Morphology and electrophysiology of the vestibular organ in the guinea pig
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Functional and anatomical alterations in the gentamicin–damaged vestibular system in the guinea pig.

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Functional and anatomical alterations in the gentamicin–damaged vestibular system in the guinea pig.
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Introduction

In the last decades, measurements of short latency vestibular evoked potentials (VsEP) to acceleration stimuli have shown to be a useful parameter of vestibular function in experimental animal research (1–6). The place of origin in the vestibular system of these potentials depends on the method of stimulation. The response from an angular acceleration stimulus is supposed to originate from the semicircular canals (7), while linear acceleration stimulus evokes a response from the otolith organs (8). Advantages of VsEP measurements compared to the calorigram are the controllability and variation of the stimulus, and the quantification of the acquired responses. The measurements can be performed longitudinally and the possibility to measure a single vestibular organ is a significant advantage over the electronystagmogram, which uses the oculomotor system and relies on both vestibular organs.

Vestibular evoked potentials measurements have been used to evaluate the functional damage afflicted to the vestibular system by aminoglycoside application. In experiments in mammals, vestibular evoked potentials have shown to diminish sharply after topical administration (9) and more gradually after systemic administration (10,11). It can be also used to differentiate between vestibulotoxic and cochleotoxic substances (12). A remarkable result was found in chicks, where a complete recovery of vestibular function was found 70 days after streptomycin application (13).
Whether the functional change is reflected in anatomical damage is still unclear. In otoconia–deficient mice, vestibular responses to linear acceleration are absent, which is a clear example how morphology and function can be related \(^{(14)}\). Morphological damage by gentamicin and other aminoglycosides to the vestibular end organs is described extensively in many studies \(^{(15–16)}\). Elidan et al. combined VsEP measurements to angular acceleration after intraperitoneal gentamicin with a light microscopy study and found morphologic changes, mainly in the cristae ampullae and milder changes in the maculae \(^{(11)}\). Sugasawa et al. found some indications that electrovestibular brainstem responses to angular acceleration might reflect morphological changes in the lateral semicircular canal in a scanning electron microscopic study with various aminoglycosides \(^{(10)}\).

The purpose of our study was to follow the alterations of the vestibular evoked potentials to linear acceleration stimuli after gentamicin application in different dosages to investigate a dosage–response effect and to investigate and quantify the morphological changes in the utricle. The first positive peak of the response was chosen as marker for vestibular function since this can be regarded as a pure vestibular response \(^{(6)}\) without the need for acoustic masking which has a dampening effect on the vestibular response \(^{(17,18)}\). Systemic gentamicin was chosen since gentamicin is more specific to the vestibular system than other aminoglycosides \(^{(19)}\) and dosages can be controlled easily. Morphological evaluation of the utricle was chosen because linear acceleration stimuli are supposed to originate from the otolith organs and the utricle is less susceptible to damage and artefacts in scanning electron microscopy preparation than the saccule, which is fixated to bone. Surface area damage calculation of the whole macula of the utricle was done to evaluate the whole utricle instead of selected parts, as in hair cell density measurements.

Materials and methods

In this study 15 female albino guinea pigs were used (bodyweight approximately 500 gram, Harlan, The Netherlands) with a normal Preyer reflex. Animal care and use was in accordance with the principles of the declaration of Helsinki and approved by the animal experiment committee (protocol number 2325). All animals were anaesthetised before every vestibular evoked response measurement using ketalazine / xylazine intramuscularly (1 mg/kg, 0.7/0.3) and received additional doses at regularly spaced intervals. The animal was sacrificed after the last measurement without regaining consciousness.
**Vestibular evoked potentials**

To stimulate the vestibular system by acceleration pulses, a steel bolt was cemented upside down to the top of the skull of the guinea pig (figure 1). After exposing the bony facial nerve canal near the bulla canal, a platinum electrode was implanted up to the first curvature. At this position only a thin bony wall separates the electrode from the vestibular nerve. A reference electrode was placed in the neck muscles.

Acceleration pulses were generated with a Bruel and Kjaer vibration exciter (type 4809) and monitored with an accelerometer, connected to a Bruel and Kjaer amplifier (type 2651). Linear acceleration pulses were applied in the direction of the earth vertical Z-axis perpendicular to the skull. Since the inner ear is fixated in the cranial bone, the acceleration measured by the accelerometer attached to the bolt is equal to the acceleration in the vestibular system. The shaker was driven by computer-generated stimuli that consisted of Haversine pulses, amplified by a power amplifier (Bruel and Kjaer type 2712) and computer-controlled with a programmable attenuator. The vestibular stimulus consisted of 500 alternating pulses with peak amplitude of 40 m/s² at 0.5 ms after onset at a rate of 51/sec.
Electrophysiologic responses to acoustic stimuli were also measured with the electrode in the facial canal to ensure a functioning cochlea. Acoustic stimuli were computer–generated and consisted of 1 kHz Haver sine pulses that were presented at a level of 70 dB with a speaker situated 15 cm in front of the animal’s head. The signals of the active electrodes were amplified (Disa, type 15 c01) and band–pass filtered (100 Hz– 5 kHz). The first 10 ms of the electrode and accelerometer signals were recorded, averaged and processed, using a Tucker–Davis BioSig–stimulate/record system; version 2.0.

