Ototoxic effects of kanamycin and the possible protective role of salicylate in adult albino rat

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Abstract: The aim of this study was to determine the ototoxic effects of aminoglycoside, kanamycin, and the possible protective role of salicylate in the adult albino rats. As aminoglycosides are indispensable agents both in treatment of infections and Meniere's disease, a great effort has been made to develop strategies to prevent aminoglycoside ototoxicity. Anti-free radical agents, such as salicylate, have been shown to attenuate the ototoxic effects of aminoglycosides. The animals, in this study, were divided into three groups, group I (control),group II (treated) which was divided into 3 subgroups, subgroup (I) received kanamycin for 7 successive days, subgroup (II) received kanamycin for 14 successive days, subgroup (III) received kanamycin for 14 successive days followed by 7 days of rest. Group III (treated protected) received kanamycin and salicylate for 14 successive days followed by 7 days of rest. Cochleaewere removed and processedfor histological, morphometrical and statistical analysis. This study showed damage of the organ of Corti, apoptotic cells with dense nuclei in spiral ganglion, excess collagen deposition in basilar membrane and in area underlying striavascularis. These effects were greatly reversed by addition of salicylate.

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Keywords: ototoxicity; kanamycin; salicylate;otoprotection; rat

1. Introduction

Aminoglycosides are incorporated into the regimen against multi-drug resistant tuberculosis as per suggestion of the World Health Organizaton (Chen *et al.,* 2007).It is also used for treating gram-negative bacterial infections (Hochman*et al.,*2006). These drugs may be the most commonly used antibiotics worldwide, especially in developing countries (Sha*et al.,*2006).Although these drugs are extremely efficacious, they can result in ototoxicity (Hochman*et al.,*2006).

The incidence of cochleotoxicity has been reported up to 33% of patients, while the balance apparatus may be affected in approximately 15% (Chen *et al.,*2007).The incidence of side effects may be higher in developing countries where the drugs are generally available over the counter, and the drug serum levels in patients are not routinely obtained for an adjustment of dosing, to avoid levels that may be associated with a higher risk of ototoxicity (Sha*et al.,*2006).

The biochemical mechanisms leading to aminoglycosides ototoxicity are not fully understood,but several evidences suggest that damage may result from the formation of reactive oxygen species that overwhelm the cellular antioxidant defense systems of the inner ear (Corbacella*et al.,* 2004).The resulting cellular redoximbalance causes apoptotic or necrotic cell death (Ruiz *et al.,*2006).In fact, the use of aminoglycosides lead to chromatin condensation and DNA fragmentation (Corbacella*et al.,*2004).According to this mechanism, several agents have been shown to reduce ototoxicity, mostly focusing on antioxidant therapy for example, aspirin (Sha*et al.,* 2006). Salicylate protects the cochlea against ototoxicity induced by aminoglycoside or cisplatin (Hyppolito*et al.,*2006).

The aim of this study is to evaluate the ototoxic effects of kanamycin and the possible protective role of salicylate in the adult albino rats.

2. Material and methods

2.1. Chemicals

Kanamycin sulphate was purchased from USB corporation (Cleveland, OH, USA) and prepared as 45 mg/ml in saline.

Acetylsalicylic acid was a product of EuropianEgyp. Ph., ind.Amriyacompany was obtained as tablets 80 mg which are dissolved in saline.

2.2. Experimental animals

A total of 50 adult albino rats weighing (200 grams) were used in the experiment. The ratswere housed individually during the whole experiment in isolated cages at the room temperature under pathogen free conditions to keep them in normal and healthy conditions with free access to a standard diet and water.

2.3. Experimental design

The rats were randomly divided into 3 groups, Group I (control group) included 10 rats which received normal saline.Group II (treated group) included30 rats which were divided into 3 subgroups,

10 rats for each subgroup. All rats were subjected daily to subcutaneous injection of kanamycin with a dose of 700 mg/kg (Jiang *et al.,* 2006) according to the following time schedule: Subgroup I were injected for 7 successive days, subgroup II were injected for 14 successive days andsubgroup III were injected for 14 successive days followed by 7 days of rest.Group III (treated protected group) included10 rats which were injected subcutaneously by kanamycin concurrently with 200 mg/kg of salicylate for 14 successive days followed by 7 days of rest.

