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Macrophage-derived cytokine and nitric oxide profiles in type I and type II diabetes mellitus: effect of thymoquinone

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Abstract Comparing macrophage-derived cytokine and nitric oxide (NO) profiles in type I and type II diabetes mellitus (DM); and determining whether thymoquinone (TQ) has any modulatory effect were the main objectives of the present study. Peritoneal macrophages have been collected from Otsuka Long-Evans Tokushima Fatty (OLETF) as a model for type II DM and its control Long-Evans Tokushima Otsuka (LETO) rats, as well as from streptozotocin (STZ)-injected LETO ones as a model for type I DM. The cells were cultured and incubated with or without TQ (10 μM) in the absence or presence of lipopolysaccharide (LPS; 1 μg/ml). The same parameters have been also assessed in sera of the used animals with or without TQ treatment (3 mg/kg) under both LPS-stimulated (10 mg/kg) and unstimulated conditions. Nitrite, IL-1β and TNF-α were significantly higher in macrophage supernatants and sera of the acutely affected STZ-LETO rats either with or without LPS stimulation compared to corresponding controls. On the other hand, chronically diabetic OLETF rats' macrophage supernatants showed significant decreases of IL-1β and TNF-α levels upon LPS stimulation or even without stimulation (IL-1β); and insignificant increase in nitrite concentration, which turned significant upon LPS stimulation. Sera of these animals, however, showed significant increase in TNF-α level. TQ normalised the elevated nitrite and cytokine profiles both *in vitro* and *in vivo*, yet had no significant effect on the already decreased parameters in chronically affected OLETF rats. These data suggest that there is a tendency for macrophage inflammatory products to increase in acute type I and to decrease in chronic type II DM; and that TQ has the potential to normalise the elevated levels of these macrophage-derived inflammatory mediators.

Key words Diabetes • Macrophage • Nitric oxide • Cytokine • Thymoquinone

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Introduction

Macrophage activation and dysfunction underlie development of type I diabetes mellitus (type I DM; insulin-dependent diabetes mellitus, IDDM) and are associated with complications in type I and type II DM (non-insulin-dependent diabetes mellitus, NIDDM), respectively. Cytokines derived from macrophages, namely IL-1β and TNF-α, and free radicals, namely nitric oxide (NO), play a critical role in the development of IDDM in rats [1, 2]. These inflammatory products are the result of mononuclear cell infiltration, including macrophages and T lymphocytes into islets of Langerhans and initiation of insulitis process [3]. From another point of view, subjects affected chronically either by type I or type II of the disease

exhibit various forms of macrophage dysfunction. Studies on DP-BB rats, streptozotocin (STZ)-treated rats, and alloxan-treated mice as models for IDDM have revealed altered (mostly decreased) release of cytokines and growth factors from macrophages [4–6]. A few investigations have been performed on macrophages from animals with type II DM. Tissue macrophages from db/db mice displayed reduced phagocytosis and intracellular killing of microbes [7] as well as changed morphology during experimental inflammation *in vivo* [8]. Thymoquinone (TQ), the active principle extracted from the seeds' oil of the herbaceous plant *Nigella sativa* [9], was reported by El-Mahmoudy et al. [10] to suppress the expression of the inducible isoform of nitric oxide synthase enzyme (iNOS) and NO production by lipopolysaccharide (LPS)-induced peritoneal macrophages in normal rats. The oil of the plant and its major component, TQ, and nigellone, the polymer of the later, were evidenced to have a hypoglycaemic effect [11]. The present study was undertaken to compare the NO and proinflammatory cytokine profiles in recently STZ-induced type I DM and chronically spontaneous type II DM both *in vitro* and *in vivo* and assessing any possible beneficial modulatory role of TQ depending on the previous findings of its hypoglycaemic and anti-inflammatory properties.

Materials and methods

Animal models

Normal male Long-Evans Tokushima Otsuka (LETO) and spontaneously diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats, 50 weeks of age, specific pathogen free were used for the present investigation. The animals were kindly supplied by Tokushima Research Institute (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan). Upon receiving them, the rats were housed in our colony at 22°C, with a 12-h dark, 12-h light cycle and food and water freely available except during the time of experiments. Care and experimental procedures were approved by Gifu University, Animal Care and Use Committee and were in accordance with the Japanese Department of Agriculture guidelines and with the "Principles of laboratory animal care" (NIH publication no. 83–25, revised 1985). Freshly prepared STZ (Sigma, St. Louis, MO, USA) was used to induce type I DM in LETO rats as mentioned below.

