

Streptidine, a metabolic derivative produced after administration of streptomycin *in vivo*, is vestibulotoxic in rats

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Summary. Streptomycin is the treatment of choice in developing countries for patients suffering from tuberculosis or other infectious diseases. However, it produces incapacitating vestibular symptoms whose onset is delayed and gradual. This observation led to the notion that a streptomycin metabolic derivative and not the antibiotic itself is the damaging agent for the inner ear.

To study further the existence of this ototoxic metabolite, chronic treatment with streptomycin or its putative derivative streptidine was carried out in young male Long Evans rats. The presence of streptomycin or streptidine in the blood of animals of either experimental group was assessed by high performance liquid chromatography and analysis of swimming behavior was used to evaluate vestibular damage. Features of the sensory epithelium and quantification of hair cells were attained in sections of the utricular organ of all groups by light microscopy.

After 25, 35 and 45 days of treatment with streptomycin, a metabolite with the same chromatographic properties as the streptidine standard run in parallel was identified in the blood of rats. Concentrations of the metabolite were 2.26 µg/ml on the 25th day and around 8.0 µg/ml in both the 35th and the 45th day of treatment, while streptomycin was below its detection level at either period. In streptidine-treated rats, the concentration of this compound was 1.0, 1.84 and 4.94 µg/ml on the 25th, 35th and 45th treatment days, respectively. Treatment with either streptomycin or streptidine resulted in similar abnormal swimming patterns and histological alterations of the utricular epithelium. Loss of hair cells was roughly equivalent even though streptidine was administered in a dose 90% lower than streptomycin.

The gradual appearance of streptidine as a metabolic

derivative of the antibiotic in the blood of rats or the administration of this compound alone, causing similar functional and structural vestibular deterioration seen in streptomycin-treated animals, supports the notion that streptidine is a potential contributor to ototoxicity after prolonged antibiotic administration.

Key words: Ototoxicity, Streptidine, Streptomycin, Streptomycin derivatives

Introduction

Primary tuberculosis (TB) has become a pandemic ailment in the last 20 years and it is an opportunistic disease in AIDS patients. Because streptomycin (STP) is cheap, has a rapid onset of bactericidal action and has no major side effects such as hepatitis associated with combined rifampicin-isoniazid therapy (Akano et al., 2002), STP is the main therapeutic agent used in the treatment of TB and other infections in developing countries.

The widespread use of STP is of concern, due to a potential risk of serious disruption of balance and other side effects that may arise from specific damage to the hair cells of the sensory epithelium of vestibular cristae (Meza et al., 1989, 1996) or utricular maculae (Meza et al., 1994, 1996; Fermin et al., 1998), as well as damage to vestibular and auditory function typical of aminoglycosidic antibiotics (Fee, 1980; Wersäll, 1995). However, the abnormal swimming patterns observed in rats correlate well with the disorientation behavior shown in water by mutant mice (pallid mutant mouse), which often lack otoconia in both, saccular and utricular maculae (Trune and Lim, 1983a,b).

Although the unwanted side effects of aminoglycosides have been known for an extensive period, the molecular action that results in these effects remains unknown. Because the onset of offensive side effects is delayed and gradual, some investigators have postulated a metabolic transformation or activation of

the antibiotic to produce molecular species, which either may have greater toxicity than the drug itself or may act synergistically with STP to produce ototoxicity (Crann and Schacht, 1996). Attempts to identify ototoxic metabolites derived from kanamycin (Owada, 1962), gentamicin (Huang and Schacht, 1990; Crann et al., 1992) or STP (Wang et al., 1999) have failed to characterise these putative compounds.

The STP molecule is composed of three molecular entities: streptidine (STD), an aminated cyclitol bearing two guanidine groups (in positions 1 and 5 of the ring); streptose, a five-carbon ring sugar and N-methylglucosamine. These compounds are bound to one another via respective glycosidic links (Meza et al., 2001). In chemical studies the application of either basic or acidic hydrolysis to STP always resulted in STD as one of the products, indicating that the alpha 1,4-glycosidic bond between STD and streptose is the most labile region of the molecule (Barba-Behrens et al., 1990; Meza et al., 1999).

