Targeting Autophagy Induction as A possible Protective Mechanism by Verapamil Compared to Rapamycin (Sirolimus) Against Gentamicin -Induced Ototoxicity in Guinea Pigs

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Abstract

Background: Ototoxicity is a harmful feature of the cochlea or auditory nerve and sometimes vestibular apparatus. Gentamicin is known to cause irreversible bilateral ototoxicity. Autophagy induction has been proposed as a target for prevention of gentamicin ototoxicity. **Aim of the work:** to investigate the possible protective effect of autophagy induction by verapamil compared to rapamycin. **Methods:** This experiment was conducted on 32 male guinea pigs. At the beginning each animal's hearing status was assessed using auditory brainstem response (ABR) audiometry. Then, they were divided into 4 equal groups: Group 1: control group, Group 2: untreated gentamicin-induced ototoxicity group. Group 3: gentamicin-induced ototoxicity treated concomitantly with rapamycin. Group 4: gentamicin-induced ototoxicity treated concomitantly with verapamil. At the end of the experiment, ABR was repeated then the animals were sacrificed, and blood samples were obtained for assaying of reduced glutathione and malondialdehyde levels. The left cochlea was processed for scanning electron microscope, while the right cochlea was processed for histopathology and LC3-II immunohistochemistry. **Results:** Verapamil revealed superiority compared to rapamycin proved by significant improvement in ABR, histopathological results, in addition to its antioxidant effect. **Conclusion:** verapamil could be suggested as a potential therapeutic approach to decrease gentamicin ototoxicity.

Keywords: *Autophagy, Gentamicin, Ototoxicity, Rapamycin, Verapamil.*

Introduction

The most common sensory condition in humans is hearing loss [1]**.** Ototoxicity is the functional damage of the inner ear contributing to hearing and/or vestibular function loss. Aminoglycosides, cisplatin, macrolides, loop diuretics are the most common ototoxic drugs [2]. Gentamicin is one of the most frequently used aminoglycosides, but it causes nephrotoxicity and ototoxicity [3].Gentamicin ototoxicity is frequently bilateral, symmetrical. It affects mainly the basal hair cells of organ of corti. The incidence of aminoglycoside hearing loss is up to 25% [4, 5]**.**There are multiple mechanisms of aminoglycosides ototoxicity; mutation in mitochondrial DNA [6], oxidative stress [7], phosphoinositide lipids involvement [8], and autophagy has recently been proposed to play a crucial role [9]. There are two pathways for autophagy induction: rapamycin (mTOR)-dependent mammalian target, and mTOR-independent pathway [10]. Rapamycin is established as an autophagy inducer and approved clinically as immunosuppressive drug,

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but it has several side effects [11, 12]. Verapamil is a calcium channel blocker that was suggested to be an autophagy inducer through mTOR-independent pathway. Moreover, verapamil might have antioxidant properties [13, 14], besides having considerable safety profile.

Materials and Methods

The study protocol has been approved by the research ethics committee, Quality Assurance Unit, Faculty of medicine, Tanta University, Egypt (Approval no. #32511/ 08/18).

Drugs and chemicals

Gentamicin sulphate (80 mg /2ml ampoule; Memphis Co. for Pharm. & Chem. Ind. Egypt). Rapamycin (1 mg tablet; a product of Wyeth Medica Ireland. United Kingdom). Verapamil hydrochloride (240mg Sustained-Release tablet; a product of Kahira Pharmaceuticals & Chemical Industries Co). Ketamine (50 mg/ml solution; Rotexmedica GmbH, Germany). Xylazine (100 mg/ml vial; BIMEDA Company).

