

Electrospun nanofiber scaffolds loaded with copper oxide for repairing traumatic brain injury through restoring copper homeostasis and regulating pyroptosis pathway

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Abstract

Background: Traumatic brain injury (TBI) is one of the leading causes of injury and disability worldwide. Pyroptosis, a specific type of programmed cell death (PCD) triggered by inflammatory signals, plays a significant part in the pathological process after TBI. Copper ions play an important role in anti-inflammation and anti-oxidative stress. There is a more active copper metabolism in neurons after injury, and that neurons may require more copper ions and downstream copper-based enzymes to maintain normal physiological functions. Given these understandings of the roles of pyroptosis and copper ions in TBI pathology, this study aims to elucidate the interplay between copper ions and pyroptosis following TBI, with the goal of identifying novel therapeutic targets for TBI management.

Methods: We developed an electrostatic spinning scaffold loaded with copper oxide (CuO@PCL/gelatin, CuO@PG) to achieve small-dose local administration and avoid toxic side effects. The membranes underwent preparation and characterization through various techniques including Fourier transform infrared spectroscopy, measurement of water contact angle, antibacterial experiment, scanning electron microscopy, and assessment of in vitro release of copper. In addition, we used a controlled cortical impact to establish a TBI model in mice to examine the effect of CuO@PG on TBI-induced pyroptosis and the ability of the membranes to heal brain injury.

Results: CuO@PG inhibited TBI-induced neuronal pyroptosis. CuO@PG can inhibit the expression of the pyroptosis-related proteins. Moreover, CuO@PG also alleviates brain edema and the degree of neurodegeneration in the acute phase of TBI. The neuroprotective effect of CuO@PG was further confirmed by wire-grip test, open field test, Morris water maze test. Lastly, the beneficial results were significantly inhibited by the use of the copper chelator tetrathiomolybdate.

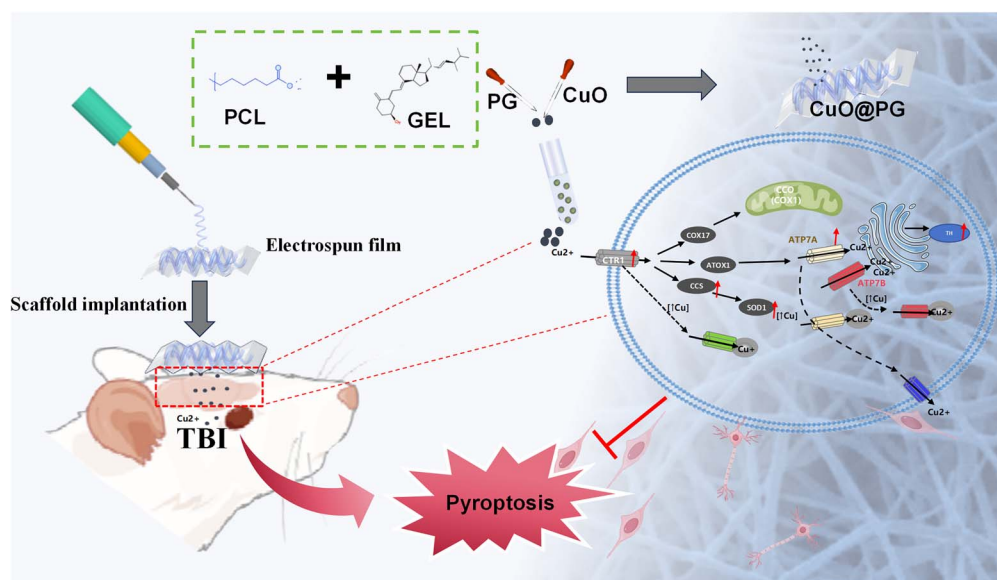
Conclusions: In this study, we successfully constructed electrostatically spun scaffolds loaded with copper oxide to achieve slow, continuous and low-dose copper supply to the local brain, which provides a new theoretical basis for the imbalance of copper homeostasis in the brain after TBI.

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Graphical Abstract



Keywords: Traumatic brain injury; Electrospun scaffold; Copper homeostasis; Pyroptosis; Neuroprotection

Highlights

- Electrostatically spun scaffolds loaded with copper oxide enable a slow, continuous, and low-dose supply of copper.
- Electrospun nanofiber scaffolds with sustained copper delivery are an effective strategy for brain tissue engineering to attenuate brain injury.
- Therapeutic effect of copper-loaded electrospun nanofiber scaffolds may be suppressing TBI-induced pyroptosis in brain regions.

Background

Traumatic brain injury (TBI) is a major international health problem with multiple etiological and epidemiological patterns and is a leading cause of death and disability in children and adults 1 to 44 years of age [1]. Common causes of TBI include motor vehicle accidents, occupational hazards, physical violence, and falls, and its complications impose a significant public health burden [2]. The development factors, pathogenesis and objective diagnosis of cerebral trauma and its concomitant neurological dysfunction are urgent problems to be solved in clinical and forensic medicine. To date, methods for preventing or relieving neurological dysfunction after TBI have yet to be explored.

Pyroptosis is an important form of programmed cell death. In the classical pathway of pyroptosis, pattern recognition receptors (PRRs) recognize damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). This recognition leads to the formation of inflammasome complexes in the cytoplasm, involving the interaction of NLR family sensors with the adaptor protein ASC. This complex, known as the inflammasome, activates caspase-1 and IL-1 β , leading to the cleavage of gasdermin D (GSDMD). The N-terminal domain of GSDMD is then integrated into the cell membrane, forming pores that disrupt plasma membrane integrity and cellular homeostasis [3]. Pyroptosis has been shown to play a significant role in central nervous system injuries [4], suggesting that the inhibition of post-injury neuroinflammation and pyroptosis is potential

therapeutic targets for diseases associated with traumatic brain injury (TBI) [5].

Copper is a transition metal and an essential trace element that has been found to be involved in various physiological processes for centuries. These processes include cellular respiration [6], antioxidant defense [7], angiogenesis [8], nerve conduction [9], lipid metabolism [10], and melanin production [11]. Mammals acquire copper through diet, which is absorbed by intestinal epithelial cells. Extracellular copper ions exist in the form of Cu²⁺ and can bind to divalent metal transporter 1 (DMT1) [12]. However, it is only after reduction by cell surface reductases that divalent copper can be transported into the cell by copper transporter 1 (CTR1) [13]. Once inside the cell, copper ions are utilized through three main pathways: in the cytoplasm, copper chaperone for superoxide dismutase (CCS) delivers copper ions to SOD1, facilitating its activation and playing a crucial role in eliminating reactive oxygen species (ROS) in neurons [14]. Cox17 shuttles between the cytoplasm and the mitochondrial intermembrane space, delivering cytosolic copper ions to Sco1 and Sco2 on the inner mitochondrial membrane, which then transfer the copper ions to the downstream subunits COX2 and COX1 of cytochrome c oxidase, participating in the electron transport chain [15]. Atox1 is responsible for the main pathway of copper utilization in the Golgi apparatus, transporting copper to ATP7A/ATP7B, ATPases located in the trans-Golgi network that transfer copper to the secretory pathway, where it binds to

copper-dependent enzymes and activates their catalytic sites. Besides its role in the secretory pathway, ATPases can also translocate to vesicles and promote copper efflux in a high-copper environment [16]. Once a threshold concentration is reached, ATPases leave the Golgi apparatus in vesicles and are guided to the plasma membrane for cell extrusion through a PKD or PKA-dependent mechanism [17,18]. Most of the copper ions in the blood plasma are bound and enter the brain mainly by regulating copper uptake, intracellular transport, and export in the brain–blood barrier endothelial cells [19].

Copper plays a critical role in maintaining normal central nervous system function, and the imbalance of copper homeostasis is associated with the occurrence of various diseases. Copper is therefore considered a potential target for the treatment of neurodegenerative disorders. Studies have reported changes in the baseline levels of copper ions in brain tissue and blood after TBI but the specific mechanisms and pathways of copper and related proteins in TBI are still poorly understood [20]. Therefore, investigating the role of copper in TBI is of great significance for the prevention and alleviation of cognitive dysfunction after TBI and the objective assessment of brain damage.

Polycaprolactone (PCL) is an ideal material in the field of biotechnology due to its excellent mechanical properties, biocompatibility, and biodegradability, making it a preferred choice for tissue regeneration and repair scaffolds [21]. However, the hydrophobic nature of PCL hinders cell adhesion and proliferation on the scaffold [22]. Gelatin, derived from hydrolyzed collagen, is a protein that can enhance the hydrophilicity of PCL while promoting cell proliferation [23,24]. Electrospinning of PCL and gelatin has been shown to maintain the mechanical strength of the scaffold and has demonstrated significant potential in tendon repair [25], vascular regeneration [26], and drug delivery [27]. However, its application in TBI is rarely reported. Studying the application of PCL/gelatin in TBI could provide new possibilities for the development of novel drug delivery systems and shed light on the mechanisms of drug interaction with TBI.

Methods

Preparation of CuO@polycaprolactone/gelatin (CuO@PG)

To prepare the polycaprolactone/gelatin (PG) solution, polycaprolactone (PCL) and gelatin (Gel) were dissolved separately in trifluoroethanol, each at a concentration of 20% (w/v). These solutions were then combined in a 3:2 volume ratio to create a spinning solution. However, due to phase separation between PCL and Gel, the mixture appeared cloudy. To resolve this, 0.2% (v/v) acetic acid was added, resulting in a clear, homogeneous PG solution.

Next, copper (II) oxide (CuO) powder was incorporated into the PG solution to prepare the PG/CuO solution, referred to as CuO@PG. Specific formulations were created, the PG solution with 0.3% CuO is labeled as 0.3% CuO@PG, and the one with 0.5% CuO is labeled as 0.5% CuO@PG. To ensure even dispersion of CuO, the mixture underwent ultrasound treatment for 30 minutes and magnetic stirring at room temperature overnight.

For the electrospinning process, a stainless-steel needle was connected to a positive high-voltage power supply. The needle tip was positioned 16 cm from the collector. The system was activated with a negative voltage of -2.0 kV and a positive voltage of $+18$ kV. During the spinning of PG, 0.3% CuO@PG, and 0.5% CuO@PG solutions, the feed rate was set to 1.5 mL/h, maintaining other parameters constant. All fibers were collected on aluminum foil and subsequently dried under vacuum at 37°C for 48 hours to remove residual solvent, yielding the final samples.

Scanning electron microscopy

5 mm $\times$ 5 mm squares of PG and 0.5% CuO@PG membranes were prepared. Place the materials on a metal sample holder, fix them with conductive adhesive, sputter coat with gold, and observe the surface morphology using SEM with an electron acceleration rate of 15 kV.

Fourier-transform infrared spectroscopy

Cut PG and 0.5% CuO@PG scaffold into 4 mm $\times$ 4 mm squares and place them on the sample stage. Use Fourier-transform infrared spectroscopy (FTIR) in ATR mode to analyze the surface functional groups of PG and 0.5% CuO@PG membranes. Test conditions: resolution of 4 cm^{-1} , measurement range from 4000 to 400 cm^{-1} .

Surface water contact angle

Cut PG and 0.5% CuO@PG membranes into 5 cm $\times$ 5 cm squares and stick them on glass slides with double-sided tape. Measure the water contact angle of the samples using a water contact angle meter at six different positions on each membrane.

Antibacterial experiment

Cut PG and 0.5% CuO@PG scaffold into 6 mm diameter and place them in culture dishes containing *Escherichia coli* or *Staphylococcus aureus* was incubated at 37°C for 24 hours. After bacterial cultivation, fix the materials with glutaraldehyde, perform alcohol gradient washing, and observe them under a gold-sputtered electron microscope. The antibacterial activities of samples studied were evaluated by Bauer-Kirby disk diffusion method. A bacterial suspension of 1×10^5 CFU/mL was evenly spread over the surface of the solid agar plates, followed by placing sterilized disk specimens (6 mm diameter) onto them. Following incubation agar plates at 37°C for 24 h, bactericidal efficiency of each specimen against *E. coli* and *S. aureus* was assessed.

Copper ion release experiment

A PG scaffold with a diameter of 5 mm, along with 0.3% CuO@PG scaffold and 0.5% CuO@PG scaffold, were placed into individual EP tubes. Subsequently, 3 ml of $1 \times$ phosphate-buffered saline (PBS) was added to each tube. The assemblies were then positioned on an orbital shaker at 37°C . At 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h after the addition of PBS, a single exchange of the liquid was conducted. The resulting liquid samples were collected and subjected to analysis using ICP-MS to determine the concentration of copper ions.