Rats were sacrificed in the day next to the time schedule mentioned before. The rats were anaesthetized lightly by diethyl ether inhalation and their cochleae were extracted from the temporal bones and preserved in formaldehyde 10 % and then decalcified with 4 % EDTA for 3 days. Then paraffin sections were prepared. The thickness of sections used was 5 microns. The sections were then subjected to histological techniques using H&E and Mallory trichrome stains.

2.4. Image analysis

Cochlear sections of mallorytrichrome stain were used to measure the percentage of surface area of collagen fibers in the basilar membrane and in the area underlying striavascularis. The data were obtained by using LeciaQwin 500 image analyzer computer system (England). The image analyzer consisted of a colored video camera, colored monitor, hard disc of IBM personal computer connected to microscopic and controlled by LeciaQwin 500 software. The image analyzer was calibrated automatically using the measurements units (pixels) produced by image analyzer program.

2.5. Statistical analysis

The collected data were tabulated and analyzed by SPSS (statistical package for social science) version 17.0 on IBM compatible computer. The results were expressed as mean (x) ± standard deviation (SD).ANOVA test is a test of significance used for statistical analysis of the different groups normally distributed having quantitative variables using graph pad for instant software and probability of chance (Saunders and Robert, 1994).

3. Results

3.1. Haematoxylin and Eosin stained sections

The control group sections showed that three fluid chambers; scala media, scalavestibuli and scala tympani (Fig. 1). Scala media (cochlear duct) is triangular in shape. The basilar membrane forms the floor of scala media separating it from scala tympani (Fig. 2). Organ of Corti sits on top of basilar membrane containing outer and inner hair cells. The outer hair cells are covered by tectorial membrane (Fig. 3). The vestibular membrane forms the superior limb of scala media that separates it from scalavestibuli (Figs. 2,3). Scala media is bounded medially by spiral ganglion (Fig. 1) while it is bounded laterally by striavascularis which is formed of intact cubical cells and vascular connective tissue underlying (Fig. 4).

The treated sections of subgroup I showed sloughing of the cells of organ of Corti leaving cellular debris covered by tectorial membrane(Fig. 5).Bulging and distortion of striavascularis were noted in the lateral wall of scala media (Fig. 6).

The treated sections of subgroup II showed markedly damaged cells of organ of Corti which are replaced by cytoplasmic vacuoles (Fig. 7). Apoptotic cells with dense nuclei were noted surrounded by clear halos in spiral ganglion (Fig. 8). Degeneration of the vascular connective tissue underlying striavascularis was noted in the lateral wall of scala media (Fig. 9).

The treated sections of subgroup III showed complete disappearance of organ of corti replaced by cellular debris and shrinkage of tectorial membrane (Fig. 10). Apoptotic cells with dense nuclei were noted in spiral ganglion (Figs. 11,12).

The treated protected sections showed maintainance of most of the normal architecture of organ of Corti compared with treated subgroups (Figs. 13,14).

3.2. Mallory trichrome stained sections

The control group sectionsshowed minimal collagen fibers deposition in basilar membrane and in the area underlying striavascularis (Fig. 15).The treated sections of subgroup I showedsome increase in collagen fibers deposition in the basilar membraneand in the area underlying striavascularis compared with the control group (Fig. 16).The treated sections of subgroup IIshowed more increase in collagen fibers deposition in basilar membrane and in area underlying striavascularis compared with subgroup I (Fig. 17).The treated sections of subgroup IIIshowed massive increase in collagen fibers deposition in basilar membraneand in area underlying striavascularis compared with the subgroup II (Fig. 18).The treated protected sectionsshowed decrease in collagen fibers deposition in basilar membrane and in area underlying striavascularis compared with subgroup III (Fig. 19).

Table 1 and Fig. 20 showed a highly significant increase in collagen fibers deposition in subgroup I ($P1< 0.001$) with a mean value of 9.69±0.46 compared with control group with a mean value of 4.06±0.29.Highly significant increase in collagen fibers deposition was observed in subgroup II ($P2< 0.001$) with a mean value of 13.32±0.56 compared with subgroup I with a mean value of 9.69±0.46.Highly significant increase in collagen fibers deposition was observed in subgroup III ($P3<$

0.001) with a mean value of 17.31±0.50 compared with subgroup II with a mean value of 13.32±0.56.Highly significant decrease in collagen fibers deposition was observed in treated protected group (*P*4< 0.001) with a mean value of 7.35±0.45 compared with subgroup III with a mean value of 17.31±0.50.

Table 1: Statistical comparison between the studied groups in the mean level and standard deviation (SD) of Mallory trichrome.

