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Effect of Nicotinamide on Experimental Induced Diabetes

Faris Q. Alenzi

*Department of Clinical Laboratory Sciences, College of Applied Medical Sciences,
King Saud University, Saudi Arabia*

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ABSTRACT

Insulin dependent diabetes mellitus (IDDM) results from irreversible loss of beta cells (β -cells) of the pancreas. A Streptozotocin (STZ)-induced diabetes in animal model mimics, in some aspects, recent onset IDDM. This study was conducted to investigate the effect of nicotinamide on experimentally-induced IDDM.

Thirty Sprague Dawley rats were divided into 3 groups; a control group, a diabetic group which received an intraperitoneal (i.p.) injection of 55 mg/kg STZ and a nicotinamide group (1g/kg/day) which were dosed orally for 3 days followed by (i.p.) STZ (55 mg/kg) with the nicotinamide treatment continuing for an additional 14 days.

Rats receiving STZ became diabetic after 2 weeks. This diabetic group showed hyperglycemia, and a very low level of C-peptide. Furthermore, pancreatic islets exhibited increased nitric oxide (NO) production together with an increased apoptotic index (as detected by TUNEL and electron microscopy). Nicotinamide treatment prevented STZ-induced diabetes, it also antagonized an increase in NO, and inhibited β -cell apoptosis. Fasting blood glucose, serum insulin and serum C-peptide were all within the normal range in the nicotinamide group.

The nicotinamide protection of β -cells may be facilitated via inhibition of apoptosis and nitric oxide generation. It is suggested that nicotinamide might be considered an effective agent for the prevention and treatment of IDDM in prediabetic, and early stages, of IDDM.

Key words: Apoptosis; Insulin dependent diabetes mellitus; Nicotinamide; Nitric oxide; Streptozotocin

INTRODUCTION

Insulin dependent diabetes mellitus (IDDM) is one of the most common chronic diseases and a major cause of

morbidity and mortality. It is manifested by a set of metabolic abnormalities, all of which are attributed to insulin deficiency. In diabetes, activation of the nuclear enzyme poly ADP-ribose polymerase (PARP) is an important factor in oxidative-nitrosative injury, which contributes to the development of experimental IDDM.

IDDM is likely to be associated with dysregulation of apoptosis or an increase in apoptosis. The non-obese diabetic (NOD) mice, a widely used model of human autoimmune IDDM, were used to establish the mode of

Corresponding Author: Faris Q Alenzi, PhD;
Associate Professor of Immunology and Consultant Immunologist,
Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, P.O. Box 422 AlKharaj 11942, Saudi Arabia. Tel: (+966 1) 545 3817, Fax: (+966 1) 545 4586, E-mail: fqalenzi@ksu.edu.sa

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beta-cell death (β -cell) responsible for the development of IDDM. It is a cytokine-mediated, T-helper cell and macrophage-dependent disease. Activated macrophages produce a variety of free radicals, nitric oxide (NO), and also produce interleukin-1 (IL-1). IL-1 β has been found to activate the inducible form of nitric oxide synthase (iNOS) and thus cause increased production of NO within the β -cell.^{1,2}

Nicotinamide (vitamin B3), a water-soluble vitamin, is a weak PARP inhibitor and a biochemical precursor of nicotinamide adenine dinucleotide (NAD). It has been shown to improve energy status in ischemic tissues,³ exhibit antioxidant properties and metabolic improvements^{4,5} and inhibit apoptosis.⁶ This makes it an attractive potential agent for the treatment of IDDM. Nicotinamide has no serious side effects, and plays a beneficial role in delaying the onset of IDDM in NOD mice⁷ and has shown promising results in humans.⁸ Treatment of prediabetic with nicotinamide improves diabetic metabolic alterations, most likely by counteracting beta-cell dysfunction and loss associated with oxidative stress.⁹ It is also not known whether the effects of nicotinamide on rodent beta cells can be reproduced in human β -cells. Protection was induced by giving the agent after the cells had been exposed to cytotoxic compounds such as Streptozotocin (STZ).¹⁰ Treatment with nicotinamide provides protection against free radicals and oxidative stress,^{11,12} improves neurological outcome and reduces infarct volume in transient and ischemia in vivo.^{13,14}

However, the exact mechanism of action of nicotinamide in diabetes is still under investigation. The main objectives of the present study were to evaluate the effect of nicotinamide in experimentally-induced diabetes, and to investigate the mechanism of action of nicotinamide especially aspects of β -cell, NO metabolism and apoptosis, using a variety of biochemical and histological approaches.