Animal handling

For all experiments, adult male ICR mice (6–8 weeks old, weighing 20–25 g) were purchased from the Experimental Animal Center of Soochow University. The mice were

maintained under controlled conditions with unrestricted access to food and water within a 12-hour dark–light cycle, at a regulated humidity of 50% and a temperature of 25°C. A computer-generated random number table was utilized to allocate mice into distinct subgroups, each uniquely numbered, for every experiment. The Institutional Animal Use and Care Committee at Soochow University granted approval for all animal-related procedures (reference number = SZUM2008031233), and the research was executed in accordance with the guidelines outlined in the Animal Use and Care of the National Institutes of Health, as well as the standards provided by the Animal Research: Reporting In Vivo Experiments protocol.

Control cortical impact (CCI)

A mild TBI mouse model was established through a controlled cortical impact using the Pneumatic Impact Device (AMS 201, AmScience). The specific methodology followed established protocols as previously described [28]. Mice were first anesthetized with Isoflurane and then positioned within a stereotaxic apparatus (David Kopf Instruments, Tujunga, California). A midline incision was made on the scalp to uncover the skull. Subsequently, a 5.0 mm craniotomy was carried out between the anterior fontanelle and the sagittal suture, ensuring the dura remained intact. Using fine micro-dissection forceps, carefully remove the bone flap from the craniotomy. The controlled cortical impact device was used to deliver a controlled impact to the exposed cortex. The device parameters were set as follows: high pressure 200 kPa, low pressure 100 kPa, uses a velocity of 2.5 m/s, dwell time of 250 ms and injury depths of 1 mm (mild injury). After the impact, the scalp incision was sutured closed. The mice were allowed to recover in a warm, clean cage.

Therapeutic intervention

We used a hole puncher to fabricate circular scaffolds with a diameter of 5 mm from electrospun fiber membranes loaded with CuO. The circular scaffolds were placed in a 16-well plate and soaked in 75% ethanol for 1 hour for sterilization. After removing the ethanol, the plate containing the scaffolds was transferred to a laminar flow hood. The scaffolds were exposed to UV light for 30 minutes for further sterilization, while the ventilation system was activated to facilitate the evaporation of residual ethanol. Establish a mild traumatic brain injury (mTBI) model, and place a 5 mm circular electrospinning scaffold infiltrated with sterile physiological saline at the site of injury. And allow copper oxide to be released into the injured brain area and implanted on the surface of the brain with sterilized tweezers. During the suturing process, cautious efforts were made to prevent any contact with the scaffold.

Experimental design

Experiment 1: To investigate alteration of copper in the ipsilateral cortex after mild TBI, mice were randomly allocated into the sham group (Sham) and the TBI group. The concentration of copper in the cortex were examined. Each subgroup consisted of $n = 7$ mice.

Experiment 2: In order to investigate the temporal changes of copper-associated proteins, mice were randomly allocated into the sham group and the TBI group. Within the TBI group, further subdivisions were made based on the duration of post-injury, namely the 6-h group, 12-h group, 1-d group, 2-d

group, 3-d group, 7-d group, and 14-d group. Furthermore, Sham, 1-d group, 3-d group, and 7-d group were also designated as temporal grouping for determining the cell types in which the changes occurred. The cellular localization and alterations in expression of copper proteins were individually investigated. Each subgroup consisted of $n = 5$ mice.

Experiment 3: To explore the impact of CuO@PG on pyroptosis, mice were randomly allocated into the Sham, TBI group, and CuO@PG group. Within the CuO@PG group, further subdivisions were made based on the time point of post-injury implantation to investigate the optimal time point for scaffold implantation firstly, which namely the 0 h group (Direct treat with the scaffold post-injury), 1 h group, 3 h group and 6 h group. Upon establishing the optimal time window for implantation, validation of the optimal copper concentration within the implant material was performed, the experimental groups were implanted with scaffolds of different concentration of copper within the selected optimal time window, which included Sham group (Sham), TBI group (TBI), TBI+PG group (TBI+PG), TBI+0.3% CuO@PG group (TBI+0.3% CuO@PG), and TBI+0.5% CuO@PG group (TBI+0.5% CuO@PG). The alterations in the expression of pyroptosis-related proteins were assessed in each group ($n = 5$ /group).

Experiment 4: To further validate the role of CuO@PG following mild TBI, mice were randomly allocated into the Sham group, TBI group, TBI+tetrathiomolybdate (TTM) group, TBI+CuO@PG group, and TBI+CuO@PG+TTM group. The TTM solution was intraperitoneally injected into the mice after scaffold implantation. Each subgroup consisted of $n = 5$ mice, which were respectively examined the cellular localization and expression changes of pyroptosis-related and copper proteins, the degree of neurodegeneration as well as the concentration of copper in the ipsilateral.

Experiment 5: To comprehensively investigate the impact of CuO@PG on neurological dysfunction after TBI, mice were randomly allocated into five distinct groups ($n = 7$ /group): Sham, TBI, TBI+TTM, TBI+CuO@PG, and TBI+CuO@PG+TTM. A series of behavioral experiments encompassing the wire-grip test, open field test, and Morris water maze (MWM) were conducted to assess the influence of the CuO@PG on motor function recovery, memory impairment, and anxiety-like behavior in mice following TBI.

Western blot

Fresh brain tissue was homogenized using sonication in a mixture of Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime) containing protease inhibitors (Phenylmethanesulfonyl fluoride, PMSF, Beyotime) on ice. The resultant tissue homogenate was centrifuged at $13\,523 \times g$ (4°C, 25 minutes), and the supernatant was collected. The protein concentration was determined using NanoDrop 2000 spectrophotometers (Thermo Fisher Scientific, USA). To achieve the desired concentration, different volumes of the RIPA and PMSF mixture were added to the samples. Subsequently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) Sample loading buffer (Beyotime) was added to the samples and boiled for 10 minutes. A protein amount of 20 μ g was electrophoresed on 10–12.5% SDS/PAGE gel, followed by electroblotting onto pre-soaked polyvinylidene fluoride (PVDF) membranes (Millipore). The PVDF membranes were blocked with 5% bovine albumin (BSA) in Tris Buffered Saline with Tween (TBST) for 2 hours at room temperature, and

subsequently incubated overnight at 4°C with primary antibodies. After washing, the PVDF membranes were incubated with horse-radish peroxidase conjugated secondary antibodies. Immunoreactive bands were detected using the ECL chemiluminescence system (Clinx Science Instruments, China).

Immunofluorescence staining

Mice were deeply anesthetized and subsequently underwent transcardial perfusion with ice-cold 0.01 M PBS, followed by ice-cold 4% paraformaldehyde in PBS. After perfusion, the brains were carefully extracted and immersed in 4% paraformaldehyde for 24 hours. Subsequently, a gradient sucrose dehydration process was executed. The brains were then embedded in optimal cutting temperature compound and sectioned into 10 μ m thick coronal slices using a freeze slicer. These slices were further fixed with 4% paraformaldehyde for 30 minutes, followed by a 10-minute wash in phosphate-buffered saline plus Tween-20 (PBST), and subsequently blocked using BSA for a span of 2 hours. Next, the slices were exposed to the primary antibody, diluted in BSA, and incubated at 4°C overnight. After being washed three times with PBST, the slices were treated with Alexa Fluor 488/594 conjugated secondary antibodies at room temperature for 2 hours. Following this, the sections were stained with DAPI (1:10 000, Beyotime) for 15 seconds and washed with PBST thrice. The fluorescent microscope (Nikon, DS-Ri2) was used to visualize the whole-brain tissue section images, which were subsequently processed using ImageJ software. The images predominantly illustrate the region surrounding the injury.

Nissl staining

Nissl staining was conducted to evaluate the extent of neurodegeneration within the vicinity of the injury. Following the previously described procedures, the slices were prepared and subsequently subjected to dehydration using an alcohol gradient: 75% alcohol for 15 minutes and 90% alcohol for 25 minutes. Following this, the slices were rinsed for two minutes with distilled water. Subsequently, the slices were exposed to Nissl staining solution (Beyotime) at room temperature for 5 minutes. After rinsing with distilled water, the slices were examined using a microscope (Nikon, DS-Ri2). The acquired images were processed using ImageJ software.

Behavioral analysis

As previously described, a wire-grip test was evaluated starting 1st day before TBI and continuing until 7th day after TBI.

In the wire-grip test, mice were positioned on a 45 cm long wire suspended 45 cm above a foam cushion for a duration of 60 seconds. Their performance was observed and evaluated during this interval. A five-point scale was employed to evaluate the performance of the mice on the rod, as follows: Score 0 indicated an inability to remain on the rod for 30 seconds. Score 1 denoted the ability to stay on the rod for 30 seconds but without using all four limbs simultaneously. Score 2 signified the ability to stay on the rod for 30 seconds using all four limbs, excluding the tail. Score 3 represented the capability to remain on the rod for 30 seconds using all four limbs and the tail, while remaining stationary. Score 4 indicated the capacity to stay on the rod for 30 seconds, utilizing all four limbs and the tail, and being capable of

movement along the rod. Score 5 was achieved by meeting the criteria for a score of 4 and additionally demonstrating the ability to move to the edge of the rod and descend to the bottom. The procedure is carried out three times daily until no statistically significant differences are observed among the average of different groups.

The water maze test is conducted to evaluate the memory and spatial learning abilities of mice, following the restoration of their basic motor functions. A circular water pool with a diameter of 1.3 meters is divided into four quadrants based on cardinal directions. In the center of one of these quadrants, a transparent circular platform with a diameter of 5 centimeters is placed. The water in the pool is filled to a depth of 1 centimeter above the platform, and the temperature of the water is maintained at approximately $23 \pm 2^\circ\text{C}$ throughout the experiment. The escape latency, which denotes the duration taken by the mice to reach the platform location subsequent to water entry, and the count of platform crossings, referred to as the number of crossings, were meticulously documented. The mice were distributed randomly, as outlined earlier, and the mean value derived from the accumulation of data from the four experimental trials was subsequently computed.

The open field test is conducted to investigate the anxiety-like behavior of mice through their spontaneous activity. The apparatus for the open field test consists of a square black box with dimensions of 1 meter in length, width, and height. The mice are placed in the center of the box, and their movement within the open field is recorded using a computer for a duration of 5 minutes. Prior to each experiment, the box is cleaned using 75% ethanol to eliminate any odors and fecal influences. The center time and total moving distance of each mouse were analyzed by the automated behavioral tracking system (#JLBehv-OFG-4, Shanghai Jiliang Software Technology, Shanghai, China).

Gait analysis: To measure the recovery of motor nerve function in mice, mouse footprint measurement can reflect the muscle function status of the four limbs. The experimental group was measured on the 2 days after injury. The hind feet were soaked in red ink, and the footprints were recorded on white paper. Meanwhile, the mice passed through a self-made wooden trough that was 80 cm long, 7 cm wide, and 10 cm high. At the end of the path, a dark room was set up, and white paper was placed at the bottom of the wooden trough to record the paw prints. The animals were allowed to move forward, and the experiment was repeated three times by the same researcher.

Quantification of metal ions

At 1 day post-injury, blood samples were collected through orbital venipuncture. Blood was collected into sodium heparin anticoagulant tubes and mixed with 1% concentrated nitric acid to a final volume of 10 ml. The ipsilateral cortex was weighed, placed into a glass digestion vessel, and treated with 5 ml of 75% nitric acid. The mixture was left to digest in darkness for 18 hours, followed by heating to boiling until the solution became colorless and transparent, with no further release of brown-red gas. The resulting solution was filtered using a syringe with a 220 nm filter head and then diluted to 10 ml with deionized water. Cu and Mo concentration was determined using ICPMS. The generated standard curve exhibited a fitting degree of 0.998, with a measuring wavelength of 324.7 nm.

Statistical analysis

All data were processed by GraphPad Prism 9 software and expressed as mean $\pm$ SD. The wire-grip test, analyzed with a two-way analysis of variance (ANOVA) by post hoc analysis (Tukey's multiple comparisons test). Other experiments data were analyzed with a one-way ANOVA followed by post hoc analysis (Dunnett's multiple comparisons test). The differences were given in the bar graphs.

