

The potential protective antidiabetic effect of inosine in type 1 diabetic mice

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ABSTRACT

Inosine –a naturally occurring purine- was long considered to be an inactive metabolite of adenosine. However, recently inosine has been shown to be an immunomodulator and anti-inflammatory agent. The aim of the present study was to determine whether inosine can affect the development of type 1 diabetes in mice. Type 1 diabetes was induced chemically by multiple low doses of streptozotocin. (MLDS). Mice were treated with inosine (100 or 200 mg/kg/day) and diabetes incidence was monitored. The effect of inosine on oxidative stress also was determined. The results showed that inosine reduced the incidence of diabetes in streptozotocin-induced diabetes and also decreased the oxidative stress. The purine exerts anti-inflammatory effects in the pancreas, which is its likely mode of action. The use of inosine should be considered as a potential preventive therapy in humans susceptible to develop Type 1 diabetes.

INTRODUCTION

It is well known that certain naturally occurring purines can exert a powerful modulatory effects on the immune system. The nucleoside adenosine is the best characterized of these purines and has been shown to affect almost all aspects of an immune response⁽¹⁻³⁾. Adenosine and its analogs can affect the development of a variety of inflammatory diseases including endotoxic shock⁽⁴⁾, rheumatoid arthritis⁽⁵⁾, pleural inflammation⁽⁶⁾, nephritis⁽⁷⁾, uveitis⁽⁸⁾, and colitis⁽⁹⁾. Adenosine's effects are partly mediated by the inhibition of deleterious immune-mediated processes, including the release of pro-inflammatory cytokines and free radicals⁽¹⁰⁾. Inosine is a naturally

occurring purine, formed from the breakdown of adenosine by adenosine deaminase⁽¹¹⁾.

Although inosine was widely believed to be inert, it had been demonstrated that inosine inhibits the release of pro-inflammatory cytokines and chemokines by activated murine macrophages⁽¹²⁾, and that such compound exerts a powerful -in vivo- anti-inflammatory effects in murine endotoxic shock^(12, 13), colitis⁽⁹⁾, septic shock⁽¹⁴⁾, and severe lung inflammation⁽¹⁵⁾. Inosine also has anti-inflammatory effects on human cells in vitro, reducing tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β production by monocytes and epithelial cells in response to lipopolysaccharide treatment, as well as inhibiting super oxide radical

production by activated human neutrophils⁽¹⁶⁾.

Type 1 diabetes is a disease characterized by the specific destruction of insulin-producing β cells in the pancreatic islets of Langerhans by the immune system⁽¹⁷⁾. The islet is invaded by immune cells, particularly macrophages and T cells, and these cells are cytotoxic to islet β cells, in part by generating cytokines and free radicals (18). It has been proposed that the insulinitis lesion is β -cell destructive when Th1 cytokines (IL-12, interferon (IFN)- γ , IL-1, and TNF- α) produced by islet infiltrating macrophages and T cells dominate over Th2 cytokines (IL-4, IL-10)⁽¹⁹⁾.

There are 2 murine models of autoimmune diabetes: the multiple low doses streptozotocin (MLDS) model and the spontaneous non obese diabetic (NOD) mouse model. The MLDS model of diabetes is characterized by a progressive hyperglycemia and insulinitis similar to that observed in human subjects with recent-onset type 1 diabetes^(20,21). The NOD mouse model also shares clinical, serological, and hist-immunological features with human type 1 diabetes⁽²²⁾. Both models have been used extensively to study the preventive therapies for type 1 diabetes^(22,23). A variety of procedures and therapies that delete, suppress, or modulate the functions of the immune system cells can block the autoimmune response against islet β cells and prevent β -cell destruction and may even reverse established diabetes in the NOD mouse^(24,25).

In view of the finding that inosine is a potent immuno-modulating agent^(9,12-16); the present study was

designed to test the potential effects of inosine in MLDS model of type 1 diabetes.

MATERIALS & METHODS

Materials

Reagents were obtained from the following sources. Streptozotocin and sodium citrate were obtained from Sigma (St. Louis, MO, USA). Insulin was measured by immunometric assay according to the protocol of Immulite (Diagnostic Product Corporation, U.S.A) using Immulite Automated Immunoassay Analyzer (Diagnostic Product Corporation, U.S.A) which automates the entire assay process.