Animals and study design

32 male guinea pigs (350-500 gm) were allowed one week of acclimatization and unrestricted access to food and water was provided *ad libitum*. At the start of the research and after its completion, each guinea pig's auditory brainstem response audiometry (ABR) was assessed under anesthesia. Only guinea pigs with normal ABR were included and divided randomly into four equal groups (n = 8 for each) where group 1; served as control group, received vehicle of 0.5% CMC orally by gastric gavage daily. Ototoxicity was induced in the other three groups using gentamicin by intraperitoneal (*ip*) injection at a dosage of 120 mg/kg/day [15] where group 2; served as untreated gentamicin ototoxicity group, group 3; was treated concomitantly with rapamycin suspended in 0.5% CMC [16] in a dose of 0.13 mg/kg/day orally by gastric gavage, group 4; was treated concomitantly with verapamil hydrochloride dissolved in distilled water [17] in a dose of 30mg/kg/day orally by gastric gavage. Rapamycin and verapamil doses were calculated according to human equivalent dose equation [18]. Treatment protocol in all groups started from 1st day of the experiment (zero point) and continued for 14 consecutive days. The ABR was reevaluated after completion of the experiment. The animals were ketamine and xylazine anesthetized by intraperitoneal (*ip*) injection in a dose of (40 mg/kg, *ip*) and (5mg/kg) respectively [15] and blood was obtained through intracardiac blood sampling and processed for assay of reduced glutathione and malondialdehyde levels. The animals were sacrificed, and each cochlea was extracted and infiltrated with a fixative solution immediately. Left cochlea was prepared for scanning electron microscopy, while right cochlea was prepared for histopathology and immunohistochemistry.

ABR audiometry

ABR was achieved while animals were anesthetized through two channel tracking using three electrodes connected to preamplifier: two electrodes were placed as negative electrode on the left and right mastoid and high frontal Fz site as a positive electrode. In response to click stimuli introduced at 90 dBnHL, ABR was registered ipsilaterally, and when response was recorded, the strength was decreased in steps of 10 dB until a threshold was detected. Using alternating polarity, 19.3 / second repeat rate, 150–1500 Hz filter setting, 50,000 gain factor, and 0–10 ms time span, ER3A-insertphone transmitted click stimuli.

Biochemical assays

Blood level determination of reduced glutathione (GSH) (mg/dl)

GSH level was performed using Biodiagnostic supplied Kit (Cat. No TA 2511.), based on the Beutler spectrophotometric process [19].

Serum level determination of malondialdehyde (MDA) (nmol / ml)

Lipid peroxidation was assessed by calculating

serum (MDA) levels according to the Ohkawa et al. method [20] using Biodiagnostic supplied kit (Cat. No. MD 2529).

Histopathological examinations of hematoxylin and eosin (H&E) stained tissues

The right cochlea of each animal was fixed by EDTA formalin 10% for 4 weeks [21]. Paraffin sections (4µm) were examined by light microscope for histopathology.

Scanning electron microscope (SEM)

Fenestration was made at left cochlear apex for infiltration with 2.5% glutaraldehyde. The cochlea was processed for examination then dried using critical point dryer then examined with a JEOL (SEM) [22].

Immunohistochemical examination of LC3-II

It was carried out in the right cochlea using a rabbit polyclonal antibody (purchased from Sun Red Bio Laboratories Cat. No. 201r-1501) according to scoring method of Schläfli et al [23].

Statistical Analysis

Values are interpreted as mean ± standard error of mean (SEM), while in immunohistochemical (IHC) expression of LC3-II scores, median was used. Differences were found to be significant at $p < 0.05$.

Results

Results of ABR measures

Detectability of ABR waves

At zero point all waves of the ABR were detectable in 100% of animals. Then at the end point ABR showed these results: group 1: ABR was present in all animals (n=8) and all waves of ABR were detectable in 100% of animals while, in group 2 ABR was detectable in 25% of the animals (n=2) and absent in 6 animals indicating severe hearing affection. In group 3 and 4, drug treatment by rapamycin and verapamil respectively led to detection of ABR was in 75% of the animals (n=6) and its absence in 2 animals (Fig.1).