Results

Characteristics analysis of the CuO@PG

The microstructures of PG and CuO@PG were observed with open and interconnected porous morphological features by field emission scanning electron microscopy (FESEM), SEM images of the surfaces of PG and CuO@PG revealed that the introduction of CuO had no significant effect on the morphology of the electrostatically spun filaments, and both exhibited uniform fiber distribution and suitable pore size, which may be beneficial for cell growth (Figure 1a). The results of the bacterial inhibition experiments showed that the PG scaffold had no inhibitory effect on *Escherichia coli* and *Staphylococcus aureus*, and 0.5% CuO@PG could inhibit their growth (Figure 1b). Afterwards, the surface of PG and 0.5% CuO@PG scaffolds were analysed using Fourier transform infrared spectroscopy (FTIR) in ATR mode groups (Figure 1c). And infrared spectroscopy analysis showed that all the scaffolds showed stretching at 2950 and 2865 cm^{-1} belonging to the CH_2 group in PCL. group in PCL, and the stretching of carbonyl group ($\text{C}=\text{O}$) showed a sharp peak at 1727 cm^{-1} in PCL group. In addition, a sharp peak at 1727 cm^{-1} was observed in the PG and CuO@PG scaffolds, two different amide bonds (1650 and 1540 cm^{-1}) were observed for gelatin, thus confirming that the amide bonding of gelatin is not a significant factor in the formation of PG and CuO@PG, thus confirming the successful preparation of PCL/Gel hybrid fibers (Figure 1c). The hydrophilic angle of the samples was measured by a water contact angle meter and was about 47° for PG and about 67° for CuO@PG. The magnitude of hydrophilic angle is negatively correlated with hydrophilicity. This indicates that the loading of CuO reduces the hydrophilicity of PG (Figure 1d). Copper ion release experiments demonstrated that CuO@PG can release copper ions at a sustained low dose (Figure 1e). At both 6 h and 12 h, 0.3% CuO@PG and 0.5% CuO@PG groups showed a significant difference in total copper release ($*P < 0.05$). Moreover, 0.5% CuO@PG can better protect HT22 cells from hydrogen peroxide-induced damage (Figure 1f-g). The above results indicate that we successfully constructed an electrostatically spun scaffold with slowly released copper ions.

CuO@PG inhibits TBI-induced neuronal pyroptosis and inflammation

Firstly, we examined the changes in copper-associated proteins, and the localization of copper proteins after TBI (Figure S1-S4). In order to explore the effect of CuO@PG on the process of pyroptosis induced by TBI, we chose to detect pyroptosis-related proteins at 24 h post-TBI, which is considered as the peak of pyroptosis induced by TBI [29]. Firstly, in order to test the optimal time point for implantation of CuO@PG after mTBI, we used WB assay

to implant scaffolds loaded with 0.5% CuO at different time windows of 0, 1, 3, and 6 h post-injury, and the results showed that the administration of exogenous copper ions at 6 h had the strongest down-regulating effect on pyroptosis, so we chose 6 h post-injury as the optimal time point for implantation of CuO@PG (Figure 2a-d). Then, in order to determine the optimal concentration of electrostatically spun scaffolds loaded with copper oxide, we chose three different concentrations of scaffolds, 0% (unloaded with CuO), 0.3%, and 0.5%, to be implanted into the wounds after mild TBI, and the scaffolds loaded with 0.5% CuO had the strongest down-regulation of NLRP3, GSDMD, Caspase1, and ASC in the cortical tissues, and 0.3% had some downregulation, but could not prevent the occurrence of pyroptosis, and implantation of PG membranes alone had no modulatory effect on pyroptosis (Figure 2e-i). Next, we tried to chelate copper ions in CuO@PG with the copper chelator TTM. 1 d after mild TBI, there was no change in cortical tissue scorch death-related proteins after administration of TTM alone compared to the TBI group. Administration of TTM after implantation of a scaffold loaded with 0.5% CuO showed an increase in the expression of pyroptosis-related proteins compared with the 0.5% CuO@PG group (Figure 2j-n). To further explore the effect of CuO@PG treatment on neuronal expression of pyroptosis-related proteins and pro-inflammatory factors, we performed double immunostaining and found that neuronal expression of NLRP3, GSDMD, Caspase-1, ASC, NLRP 3, increased after TBI. Whereas, all were significantly decreased after treatment with CuO@PG. After chelating copper with TTM, the above results were changed (Figure 3).

CuO@PG alleviates TBI-induced neurodegeneration

To further investigate the effect of CuO@PG on the pathophysiological processes in the acute phase of TBI, we performed Nissl staining at 24 h after TBI to assess the degree of neurodegeneration, which was consistent with previous reports [30], where injured neurons were shrunken and darkened, whereas normal neurons appeared rounded and mauve. As shown in Figure 4a-b, CuO@PG treatment significantly improved the shrinkage and dark staining morphology of Nissl.

CuO@PG improves TBI-induced motor dysfunction, anxiety behavior and memory deficits

We next performed behavioral experiments to determine the effects of CuO@PG on long-term neurological function after TBI. We performed a wire grip test from day 1 pre-TBI to day 7 post-TBI to assess the effect of CuO@PG on motor function. We found that the scores of all groups (except the sham-operated group) gradually increased with the number of days after TBI, suggesting that the motor dysfunction of mice would gradually recover over time (Figure 5a). Compared with Sham group, mice after mild TBI showed significant impairment in motor function within one week of injury, 3d after implantation of CuO@PG, mice showed significant improvement in motor function, this effect was attenuated by concurrent administration of TTM. The open field test was then performed to assess anxiety-like behavior; rodents instinctively avoid the central region and move along the edges during spatial activity, and when cognitive function is affected mice prolong activity in the central region, which is

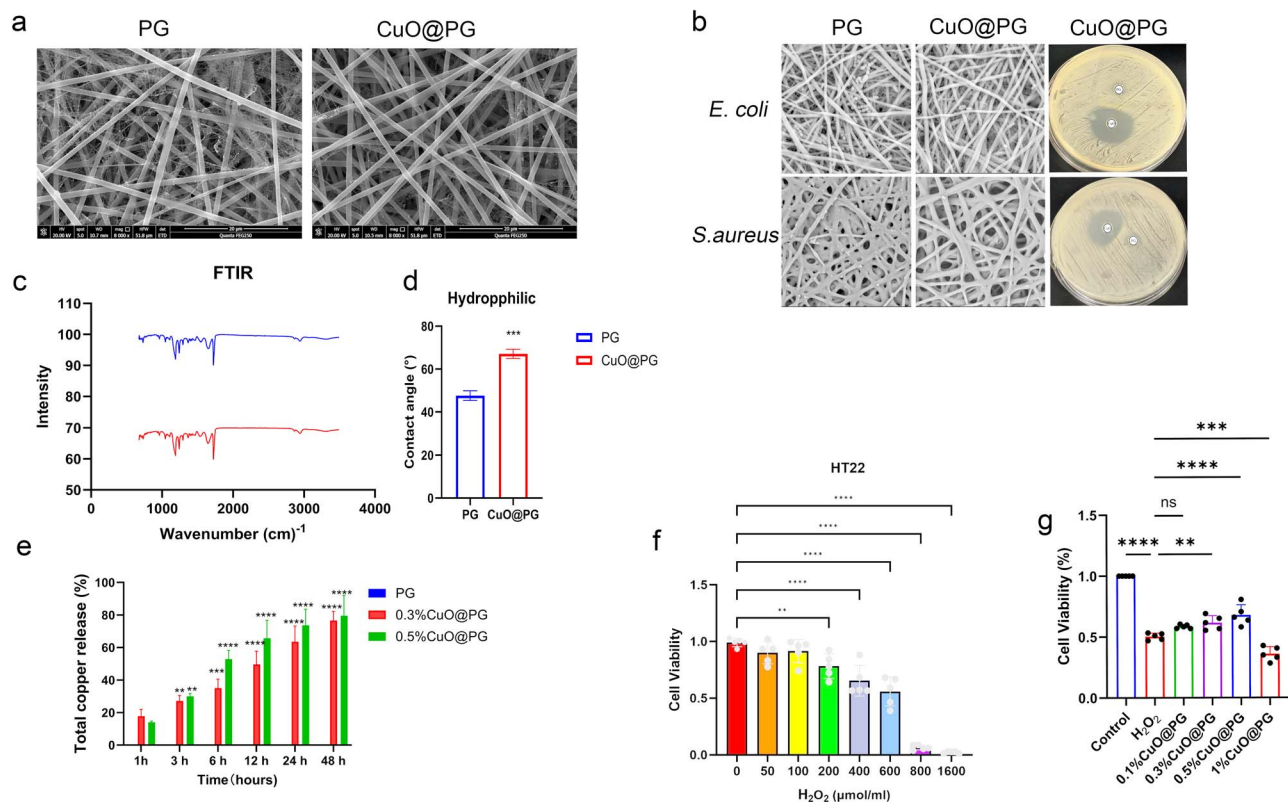


Figure 1. Characteristics analysis of the CuO@PG. (a) Morphological characterization of PG and CuO@PG. (b) Bacteriostatic test of PG and CuO@PG. (c) Surface infrared spectral analysis of PG and CuO@PG. (d) Water contact angle between PG and CuO@PG. (e) Copper ion release experiment. (f) the damage of HT22 caused by hydrogen peroxide and the rescue effect of CuO@PG with different concentrations on hydrogen peroxide-induced HT22 injury. (g) The protective effect of CuO@PG at varying concentrations against H₂O₂-induced HT22 cell damage. Significance was set at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. PG, polycaprolactone/gelatin; CuO@PG, CuO@polycaprolactone/gelatin; *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*; FTIR, Fourier transform infrared spectroscopy; HT22, mouse hippocampal neuronal cell line

associated with anxiety-like depression in mice. In open-field experiments, mice after TBI increased the distance travelled in the central region and decreased the distance travelled in the peripheral region, which was ameliorated by implantation of CuO-loaded electrospun fiber scaffolds; this effect was weakened by concomitant administration of TTM. (Figure 5b-d).

MWM was performed 14–23 days post-injury to test the spatial memory capacity of rats. Representative swimming paths showed a significant reduction in platform-seeking trajectories in all groups of mice after training. However, mice showed significant impairment in spatial memory and learning ability after TBI compared to the Sham group, with an increased latency to emergence, which was significantly shorter after administration of exogenous copper ions. After removing the platform, mice in the TBI + CuO@PG group crossed the platform more often than the TBI group in the same time period; this effect was weakened by the simultaneous administration of TTM, and was only reflected in the slight shortening of the latency period (Figure 5e-g). Gait analysis experiments showed that CuO@PG can improve the neuromuscular function of mice, while the addition of TTM inhibited the recovery of neurological function (Figure 5h-j).

CuO@PG regulates copper homeostasis after mTBI

Finally, we used WB in order to verify the effect of CuO@PG on copper homeostasis after mild TBI, where the expression of ATP7A was decreased by administration of TTM after TBI, and after administration of exogenous copper ions, the

expression level of ATP7A was decreased in comparison with the TBI group, but increased by simultaneous administration of TTM. The expression of CTR1 was increased by the administration of TTM after TBI; after the administration of exogenous copper ions, there was no significant change in the expression level of ATP7A compared with that of the TBI group, but the expression was increased by the concomitant administration of TTM (Figure 6a–c).

When TTM was given after TBI, there was no significant change in the expression of the copper chaperone proteins CCS, and a decrease in the expression of SOD1 and TH. After the administration of exogenous copper ions, the expression levels of CCS, SOD1, and TH were decreased compared with those of the TBI group, but the expression was increased by concurrent administration of TTM (Figure 6a and d-f). Immunofluorescence results were consistent with the above results, the number of co-localized ATP7A-positive neurons was reduced in the TBI + CuO@PG group compared to the TBI group and increased after the administration of TTM; whereas the expression of CTR1 in neurons did not change significantly between the TBI group and the TBI + CuO@PG group, and the number of neurons that were CTR1-positive increased after the administration of TTM (Figure 7a-d).

