NOTE

Direct Perfusion Fixation of Cochlear Organ — A Practical Method for Histopathological Examination

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Intravital perfusion, as the method of fixation, has generally been recommended in the histological study of the inner ear (1, 2). This method of fixation is excellent but not simple as the routine procedure. The purpose of the present paper is to describe a direct perfusion technique of the isolated inner ear with Wittmaack's fluid that is simple and applicable to the routine histological study with excellent preservation.

The apparatus used for the direct perfusion is shown in Figs. 1 and 2. Procedures of the technique are as follows. Guinea pigs (Hartley strain) weighing approximately 300 g were bled to death under ether anesthesia and the inner ear including the cochlea, vestibulum and semicircular duct was excised. The stapes was removed from the oval window under a stereomicroscope and a 1/6 inch gauge dental needle connected with a transfusion set was inserted to the cochlea window (Fig. 2). Perfusion was done for six hours with fixatives shown in Table 1. For comparison, the intravital perfusion [1–3] with Wittmaack's fluid [4] were performed for six hours. The experimental groups and steps of processing prior to the paraffin embedding are shown in Table 1. Quasi serial sections including the axis of cochlea were prepared and stained with hematoxylin-eosin.

Histopathological examination on the inner ear specimens fixed by the intravital perfusion with Wittmaack's fluid revealed good preservation of the organs of Corti and structural components of the organ were clearly identified each other (Fig. 3). In the inner ear specimens fixed by the direct perfusion with
<table>
<thead>
<tr>
<th>Primary fixative (Perfusion)</th>
<th>Post fixation (dipping)</th>
<th>Neutralization*</th>
<th>Defatting**</th>
<th>Decalcification (48 hrs)</th>
<th>Neutralization***</th>
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</thead>
<tbody>
<tr>
<td>Wittmaack's fluid</td>
<td>Intravital</td>
<td>Primary fixative (24 hrs) and 10% neutral buffered formalin (14 days)</td>
<td>+</td>
<td>+</td>
<td>Plank. Rychlo solution</td>
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<tr>
<td></td>
<td>Direct</td>
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<tr>
<td>Perfex*</td>
<td>Direct</td>
<td>Primary fixative (14 days)</td>
<td>-</td>
<td>-</td>
<td>Cal-EX® fluid</td>
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<tr>
<td>2% paraformaldehyde and 2.5% glutaraldehyde</td>
<td>Direct</td>
<td>Primary fixative (14 days)</td>
<td>-</td>
<td>+</td>
<td>Plank. Rychlo solution</td>
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<tr>
<td>Cal-EX II®</td>
<td>Direct</td>
<td>Primary fixative (48 hrs) Perfix* fluid (14 days)</td>
<td>-</td>
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<tr>
<td>10% neutral buffered formalin</td>
<td>Direct</td>
<td>Primary fixative (14 days)</td>
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<td>+</td>
<td>Plank. Rychlo solution</td>
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5 animals/group, +: applied, -: not applied, *: In 5% sodium sulfate (24 hrs) and washing in tap water (12 hrs), **: In alcohol (24 hrs) washing in tap water (6 hrs) before and after, ***: In 5% sodium sulfate (24 hrs) washing in tap water (12 hrs).
Wittmaack's fluid, overall findings were better than in those fixed by the intravital perfusion, and the hair cells and supporting cells were distinguishable from one another by the difference in staining characteristics of their cytoplasm: the hair cells were more acidophilic than the supporting cells (Fig. 4). In these preparations, the ampullary crest, vestibulum, cochlear ganglion and stria vascularis were also well preserved and their structure were clearly discernible. In the inner ear specimens fixed by direct perfusion with 10% neutral buffered formalin, on the other hand, the organ of Corti was destroyed and the hair cells and supporting cells were shrunken and separated from the basilar membrane (Fig. 5). In preparations of the inner ear fixed by direct perfusion with Cal-Ex II® fluid (Fisher Scientific Co.), though the organ of Corti was deformed, the hair cells and supporting cells were seen to be located on the original position, the basilar membrane, anyhow these two cells were indistinguishable (Fig. 6). In the specimens of the inner ear fixed by direct perfusion with paraformaldehyde and glutaraldehyde solution, both the hair cells and the supporting cells were shrunken and eosinophilic, and the organ of Corti was deformed (Fig. 7). In the specimens of the inner ear fixed by direct perfusion with Perfix fluid® (Fisher Scientific Co.), the structure of the organ of Corti was preserved but the cytoplasm of both hair cells and supporting cells was stained equally eosinophilic. Judging from the abovementioned results, the method of direct perfusion with Wittmaack’s fluid was most effective and simple among the methods and fixatives tested to preserve delicate structure of the inner ear.

