Pancreatic islet-cell viability, functionality and oxidative status remain unaffected at pharmacological concentrations of commonly used antibiotics *in vitro*

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Environmental factors such as diet, physical activity, drugs, pollution and life style play an important role in the progression and/or precipitation of diseases like diabetes, hypertension, obesity and cardiovascular disorders. Indiscriminate use of antibiotics to combat infectious diseases is one of the commonest forms of misuse of drugs. Antibiotics seem to have a correlation with diabetes and pancreatic function. There are controversial reports about the effect of antibiotics on the pancreatic islets; some suggesting their harmless action, some depicting a beneficial role and others indicating deleterious effect. Moreover, use of antibiotics is mandatory during islet isolation and cultivation to reduce incidences of microbial contamination. It is likely that antibiotic treatment may adversely affect islet viability and its functioning leading to failure of islet transplantation. The present *in vitro* study was undertaken to examine the effect of commonly used antibiotics such as gentamycin, penicillin, streptomycin, tetracycline, neomycin, erythromycin and chloramphenicol on islet viability, its functioning and induction of oxidative stress if any. The viability and insulin production data showed that none of the antibiotics used in the present study affect the viability and the functioning of the islets at their pharmacological concentrations. Free radical levels measured in terms of melonyldialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) reveal that except for a marginal increase in lipid peroxidation with tetracycline and slight increase in NO levels with streptomycin, none of these antibiotics affect the oxidative status of the cells. Antioxidant enzymes such as superoxide dismutase and catalase remain unaffected after this treatment. Our results reveal the innocuous nature of the antibiotics used at pharmacological concentrations, suggesting their safety whenever prescribed to combat infections and also during islet isolation procedures.

1. Introduction

Several genetic and environmental factors including infections have been known to affect the development, morbidity and mortality of diabetes mellitus. These factors may either enhance or prolong the progression of the diabetes. A wide variety of frequently prescribed medicines are known to cause glucose intolerance and/or to precipitate overt diabetes mellitus in non-diabetic individuals or to worsen glycemic control in subjects with established disease (Bressler and Defronzo 1994). Antibiotics form a major group among these commonly used medicines, to combat infectious diseases. Certain antibiotics are known to induce free radical generation (Vijaylakshmi *et al* 1992). On the other hand, certain others have been suggested to reduce the incidences of experimentally induced diabetes (Thelvaris *et al* 2000). Cyclophosphamide an alkylating cytotoxic drug is known for its ability to accelerate a number of autoimmune diseases including spontaneous diabetes in non obese diabetic (NOD) mice

Keywords. Antibiotics; diabetes; pancreatic islet-cell

Abbreviations used: DTNB, 5,5-dithiobis-2-nitrobenzoic acid; DTZ, dithiozone; FCS, foetal calf serum; GSH, glutathione; KRBH, Krebs–Ringer bicarbonate buffer (pH 7·4) with HEPES; MDA, melonyldialdehyde; NO, nitric oxide; SOD, superoxide dismutase; STZ, streptozotocin.

