Comparison of the carcinogenic potential of streptozotocin by polarography and alkaline elution*

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The carcinogenic potential of streptozotocin (STZ) was evaluated by the polarographic determination of its reduction potential in the presence of α-lipoic acid and detection of DNA single-strand brakes by alkaline elution. After STZ electrochemical reduction in an anhydrous solvent, the half-wave potential ($E_{1/2}$) was determined to be –1.340 V. The parameter of the carcinogenic potential (tg α) for STZ was 0.400. This is in good agreement with WHO data regarding STZ carcinogenicity. Additionally, it is in the good agreement with the tg α value determined for the positive control used, N-nitroso-N-methylurea (NMU), which was found to be 0.459.

The 3 hours exposure of A549 human lung tumor cells to 250, 500, and 1000 nmol/ml STZ was followed by DNA single-strand brakes detection using the alkaline elution method. NMU, the positive control, was tested under identical experimental conditions at the same concentrations. Without metabolic activation, NMU induced a significant formation of DNA single-strand brakes only at 1000 nmol/ml. In the presence of the metabolic activation, NMU caused a significant, concentration-dependent formation of DNA single-strand brakes. In the absence of metabolic activation, STZ induced no significant formation of DNA single-strand brakes at any concentration used. In the presence of metabolic activation, STZ caused a significant, concentration-dependent formation of DNA single-strand brakes.

The results of this study underline the crucial role of using a metabolic activation system when carcinogenic potential of drugs and chemicals is investigated *in vitro* studies. Results of polarographic experiments and alkaline elution correlate well with each other and they indicate that these methods are useful to early predict the carcinogenic potential of STZ and other xenobiotics.

Key words: Streptozotocin, polarography, alkaline elution, single-stranded DNA, carcinogenicity.

Streptozotocin (2-Deoxy-2{[methyl-nitrosoamino)carbonyl]amino}-D-glucopyranose, CAS No.18883-66-4) (STZ) is an antibiotic originally derived from the soil microorganism *Streptomyces achromogenes*.

The principal therapeutic use for STZ is in the treatment of metastasizing pancreatic islet cell tumors. It is also effective in treating malignant carcinoid tumors, especially of the small intestine. It has been investigated for use in diabetes, since it has specific toxic action on pancreatic beta-cells. Moreover, the compound has been shown to artificially induce diabetes in rats [4]. STZ has been investigated as a potential antibacterial agent but has never been used commercially for this purpose. There is evidence for the carcinogenicity of STZ in several experimental animal species [9, 10]. No adequate data on humans are available (group 2B), but the chemotherapeutic use of STZ indicates the existence of an exposed group. Routes of potential exposure to STZ are inhalation, injection and dermal contact. Health professionals such as pharmacists, doctors and nurses may be exposed. Potential occupational exposure may also occur during STZ production.

The aim of the present study was to evaluate the carcino-

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genic potential of STZ by measuring its reduction potential in the presence of α-lipoic acid (α-LA) using the polarographic method and detection of DNA single-strand brakes by alkaline elution.

Material and methods

Chemicals. N,N-dimethylformamide (DMF) was of a commercial origin from Fluka (Fluka, Buchs, Switzerland). DMF was additionally purified prior to all electrochemical measurements by double vacuum distillation in a dry nitrogen atmosphere [26]. DMF water content did not exceed 0.01 wt. %. Tetrabutylammonium perchlorate (TBAP), was used as the supporting electrolyte at a concentration of 0.15 mol/l, was the product of Fluka. α-LA (D,L-6,8-thioctic acid) was purchased from Koch Light Laboratories, Colnbrook, United Kingdom. STZ and N-nitroso-N-methylurea (NMU) were purchased from Sigma, Munich, Germany.

Polarographic measurements. All polarographic measurements were performed using the three-electrode setting at the polarographic analyzer PA 4 equipped with the two-line recorder XY 4106 from Laboratorní přístroje Prague, the Czech Republic. Polarographic experiments were performed in a polarographic cell adapted for the work in anhydrous system. As the indicating electrode, a dropping mercury electrode (DME) was used with a drop time of 3 s and a flow rate of 2.27 mg/s at a mercury column height h_{Hg} of 81 cm. As the reference electrode, a saturated calomel electrode (SCE) modified for anhydrous conditions was used. The auxiliary electrode was a platinum electrode.