In vitro incubation of STP in human serum gave rise to STD which could be metabolically separated from the rest of the molecule (Granados et al., 2002). Other studies suggested that STD might be the active ototoxic part of the molecule because when isolated cell membranes of rat (kidney, liver, brain or vestibular hair cells) were incubated with either STP or any of its three components, only the STD portion of STP was bound to hair cell membranes of the vestibule to a highly specific cationic site through its guanidine groups (Meza et al., 1999).

Cytofluorescence experiments in vestibular tissue of guinea pigs acutely treated with STP have demonstrated fluorescence attributed to the antibiotic accumulation in the vestibular sensory cells and nerve fibers of the stroma (Bareggi et al., 1986; Meza et al., 2001). Fluorescence was very intense after 1 hour of treatment with STP and increased after 12 hours, decreasing to control levels 24 hours after treatment (Meza et al., 2001). STP reaches the ear through blood and its entry to the cerebrospinal fluid is very limited (Strausbaugh et al., 1977; Sande and Mandell, 1996). In turn, the half-life of STP is 4 hours in the inner ear perilymph and its concentration decreases until being completely cleared out after 24 h (Voldrich, 1965; Tran Ba Huy et al., 1981), suggesting, in our experiments, an additional fluorescent agent (probably STD) had reached the ear and accumulated during a 4-12 hour period.

We could corroborate these observations when STP and STD were incubated individually or together in a test tube in various buffer solutions and spectrofluorescent observations detected an increase of the signal only when both compounds were together (Meza et al., 2001).

To study further the proposed existence of STD as a putative ototoxic metabolic derivative of STP we conducted parallel biochemical, behavioral and morphological studies of the effects of chronic treatment with STP or STD on male rats.

Materials and methods

Animals

Young male Long-Evans rats weighing around 70-80 g were used in this study. Animals were housed individually under a standard 12-h light/12-h dark cycle at room temperature of 22 °C with food and water *ad libitum*. All experimental manipulations were performed after each experimentation period during the light phase of the cycle. The general good health of the animals throughout experimentation time was ascertained by the estimation of body weight increase. All methods used were in compliance with the Rules in Health Matter (Ministry of Health, México) with the approval of local Animal Care Committee.

Treatment

Rats were given daily intramuscular injections of STP (400 mg/kg body weight) (Pisa Laboratories, México) or 50 mg/kg of STD, which was obtained by acid hydrolysis of STP and purified and identified, as already described (Barba-Behrens et al., 1990; Meza et al., 2001) in alternate right or left hind leg. Due to STD partial water insolubility, both compounds were dissolved in 70% glycerol for administration. Control rats received 70% glycerol alone for the same time interval and conditions as their experimental mates.

Experimental groups

Data from 3 experimental conditions are used in this report. The animals in the first experimental paradigm (Group 1) were used to study the possible transformation of STP into a derivative in the blood of animals during STP treatment. This group was subdivided into 4 experimental subgroups: 3 of them were treated with STP and the animals were sacrificed 25, 35 or 45 days after treatment (n=4 to 9 each) and a group of normal glycerol-treated animals which served as controls (n=4 each) sacrificed on the respective STP treatment days.

In the second experimental approach (Group 2), the animals were subdivided into 4 experimental subgroups: 3 subgroups were treated with streptidine (STD), putative metabolic derivative of STP and the rats were sacrificed 25, 35 and 45 days after treatment (n=5 to 11 each). The fourth group was the control-glycerol-treated animals (n=4 each) sacrificed on respective 25, 35 and 45 days after treatment.

The third experimental condition (Group 3) was designed to assess whether alterations in swimming behaviour (putatively attributable to vestibular utricular damage) were present in the rats 25, 35 and 45 days after treatment with either STP or STD and whether histological damage occurred in either treatment group.

This group of animals was subdivided in turn into 9 experimental subgroups: 3 subgroups (n=9 to 14 each) were treated with STP for 25, 35 or 45 days,

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respectively. 3 subgroups (n=7 to 17 each) underwent 25, 35 or 45 days of respective STD administration, and 3 subgroups of control glycerol-treated animals (n=5 each) for respective STP or STD treatment days.