General protocol and animal grouping

The effect of TQ (Aldrich, Milwaukee, WI, USA; purity 99%) was examined on macrophage cells isolated from acutely induced STZ-LETO and chronically affected OLETF rats as examples for type I and type II DM, respectively, as well as from LETO rats as control. Animals were allocated to the experimental groups as follows: the first group of LETO rats was injected with 65 mg/kg of STZ dissolved immediately before administration in pH 4.5 citrate buffer. The animals of the group were checked 48 h later for successful induction of diabetes; most of the injected animals exhibited severe hyperglycaemia of more than 500 mg/dl. The second group was the spontaneously diabetic OLETF rats; only those of glycaemia level around 200 mg/dl were used for comparison with the STZ-LETO diabetic rats. The third group was the control LETO rats which were administered only citrate buffer. STZ and citrate buffer injections were carried out after overnight fasting and through the intraperitoneal route. Each group of animals was further subdivided into subgroups treated or not with TQ (3 mg/kg) single dose daily for a total of 3 days; and stimulated or not with LPS (10 mg/kg; once just before sampling with 12 h). On the third day of treatment with TQ (the fifth from the start of the experiment) serum samples and peritoneal macrophages were collected from all animals and subjected to measurement of the parameters indicated.

Metabolic parameters

Glucose levels were measured in fresh blood directly using a glucose test meter (GT 1640, KDK Corporation, Kyoto, Japan) using the glucose oxidase method. Insulin levels were measured in serum where freshly collected blood samples were allowed to clot at room temperature and sera immediately separated by centrifugation at 1000 g and stored at -30°C. Insulin levels were determined using a commercially available ELISA insulin kit (Morinaga, Tokyo, Japan) in accordance with the manufacturer's instructions.

Macrophage isolation and culturing

After fasting overnight, animals were sacrificed and peritoneal macrophages were collected as described previously [10]. Cells were washed, re-suspended in fresh Dulbecco's modified Eagle's medium (DMEM), and seeded at $2x10^5$ cells in 200 µl per well of 96-well flat-bottom tissue culture plates. Cells were incubated for 2 h at 37°C, 5% CO_2, in a humidified chamber to allow macrophages to adhere and spread. Non-adherent cells were removed by washing of the wells 3 times by DMEM and the remaining adherent cells (>97% macrophages as assessed by morphologic examination) were used. The macrophage cells prepared for experimentation from control LETO, STZ-LETO and OLETF rats were then incubated either unstimulated or stimulated with LPS (1 µg/ml) for 24 (for NO and TNF-α) or 48 h (for IL-1β) in the absence or presence of 10 µM TQ dissolved in 10% DMSO. Culture-conditioned medium was collected at the appropriate times in fresh plates and stored at -30°C for assessment of nitrite and TNF-α but not IL-1β. The latter levels were assessed in macrophage lysates because substantial IL-1β is retained in the cell [12]. Macrophage lysates were prepared by 3 rapid cycles of freeze–thawing at -70 and 37°C, respectively and stored at -30°C. The 24 and 48 h incubation times were selected to correlate with the maximum productions for NO, TNF-α and IL-1β, respectively.

The viability of the cells was examined by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma), which is based on the ability of mitochondrial enzyme, succinate dehydrogenase, to cleave MTT to the blue compound formazan as previously described [10]. Insignificant difference in viability was observed among cells of all groups.