All polarographic mesurements were carried out at room temperature in a stream of dry nitrogen in order to exclude atmospheric oxygen and humidity from the polarographic cell as previously described [20, 32]. The potential carcinogenic activity of STZ was determined based on its ability to form complexes with α-LA. α-LA does not provide polarographic wave, but has a strong effect on the polarograhic reduction of carcinogens regardless the necessity of their enzymatic activation [20]. During the simultaneus determination of a potential carcinogen with α-LA, the height of the polarographic wave is increasing linearly depending on the α-LA concentration. The wave height versus α-LA concentration linear plot can be characterized by its slope as tg α . The higher is the tg α value, the greater is the carcinogenic activity of a particular compound [20]. The tg α value was used in the present study as a prescreening criterium of potential carcinogenecity of STZ and of the positive control NMU.

Cell culture for alkaline elution experiments. A549 (human type II lung tumor derived) cells were used for the *in vitro* experiments carried out with STZ in alkaline elution tests. The A549 cells were a subclone (ATCC No. CCL 185) of the line described [6]. A549 cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

Cells were routinely cultured using 75 cm^2 flasks in 13 ml Dulbecco's modified Eagle medium containing 10% newborn calf serum (Gibco, Karlsruhe, Germany) and 200 mmol/l glutamine. Cells used in early passages were determined to be mycoplasma-free and cultured in monolayer in 10% CO_2 in a humid atmosphere at 37 °C. Cells were passed by trypsinization when approaching confluence. For determination of the cell number, cells were removed by trypsinization and counted with a Coulter counter (Coulter Electronics, Kempen-Huls, Germany). For experiments, $4x10^5$ viable cells/35 mm dish were seeded in 2 ml Dulbecco's modified Eagle medium containing 10% newborn calf serum and 200 mmol/l glutamine and were incubated in 10% CO_2 at 37 °C for 24 hours to allow attachment. At the end of the 24 hours attachment interval, the incubation medium was discarded and cells were cultured for additional 48 hours. During this incubation time cell cultures were washed twice (every 24 hours) with an arginine-free Dulbecco's modified Eagle medium supplemented with 2% dialyzed fetal calf serum and 200 mmol/l glutamine. At the end of the additional 48 hours incubation time, the arginine-free Dulbecco's modified Eagle medium was discarded and replaced, just prior to the cell treatment, with 2 ml Dulbecco's Eagle medium supplemented with 2% dialyzed fetal calf serum, 10 mmol/l hydroxyurea and 200 mmol/l glutamine. Reduction of the serum concentration, addition of hydroxyurea and arginine-free medium were used in order to inhibit the semi-conservative DNA replication (S phase). This test system for human cell cultures has been described by MARTIN et al [16]. The use of the human cell line A549 (ATCC No. CCL 185) to determine DNA damage and repair was carried out as described and recommended by the OECD guidelines [21] and MITCHELL et al [18]. The present experiments were conducted according to these guidelines.

Treatment of cultures. Stock solutions of the positive control NMU and of STZ were prepared in dimethylsulfoxide (DMSO). Stock solutions were filtered sterile through a 0.2 μm filter (Schleicher & Schull, Dassel, Germany) before use. To achieve the final concentrations, 20 μl from each stock solution was added to each of the dishes used to test a certain concentration of the test substance.

At least 4 cell cultures (dishes) were used in each experiment per tested concentration. In the present study concentrations of 250, 500, and 1000 nmol/ml for both NMU and STZ were tested.

In the experiments with metabolic activation, 100 μl of rat liver S9 mix (3 mg/ml protein) were added to each dish in the alkaline elution assays. Cells were incubated with the test substance in 10% CO_2 and at 37 °C for 3 hours. At the end of the 3 hours treatment, incubation medium was discarded and cells were harvested by gentle policing into 1 ml cold,

phosphate-buffered saline (PBS, pH 7.4) using a rubber policeman.

Alkaline elution. The alkaline elution assay measures the rate of DNA single-strand brakes elution through a filter membrane under alkaline conditions. The amount of DNA single-strand brakes under alkaline conditions is determined on the basis of the increase in DNA elution rate.