HPLC conditions for groups 1 and 2

The HPLC method was performed essentially as described by Whall (1981). The chromatographic system consisted of a Pump Waters 600E (Waters Assoc., Milford, MA, USA) equipped with an autosampler Waters 717plus and a Photo diode array (PDA) detector Waters 996. The analytical column was a Prodigy ODS3 reversed-phase, 5 μ m, 250x4.6 mm (Phenomenex, Torrance, CA, USA). The column flow-rate was maintained at 1.3 ml/min and its temperature was set to 25 \pm 0.1 $^{\circ}$ C. The detector wavelength was of 200 \pm 0.2 nm. Upon completion of daily analysis, the column was washed with a mixture of acetonitrile: water (65:35).

The mobile phase to separate and estimate STD and STP contained 20 mM sodium 1-hexanesulfonate (REGIS, Morton Grove, IL, U.S.A) and 25 mM tribasic sodium phosphate (J.T. Baker, Xalostoc, México), in a water:acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) mixture (85:12-15 v/v). The solutions were adjusted to pH 6.0 with 85% phosphoric acid (J.T. Baker, Xalostoc, México) and filtered through a 0.22 μ m filter (Millipore Corp., Bedford, MA, USA) prior to use.

Rat serum

Trunk blood was collected by decapitation and serum was obtained by centrifugation for 12 min at 12,000 rpm at room temperature. Procedures were conducted individually on controls, STP or STD-treated rats at treatment days of 25, 35 and 45 days.

Extraction of STP and STD and HPLC determination

Individual sera were deproteinized by addition of 20% trichloroacetic acid (TCA). Supernatant was used for chromatography after separation from precipitate by centrifugation at 14,000 rpm for 40 min.

Concentration of either STP or STD present in serum of treated rats was calculated from standard curves derived from analysis performed in deproteinized serum of control rats to which a mixture of 5 increasing amounts (from 5-80 μ g/ml) of STP or STD (from 5-16 μ g/ml) were added.

Swimming analysis and histology for group 3

Swimming behaviour was assessed for each animal of the nine subgroups of group 3, starting on the 25th day of treatment and continuing at approximately one-week intervals, with animals tested repeatedly as the duration of treatment was extended. Rats were placed in a water tank at 27 $^{\circ}$ C and swimming activity was recorded on videotape for 45 sec as described by Meza et al. (1996).

Analysis and classification of swimming patterns were performed after the test.

Fixation and embedding procedures for histological studies

One day after treatment was interrupted, each individual animal which presented abnormal swimming patterns throughout STD or STP administration and their respective controls were deeply anaesthetised with 475 mg/kg chloral hydrate (Sigma, St. Louis, MO, USA) and processed essentially as already reported (Meza et al., 1996; Fermin et al., 1998). They were transcardially perfused, first with isotonic saline solution (0.85% NaCl, Sigma) and secondly with 4% paraformaldehyde (Polysciences Inc.) in 0.1 M sodium cacodylate (Electron Microscopy Sciences) buffer (SCB) pH 7.4 at 4 $^{\circ}$ C (fixative solution). After perfusion, the cranium was opened and auditory bullae were dissected and placed in the same cold fixative solution for a 2-hour post-fixation under slight agitation. After that, bullae were opened under a dissecting microscope to expose vestibular organs and the utricular macula was extracted. The utricular macula was then thoroughly rinsed, dehydrated and embedded in paraffin.

The maculae were then sectioned at 3 μ m until the medial border was reached. After sectioning into the region further to reach the targeted area a minimum of 80 serial sections were obtained from within the medial part of the macula (95 to 115 μ m). Sections were then mounted on gelatin-coated slides, stained with 0.2% toluidine blue and examined under light microscopy with Nomarski illumination.

Light microscopy

Sections from each group were observed using a Polyvar light microscope (Reichert-Jung, Wien, Austria). The specimens to be analysed were located using a 10x objective and later focused with a 40x objective to ensure maximum detail. Permanent record images were captured with a digital video camera (Jai, Tokyo, Japan) coupled to the microscope using Matrox PC-VCR software. For publication, they were edited and labelled with Adobe Photoshop 5.0 LE program after which they were converted to TIFF format using GIMP software.