Groups	Mallory trichrome		ANOVA	*P* value	Post hoc test LSD
	Mean	SD			
Control group (group I)	4.06	0.29			
Treated group (subgroup I)	9.69	0.46			**P1: <0.001**
Treated group (subgroup II)	13.32	0.56			**P2: <0.001**
Treated group (subgroup III)	17.31	0.50			**P3: <0.001**
Treated protected group (group III)	7.35	0.45	614.1	**<0.001**	**P4: <0.001**

P1: comparison between control and subgroup I. P2: comparison between subgroup I and subgroup II.
P3: comparison between subgroup II and subgroup III. P4: comparison between subgroup III and group III (treated protected).
P-value > 0.05 non significant. P-value < 0.05 significant. P-value < 0.001 highly significant.

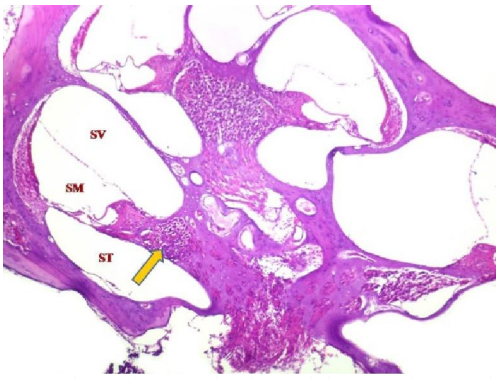

Fig. 1: A photomicrograph of an adult control rat cochlear section showing the membranous cochlear duct which is triangular in shape called scala media (SM). It divides the bony canal into two spaces; one above called scalavestibuli (SV) and one below called scala tympani (ST). The cochlear duct is bounded medially by spiral ganglion (arrow) **(Hx. & E., X100).**

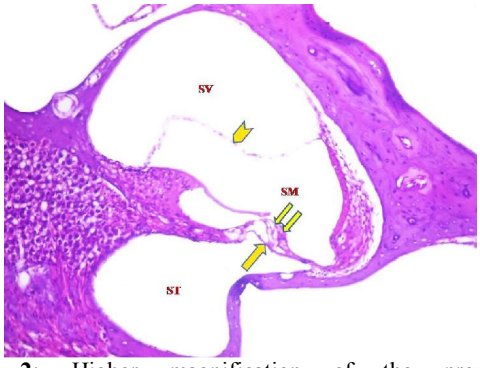

Fig. 2: Higher magnification of the previous photomicrograph showing scalavestibuli (SV), scala tympani (ST) and scala media (SM). The floor of the scala media (cochlear duct) is formed by basilar membrane (arrow).Organ of Corti (double arrow) lies on basilar membrane. Note that vestibular membrane (arrow head) separates scala media from scalavestibuli**(Hx. & E., X200).**

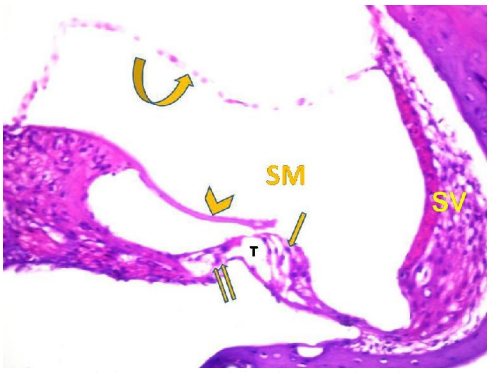

Fig. 3: Higher magnification of the previous photomicrograph showing outer hair cells (arrow) and inner hair cells (double arrow) separarted by tunnel of Corti. They are covered by tectorial membrane (arrow head). Scala media (SM) is bounded laterally by striavascularis (SV) and above by vestibular membrane (curved arrow) **(Hx. & E., X400).**

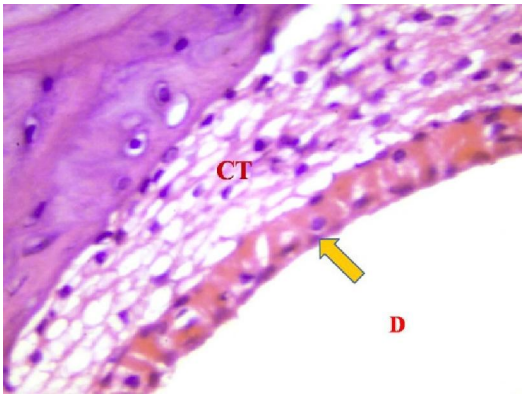

Fig. 4: A photomicrograph of an adult control rat cochlear sectionshowing striavascularis which is formed of intact cubical cells (arrow), facing the endolymph of the cochlear duct (D), and vascular connective tissue underlying (CT) **(Hx. & E., X1000).**