In vitro assessment of cytokines and nitric oxide

IL-1β and TNF-α concentration in culture supernatants were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits purchased from Amersham Pharmacia Biotech, UK and according to the instructions of the manufacturer employing a quantitative "sandwich" ELISA technique. Briefly, anti-rat monoclonal antibody (capture antibody) specific for the cytokine to be measured (IL-1β/TNF-α) was precoated onto the 96-well microtitre plate. All reagents were brought to room temperature and an assay diluent was added to each well. The standards or unknown samples were then added to the wells in duplicates. After a 2-h incubation period, the wells were washed 3 times with the provided wash buffer followed by addition of a second cytokine-specific biotinylated antibody (detection antibody). The plate was then incubated at room temperature for a further hour and the wash was repeated. This was followed by addition of HRP streptavidin conjugate solution and incubation for 30 min and then wash. A TMP substrate solution was then added to all wells and incubated for 30 min followed by the stop solution. Colour intensity was measured at 450 nm using ELISA microplate reader. A standard curve was prepared by plotting the optical density *vs.* concentration of the cytokine expressed as pg/ml. Values for the unknown experimental samples were determined *via* extrapolation.

NO, as a free radical, is relatively unstable in oxygenated solutions where it rapidly and spontaneously reacts with molecular oxygen to yield a variety of nitrogen oxides. It was demonstrated that the only stable products formed by spontaneous decomposition of NO in oxygenated solutions are nitrites, and upon long time exposure, nitrates. Thus nitrite was measured as an indicator for NO production using Griess reaction [13]. Nitrite level was measured spectrophotometrically (540 nm, MPR.A4i Microplate Reader, Tosoh, Japan) after mixing equal amounts (100 µl) of centrifuged culture supernatants and Griess reagent (solution of 1 part of 1.32% sulfanilamide in 60% acetic acid and 1 part of 0.1% naphthyl-ethylene-diamine HCl) in a 96-well plate and incubation for 10 min at room temperature in the dark. Nitrite concentrations were then interpolated from sodium nitrite (Sigma) standard curve (10–250 nM).

In vivo assessment of cytokines and nitric oxide

Serum samples collected from all experimental animals under stimulated or unstimulated condition have been subjected to cytokine and nitrite assessments. The used *in vitro* methods have been applied for such assessments; however, in the case of nitrite, the serum nitrates have been firstly reduced into nitrites using Aspergillus reductase enzyme (Sigma).

Statistical analysis

Tests were performed in duplicates; the inter- and intra-assay coefficients of variation of the assays did not exceed 7 and 4%, respectively. Data were expressed as means±SE. The diabetic STZ-LETO and OLETF rats were compared to normal LETO ones as control under the same corresponding condition either with or without TQ treatment under LPS stimulation or not. Statistical analysis was performed by the Student's *t*-test to express the difference between two groups. Multiple comparisons were carried out using one way analysis of variance (ANOVA) followed by Dunnett's test. Results in which the p value is <0.05 were considered significantly different.

Results

Characteristics of experimental models of diabetes

Fifty week-old LETO rats injected with STZ exhibited clinical signs, including polyurea and polyphagia and metabolic characteristics, including severe hyperglycaemia and hypoinsulinaemia relevant to IDDM. Age-matched OLETF animals exhibited gross appearance including over body weight and metabolic characteristics including moderate hyperglycaemia and hyperinsulinaemia with insulin resistance relevant to NIDDM (Table 1).

Table 1 General characteristics of LETO, STZ-LETO and OLETF rats and effects of TQ

	LETO		STZ-LETO		OLETF	
	Untreated	TQ-treated	Untreated	TQ-treated	Untreated	TQ-treated
Body weight, g	535.7±15	540.9±17	515.4±12	520.3±14	665.2±17	655.5±21
Blood glucose, mg/dl	115.7±8.3	112.8±7.5	530.2±21.5**	370.9±25.5†	210.9±16.7*	183.1±15.3
Serum insulin, ng/ml	1.53±0.16	1.68±0.12	0.6±0.1**	0.7±0.19	3.73±0.32**	3.23±0.4

Data are mean±SE. After overnight fasting, blood was collected during exsanguinations of the animals
*$p<0.05$; **$p<0.01$ *vs.* untreated LETO; $^{\dagger}p<0.05$ *vs.* corresponding untreated (n=5)

Interleukin-1β profile

As illustrated in Figure 1a, concentrations of IL-1β were significantly higher in cultures of peritoneal macrophages from STZ-LETO rats compared with those from controls. This result occurred either without or with LPS *in vitro* stimulation. Macrophages derived from OLETF diabetic rats showed significant decrease in IL-1β concentration compared with corresponding controls either without or with *in vitro* LPS stimulation. Figure 1b shows that the concentrations of IL-1β in sera were under the detectable level in normal control LETO and chronically affected OLETF groups. It was only detectable upon LPS stimulation in the control and OLETF groups; and in the STZ-LETO diabetic group either with or without LPS stimulation. By comparison, STZ-LETO group exhibited significantly higher levels of IL-1β, while OLETF rats showed significantly lower levels of the same cytokine compared to corresponding controls.

