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ALLOXAN TOXICITY IN HUMAN AND CANINE SPERMATOZOA

POSSIBLE BIOCHEMICAL BASIS FOR A SPECIES DIFFERENCE IN SENSITIVITY

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Abstract—In view of the well known species differences in the sensitivity of pancreatic B-cells to the toxic glucose analogue alloxan, it was tested whether spermatozoa from two species with a different diabetogenic effect of alloxan displayed a similar difference in their sensitivity to this drug. In canine spermatozoa, less than 2 mM alloxan profoundly reduced the rate of glucose oxidation and cellular motility whereas more than 5 mM was required to significantly alter these parameters in human spermatozoa. Such species difference was not observed in spermatozoal sensitivity towards the inhibitory effects of *tert*-butyl hydroperoxide. The phenomenon is not attributable to a different rate of alloxan uptake since the drug is not incorporated by dog or human spermatozoa. The alloxan toxicity was counteracted by D-glucose and its 3-*O*-methyl analogue in both species, and was potentiated by ascorbic acid; however, only in man. The protective effect of D-glucose was much less marked in *tert*-butyl hydroperoxide-cytotoxicity.

It is concluded that the observed species difference in spermatozoal alloxan sensitivity is not related to differences in alloxan uptake or in sensitivity to organic peroxides; differences in cellular scavenging of superoxide anion radicals and/or ascorbic acid metabolism may explain the lower sensitivity of human spermatozoa for alloxan.

Alloxan—a molecule which structurally resembles D-glucose [1]—has the intriguing property of selectively killing the glucose sensing pancreatic B-cell in several mammal species. The drug has therefore been promoted to a frequently used agent for the induction of experimental insulinopenic diabetes [2]. Despite intensive research over the past decades the action mechanisms by which alloxan destroys insulin producing B-cells remain a matter of debate. According to an early hypothesis the B-cell membrane represents the primary target site for alloxan [3]. More recently, however, it was proposed by Malaisse that the destructive effects of the drug upon B-cells result from a rapid cellular uptake coinciding with an inadequate scavenging of toxic oxygen containing radicals and hydrogen peroxide which are believed to be generated *in situ* during the alloxan-dialuric acid redox cycle [2]. *In vitro* experiments furthermore indicated a close but complex interplay between cellular glucose handling and alloxan cytotoxicity, which is best illustrated by the parallelism between the uptake of glucose and of alloxan [4, 5] and the protecting effect of D-glucose and other substances known to interact with the glucose carrier [6, 7].

The hypothesis of Malaisse [2] offers a plausible explanation for the differences in alloxan sensitivity which have been noted between islets of Langerhans and other tissues in the rat [4] as well as between different cell types within the endocrine pancreas [5]. This hypothesis also suggested a cellular basis for the markedly lower alloxan sensitivity of guinea-pig B-cells [8]. However, it remains unknown why human pancreatic B-cells are more resistant to alloxan than dog or rodent insulin containing cells [9]. As the answer to this question is hampered by the inability to prepare human B-cells in sufficient quantities for *in vitro* experiments, we considered the use of human spermatozoa as a model for this investigation. This choice has been inspired by the following reflections. Firstly, at variance with other human tissues, large numbers of functionally intact spermatozoa can be isolated without the need for traumatic intervention or aggressive tissue dissociation [10–13]. Secondly, spermatozoal motility represents a readily quantifiable index of the functional integrity of the cells. Thirdly, spermatozoa possess an efficient glucose transport system (Gorus and Pipeleers, unpublished work) in conjunction with a pronounced sensitivity to peroxidative attack [13, 14]. As the isolated human spermatozoa were found to be relatively resistant to the toxic effects of alloxan (Gorus and Pipeleers, *J. cell. Physiol.* in press), we undertook a comparative analysis of the alloxan-induced changes in the motility and glucose handling of human cells with that of cells from an alloxan-sensitive species, such as the dog.

