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Effect of minocycline and its nano-formulation on central auditory system in blast-induced hearing loss rat model

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ABSTRACT

Blast injuries are common among the military service members and veterans. One of the devastating effects of blast wave induced TBI is either temporary or permanent hearing loss. Treating hearing loss using minocycline is restricted by optimal drug concentration, route of administration, and its half-life. Therefore, therapeutic approach using novel therapeutic delivery method is in great need. Among the different delivery methods, nanotechnology-based drug delivery is desirable, which can achieve longer systemic circulation, pass through some biological barriers and specifically targets desired sites. The current study aimed to examine therapeutic effect of minocycline and its nanoparticle formulation in moderate blast induced hearing loss rat model through central auditory system. The I.v. administered nanoparticle at reduced dose and frequency than regularly administered toxic dose. After moderate blast exposure, rats had hearing impairment as determined by ABR at 7- and 30-days post exposure. In chronic condition, free minocycline also showed the significant reduction in ABR threshold. In central auditory system, it is found in this study that minocycline nanoparticles ameliorate excitation in inferior colliculus; and astrocytes and microglia activation after the blast exposure is reduced by minocycline nanoparticles administration. The study demonstrated that in moderate blast induced hearing loss, minocycline and its nanoparticle formulation exhibited the optimal therapeutic effect on the recovery of the ABR impairment and a protective effect through central auditory system. In conclusion, targeted and non-targeted nanoparticle formulation have therapeutic effect on blast induced hearing loss.

1. Introduction

Primary blast injury by direct effect of the shock wave can cause diffuse injury (multifocal injuries) (Schwartz et al., 2008). Earlier publications reported that the blast wave has more impact on ear first compare to other organs (Cernak, 2010; Pham et al., 2015; Sundaramurthy et al., 2012; Lew et al., 2007). Hearing loss (HL) and tinnitus are highly prevalent in the growing population of returning soldiers, while no effective and safe treatment is revealed (Lew et al., 2007; Kuchinsky et al., 2020; Chadha and Cieza, 2017). In United States, the occurrence of noise induced hearing loss among noise exposed population is 23% with hearing loss, 15% with tinnitus and 9% with both illnesses (Masterson et al., 2016). A projected estimation of 900 million people will lose their hearing by 2050 and treatment cost estimated as $750 billion globally (World Health Organization). Till date, the most popular mechanisms of blast induced hearing loss are injured hair cells, cranial nerve VIII and abnormal neural plasticity. A significant number of researches have been done on the peripheral auditory system (PAS) malfunction mechanism, including tympanic membrane rupture, cochlea damages and auditory nerve malfunction. While only recently central auditory system (CAS) abnormalities evoked investigators' interest (Shao et al., 2021a). High intensity blast over pressures influence on the auditory system (peripheral and central nervous system) and subsequently induce hearing loss (Shao et al.,

Abbreviations: ABR, auditory brainstem response; AC, auditory cortex; CAS, central auditory system; GABA, gamma-aminobutyric acid; 5-HsT, 5-hydroxytryptamine; NMDAR1, N-methyl-D-aspartate receptor 1; PAS, peripheral auditory system; DAI, (diffuse axonal injury); HL, (Hearing loss); bTBI, blast traumatic brain injury.

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2021a; Aravind et al., 2020; Ravula et al., 2022a) and even if the blast overpressure is at relatively mild level (Smith et al., 2020; Kallakuri et al., 2018; Lasak et al., 2014). Hearing loss can directly damage both PAS and CAS components (Lasak et al., 2014; Basta et al., 2018). As per earlier studies, bTBI exposed patients have a hearing problem in complex auditory environments where peripheral hearing system still remain normal but there could be an involvement of central auditory system (Gallun et al., 2012). For instance, Race et al., and others reported that both peripheral and central auditory system vulnerability to blast wave (Race et al., 2017; Lu et al., 2021). Similarly, other works also observed tympanic membrane damage, altered neurotransmitter receptors expression and impairment in direct signal transmission between CAS and PAS after moderate blast (Shao et al., 2021a; Younger et al., 2019).

It is revealed that the existence of hyperactivity in the dorsal cochlear nucleus (DCN), inferior colliculus (IC), and auditory cortex (AC) along with increased neuronal synchronization and tonotopic remodeling in the AC is identified (Mao et al., 2012; Luo et al., 2014, 2017). These findings shed light on that both PAS and CAS are both susceptible to blast trauma.

Even though there is no approved treatment for blast induced hearing loss so far, there have been many potential treatments for hearing loss. According to the previous research, stem cell therapy and neurotrophic factors were used in animal models to repair or regenerate the damaged structure. Also therapeutics like steroids, etanercept, D and L-methionine, and kenpaullone (a cyclin dependent kinase 2 (CDK2) inhibitor) have shown efficacy against cisplatin induced ototoxicity in in vivo models (Rybak et al., 2019). Allopurinol, a free radical scavenger, has showed short term protection from hearing loss (Robinson et al., 2015). Administration of 2,4-disulfonylalpha-phenyl tertiary butyl nitrone (HPN-07) and N-acetylcysteine (NAC) prevented bTBI induced auditory pathological changes in animal model. In addition, a combinatorial regimen HPN-07 and NAC can significantly reduce pathologic Tau accumulation and alleviate the neurodegeneration in the cochlea (Lu et al., 2021; Du et al., 2017). Jianzhong Lu et al. also demonstrated reduction in blast-induced neurodegeneration risk of tinnitus (Lu et al., 2021).

Another potential drug for treating hearing loss is Minocycline, an FDA approved semi-synthetic tetracycline derivative, has both anti-inflammatory and neuroprotective properties (Zhang et al., 2017; Gupta et al., 2020). It is tetracycline derivative that has antimicrobial, anti-inflammatory, anti-oxidant, antiapoptotic properties and identified with various neuroprotective properties (Camargos et al., 2020; Hiskens et al., 2021). It easily cross BBB barrier due to lipophilic in nature and also have the ability to restore BBB breakage (Shi et al., 2011). Because of this property, minocycline can have systemic effect both in the peripheral and also central systems, making this a promising and efficient drug for blast induced hearing problems. Earlier study showed that neuroprotective effects of minocycline against a wide variety of neurodegenerative diseases (Garrido-Mesa et al., 2013). More importantly, several other works revealed that minocycline attenuated noise-induced and ototoxic drug (cisplatin, neomycin and gentamycin) hair cell loss (in a Guinea pig model) and in *vitro models* (Hou et al., 2018; Corbacella et al., 2004; Sun et al., 2015; Du et al., 2011; Lee et al., 2011; Shi, 2016). Alan M. Robinson et al. also suggested applying combination of minocycline and aminoglycoside antibiotics to inhibit ototoxic hearing loss (Robinson et al., 2015). Besides as a promising drug in treating hearing loss, the optimal drug delivery method is another focus of current otology research in treating ontological diseases. Targeted delivery to cochlear protecting drugs to the brain and inner ear is the vital to protecting hearing loss. Hence minocycline and its nano formulation evaluated to ameliorate hearing loss in rat model after intratympanic neomycin administration (Robinson et al., 2015). However, blood labyrinth barrier (BLB) is main hindrance in delivering drugs to the inner ear (Shi, 2016; Cohen-Salmon et al., 2007)and also other obstacle is BBB for reaching optimal drug concentration. Besides synthetic compounds, natural compounds been applied for hearing loss therapy (Li et al., 2018; Ravula et al., 2021).