Criteria for hair (sensory) cell identification

Hair cells were identified by the presence of well-defined stereociliary tufts emerging from a smooth upper surface located at the luminal portion of the sensory epithelia.

Quantitative estimation of number of sensory cells in the utricular macula

The methodology followed was essentially as

described by Ross (1997) and López et al. (2002). An area of about 3.9 mm² in the central part of the macular section was chosen for quantitative estimation of the sensory cells (Fig. 3A,B). To obtain total hair cell count the average cell counts of sections within 20 µm were multiplied by the ratio of all sections in the interval (20 µm) to the thickness of the counted sections (3 µm).

Results

Determination of STD in blood of STP or STD-treated rats by HPLC

A mixture of 7.2 µg/ml of STD plus 77 µg/ml of

STP in deproteinized control rat serum rendered satisfactory separation of STD from STP, with retention times of 3.6 min and 6.6 min, respectively (Fig. 1A).

After chromatographic analysis of blood derived from rats treated for 25, 35 or 45 days with STP, no peak was observed in the STP region; however, a peak that increased in height with increasing treatment period was observed at the region of STD (Fig. 1B,C and D).

Similarly, in animals treated with STD a discrete STD peak was evident on the 25th or 35th treatment days which increased in height 45 days after treatment (Fig. 1E). In serum of control animals, no STD or STP peaks were found (not shown).

The concentrations of STD derived *in vivo* from STP

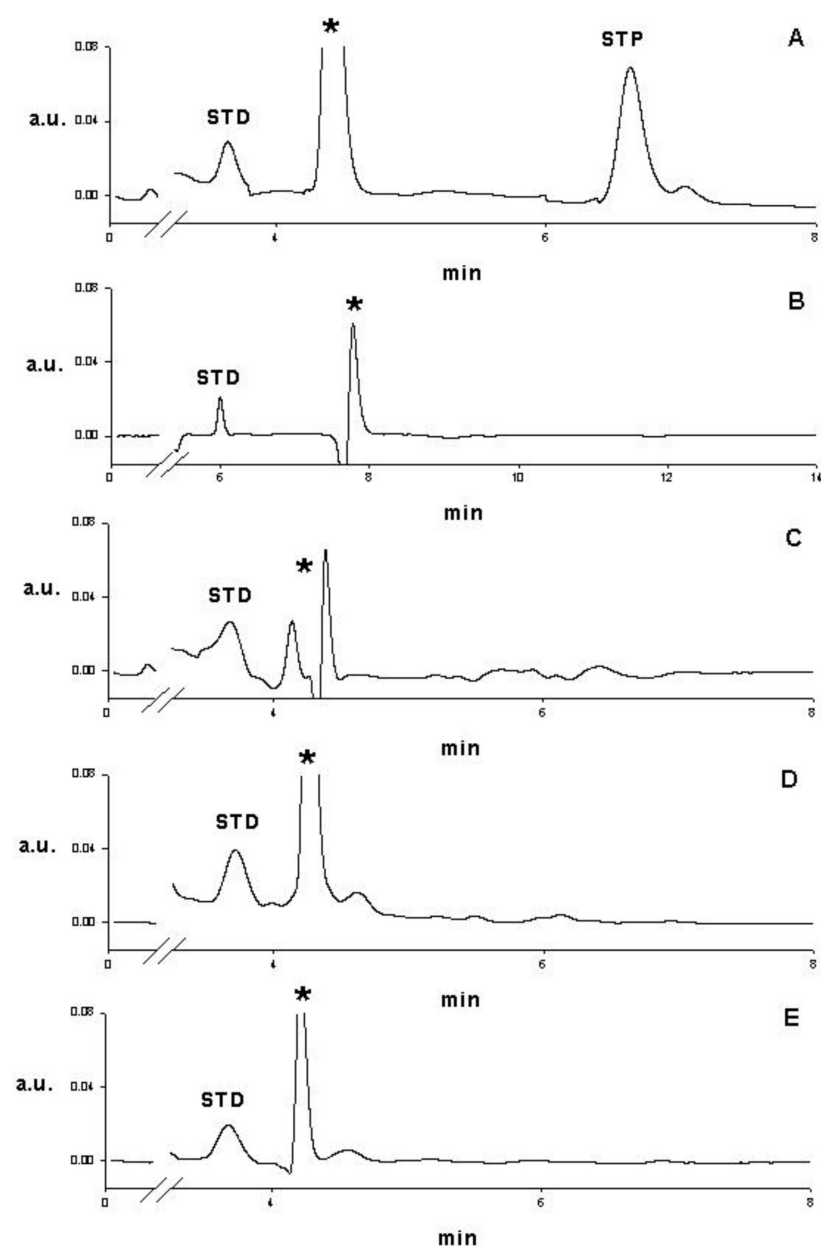
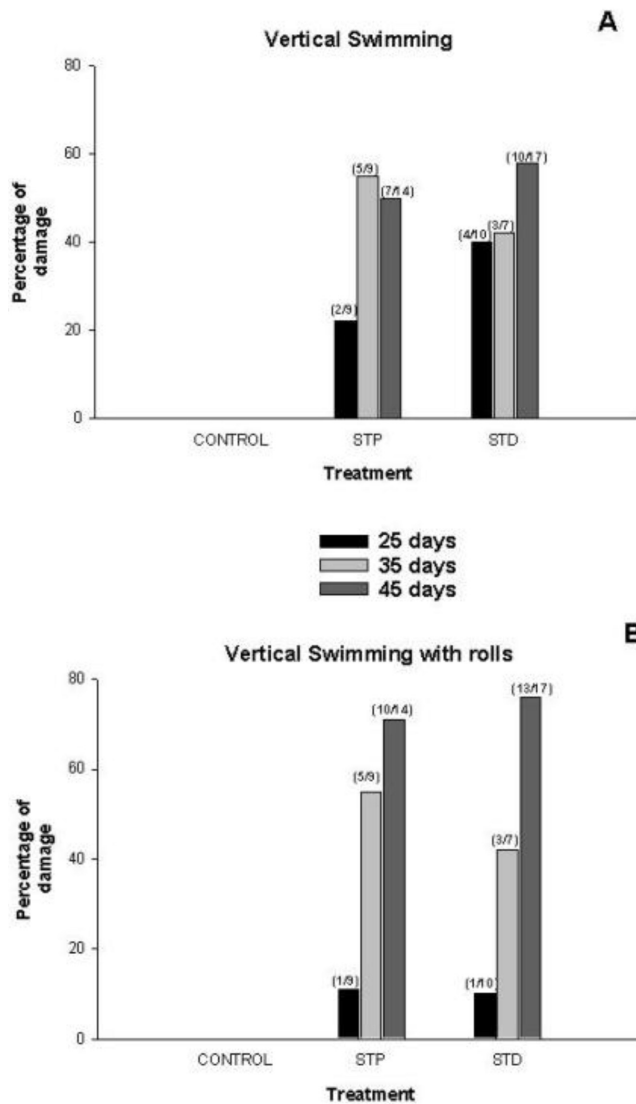