Discussion

TBI is one of the risk factors for structural damage, disturbances in homeostasis and functional impairment of the

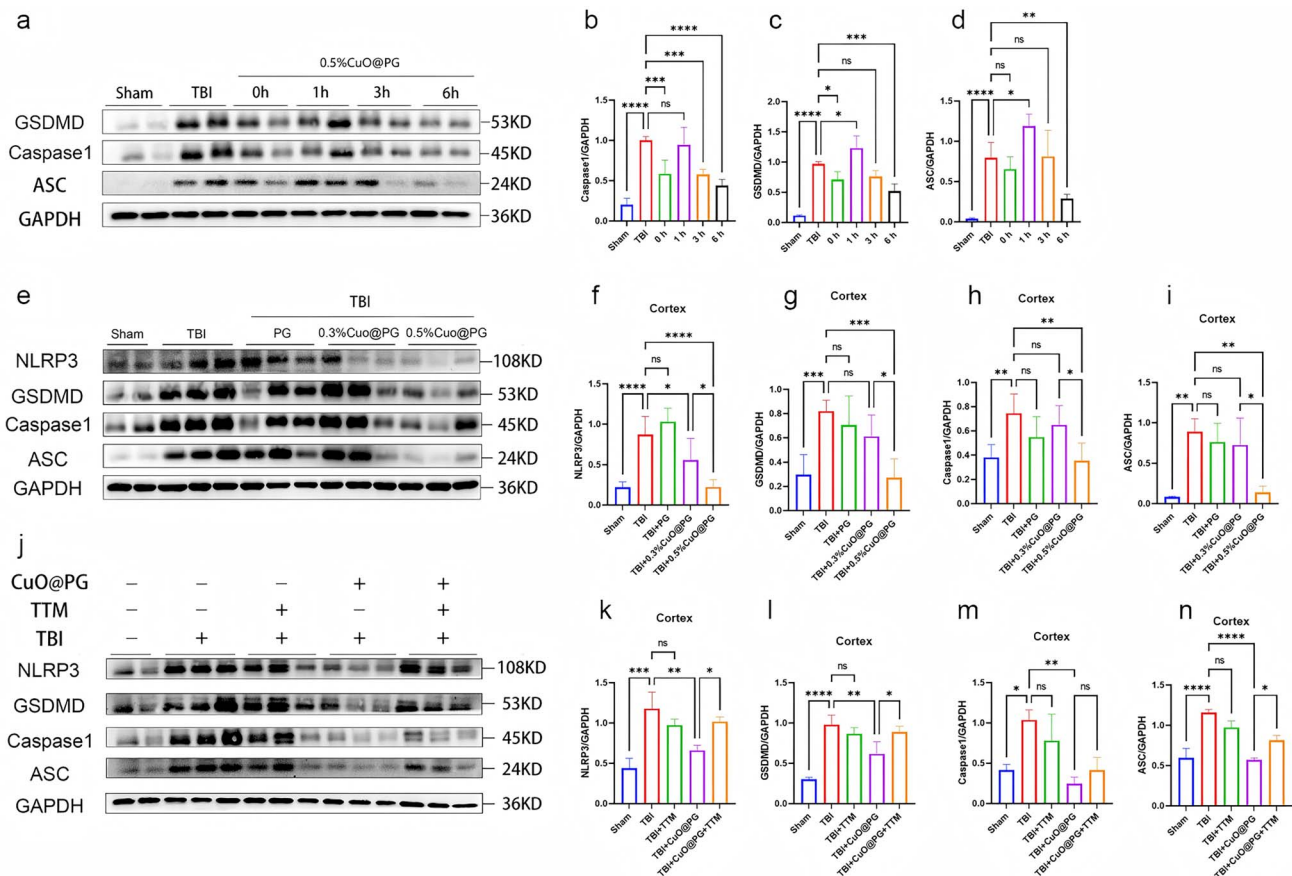


Figure 2. CuO@PG inhibited the expression of pyroptosis-related proteins after TBI. Representative western blots indicating the expression changes of GSDMD, Caspase-1, ASC, NLRP3 in the injured cortex. (a-d) Western blot shows the effect of exogenous copper ions administered at different time points on the expression levels of pyroptosis-related proteins in cortical tissues after TBI. (e-i) Western blot shows the effect of different concentrations of exogenous copper ions on the expression levels of pyroptosis-related proteins in cortical tissues after TBI. (j-n) Western blot showing the effect of TTM on the expression levels of pyroptosis-related proteins in cortical tissues after TBI. The data were analyzed with a one-way ANOVA followed by post hoc analysis (Dunnett's multiple comparisons test). Significance was set at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The differences are shown in the bar graphs. $n = 5$ animals per group. CuO@PG, CuO@polycaprolactone/gelatin; TTM, tetrathiomolybdate; GSDMD, Gasdermin D; Caspase-1, cysteine-dependent aspartate-directed protease 1; ASC, apoptosis-associated speck-like protein containing a CARD; NLRP3, NOD-like receptor family pyrin domain containing 3

brain, and a variety of metal ions are involved in the process of TBI. Copper acts as an antioxidant and pro-oxidant and is involved in a variety of physiological reactions. Cu^{2+} is an essential component of cytochrome c oxidase (complex IV), which catalyzes the final stage of the electron-to-oxygen transfer in the process of oxidative phosphorylation in the mitochondrion [31]. Cu^{2+} constitutes the ceruloplasmin that transports itself and participates in a variety of redox reactions with plasma transport [32]. Also, copper is the center of activity of important antioxidant molecules such as superoxide dismutase SOD1 [31,32]. In the central system, neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Menkes and Wilson genetic disorders such as Menkes and Wilson are associated with copper homeostatic imbalances in the central nervous system [33].

Studies on rodent TBI models have shown that dysregulation of brain copper homeostasis persists for a long time after brain injury, with a chronic increase in Cu^{2+} content in the cortex on the damaged side [20]. It has also been documented that copper levels are significantly higher in the cortex bilaterally after mild TBI [34]. All of this suggests that copper may be involved in the progression of injury after TBI, but there are no definitive studies on its specific mechanisms.

Therefore, we first investigated the temporal expression of Cu in cortical tissues on the injured side after mild TBI, and found that the expression of both CTR1 and ATP7A tended to increase 14 d after injury, which was consistent with the long-term increase in cortical Cu levels after TBI. At the same time, another study found, by PET/CT Cu64 tracing, that increased copper uptake by traumatized brain tissue [35]. Examination of the cortex on the injured side after mild TBI revealed a decrease in total copper levels. Therefore, we speculate that the increased demand for copper in brain tissue after mild TBI increases copper utilization by up-regulating CTR1 expression, mobilizing more copper from the liver and blood to brain tissue and contributing to the activation of downstream ATP7A-mediated activation of specific enzymes, such as tyrosinase, which is translocated to the melanosomes in the melanophore, dopamine- β -hydroxylase, extracellular superoxide dismutase 3, peptidyl glycine α -amidated monooxygenase (PAM) and lysyl oxidase family [36–40]. The up-regulation of ATP7A after TBI may result from a combination of feedback caused by increased demand for downstream copper-based enzymes and competitive binding of copper ions by Atox1-ATP7A with copper proteins from other pathways, such as CCS and COX17, with

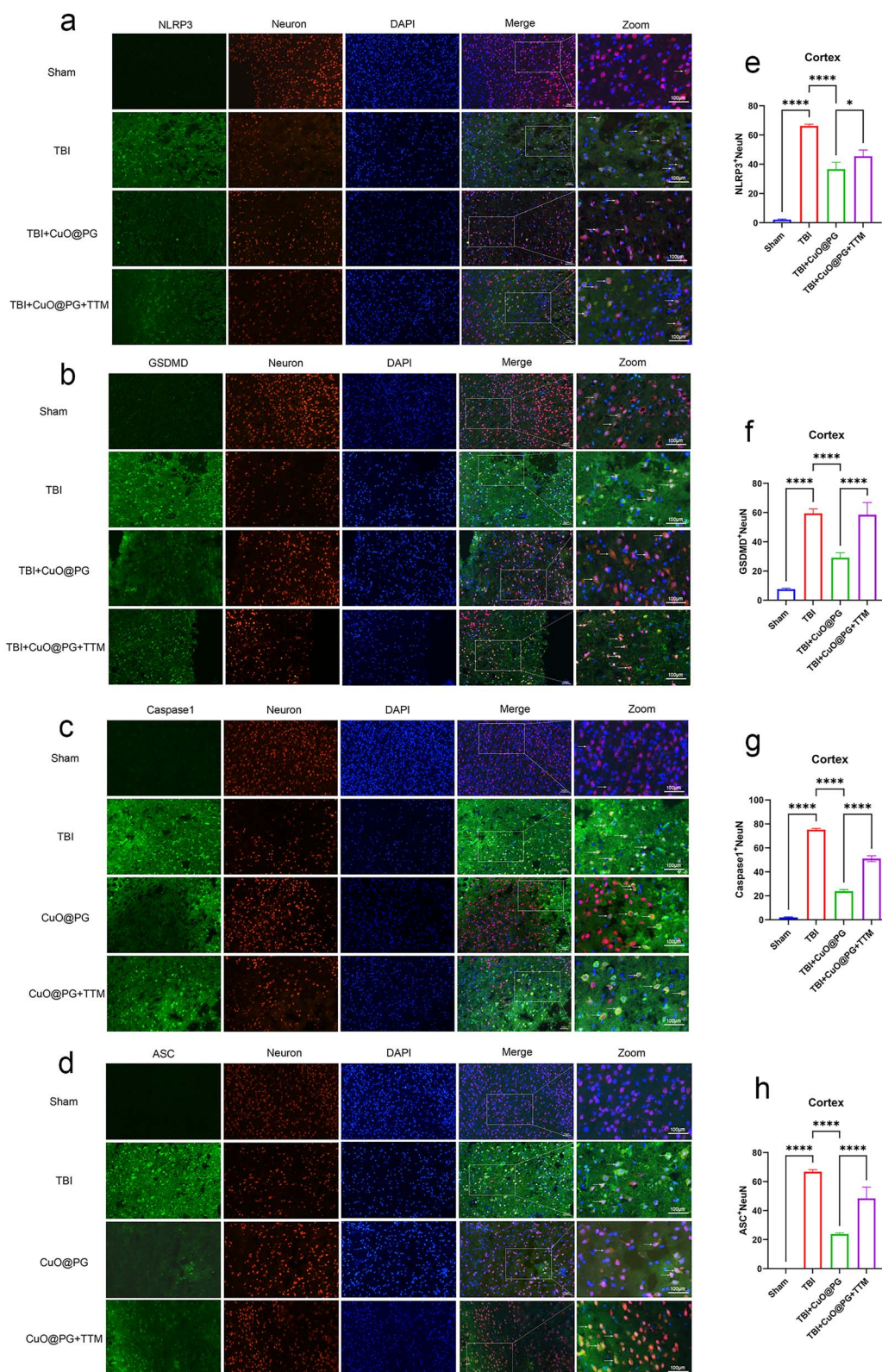


Figure 3. TTM inhibited the expression of TBI pyroptosis-related proteins improved by CuO@PG. **(a)** Immunofluorescence staining for NLRP3 protein in cortical tissues after TBI, **(b)** Immunofluorescence staining for GSDMD protein in cortical tissues after TBI, **(c)** Immunofluorescence staining for Caspase1 protein in cortical tissues after TBI, **(d)** Immunofluorescence staining for ASC protein in cortical tissues after TBI. Counter-staining with DAPI (blue) is used to visualize the location of cell nuclei. The magnification is 100 $\times$, and the scale bar is 100 μ m. ImageJ software was used for quantitative analysis **(e-h)** of the fluorescence images. The data were analyzed with a one-way ANOVA followed by post hoc analysis (Dunnett's multiple comparisons test). Significance was set at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The differences are shown in the bar graphs. $n = 5$ animals per group. CuO@PG, CuO@polylactone/gelatin; TTM, tetrathiomolybdate; GSDMD, Gasdermin D; Caspase-1, cysteine-dependent aspartate-directed protease 1; ASC, apoptosis-associated speck-like protein containing a CARD; NLRP3, NOD-like receptor family pyrin domain containing 3; NeuN, neuron neuronal nuclei; DAPI 4',6-diamidino-2-phenylindole; Sham refers to a control procedure in which animals undergo all steps of an experimental protocol except the actual intervention; TBI, traumatic brain injury