Fig. 1. Schematic shows blast tube setup, moderate blast pressure, administration of free minocycline and its Nano formulations through i.v. In bTBI induced rat hearing loss model and subsequent ABR and immunohistochemical analysis in central auditory systems.

Drug delivery to brain is limited by blood brain barrier (BBB) (Kuriakose et al., 2019; Murugan et al., 2020). Correspondingly, BLB plays a vital role in maintaining the unique fluid composition within the cochlea and limit the rapid delivery of therapeutic drugs to the cochlea (Juhn et al., 1982; Chen et al., 2010). In addition, systemic delivery of high doses to treat inner ear cause severe adverse effects in other organs (Lee et al., 2018). Due to these restrictions, the traditional route administration is not viable, hence there is requirement of designing novel delivery system to treat hearing loss. Nanoparticles (NPs) have been applied for the past five decades in treating various disease condition (Wu et al., 2014; Eshraghi et al., 2012). Specifically, stealth nanoparticles, C. Sun et al., encapsulated dexamethasone in PEG-coated PLA nanoparticles for delivering compounds to the cochleae (Kuchinsky et al., 2020; Horie et al., 2010; Sun et al., 2016). Nanoparticle (NP)-have showed rewards of sustained release and comfort of surface modification, therefore the NP-based delivery systems have become a current research attention[(An and Zha, 2020).

Even though minocycline crosses BBB, achieving therapeutic concentration to elicit an effect is challenging and limiting its use in various brain related diseases (Elewa et al., 2006). In particular, to understand the CAS based mechanism of mitigating drug/noised induced hearing loss, there is great demand for targeted delivery approach of therapeutics including minocycline via BBB. Therefore, new approaches to the delivery of drug molecules across these barriers for hearing loss are highly demanded. Mark Pretorius et al., shown Cy3-labeled silica nanoparticles delivery to distal part of the central auditory pathway (Praetorius et al., 2007). In particular, albumin-based nanoparticle has been explored widely for the effective delivery of therapeutics to cancer, brain disorders and more (Karimi et al., 2016). Accordingly, we speculated that minocycline, BBB targeted transferrin tagged and PEGylated(non-targeted) formulation should facilitate sustained release of the drug after systemic (i.v) administration, leading to a protection against bTBI induced hearing loss through peripheral and central mechanism. In the present study, we systemically administered minocycline, BBB targeted transferrin tagged and PEGylated(non-targeted) formulation to bTBI induced hearing loss in rats, and examined the function and histology of the rat's cochleae to evaluate their potential protective effect against bTBI induced hearing loss. The aim of the study was to examine if administration of a minocycline and its targeted and non-targeted nano formulation with four days of consecutive injection after blast exposure could reduce both acute and chronic hearing loss through peripheral and central mechanism. To our knowledge this was the first study evaluating whether minocycline and its nano formulation has otoprotective effects in moderate blast induced hearing loss rat model through peripheral and central mechanism.

2. Materials and methods

2.1. Chemicals, reagents and instruments

Minocycline, Bovine Serum Albumin (lyophilized powder, ≥96% agarose gel electrophoresis), anhydrous ethanol (200 proof,

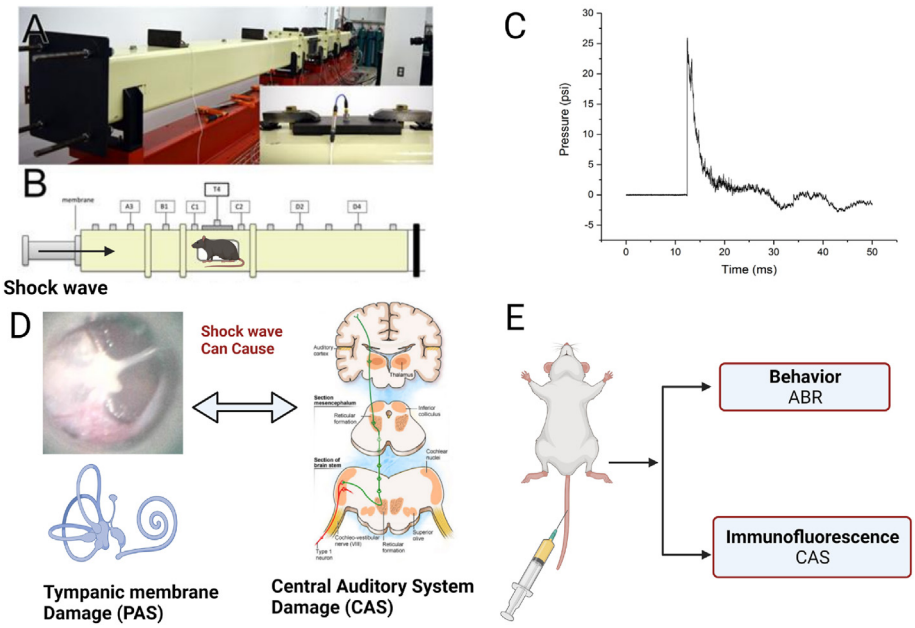

Fig. 1. Schematic shows (A). Photo of the 9-inch square cross section, 22 ft long shock tube instrumented with pressure sensors; (B) Aerodynamic rat holder mounted in the test section (top), with rat placed on top (middle) and animal wrapped in a harness to minimize head and body motion during blast exposure (bottom); (C) Representative moderate blast overpressure profiles as measured in the test section at the location of the animal's head for 10 experimental groups used in our study. (D) Subsequent blast mediated tympanic membrane and central auditory system (CAS) damage; (E) Free minocycline and its nanoparticle iv administration in bTBI induced rat hearing loss model.

anhydrous, ≥99.5%) crosslinker NHS-PEG-MAL-5000 and transferrin (human: minimum 98%) were purchased from Sigma Aldrich U.S.A. HPLC Grade Ammonium acetate was purchased from VWR. HPLC grade water, Methanol and Acetonitrile were purchased from Fisher Scientific. HPLC-grade glutaraldehyde (100%) were obtained from Alfa Aesar (USA). The Millipore Milli-Q Plus apparatus was used to procure ultrapure water. Traut's reagent (2-Iminothiolane) and Ellman's reagent was obtained from Pierce (Rockford, USA). The PD-10 Columns SephadexTM G-25 M were from GE Healthcare. Lyophilizer, Centrifuge (Eppendorf centrifuge 5810R, Thermo scientific sorvall RC 6+), Zeta sizer (malvern), HPLC (Thermo Fischer), Sonicator and Rota evaporator (Rotaevapor Buchi, R-210) instruments were used in the study.