Animals

The current work was done on 50 male mice, weighing 150-250 g and average 12 weeks old. They were classified into 5 groups, 10 mice in each group. Group 1 (control group), were given water orally (Vehicle) starting on day one. Group II was given inosine alone. Group III was treated with streptozotocin (40 mg/kg dissolved in citrate buffer, pH 4.5) intraperitoneally for 5 consecutive days. Groups IV and V were treated as group III and at the same time were treated every day starting on day one with inosine 100 and 200 mg/ kg/day respectively.

Blood glucose was measured on days 1, 7, 14, and 21. Hyperglycemia was defined as a non fasting blood glucose level ≥ 140 mg/dl.

Determination of Pancreatic Insulin and Malondialdehyde.

Insulin contents in pancreas of mice were determined from a pancreas biopsy, which was homogenized in acidified ethanol

(75% ethanol, 1.5% 12 mol/L HCl, and 23.5% H₂O), and then incubated for 72 h at 4 °C and centrifuged. Care was taken to remove biopsies from the same location of the pancreas (body) to avoid differences between the regions of the pancreas in regards to insulin content. The insulin content of the supernatant was determined using immulite insulin kit (Diagnostic Product Corporation, U.S.A). Pancreatic insulin content was expressed as ng insulin/mg protein, which was determined by the Bradford assay⁽²⁶⁾.

Malondialdehyde (MDA) content was determined from a pancreatic biopsy homogenized in 1.15% KCl buffer. Homogenate (200 µL) was added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 µL 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5), and 600 µL distilled H₂O. The mixture was incubated at 90 °C for 45 min. After cooling to room temperature, the

sample was cleared by centrifugation (10000 × g, 10 min) and absorbance was measured at 532 nm, using 1,1,3,3-tetra-methoxypropane as an external standard (14). Results were expressed as pmol MDA/mg protein.

Statistical Analyses

Data were presented as means ± SD. Statistical analysis was performed using the t-test as appropriate; *P* < 0.05 was considered significant.

RESULTS

Table 1 and Figure 1 showed that MLDS treatment induced a progressive hyperglycemia over a 21-day period (blood glucose ≥ 140 mg/dL) in mice. Inosine at 100 and 200 mg/kg/day significantly reduced the rise in mean blood glucose level at 21 day from 209.4±11.78 to 156.85±9.1 (*P* < 0.001) & and 127.6±9.3 (*P* < 0.001) respectively. Inosine alone had no effect on blood glucose (60.9±7.8).

Table 1: Blood Glucose Level (mg/ dl)

	Day 1	Day 7	Day 14	Day 21
Group I	61.3±6.4	55.2±5.4	50.5±5.7	49.05±5.1
Group II	61.1±6.13	59.6±8.2	60.7±7.9	60.9±7.81
Group III	61.35±6.4	126.6±16.32	153.35±11.2	209.4±11.78
Group IV	60.65±5.8	97.95±8.7	124.9±9.3	156.85±9.1*
Group V	60.1±5.17	80.05±7.65	118.3±8.7	127.6±9.3**