The procedures followed in this study were essentially those described by KOHN et al [12]. The apparatus consisted of 50 ml syringe barrels attached to filter units (Millipore, Eschborn, F.R.G.) containing 2 μm polycarbonate filters (Nucleopore, Pleasanton, USA). The outlet portion of the filters was connected to 16-channel peristaltic pumps (Ismatec, Zurich, Switzerland) by flexible tubing. Cell suspensions removed from the dishes at the end of the 3 hours treatment interval (approximately $1x10^6$ cells from one dish) were placed in the corresponding barrels containing 2 ml PBS and pulled into filters at a rate of 0.6 ml/min during 5 min. Cells were lysed by filling the filter holder with 2 ml of warm (37 °C) solution containing 1.5% sodium dodecyl sulfate (SDS), 0.025 mol/l EDTA, 0.1 mol/l glycine and 0.5 mg/ml proteinase K, pH 10. After 30 min at room temperature, lysis was completed and the DNA on the filter was washed twice with 5 ml 0.02 mol/l EDTA, pH 10. At no time were the filters allowed to pump to dryness or to have bubbles from within the filter holder. Elution of single-stranded DNA was accomplished by pumping a solution of 0.02 mol/l EDTA, adjusted to pH 12.1 with tetraethylammonium hydroxide, through the filter at 0.035 ml/min. Fractions were collected at 3 hours intervals for 21 hours. A modification of the method of HINEGARDNER [8] was used for the fluorometric assay of DNA eluted and remaining on the filter. Freshly prepared 3,5-diaminobenzoic acid hydrochloride (Fluka, Buchs, Switzerland) was added to each sample and fluorescence was read in a Hitachi model F-300 fluorescence spectrophotometer at an emission wavelength of 520 nm with excitation at 420 nm. Samples were compared to DNA standards, which were also processed by the same procedure. Two independent experiments in the absence or in the presence of a metabolic activation system (liver S9 mix) were performed. All data were expressed as percent of the amount of DNA retained on the filters.

The S9 mix. A preparation of rat liver 9000 x *g* supernatant (S9) was used as a metabolizing mixture for all test systems. Male Sprague-Dawley rats were pretreated with Aroclor 1254 (500 mg/kg). Preparation of the S9 fractions and of the cofactor solution (mix) was carried out as described by MARON and AMES [15]. Protein concentration was determined by the method of SCHACTERLE and POLLACK [29] using bovine serum albumin as standard.

Calculations. Mean value, standard deviation (SD), Student's t-test and the 1-way analysis of variance were used for statistical analysis of the data. The 0.05 level of probability was used as the criterion for significance.

Results

Polarography. STZ is reduced at the mercurry dropping electrode during one two-electron steps in anhydrous DMF. STZ half-wave potential $E_{1/2}$ is –1.340 V vs. SCE. Figure 1 diplays the polarographic wave mas a result of STZ (500 μmol/l) reduction in the absence of α-LA. The difusse current of the STZ reduction wave increased linearely with the increasing of the α-LA concentration (Fig. 1). When STZ reduction was measured in the presence of 500 μmol/l STZ and 520 μmol/l α-LA, the difusse curent increase was more than ten times higher than that of STZ polarographic wave, measured in the absence of α-LA. The tg α value of the potential carcinogenic activity was determined as 0.400 for STZ (Fig. 2) and 0.459 for NMU.