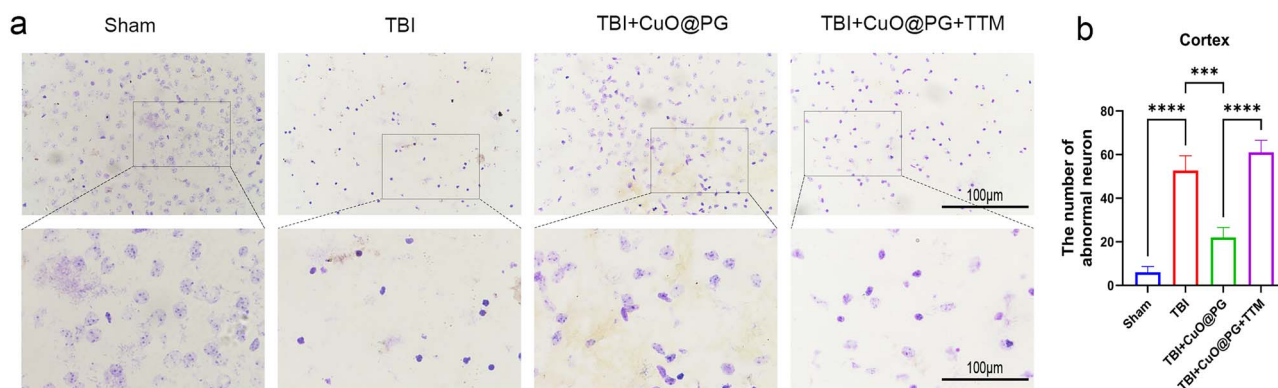


Figure 4. Abnormal number and morphological changes of neuronal cells with Nissl staining in the cortex after administration of exogenous copper ions and TTM. (a) Representative pictures showing the results of Nissl staining in the sham, TBI, TBI + CuO@PG, TBI + CuO@PG + TTM groups. (b) the data were analyzed with a one-way ANOVA followed by post hoc analysis (Dunnett's multiple comparisons test). Significance was set at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. The differences are shown in the bar graphs. $n = 5$ animals per group. CuO@PG, CuO@polycaprolactone/gelatin; TTM, tetrathiomolybdate; Sham refers to a control procedure in which animals undergo all steps of an experimental protocol except the actual intervention; TBI, traumatic brain injury

the aim of translocating sufficient raw materials to synthesize copper enzymes that activate downstream proteins in the Golgi, which are involved in post-traumatic vasculogenesis, the promotion of myelin sheath formation, and the processes such as neurotransmitter secretion [41]. The temporal expression of TH confirms this hypothesis to a certain extent, with TH expression increasing at 1 d after injury and returning to baseline levels at 14 d. The expression of TH in neuronal cells after TBI has been reported to increase at 1 d after injury. It has been reported that neuronal necrosis and phosphorylation levels of TH after TBI. It has been reported that necrosis of neuronal cells after TBI and a decrease in the phosphorylation level of TH caused a defect in enzyme activity, but there was no significant change in the level of dopamine in the short term, which may be one of the mechanisms for the compensatory increase in ATP7A and TH [42,43]. To clarify in which cell type the copper transporter protein upregulation occurs mainly in which cell type, we co-localized ATP7A in the cortex with neurons, microglia and delivery astrocytes. In normal cortical tissues, we found that ATP7A was mainly located in microglia in normal cortical tissues, whereas the up-regulation of ATP7A expression 1–3 d after TBI mainly occurred in neurons, suggesting that ATP7A may be involved in the pathological progression of neurons in the acute phase after TBI. The up-regulation of ATP7A expression mainly occurred in glia 3 d–7 d after TBI, which may be related to the activation of microglia and proliferation of astrocytes at different times after TBI [44]. Subsequently, we explored the temporal expression of the copper chaperonin CCS and the downstream copper-based enzyme SOD1.

In the realm of TBI research, the maintenance of intracellular metal ion homeostasis holds paramount significance. ATP7A and CTR1, as pivotal proteins in copper ion metabolism, exhibit expression alterations that are intricately associated with the pathophysiological processes subsequent to TBI. ATP7A, a copper—transporting ATPase, principally undertakes the task of translocating copper ions across the cell membrane and out of the cell. It plays an indispensable and pivotal role in upholding the dynamic equilibrium of intracellular copper ions [45]. Conversely, CTR1 serves as the primary transporter protein for cellular copper ion uptake, governing the process by which copper ions enter the cell.

Under normal physiological conditions, these two proteins collaborate synergistically to ensure that the intracellular copper ion concentration remains at an appropriate level, thereby buttressing the normal physiological functions of cells [46,47].

In the present study, through experimental investigations, we discerned that the expressions of ATP7A and CTR1 underwent dynamic fluctuations at diverse time points following TBI. Evidently observable from Figure S1a & d of the results, within the time frame spanning from 6 hours to 14 days post-TBI, the expression levels of ATP7A and CTR1 demonstrated conspicuous oscillations in comparison to those of the Sham (Figure S1a & S1c–d). Specifically, the expression level of ATP7A manifested a marked upsurge 1–2 days after TBI. This phenomenon is likely ascribable to pathological processes, such as the inflammatory response and oxidative stress engendered by TBI (Figure 2a & S1). These processes impel cells to upregulate the expression of ATP7A in an effort to contend with the perturbations in copper ion metabolism and endeavor to maintain the stability of the intracellular milieu. The expression of CTR1 persisted at a relatively elevated level 3–7 days after TBI, presumably because of the augmented demand for copper ions during the repair process of nerve cells after TBI.

This expression changes bear substantial guiding implications for ascertaining the optimal timing of therapeutic interventions post-TBI. In light of the elevated expression of ATP7A after TBI, it is plausible that conducting therapeutic interventions targeting the ATP7A—related pathway in the early phase of injury could be a potential strategy. Drugs can be rationally designed to modulate the activity or expression level of ATP7A, thereby optimizing the distribution of intracellular copper ions and mitigating nerve damage induced by copper ion metabolic derangements. With respect to CTR1, its elevated expression 3–7 days after TBI intimates that implementing treatment measures aimed at promoting the normal function of CTR1 or curbing its overexpression at this juncture may facilitate the repair and regeneration of nerve cells. As exemplified in our subsequent research, it was discovered that the administration of 0.5% CuO@PG 6 hours after TBI could effectively attenuate neuronal pyroptosis following TBI.

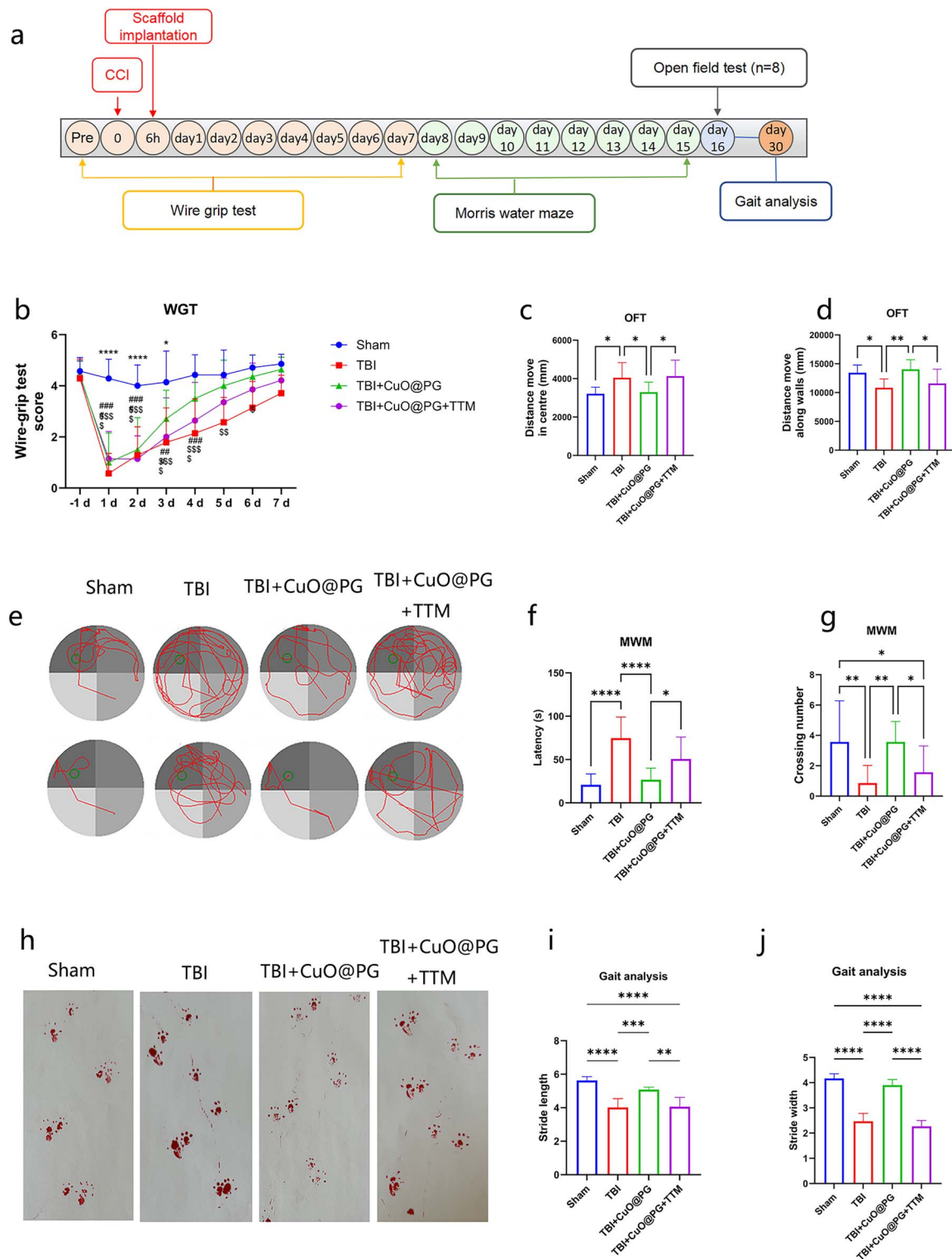


Figure 5. CuO@PG improves TBI-induced motor dysfunction, anxiety/depression behavior and memory deficits **(a)** timeline of various behavioral experiments. **(b)** Results of the wire grip test (WGT). The graph shows the recovery of motor function in animals at different times after injury. **(c and d)** The open field experiment: detection of spontaneous activity in different groups of mice. **(c)** Distance move in centre; **(d)** Distance move along walls. **(e)** Representative figures of water maze test. **(f)** Statistical results of the water maze test latency. **(g)** Number of trips across the platform in water maze. **(h)** Representative diagram of Gait analysis. **(i)** Statistical results of the stride length. **(j)** Statistical results of the stride width. The wire-grip test was analyzed with a two-way analysis of variance (ANOVA) by posthoc analysis (Tukey's multiple comparisons test). Significance was set at \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$, \$\$\$\$ $p < 0.0001$ sham vs TBI; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ sham vs TBI + CuO@PG + TTM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ sham vs TBI + CuO@PG. Other experiments data were analyzed with a one-way ANOVA followed by posthoc analysis (Dunnett's multiple comparisons test). Significance was set at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The differences are shown in the bar graphs. $n = 5$ animals per group. CCI, controlled cortical impact; CuO@PG, CuO@polycaprolactone/gelatin; TTM, tetrathiomolybdate; Sham refers to a control procedure in which animals undergo all steps of an experimental protocol except the actual intervention; TBI, traumatic brain injury; WGT, wire-grip test; OFT, open field test; MWM, Morris water maze test