MATERIALS AND METHODS

Animals and Treatment

Thirty male Sprague Dawley rats weighing 200-250g were used in this study. Rats were obtained from the Jackson Laboratory (ME, USA) and were kept in metallic cages with good ventilation. Animals were fed a standard laboratory chow, with water and libitum.

Rats were divided into three equal groups, with 10 animals in each. The first group received a single intraperitoneal (i.p.) dose of 55 mg/kg b.w. (ref15) of the dia-

betogenic agent streptozotocin (STZ; Sigma, USA) which had been dissolved in citrate buffer at pH 4.5. The second group received a single i.p injection of citrate buffer and served as control group. The third group received oral nicotinamide (Sigma, USA) dissolved in distilled water, at a dose of 1 g/kg b.w. (ref15) given daily for 3 consecutive days, followed by a single i.p. injection of 55 mg/kg b.w. of STZ, after which oral nicotinamide administration continued for an additional 14 days.

Urine glucose content and body weight were monitored throughout the 14 days for each of the three animal groups. At the end of the treatment protocol, rats were fasted overnight, and then anaesthetized with sodium pentobarbitone (60 mg/kg, ip.). Blood samples were taken from the heart, and serum was separated by centrifugation at 3000g for 15 min, and then stored at -20°C until used. The pancreas from each animal was removed using the technique described by Lacy and Kostianovsky,¹⁶ and was then divided into three parts.

Isolation of Pancreatic Islets

Islets were isolated from one portion of the pancreas using a Ficoll gradient after collagenase digestion.¹⁷ Islet cells then were counted and plated at a concentration of 10^4 cells/ml into tissue culture dishes containing RPMI-1640 (Sigma, USA) culture medium supplemented with 10% fetal calf serum.

Histological Examination

The second portion of pancreas was fixed in Bouin's solution, embedded in paraffin and serial sections (4 μ m) were cut. The sections were stained with hematoxylin and eosin (H & E) and aldehyde fuchsin for the differentiation of β -and α -cells.¹⁸ To demonstrate apoptosis, sections were stained immunohistochemically using the terminal deoxy- nucleotidyl transferase deoxyuridine triphosphate nick end labeling method (TUNEL) (R&D, USA). TUNEL-stained apoptotic cells were counted and the apoptotic index was calculated as the number of apoptotic cells per 100 islets. Viable cells were identified by their intact nuclei with blue fluorescence; necrotic cells by their intact nuclei with yellow fluorescence (positive for fragmented nuclei with blue fluorescence).

Electron Microscopy

The third portion of the pancreas was immediately cut into small cubes and transferred to ice-cold fixation buffer (1.25% v/v glutaraldehyde in 0.1 mM cacodylate-HCl buffer, 0.1 M sucrose, and 2 mM calcium chloride;

pH 7.2) and prepared for transmission electron microscopy.¹⁹

Biochemical Analysis

Blood glucose was measured by the glucose oxidase method using Haemoglucotest strips (Boehringer Mannheim, Germany). Serum insulin was determined by Immulite Insulin (Diagnostic Products Corporation, Los Angeles) which depends on a two-site chemiluminescent enzyme-labeled immunometric assay²⁰ Serum C-peptide was measured by radioimmunoassay (Medgenix Diagnostics) as described by Kumar et al.²¹ Nitric oxide in islet cells cultured in vitro was determined spectrophotometrically by measuring the accumulation of its stable oxidized product, nitrite. Nitrite was estimated in cell culture by using Griess reagent and was quantified using sodium nitrite as standard.²² All chemicals and reagents were of pure analytical grade.