Fig. 1. Chromatograms of a typical assay of rat serum of the various experimental groups run in parallel. **(A)** STD, 7.2 µg/ml and STP, 77 µg/ml standards in rat serum extract. **(B)** Serum extract of STP-treated rats (400 mg/kg) at 25 days, **(C)** 35 days and **(D)** 45 days of treatment. **(E)** Serum extract of STD-treated rats (50 mg/kg) at 45 days of treatment. Asterisks indicate a peak of absorbance of the serum contents. Retention time (RT) for STD at 25 days of treatment in 12% acetonitrile was of 6.0 min. RT for STD and STP in 15% acetonitrile were 3.6 to 6.6 min, respectively. a.u.: arbitrary units.

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are presented in Table 1. After 25 days of treatment, a concentration of 2.26 $\mu\text{g/ml}$ of STD was found and concentrations of 8.69 and 7.70 $\mu\text{g/ml}$ of STD were present at 35 or 45 days of STP treatment, respectively. By the same token, in STD-treated animals, the respective STD concentrations at 25, 35 and 45 days after treatment were of 1.0, 1.84 and 4.94 $\mu\text{g/ml}$ (Table 1).

Swimming behavior

Abnormal swimming patterns, already described for STP treated rats (Meza et al., 1994, 1996) which consisted of vertical swimming and vertical swimming

Table 1. Concentration of STD in serum of rats after treatment with STP (400mg/kg) or STD (50 mg/kg)*.