EXPERIMENTAL

Materials

Siliconized (Silicon oil, Serva, Heidelberg, F.R.G.) glassware and disposable plastics were used

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throughout. Bovine serum albumin fraction V (BSA) was purchased from Armour (Armour, Bucks, U.K.); *tert*-butyl hydroperoxide (*tert*-butOOH) and 3-*O*-methyl-D-glucose (3-*O*-MG) from Aldrich-Europe (Beerse, Belgium), L-glucose and alloxan from Sigma Chem. Co (St. Louis, MO); L-ascorbic acid and 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) from Merck (Darmstadt, F.R.G.); Percoll from Pharmacia (Uppsala, Sweden); antimycin A, rotenone and superoxide dismutase from bovine erythrocytes (SOD) from Boehringer (Mannheim, F.R.G.); D-[1-^{3}H(n)] mannitol (15 Ci/mmol) from New England Nuclear (Boston, MA); D-[U-^{14}C] glucose (230 mCi/mmol) and [^{14}C]urea (50 mCi/mmol) from Amersham (Amersham, Bucks, U.K.); [2-^{14}C]alloxan (3 mCi/mmol) from Californian Bionuclear Corp. (Sun Valley, CA). Unless otherwise stated the incubation medium consisted of a Krebs Medium (KRBH) buffered with Hepes and bicarbonate at pH 7.4 and containing 0.5% (w/v) BSA [15]. Both labelled and unlabelled alloxan preparations contained less than 5% alloxanic acid as judged by thin layer chromatography [6].

Methods

Sperm cell isolation. Human semen was obtained from fertile volunteers, sperm bank donors and prevasectomy patients (age 25–40 years) by masturbation following a 2–4 days abstinence period while canine semen was obtained from mongrel dogs by a massage technique [16]. Semen samples were collected in clean plastic disposable recipients (Distrimedia, Brussels, Belgium) and further processed within the first hour following ejaculation. Spermatozoa were purified and isolated according to their progressive motility using a previously described Percoll gradient centrifugation (ref. 11 modified according to ref. 12). Further experiments were performed exclusively on gradient fraction 6 which contains only cells with good progressive motility.

Motility assay. Canine sperm heads displayed a tendency to stick to microscopic glass slides and to the surface of Makler's counting chamber [17] so that no accurate stroboscopic measurements could be performed [12]. Hence, both human and canine sperm motility characteristics were determined at 23° by photon correlation spectroscopy (PCS) measurements [12] and by visual estimation of the percent beating flagella in a Makler chamber. Both labelled and unlabelled alloxan were kept at 4° in 1 mM HCl until 15 sec prior to the start of cell incubations. The half life of alloxan at pH 7.4 and 23° does not exceed 5 min [6]; hence, the cells are only briefly exposed to alloxan after addition of the drug.

Metabolic studies. Uptake and oxidation experiments were conducted on samples of 10^7 cells according to previously described procedures except that D-[^{3}H] mannitol was used instead of [^{3}H]sucrose as extracellular space marker during uptake experiments [19]. In view of the well known instability of alloxan at 37° and pH 7.4 all uptake experiments were carried out at 23° [6]. Briefly, the cells were preincubated for 30 min at 23° in glucose-free KRBM medium whereafter 1 mM D-[^{3}H] mannitol (25 μCi/ml) and 5 mM ^{14}C urea (12.5 μCi/ml) or 5 mM [^{14}C]alloxan (15 μCi/ml) were added to the medium. Following a 5 min incubation at 23° the cells were separated from the medium by a 4 sec centrifugation at 8000 *g* (Microfuge B; Beckman Instruments, Fullerton, CA) through a layer of di-n-butyl phthalate (BDH Chemicals, Poole, Dorset, U.K.; d 1.045) and the radioactivity of the cell pellet was counted. From these data the apparent distribution space of the [^{14}C]-labelled compounds was calculated; results were always corrected for extracellular contamination as judged from the [^{3}H]-mannitol space measured in the same sample. Extracellular contamination was variable for different experimental conditions but as a rule did not exceed 3 times the cellular waterspace (estimated by urea distribution). Oxidation rates were determined at 37° during 30 min incubations.

Statistical analysis. Results were expressed as means ± S.E.M. for the number of experiments indicated in the figure legends. The statistical significance of differences between experimental groups was assessed by Student's *t*-test for unpaired data.

RESULTS

Alloxan toxicity

The cytotoxic effect of alloxan was evaluated by measuring the percent immobilized cells 3 hr following addition of the drug. In human spermatozoa, alloxan concentrations above 5 mM were required to block flagellar activity in 50% of the cells (Fig. 1). Canine spermatozoa were more susceptible to alloxan as a drug concentration of less than 2 mM was sufficient to arrest flagellar beating in 50% of the cells (Fig. 1). Despite their different sensitivity towards alloxan, human and canine sperm cells appeared equally sensitive to *tert*-but OOH which induced a 50% immobilization in both species at 1–2 mM concentrations (Fig. 1).