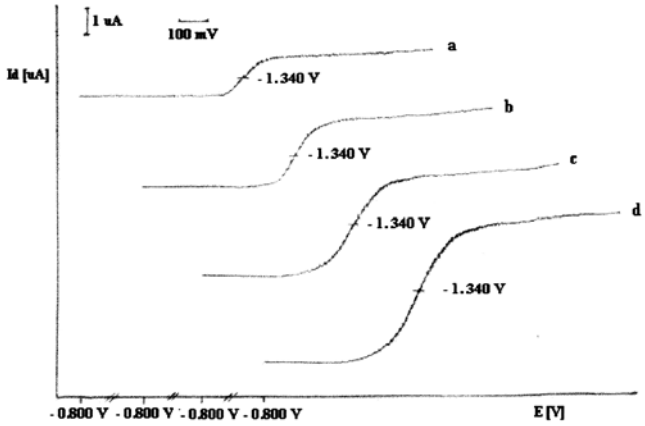

Figure 1. Polarographic reduction of streptozotocin in anhydrous DMF in the absence and in the presence of α-lipoic acid. Supporting electrolyte: 150 mmol/l TBAP in DMF. a = streptozotocin concentration 500 μmol/l; Concentration of α-LA: b=200 μmol/l; c=320 μmol/l; d=520 μmol/l. Scanning range ($E_{[V]}$) from –0.800 V to –2.200 V at a scanning rate of 5 $mV.s^{-1}$. I_d = the diffuse current [μA]. The half-wave potential ($E_{1/2}$) for streptozotocin was –1.340 V.

Alkaline elution. As shown in Figure 3 the positive control NMU induced some formation of DNA single-strand breaks, without metabolic activation. However, in the presence of metabolic activation, NMU caused single-strand brakes of DNA in A549 cells, which were detected at all investigated concentrations (Fig. 4). Treatment of A549 cells with STZ in the absence of metabolic activation cause no significant increase in the formation of DNA single-strand brakes at any of the investigated concentrations (Fig. 5). Moreover, treatment of A549 cells with STZ in the presence of metabolic activation induced a concentration-dependent increase in the formation of DNA single-strand breaks (Fig. 6). The DNA damaging effect of both NMU and STZ requires metabolic activation by exogenously added S9 microsomal enzymes.

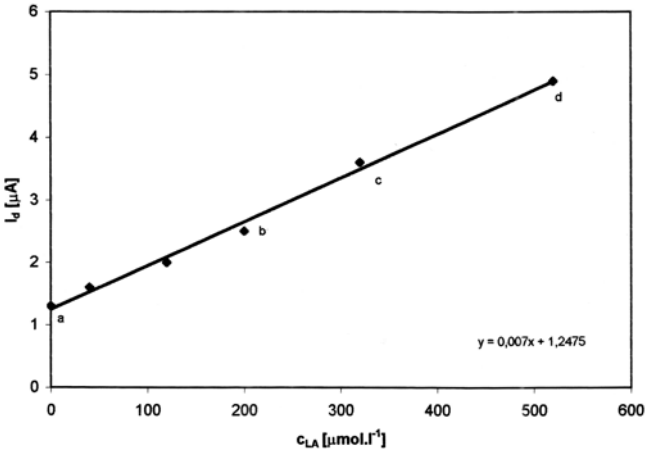

Figure 2. Determination of the potential carcinogenic activity for streptozotocin. The parameter of the carcinogenic potential (tg α) for streptozotocin was 0.400. I_d [μA] = the diffuse current. c = concentration of α-lipoic acid [μmol.l^{-1}].

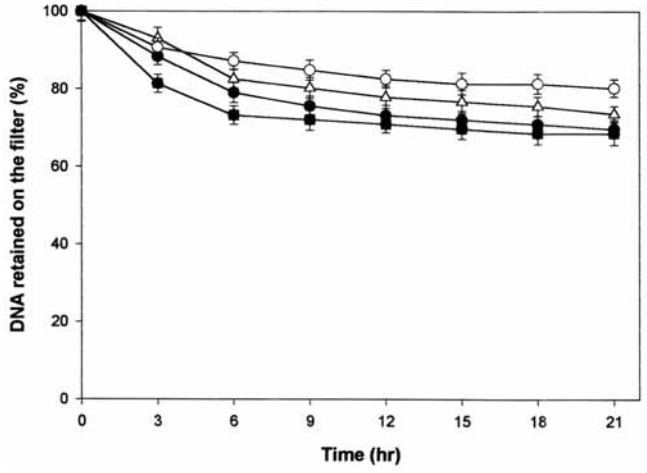

Figure 3. Alkaline elution of DNA single-strand brakes after a 3 hours incubation of human lung tumor cells with different concentration of N-nitroso-N-methylurea (NMU) without metabolic activation. (O) Control (DMSO); NMU, (■) 250 nmol/ml; (Δ) 500 nmol/ml; (●) 1000 nmol/ml. Each point represents ±SD of 8 experiments. 100% DNA retained on the filter corresponds to the total amount of the sample. *Significantly different from controls.