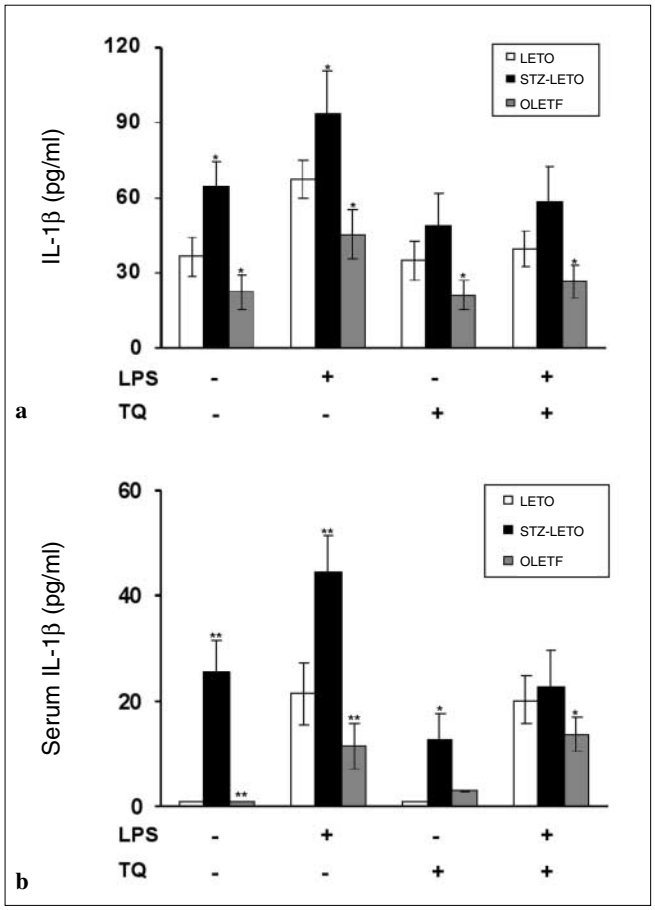

Fig. 1 IL-1β levels in culture supernatants of peritoneal macrophages (**a**) and sera (**b**) of LETO, STZ-LETO (65 mg/kg, i.p) and OLETF rats: **a** The cells are incubated with or without LPS (1 μg/ml) in the absence or presence of 10 μM TQ. IL-1β was measured by ELISA in the cell lysates after 48 h incubation. **b** Serum samples were taken from the same groups of animals either unstimulated or after LPS (10 mg/kg) stimulation, treated or not with TQ (3 mg/kg), intraperitoneally. IL-1β levels were measured directly using the same ELISA kit. *p<0.05 *vs*. control; **p<0.01 vs. control

Tumour necrosis factor-α profile

Concentrations of TNF-α (Fig. 2a) were significantly higher in cultures of peritoneal macrophages collected from STZ-LETO rats compared with those from controls. This result occurred either without or with LPS *in vitro* stimulation. Macrophages derived from OLETF rats showed an insignificant increase of TNF-α level in their culture supernatants in unstimulated conditions which turned to a significant decrease in the case of stimulation with LPS, compared with stimulated control.