2.2. Animals

10-week-old adult male Sprague-Dawley (Charles River Laboratories) rats weighing 250 ± 50 g were used in this study. The animals were housed with free access to food and water in a 12-hdark-light cycle at constant monitoring at 25 °C. All conducted experimental procedures followed the guidelines of Care and Use of Laboratory Animals approved by Rutgers University Institutional Animal Care and Use Committee before experiments. Rats were divided into five groups: 1) Control, 2) Blast, 3) Blast + Minocycline injection 4) Blast + BBB targeted transferrin tagged nanoparticles and 5) Blast + and PEGylated(non-targeted) nanoparticles. Animals in the control group were placed outside of the shock tube exposed to only the sound (no shock wave). While animals in 180 kPa blast group, they were exposed to both sound and shock wave as detailed explained in our previous publications (Skotak et al., 2013; Li et al., 2013a).

2.3. Blast injury

Rats were exposed to a single 180 kPa moderate blast using shock tube at the NJIT. All animals were anesthetized with 5% isoflurane, released in a chamber containing 95% air and 5% CO_2. Then animals in blast group were fastened on the stage facing the direction of the blast wave as illustrated in Fig. 1(i). The control group was placed outside of the shock tube to avoid shock wave, only the blast sound was exposed as shown inFig. 1B (Murugan et al., 2020; Rana et al., 2020).

2.4. Blast overpressure measurement

The real time pressure was recorded along the shock tube. The position of the pressure sensors is listed in Fig. 1 as B1, C1, T4, C2, D2 and D4. Typical 180 kPa blast profile with all six pressure sensors recordings is displayed in Figure, the T4 sensor recorded what the animal was experiencing during the blast. The sensor displayed the typical" overpressure-under pressure" profile, and our shock tube trustworthily generated 180 kPa (~26.107psi) in peak pressure and within a millisecond time duration.

2.5. Preparation of BBB targeted transferrin tagged and PEGylated(non-targeted) minocycline loaded nanoparticles

BBB targeted transferrin tagged Nanoparticle was formulated in three steps (Michaelis et al., 2006; Ulbrich et al., 2009). In the first step, minocycline encapsulated albumin nanoparticle was prepared using a modified desolvation method (Li et al., 2009, 2013b).Briefly, 10% BSA (w/v) in HPLC grade water was stirred (at 600 rpm) with 7.5% of minocycline (w/v) at room temperature for drug absorption onto albumin. After 1 h of continuous stirring, the pH value was adjusted from 8.5 using 0.1 M NaOH. The mixture was then desolvated through addition of a suitable amount of ethanol, using a peristaltic pump at a rate of 1 ml/min under stirring (at 600 rpm). Ethanol addition was sustained till turbidity point and residual ethanol was removed by a rotary evaporator at 4 °C. Then, the formulated minocycline-loaded nanoparticle was stabilized by crosslinking with 8% glutaraldehyde solution for 18 h. The nanoparticle in solution was ultra-centrifuged (Sorvall LYNX 6000, Superspeed Centrifuges) at 36,288 g force for 40 min.

Secondly, 2-iminothiolane solution was added to bind a sulfhydryl group to the transferrin, and was quantified through use of Ellman's reagent. Briefly, transferrin was dissolved in phosphate buffer (1 mg/ml at pH 8.0) and incubated with 12.8 μl (50.85-fold molar excess) of 2-iminothiolane solution (6.9 mg in 1.0 ml phosphate buffer, pH 8.0) in the dark for 2 h at 20 °C under continuous shaking (500 rpm). Thereafter, the thiolated transferrin was purified by PD-10 Columns SephadexTM G-25 M, using phosphate buffer (pH 8.0) as eluent.

Thirdly, NHS-PEG-MAL-5000 solution in 10-fold molar excess was introduced to the nanoparticles to cross-link activate them. To conjugate the NPs, 500 μl of thiolated and purified transferrin solution was added to 500 μl of reactive BSA NPs and kept under shaking for 24 h at room temperature. Thiolated transferrin excess was removed by 2-fold nanoparticle centrifugation, redispersed in water and lyophilized. Precipitate obtained from the centrifugation was washed with pure water three times and then freeze dried with addition of 50 mg mannitol to obtain brownish fine powder of Tf conjugated MANP. For further characterization, a stock suspension of NP was used. Similarly, non-targeted nanoparticle was also prepared without the transferrin ligand conjugation.

Two separate approaches were utilized to redisperse the lyophilized MANP, physical shaking and sonication (Yadav and Sawant, 2010). Manually shaking method was applied using weighed quantity of lyophilized NP with phosphate buffer saline pH 7.4. The nanosuspension was subject to gentle shaking for 2 min to redisperse the solution and then immediately measured for particle size using a Malvern zetasizer (Venkatesan et al., 2011; Perumal et al., 2011). Micrometer sized particles were considered to non-dispersible. Sonication method was applied with lyophilized NP in phosphate buffer saline pH 7.4 for 2 min using a bath sonicator and redispersibility.

2.6. Drug administration

Animals in the treated group were tail vein injected intravenously (i.v.) with a 3 mg/kg minocycline, BBB targeted transferrin tagged and PEGylated(non-targeted), which were dispersed in 0.5 ml of physiological saline solution. Free minocycline and nanoparticle were administered 4 h after blast overexposure and then continued once a day for three days whereas, control group of animals were injected with similar amount of saline.

2.7. Auditory brainstem response (ABR)

Cochlea conditions and inferior colliculus integrity were examined by ABR at post day 7 and 30 in control, blast, minocycline, BBB targeted transferrin tagged and PEGylated(non-targeted) nanoparticle group. ABR thresholds (Sound pressure level (SPL) measured in decibels (dB)) were recorded as the parameter of functional evaluation of neuronal circuit between cochlea and inferior colliculus. For each above-mentioned time points, animals were examined for tympanic membrane (TM) rupture conditions by using otoscope. After the blast exposure, when animals were still under the effect of anesthesia, a commercialized Teslong Ultra-Thin Otoscope (3.9 vmm HD Visual Ear Cleaner Ear Scope Camera with six LED Lights) were inserted in the pre-cleaned (with Q tips and alcohol) ear canal of the animals. Animals were placed on the heating pad to avoid hypothermia. The ear tip was used for the guidance of the otoscope camera. Tympanic membrane pictures were taken when the camera was capturing the most range of the tympanic membrane (TM). Animals were anesthetized for 1 min then placed on the heating pad when doing TM examination in other time points.

TM ruptured animals were excluded from the ABR examination. Then animals were initially anesthetized. Three platinum-coated tungsten electrodes were inserted in the vertex, below the ipsilateral pinna, and in the hind leg muscles for the positive, negative and ground positions, respectively. Click and tone-burst stimuli at 4, 6, 8, 10, 12, 14, 16, 18, 20 kHz were delivered through TDT M1 for free field operation. Stimuli were played from 100 to 5 dB with 5 dB stepwise decrease. ABR signals were amplified, band-filtered from 0.3 to 3 kHz, notch-filtered at 60 Hz, and averaged 300 times for click and tone-burst stimuli, respectively. The signal response collection was conducted using the E-prime software (Psychological Software Tools, 2002). For each set of experiments, calibration was performed by playing continuous sound at different dB level with dB meter (Bruel & Kjaer 2250 sound level meter) in the sound attenuated chamber.