(Albamunits *et al* 1999). Formycin-A augments insulin release evoked by glucose, (Malaissie *et al* 1996). Gramicidin-D, a broad-spectrum antibiotic is identified as an insulin secretagogue in mouse *b*-cell line TC3. This induction of insulin secretion is mediated via promotion of Ca^{2+} influx (Dibas *et al* 1995). Some other antibiotics or their derivatives are known to improve *b*-cell function of the transplanted islets (Hiramatsu *et al* 2000). In case of acute pancreatitis, prophylactic antibiotics appear to be helpful in avoiding, delaying and/or lessening secondary lepsis. The antibiotics exert differential effects on the different tissues depending upon the various factors. An antibiotic may altogether have a different effect *in vitro* than what is seen *in vivo*. Gentamycin, is known to exert its nephrotoxic and ototoxic effect by free radical generation (Song *et al* 1997). However islets cultured in the Hams F-12 containing gentamycin were unaltered with respect to their viability and functioning (White *et al* 1997). Gentamycin, in certain other cases is claimed to reduce insulin release in the absence or presence of increased glucose without affecting further glucose metabolism (Boschero and Delattre 1985). Antibiotics like oxytetracyclin are known to affect the morphology of rat pancreas (Lorenzo *et al* 1999). Oxytetracycline treatment on the other hand did not affect the functionality of pancreas (islets in particular) in case of lean and obese mice (Dalpe-Scott *et al* 1983). Antibiotics also show concentration dependant effect on the islet functionality. Injection of tetracycline hydrochloride at the dose of 25 mg/kg body weight in mice did not change the functional activity of the *b*-cells and the rate of insulin formation and secretion for 10 days. Higher doses of the same antibiotic applied *in vivo* (100 mg/kg body weight) and *in vitro* (100 μM for 2 h) inhibited glucose stimulated insulin secretion but exerted no effect on insulin biosynthesis (Poltorak 1984). Pentamide was tolerated at the dose of 7·5 mg/day whereas severe, relapsing and eventually lethal hypoglycemia was observed within a few days with a dose of 15 mg/day (Assan *et al* 1993).

Antibiotics are also used in culture media for cultivation and maintenance of islets. Islets from rodents are routinely cultured in the medium containing 100 μg/ml streptomycin and 100 U/ml penicillin for 80 days duration of culture without affecting their viability and functionality (Beurer and Noe 1985). Islets cultured in the medium with penicillin, gentamycin and amphotericin B were free of any bacterial contamination. None of the antibiotics compromised stimulated insulin release (White *et al* 1997).

In the light of such a contrasting effects of the antibiotics on the various tissues, including endocrine pancreas, *in vitro* as well as *in vivo* we took up the present study to find out whether antibiotic treatment to islets *in vitro* affect: (i) their viability and functionality in terms of exerting direct cytotoxic effect if any, compared to that obtained by streptozotocin – a known diabetogen – treated islets; (ii) whether any oxidative stress is observed, in terms of levels of lipid peroxidation measured by the melonyldialdehyde (MDA) formed, the levels of nitric oxide (NO) generated and the amounts of reduced glutathione obtained; (iii) whether any changes in antioxidant enzyme profiles are brought about with respect to the activities of superoxide dismutase (SOD) and catalase.

Therefore in order to ascertain the role of antibiotics on the endocrine pancreas, we examined the effect of pharmacological concentrations of some commonly used antibiotics viz. gentamycin, penicillin, streptomycin, tetracycline, neomycin, erythromycin and chloramphenicol on the viability, functionality, oxidative status and activities of various antioxidant enzymes viz. SOD and catalase on the islets of Langerhans, *in vitro*. We compared the results obtained with *b*-cell toxin streptozotocin treated islets to reveal the degree of damage obtained.

2. Materials and methods

2.1 *Islet isolation*

Islets were isolated from six to eight week old Balb/c mice (Animal Facility NCCS) by using protocol of Shewade *et al* (1999). In short aseptically removed pancreata were digested using digestion medium with bovine serum albumin (BSA), soybean trypsin inhibitor and type IV collagenase. The digestion was followed by centrifugation at 200 *g* for 10 min. The vortexed pellets were seeded in the bottles containing RPMI-1640 with 10% foetal calf serum (FCS). Islets were ready after 48 h. Fixed number of islets was handpicked and was given treatment with antibiotics.