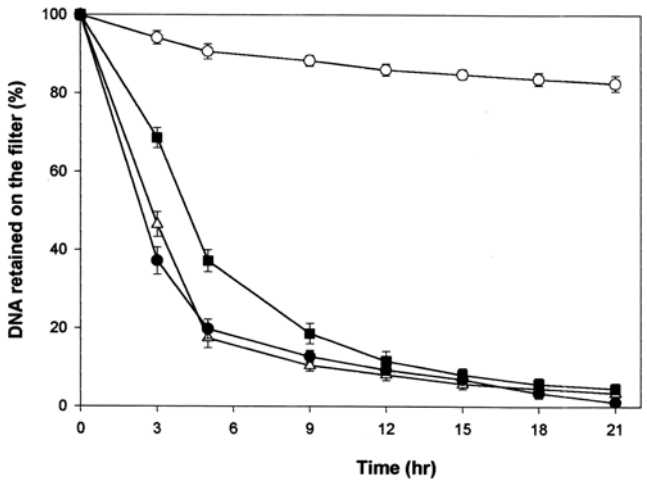

Figure 4. Alkaline elution of DNA single-strand brakes after a 3 hours incubation of human lung tumor cells with different concentration of N-nitroso-N-methylurea (NMU) with metabolic activation. (O) Control (DMSO); NMU, (■) 250 nmol/ml, (Δ) 500 nmol/ml; (●) 1000 nmol/ml. Each point represents ±SD of 8 experiments. 100% DNA retained on the filter corresponds to the total amount of the sample. *Significantly different from controls.

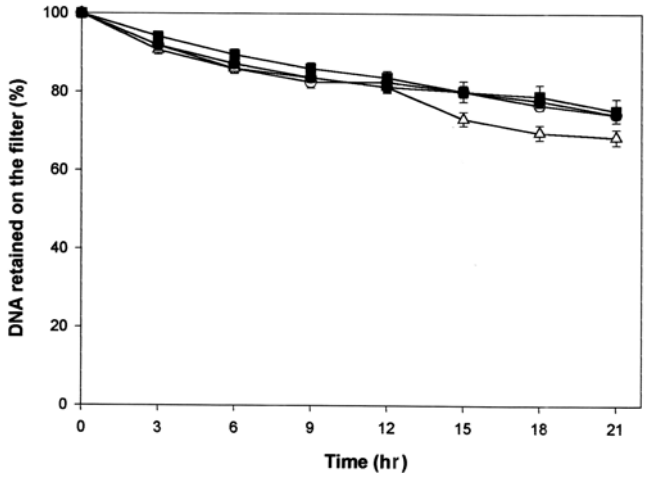

Figure 5. Alkaline elution of DNA single-strand brakes after a 3 hours incubation of human lung tumor cells with different concentration of streptozotocin (STZ) without metabolic activation. (O) Control (DMSO); STZ, (■) 250 nmol/ml; (Δ) 500 nmol/ml; (●) 1000 nmol/ml. Each point represents ±SD of 8 experiments. 100% DNA retained on the filter corresponds to the total amount of the sample. *Significantly different from controls.

Discussion

STZ-induced diabetes in mice and rats has been used widely as an experimental model to study type I diabetes [1, 25]. A single large dose of STZ is sufficient to induce hyperglycemia resulting from loss of pancreatic beta-cells. STZ is one of the older chemotherapy drugs, and has been in use for many years for treatment of carcinoid tumor and endocrine tumor of pancreas. Human exposure to environmental nitroso agents such as STZ a cycasin, a toxin obtained from the cycad plant (Cycas spp.), could trigger a cascade of cellular events leading to cell dysfunction and cell loss, and occurrence of diabetes mellitus and neurodegenerative diseases [5].

