gene expression in the diabetic-only group.

Granulosa cells are the main source of estrogen and a good supplier for progesterone when luteinization is initiated. The ovarian expression of the *FSHr* gene is crucial for the production of these hormones and has been involved in follicular development and ovulation [11,12]. In the current study, the mRNA expression of the *FSHr* gene was decreased in both diabetic-only animals and DOX-treated diabetic rats when compared to the control. So, the granulosa cell degenerative and apoptotic changes seen in H&E sections could be correlated with the decreased *FSHr* gene expression and decreased serum estrogen. However, Zhang et al. [11] showed an equivocal effect of DOX on mRNA expression of *FSHr* in a mouse model of DOX-induced ovarian toxicity. This difference might be explained by different animal species and could be related to different in vitro experimental design that utilized cultured granulosa cells and smaller doses of doxorubicin.

In DOX/quercetin-treated diabetic, DOX/sitagliptin-treated diabetic, and DOX/quercetin/sitagliptin-treated diabetic groups, the hormonal profile assay showed an increase in the serum estrogen, progesterone, and insulin when compared to the DOX-treated diabetic rats with complete restoration of the hormonal assay in the DOX/quercetin/sitagliptin-treated diabetic group. In correlation, the gene expression level of *FSHr* showed a parallel increase in these groups, with the best efficacy using the combination treatment. This confirmed the *FSHr* significance in estrogen and progesterone production, previously reported by Zhang et al. [11] and Kishi et al. [12]. Also, DOX/QCT-treated diabetic and DOX/SIT-treated diabetic groups showed an improved blood glucose and serum insulin levels with a better response in the QCT/SIT combination therapy group. Consistent with these observations, the histological findings displayed major improvement in the cortical stroma and ovarian folliculogenesis with the smallest percentage of follicular atresia in the QCT/SIT combination treated diabetic group. There was no statistical difference between QCT and sitagliptin in the ameliorative anti-inflammatory and antioxidant effects. The maximal

anti-inflammatory and antioxidant efficacy was seen in combination therapy group that was indicated by the maximal improvement of the ovarian concentrations of NO, MDA, and SOD. Moreover, the improvement in the iNOS, 8-OHdG immunoexpression in DOX/QCT-treated diabetic, DOX/SIT-treated diabetic, and DOX/QCT/SIT-treated diabetic groups was consistent with the histological and biochemical findings. This harmony indicated anti-inflammatory, antioxidant, and anti-DNA damage properties for QCT and SIT. In comparison to sitagliptin, QCT was superior in protecting DNA, and the best protection was in the combination therapy group.

Furthermore, the *NOBOX* and *iNOS* gene findings confirmed the above-mentioned observations. The same three QCT and SIT treatment groups showed an increased *NOBOX* gene expression as compared to the DOX-treated diabetic group and with a greater gene effect in the combination therapy group. However, quercetin and/or sitagliptin treatment decreased the *iNOS* mRNA when compared to the DOX-treated diabetic group with almost complete restorative effect in combination-treated diabetic animals.

Taken together, both quercetin and sitagliptin improved the DOX-induced functional and histological changes in diabetic rat ovaries and the combination of both QCT/SIT had an additional synergistic effect. The present study suggested anti-inflammatory, antioxidant, and anti-DNA damage activities as postulated mechanisms were elicited by sitagliptin. In addition, its hypoglycemic effect added to the antioxidant action due to abolishing hyperglycemia, a cornerstone in ROS generation and oxidative stress [32]. In contrast, a clinical study [42] demonstrated no change in the urinary excretion of 8-OHdG in sitagliptin-treated participants when compared to those teneligliptin-treated participants. Also, a more recent study [43] showed a similar finding in linagliptin-treated patients, a different DPP4 inhibitor. This suggests that the potential anti-DNA oxidative damage exerted by DPP4 inhibitors is context-dependent and may vary depending on animal or human participant, the methods used, the type of DPP4 inhibitor medication, dosage, preexisting clinical/drug history, and may be tissue-specific.

Quercetin alone was proven to have both anti-inflammatory and antioxidant capabilities as demonstrated in vitro and in vivo clinical and animal studies [44]. In addition, Carlos-Reyes et al. suggested that quercetin had anti-tumor activity and anti-DNA damage function by reversal of the epigenetic alterations associated with oncogene activation [45]. Quercetin could potentiate the anticancer function of doxorubicin and minimize its toxic effects in human breast cancer

cells as demonstrated by Li et al. [46]. Combining the effects of both quercetin and sitagliptin, in the QCT/SIT therapy group can account for the similar results to the control.

## Conclusion

The findings of this study revealed a gonadoprotective effect with individual administration of quercetin and sitagliptin against the ovarian toxicity induced by doxorubicin in the STZ-induced diabetic rat model. Most interesting was that the combination of quercetin and sitagliptin showed more curative leverage than individual drug treatment that could be due to the additive, synergistic actions of this combination through antioxidant, anti-inflammatory, and anti-DNA damage mechanisms. Furthermore, their antidiabetic properties made them the first treatment priority in diabetic cancer patients to control diabetes and to protect against DOX-induced toxicity at the same time. This consideration might decrease the burden of multiple drugs intake, thus improving the quality of life of cancer survivors.

## Data availability statement

All generated or analyzed data in the current study were included in the published version of the article and any query will be upon request to the corresponding author.

## Disclosure statement

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## Author contributions

Ahmed Morsi interpreted and wrote initial draft, figure legends, text descriptions of histological observations, and participated in the discussion. Eman Faruk participated in writing the paper, data search, preparing histological sections, and photomicrography. Engy Medhat carried out biochemical and gene studies, analysis, interpretation, and participated in manuscript writing. Neama Taha participated in the analysis of biochemical results, correlated research findings, and wrote the final draft of the paper. Usama Fouad collected literature data, performed morphometric study, analysis, and results correlation. All authors approved the final version of the manuscript.

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