These differences in sensitivity to alloxan were also noted when sperm motility was measured by photon correlation spectroscopy (PCS) or when the rate of glucose oxidation was measured (Fig. 2). Incubation of canine spermatozoa with 5 mM alloxan reduced their rate of glucose oxidation by 50% and their PCS motility index by 80%. In human cells, this condition produced only a marginal inhibition in these parameters (Fig. 2). In contrast, the inhibitory effect of 1 mM *tert*-butyl OOH was equally potent in both species (Fig. 2).

Alloxan uptake

In order to test whether the higher sensitivity of canine spermatozoa to alloxan is caused by a higher degree of internalization of the drug, alloxan uptake by human and canine cells was compared at a 5 mM drug concentration which produces marked toxic effects in canine but not in human cells (see above, Fig. 1). Under these conditions spermatozoa of both species failed to exhibit any significant degree of alloxan uptake as the apparent alloxan space did never exceed 5% of the cellular waterspace, estimated by urea distribution [18] (Fig. 3). The presence of 5 mM unlabelled alloxan did not significantly alter the cellular urea space in both species (results not

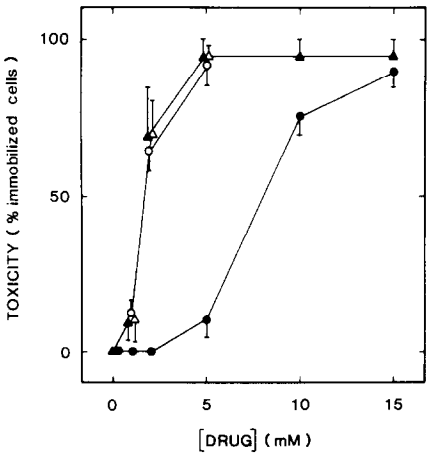

Fig. 1. Concentration dependence of alloxan- and *tert*-butyl OOH toxicity in human and canine spermatozoa (Percoll fraction 6). Experimental conditions for semen collection and cell isolation are described in the Methods section. The percent immotile cells was visually estimated in Makler counting chambers [17] after a 3 hr incubation at 23° in KRBH medium supplemented with various concentrations of alloxan or *tert*-but OOH. The degree of toxicity of a given compound was computed by correcting the percent immotile cells in presence of this compound for the percent immotile cells in a control condition without it and subsequently dividing the obtained difference by the fraction motile cells in the control condition. Results represent mean values ± S.E.M. for 3 independent observations. The presence of alloxan is denoted by circles and that of *tert*-but OOH by triangles, whereas filled symbols refer to human cells and open symbols to canine cells.

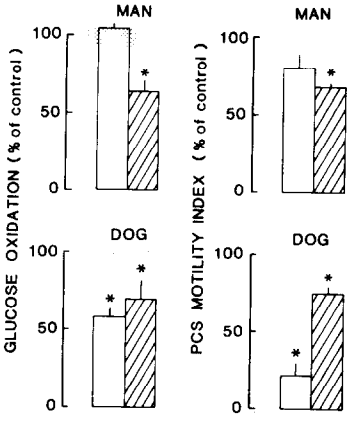

Fig. 2. Effect of alloxan and *tert*-but OOH on motility and glucose oxidation rates of human and canine spermatozoa (Percoll fraction 6). Sperm cells were incubated during 3 hr at 23° in glucose-free KRBH medium (control) or in the same medium supplemented with 5 mM alloxan (open bars) or 1 mM *tert*-but OOH (hatched bars). PCS motility parameters (right panels) and glucose oxidation rates (left panels) were subsequently determined both in human (upper panels) and canine spermatozoa (lower panels) and expressed as percentage of the values obtained in the absence of alloxan and *tert*-but OOH. PCS motility index was computed as the product of the percent motile cells and the motility parameter C determined by PCS measurements [12]. Results represent mean values ± S.E.M. for 3 independent observations. For comparison the mean value ± S.E.M. of the control (no alloxan nor *tert*-but OOH) is indicated by the dotted area. * $P < 0.01$ vs. control.

shown) indicating that the apparent lack of alloxan uptake was not due to an instantaneous permeability change of the plasma membrane, leading to a falsely increased D-mannitol space and hence a falsely decreased urea space after correction for extracellular radioactive contamination, as judged by the D-mannitol space.

Protection

a. *By sugars.* Both D-glucose and—to a lesser extent—3-*O*-MG provided a significant level of protection against alloxan-induced cellular damage as judged from PCS motility ratings (Fig. 4). The presence of L-glucose and uric acid had no influence whatsoever on the action of the drug (results not shown). In contrast to alloxan toxicity, the adverse effects of *tert*-butyl OOH could not be prevented by previous addition of 3-*O*-MG whereas D-glucose provided a significant but partial protection (Fig. 4).