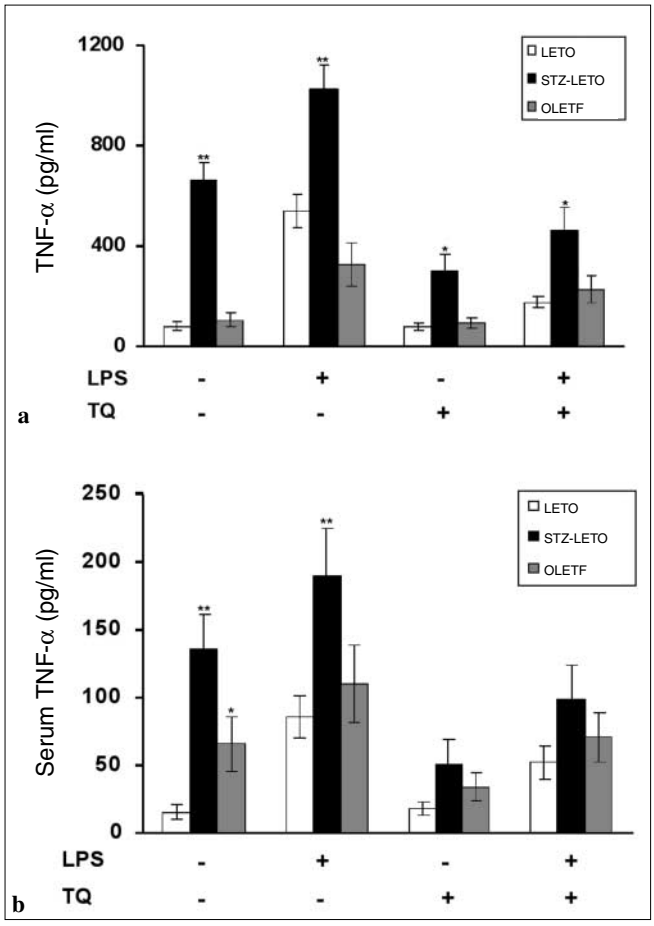

Fig. 2 TNF-α levels in culture supernatants of peritoneal macrophages (**a**) and sera (**b**) of LETO, STZ-LETO (65 mg/kg, i.p.) and OLETF rats: **a** The cells are incubated with or without LPS (1 μg/ml) in the absence or presence of 10 μM TQ. TNF-α was measured by ELISA in the cell culture supernatants after 24 h incubation. **b** Serum samples were taken from the same groups of animals either unstimulated or after LPS (10 mg/kg) stimulation, treated or not with TQ (3 mg/kg), intraperitoneally. TNF-α levels were measured directly using the same ELISA kit. *p<0.05 and **p<0.01 *vs*. control

As illustrated in Figure 2b, serum levels of TNF-α in STZ-LETO rats were significantly higher with or without LPS stimulation compared to normal controls. In contrast to macrophage culture data, OLETF group showed significant rise of serum TNF-α level without stimulation. Such rise turned insignificant upon LPS stimulation when compared with the corresponding stimulated control.

Nitric oxide profile

As shown in Figure 3a, unstimulated rat peritoneal macrophages released negligible amounts of nitrite. *In vitro* stimulation of macrophage cells with LPS resulted in release of relatively large amounts of nitrite. The levels of nitrite in culture supernatants were significantly higher in STZ-LETO diabetic macrophages either in unstimulated or stimulated conditions. OLETF rat-derived macrophage cells exhibited no significant difference between nitrite levels in culture supernatants in unstimulated conditions; however, stimulated diabetic macrophages showed a slight significant increase in nitrite level when compared with the stimulated control cells.