DAYS OF TREATMENT	STD ($\mu\text{g/ml}$) IN STP-TREATED	STD ($\mu\text{g/ml}$) IN STD-TREATED
25	2.26 \pm 0.11 (4)	1.00 \pm 0.20 (5)
35	8.69 \pm 0.52 (9)	1.84 \pm 0.30 (7)
45	7.70 \pm 0.29 (9)	4.94 \pm 0.35 (11)

*: Figures are mean values \pm standard error of the mean for the number of rats in parenthesis whose extract was run in triplicate.

Fig. 2. Histograms of the percentage of rats displaying the disrupted swimming patterns (Vertical swimming, **A**; Vertical swimming with rolls, **B**) in control, STP and STD-treated rats at 25, 35 and 45 days of treatment. The number of damaged rats/total number of rats is indicated in parenthesis above the respective columns.

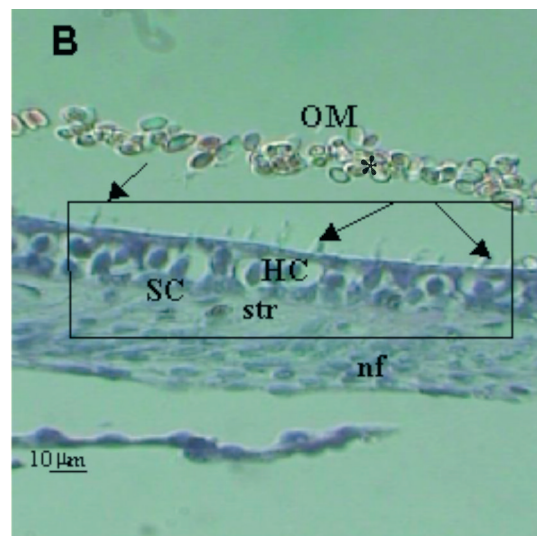
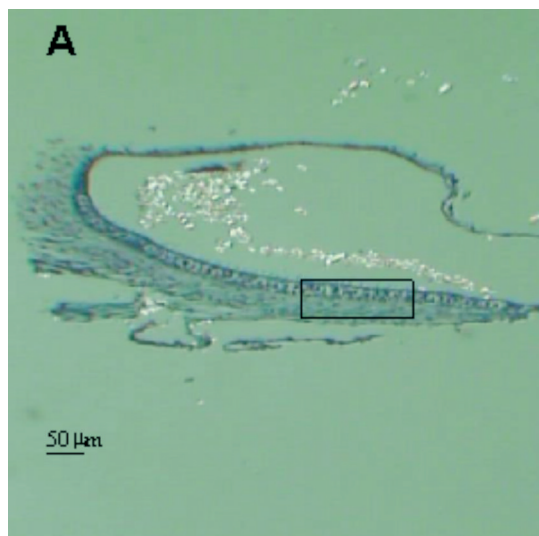


Fig. 3. **A.** Light microscopy of the normal rat utricular macula (control rats). **B.** A higher magnification view of area within the rectangle in **A**. OM, otolith membrane; HC: hair cells; SC: supporting cells; str: stroma; nf: nerve fibres. Asterisks indicate otoconia and hair bundles (arrows). Sections were observed under Nomarski illumination.