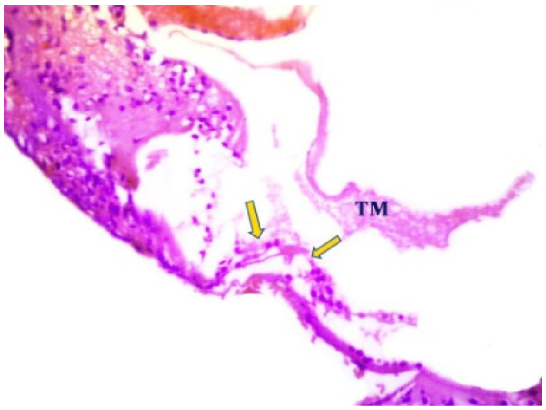

Fig. 5: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 7 days (subgroup I) showing bulging and distortion of striavascularis (arrow) **(Hx. & E., X400).**

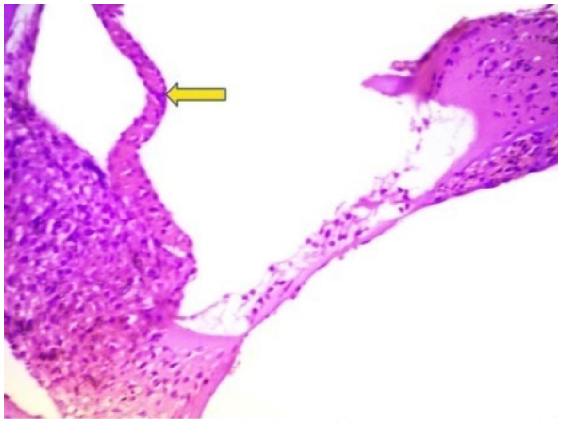

Fig. 6: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 7 days (subgroup I) showing sloughing of the cells of organ of Corti leaving cellular debris (arrows) covered by tectorial membrane (TM) **(Hx. & E., X400).**

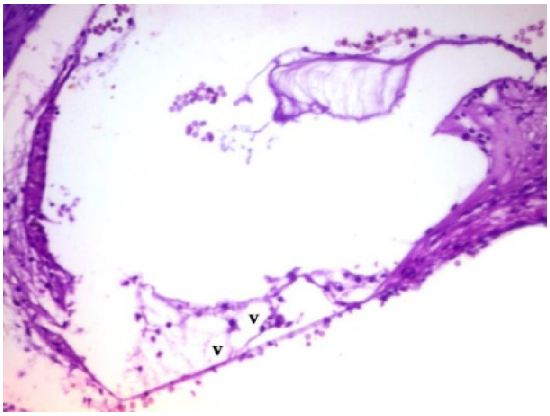

Fig. 7: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days (subgroup II) showing markedly damaged cells of organ of Corti which are replaced by cytoplasmic vacuoles (V) **(Hx. & E., X400).**

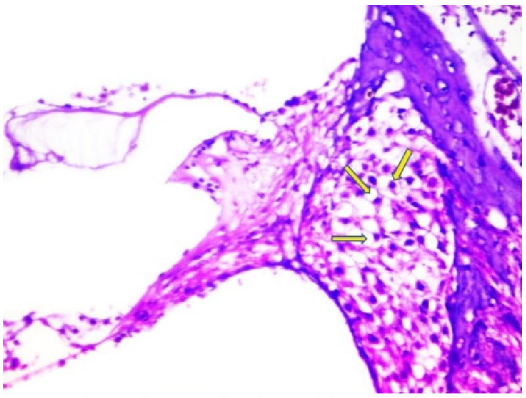

Fig. 8: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days (subgroup II) showing apoptotic cells with dense nuclei surrounded by clear halo in spiral ganglion (arrows) **(Hx. & E., X400).**