The polarographic reduction of STZ in anhydrous solution was not reported so far. The STZ polarographic reduction in the presence of the α-LA allows determination of the value of the tg α parameter for its potential carcinogenic activity. The potential carcinogenic activity of STZ determined in the present study as tg α was 0.400. This value is

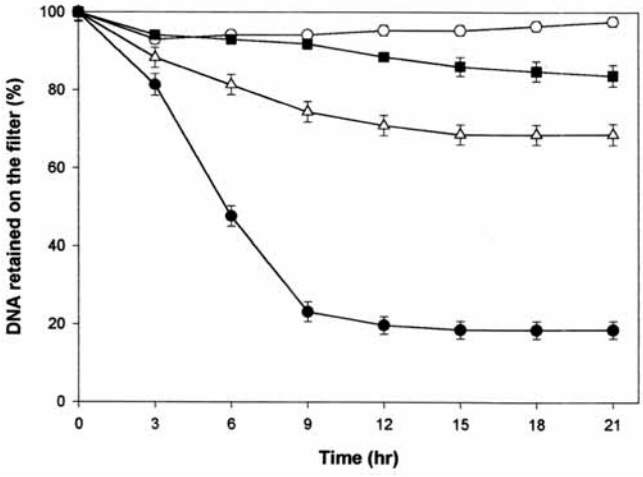

Figure 6. Alkaline elution of DNA single-strand brakes after a 3 hours incubation of human lung tumor cells with different concentration of streptozotocin (STZ) with metabolic activation. (O) Control (DMSO); STZ, (■) 250 nmol/ml; (Δ) 500 nmol/ml; (●) 1000 nmol/ml. Each point represents ±SD of 8 experiments. 100% DNA retained on the filter corresponds to the total amount of the sample. *Significantly different from controls.

close to those of known carcinogens such chloramphenicol (tg α 0.410), NMU (tg α 0.459), benzo(a)pyrene (tg α 0.505) and adriamycin (tg α 0.575) [20, 32]. Other compounds such as chrysene (tg α 0.105), 5-nitrofurantoin (tg α 0.290) and erythromycin (tg α 0.320) have lower tg α values than STZ [20, 32]. The carcinogenic activity of STZ is in an excellent agreement with WHO data regarding its possible carcinogenicity. STZ, classified as a teratogenic compound by IARC during the year 2001, is included into the group 2B according to its carcinogenic properties. The results of this and previous studies [20, 32] confirm the possibility of the use of this method for pre-screening and early detection of the carcinogenic potential of various xenobiotics. However, the use of the polarogrphic method cannot replace the *in vitro* and *in vivo* mutagenicity and carcinogenicity tests recommended by WHO.

The mechanism of STZ-induced death was investigated in a murine pancreatic beta-cell line [27]. The results of this study suggest that higher rate of apoptosis, as compared to necrosis, were observed when cells were exposed for 1 hour to low doses of STZ (15 mmol/l); higher doses of STZ (30 mmol/l) caused the murine pancreatic beta-cells to undergo necrosis (22%) as well as apoptosis (17%). In another study, the mechanism of DNA damage caused by STZ was investigated *in vitro* using a human cell line [19]. The results of this study showed that STZ induced DNA damage and apoptosis, and frequently initiated DNA modification at guanines, similar to N-methyl-N-nitrosourea (NMU), a typical methylating agent. Further, the results of the same study [19] suggest that STZ caused damage may be due to the methylation of guanines via methyl cations. This alkylation may be responsible for triggering apoptosis, necrosis of the pancreatic cells, and subsequently diabetes.

Exposure of a clonal isolate from a rat insulinoma cell line to the nitrosourea STZ caused appreciable mitochondrial DNA damage in a dose-dependent manner [23]. Using cultured J774 cells as a model, it was shown that the antioxidant α-LA stabilize lysosomes against oxidative stress, probably by chelating intralysosomal iron and, consequently, preventing the intralysosomal Fenton reactions and subsequent oxidant-mediated apoptosis. Further, the iron-chelator, desferrioxamine also provided protection against oxidant-mediated cell death [22].

The mechanisms of the induction of diabetes are not well elucidated, but it was proposed that they may be related to the generation of reactive species such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) which result in DNA fragmentation. However, NO and reactive oxygen species can act separately or form the highly toxic peroxinitrite (ONOO). Therefore, antioxidants and NO scavengers may substantially depress STZ toxicity.