Determination of Apoptotic Cells by Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

Slides were air dried overnight, rehydrated in TBS for 15 minutes at room temperature and dried. The slides were covered by a 5 ml droplet of protein K diluted 1:100 in 10mM Tris (pH 8), incubated 5 minutes at room temperature then dipped three times into TBS and dried. The slide was covered with 100 µl of supplied equilibration buffer and incubated for 30 minutes at RT. Excess buffer was poured off and freshly prepared TdT labelling mixture (3 µl TdT enzyme in 57 µl TdT labelling reaction mix (Frag EL:Calbiochem, Nottingham, UK) was layered on to the cells. The slide was incubated at 37°C

in humidified chamber for 1.5 hour then washed x3 in TBS at room temperature. A coverslip was applied over mounting medium (Frag EL) and sealed with nail varnish to prevent evaporation. Apoptotic cells were scored by fluorescent light microscopy (494 nm).

Statistical Analysis

Statistical analysis was carried out using Microsoft Excel spreadsheet and the StatView SE +graphics software. The probability of a significant difference between groups was determined by Mann-Whitney U test and Wilcoxon Signed rank test. Graphs were plotted using Cricket graph graphics package. All software was run on a Macintosh computer.

RESULTS

Biochemical Results

The results illustrated in Table 1 and Figure 1 show that STZ injection (55 mg/kg b.w.) into rats induced severe diabetes. This was manifested as a significant elevation in fasting blood glucose (+ 146.1%), and significant reductions in serum insulin (-82.2%) and C-peptide (-90.5%). These changes were accompanied by a significant decrease in body weight (-24.9%) as compared with the control group.

Nicotinamide treatment (oral 1 g/kg b.w.) for 18 days (starting from 3 days prior to STZ injection), significantly prevented the diabetogenic effect of STZ as compared with the STZ group. In the nicotinamide treated group, body weight, fasting blood glucose, serum insulin and C-peptide were similar to the normal values seen in the control group (Table 1, Figure 1).

Table 1. Body weight, blood glucose, serum insulin, serum C-peptide and β-cell nitrite in the control and the treated rat groups.

Parameter	Control group	STZ group	Nic+STZ group
Body weight (g)	229.4±4	172.3±5.1*	227.3±4*
Blood glucose (mg/dL)	72.3±4	177.9±15.4*	81±3.4**
Serum insulin (uIU/mL)	25.3±1	4.5±0.6*	25±1.5**
Serum C-peptide (pmol/mL)	0.7±0.1	0.1±0.01*	0.7±0.1**
B-cell nitrite (pmol/10 cells)	33.7±3.2	161.5±5.5	40±4**

Values are means ± SEM; n=10

STZ was injected i.p. as a single dose of 55 mg/kg b.w.

Nicotinamide was administered orally 1 g/kg b.w. daily for 3 days prior to STZ injection and continued for additional 14 days.

* Significant at P<0.001 compared with the control group.

**Significant at P<0.001 compared with the STZ group.

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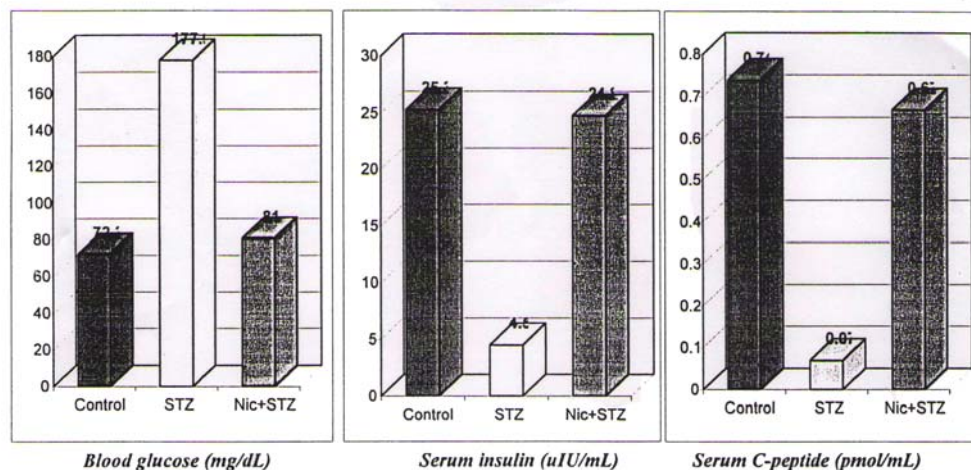


Figure 1. Blood glucose, serum insulin and serum C-peptide 14 days post STZ injection in various experimental groups. Values are means of 10 experiments.