Cultured islets were placed in each well of 24 well-plate (Nunc-Denmark) containing 1 ml of RPMI-1640 with 10% FCS. Antibiotics used and their concentrations were as follows: gentamycin (25 μg/ml), penicillin (25 μg/ml), streptomycin (25 μg/ml) tetracycline (2·5 μg/ml), neomycin (25 μg/ml), erythromycin (25 μg/ml) and chloramphenicol (25 μg/ml). Each group of islets was treated with each of the seven antibiotics for 24 h to 30 days (fresh antibiotics were added every third day to maintain the level of the antibiotics for all the 30 days). The islets treated with 1 mM streptozotocin (STZ) were used as positive control. The islets were then sonicated at 20 blasts for 20 duty cycles and were then used for further biochemical analyses. Islets were then tested for their viability by Trypan blue dye exclusion test and specificity by dithiozine (DTZ) staining.

2.2 *Assessment of islet viability using Trypan blue dye exclusion test*

The viability of the islets was checked by trypan blue dye exclusion test (Warburton and James 1995) using 0·4%

(W/V) Trypan blue (ICN Pharmaceuticals, USA). Blue stained islets were scored as non-viable and the unstained were scored as viable islets.

2.3 *Assessment of islet specificity using DTZ staining*

Specificity of islets was determined by dithiocarbazone staining (Samual *et al* 1994). DTZ staining was carried out by adding 10 µl DTZ stock to islets suspended in 1 ml Krebs–Ringer bicarbonate buffer (pH 7·4), with HEPES (10 mM) (KRBH) and incubated at 37°C for 10 15 min. The stained islets looked bright red under the inverted microscope (Olympus, Japan). Non-islet tissue remained unstained.

2.4 *Assessment of islets functionality (insulin release assay)*

Triplicate groups of 10 islets each were placed in single well of 24 well plate in KRBH. The plates were incubated at 37°C in a CO_2 incubator for 1 h with 5·5 mM and 16 mM glucose respectively. The supernatant was collected and stored at – 20°C and were assayed for basal insulin level. Radioimmuno assay was carried out using Radioimmuno assay kit (Diagnostic Products Corporation, Los Angles, USA) and insulin content of all the stored samples was determined.

2.5 *Measurement of lipid peroxidation*

Lipid peroxidation was measured in terms of melonyldialdehyde (MDA) (Konings and Drijver 1979). Mixture of sample with KCI, HCl, TBA and TCA was heated in water bath at 90°C. The mixture was then cooled on ice for 2 min and centrifuged at 200 *g* for 15 min. The optical density was read at 320 nm and values were expressed as MDA formed in nM/50 islets.

2.6 *Estimation of catalase*

Catalase activity was measured using method of Aebi (1984). The reaction was initiated by addition of H_2O_2. The time scan was performed for 2 min. The optical density was measured at 240 nm. The values were expressed as µcat/50 islets.

2.7 *Estimation of SOD*

SOD levels were measured (Beauchamp and Friddoivich 1971) using methionine (Sigma, USA) and nitro blue tetrazolium (NBT) (Sigma, USA) in phosphate buffer (pH 7·8). The reaction was started using 0·3 ml of riboflavin (20 µM). Absorbance was read at 560 nm. SOD activity was expressed as the µU/50 islets.

2.8 *Measurement of reduced glutathione levels*

Reduced glutathione levels were measured by using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Ames *et al* 1993). Sample (100 µl) was mixed with 900 µl PO_4 buffer and 2 ml of DTNB. Optical density was taken at 412 nm. Reduced glutathione levels were expressed in terms µM per 100 islets.

2.9 *Measurement of nitric oxide levels*

Nitric oxide (NO) estimation was done using Griess reagent (0·01 g sulphanilamide and 0·001 g naphthylethylenediamine was dissolved in 1 ml of 0·1 M HCl). Fifty µl of the cell/islet supernatant ± 50 µl Griess's reagent was mixed and mixture was incubated at room temperature for 10 min. Absorbance was taken at 546 nm. Values were expressed as nM of NO formed/50 islets (Ignnaaro *et al* 1987).