The latency and amplitude of the first positive peak (P1) of the VsEP was used as a measure of vestibular function (6). The value on the first day of the measurement series was used as a reference. All subsequent values were expressed normalized with respect to this reference value. Statistical evaluation was performed using SPSS 10.5. The non–parametric Kruskal–Wallis test was used.

The fifteen animals were allowed to recover from the operation for several days and divided in three equal groups; two gentamicin and one control group. Intra–muscular gentamicin injections were given daily in the gentamicin groups, over three periods of five days, with a pause of two days between the periods. Gentamicin (gentamicinsulphate 40mg/ml, Centrafarm) was given in two dosages. One group received 50 mg/kg (G50) daily, and the other group 100 mg/kg (G100). VsEP measurements were then performed on the first, third and fifth day during the three gentamicin administration 5–day periods. To monitor the general condition, all guinea pigs were weighed before every VsEP measurement. One week after the last administration of gentamicin, the VsEPs were measured for the last time and afterwards the guinea pigs were sacrificed by an intracardial injection of sodium pentobarbital (60 mg/kg i.p.) and prepared for scanning and transmission electron microscopy.

**Scanning and transmission electron microscopy**

After sacrifice, the animals were decapitated and the bullae were removed and placed in ice–cold HBSS (Hanks’ buffered salt solution) (pH 7.4; 320 Mosm; 0–40°C). The vestibulum was opened, and the otolith organs were located. With a fine pair of tweezers the otolithic membrane was gently removed and the otolith organs were then fixated in a solution of 2.5% glutaraldehyde in 0.1M Na–cacodylate buffer (pH 7.4; 4EC; 400 Mosm) and 2mM calcium chloride. Eleven gentamicin utricles were prepared for scanning electron microscopy.
(SEM) and nine for transmission electron microscopy (TEM). A more detailed description of preparation and fixation is published elsewhere (20).

**Surface area damage ratio**

The surface area damage ratio measurements were performed by a technician who was not involved in the treatment with gentamicin, nor the electrophysiological measurements, preparation and examination of the specimens for morphological evaluation. He was not aware whether the utricles were from gentamicin treated subjects or from the control group. Several specimens were severely damaged during preparation, which made outlining of the utricle impossible. These were excluded from statistical evaluation. Three of the control group, four of the G50 and five of the G100 specimens remained for analysis.

To assess the surface area damage ratio, an overview picture of the surface of the whole macula of the utricle was taken. The outline of the utricle was drawn and the surface computer–calculated in $\mu m^2$. Then damaged areas were marked. These were defined as areas with a clear reduced density of hair cell bundles, areas with degeneration signs like fusion or holes, or areas within the outline with no hair cell bundles but only supporting cells. The surface of all marked damaged areas per utricle was calculated, added up and divided by the whole surface area of the utricle to obtain the surface area damage ratio. A ratio was used since the surface of the utricle is not always positioned and fixated in the same angle and plane. Any differences in surface area due to different angles.

**Figure 2** Typical traces of VsEP development during measurement series in gentamicin injected guinea pig (left) and control guinea pig (right). The bottom trace shows the acceleration pulse. Only the first 5 ms are shown. At first, the amplitude of the first positive peak (P1) remains stable, but decreases visibly after two weeks. Note that no acoustic masking was used. Therefore, after the P1 the response is a combined response from the cochlea and vestibular system. Since the response of the cochlea is high and mostly undamaged by gentamicin the response looks visually intact.
are largely compensated when expressed in ratio of the surface. The non-parametric Kruskal–Wallis test in SPSS was used for statistical analysis with standard significance values (p=0.05).

Results

The pre- and post-experiment weights and weight gain of all subjects showed no statistically significant differences (p=0.29). All subjects had functioning cochleas. In two of the fifteen guinea pigs, the electrodes became dysfunctional during the measurement series and these two measurement series were excluded. Four G50, four G100 and five control guinea pig ears were available for electrophysiological evaluation.

The values of the amplitude and latency of the first measurements (base values) were not statistically significantly different in the three groups. During the weeks of gentamicin application until the pre-sacrifice measurement, the P1 amplitude of the gentamicin treated subjects decreased gradually (figure 2), and the P1 latency increased significantly compared to the control group. The changes of P1 in the G100 group were higher than in the G50 group (figure 3b). After day 12, the VsEPs became statistically significantly different and remained so until the last measurements (table 1). Both amplitude and latency of both gentamicin groups were statistically significantly different from the control group.