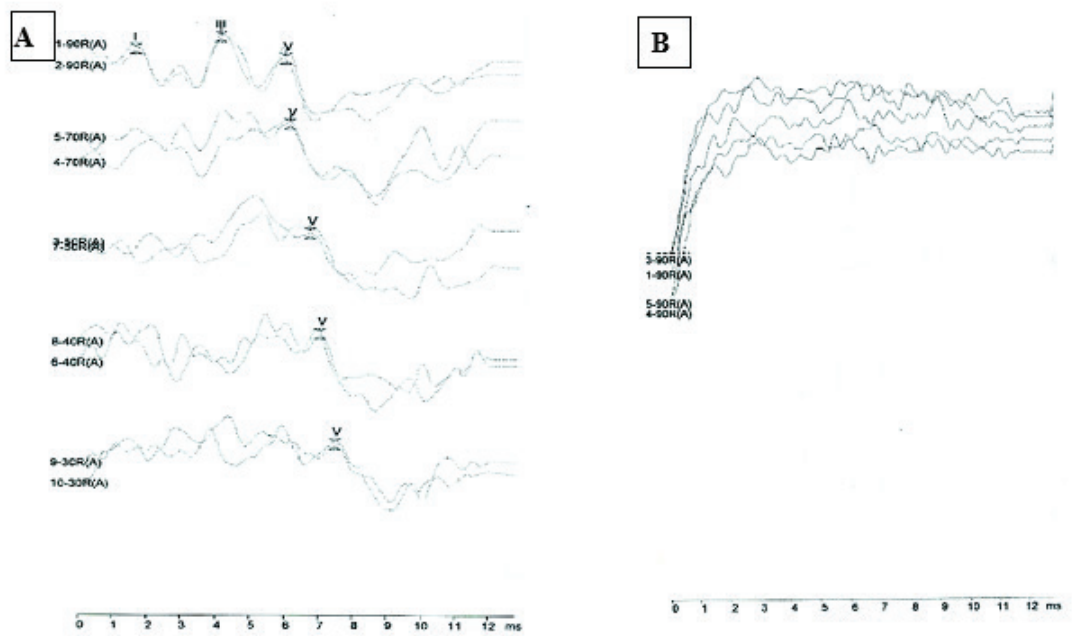
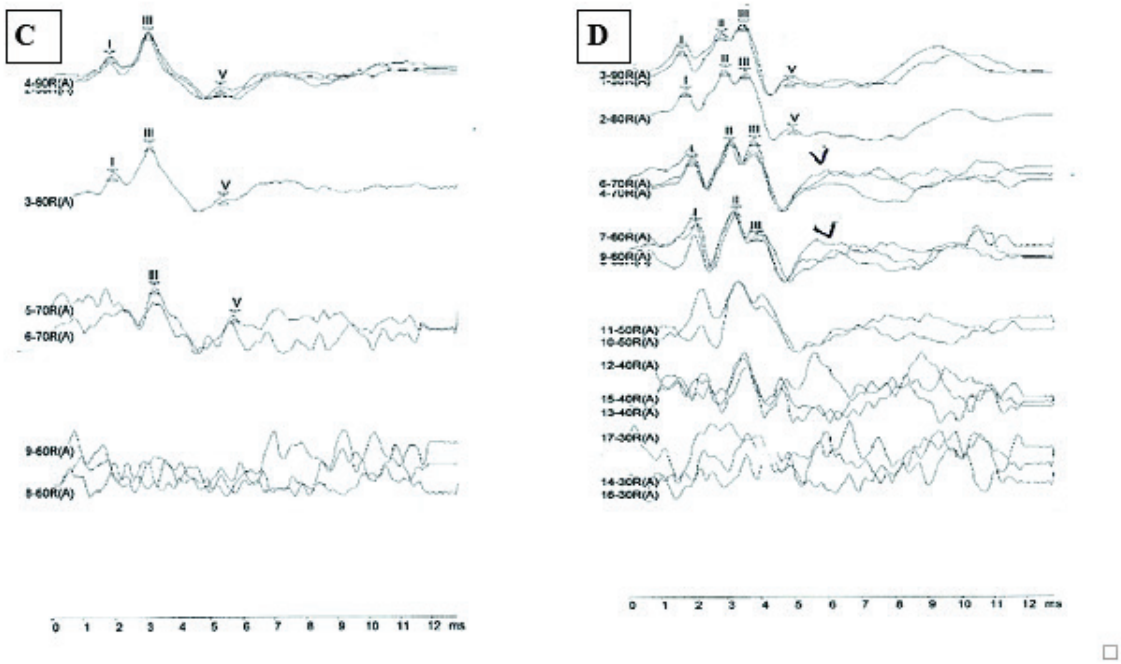


Fig.1. Results of ABR (A) control group, (B) gentamicin induced ototoxicity group, (C) gentamicin induced ototoxicity group treated with rapamycin, (D) gentamicin induced ototoxicity group treated with verapamil.