STZ-induced diabetes was associated with a significant increase in the nitric oxide content in the β -cell culture (measured as nitrite). This increase was counteracted by nicotinamide treatment, as shown in Table-1. We found a significant negative correlation between β -cell nitrite and fasting serum insulin ($r = -0.9768$, $P < 0.001$) (data not shown). We also found a significant positive correlation between β -cell nitrite and fasting blood glucose ($r = + 0.9262$, $P < 0.001$) (data not shown). These results indicate that nitric oxide plays a central role in β -cell damage induced by STZ. This notion was further confirmed by histological and electron microscopic examinations of the pancreatic islets.

Histological Findings

i- Light microscopy

Pancreatic sections from the control group showed the cells of the islets of Langerhans arranged as anastomosing cords that were profusely supplied by fenestrated capillaries (Figure 2A). Aldehyde fuchsin stain showed the β -cells, in control group, characterized by specific fine red granules distributed in the cytoplasm and located mainly in the center of the islets forming the main mass of islets (Figure 2B). In STZ group, there was extensive degeneration of most of the β -cells, and this was manifested by the vacuolated cytoplasm, the absence of secretory granules, and furthermore, the nuclei were small and pyknotic (Figure 2C). Light microscopic examination of the third group of animals (nicotinamide+STZ group) showed no pathological changes and β -cells appeared normal (data not shown). Figure 2D shows normal β -cells on nicotinamide-treated rats on light microscopy.

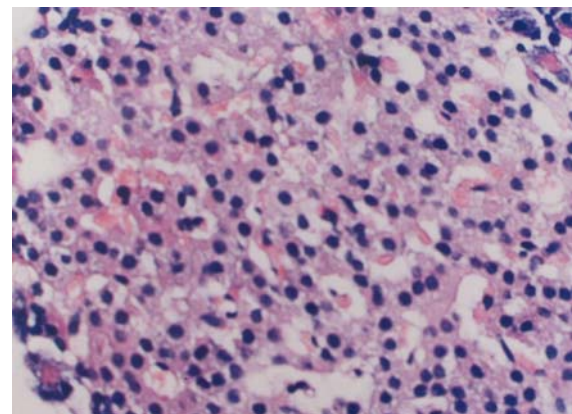


Figure 2A. A photomicrograph of islet of Langerhans of normal non-diabetic rat showing islet cells arranged as anastomosing cords profusely by fenestrating capillaries (H & E $\times 400$).

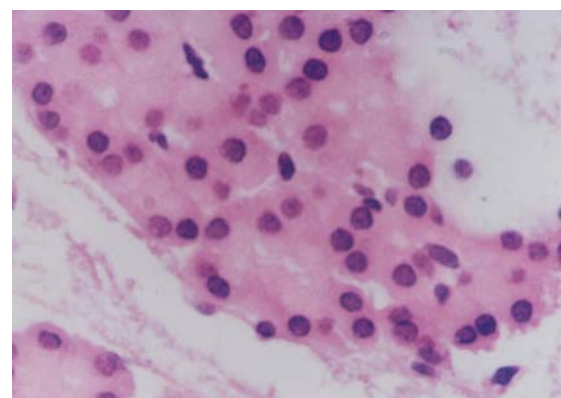


Figure 2B. A photomicrograph of islet of Langerhans showing β cells of control rat characterized by specific fine red granules distributed in the cytoplasm and located mainly in the center of islets forming the main mass of islets (Aldehyde fuchsin $\times 400$).