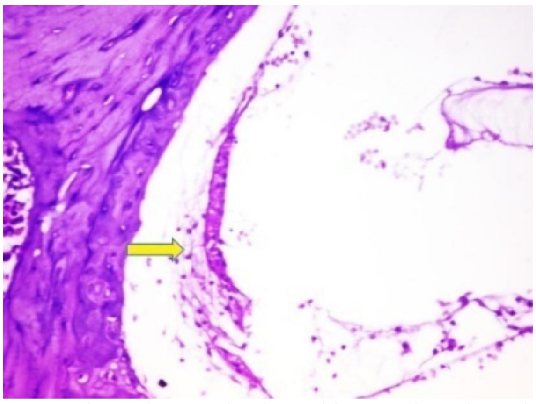

Fig. 9: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days (subgroup II) showing degeneration of vascular connective tissue underlying striavascularis (arrow) **(Hx. & E., X400).**

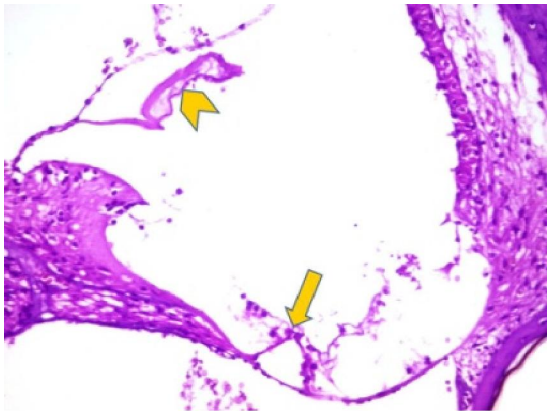

Fig. 10: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days followed by rest for 7 days (subgroup III) showing complete disappearance of organ of Corti (arrow). Note that tectorial membrane is shrunken (arrow head) **(Hx. & E., X400).**

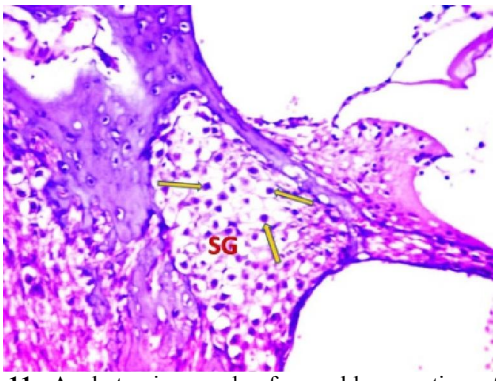

Fig. 11: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days followed by rest for 7 days (subgroup III) showing apoptotic cells (arrows) with dense nuclei surrounded by clear halo in spiral ganglion (SG) **(Hx. & E., X400).**

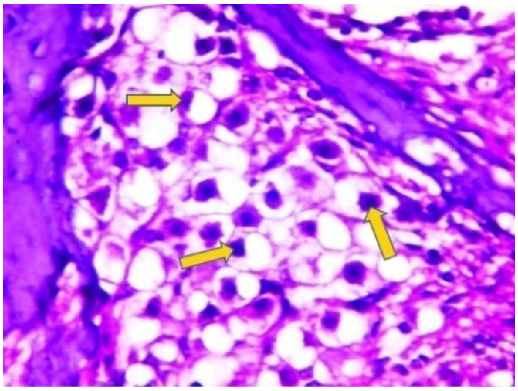

Fig. 12: Higher magnification of previous photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days followed by rest for 7 days (subgroup III) showing apoptotic cells (arrows)with dense nuclei surrounded by clear halo in spiral ganglion **(Hx. & E., X1000).**

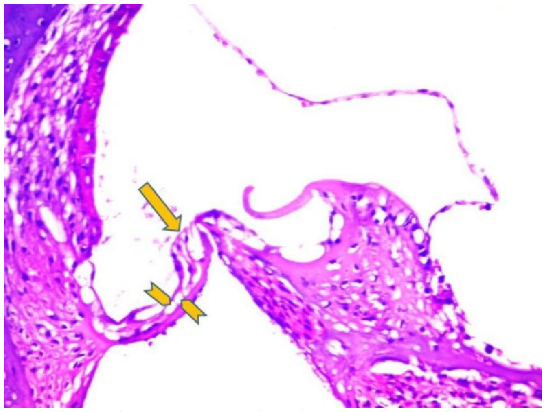

Fig. 13: A photomicrograph of a cochlear section of an adult rat injected by kanamycin and salicylate for 14 days followed by rest for 7 days (treated protected group) showing maintainance of most of normal architecture of the organ of Corti (arrow). Note that few cytoplasmic vacuoles (arrow heads) are still present **(Hx. & E., X400).**