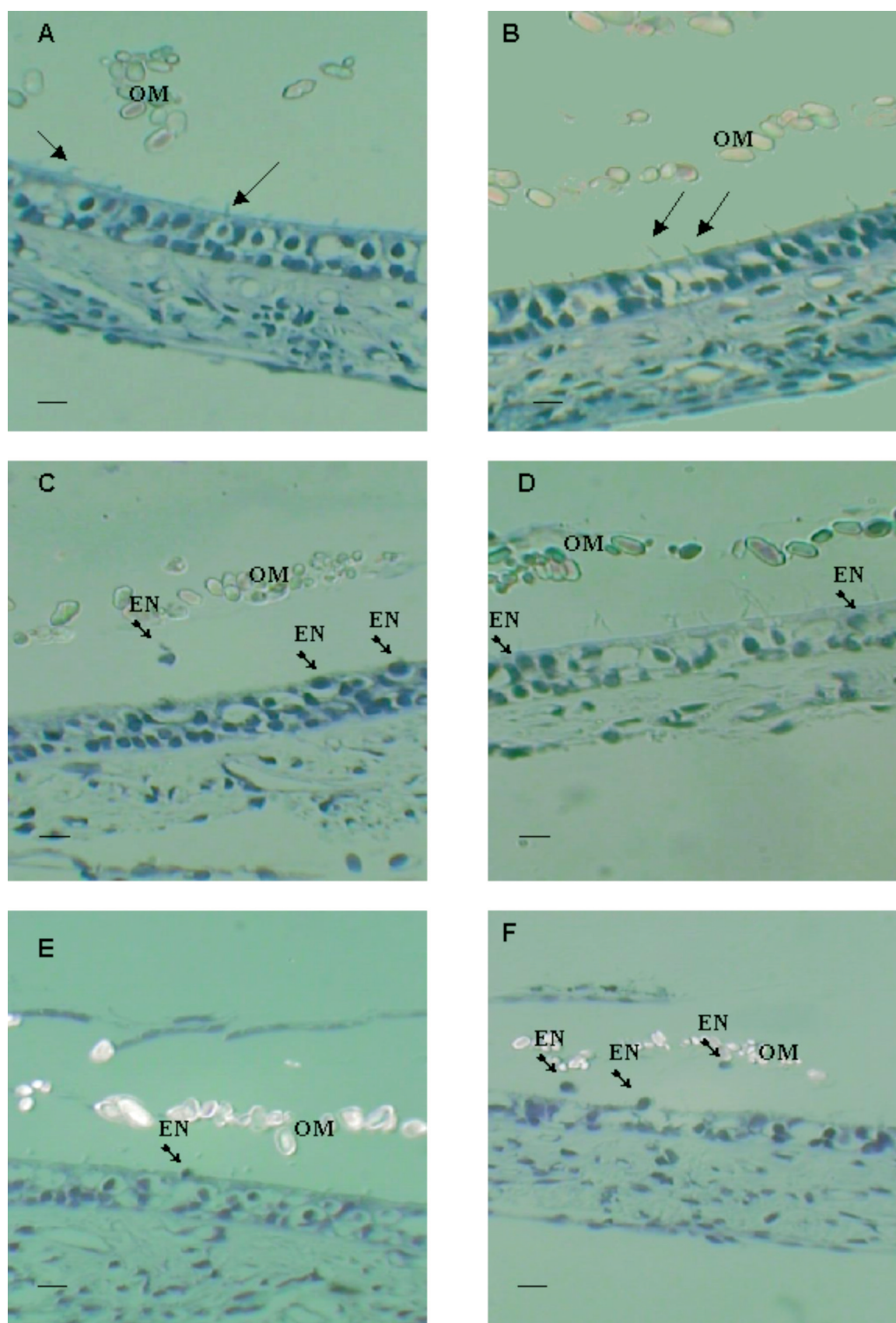


Fig. 4. Typical digital light micrographs of 3 μ m utricular epithelium sections. **A, C and E.** STP-treated rats (400 mg/kg) after 25, 35 and 45 days of administration, respectively. **B, D and F.** STD-treated rats (50 mg/kg) at 25, 35 and 45 days, respectively. OM: otolithic membrane; EN: extruded nuclei and hair bundles (arrows). Sections were observed under Nomarski illumination. Bars: 10 μ m.

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with rolls, the former appearing earlier than the latter. These patterns were observed in both STP and STD-treated animals (Fig. 2). However, the effects increased in magnitude on subsequent days of STP or STD administration (Fig. 2).

The percentage of rats in either treatment showing vertical swimming was 40%, 42% and 58% for 25, 35 and 45 day-STD-treated animals; 22%, 55% and 50% for STP-treated rats for respective 25, 35 and 45 days of treatment (Fig 2A). Whereas animals presenting vertical swimming with rolls was 10%, 42% and 76% for STD-treated animals; 11%, 55% and 71% for STP-treated rats (Fig 2B), respectively.