Results of ABR latencies

Cont... **Fig.1. Results of ABR (A) control group, (B) gentamicin induced ototoxicity group, (C) gentamicin induced ototoxicity group treated with rapamycin, (D) gentamicin induced ototoxicity group treated with verapamil.**



Regarding latency of waves (I, III, V); there was non-significant difference between all groups at the start point of the study. Moreover, group 1 showed non-significant difference in all latency of waves (I, III, V) at the start and the end point of the study. Regarding Group 2, latency of wave (I) showed non-significant difference comparing zero and end points of the experiment, while latency of wave III showed significant increase at end point compared to zero point (P=0.02), and similarly latency of wave V (P=0.001). On comparing latency of the three waves in group 2 with control group at the end point; there was significant increase in latency of both waves III (P=0.004), and V (P=0.001). Group 3 showed significant increase in latency of wave I and wave V at the end point compared to zero point (P=0.02) and (P=0.05) respectively, Moreover, latency of wave III showed a significant decrease on comparing group 3 with group 2 (P=0.03). On verapamil treatment in group 4 there was a significant increase in latency of wave I (P=0.002), and latency of wave V at the end point compared to the zero point (P=0.003) (Table 1).

Table (1): Latencies in different studied groups at zero point and the end point.

Latency **Group**	N (%detectability)	**Latency of wave I (Mean ± SEM)**	**Latency of wave III (Mean ± SEM)**	**Latency of wave V (Mean ± SEM)**
		F (P value)		
		F (3.02) (P NS)	**F (6.09) (P=0.006)**	**F (13.83) (P=0.001)**
Group 1 At zero point At end point	8 (100%) 8 (100%)	1.3 ± 0.0 1.3 ± 0.0 Ppaired NS	3.1 ± 0.1 3.1 ± 0.1 Ppaired NS	5.0± 0.1 5.0± 0.1 Ppaired NS
Group 2 At zero point At end point	8 (100%) 2 (25%)	1.4 ± 0.1 1.8 ± 0.2 Ppaired NS	3.0 ± 0.2 4.2 ± 0.1 Ppaired=0.02 P1=0.004	5.0 ± 0.2 6.2 ± 0.1 Ppaired=0.001 P1=0.001
Group 3 At zero point At end point	8 (100%) 6 (75%)	1.3 ± 0.1 1.6 ± 0.1 Ppaired=0.02	2.8 ± 0.2 3.4 ± 0.2 Ppaired NS P2=0.03	4.7 ± 0.3 5.8 ± 0.4 Ppaired=0.04 P2 NS
Group4 At zero point At end point	8 (100%) 6 (75%)	1.3± 0.1 1.7± 0.1 Ppaired=0.002	3.0 ± 0.2 3.5 ± 0.1 Ppaired NS P2 NS, P3 NS	4.8 ± 0.2 6.1 ± 0.1 Ppaired=0.003 P2 NS, P3 NS

Values expressed as mean ± SEM, n: number, NS: non-significant (P>0.05).

Ppaired: P value of paired sample t- test.

F (P value): F value (P value) of one-Way ANOVA.

Post- hoc Tukey test:

- P1: group 2 vs group 1
- P2: group 3 & group 4 vs group 2
- P3: group 4 vs group 3

Results of ABR threshold

Regarding threshold there was no significant difference between all the studied groups at zero point and in group 1 till the end of the study while, in group 2 gentamicin administration induced significant increase in the threshold at the end point compared to zero point (P=0.02). On rapamycin treatment ABR threshold elevated significantly at the end point (P=0.001) and the same change was found on treatment with verapamil (P=0.002). In addition, group 2 showed significant increase in the threshold

in comparison to group 1 at the end point (P=0.001), Co-treatment with both rapamycin and verapamil exhibited decrease in ABR threshold when compared to group 2 treated with gentamicin alone. This decrease was significant only in case of verapamil (P=0.004) which also exhibited a significant decrease in the threshold compared to group 3 (P=0.05) (Table 2).

Table (2): Threshold in different studied groups at zero point and the end point

Threshold	n (%detectability)	Mean ± SEM	F value P value of different groups at the end point
Group 1 At zero point At end point	8 (100%) 8 (100%)	32.5 ± 1.6 32.5 ± 1.6 Ppaired NS	F (45.2) P value =0.001
Group 2 At zero point At end point	8 (100%) 2 (25%)	40.0 ± 0.0 80.0 ±5.8 Ppaired=0.02 P1=0.001	
Group 3 At zero point At end point	8 (100%) 6 (75%)	32.0 ±2.0 72.0 ±3.7 Ppaired=0.001 P2 NS	
Group 4 At zero point At end point	8 (100%) 6 (75%)	35.0 ±2.2 60.0 ±2.6 Ppaired=0.002 P2=0.004 P3=0.04	

Values expressed as mean ± SEM, n: number, NS: non-significant (P>0.05).

Ppaired: P value of paired sample t- test.