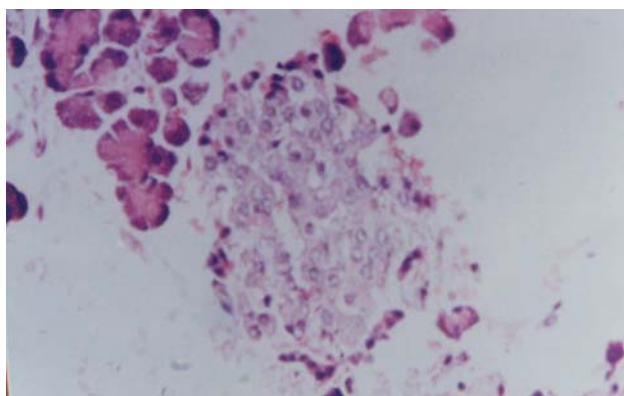


Figure 2C. A photomicrograph of islet of Langerhans of diabetic rat showing vacuolated cytoplasm, absence of secretory granules and the nuclei are small and pyknotic (H & E $\times 400$).

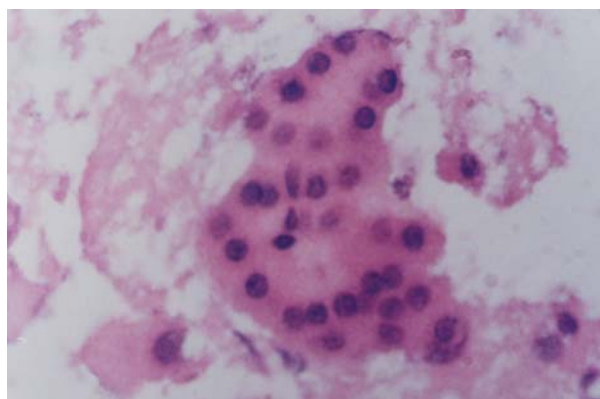


Figure 2D. A photomicrograph of islet of Langerhans of nicotinamide-treated group showing normal β cells more or less as the control (Aldehyde fuchsin $\times 400$).

ii- Electron microscopy

β -cells of both the control and nicotinamide-STZ groups appeared normal with a single nucleus surrounded by a cytoplasm which contained mitochondria, Golgi apparatus, endoplasmic reticulum and insulin secretory granules (Figure 3A).

On the other hand, β -cells of STZ group showed electron -translucent areas in the cytoplasm, nuclear pyknosis and indentation of the nuclear membrane. Furthermore, the nuclear membrane of some cells appeared dissolved with the chromatin content spilled into the cytoplasm. The cytoplasmic organelles showed severe pathological changes when the mitochondria were swollen; they had lost their cristae, and the vesicles of the Golgi apparatus were dilated. The secretory granules were fewer in number, and sometimes had even completely disappeared (Figure 3B).

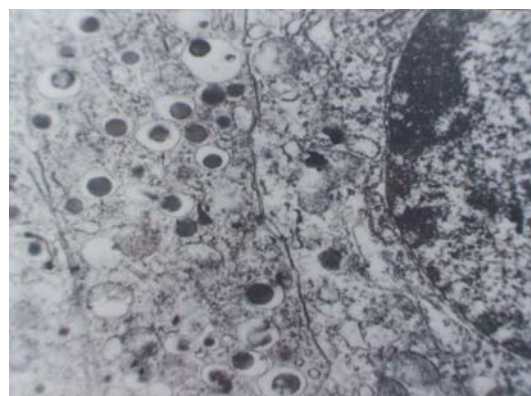


Figure 3A. Electronmicrograph of β cells of control rat showing the single nucleus surrounded by a cytoplasm containing mitochondria, Golgi apparatus, endoplasmic reticulum and insulin secretory granules.

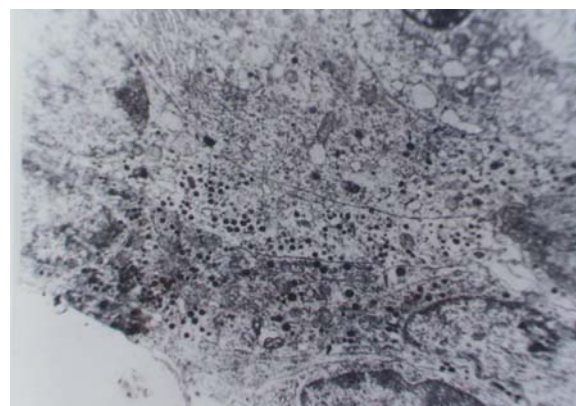


Figure 3B. Electronmicrograph of β cells of diabetic rat showing degenerating β cells, absence of secretory granules and degeneration of organelles.

iii- TUNEL immunohistochemistry:

Apoptotic cells appeared as blue-stained and were detected by TUNEL assay (Figure 4A). They were counted and the apoptotic index was calculated (Figure 4B).