Morphological damage in the utricular epithelium after STD or STP treatment

Three-micrometer sections of utricular organs of control animals injected with glycerol alone (Fig. 3B) showed the typical features of the utricular macula were a pseudostratified epithelium composed of hair cells with their nuclei in an upper layer, and supporting cells with their nuclei forming a lower layer. The upper surface of the epithelium was smooth with hair bundles projecting from it into the overlying otolithic membrane that contained many polymorphous otoconia of relatively homogeneous size.

The number of hair cells in utricular sections of glycerol-control animals stayed unchanged throughout treatment (n=166, 173 and 176) (Table 2). These features were followed in sections of treated animals.

After STP treatment for 25 days, the epithelium had a similar appearance to the control (Fig. 4A), but after 35 and 45 days there was increasing levels of disorganisation and hair cell loss (Fig. 4C,E). Thus numbers of hair cells were 151, 88 and 79 after each treatment period, respectively (Table 2). This loss of hair cells was accompanied morphologically by a reduction in visible hair bundles, some evidence of extruded hair cells nuclei and irregularities in the surface of the epithelium and in the pseudostratification.

After STD treatment, the changes of the utricular epithelium at each stage were similar as STP-treated animals (Fig. 4B,D,F) and the hair cells loss was 157, 95

and 49 after 25, 35 and 45 days, respectively (Table 2). One further alteration noted in the latter treatment was reduction in the apparent size of the otoconia.

Discussion

Our results demonstrate the presence of STD *in vivo* following chronic treatment with STP. Further, we show that chronic treatment with STD, as assessed by swimming behaviour observations, produces disruption of utricular organ-related vestibular function earlier described by us in rats treated with STP (Meza et al., 1994, 1996; Fermin et al., 1998). These effects of STD on swimming are similar as those produced by STP, even when the dose of STD is only 10% that of the STP dose.

The putative source *in vivo* of STD is hydrolysis of the aminoglycoside in the rat blood, as the metabolic transformation that has been previously suggested for other aminoglycosides (Huang and Schacht, 1990; Crann et al., 1992; Crann and Schacht, 1996).

These findings, in concert with a similar specific decrease in the number of sensory cells in the utricular macula produced in either STP-treated animals or by a lesser dose of STD, support our hypothesis that blood-borne STD is the major ototoxic agent producing disruption of vestibular function in STP therapy (Meza et al., 2001). Other studies have demonstrated that this antibiotic accumulates in the perilymph and remains there for a short time period (0 to 4 hours) after which it is excreted (Voldrich, 1965; Tran Ba Huy et al., 1981).

The *in vivo* presence of STD in the blood of STP-treated animals contradicts the assertion that STP, and possibly other aminoglycoside antibiotics, are excreted in urine essentially unchanged (Schentag and Jusko, 1977). This finding also corroborates the observation of specific ototoxic effects of STP without any nephrotoxic effects reported for other antibiotics (Williams et al., 1987; Begg and Barclay, 1995) as the general health of the animals in our study was preserved.

This is the first report to our knowledge to identify STD as a vestibulotoxic compound derived *in vivo* from STP. Wang and collaborators (1999) described a cytotoxic metabolite that was converted from STP in serum of patients with hearing loss, resulting from treatment with STP. The HPLC technique in Wang et al. (1999) was similar to that used here, and the compound derived from STP produced an HPLC peak at a retention time similar to that of the STD on this study. However, Wang et al. (1999) did not use procedures that identified the specific compound that derived from STP.

These results confirm that there is a longstanding metabolic capability in the rat blood to metabolise systemically administered STP and convert it to STD. Further, these data suggest this metabolite is potentially contributing together with STP for damage to the inner ear. However, the precise biochemical mechanism and the possible blocking of this conversion, which may lead to a safer treatment to patients requiring STP, is presently being investigated.

Table 2. Estimation of the number of sensory cells in the utricular macula after treatment with STP or STD*.

DAYS OF TREATMENT	CONTROL	STP	STD
25	166±6.38 (3)	151±12.87 (3)	157±9.18 (4)
35	173±7.57 (3)	88±10.48 (5)	95±11.20 (5)
45	176±10.31 (3)	79±9.89 (5)	49±8.10 (5)

*: Figures are values ± standard error of the mean of cell number estimated as described in Methods for rats indicated in parenthesis.

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