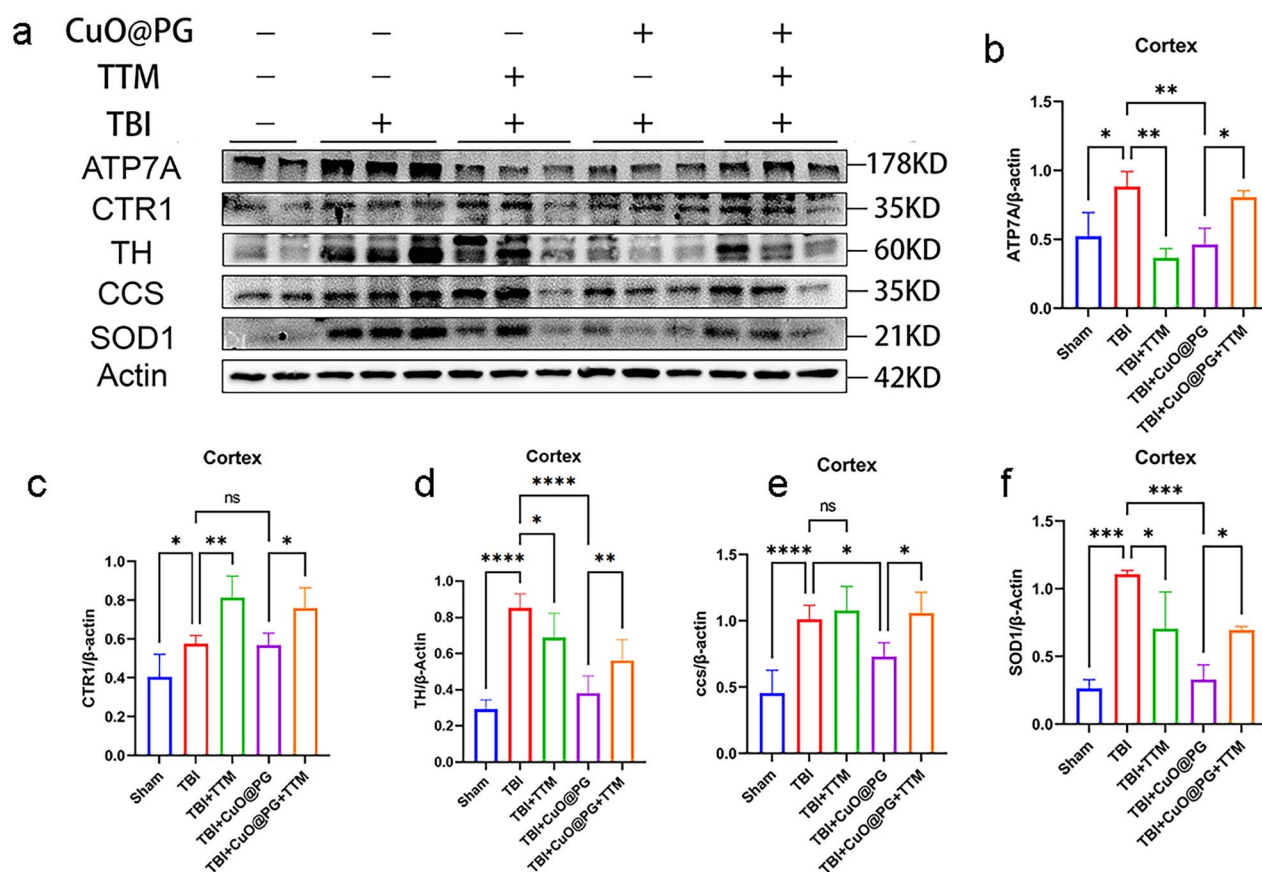


Figure 6. Effect of exogenous copper ions on copper metabolism after mild traumatic brain injury. Copper proteins in the injured side cortex 1d after mild TBI in the Sham, TBI group, the TBI + CuO@PG, TBI + CuO@PG + TTM groups. (a) Western-blot analysis of copper-related proteins in the injured-side cortex 1 day after mild TBI in the Sham group, TBI group, TBI+CuO@PG group, and TBI+CuO@PG+TTM group. (b-f) Quantitative analysis of the Western-blot results. The data were analyzed with a one-way ANOVA followed by posthoc analysis (Dunnett's multiple comparisons test). Significance was set at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The differences are shown in the bar graphs. $n = 5$ animals per group. CuO@PG, CuO@polycaprolactone/gelatin; TTM, tetrathiomolybdate; TBI, traumatic brain injury; ATP7A, ATPase copper transporting alpha; CTR1, copper transporter 1; TH, tyrosine hydroxylase; CCS, copper chaperone for superoxide dismutase; SOD1, superoxide dismutase 1

This study uncovers the expression change patterns of ATP7A and CTR1 at distinct time points after TBI. These findings furnish a crucial theoretical foundation for further probing into the optimal timing of therapeutic interventions post-TBI and are anticipated to proffer novel insights and directions for the optimization and development of future TBI treatment strategies.

We then explored the temporal expression of the copper chaperone protein CCS and the downstream copper-based enzyme SOD1, and we found that the expression of these proteins increased in a transient manner at 12 h-1 d post-injury, and then declined to the baseline level. The up-regulation of CCS and SOD1 was related to the cellular oxidative stress after TBI injury; this further verified the important role of copper in the acute phase of the post-traumatic brain injury (PBI) process. In the localization of CCS and SOD1, as well as the copper chaperone proteins Atox1 and COX17 in neurons after TBI, we found that all the above proteins were highly expressed in neurons. All these results suggest that there is a more active copper metabolism in neurons after injury than in normal tissues, and neurons may require more copper ions and downstream copper-based enzymes to maintain normal physiological functions, leading to an impact on intracellular copper ion homeostasis, which is particularly evident in the acute phase of injury. Meanwhile, this study explored the

differential schedule of copper proteins at different times after TBI, which is important for the objective determination of the time of TBI in the process of forensic identification.

Mechanical violence in TBI disrupts cell membranes, leading to the release of cellular contents, which can result in the aggregation of inflammatory vesicles and activation [48]. ROS accumulation triggered by mitochondrial dysfunction, leakage of inflammatory components because of blood-brain barrier damage and activation of inflammatory vesicles due to secondary bacterial infections all contribute to the onset and exacerbation of pyroptosis after TBI [49,50]. Compared to apoptosis, cellular pyroptosis occurs more rapidly, and it has been shown that pyroptosis-related proteins are significantly elevated 1 d after mild TBI, which is more in line with copper metabolism in the acute phase of TBI [5]. Ceruloplasmin (CP) is a plasma protein synthesized in the liver, responsible for transporting copper in the blood, and its synthesis and secretion can be increased during inflammation, and hepatic CP synthesis can be up-regulated by inflammatory cytokines and hypoxia-inducible factor (HIF1) [51]. Under pathological inflammatory conditions, tissue copper levels are significantly elevated and copper metabolism is significantly enhanced, which also increases CP synthesis [52]. Previous studies have shown that plasma ceruloplasmin are significantly higher in patients 3 days after TBI compared to healthy

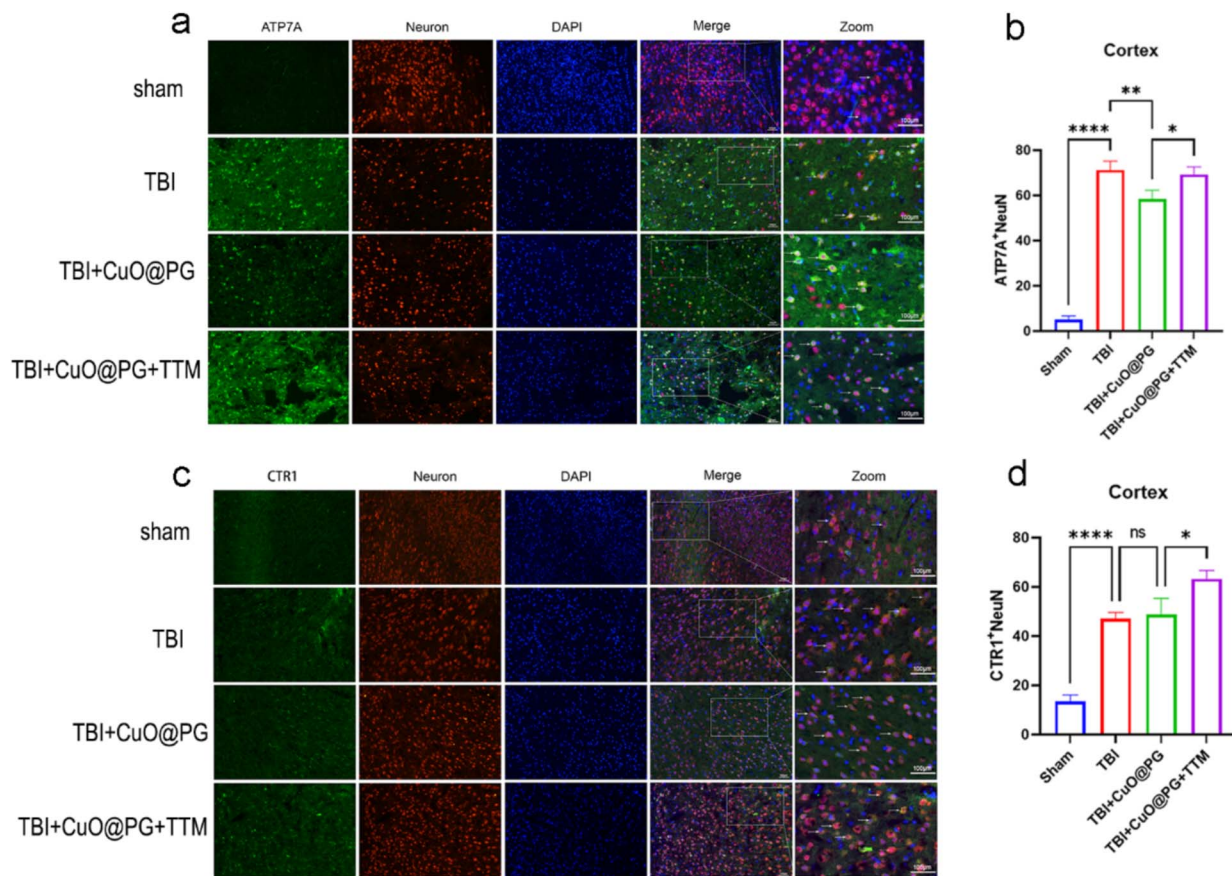


Figure 7. Effect of exogenous copper ions on neuronal copper transporter proteins immunofluorescence showed the co-localization of the copper ion transport proteins CTR1 and ATP7A with neurons in the sham, TBI, TBI + CuO@PG, and TBI + CuO@PG + TM groups in the injured cortex 1 d after mild TBI. Status. Immunofluorescence images (a) and quantitative analysis (b) of ATP7A in the injured cortex 1 day after mild TBI in the Sham, TBI, TBI+CuO@PG, and TBI+CuO@PG+TM groups. Immunofluorescence images (c) and quantitative analysis (d) of CTR1 in the injured cortex 1 day after mild TBI in the above groups. Counter-staining with DAPI (blue) is used to visualize the location of cell nuclei. The magnification is 100 $\times$, and the scale bar is 100 μ m. ImageJ software was used for quantitative analysis of the fluorescence images. The data were analyzed with a one-way ANOVA followed by posthoc analysis (Dunnett's multiple comparisons test). Significance was set at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The differences are shown in the bar graphs. $n = 5$ animals per group. CuO@PG, CuO@polycaprolactone/gelatin; TTM, tetrathiomolybdate; ATP7A, ATPase copper transporting alpha; CTR1, copper transporter 1; NeuN, neuron neuronal nuclei, DAPI 4',6-diamidino-2-phenylindole; Sham refers to a control procedure in which animals undergo all steps of an experimental protocol except the actual intervention; TBI, traumatic brain injury

individuals, and that in patients who developed high intracranial pressure, plasma ceruloplasmin levels and serum copper were significantly lower within 24 hours after injury [53]. The copper complex CuII (atasm) has demonstrated anti-inflammatory effects in a mouse model of cerebral ischemia [54]. Additionally, research indicates that oral administration of diacetylbis(4-methylthiosemicarbamide) copper (II) [Cu(II)(atasm)] enhances motor function and survival in mice models of ALS [55]. As a well-established regulatory compound, this copper complex holds significant potential for treating brain injuries [54]. However, excessive copper ions lead to oxidative stress and mitochondrial dysfunction in cells, rather exacerbating inflammatory damage; meanwhile, since copper ions cannot freely cross the blood-brain barrier, traditional routes of administration (orally or intravenously) are limited by the rate of transit and require large doses to deliver the target concentration of copper ions to the brain, whereas direct administration through the brain parenchyma or lateral ventricles is invasive [56,57]. Therefore, we constructed CuO-loaded electrostatically spun scaffolds for in situ delivery of exogenous copper ions, which have a slow release of exogenous copper ions, enabling small-dose

local administration and avoiding the cytotoxicity of copper. The assay showed that the CuO-loaded scaffolds also had a good morphological structure, and their uniform fiber arrangement and pore size contributed to cell growth. After loading with CuO, the scaffold still showed high hydrophilicity, which did not have much effect on cell adhesion within the adhesion within the scaffolds; at the same time, CuO showed excellent antimicrobial function, which may have a certain effect on the anti-infection of wounds. It may have a certain effect on the anti-infection of wounds.

Copper, an essential trace element in the human body, functions as a redox-active metal. Disruption in copper homeostasis can undermine the oxidoreductase system, as enzymes like CuZn-SOD and ceruloplasmin, which use copper as a catalytic cofactor, are highly sensitive to tissue copper levels [58]. Additionally, copper balance affects the activity of non-copper-containing enzymes such as catalase and selenium-dependent glutathione peroxidase (Se-GPx) [59]. Excess copper can lead to the production of reactive oxygen species (ROS), and a variety of copper transporters and copper-binding proteins have evolved to protect cells by regulating copper uptake, distribution, efflux, and delivery [58].