3. Results

3.1 *Effect of antibiotic treatment on the viability of islets*

The viability of the control islets recorded was 85%, whereas those treated with STZ – a potent diabetogen – was found to be 47% only (figure 1). There was no major decrease in the viability of islets treated with gentamycin (84·72%), penicillin (83·59%) and streptomycin (85·11), tetracycline (81·12%), neomycin (84·11%), erythromycin

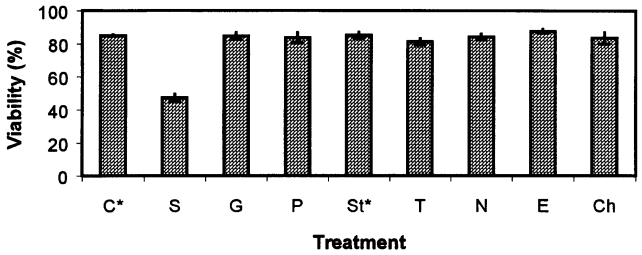

Figure 1. Effect of 24 h antibiotic treatment on the viability of islets of Langerhans. Islets were treated with antibiotics for 24 h. Hatched columns represent the % viability taken by using Trypan blue staining. The values are represented as mean ± SE. **P* < 0·05 when compared with STZ treated islets.

(C, Control; S, streptozotocin; G., gentamycin; P, penicillin; St, streptomycin; T, tetracyclin; N, neomycin; E, erythromycin; Ch, chloramphenicol.)

Table 1. Effect of antibiotic treatment on islet viability and insulin secretion after 30 days treatment.

Treatment	Viability (%)	Insulin secretion in μIU/10 islets	
		Basal	Stimulated
Control	93·25 ± 7·24	39·50 ± 0·356	110·5 ± 0·522
STZ	43·52 ± 8·20	20·77 ± 0·785	68·932 ± 0·347
Gentamycin	88·34 ± 8·23	36·32 ± 0·669	102·40 ± 0·565
Penicillin	90·24 ± 5·86	36·92 ± 0·435	101·3 ± 0·237
Streptomycin	86·53 ± 6·83	37·88 ± 0·535	110·73 ± 0·546
Tetracycline	80·93 ± 9·83	32·45 ± 0·739	98·78 ± 0·593
Neomycin	83·27 ± 5·25	33·41 ± 0·191	101·23 ± 0·423
Erythromycin	88·37 ± 8·82	30·19 ± 0·119	97·23 ± 0·523
Chloramphenicol	85·45 ± 7·82	31·23 ± 0·023	95·30 ± 0·321

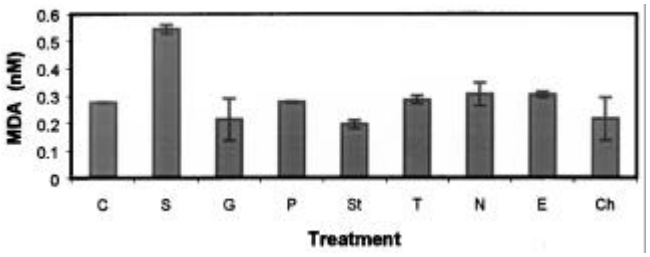

Figure 2. Effect of 24 h antibiotic treatment on the activity of catalase in the islets of Langerhans. Catalase activity is represented in μcat. The columns represent the μcat of catalase activity/50 islets. The values are represented as mean ± SE. All the values showed $P < 0·05$ when compared with STZ treated islets.

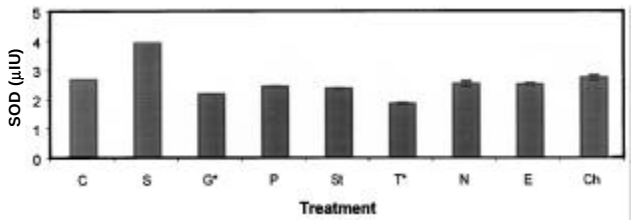

Figure 3. Effect of 24 h antibiotic treatment on the activity of super oxide dismutase in the islets of Langerhans. Super oxide dismutase activity is represented in μIU. The columns represent the μIU of SOD activity/50 islets. The values are represented as mean ± SE. *$P < 0·05$ when compared with STZ treated islets.