2.8. Immunohistochemistry and microscopy

Rats were cardiac perfused with 4% Paraformaldehyde in 9.6 g/L PBS after injury. The heads of the animal were harvested, and the skin was removed to expose the dorsal surface of the skull. The cerebrum, cerebellum, and brain stem were exposed and harvested by breaking the occipital bone and parietal bone. The brain specimen then was fixed in 4% Paraformaldehyde in 9.6 g/L PBS for 2–4 days, rinsed in 9.6 g/L PBS and stored in 30% sucrose 9.6 g/L PBS solution. Desired brain regions were then dissected into 20-μm thickness sections using vibratome. Auditory cortex (AC) and inferior colliculus (IC) were identified by referring to Rat Atlas.

Tissue sections (20 μm thick, freshly cut by vibratome) were fixed in ice-cold methanol (100%) solution for 10 min at −20 °C, blocked in 10% donkey serum at room temperature for 1 h in PBS containing 0.03% Triton X-100. Fixed tissues were incubated overnight at 4 °C with respective primary antibodies to NMDA-R1 (1:150, abcam68144), GABAAR alpha 1(1:500, abcam33299), NeuN (1:200, abcam104224). NMDA-R1, GABAAR alpha 1 were double stained with NeuN respectively. IBA 1(1:300, invitrogen, PA5-18039) and GFAP (1:300, abcam53554) were single stained, respectively. Secondary antibodies conjugated with AlexaFluor 594 (red) were used for both NMDAR, GABAA receptors and GFAP separately. AlexaFluor 488(green) were used for NeuN and IBA1. Anti-fading reagent with DAPI (ProLong™ Gold Antifade Mountant with DAPI, Invitrogen) was used before putting cover slide (Kuriakose et al., 2019; Murugan et al., 2020; Rana et al., 2020; Ravula et al., 2022b). Subsequent immunostaining, followed Fluorescence intensities analysis as described in our previous study (Sundaramurthy et al., 2012; Younger et al., 2019; Kuriakose et al., 2018; Chandra et al., 2015).

3. Results and discussion

Various nano drug delivery systems are in preclinical stage before reaching therapeutic application (Maples et al., 2015; Perumal et al., 2019; Saravanan et al., 2021). Previous studies of nano targeted delivery has displayed potential for treating hearing loss. Cyclodextrin as nanocarrier solubilizes the plasma membrane by releasing glycosylphosphatidylinositol-anchored proteins and sphingolipid domains (Ilangumaran and Hoessli, 1998)and potentially enter through BLB However, high doses of cyclodextrin are toxic to outer hair cells, causing moderate to severe hearing loss (Crumling et al., 2017). Though systemic drug delivery approach is promising, commonly exert toxicity and its potency is limited hampered by the blood-cochlear barrier. In our recent works, we have demonstrated that blast-induced traumatic brain injury altered brain functionality including central auditory pathways (Sundaramurthy et al., 2012; Kuriakose et al., 2018; Chandra et al., 2015). Similar to our previous published work, CAS damage could

be a direct mechanical disturbance in neuronal cells within the brain such as disturbed neuronal synapses connectivity and altered central neurotransmitter and its receptor levels, oxidative stress and neuroinflammation can contribute to permanent hearing loss after blast exposure (Rama Rao et al., 2018) (Shao et al., 2021b). In another work demonstrated severe and persistant CAS and choclear damage after blast expsure (Race et al., 2017). Our previous work has also estabilshed that BBB dmagae, oxidative stress and neuroinflamation by moderate blast exposure in frontal cortex, striatum, hipocampus, thalamus and cerebellum (Younger et al., 2019). In the current study we explored neuroinflammation, neurotransmitor alteration in CAS after moderate blast exposure. The major focus of the study is to provide the needed bioavailability/cell targeting in the injured brain at a significantly lower dose to achieve maximum bioavailability of minocycline through a targeted nanoparticle and subsequent effect on hearing loss. Our proposed research of (BBB) targeted transferrin tagged (tfr -transferrin receptor) and PEGylated(non-targeted) minocycline entrapped albumin nanoparticle may deliver the minocycline to brain in mitigating the blast/noise induced hearing loss in rat model through CAS mediated effect.

In our study, albumin protein-based NPs was attained by desolvation method (Torchilin, 2000) that can be achieved through precise addition of desolvating agent (ethanol) to albumin solution at an optimum pH (optimal size and encapsulation) with constant stirring until turbidity. A decrease in the solubility of albumin followed by phase separation in water during the desolvation process leads to nanoparticle formation. Further the nanoparticles can be stabilized by crosslinking lysine and guanidino side chains of albumin with the crosslinking agent glutaraldehyde (Li et al., 2009). When crosslinking is increased, the rigidity of the nanoparticle can lead to successive decreases in particle size due to the formation of more compact particles. Minocycline react with hydrophobic regions of the BSA nanoparticle, subsequently stabilized by glutaraldehyde and NH_2 group OF the protein nanoparticle (Motevalli et al., 2019). Thereby amine and CO combine to give −N═C- bond to obtain the nanoparticles of albumin (Mahobia et al., 2016). The crosslinked nanoparticle has no toxicity (Bronze-Uhle et al., 2017). The free the glutaraldehyde was separated by ultracentrifugation to evade the toxicity.

Minocycline-loaded albumin nanoparticles (both BLB/BBB targeted and PEGylated) were freeze dried with 0.05% of mannitol, resulting in a brownish powder that can also be dispersed in PBS or 0.9% saline solution. Nanoparticles with uniform size allow increased cellular interaction and possess enhanced toxicity (Nan et al., 2008). Our patented (US patent) work has detailed characterization of nanoparticles and in vivo studies on rat bTBI model in ameliorating the brain injury and neuroinflammation. Lyophilization may increase the particle size of the nanoparticle, possibly due to aggregation; hence we checked the redispersibility of the particles after lyophilization using mechanical shaking and bath sonication methods.

3.1. Minocycline, BBB targeted transferrin tagged and PEGylated(non-targeted) minocycline nanoparticles on auditory brainstem response (ABR)

It was revealed in Fig. 2 that the tympanic membrane was intact before the ABR examination.

As shown in Fig. 2, in acute condition, the blast treated animal displayed significant increase in ABR threshold. However, the all 3 different drug treatments, groups on an average reduced the ABR threshold level. What is interesting is that the free minocycline drug has the significant effect of reducing the ABR threshold compared with other two nanoparticle treatments (ANOVA post hoc, compare Blast exposed group with free minocycline treated group, P value = 0.02205; compare to Blast exposed group with BBB targeted transferrin tagged nanoparticles treated group, P value = 0.1572, compare Blast exposed group with non-targeted nanoparticles treated group, P = 0.8914). In chronic condition minocycline free drug also showed the promising reduction effect on ABR threshold. In the individual pitched tone ABR at 6 kHz, 8 kHz, 16 kHz, 18 kHz and 20 kHz, blast caused increase ABR threshold in acute level, whereas the increased threshold persisted at 6, 8 and 20 kHz in the chronic condition. While with the minocycline drug treatments showed significant recovery compared with acute condition at 6 kHz, in the chronic condition at 16 kHz, in both acute and chronic conditions, all three drug treatments had no significant effect on ABR threshold recovery. At 18 kHz, both minocycline free drug (compare Blast exposed group with free minocycline treated group P value = 0.00333) and PEGylated non-targeted treatments (compare Blast exposed group with non-targeted treated group, P value = 4.9e-5) significantly reversed the increase in ABR threshold caused by blast in the acute condition. The results show that the nanoparticle designed to be delivered to brain have not reduced blast induced hearing loss compared to minocycline and PEGylated(non-targeted) nanoparticle. This is possibly due to maximal delivery of BBB targeted transferrin tagged nanoparticle in brain which in turn resulted in less effect on auditory brain response. However, free minocycline shows significant recovery in hearing loss both in acute and chronic timepoints compared to PEGylated(non-targeted) nanoparticle.