In TBI, the integrity of the blood–brain barrier (BBB) is compromised. Normally, the BBB restricts the exchange of substances, including copper, between the blood and brain tissue [60]. Post-injury, copper ions are more likely to enter brain tissue from the bloodstream, causing abnormally high copper levels. Furthermore, copper stored within brain tissue may be released into the extracellular space because of cellular damage, further disrupting normal copper distribution [61]. When copper homeostasis is disrupted, especially with an excess of copper ions, the production of ROS through Fenton or Haber-Weiss reactions increases, attacking the lipid bilayer of neuronal membranes and initiating lipid peroxidation. This process alters membrane fluidity, increases permeability, and ultimately leads to structural and functional impairment of neurons [33].

Oxidative stress activates glial cells, prompting them to release inflammatory factors. For instance, oxidative stress-induced activation of microglia results in the release of inflammatory mediators such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), intensifying the inflammatory response and oxidative damage to brain tissue. This also disrupts the normal support and repair functions of glial cells [62]. Furthermore, ROS can damage cerebral vascular endothelial cells, increasing vascular permeability and allowing blood components to leak into brain tissue, thereby exacerbating brain edema. Injury to vascular endothelial cells also impairs the normal regulatory functions of cerebral vessels, such as vasodilation and vasoconstriction, leading to abnormal local cerebral blood perfusion. These changes further impair BBB function, creating a vicious cycle that exacerbates the pathological process following TBI [63,64].

We have successfully established a nerve cell injury model using hydrogen-peroxide-induced injury in HT22 cells. We further evaluated the therapeutic effects of different doses of CuO@PG and found that the doses of 0.3% and 0.5% CuO@PG had the better reversal effect on cell viability, while a dose of 1% CuO@PG exacerbated cell damage. The results of *in vivo* experiments show that 0.5% CuO@PG is better than 0.3%. On this basis, we chose an electrospun silk scaffold loaded with a concentration of 0.5% CuO as an implantation experiment. The scaffolds were implanted with a radius of approximately 5 mm to cover the TBI wound. When exploring the time window of treatment, we found that at 0 h, 1 h and 3 h after TBI, the scaffolds were implanted at the same time. When exploring the time window of treatment, we found that implantation of 0.5% CuO@PG at 0 h, 1 h, 3 h, and 6 h after TBI could alleviate the occurrence of scotoma to a certain extent, but the best effect of implantation was at 6 h after injury. However, implantation at 6 h after TBI had the best effect in inhibiting pyroptosis, which may be related to the intracranial pressure changes after mild TBI. After determining the time window for drug administration, we implanted scaffolds with different concentrations of CuO in the wound 6 h after TBI. After determining the time window of administration, we implanted scaffolds with different concentrations of CuO into the wound 6 h after TBI, and found that only 0.5% of CuO@PG had an inhibitory effect on pyroptosis; 0.3% of CuO@PG could down-regulate NLRP3 but could not prevent the onset of pyroptosis; implantation of electrospun scaffolds alone did not down-regulate pyroptosis-related proteins, confirming the treatment modality was to attenuate cellular pyroptosis by administering exogenous

copper ions to the injured cortex. In confirming the time window and optimal concentration for implantation, we chelated copper ions *in vivo* by tetrathiomolybdate ammonium (TTM). Simultaneous administration of TTM and CuO@PG revealed no significant changes in pyroptosis-associated proteins compared to the mild TBI group, directly indicating that the inhibitory effect of exogenous copper ions on pyroptosis. Meanwhile, we found that the pyroptosis occurred mainly in neurons 1 d after TBI by immunofluorescence, and the administration of exogenous copper ions attenuated the pyroptosis of neurons. The results of Nissl-staining showed that exogenous copper ions reduced neuronal deformation. Based on the above findings, we conclude that exogenous copper ions played a neuroprotective role in the acute stage after TBI.

TBI-induced neurological damage can lead to long-term complications and sequelae, resulting in motor, memory, and cognitive-emotional dysfunction. To verify the long-term neuroprotective effects of exogenous copper ions after TBI, we further evaluated the changes in motor, memory, and cognitive functions in mice. The results showed that compared with the TBI group, mice implanted with CuO@PG had higher motor function scores, stronger spatial memory and better cognitive-emotional levels in behavioral tests compared with the TBI group, which reflected the important role of exogenous copper ions in improving long-term prognosis after mild TBI.

Finally, we examined total intracortical copper concentrations 1 d after mild TBI, copper ion concentrations in the cortex were reduced, and administration of exogenous copper ions restored copper levels in the injured side of the cortex, restored copper homeostasis in the injured tissue and elevate the copper ion concentration in the blood. At the same time, the copper proteins were examined 1 d after the exogenous copper intervention, and the results showed that the copper levels in the cortex of the injured side could be restored by the administration of TBI. The results showed that the decrease in ATP7A expression after the administration of TTM after TBI was due to the decrease in the expression level of ATP7A to reduce copper after chelating intracellular copper ions. The decreased expression level of ATP7A reduced copper efflux to maintain intracellular copper homeostasis. However, we found that administration of exogenous copper ions also decreased ATP7A expression, which may be due to the increase of intracellularly available copper ions and the increase of ATP7A expression on the Golgi apparatus competitively binding copper ions. At the same time, exogenous copper ions reduced the damage, maintained the homeostasis of the nervous system, and improved the efficiency of dopaminergic transmission, which led to a decrease in the negative feedback of downstream copper-based enzymes on ATP7A. After the concomitant administration of TTM, the expression of ATP7A increased, and the effect of exogenous copper ions was showed similar expression profiles for TH, which could confirm the above hypothesis that the neuroprotective effects of exogenous copper ions are not limited by TTM. The neuroprotective effect of exogenous copper ions restored dopamine secretion and neurotransmission without the compensatory effects of TH and ATP7A. The above results suggest that the expression of ATP7A after TBI may be affected by both downstream feedback regulation and intracellular available copper concentration after injury.

The expression of CTR1 did not change significantly before and after the administration of exogenous copper ions, but after the administration of TTM, the expression level of CTR1 was up-regulated. The expression of CTR1 was also up-regulated in both the TBI + TTM group and the TBI + CuO@PG + TTM group. This suggests that lowering the concentration of copper ions will cause a compensatory increase in the expression of CTR1, while the administration of low concentrations of exogenous copper ions will not affect the expression of CTR1. This phenomenon may explain the reported chronic accumulation of copper ions in the cortex after TBI: CTR1 expression remained at a high level at 14 d, and did not decrease to the baseline level with the restoration of copper homeostasis, resulting in high levels of copper inward flow even after repair of damage in the acute phase of TBI. The compensatory rise in ATP7A reduces the intracellular copper concentration. After the loss of compensation, intracellular copper ions begin to accumulate, and the imbalance in body copper homeostasis caused by the increase in cellular copper requirement in the short term may have affected copper homeostasis in the chronic phase. This may be one of the possible mechanisms of post-TBI neurodegeneration: copper can be observed in the brains of patients with Alzheimer's disease, where overloaded with copper was in some brain regions. Excess copper induces insoluble A β production and generation of reactive oxygen species, which leads to neuronal damage [65]. This may suggest that exogenous copper ion therapy may be limited to within 14 days after TBI, whereas in the chronic phase of TBI where intracellular copper accumulates, chelated copper reduces Alzheimer's disease [66]. This also provides a new idea for our follow-up study: whether chelating copper can regulate the chronic copper homeostatic imbalance after TBI.

Compared to the TBI group, both CCS and SOD1 showed decreased expression after administration of exogenous copper ions. This suggests that exogenous copper ions attenuated the intracellular oxidative stress level without directly activating the CCS-SOD1 pathway, and that homeostasis may be reached through other antioxidant pathways. It has been reported that copper ions can promote iron exocytosis by decreasing intracellular iron content, then inhibit iron-induced lipid peroxidation [67].

In summary, in this paper, by detecting copper proteins in a mild TBI model, we found that abnormal copper metabolism and cellular distribution of copper proteins were in the cortex after TBI, and the imbalance of copper homeostasis after mild TBI. On this basis, we constructed an electrostatically spun scaffold with the performance of continuous and low-dose copper supply, explored the optimal time window and concentration, and found that exogenous copper ions can down-regulate pyroptosis-associated proteins, which can reduce neuronal cell damage and thereby restoring the copper metabolism and maintaining the intracellular copper homeostasis by reducing the damage to the nerve cells and increasing the concentration of available copper in the cells. However, there are limitations to this study. Although we demonstrated the inhibitory effect of copper on cellular pyroptosis, the exact link between copper ions on cellular pyroptosis pathway and inflammation needs to be further explored. In addition, the present experiment only demonstrated that exogenous copper ions partially restored

intracellular copper homeostasis, but the exact mode of action still needs to be further investigated. The chronic accumulation of copper in cells after TBI also suggests an increase in other copper metabolic pathways, such as the copper-dependent kinase lysyl oxidase (LOX), which is involved in angiogenesis, and the COX17- cytochrome oxidase pathway, and there is a lack of studies on the changes in these metabolic pathways that exist. We will further investigate more downstream copper protein expression and subcellular structure changes in copper metabolism and homeostasis in TBI brain tissue samples to provide more targets for clinical treatment of craniocerebral injury and more ideas for forensic identification after TBI.

Conclusions

In summary, we developed an electrostatically spun scaffold loaded with copper oxide to inhibit neuronal pyroptosis by improving copper homeostasis after TBI alleviate neurodegenerative lesions, promote neural recovery, and reduce chronic neuroinflammation in order to exert neuroprotective effects after TBI. This study provides a new theoretical basis for the treatment of exogenous copper and the clinical application of electrostatically spun scaffolds after TBI. However, we primarily focus on neuronal pyroptosis in the early stage of TBI and further exploration and validation on long-term effect of CuO@PG on neuronal regeneration, synaptic plasticity, the proliferation and differentiation of neural stem cells, and the interaction between neurons and glial cells are needed in the future.

Author contributions

YA, SL, HS, LT, MY contributed to conceptualization; YA, SL, XH, ZW, YZ, JL contributed to investigation; XC, MX, CC, XZ contributed to formal analysis. All authors read and approved the final manuscript.

Supplementary data

Supplementary data is available at *Burns & Trauma Journal* online.

Ethic approval

All animal experiments were approved by the Institutional Animal Use and Care Committee at Soochow University (reference number = SZUM2008031233) and conducted in accordance with Animal Use and Care of National Institutes of Health and the Animal Research: Reporting *In Vivo* Experiments.

Conflict of interest

The authors declare no known competing financial interests.

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Data availability

The data are available from the corresponding author upon reasonable request.