F (P value): F value (P value) of one-Way ANOVA.

Post- hoc Tukey test:

- P1: group 2 vs group 1
- P2: group 3 & group 4 vs group 2
- P3: group 4 vs group 3

GSH levels in the blood (mg/dl)

GSH level declined significantly in group 2 as compared to group 1 (P<0.001) while rapamycin

treatment revealed non-significant increase in GSH level as compared to group 2. On the other hand, group 4 revealed significant increase as compared to group2 and group 3 ($p<0.05$) (Table 3).

Table (3): GSH levels (mg/dl) in the blood in different studied groups

Groups	Group 1 (n=8)	Group 2 (n=8)	Group 3 (n=8)	Group 4 (n=8)	One-way ANOVA F value (P value)
(GSH) mg/dl	39.37± 3.59	13.49±1.23 P1<0.001	15.67±1.13 P2 NS	28.14±4.88 P2<0.05 P3<0.05	14.64 (P<0.001)

-Values expressed as mean ± SEM, n: number, NS=non-significant

-Post -hoc Tukey test:

- P1: group 2 vs group 1
- P2: group 3 & group 4 vs group 2
- P3: group 4 vs group 3

MDA levels in the serum (nmol/ml)

In group 2 there was significant increase in MDA level as compared to the control (P<0.01). Group 3 showed non-significant increase as compared to group 2. However, Group 4 showed significant decrease as compared to both groups 2 and 3 (P<0.05) (Table 4).

Table (4): MDA (nmol/ml) level in the serum in different studied groups

Groups	Group 1 (n=8)	**Group 2 (n=8)**	**Group 3 (n=8)**	**Group 4 (n=8)**	**One-way ANOVA F value (P value)**
(MDA) (nmol/ml)	6.77±1.23	15.53±1.44 P1<0.01	16.51±1.79 P2NS	9.72±1.41 P2 <0.05 P3 <0.05	9.870 (P<0.001)

-Values expressed as mean± SEM, n: number, NS=non-significant

-Post- hoc Tukey test:

- P1: group 2 vs group 1
- P2: group 3 & group 4 vs group 2

- P3: group 4 vs group 3

H&E-stained histopathology sections of right cochlea

Group 1 showed normal outer, inner hair cells (IHCs), normal straight stria vascularis without vacuolations and normal spiral ganglion. Vestibular membrane was intact without vacuolations. In group 2 there was loss or total absence of organ of corti (OC). Others showed hydropic degenerations in outer hair cells (OHCs), broken vestibular membrane, vacuolations and cracking in the stria vascularis and spiral ganglion. Group 3 revealed preservation of hair cells with mild hydropic degeneration, waved basilar membrane in addition to focal degeneration in spiral ganglion. Group 4 showed preservation of normal OC, stria vascularis, spiral ganglion neurons with less degeneration (Fig.2).

SEM of left cochlea

Group 2 revealed complete fusion of stereocilia, and massive damage of the normal u or v shaped order. While group 3 showed to some extent preservation of stereocilia with focal fusion or complete loss. Group 4 showed minimal loss of stereocilia with preservation of normal shape (Fig.2).