Similar to our finding, other study also reported minocycline protecting cochlear damage in gerbils in chemical induced hearing loss (Robinson et al., 2015) and also in noise-induced hearing loss (NIHL) (Zhang et al., 2017). What's more, minocycline treatment can also reduce noisy environment induces neuroinflammation (Lin et al., 2020). In our study we have administered all 3 formulations via intravenous route with reduced dose and exhibited similar results compared to previous study. There was also minocycline nanoparticles usage for neuronal implants in order to quench the adverse inflammation (Fried et al., 2018). It was reported that minocycline-loaded PLGA nanoparticles on gelatin-coated neural implants decreased inflammation and immune responses in mice. This nanoparticle significantly reduced the activation of microglial cells and the astrocytic response. Sun et al. (2016) used dexamethasone loaded PEG-PLA NPs in cisplatin induce hearing loss guinea pigs, protected auditory function at 4 and 8 kHz. Similarly, Mart'ınsaldāna et al. (Martin-Saldana et al., 2016) demonstrated that 6a-methylprednisolone loaded NPs protected auditory functions at 10, 14 and 16 kHz.

3.2. Reduction of microglia and astrocytes activation in both AC and IC

In Fig. 3, IBA immunohistochemistry staining intensity is displayed. The graph revealed that after blast trauma, the intensity of microglia was elevated in AC which indicates the microglia activation in response to the blast induced tissue damages. After free drug minocycline and BBB targeted transferrin tagged minocycline nanoparticle treatment, the activated microglia level is significantly reduced. The group treated with PEGylated(non-targeted) nanoparticle treatment shows no change compared with that of the blast condition. Blast group showed significant increase (p value = 0.002) in microglial activation compared to control groups. Minocycline and targeted NP treatments show significant decrease (p value = 0.003, and p value = 0.03, respectively) compared to blast groups. Targeted treatment is still significantly different from the controls(p = 0.000000783), suggesting no treatment efficacy. In Inferior colliculus, no significant change is occurred after the blast

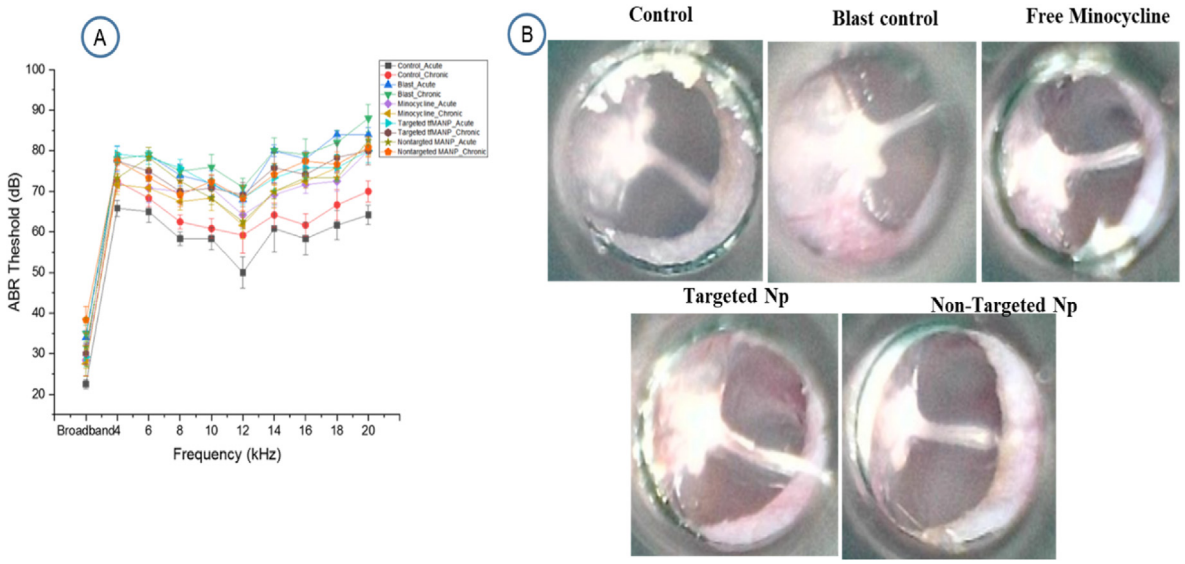

Fig. 2. (A) Mean ABR thresholds (Sound pressure level (SPL) measured in decibels (dB)) and shifts after blast TBI in rat model at acute and chronic conditions. The rats were treated with sham, blast, minocycline, non-targeted MANP and targeted tfMANP group. Error bars represent SEMs. *P < 0.05. (B) Representative tympanic membrane images by otoscope with different conditions.