In an *in vitro* model of isolated rat pancreatic islets, it was shown that alloxan (ALX) and STZ stimulated H_2O_2 generation and DNA fragmentation, which was minimal at a concentration of 0.1 mmol/l and maximal at 1.0 mmol/l [30]. In the same study, administration of STZ or ALX to rats stimulated H_2O_2 generation and caused DNA fragmentation in pancreatic islets. The following biochemical sequence of events was proposed: STZ and ALX stimulate H_2O_2 generation which, in turn, causes DNA fragmentation followed by destruction of beta-cells and diabetes [30].

The effects of the antioxidant melatonin in diabetes were investigated in rats [33] and mice [2]. Six weeks after initiation of diabetes with STZ, the levels of malondialdehyde (MDA), a stabile lipid peroxidation product, in untreated diabetic rats were higher than those in control group, whereas MDA levels in diabetic rats treated with melatonin were not different from control [33].

Melatonin treatment of diabetic rats counteracted the decrease in the levels of reduced glutathione, glutathione peroxidase and superoxide dismutase observed in untreated diabetic rats [33]. Interestingly, diabetes induced by STZ in mice was effectively prevented by administration of melatonin 30 min prior to STZ injection [2]. The results of these studies suggest the presence of oxidative stress in STZ induced DNA damage and diabetes and that antioxidants such as melatonin may play a role in alleviating diabetes complications.

In the present study, the exposure of A459 cells to NMU or STZ for 3 hours, without metabolic activation, caused some formation of DNA single-strand brakes or did not induce significant production, respectively. However, N-methyl-N-nitroso-N-nitrosoguanidine (MNNG) caused significant formation of DNA single-strand brakes in the absence of metabolic activation (unpublished data). After a 3

hours exposure of A459 cells, in the presence of metabolic activation, both NMU and STZ caused significant DNA single-strand brakes production whereas with MNNG formation of DNA single-strand brakes was greatly increased at all investigated concentrations. The alkaline elution results of the present study underline the crucial role of using a metabolic activation system when the carcinogenic potential of various xenobiotics is investigated in *in vitro* studies. Measurement of unscheduled DNA synthesis (UDS) (repair) in rat kidney cells following *in vivo* treatment with NMU and STZ yielded strong UDS responses [31]. Taken together, the results of these studies suggest the mutagenic and carcinogenic potential of MNNG, NMU and STZ.

NAD^+ is a substrate of the enzyme poly(ADP-ribose) polymerase (PARP) that plays a role in DNA repair. In the presence of excessive DNA damage, PARP is highly activated [28] and NAD^+ critically depleted resulting thus in ATP production depletion, leading to energy loss and cell death [24]. Toxicity tests revealed that STZ and NMU were not toxic at equimolar concentrations. However, at equimolar concentrations both compounds caused comparable DNA-strand brakes [14].

Cotreatment of mice [11] or rats [7, 11] with nicotinamide, a precursor of NAD^+, attenuates STZ-induced DNA damage and diabetes complications. The optimum dose of nicotinamide to achieve significant protection against STZ induced beta-cell destruction in rats appears to be 500 mg/kg/body weight [7]. Thymidine, like nicotinamide, was also found to be an effective scavenger of hydroxyl radicals [13]. Overexpression of metallothionein, an inducible antioxidant protein, in pancreatic beta-cells protects from DNA breakage and depletion of NAD^+ [3]. Further, 3-aminobenzamide, a strong inhibitor of PARP, protected against STZ toxicity in rats [17]. Results of investigations in PARP-deficient mice indicate that lack of PARP activity in mutant mice prevents STZ-induced beta-cell destruction and hyperglycemia [24]. Thus it appears that generation of free radicals and resulting DNA damage along with activation PARP play an important role in STZ toxicity.

In conclusion, polarographic reduction of STZ indicates that this alkylating agent has a potential carcinogenic activity. This correlates well with the *in vitro* results of alkaline elution, which showed that STZ caused significant DNA single-strand brakes formation in the presence of metabolic activation. Polarographic measurements together with alkaline elution are useful tools for early, cost-effectively detection and pre-screening of a large number of environmental agents for their potential carcinogenic activity.

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