Figure 3: Development of the means of the VsEP in the three groups in P1 amplitude (a) and latency (b). Note the difference between the effects in the high dosage group and low dosage group. (G50 standard deviations are shown on an earlier day to avoid overlap)
Morphology

In the scanning microscopic views of the whole utricle, the surface of the utricle of all control animals was undamaged (figure 4a). In the gentamicin treated subjects, sensory hair cell density reduction is seen in large areas. This occurs especially in the striolar region, which seems enlarged (figure 4b). However, other parts of the utricle are sometimes affected as well without a clear preference to a specific part. In higher magnification views (figure 5), the structure of the normal hair cells are in clear disarray in the gentamicin treated guinea pigs. The hair cell bundles are damaged in different ways. In some, the stereocilia seem to have lost their interconnections and are spread apart, whilst others are fused forming a thick bundle. The number of stereocilia in the hair cell bundle is diminished, beginning with the short stereocilia. Blebs are seen far more often in the gentamicin treated group than in the control subjects. In some areas, heavily damaged sensory cells have lost their hair cell bundle and there are holes where the hair cell bundle and sensory cell previously was. In other places supporting cells have replaced the original hair cells. In some of the control specimens, in high magnification, fusion or dissociation of a hair cell bundle was also seen, but these were rare and isolated. The global degeneration with different stages as in the gentamicin treated specimens was not found.

In transmission electron microscopy (figure 6), the differences between the vestibular epithelia of the normal and treated groups are very clear. There

<table>
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<th>Group</th>
<th>day 1</th>
<th>day 8</th>
<th>day 15</th>
<th>day 22</th>
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<tbody>
<tr>
<td></td>
<td>mean amp. P1(%)</td>
<td>p–value (K–W)</td>
<td>mean latency P1</td>
<td>p–value (K–W)</td>
</tr>
<tr>
<td>G 100</td>
<td>4.87 (100)</td>
<td>0.98</td>
<td>5.09 (112)</td>
<td>0.99</td>
</tr>
<tr>
<td>G 50</td>
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<td>0.57</td>
<td>4.57 (93)</td>
<td>0.54</td>
</tr>
<tr>
<td>Control</td>
<td>4.00 (100)</td>
<td>1.03</td>
<td>4.01 (103)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

G 100: gentamicin 100 mg/kg   G 50: gentamicin 50 mg/kg (K–W): non parametric Kruskal–Wallis test

Table 1  Amplitudes and latencies of the first positive peak (P1) of the VSEP
Figure 4. Scanning electron photomicrograph of a control (A) and gentamicin injected guinea pig (B) (S: striola). The damaged surface area is marked for calculation of the surface area damage ratio.
Figure 5  High magnification scanning electron photomicrograph of the macula of the utricle in normal (A) and gentamicin treated (B) guinea pig. Different stages of degeneration are visible. At R the reduction of stereocilia in the sensory hair cell bundle is seen. D shows the dissociation of the bundle and F, the fusion into one thick bundle. At H the sensory cell has disappeared and a hole is seen. Also note the irregular pattern of the supporting cells and the abundance of microvilli in the gentamicin utricle compared to the normal utricle.
is an abundance of normal and bloated mitochondria, indicating a highly active process, and increased vacuolization. In detail pictures we see the disappearance of the structural integrity of the hair cell bundle. The side and tip links lose their integrity and the stereocilia fall apart. The fusion of the stereocilia and kinocilium into one thick bundle is seen. The hair cell bundle of the sensory hair cell and the contents of the cell are extruded, and overgrowth by supporting cells is observed. In normal specimens no fusion or degeneration signs were found.

In the damaged surface area ratio calculations, the general surface area of all utricles was not statistically significantly different between groups ($p=0.49$). Control animals showed no damaged areas at all. The average damaged ratio in gentamicin injected subjects was 0.09. Damaged areas were clearly larger in the group treated with 100 mg/kg than in the 50 mg/kg group (0.12 respectively 0.05), and the difference between groups was significantly different ($p=0.01$) (figure 7).
Discussion

Our results clearly demonstrate the vestibulotoxicity of gentamicin on the otolith organs in the guinea pig vestibular system, both functionally as anatomically. Since we used intramuscular gentamicin, the change in VsEP was gradual. It was only after two weeks that the amplitude and latency of the VsEP changed significantly in comparison with the control group, implicating that the damaging effect on the vestibular function of systemic gentamicin develops slowly. This corresponds with the effects found on the vestibular evoked response in cats after intraperitoneal gentamicin. Sugasawa et al. found a similar gradual influence on the electrovestibular brainstem responses with angular acceleration after intramuscular gentamicin, which suggests that all vestibular end organs are affected. When gentamicin is administered topically, the effect is quicker and more severe. In the VsEP measurements, we also found a dosage–response effect. The amplitude of P1 of guinea pigs who received 100 mg/kg gentamicin decreased earlier and worse than those who received 50 mg/kg.