The apoptotic index in the STZ (diabetic) group was 12.6 ± 1.5 , whereas the apoptotic indices of the control group, and of the nicotinamide-STZ group, were 1.8 ± 0.1 and 2.2 ± 0.2 , respectively. The STZ group showed a significant increase in apoptosis as compared with the control group ($P < 0.001$). There was no significant difference in β -cell apoptosis between the nicotinamide-STZ group and the control group.

Apoptosis index correlated with the rate of nitric oxide production in β -cells ($r = +0.922$, $P < 0.001$) (data not shown), thereby supporting our notion of a central role of nitric oxide in STZ-induced apoptosis.

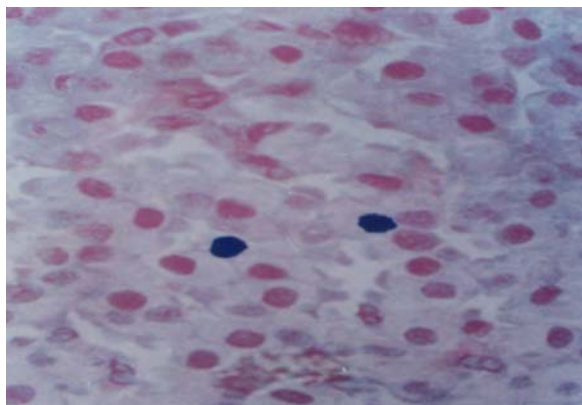


Figure 4A. A photomicrograph of islet of diabetic rat showing apoptotic β cells stained blue (TUNEL $\times 400$).

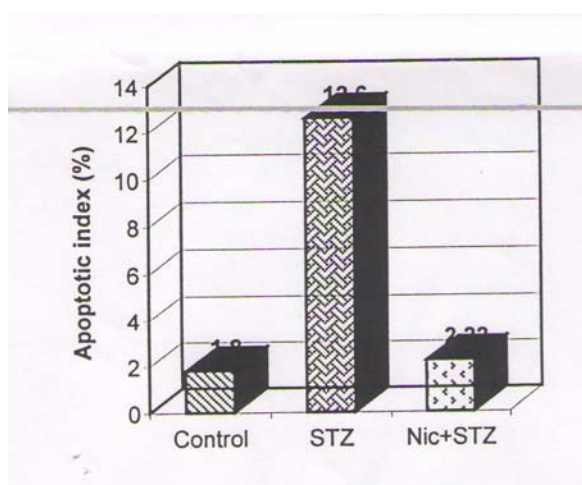


Figure 4B. Apoptotic index of β -cells in various experimental groups (expressed as mean number of apoptotic cells per 100 islets) 14 days post STZ injection. Values are means of 10 experiments.

DISCUSSION

STZ contains a nitroso moiety and can liberate NO which may, at least in part, be responsible for the STZ-induced damage of pancreatic β -cells of rodents.¹² Generation of free radicals, DNA strand breaks, activation of the PARP and depletion of intracellular NAD appear to be common factors in β -cell death, whether mediated by oxygen radicals, nitric oxide, or STZ.²³⁻²⁵

It has been demonstrated that, in a multiple low dose STZ model of IDDM, apoptosis of mouse β -cells was the mode of death in these cells.²⁵ In the present study, STZ (55 mg/kg b.w. single dose) was used to induce diabetes in rats. This was characterized by hyperglycemia, hypoinsulinemia, and a very low level of C-peptide. Moreover, TUNEL immunohistochemistry of pancreatic islets

showed enhanced apoptosis as compared to normal control rats. Electron microscopic examination confirmed the presence of apoptotic changes manifested as nuclear pyknosis, indentation of nuclear membrane, chromatin release into the cytoplasm, swollen mitochondria, dilation of Golgi apparatus vesicles and disappearance of secretory granules.