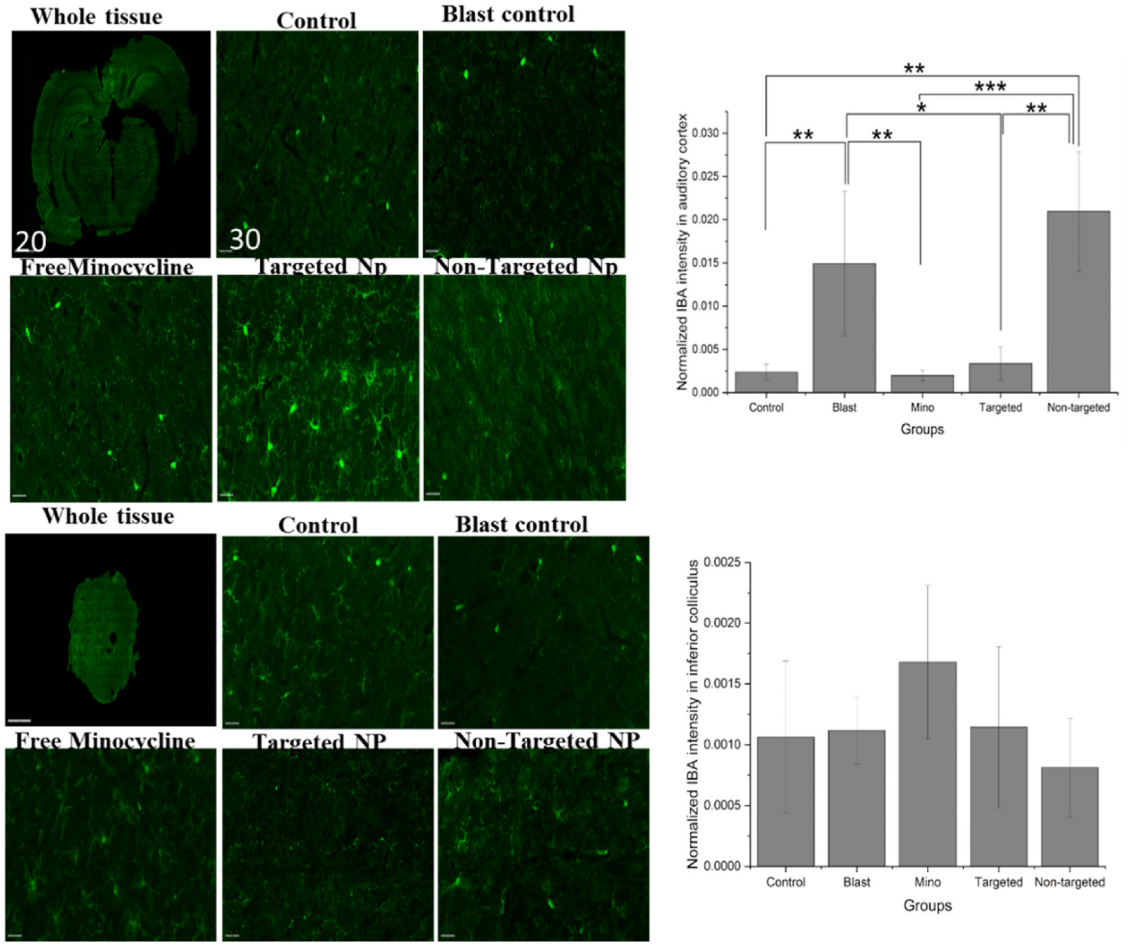

Fig. 3. The fluorescence of IBA stained brain sections harvested from post-bTBI induced hearing loss rat model. Quantification of fluorescence intensities of IBA in different brain regions (A) auditory cortex (AC) and (B) inferior colliculus (IC) are showing a differential degree of staining n = 5. *p < 0.05. Scale bar = 30 μm.

and all three treatments displayed the same intensity level indicating no significant activation of microglial level.

In Fig. 4, GFAP immunostaining intensity with quantification among all five groups are displayed. It shows that after the blast trauma, the astrocytes level is elevated both in AC and IC. Both minocycline and targeted treatments are displaying promising recovery after blast injury except non-targeted treatment. In AC, compared with control, the blast group showed significant increase($P = 0.005$) in astrocytes level. All three treatment groups significantly reduced astrocytes level compared to blast group. In IC, both minocycline (P value = 0.01) and targeted nanoparticle (P value = 0.001) treatment is showing decrease in astrocyte level compared to blasts. Compared with blast, minocycline and targeted nanoparticle treatment show significant decrease (P value = 0.0000163 and P value = 0.000000510, respectively) while the non-targeted nanoparticle treatment shows no significant change.

Our study shows that the minocycline nanoparticle and free minocycline treatment can reverse the increased in positive astrocytes and also microglia in AC. Similarly, it was previously revealed that minocycline has the inhibition effect on microglia activation (Acharjee et al., 2018)It showed in detail that (Beheshti Nasr et al., 2013) minocycline treatment 24 h after moderate to severe controlled cortical impact injury (CCI) attenuates nuclear to cytosolic translocation of high mobility group box-1 (HMGB) and reduced microglial activation in the ipsilateral cortex, hippocampus, and thalamus after 7 days in rats. Similarly, other works were also demonstrated (Bulduk et al., 2019) minocycline suppressive effect on microglial/astrocytic activation, in CA1 and dentate gyrus (DG) in epileptic rat model (Fried et al., 2018). Minocycline treatment can also reduce noisy environment induces neuroinflammation (Bulduk et al., 2019). In our study low dose of free minocycline and targeted or non-targeted nanoparticle was administered via IV to reduce the possibility of any toxicity associated with minocycline high dose treatment to make sure the safety, besides, the results favor that it also displays the efficacy of reducing the neuroinflammation.

3.3. Recovery of excitatory but not the inhibitory neurotransmitter receptors

In Fig. 5, it shows the colocalization of NMDAR1 and NeuN intensity quantification. It shows that after the blast trauma, the intensity of the colocalization of NMDAR1 and NeuN intensity decreases in AC, while minocycline and targeted treatment have no effect on blast damage. In AC, blast caused significant decrease (P value = 0.00707) in colocalization of NMDAR1 and NeuN intensity