(87·63%) and chloramphenicol (83·45%) which was comparable to that of the controls.

3.2 *Effect of antibiotic treatment for 30 days on islet viability and insulin secretion*

Table 1 shows that there is no significant change in the per cent viability of islets treated with antibiotics for 30 days. The control islets exhibited the levels of insulin secretion as (39·50 ± 0·356 μIU) for basal and (110·5 ± 0·522 μIU of insulin/10 islets) for glucose stimulated conditions. Islets treated with STZ exhibited insulin secretion of (20·77 ± 0·785 μIU of insulin/10 islets) for basal and (68·932 ± 0·347 μIU of insulin/10 islets) for glucose stimulated insulin secretion. No significant change in the levels of insulin secretion (neither basal nor stimulated) was seen with any of the antibiotics except for tetracycline which showed slightly lower levels of insulin secretion as (30·45 ± 0·639 μIU of insulin/10 islets) for basal and (93·78 ± 0·93 μIU of insulin/10 islets) for stimulated state.

3.3 *Effect of antibiotic treatment on the lipid peroxidation in islets*

Lipid peroxidation was measured as the levels of MDA formed per 50 islets, which was (0·270 ± 0·003 nM/50 islets) in control islets (figure 2). Elevated levels of MDA (0·539 ± 0·0152 nM/50 islets) was seen after STZ treatment. A marginal increase over control was seen with tetracyclin (0·28 ± 0·0152 nM/50 islets), neomycin (0·301 ± 0·043) and erythromycin (0·299 ± 0·011). There was no increase in the MDA levels after treatment with gentamycin (0·209 ± 0·016 nM/50 islets.), penicillin (0·272 ± 0·076 nM/50 islets) and chloramphenicol (0·211 ± 0·078).

3.4 *Effect of antibiotic treatment on the SOD activity in islets*

The SOD activity of control islets was 2·7 ± 0·0059 μIU/50 islets (figure 3). STZ treatment brought about increase in SOD activity (3·98 ± 0·0192 μIU/50 islets) compared to that of controls. No significant change was seen in SOD activity of islets treated with gentamycin (2·21 ±

0·0121 μIU/50 islets), penicillin (2·47 ± 0·007 μIU/50 islets) and streptomycin (2·40 ± 0·0125 μIU/50 islets). A slight decrease in the SOD activity (1·89 ± 0·007 μIU/50 islets) was found in islets treated with tetracycline.

3.5 *Effect of antibiotic treatment on the catalase activity in islets*

The catalase activity in the control islets was 1·54 ± 0·0021 μcat/50 islets (figure 4). The islets after STZ treatment exhibited significant increase in the catalase (1·17 ± 0·0108 μcat/50 islets) activity. There was further increase in catalase activity after gentamycin treatment (1·84 ± 0·073 μcat/50 islets). No change in the catalase activity was observed when islets were treated with penicillin (1·54 ± 0·019 μcat/50 islets). A decrease in the catalase activity was found in streptomycin (0·982 ± 0·0015 μcat/50 islets) and tetracycline (1·03 ± 0·057 μcat/50 islets) treated islets. However increased catalase activity was seen in chloramphenicol treated islets (1·77 ± 0·034).