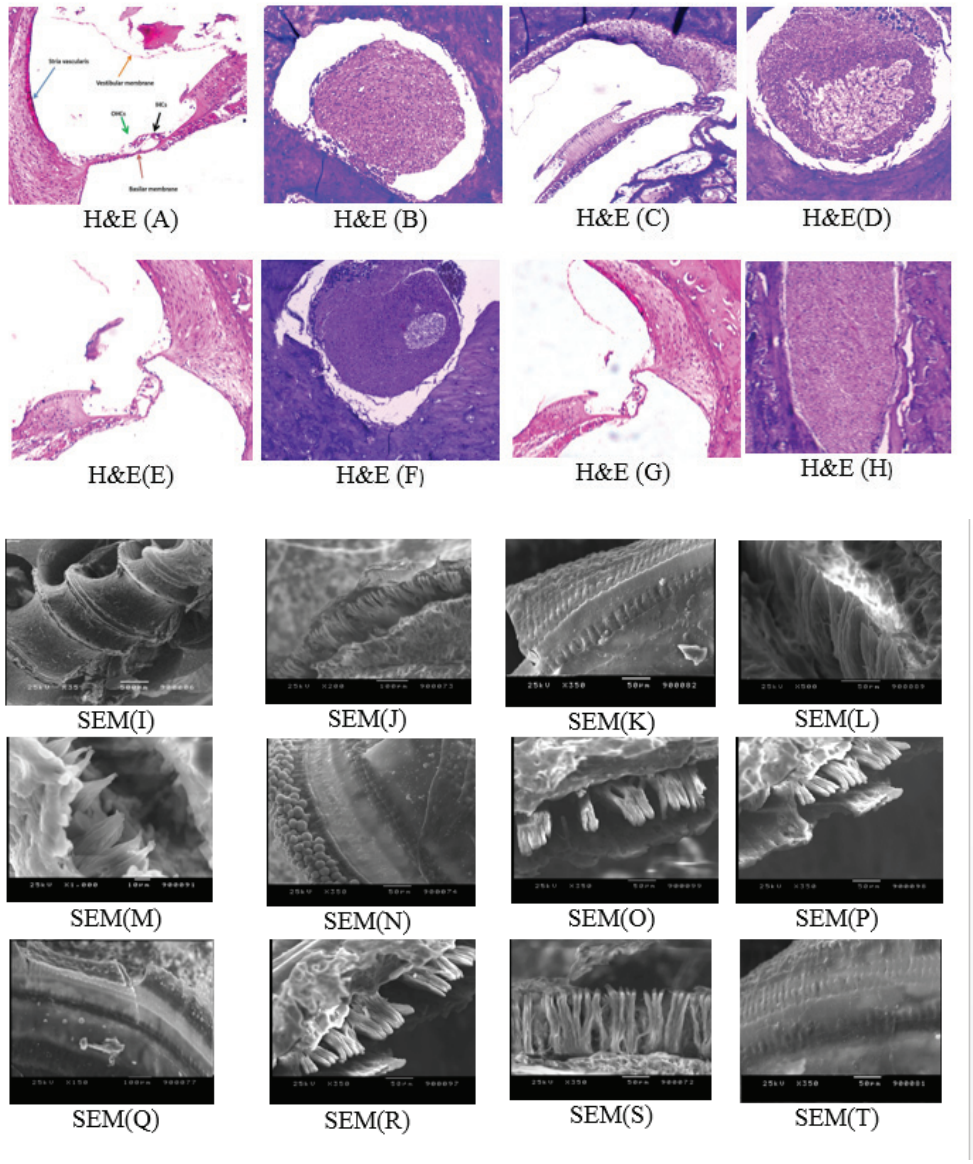


Fig. 2. H&E-stained cochlea photomicrographs (A: H) and micrographs of scanning electron microscope SEM (I: T) of different studied groups:

H&E: Group1 (A, B) where (A) showing normal (OHCs) & (IHCS), stria vascularis, straight intact basilar membrane (H& E X40) and (B) normal spiral ganglion with no degenerations (H& E X100). Group 2 (C, D) where (C) showing complete destruction of HCs and basilar membrane with hydropic degeneration, edema in stria vascularis (H& E X40) and (D) showing hydropic degeneration in spiral ganglion (H&E X100). Group 3 (E, F) where (E) showing preservation of HCs with waved basilar membrane, hydropic degeneration in stria vascularis (H&E X40) and (F) showing focal degeneration in spiral ganglion (H&EX100). Group 4 (G, H) where (G) showing preservation of OC architecture and waved basilar membrane with mild hydropic degeneration in stria vascularis (H&E X40) and (H) showing no degeneration of the spiral ganglion (H&E X100).

SEM: Group1 (I: K) where (I) showing panoramic overview of the three and half turns of the cochlea (SEM x35), (J) showing normal stereocilia of HCs of OC (SEM x200), (K) showing (u or v) shaped rows of HCs (SEM x350). Group 2 (L: N) where (L, M) showing complete fusion of stereocilia of HCs (SEM x500-1000), and (N) showing complete damage of the normal order (SEM x350). Group 3 (O: Q) where (O, P) showing to some extent preservation of stereocilia with focal loss of stereocilia in others (SEM x350), (Q) showing preservation to less extent of (U or V) shaped in some areas (SEM x150). Group 4 (R: T) showing minimal loss of stereocilia with preservation of appearance of normal order of HCs (SEM x350).

LC3-II Immunohistochemical scores

Group 2 revealed significant increase in LC3-II score as compared to group1 (P=0.001) while, in both groups 3 and 4 a significant decline in LC3-II scoring as compared to group 2 was found (P=0.03) and (P=0.006) respectively (Fig.3, Table 5).