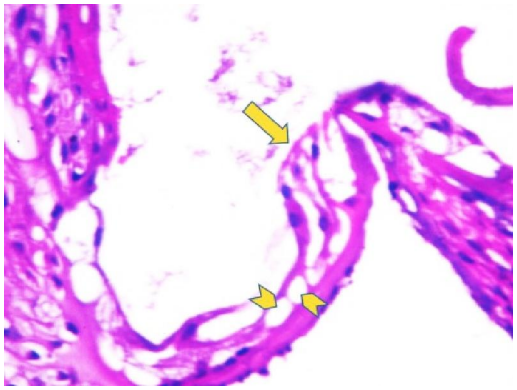

Fig. 14: Higher magnification of previous photomicrograph of a cochlear section of an adult rat injected by kanamycin and salicylate for 14 days followed by rest for 7 days (treated protected group) showing maintainance of most of normal architecture of the organ of Corti (arrow). Note that few cytoplasmic vacuoles (arrow heads) are still present **(Hx. & E., X1000).**

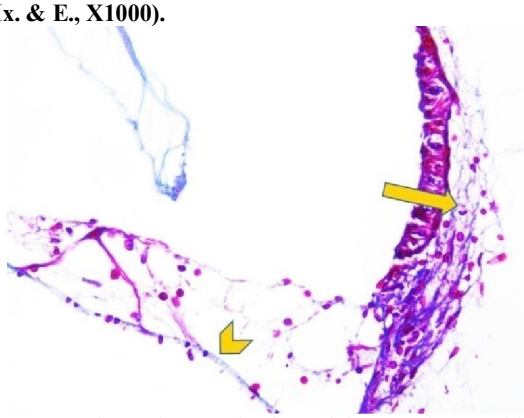

Fig. 15: A photomicrograph of an adult control rat cochlear section showing minimal collagen fibers deposition in the basilar membrane (arrow head) and in the area underlying striavascularis (arrow) **(Mallory trichrome, X400).**

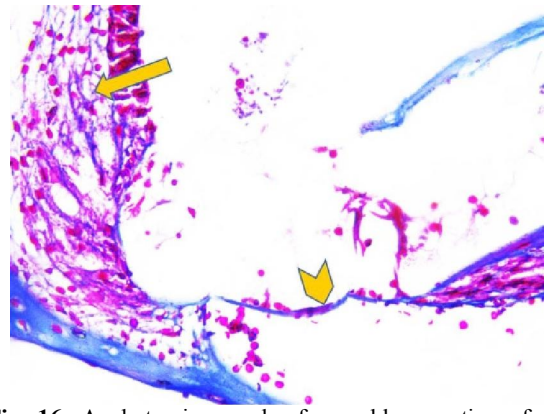

Fig. 16: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 7 days (subgroup I) showing some increase in collagen fibers deposition in the basilar membrane (arrow head) and in the area underlying striavascularis (arrow) compared with control group **(Mallory trichrome, X400).**

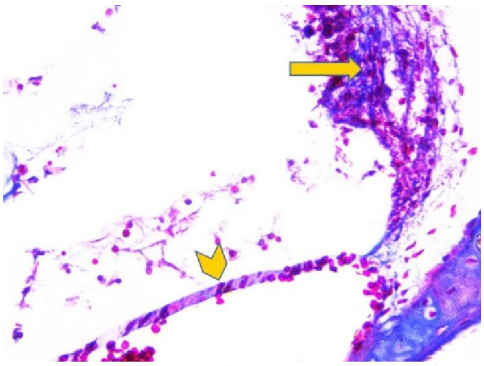

Fig. 17: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days (subgroup II) showing more increase in collagen fibers deposition in basilar membrane (arrow head) and in area underlying striavascularis(arrow) compared with subgroup I **(Mallory trichrome, X400).**

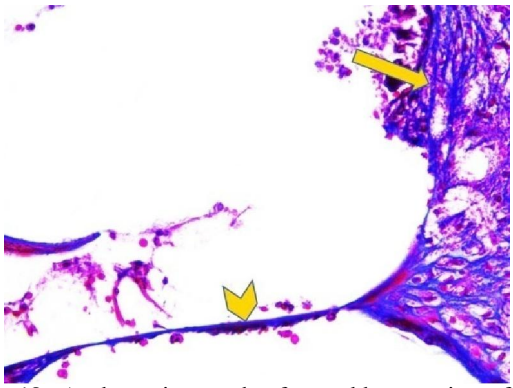

Fig. 18: A photomicrograph of a cochlear section of an adult rat injected by kanamycin for 14 days followed by rest for 7 days (subgroup III) showing massive increase in collagen fibers deposition in basilar membrane (arrow) compared with subgroup II **(Mallory trichrome, X400).**