**P* < 0.001 significant difference between (group IV and III).

***P* < 0.001 significant difference between (group V and III).

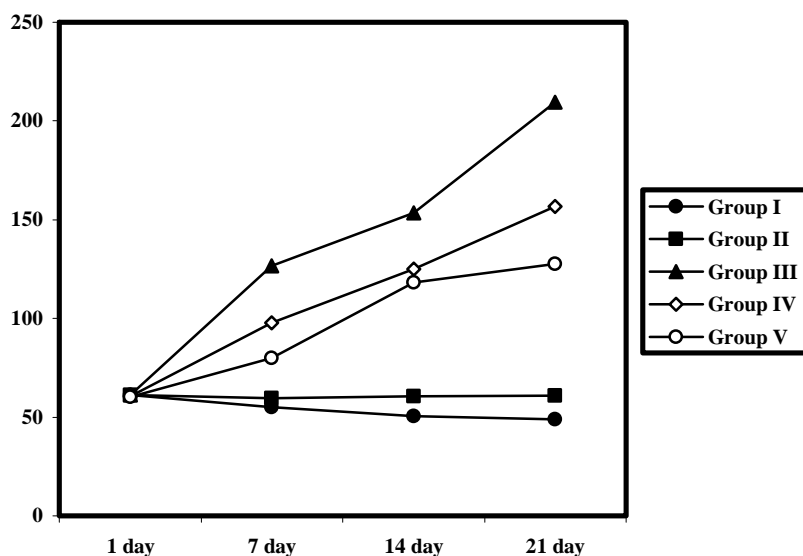


Figure 1: Daily treatment with inosine (100 or 200 mg/kg/day) for 21 day decreases hyperglycemia following MLDS treatment of mice on days 1 to 7.

Figure 2 & Table 2 showed that MLDS treatment significantly decreased the pancreatic insulin content in mice on day 21 from 79.8±8.65 to 16.5± 3.94 (P < 0.0003). This was significantly increased by inosine treatment (100 mg/kg /day and 200 mg/kg/day) from 6.5± 3.94 to

34.2±4.99 and 53.7±4.45 respectively (P < 0.0001 & 0.0004 respectively). Also, the effect of 200 mg/kg/day of inosine is significantly higher than the effect of 100 mg/kg/day of inosine on the pancreatic insulin effect (from 34.2±4.99 to 53.7±4.45) (P < 0.001).

Table 2: Pacreatic Insulin Content (ng insulin / mg protein)

	GROUP 1	IIROUPG	IIIGROUP	GROUP IV	GROUP V
MEAN	79.8	74.8	16.5*	34.2**	53.7***†
SD	8.65	7.64	3.94	4.99	4.45

* P < 0.003 significant difference between (III and I)

** P < 0.0001 significant difference between (IV and III)

*** P < 0.0004 significant difference between (V and III)

† P < 0.001 significant difference between (V and IV)

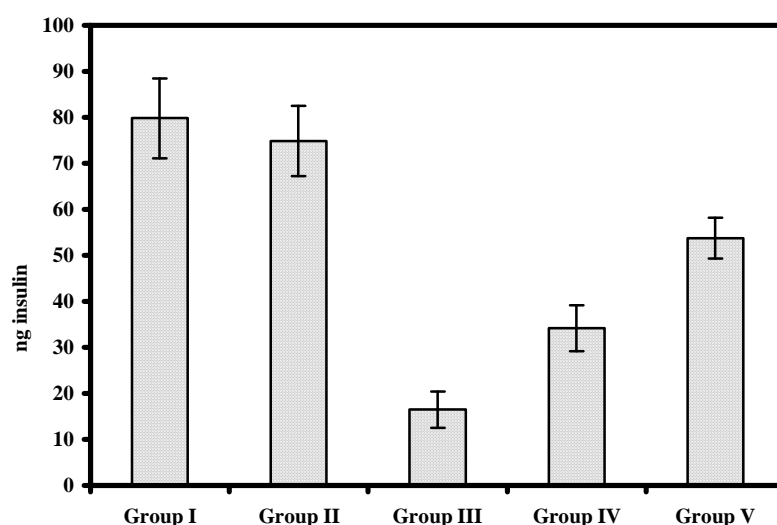


Figure 2. Inosine treatment attenuates the effects of MLDS treatment on pancreatic insulin content.

Table 3 and Figure 3 use MDA formation to quantify lipid peroxidation in the pancreas, a measure of oxidative stress showed that MDA was significantly elevated by MLDS from 204.9 ± 34.3 to 698.5 ± 9.47 ($P < 0.0001$) and the treatment with inosine 100 mg/kg/day

had significantly decreased the level of MDA from 698.5 ± 9.47 to 455.7 ± 8.3 ($P < 0.0002$). Increasing inosine dose from 100 mg/kg/day to 200 mg/kg/day had significantly more decreasing effect on the level of MDA. It decreased MDA level from 455.7 ± 8.3 to 413 ± 11.77 ($P < 0.001$).