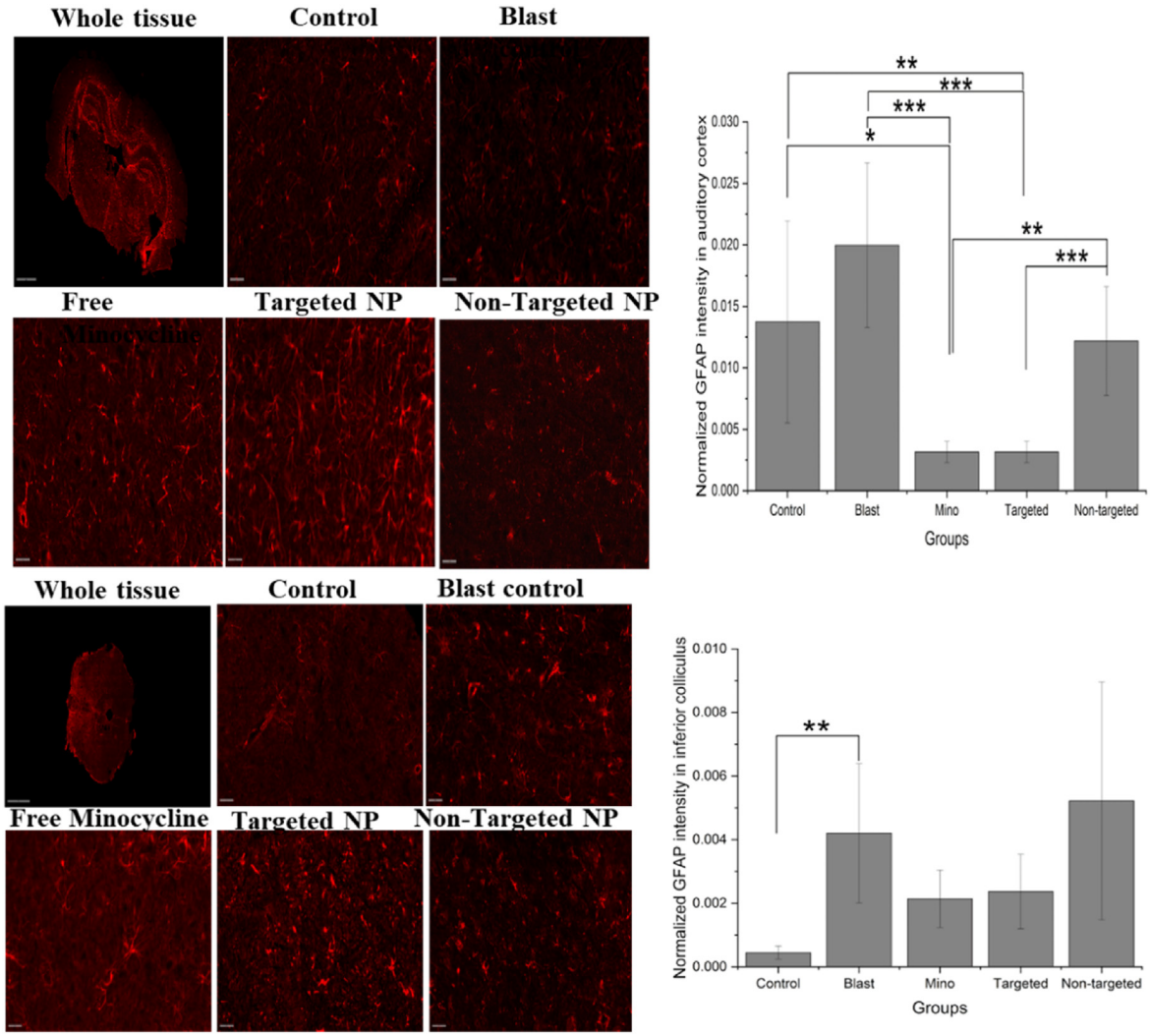

Fig. 4. The fluorescence of GFAF stained brain sections harvested from post-bTBI induced hearing loss rat model. Quantification of fluorescence intensities of GFAF in different brain regions (A) auditory cortex (AC) and (B) inferior colliculus (IC) are showing a differential degree of staining n = 5. *p < 0.05. Scale bar = 30 μm.

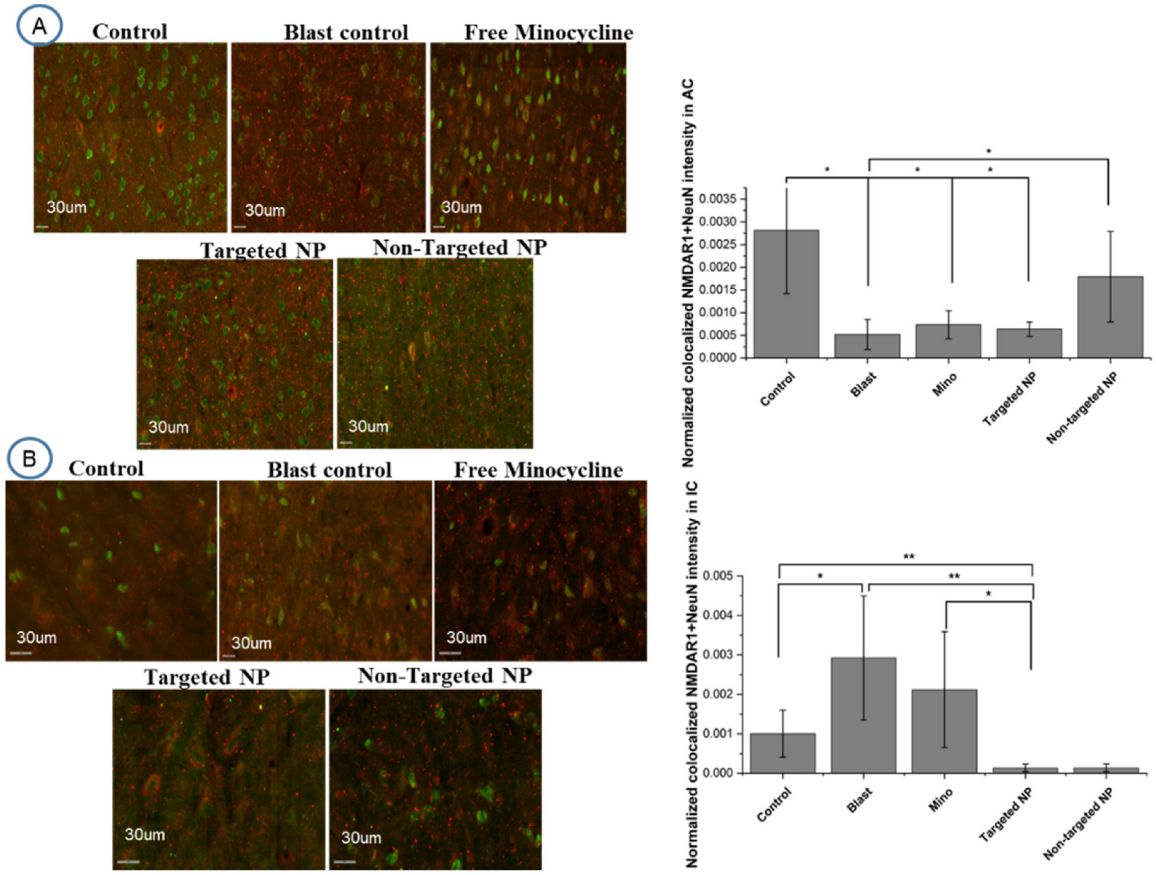

Fig. 5. The fluorescence of NMDAR + NeuN stained brain sections harvested from post-bTBI induced hearing loss rat model. Quantification of fluorescence intensities of NMDAR + NeuN in different brain regions (A) auditory cortex (AC) and (B) inferior colliculus (IC) are showing a differential degree of staining n = 5. *p < 0.05. Scale bar = 30 μm.

compared with control's, but both minocycline and targeted treatments also caused decrease compared with control's (P value = 0.0304, P value = 0.0318, respectively). While in non-targeted group, it shows significant increase compared with blast group (p = 0.0397). Interestingly, non-targeted treatment shows the better effect in comparison to other treated groups. This result is possibly due to our PEGylated stealth nanoparticle reaching cochlea via compromised BLB. Previous studies also support our findings. Stealth nanoparticle is a strategy for sustained delivery of drugs to cochleae after systemic application (Kuchinsky et al., 2020). PLA nanoparticles with PEG coating efficiently escaped from the mononuclear phagocyte system in the liver and spleen which resulted in prolonged circulation of PLA nanoparticles and the surface modification of the NPs was reported for overcoming the barriers to inner ear delivery (Masserini, 2013; Suh et al., 2007). In IC, all three treatments revealed the positive effect of recovery after the blast. Targeted treatment displays significant decrease in colocalization of NMDAR1 and NeuN compared to control group's (P value = 0.000761). Compared to blast, both targeted and non-targeted treatment displayed the significant decrease (P value = 0.0000470, p value = 0.0328, respectively) in colocalized intensity of NMDAR1 and NeuN. Moreover, the targeted treatment showed significant decrease in colocalization of NMDAR1 and NeuN compared to the minocycline treatment.

In Fig. 6, displayed the colocalization of GABAA and NeuN intensity quantification. It shows that in AC, there is no significant decrease after blast. Minocycline and targeted nanoparticle treated group are both showing significant decrease in colocalization of NMDAR1 and NeuN, revealing no effect of recovery (p = 0.00740, and P = 0.00855, respectively). Whereas in IC, it shows the intensity elevation after the blast(P = 0.0427), and the non-targeted NP treatment shows no significant change in colocalization of NMDAR1 and NeuN compared with control's, indicating non-targeted nanoparticle of minocycline reduces the blast induced NMDAR1 elevation. This observation is corresponding to the previous study that minocycline also has seizure reduction and prevention effect. Earlier work demonstrated that an upregulation of NMDA receptor expression to epilepsy which was administration of minocycline mitigated epilepsy (Beheshti Nasr et al., 2013). Similarly, Our previous study also showed that blast exposure reduces both NMDAR1 and GABAA receptor levels in acute condition in AC and IC (Shao et al., 2021a).