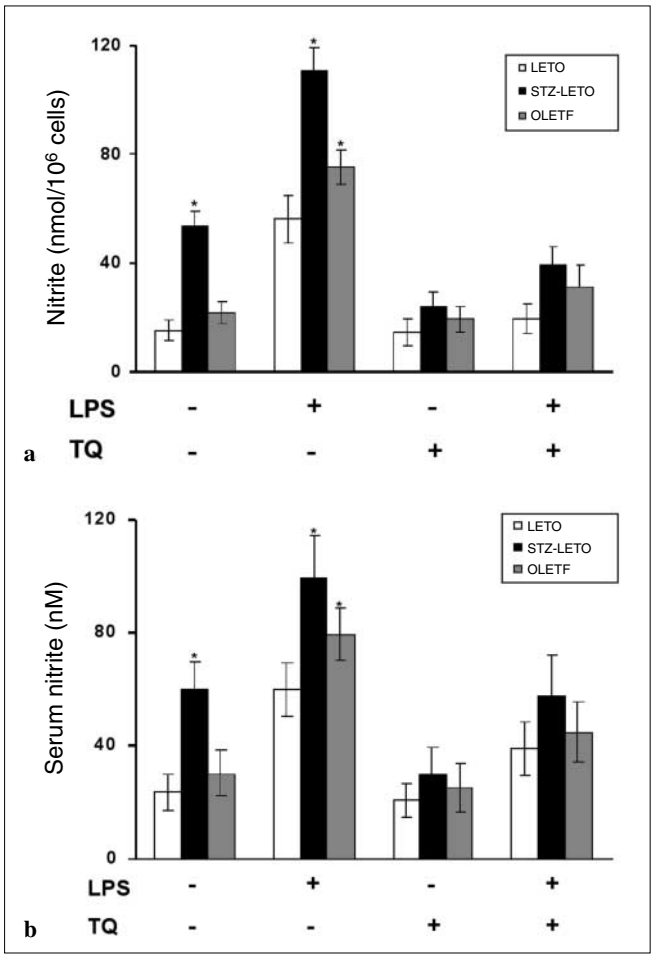

Fig. 3 Nitrite levels in culture supernatants of peritoneal macrophages (**a**) and sera (**b**) of LETO, STZ-LETO (65 mg/kg, i.p) and OLETF rats: **a** The cells are incubated with or without LPS (1 μg/ml) in the absence or presence of 10 μM TQ. Nitrite was measured by Griess reaction in the cell culture supernatants after 24 h incubation. **b** Serum samples were taken from the same groups of animals either unstimulated or after LPS (10 mg/kg) stimulation, treated or not with TQ (3 mg/kg), intraperitoneally. Nitrite levels were measured after reduction of nitrates with Aspergillus reductase enzyme and then using Griess reaction. *$p<0.05$ *vs*. control

Mean serum nitrite concentrations in control, STZ-LETO and OLETF rats are presented in Figure 3b. In unstimulated conditions, serum nitrite concentration was significantly increased in STZ-LETO rats; however, OLETF rats' serum nitrite was insignificantly higher than that of the corresponding control. Induction of NO production by intraperitoneal injection of LPS resulted in elevation of serum nitrite in all groups of rats. When the LPS-stimulated nitrite levels were evaluated together, a highly significant increase in STZ-LETO diabetic rats was found, while there was slight significant increase in OLETF rats compared to controls.

Modulatory effect of thymoquinone

TQ-treated STZ-LETO rat-derived macrophages exhibited significant effects towards the normal levels of cytokine profiles when compared with corresponding stimulated and unstimulated controls; in other words, the abnormally elevated productions of TNF-α and IL-1β recorded from macrophages due to STZ injection were significantly inhibited by TQ in either the absence or presence of *in vitro* LPS stimulation, and thus the development of diabetic condition could be abrogated. TQ had no significant effect on the already decreased IL-1β and TNF-α levels exhibited by OLETF rat-derived macrophages. Serum levels of IL-1β and TNF-α in TQ-treated groups showed parallel results to those of macrophage cultures where the elevated levels of IL-1β and TNF-α observed in STZ-LETO were normalised. While in the OLETF group there was no effect on the reduced levels of IL-1β but the significantly higher concentration of TNF-α turned insignificant upon treatment with TQ.

In comparison with macrophages derived from STZ-LETO rats, it could be stated that TQ was able to prevent the enhanced release of nitrite by macrophages due to the diabetic condition both in the absence and presence of LPS. Also, TQ significantly decreased the moderately elevated nitrite level in the presence of LPS and almost normalised its release in cultures of macrophages derived from OLETF rats. Serum nitrites in TQ-treated rats showed parallel results to those of macrophage cultures where the elevated levels of nitrites due to the diabetic condition were preserved at the normal range. It should be

mentioned that TQ had an insignificant effect on unstimulated negligible release of NO in control cells; however, it significantly decreased LPS-induced NO production in the same cells.

Discussion

In the present study we tried to compare macrophage secretory profiles in both induced acute type I and spontaneous long standing type II diabetes in the same species of animal; the point which may exclude any species-related effectors on the results. Also, the possible modulatory effect of TQ on the diabetic-induced alterations has been investigated.