Table 3: Pancreatic MDA (pmol/mg protein)

	Control (GP I)	Inosine 200mg/kg/day (GPII)	Streptozotocin +Vehicle (GPIII)	SZT+Inosine 100 mg/kg/day (GPIV)	SZT+Inosine 200 mg/kg/day (GPV)
MEAN	216.7	204.9	698.5*	455.7**	413***
SD	12.3	34.3	9.47	8.3	11.77

* $P < 0.0001$ significant difference between (IV and III)

** $P < 0.0002$ significant difference between (III and I)

*** $P < 0.001$ significant difference between (V and IV)

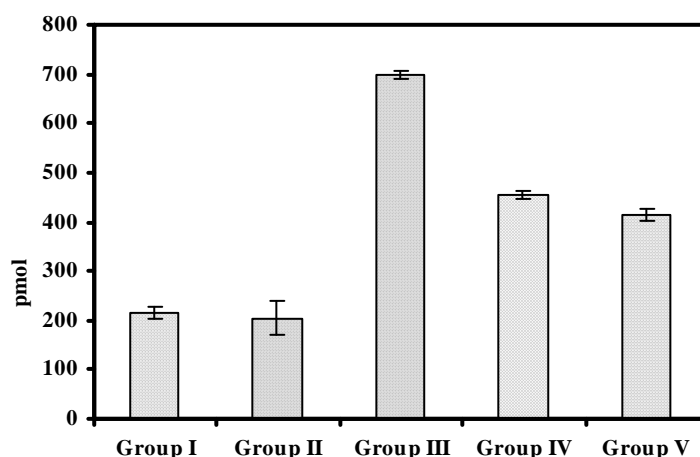


Figure 3. MDA formation was used to quantify lipid peroxidation in the pancreas.

DISCUSSION

The present study in the MLDS-induced model of type 1 diabetes demonstrated that inosine dose-dependently protects against hyperglycemia and significantly reduces the incidence of diabetes. Doses of inosine under 100 mg/kg/day appear to be ineffective in protecting against MLDS-induced diabetes. Previous studies showed similar effects on colitis⁽⁹⁾, endotoxic or septic shock^(12,14), and lung inflammation⁽¹⁵⁾. Inosine was effective only at dosages greater than 100 mg/kg/day. Previous reports revealed that inosine had marked anti-inflammatory effects, both in vitro and in vivo in acute inflammatory conditions^(9,12-16). Inosine reduced pancreatic infiltration by polymorphonuclear leukocytes and monocytes resulting in decreased pancreatic damage (lipid peroxidation) and a greater survival of

β cells. The effects of inosine appeared to be via a reduction of the pancreatic levels of the Th1 cytokines (IL-12, IFN- γ , TNF- α). However, inosine failed to reduce pancreatic levels of IL-1. The cytokines IFN- γ , TNF- α , and IL-1 all have been implicated in β -cell functional inhibition, destruction, and autoimmune diabetes^(18,28).

Also, IL-12 expression has been correlated with development of diabetes in the NOD mouse⁽²⁹⁾ and treatment of NOD mice with an IL-12 antagonist-suppressed diabetes development and decreased pancreatic expression of mRNA for IFN- γ ⁽³⁰⁾.

Previous -in vitro- experiments have demonstrated inhibition of Th1 cytokines by inosine, without any increase in Th2 cytokine production from macrophages stimulated with lipopolysaccharide⁽¹²⁾. In contrast, in endotoxemic mice inosine not only significantly reduced Th1 cytokine

levels but also increased the levels of Th2 cytokines⁽¹²⁾, suggesting that inosine's overall effect is a shift from a Th1- to a Th2-type cytokine profile.

A chemical form of inosine, inosine pranobex, has been reported previously to be partially protective against hyperglycemia in the MLDS-induced model of diabetes⁽³¹⁾. Based on the current results, we believe that the protective effects of inosine pranobex⁽³¹⁾ are related to the inosine component. It is noteworthy that in some countries inosine pranobex has been approved for the treatment of humans with various inflammatory conditions including hepatitis and arthritis, and in humans, the doses of the compound range from 3 g/d to 200 mg /kg/day^(32,33).