A puzzling effect was found in the responses of the normal group. During the measurement series the amplitude rose and the latency shortened. This
could not be attributed to a direct postoperative effect, since the subjects were allowed to recover for at least several days. The general condition as monitored by the weight and by observation, did not give an explanation either. Speculatively, this might be a late operative effect or an aging effect. In the guinea pig, data lack on the development of VsEPs, but in rats a rise in amplitude and shortening of latency was found in the postnatal development until 90 days (21). In any case, the gentamicin groups should follow the same development which was clearly not the case.

The results of scanning electron microscopy in this study shows extensive damage in the hair cell bundle and the striolar region, which is consistent with other studies (22). Other damaged areas lie in the peripheral parts of the macula, without a clear pattern or preferred location. At high magnification, damaged sensory hair cells especially in gentamicin 100mg/kg treated subjects can be found in most parts of the utricle.

The process of the degeneration is seen in its different stages. A diminishing number of stereocilia in the hair cell bundle and fusion or dissociation is seen. It is difficult to ascertain whether these are successive phases of degeneration or different paths. Finally the disappearance of the whole hair cell bundle and sensory hair cell can occur, resulting in a hole in the surface. Whether this breach of surface integrity is an incidental process or a normal phenomenon is unclear. In both cases, a supporting cell probably replaces the sensory hair cell. In several parts of the damaged macula, the surface consists almost exclusively of supporting cells.

These changes in sensory hair cells, surprisingly, are not solely confined to gentamicin treated utricles. In high magnification scanning electron photomicrographs of normal utricles, the phenomenon of hair cell bundles falling apart and fusion is occasionally seen, especially in the striolar region, although the difference with the gentamicin treated subjects was obvious. Lambert et al. found in an analysis of small hair cell bundles in mature guinea pigs that “new–born” bundles can be found as a part of a normal regeneration process (23). This regeneration process has also been found in aminoglycoside damaged avian vestibular epithelium (24) and in mature guinea pigs (25). Our findings in the control specimens may be the deterioration part of a normal degeneration and repair process.

The surface area damage ratio gives us the possibility to quantify the large areas with extensive damage in gentamicin treated guinea pigs in low magnification. Since the normal utricles show no damage, marking the damaged areas was remarkably easy. Obviously, the difference in comparison with the control
group is highly significant. Furthermore, the dosage–response effect as seen in the VsEP is reflected in the surface area damage ratio. Like in the VsEP, there is a dosage–response effect; the ratio in the G100 group is higher than in the G50 group. The surface area damage ratio can be regarded as a useful indicator of morphological damage. In the future it would be interesting to find out whether functional recovery as found in avians (13) and anatomical recovery as found in guinea pigs (26) occurs and whether these are related.

We consider both the VsEP to linear alternating acceleration stimuli and surface area damage ratio of the utricle to be general parameters of vestibular damage, not specified to a single organ of the vestibular system. A direct quantitative link between VsEP and surface area damage ratio connection should not be presumed. Although the sensory hair cell bundles on the surface are an essential element in generating the vestibular action potentials, the inner cell structures and the ionic and synaptic processes are at least as important. Plotnik et al. (8) found the origins of the response to linear acceleration to be mainly in the otolith organs, although a contribution of the semicircular canals could not be excluded. In linear acceleration along the earth vertical axis, the main origin of the VsEP is the saccule. However, since the macula of the utricle is not exactly positioned in the horizontal plane and perpendicular to the saccule, any linear acceleration will originate a combined response from both organs. In a previous study (6) the use of P1 as pure vestibular parameter of the response to Z–axis earth vertical alternating acceleration stimuli without acoustic masking has been validated. The preferred X– or Y–axis, considering the morphometry of the utricle, acceleration would have harmed the validity.

**Conclusion**

We found that intramuscularly gentamicin application has a gradual damaging effect on the vestibular function as measured in the VsEP to linear acceleration stimuli. The onset and severity of the response varies with the dosage. This functional damage is reflected in the morphological changes. The surface area damage ratio seems to be a useful semi–objective method of assessing the gross morphological damage. With the use of these techniques it is possible to assess the functional damage to the vestibular system longitudinally, and then observe and quantify morphological changes.
Reference List


MORPHOLOGY AND ELECTRICAL PHYSIOLOGY OF THE VESTIBULAR ORGAN IN THE GUINEA PIG