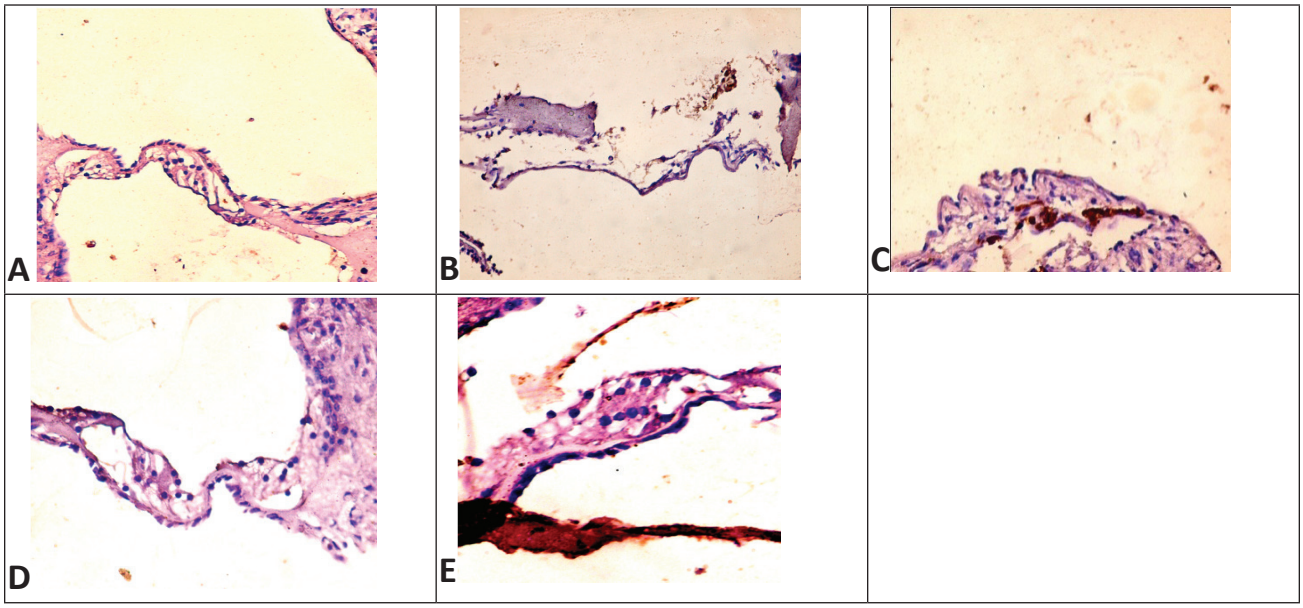


Fig.3. A photomicrograph of cochlea from studied groups, (A) group 1 showing barely seen staining for LC3-II expression score (0) (IHC X100). (B, C) group 2 showing destruction of hair cells of OC with expulsion of contents and high expression of the LC3-II with score (2) (IHC X 200). (D) group3 showing mild expression of the LC3-II with score (1) (IHC X 100). (E) group 4 showing mild expression of the LC3-II

with score (1) (group IHC X200).

Table (5): Immunohistochemical (IHC) expression of LC3- II scores in different studied groups

GROUPS	LC3-II score (median)	Kruskal–Wallis test & Mann-Whitney test (P value)
Group1	0	P< 0.001
Group2	2**	
Group3	1*	
Group4	1*	

Values expressed as median, Kruskal–Wallis and Mann-Whitney test:

** Significant; group 2 vs group 1.

*Significant; group 3, group 4 Vs group 2.