The effects of inosine on inflammatory cytokine and chemokine production were proposed to be mediated, at least in part, by adenosine-receptor related mechanisms⁽¹²⁾. Activation of the adenosine A2a receptor has been shown to down regulate inflammation and protect against tissue damage⁽³⁴⁾. Inosine treatment markedly reduced the early inflammatory reaction elicited by an islet xenograft and substantially prolonged the graft survival. Therefore, such an approach may form an important future component of therapeutic regimens applied in clinical islet xenotransplantation⁽³⁵⁾. Inosine may deserve further evaluation for its potential hyperoxic. Inosine treatment during hyperoxic exposure reduce damage to the pulmonary alveolar epithelium⁽³⁶⁾. Inosine reduced the severity of acute pancreatitis, suggesting a possible application of

that compound in the treatment of acute pancreatitis⁽³⁷⁾.

The use of specific inhibitors for that receptor subtype has demonstrated that inosine's inhibitory effect on inflammatory cytokine production is mediated in part by activation of the adenosine receptor⁽¹²⁾. It is also possible that inosine produces its inhibitory effects on cytokine production via binding to adenosine A3 receptors shown to be present on monocytes and macrophages⁽³⁸⁾. However, no specific rodent A3 receptor antagonist has been tested so far to investigate that possibility⁽¹²⁾. It is, also, possible that part of the anti-inflammatory effects of inosine are not mediated by adenosine receptor activation but, rather, by other mechanisms⁽¹²⁾. It has been established that production of proinflammatory cytokines can be regulated at the translational level. For example, tetracycline⁽³⁹⁾, metalloproteinase inhibitors⁽⁴⁰⁾, and polyamines⁽⁴¹⁾ all suppress the production of inflammatory mediators without affecting transcriptional events. The post-transcriptional nature of inosine's mechanism of action would be preferable to transcriptional inhibitors, because it would be expected to increase the window of therapeutic opportunity, and may remain effective even in a post-treatment paradigm.

Other data has, also, indicated that inosine at millimolar concentrations in vitro can inhibit the enzyme, poly (ADP-ribose) polymerase⁽⁴²⁾, an enzyme implicated in the pathogenesis of various forms of inflammation including autoimmune diabetes^(23,43). We do not

know whether the concentrations of inosine achieved in this study in vivo reached levels at which a significant inhibition of poly (ADP-ribose) polymerase could have accounted for the protective effects of inosine against diabetes.

Inosine has been shown to be more than just an "inactive" metabolite of adenosine, acting not only as an anti-inflammatory agent^(9,12-16), but also, preventing glial cell death during glucose deprivation⁽⁴⁴⁾, decreasing the release of intracellular enzymes from hypoxic lymphocytes⁽⁴⁵⁾, improving renal function during ischemia⁽⁴⁶⁾, and removing the harmful effects of total hepatic ischemia⁽⁴⁷⁾. Inosine, also, reduced the severity of acute pancreatitis, suggesting a possible application of that compound in the treatment of acute pancreatitis⁽⁴⁸⁾ and protecting against allergic encephalomyelitis⁽⁴⁹⁾.

As described above, the mechanism through which inosine exerts its anti-inflammatory effect is still unclear, and likely to employ multiple mechanisms. An additional mechanism, recently highlighted by studies in murine encephalomyelitis models, may be related to direct antioxidant effects: inosine is broken down in vivo to produce uric acid, and uric acid has potent antioxidant properties against peroxynitrite and other reactive oxygen species⁽⁴⁹⁾. Therefore, at least some of the therapeutic effects, particularly on the inflammatory processes and possibly islet cell damage could be mediated by the antioxidant action of uric acid. In fact, the protective effects of uric acid in a variety of models of

inflammation are now well established⁽⁵⁰⁾. Future testing of the effect of uric acid in the NOD model of diabetes may directly address that question.

Inosine is a safe, naturally occurring purine, which appears to be nontoxic to humans, even when ingested at high doses⁽⁵¹⁾. Inosine has recently been used in small patient populations for the therapy of multiple sclerosis⁽⁵²⁾. With an increasing body of pre clinical evidence showing that inosine is effective in a wide variety of inflammatory diseases, it may be worthwhile re-evaluating its therapeutic potential in humans suffering from a variety of inflammatory and autoimmune diseases.