Both types of DM are often associated with altered function(s) of macrophage system. This alteration may be in the form of hyperactivation as evidenced in onset of type I DM including release of excessive proinflammatory mediators and contribution to the process of insulitis [2–3, 14–21] or hypoperformance as reported in long-standing DM of both types including decreased secretory potential, phagocytosis and antigen presentation [5, 7, 22]. In the present study we investigated the hypothesis of improving the diabetic condition by TQ depending on its modulating effect on proinflammatory mediators.

In spite of the extensive use and established evidence of *N. sativa* oil [23] and TQ [11], respectively, in treatment of DM, there is not yet a report describing the possible modulating effect of the oil or its major component, TQ, on alterations in macrophage function associated with the disease. The current data showed that there is a general rise in the inflammatory mediators, namely NO, IL-1β and TNF-α, produced by macrophage cells at the developing phase of STZ-induced IDDM. TQ inhibited this elevated production by activated macrophage cells. Such effects may have a protective effect on β-cells from further damage by the autoimmune response that has been initiated after the toxic effect of STZ injection on β-cells. The indirect protection from this auto-attack may abrogate development of the diabetic condition with consequent limitation of the disease in a mild state. As previously described [10], the inhibitory effect of TQ on the NO production may be accomplished by inhibiting the transcription machinery of macrophage iNOS and probably of other cells. The mechanisms of TQ normalizing action on IL-1β and TNF-α remain to be investigated, however, it could be explained on the basis of the anti-oxidizing and free radical scavenging properties of TQ [24, 25], which may lead to the reduction of toxic effect of STZ on β-cells with consequent cytoprotection and lesser amounts of released β-cell intracellular proteins (autoantigens) and thus lower responsive proinflammatory cytokine release from macrophage cells therein.

On the other hand, the current data have shown that there is a tendency for proinflammatory mediators to decrease in both longstanding type II DM as well as in type I DM (unpublished data). The discrepancy between TNF-α levels in *in vitro* and *in vivo* experiments, where there is elevation in the latter, may be explained on the basis that, *in vivo*, there are other sources for TNF-α, especially adipocytes, which are enormous in the obese OLETF rats.

Diminished cytokine release from chronically affected macrophages may not represent a single defect in LPS-specific signalling pathways as the amount of IL-1β in diabetic cultures was also reduced when stimulants other than LPS were applied [26]. The tendency of the cytokine profile to decrease may, therefore, be attributed to the hypofunction of macrophages that occurs as a complication associated with the disease due to intracellular accumulation of the AGEs, which is a common consequence of long-standing hyperglycaemia. Macrophages as scavenger cells possess a number of receptor species capable of binding to and internalizing such products which are resistant to degradation [27, 28]. This might impede the normal cell function together with the modification of extracellular matrix proteins by conjugation with the AGEs, which could also disturb the communication between immune cells. However, the elevated NO expression observed in macrophages isolated from chronically affected type II diabetic OLETF rats may be also explained on the basis that the glycation products could possibly cause a relative decrease in the amount of free NO in tissues [26]; the factor which activates iNOS expression in macrophages and other types of cells as a compensatory mechanism. The formation of AGEs in long-standing DM may be reduced by TQ indirectly as a consequence of its hypoglycaemic effect; therefore, the activation of iNOS induction that is associated with the condition may be normalized upon quenching glycation products being formed. Further studies are needed to investigate such a possibility and whether there is a direct relation between TQ and AGEs. In addition, the direct inhibitory effect of TQ on iNOS expression and synthesis is another important explanation.

The anti-oxidizing and free radical scavenging properties exhibited by TQ may add positively to its cytoprotective action. These properties, in addition to those reported in this study, may render TQ very effective for protecting the pancreatic islets during the pathogenetic process of DM whether occurring spontaneously or experimentally by injection of STZ, which has been reported to accumulate in the pancreatic islets [29] where it may produce damaging NO and other reactive oxygen free radicals [30, 31]. These effects could be greatly reduced by intervention with TQ because of its beneficial anti-inflammatory and anti-oxidant potentials.

In conclusion, the results of the present study suggest that TQ may be effective in impeding pathogenetic processes occurring after STZ injection that lead to IDDM, apparently by reducing the toxic effect of STZ with consequent downregulation of autoreactivity towards β-cells mediated by NO, TNF-α and IL-1β. Also, it may have an ameliorating effect in NIDDM by normalizing extra NO and TNF-α concentrations.

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