Type-1 diabetes has been accepted to be a cytokine-mediated, T-helper-cell and macrophage-dependent disease. Recruited macrophages are stimulated by IFN- γ to produce IL-1 β and TNF- α which, in synergy with IFN- γ , leads to β -cell toxicity via β -cell specific induction of NOS and apoptosis-activating pathways.²⁶ The present data demonstrated that STZ-induced diabetes was associated with an increased production of NO in β -cells.

NO has been implicated in β -cell death by inducing necrosis or apoptosis, or both. NO is known to activate Krebs cycle aconitase by nitrosylation of Fe-S groups, thereby preventing glucose oxidation and ATP generation, and thus leading to cell death by necrosis.²⁷ Moreover, NO can damage DNA through the induction of DNA strand breaks.^{26,27} DNA strand breaks may cause β -cell necrosis by themselves, or by the activation of DNA repair mechanisms, including the induction of the PARP, and thereby leading to depletion of cellular NAD and finally β -cell death.^{26,27}

In the present investigation, a significant positive correlation was found between NO production and the percentage of apoptotic cells in rat islets. NO generation has been suggested to be one of the mechanisms of STZ-induced diabetogenesis.²⁸ Besides being a strong alkylating agent that can directly damage DNA in β -cells, STZ contains a nitroso moiety that can liberate NO.²⁹ In an in vitro β experiment, NO was found to be generated from STZ in presence of ascorbate and Cu (II). Nicotinamide was shown to prevent this NO release through complex formation between nicotinamide and Cu (I) which is reduced by ascorbate.³⁰

Since the pancreatic islets of rats contain both ascorbate and copper in relatively high amounts relative to other organs, the induction of diabetes by STZ (and its protection by nicotinamide) has been attributed to NO generation, and to inhibition of NO generation, respectively.³⁰

In the present in vivo study, rats treated with nicotinamide (1g/kg b.w./day), 3 days prior to STZ and for 14 days after STZ, exhibited normal nitric oxide levels, together with a normal apoptotic index of β -cells. Further, nicotinamide prevented STZ-induced diabetes where

fasting blood glucose, serum insulin and C-peptide were all within the normal range. Thus, we believe nicotinamide protected β -cells against STZ by inhibiting NO-mediated damage. Nicotinamide may prevent islet cell nitric oxide production by inhibiting the expression of iNOS in β -cells. The role of oxygen radicals, generated during STZ-induced islet cell damage, as possible mediators of the expression of iNOS has already been suggested. NO is known to react synergistically with the superoxide anion, released by macrophages, to form a peroxynitrite anion and the far-more reactive hydroxyl radical which might play a major role in apoptotic cell death. Nicotinamide has proven to have free radical scavenging activity and consequently could well reduce DNA damage.³¹ In the present study, we feel the importance of the process whereby nicotinamide treatment inhibits β -cell apoptosis may be further supported by our finding that nicotinamide prevented the development of diabetes in a cyclophosphamide induced NOD mouse model, and that it did so by reducing β -cell apoptosis. As nicotinamide is involved in NAD biosynthesis, it can restore the islet cell content of NAD towards normality and can also inhibit the DNA repair enzyme, PARP, thereby preventing cellular NAD depletion and inhibiting apoptosis.³²⁻³⁴ Although nicotinamide can antagonize the damaging effects of STZ by suppressing glutathione depletion, the mechanism by which nicotinamide protects cells against apoptosis does not involve a reduction in oxidative stress.³⁵ It can be concluded that nicotinamide holds promise as a dietary supplement to help prevent disorders involving excessive apoptosis. As apoptosis represents the mode of β -cell death in STZ-induced IDDM, and nicotinamide effectively prevented it, we feel that nicotinamide (a readily available and regulatory approved natural substance) may be thus recommended as a promising candidate for the prevention and treatment of IDDM in prediabetic and early stages of IDDM in humans.

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