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التأثير الوقائي للمضاد لمرض البوال السكرى (الانوسين) فى فئران مصابة بمرض السكرى من النوع الاول ناتج من جرعات قليلة متعددة من الستريبتوزوتوزين

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قسمي الكيمياء الحيوية¹ و الفسيولوجي² كلية الطب جامعة المنيا

كان الانوسين -أحد أنواع البيورين- يعتبر لفترة طويلة مادة خاملة ناتجة من التمثيل الغذائى للادينوسين. ولكن اكتشف حديثا إن الانوسين له تأثير مغاير على المناعة و مضاد للالتهابات. و معروف إن مرض البوال السكرى (النوع الاول) له أسباب مناعية. أذ يتميز النوع الأول من مرض البوال السكرى بتكسير لخلايا بيتا الموجودة في جزر لانجرهانز في البنكرياس بسبب الجهاز المناعى الذى يهاجم الجزر بالخلايا المناعية. هذه الخلايا سامة للجزر وذلك عن طريق إفراز السيتوكين والشقائق الحرة.

الغرض من هذه الدراسة هو بحث دور الانوسين فى وقاية خلايا البنكرياس من التأثير السام للجهاز المناعى على خلاياه باستخدام فئران لديها مستحثة تجريبيا بمرض البوال السكرى نتيجة لجرعات قليلة متعددة من الستريبتوزوتوزين (٤٠ مج/كجم/اليوم لمدة ٥ أيام متتالية) استخدم لهذا البحث ٥ مجموعات (كل مجموعة تتكون من ١٠ فئران). المجموعة الأولى الضابطة لم تعالج باى كيماويات. المجموعات الأخرى حقنت بعقار الستريبتوزوتوزين ثم عولجت المجموعة الثالثة بماء لمدة ٢١ يوما. المجموعة الرابعة عولجت بالانوسين بتركيز ١٠٠مج/كجم/اليوم والمجموعة الخامسة بالانوسين بتركيز ٢٠٠مج/كجم/اليوم لمدة ٢١ يوم. تم اخذ عينات من الدم لمعرفة نسبة السكر و كذلك عينات من البنكرياس لقياس كمية الأنسولين وثنائي الدهيد المالوت كمقياس للأكسدة.

أدى حقن الستريبتوزوتوزين بعد ٢١ يوم الي ارتفاع السكر بالدم بدرجة ذات دلالة إحصائية من ٥.١±٤٩.٠٥الى ٢٠.٩±١١.٧ مج/١٠٠سم^٢. وانخفض نسبة السكر بعد العلاج بالانوسين فى المجموعة الرابعة والخامسة انخفاضا ذا دلالة إحصائية الى ٩.١±١٥٦.٨٥ & ٩.٣±١٢٧.٦ مج/١٠٠سم^٢ على الترتيب. كما زادت كمية الانسولين فى خلايا البنكرياس بعد العلاج بالانوسين بدرجة ذات دلالة إحصائية من ١٦.٥±٣.٩٤ (نانوانسولين/ مج بروتين) فى المجموعة الثالثة الى

٤.٤٥±٥٣.٧ & ٤.٩٩±٣٤.٢ (نانوانسولين/ مجم بروتين) على الترتيب. كما انخفضت نسبة ثنائي الدهايد المالوت فى خلايا البنكرياس بعد العلاج بالايونسين بدرجة ذات دلالة احصائية من ٦٩٨.٥ ٩.٤٧ بيكومول/مجم بروتين فى المجموعة الثالثة الى ٨.٣±٤٥٥.٧ & ١١.٧٧±٤١٣ بيكومول/مجم بروتين على الترتيب.

نستنتج من هذا البحث ان الاينوسين له تاثير وقائى ضد مرض البوال السكرى من النوع الاول و ذلك عن طريق مقاومة تاثير الاكسدة التى تحدث فى خلايا البنكرياس . و لكن الديناميكية التى يعمل عن طريقها الاينوسين مازالت غير واضحة و متعددة . لذا تحتاج إلى مواصلة دراسة كيفية عمل الاينوسين حتى يمكن الاستفادة من الاينوسين فى علاج مرض البوال السكرى وكذا الأمراض المناعية الأخرى.