Addition of sugars 20 min after alloxan or *tert*-butyl OOH exposure did not lead to significant improvement of motility characteristics (results not shown). Although higher alloxan concentrations were needed to produce toxic effects in human spermatozoa, the various sugars tested conferred roughly the same degree of protection as in dog cells (results not shown).

b. *By ascorbic acid.* Ascorbic acid alone did not significantly alter sperm motility (Fig. 5). When administered in combination with 5 mM alloxan, ascorbic acid potentiated the immobilizing effect of alloxan in human but not in canine spermatozoa, rendering both cell preparations equally susceptible to the immobilizing effect of the drug (Fig. 5). Ascorbic acid did, however, not alter the degree of motility inhibition that was induced by *tert*-but OOH (results not shown). Addition of superoxide dismutase (SOD; 1 mg/ml) effectively quenched alloxan toxicity (results not shown).

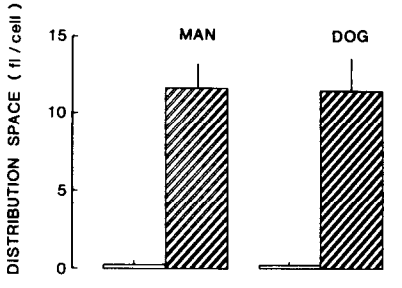

Fig. 3. Apparent distribution space of urea and alloxan in human and canine spermatozoa (Percoll fraction 6). Following a 30 min incubation at 23° in glucose-free KRBH medium, [^{14}C]urea (5 mM, 12.5 μCi/ml) or [^{14}C]alloxan (5 mM, 15 μCi/ml) were added to the medium and their cellular distribution space (hatched bars for urea and open bars for alloxan) determined both for human (left) and canine spermatozoa (right) following a 5 min incubation at 23° and after correction for extracellular contamination as judged from the D-[^{3}H] mannitol space (1 mM D-mannitol, 25 μCi/ml) determined in the same sample (see Methods section and ref. 18). Results represent mean values ± S.E.M. for 3 individual observations.

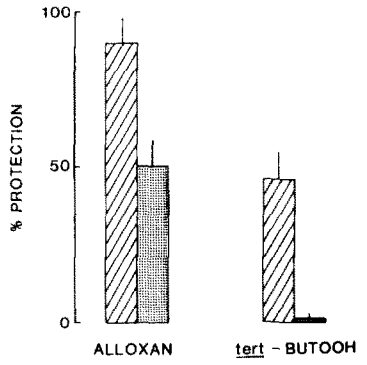

Fig. 4. Protective effect of D-glucose (hatched bars) and 3-*O*-MG (black bars) against alloxan- (left) and *tert*-butOOH-induced (right) toxicity in canine spermatozoa (Percoll fraction 6). Following a 20 min preincubation period at 23° in either sugar-free KRBH or in KRBH supplemented with 1.4 mM D-glucose or 5 mM 3-*O*-MG, alloxan (5 mM) or *tert*-butOOH (1 mM) were added to the sperm cells and after a second 3 hr incubation PCS motility parameters were determined and expressed as percentage of the values obtained in the absence of toxic substances. PCS motility index was computed as the product of the percent motile cells and the motility parameter C determined by PCS measurements [12]. The percent protection conferred by a given sugar against a given toxin was computed by dividing the percent decrease in motility index caused by the toxin in presence of the sugar by the percent decrease in motility index caused by the toxin in the absence of the sugar and substracting the obtained percent from 100%. Results represent mean values ± S.E.M. for 3 independent observations.

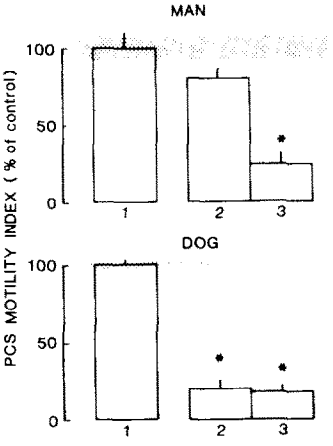

Fig. 5. Effect of L-ascorbic acid on alloxan toxicity in human and canine spermatozoa (Percoll fraction 6). Sperm cells were incubated during 3 hr at 23° in glucose-free KRBH medium (control) or in the same medium supplemented with 5 mg/dl L-ascorbic acid (column 1), 5 mM alloxan (column 2) or 5 mg/dl ascorbic acid and 5 mM alloxan (column 3). PCS motility parameters were subsequently determined both in human (upper panel) and in canine cells (lower panel) and expressed as percentage of the values obtained in control cells without added compounds. PCS motility index was computed as the product of the percent motile cells and the motility parameter C determined by PCS measurements [12]. Results represent mean values ± S.E.M. for 3 independent observations. For comparison, the mean value ± S.E.M. of the control is indicated by the dotted area. *$P < 0.01$ vs. control.