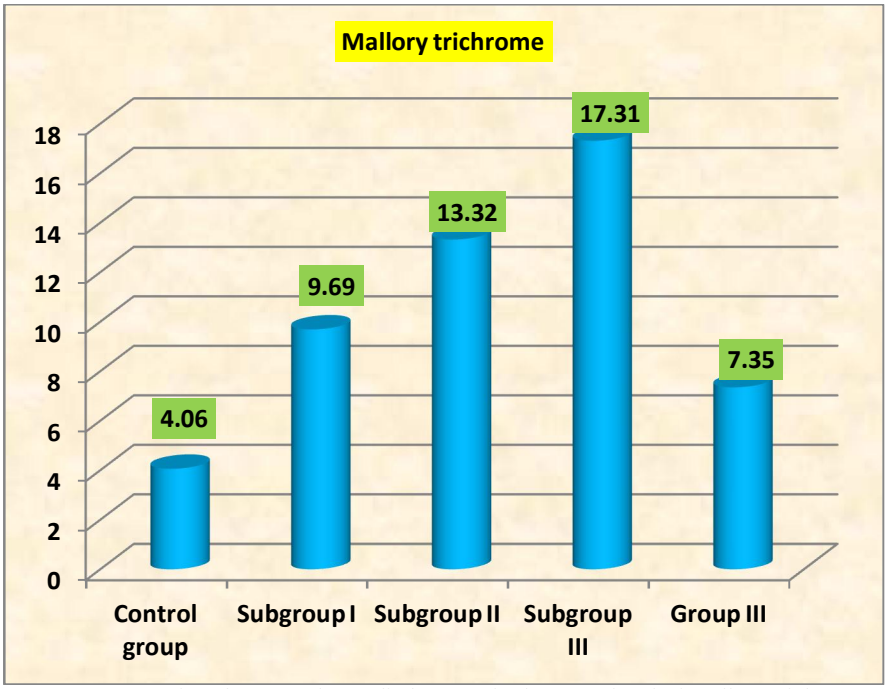

Fig. 20: Comparison between the studied groups in the mean level of Mallory trichrome.

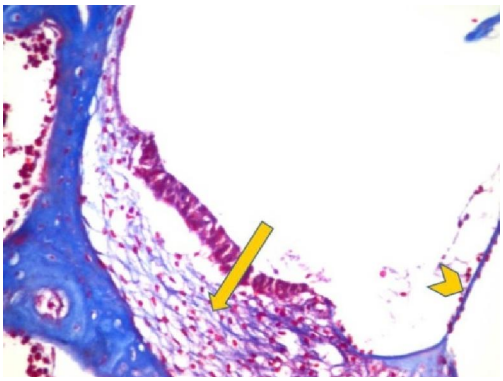

Fig. 19: A photomicrograph of a cochlear section of an adult rat injected by kanamycin and salicylate for 14 days followed by rest for 7 days (treated protected group) showing decrease in collagen fibers deposition in basilar membrane (arrow head) and in area underlying striavascularis (arrow) compared with subgroup III **(Mallory trichrome, X400).**

4. Discussion

Hearing and vestibular loss have been observed in patients treated with aminoglycoside antibiotics since the use of streptomycin for treatment of tuberculosis in 1940 (Schatz *et al.,* 1944). Since then,

research efforts to improve our understanding of ototoxicity have been performed primarily in birds, guinea pigs and in organ culture of neonatal mice (Karasawa et al., 2008). Recently scientists have turned to in vivo experiments in mice to study molecular mechanism of hair cell susceptability to ototoxicity (Hartman *et al.,*2009). Therefore, it was the aim of the present study to evaluate the ototoxic effects of kanamycin in adult albino rats and the possible protective effects of salicylate.