Then the usefulness of this method was evaluated in the animals with impaired hearing. Three groups of 5 guinea pigs each (Hartley strain) weighing approximately 300 g were used. Groups 1 and 2 were given 350 mg/kg/day of kanamycin intramuscularly for 14 and 21 consecutive days respectively, and group 3 served as intact control. As an auditory functional examination, the pinna reflex test was carried out with an audiometer at the levels of 1, 2, 3, 4, 6, 8, 12, 15 and 20 KHz. Pinna reflex thresholds in each frequency level were measured before the dosing period and the 14th and 21st day of treatment. As shown in Fig. 10, marked increase in the pinna reflex thresholds and disappearance of the pinna reflex were recorded in the animals on the 14th day of treatment. On the 21st day of treatment the pinna reflex was no longer observed in any of the treated animals. The animals of group 2 treated with kanamycin for 14 consecutive days were necropsied and the inner ear was subjected to direct perfusion with Wittmaack’s fluid. Histological examination revealed incomplete or complete loss of the outer hair cells (Fig. 8) from the basal turn to the 3rd turn of the cochlea corresponding to the degree of impaired hearing. The technique was also applicable to the inner ear of rats and similarly effective results were obtained (Fig. 9).

The probable cause of excellent preservation of the delicate inner ear structure by the present technique lies in the fact that the perilymph is expelled and replaced immediately with fixative so that the sensory cells are exposed to the fixative shortly after excision of the inner ear.

In the preparations fixed with this method, slight destruction occurred occasionally in the organ of Corti at the basal turn. This tissue destruction, which is ascribable to the mechanical damage due to insertion of needles, has
little or no effect upon microscopic examination. Since the method reported in this paper is effective as well as simple, it can be used as a practical method for examination of the ear of experimental animals used in the long-term toxicity study where many animals have to be autopsied at a time.

Acknowledgements. The authors wish to acknowledge their indebtedness to Dr. Y. Noguchi, Manager of their Laboratory, for his generous encouragement and support.

References

Explanation of Figures

Fig. 2. Isolated inner ear of a guinea pig. Needle was inserted to the cochlear window. Scale, 1 cm. Figs. 2 to 8 are preparations of inner ear of guinea pigs. Fig. 9 is of a rat. Hematoxylin and eosin stain. ×150.

Fig. 3. Intravitally perfused with Wittmaack’s fluid.

Fig. 4. Directly perfused with Wittmaack’s fluid. Hair cells (arrow heads) and supporting cells (arrow) are clearly distinguishable.

Fig. 5. Directly perfused with 10% neutral buffered formalin. Organ of Corti is destroyed.

Fig. 6. Directly perfused with Cal-Ex II® fluid.

Fig. 7. Directly perfused with paraformaldehyde and glutaraldehyde solution. Both hair cells and supporting cells are shrunken and deeply eosinophilic.

Fig. 8. Inner ear of a guinea pig treated with 350 mg/kg kanamycin intramuscularly for 14 consecutive days. Direct perfusion with Wittmaack’s fluid. (Only one outer hair cell (arrow) remained.

Fig. 9. Directly perfused with Wittmaack’s fluid. Hair cells (arrow heads) and supporting cells (arrow) are distinguishable from one another.
要約

蝦牛の直接灌流固定法——病理組織検査用の実用的な方法（短報）：和田 功・薄 良雄・武下政一・子林孝司・岡庭 柚（田辺製薬株式会社安全性研究所）——固定状態のよい内耳標本を得るためにモルモットの摘出内耳を各種の固定液で直接灌流固定し、従来の Wittmaack 液による生体灌流固定と比較検討した。Wittmaack 液で直接灌流した摘出内耳の固定状態は Corti 器の有毛細胞の保存性ならびに染色性において生体灌流固定のそれよりもすぐれており、その簡便性と有効性から聴器の形態学的検査法として有用であると考えられた。