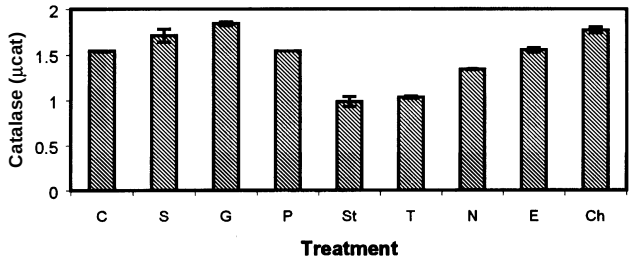

Figure 4. Effect of 24 h antibiotic treatment on the levels of lipid peroxidation in the islets of Langerhans. Lipid peroxidation is measured in terms of amounts of MDA formed. The columns represent the nM of MDA formed/50 islets. The values are represented as mean ± SE. All the values showed $P < 0.05$ when compared with STZ treated islets.

3.6 *Effect of antibiotic treatment on the reduced glutathione levels in islets*

The levels of reduced glutathione in the control islets were 32·18 ± 0·013 μM/50 islets (figure 5). Treatment with STZ brought about decrease in the levels of reduced glutathione (19·78 ± 0·0159 μM/50 islets). Significant decrease was not seen after treatment with gentamycin (29·90 ± 0·0192 μm/50 islets), penicillin (29·08 ± 0·009 μm/50 islets), streptomycin (30·85 ± 0·0732 μm/50 islets). However, neomycin treated cultures exhibited higher GSH activity (33·41 ± 0·0191) whereas treatment with tetracycline showed significant decrease of catalase (21·55 ± 0·0732 μM/50 islets) activity.

3.7 *Effect of antibiotic treatment on the NO levels in islets*

The levels of NO in the control islets was 1·76 ± 0·0089 nM/50 islets (figure 6). There was an increase in the NO levels after STZ treatment with the value of 1·98 ± 0·0592 nM/50 islets. There was no significant decrease in NO activity in all the antibiotics tested except for streptomycin (1·53 ± 0·0013 nM/50 islets).

4. Discussion

In the present investigation we have shown that exposure of islets *in vitro* to the commonly used antibiotics in pharmacological concentrations for 30 days did not affect their viability and insulin secretion.

The rationale for employing *in vitro* islet model was two-fold. (i) Such a study assesses the effect of direct exposure of the islets to these antibiotics, which are routinely used during islet isolation and transplantation. (ii) The isolated islets *in vitro* behave and respond to drug

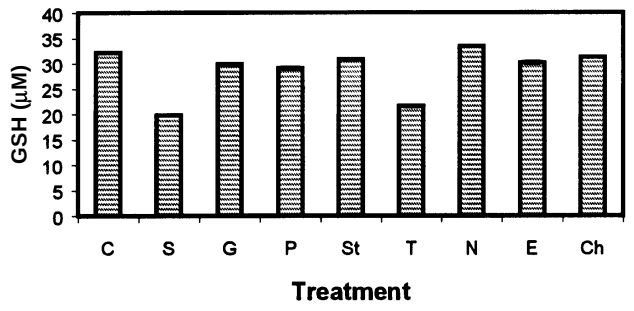

Figure 5. Effect of 24 h antibiotic treatment on the levels of reduced glutathione in the islets of Langerhans. The columns represent the μM of GSH (reduced glutathione) formed/50 islets. The values are represented as mean ± SE. All the values showed $P < 0.05$ when compared with STZ treated islets.

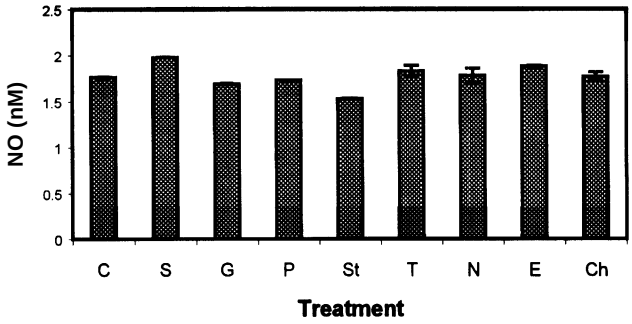

Figure 6. Effect of 24 h antibiotic treatment on the levels of nitric oxide generation in the islets of Langerhans. The columns represent the nM of nitric oxide (NO) formed/50 islets. The values are represented as mean ± SE. All the values showed $P < 0.05$ when compared with STZ treated islets.

treatment simulating *in vivo* conditions (Bhonde *et al* 1999) making it easy to extrapolate the results to *in vivo* situations.