The redundant apoptosis pathways in hair cells and species variation limits the efficacy of minocycline in mitigating hearing loss. Similarly, other agents also have a partial therapeutic efficacy (ex, Allopurinol) in treating hearing loss (Hiskens et al., 2021; Shi et al., 2011; Franzé et al., 2003). Also, drug delivery approach, optimal dosages and routes of administration, including i.v injection of nanoparticle formulation at reduced dose than toxic level, have not been investigated. Simillar to our study design, M. Chen at al reported minocycline protective role at the dose of 1.2 mg/kg (Chen et al., 2000a). Minocycline at various concentration have established different efficacy in terms of reducing apoptosis. For instance, in vitro model, 0.1 μM minocycline inhibited the apoptosis promoting enzyme activity of poly (ADP-ribose) polymerase 1 whereas in vivo mouse model 5 mg/kg intraperitoneal minocycline inhibited caspase-1 and caspase-3 expression (Chen et al., 2000a). Besides, preclinical studies, 3–10 mg/kg intravascular doses of

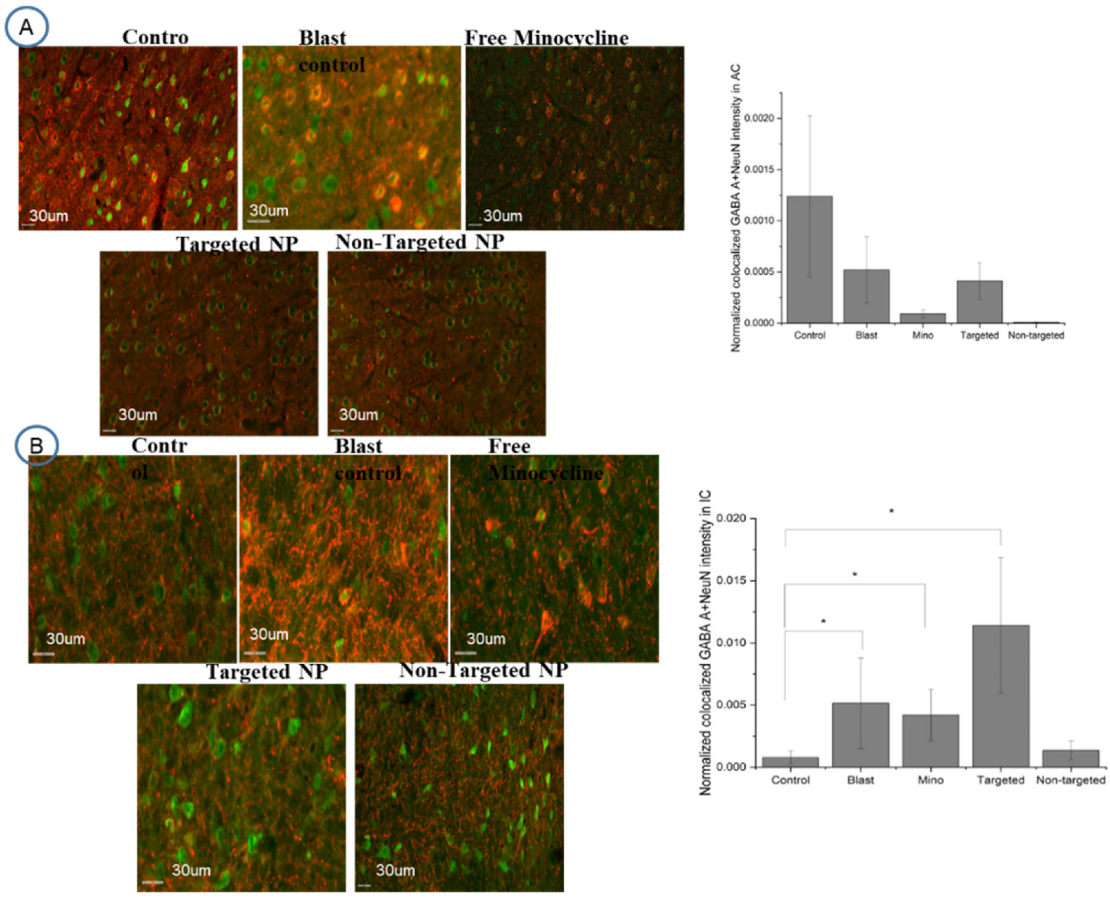

Fig. 6. The fluorescence of GABA A + NeuN stained brain sections harvested from post-bTBI induced hearing loss rat model. Quantification of fluorescence intensities of GABA A + NeuN in different brain regions (A) auditory cortex (AC) and (B) inferior colliculus (IC) are showing a differential degree of staining n = 5. *p < 0.05. Scale bar = 30 μm.

minocycline in human have been applied for the treatment of stroke. However minocycline that is promising in animal models, for example, Huntington's disease (Chen et al., 2000b), may not advance to human use (Robinson et al., 2015) due to toxicity. Despite the adverse effects, systemic drug delivery, the using nanodrugs will eventually significantly enhance drug delivery to brain in alleviate drug/noise induced hearing loss (Mittal et al., 2019). Therefore, we developed a new drug delivery system for minocycline to improve the protective efficacy against blast induced hearing loss. The speculated mechanism of targeted nanoparticle crossing BBB via transferrin receptor expressed in endothelial cells of BBB whereas, non-targeted nanoparticles reaches compromised BBB due to blast exposure and permeates free minocycline and nontargeted nanoparticles at initial hours of the blast induced in the model.

4. Conclusion

The impact of hearing loss on individuals, families and society is often underestimated. However, the degenerative processes associated with blast induced hearing loss are now well understood and many experimental therapies are being developed to halt or reverse the degenerative processes, aiming to restore hearing. During drug prevention and treatment of hearing loss, the BBB/BLB limits the effective delivery and efficacy of therapeutic drugs in the inner ear. It is inspiring that minocycline-loaded targeted albumin nanoparticle delivery systems can improve cross the BBB and BLB, and advances in treating hearing loss. We created blast induced hearing loss model and observed the results of ABR testing. Then we demonstrated that free minocycline and its loaded pEGylated nanoparticle displayed greatly enhanced the protective efficacy in bTBI induced hearing loss after systemic application through central auditory system. We believe that minocycline and its nanoformulation could be applied in variety of inner ear disorders and that it would be a promising addition to clinical study. In conclusion, the current study established that minocycline, a clinically approved drug can reduce blast induced hearing loss in rats and may possibly be used for treatment of hearing loss at reduced dose and toxicity. Future studies will include comparison of free minocycline and its nanoformulation with varied dosage matching in human dosages. Further, combination effect of nano formulation along with aminoglycoside antibiotics, minocycline for bacterial infection could also be assessed for ototoxic hearing loss. Further detailed study is needed to investigate specific mechanisms underlying the otoprotective effects of minocycline in blast induced hearing loss in rats.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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