DISCUSSION

The present results indicate that alloxan can induce functional damage in both human and canine spermatozoa as witnessed by an impaired cellular motility coinciding with a decreased glucose handling. Although it has not been demonstrated which of both events is primarily affected by alloxan, it is, however, conceivable that, by analogy to the enhanced glucose catabolism following cyclic AMP mediated stimulation of sperm motility [20], the fall in glucose oxidation rate occurs as a consequence of the altered metabolic needs which follow an impaired sperm motility. A drop in cellular motility was also found to always precede a decrease in glucose handling by the isolated spermatozoa (Gorus and Pipeleers, unpublished work).

Since the impermeability of the spermatozoal plasma membrane for alloxan does not preclude the development of cytotoxic effects, it is tempting to speculate that the cell membrane constitutes a primary target site for alloxan in spermatozoa or alternatively might act as a gating mechanism for toxic oxygen radicals [21]. The mechanisms implicated in the alloxan toxicity upon spermatozoa might bear relevance for those involved in alloxan-induced diabetes, since canine spermatozoa were found to be more susceptible to the drug than human cells as seems also the case for pancreatic B-cells. Furthermore, both D-glucose and 3-*O*-methylglucose conferred a protection against alloxan, both in pancreatic B-cells as in spermatozoa. It seemed therefore interesting to examine the specific variation in spermatozoal sensitivity to alloxan.

Differences in glutathione peroxidase activity are probably not responsible for this phenomenon, as human and canine cells exhibited a comparable susceptibility to *tert*-butOOH, an organic peroxide that penetrates—without species preference—the cells where it serves as a substrate for this enzyme [8]. Neither is the higher sensitivity of canine spermatozoa attributable to a cellular lack in uricase and hence in the capacity to form a major anti-oxidant [22], since the addition of uric acid did not prevent alloxan-induced damage in these cells. Human and canine spermatozoa might, however, differ in their sensitivity to superoxide anion radicals, which are believed to be involved in B-cell damage by alloxan [23–25] as well as in spontaneous peroxidation of membrane phospholipids in aerobically incubated spermatozoa [26]. The finding that exogenous SOD protected against the alloxan toxicity in sperm cells together with the reported resistance of human sperm cells to spontaneous lipid peroxidation [13] favors the viewpoint that differences in SOD activity may contribute to the observed species variation in sensitivity towards alloxan. A second, perhaps more crucial, cause for the different alloxan sensitivity of human and canine spermatozoa may reside in their respective cellular ascorbic acid levels. This vitamin is indeed more concentrated in tissues of animals with an intact ascorbic acid synthetizing machinery

such as the dog than in those without such as man [27]. In contrast to canine spermatozoa, addition of ascorbic acid to human spermatozoa enhanced the sperm immobilizing action of alloxan at a 5 mM concentration; this potentiating effect of ascorbic acid on alloxan cytotoxicity has been observed previously in other cell systems and has been ascribed to an acceleration of the alloxan–dialuric acid redox cycle leading to an increased production of oxygen radicals and hydrogen peroxide [2, 28, 29]. Incidently, a relative resistance to alloxan is also present in the guinea pig [8, 30] which, like man, is exclusively dependent on dietary supply of ascorbic acid [31].

The observed impermeability of sperm cells for alloxan is difficult to reconcile with the protective effects of 3-*O*-MG. In pancreatic B-cells the protective action of this poorly metabolized glucose analogue has been explained on the basis of an inhibited cellular alloxan uptake [6]. However, the reported competition between 3-*O*-MG and ascorbic acid for the glucose carrier of several cell types [32–34] raises the possibility that 3-*O*-MG and D-glucose might, at least partially, be operative by interfering with ascorbic acid transport, which could explain the higher degree of protection against alloxan toxicity than against *tert*-butyl OOH.

In conclusion, human spermatozoa were markedly less sensitive to alloxan than dog sperm cells. This finding might relate to species differences in ascorbic acid handling and/or oxygen radical scavenging. Since the observed spermatozoal sensitivity *in vitro* parallels the previously reported *in vivo* sensitivity of pancreatic B-cells, its seems likely that the present findings bear relevance for the susceptibility of pancreatic B-cells to diabetogenic drugs such as alloxan.

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