In the present study, light microscopic results revealed evidence of degenerative changes in outer and inner hair cells replaced by cytoplasmic vacuoles, atrophy and bulging of striavascularis and appearance of apoptotic cells in spiral ganglion. These data were in agreement with McFadden *et al.* (2002) who stated that kanamycin produced extensive death of cochlear hair cells, but the vestibular organs were completely unaffected. Eskelinen (2005) found that there are a few inner hair cells that appeared to be necrotic. In addition, there are cells that showed a number of features suggesting they are involved in degeneration of their own cellular material: internalization of the stereocilia and/or the presence of cytoplasmic material and cell organelles enclosed within vacuoles, some of which are double-membraned, a feature described as characteristic of autophagy. Debnath*et al.* (2005) considered that autophagy is a mechanism for turnover and recycling of cell components through the lysosomes. It is meant to promote cell survival and protection from damage. Cuervo (2004) stated that however, autophagy may also be a programmed cell death pathway, which is sometimes called type II cell death. Accumulation of autophagic vesicles in cells is seen in a number of neurodegenerative diseases. Wiegand*et al.,* (2001) noticed that necrosis among outer hair cells (OHC) was almost completely undetectable. The presence of cellular debris closely associated with condensed apoptotic-like nuclei in regions of ongoing OHC death is most likely due to OHC entering the apoptotic pathway but failing to complete it. On the other hand, Tran Ba Huy*et al.*, (1981) detected that single doses of kanamycin caused no obvious damage to the organ of Corti or striavascularis. While Campbell *et al.*(1999) reported that striavascularis in the basal turn may show edema, bulging, and depletion of intracellular organelle concentrations. These changes occur primarily in the marginal cells.

In the current study, light microscopic results revealed evidence of fibrosis in basilar membrane and in area underlying striavascularis. These results were in agreement with Ladrech*et al.* (2007) who found that both in animals and in humans, at prolonged periods following loss of all hair cells, continuing remodeling of the remaining supporting elements of the organ of Corti ultimately results in its replacement by a thin, nonspecialized epithelium covering the basilar membrane. Death of supporting cells may occur during this progression. Also Laurell and Bagger-Sjoback, (1991) reported that with more prolonged treatment, lesions spread to all cell types, eventually replacing the entire cochlear sensory epithelium with a layer of non-specialized epithelial scar tissue.

In the present study, there was an evidence of apoptosis in spiral ganglion. This was in agreement with Ladrechet al., (2004) who detected that the morphology of nuclei confirmed that outer hair cells were dying via programmed cell death. Jiang *et al.*(2006) noticed that in a previous study of OHC loss following chronic treatment of mature mice with kanamycin alone over a 14-day period, no activated caspase-3-positive OHC could be detected during the period of several days following treatment over which OHC died. Ladrech*et al.*(2004) found, not all OHC that showed apoptotic nuclei labeled for activated caspase-3 and the number of such positively labeled cells was relatively small. This may indicate that, whereas some cells are triggered to die via the classical apoptotic route, other cells are subjected to alternative signals leading to an apoptotic death by caspase-independent pathways.

Huth*et al.*(2011) declared that with increasing understanding of ototoxic cell death, a myriad of therapeutic efforts have been proposed to target various steps of the complex cascades to hair cell death. Those strategies include inhibition of apoptosis, neutralization of reactive oxygen species, and administration of neurotrophic factors. In the current study, light microscopic results revealed evidence of proliferation and regeneration after using of salicylate as a protective factor. These observations were in agreement with Lamm and Arnold, (1998) who declared that several non-steroidal anti-inflammatory drugs (NSAIDs) exhibit protective effects on the inner ear against acoustic injury in rodents. Sha and Schacht, (1999) reported that acetylsalicylate (ASA) is an iron chelator with additional direct antioxidant properties. Hyppolito*et al.* (2006) stated that salicylate protects the cochlea against ototoxicity induced by aminoglycoside. Yamashita *et al.* (2005) demonstrated that in animals subjected to acoustic injury induced by aminoglycosides, there is a window of opportunity for rescue by using salicylate after the onset of this type of injury.

On the other hand, Chen *et al.*(2010) declared that however, ASA itself is ototoxic and potentially causes tinnitus, vertigo, and hearing loss. Although these symptoms are known to be reversible, aminoglycosides remain in hair cells for months and

ototoxic damage can occur after many years. Thus chronic treatment with ASA appears necessary and ototoxic effects of both, aminoglycosides and ASA, need to be evaluated over a long period. Moreover Tomofumi*et al.* (2010) found that the usage of NSAIDs at excessively high doses will induce inner ear disturbances, causing tinnitus and mild to moderate sensorineural hearing loss. These otological side effects are often transient and reversible after the cessation of NSAID consumption. Further investigations regarding NSAIDs are necessary to clarify the mechanisms of their side effects and their potential protective actions.

Conclusion

In the present study, we approved the cochleotoxic reactions of kanamycin and the probability of recovery when salicylate was administrated.

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