Among the antibiotics used none of the antibiotics led to development of oxidative stress, except tetracycline. This is obvious by the increase in lipid peroxidation and moderate decrease in SOD and catalase activities. The slight decrease in the viability after treatment with tetracycline could be attributed to the slight increase in lipid peroxidation. However despite the increased lipid peroxidation, insulin secretion was not significantly hampered the tetracycline treated islets, which indicated the safe use of tetracycline. The viability of islets, levels of free radicals (MDA) and activities of antioxidant enzymes (SOD and catalase) in the islets treated with these antibiotics did not deviate much from the control values indicating non-toxic nature of the antibiotics used in these experiments. The variations obtained in the antioxidant enzyme profiles with individual antibiotics at the given dose did not differ significantly from those of controls; although they differ significantly from the STZ treated positive controls confirming their inert role.

There are a few reports suggesting preventive and therapeutic effects of certain antibiotics or their derivatives (Stosic *et al* 1999) in a model of mild low dose-streptozotocin (MLD-SZ) induced diabetes. Antibiotics like oxytetracycline are known to affect the morphology of the pancreas (Lorenzo *et al* 1999) and some others like gramicidin D are known to be potent insulin secretagogues (Dibas *et al* 1995). The bio breeding (BB) rats develop insulitis and IDDM with many features analogous to man. It is reported that weekly administration of 2′-deoxyconformycin for 4 months significantly reduces the incidence of insulin dependent diabetes mellitus (IDDM) in BB rats by 70% and that the animals remains free from diabetes for a minimum of two months after drug withdrawal (Thelwaris *et al* 2000). All these reports suggest beneficial effects of antibiotics on morphology and functionality of islets, thus supporting our data. Our results differ from those reported by Vijayalakshmi *et al* (1992) wherein they have shown that administration of antibiotics such as ampicillin, tetracyclin, chloramphenicol and streptomycin bring about profound alteration in lipid peroxidation levels of different tissues of rat along with decrease in SOD and catalase activity and increase in the levels of reduced glutathione especially in the kidney. However antibiotics used in the present study did not produce any oxidative stress as evidenced by low levels of MDA comparable to those of controls (figure 2). This difference could be attributed to the different tissues (liver, kidney and heart and not pancreas) used by Vijayalakshmi *et al* (1992).

The mechanism by which antibiotics exert their toxic effect may be different in each case. Different tissues of animals respond differently to oxidative stress depending on the status of their own antioxidant defense system and nature of damaging agents. STZ – a known diabetogenic agent – administration in rat has been reported to decrease glutathione (GSH) levels in liver and pancreatic tissue while exhibiting no change in the kidney of diabetic rats (Bastar *et al* 1998).

Some other antibiotics like pentamide appears to have a direct toxic effect on *b*-cells that is similar in nature as seen in STZ (Sai *et al* 1983; Jha and Sharma 1984). Pentamide and isoniazide (Zugar *et al* 1986) have been attributed to the development of impaired glucose tolerance and overt diabetes mellitus. These results suggest the detrimental action of antibiotics on *b*-cells.

In light of such contrasting reports, our data indicates that it is safe to use the antibiotics used in our present study without any detrimental effect on the islet viability and functionality. These antibiotics neither affect the islet viability nor insulin secretion. Also, with the difference in the activities of antioxidant enzymes was not significant. Our results thus show that commonly used antibiotics do not lead to oxidative stress and or affect their viability and functionality suggesting their non-toxic nature to islets.

The study further indicates that it may be safe to use these antibiotics whenever needed without any risk of developing diabetes, as well as during islet isolation and transplantation studies.

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