Discussion

Autophagy may be double edged sword. Meanwhile in many diseases, it plays an important role in cell survival, but it is still being debated if its activation increases or reduces cell death [24]. It has a protective effect in many diseases [25]. On the contrary it has been reported to cause pathological changes [26]. In addition to that, too much autophagy activation was believed to cause ischemia/reperfusion cardiac cell death [27] and promotion of hepatic fibrosis [28]. Our research aims to examine autophagy role in gentamicin- induced ototoxicity. At the start point, there was non-significant variation between groups with normal ABR in all animals, while at the end point there was significantly increased ABR threshold compared to the start one. Our results in group 2 compared to group 1 showed absence of ABR response in 75% of animals indicating sever hearing loss, while others showed significant increase in wave III latency, wave V latency and significant ABR threshold increase which coincides with many studies [5, 22, 29]. The ABR results are matched with histopathology in light and SEM where there are variable degrees of HCs death, pathological changes in stria vascularis and degeneration of spiral ganglion which are in line with many studies [22, 30, 31]. ROS role in gentamicin ototoxicity is documented in different studies [32, 33]. When there is imbalance between ROS and intrinsic antioxidants, apoptotic cell death is activated [34]. This is represented by marked elevation of MDA and marked reduction of GSH which coincide with Draz et al. [22]. To investigate the role of autophagy, LC3-II immunohistochemistry is determined using Schläfli et al. scoring (0-3)[23]. Our results in group 2 exhibited accumulation of autophagosomes represented by heavy LC3-II staining compared to group1 which shows presence of low basal level of autophagy that coincides with Kim et al. [12] who suggested that this accumulation is due to reducing lysosomes autophagosomes fusion. To investigate HCs survival autophagy role, rapamycin has been used [11, 12]. We used rapamycin orally according to Saegusa et al. who reported effectiveness of oral low dose rapamycin to induce autophagy [33]. There is no definitive oral rapamycin dose in guinea pigs was investigated to induce autophagy, so we depended on the human equivalent dose [35]. Rapamycin group as compared to group 2, it showed significant decrease regarding wave III latency, non-significant difference in wave V latency and ABR threshold. SEM and light

microscope showed to some extent preservation of normal OC with focal vacuolations or loss of some stereocilia. While there are non-significant differences in GSH, and MDA level. Regarding LC3II level there is significant reduction which indicates reduction in autophagosomes suggesting induction of autophagic flux which coincides with Cui et al. [36].Whether rapamycin enhances HCs preservation or causes cell death, it is a matter of debate because the role of mTOR in cochlea is still unclear. Our results coincides with Leitmeyer et al. [37] who found that mTOR inhibition by rapamycin treatment led to dose dependent (HCs) death due to elevation of oxidative stress [37]. On the other hand, these findings are contradictory to He et al. [38] who documented elevated survival rates of HCs after rapamycin autophagy activation, decreased levels of ROS and decreased cell death, in addition to Kim et al. [12] who found that hearing thresholds were significantly better in rapamycin injected ear group, and reduction of ABR threshold via alleviating cisplatin ototoxicity [39].This variability can be explained by that the efficacy of rapamycin can depend upon cellular type and mTOR target. Also, it is affected by the level of phosphatidic acid (PA) which is a competitive antagonist to rapamycin. The level of rapamycin which is needed to inhibit mTOR is affected by PA level in the cell [40]. Another explanation is occurrence of excessive autophagy which may lead to cell death as reported in different tissues [27]. So, the concentration of rapamycin in the cochlea is critical for cell survival. Away from the mTOR dependent pathway, verapamil was suggested in previous studies to be an autophagy inducer by decreasing the intracellular Ca^{+2} and increasing autophagosome formation, so help in clearance of mutant aggregate-prone proteins [13, 14]. It was also found to increase fusion between autophagosome and lysosome in liver, so it was tried for autophagic flux restoration and treatment of obesity [41]. It was also reported to have cytoprotective effect via induction of autophagy [42], in addition to recent study that suggested that verapamil extends lifespan by promoting autophagy [43]. As far as we know there is no definitive dose for verapamil to induce autophagy in guinea pigs, so we depended on human equivalent dose (FDA label dose is 240: 480 mg. The average daily dose is 360 mg/day)[44]. Verapamil treated group showed significant decrease in ABR threshold compared to group 2, significant reduction in MDA and significant elevation in GSH which are in line with Kedziora-Kornatowska et al. [13]. SEM and light microscope showed decline in HCs death and less pathological changes compared to group 2 and group 3. Regarding LC3-II expression there is significant decrease as compared to group 2 suggesting induction of autophagic flux. These are in consistence with previous reports which suggested that verapamil allowed lysosomes and autophagosomes to re-associate leading to autophagosome accumulation reduction [41]. Verapamil demonstrated superiority in protection of HCs against gentamicin induced ototoxicity compared to rapamycin due to its antioxidant and significant improvement ABR results.

Conclusion

Autophagy induction via Ca^{+2} channel antagonist verapamil may be a new therapeutic target to decrease gentamicin ototoxicity by its autophagy inducer effect and antioxidant properties.

Recommendations

More autophagy markers such P62 and different doses of rapamycin could be used to better present autophagy activity in gentamicin ototoxicity.

Conflict of Interest: None

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