References

1. Maas AIR, Menon DK, Adelson PD, Andelic N, Bell MJ, Belli A., et al. Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. *Lancet Neurol.* 2017;16: 987–1048. [https://doi.org/10.1016/S1474-4422\(17\)30371-X](https://doi.org/10.1016/S1474-4422(17)30371-X)
2. Pattinson CL, Gill JM. Risk of dementia after tbi - a cause of growing concern. *Nat Rev Neurol.* 2018;14:511–2. <https://doi.org/10.1038/s41582-018-0041-8>
3. Shi J, Gao W, Shao F. Pyroptosis: Gasdermin-mediated programmed necrotic cell death. *Trends Biochem Sci.* 2017;42: 245–54. <https://doi.org/10.1016/j.tibs.2016.10.004>
4. Hu X, Chen H, Xu H, Wu Y, Wu C, Jia C., et al. Role of pyroptosis in traumatic brain and spinal cord injuries. *Int J Biol Sci.* 2020;16: 2042–50. <https://doi.org/10.7150/ijbs.45467>
5. Chen X, Huang X, Liu C, Li S, Yang Z, Zhang F., et al. Surface-fill h2s-releasing silk fibroin hydrogel for brain repair through the repression of neuronal pyroptosis. *Acta Biomater.* 2022;154: 259–74. <https://doi.org/10.1016/j.actbio.2022.11.021>
6. Ruiz LM, Libedinsky A, Elorza AA. Role of copper on mitochondrial function and metabolism. *Front Mol Biosci.* 2021;8:711227. <https://doi.org/10.3389/fmolb.2021.711227>
7. Shin LJ, Lo JC, Yeh KC. Copper chaperone antioxidant protein1 is essential for copper homeostasis. *Plant Physiol.* 2012;159: 1099–110. <https://doi.org/10.1104/pp.112.195974>
8. Urso E, Maffia M. Behind the link between copper and angiogenesis: established mechanisms and an overview on the role of vascular copper transport systems. *J Vasc Res.* 2015;52:172–96. <https://doi.org/10.1159/000438485>
9. Opazo CM, Greenough MA, Bush AI. Copper: from neurotransmission to neuroproteostasis. *Front Aging Neurosci.* 2014;6:143. <https://doi.org/10.3389/fnagi.2014.00143>
10. Blades B, Ayton S, Hung YH, Bush AI, La Fontaine S. Copper and lipid metabolism: a reciprocal relationship. *Biochim Biophys Acta Gen Subj.* 2021;1865:129979. <https://doi.org/10.1016/j.bba-gen.2021.129979>
11. Wiriyaesermkul P, Moriyama S, Nagamori S. Membrane transport proteins in melanosomes: regulation of ions for pigmentation. *Biochim Biophys Acta Biomembr.* 2020;1862:183318. <https://doi.org/10.1016/j.bbamem.2020.183318>
12. Shawki A, Anthony SR, Nose Y, Engevik MA, Niespodzany EJ, Barrientos T., et al. Intestinal dmt1 is critical for iron absorption in the mouse but is not required for the absorption of copper or manganese. *Am J Physiol Gastrointest Liver Physiol.* 2015;309:G635–47. <https://doi.org/10.1152/ajpgi.00160.2015>
13. Ohgami RS, Campagna DR, McDonald A, Fleming MD. The steep proteins are metalloredutases. *Blood.* 2006;108:1388–94. <https://doi.org/10.1182/blood-2006-02-003681>
14. Wong PC, Waggoner D, Subramaniam JR, Tessarollo L, Bartnikas TB, Culotta VC., et al. Copper chaperone for superoxide dismutase is essential to activate mammalian cu/zinc superoxide dismutase. *Proc Natl Acad Sci USA.* 2000;97:2886–91. <https://doi.org/10.1073/pnas.040461197>
15. Palumaa P, Kangur L, Voronova A, Sillard R. Metal-binding mechanism of cox17, a copper chaperone for cytochrome c oxidase. *Biochem J.* 2004;382:307–14. <https://doi.org/10.1042/BJ20040360>
16. Petris MJ, Mercer JF, Culvenor JG, Lockhart P, Gleeson PA, Camakaris J. Ligand-regulated transport of the menkes copper p-type atpase efflux pump from the golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *EMBO J.* 1996;15:6084–95. <https://doi.org/10.1002/j.1460-2075.1996.tb00997.x>
17. Pilankatta R, Lewis D, Inesi G. Involvement of protein kinase d in expression and trafficking of atp7b (copper atpase). *J Biol Chem.* 2011;286:7389–96. <https://doi.org/10.1074/jbc.M110.171454>
18. Cobbold C, Ponnambalam S, Francis MJ, Monaco AP. Novel membrane traffic steps regulate the exocytosis of the menkes disease atpase. *Hum Mol Genet.* 2002;11:2855–66. <https://doi.org/10.1093/hmg/11.23.2855>
19. Zheng W, Monnot AD. Regulation of brain iron and copper homeostasis by brain barrier systems: implication in neurodegenerative diseases. *Pharmacol Ther.* 2012;133:177–88. <https://doi.org/10.1016/j.pharmthera.2011.10.006>
20. Portbury SD, Hare DJ, Sgambelloni C, Finkelstein DI, Adlard PA. A time-course analysis of changes in cerebral metal levels following a controlled cortical impact. *Metallomics.* 2016;8:193–200. <https://doi.org/10.1039/c5mt00234f>
21. Malikmammadov E, Tanir TE, Kiziltay A, Hasirci V, Hasirci N. Pcl and pcl-based materials in biomedical applications. *J Biomater Sci Polym Ed.* 2018;29:863–93. <https://doi.org/10.1080/09205063.2017.1394711>
22. Siddiqui N, Asawa S, Birru B, Baadhe R, Rao S. Pcl-based composite scaffold matrices for tissue engineering applications. *Mol Biotechnol.* 2018;60:506–32. <https://doi.org/10.1007/s12033-018-0084-5>
23. Fee T, Surianarayanan S, Downs C, Zhou Y, Berry J. Nanofiber alignment regulates nih3t3 cell orientation and cytoskeletal gene expression on electrospun pcl+gelatin nanofibers. *PLoS One.* 2016;11:e0154806. <https://doi.org/10.1371/journal.pone.0154806>
24. Rajasekar M, Lavanya M. Gel scaffolds and emerging applications in biomedicine. *RSC Adv.* 2022;12:15925–49. <https://doi.org/10.1039/d2ra00924b>
25. Sheng D, Li J, Ai C, Feng S, Ying T, Liu X., et al. Electrospun pcl/gel-aligned scaffolds enhance the biomechanical strength in tendon repair. *J Mater Chem B.* 2019;7:4801–10. <https://doi.org/10.1039/c9tb00837c>
26. Lu X, Zou H, Liao X, Xiong Y, Hu X, Cao J., et al. Construction of pcl-collagen@pcl+gelatin three-layer small diameter artificial vascular grafts by electrospinning. *Biomed Mater.* 2022;18:18. <https://doi.org/10.1088/1748-605X/aca269>
27. Suarez DF, Pinzon-Garcia AD, Sinisterra RD, Dussan A, Mesa F, Ramirez-Clavijo S. Uniaxial and coaxial nanofibers pcl/alginate or pcl/gelatine transport and release tamoxifen and curcumin affecting the viability of mcf7 cell line. *Nanomaterials (Basel).* 2022;12:3348. <https://doi.org/10.3390/nano12193348>
28. Chen X, Gao C, Yn Y, Cheng Z, Chen G, Rui T., et al. Ruxolitinib exerts neuroprotection via repressing ferroptosis in a mouse model of traumatic brain injury. *Exp Neurol.* 2021;342:113762. <https://doi.org/10.1016/j.expneurol.2021.113762>
29. Liu W, Chen Y, Meng J, Wu M, Bi F, Chang C., et al. Ablation of caspase-1 protects against tbi-induced pyroptosis in vitro and in vivo. *J Neuroinflammation.* 2018;15:48. <https://doi.org/10.1186/s12974-018-1083-y>
30. Ooigawa H, Nawashiro H, Fukui S, Otani N, Osumi A, Toyooka T., et al. The fate of nissl-stained dark neurons following traumatic brain injury in rats: difference between neocortex and hippocampus regarding survival rate. *Acta Neuropathol.* 2006;112:471–81. <https://doi.org/10.1007/s00401-006-0108-2>
31. Ekici S, Turkarslan S, Pawlik G, Dancis A, Baliga NS, Koch H-G., et al. Intracytoplasmic copper homeostasis controls cytochrome c oxidase production. *MBio.* 2014;5:e01055–13. <https://doi.org/10.1128/mBio.01055-13>
32. Hellman NE, Gitlin JD. Ceruloplasmin metabolism and function. *Annu Rev Nutr.* 2002;22:439–58. <https://doi.org/10.1146/annurev.nutr.22.012502.114457>

33. An Y, Li S, Huang X, Chen X, Shan H, Zhang M. The role of copper homeostasis in brain disease. *Int J Mol Sci.* 2022;23:23. <https://doi.org/10.3390/ijms232213850>
34. Juan SMA, Daglas M, Gunn AP, Lago L, Adlard PA. Characterization of the spatial distribution of metals and profile of metalloprotein complexes in a mouse model of repetitive mild traumatic brain injury. *Metallomics: Integrated Biometal Science.* 2022;14:mfac092. <https://doi.org/10.1093/mtomcs/mfac092>
35. Peng F, Muzik O, Gatson J, Kernie SG, Diaz-Arrastia R. Assessment of traumatic brain injury by increased 64cu uptake on 64cuc12 pet/ct. *Journal of Nuclear Medicine: Official Publication, Society of Nuclear Medicine.* 2015;56:1252–7. <https://doi.org/10.2967/jnumed.115.154575>
36. Petris MJ, Strausak D, Mercer JF. The menkes copper transporter is required for the activation of tyrosinase. *Hum Mol Genet.* 2000;9:2845–51. <https://doi.org/10.1093/hmg/9.19.2845>
37. Gerbasi V, Lutsenko S, Lewis EJ. A mutation in the atp7b copper transporter causes reduced dopamine beta-hydroxylase and norepinephrine in mouse adrenal. *Neurochem Res.* 2003;28:867–73. <https://doi.org/10.1023/A:1023219308890>
38. Qin Z, Itoh S, Jeney V, Ushio-Fukai M, Fukai T. Essential role for the menkes atpase in activation of extracellular superoxide dismutase: implication for vascular oxidative stress. *FASEB Journal: Official Publication of the Federation of American Societies For Experimental Biology.* 2006;20:334–6. <https://doi.org/10.1096/fj.05-4564fje>
39. Steveson TC, Ciccotosto GD, Ma X-M, Mueller GP, Mains RE, Eipper BA. Menkes protein contributes to the function of peptidylglycine alpha-amidating monooxygenase. *Endocrinology.* 2003;144:188–200. <https://doi.org/10.1210/en.2002-220716>
40. Shanbhag V, Jasmer-McDonald K, Zhu S, Martin AL, Gudekar N, Khan A., et al. Atp7a delivers copper to the lysyl oxidase family of enzymes and promotes tumorigenesis and metastasis. *Proc Natl Acad Sci USA.* 2019;116:6836–41. <https://doi.org/10.1073/pnas.1817473116>
41. Zirngibl M, Assinck P, Sizov A, Caprariello AV, Plemel JR. Oligodendrocyte death and myelin loss in the cuprizone model: An updated overview of the intrinsic and extrinsic causes of cuprizone demyelination. *Mol Neurodegener.* 2022;17:34. <https://doi.org/10.1186/s13024-022-00538-8>
42. Shin SS, Bray ER, Zhang CQ, Dixon CE. Traumatic brain injury reduces striatal tyrosine hydroxylase activity and potassium-evoked dopamine release in rats. *Brain Res.* 2011;1369:208–15. <https://doi.org/10.1016/j.brainres.2010.10.096>
43. Wasinski F, Pedrosa JAB, Dos Santos WO, Furigo IC, Garcia-Galiano D, Elias CF., et al. Tyrosine hydroxylase neurons regulate growth hormone secretion via short-loop negative feedback. *J Neurosci.* 2020;40:4309–22. <https://doi.org/10.1523/JNEUROSCI.2531-19.2020>
44. Michinaga S, Koyama Y. Pathophysiological responses and roles of astrocytes in traumatic brain injury. *Int J Mol Sci.* 2021;22:22. <https://doi.org/10.3390/ijms22126418>
45. Kaler SG. Atp7a-related copper transport diseases-emerging concepts and future trends. *Nat Rev Neurol.* 2011;7:15–29. <https://doi.org/10.1038/nrneurol.2010.180>
46. Xue Q, Kang R, Klionsky DJ, Tang D, Liu J, Chen X. Copper metabolism in cell death and autophagy. *Autophagy.* 2023;19:2175–95. <https://doi.org/10.1080/15548627.2023.2200554>
47. Das A, Ash D, Fouda AY, Sudhakar V, Kim Y-M, Hou Y., et al. Cysteine oxidation of copper transporter ctr1 drives vegfr2 signalling and angiogenesis. *Nat Cell Biol.* 2022;24:35–50. <https://doi.org/10.1038/s41556-021-00822-7>
48. Zhao Z, Zhou Y, Tian Y, Li M, Dong J-F, Zhang J. Cellular microparticles and pathophysiology of traumatic brain injury. *Protein & Cell.* 2017;8:801–10. <https://doi.org/10.1007/s13238-017-0414-6>
49. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK., et al. Mitochondrial cardiolipin is required for nlrp3 inflammasome activation. *Immunity.* 2013;39:311–23. <https://doi.org/10.1016/j.immuni.2013.08.001>
50. Jorgensen I, Miao EA. Pyroptotic cell death defends against intracellular pathogens. *Immunol Rev.* 2015;265:130–42. <https://doi.org/10.1111/immr.12287>
51. Bellouard M, de la GrandMaison GL, Cappy J, Grimaldi L, Lontsi-Djeagou A, Alvarez J-C. Trace elements repartition in body fluids, hair and organs in an autopsied population evaluated by icp-ms high resolution. *Environ Toxicol Pharmacol.* 2022;95:103978. <https://doi.org/10.1016/j.etap.2022.103978>
52. Vasilyev VB. Interactions of caeruloplasmin with other proteins participating in inflammation. *Biochem Soc Trans.* 2010;38:947–51. <https://doi.org/10.1042/BST0380947>
53. Dash PK, Redell JB, Hergenroeder G, Zhao J, Clifton GL, Moore A. Serum ceruloplasmin and copper are early biomarkers for traumatic brain injury-associated elevated intracranial pressure. *J Neurosci Res.* 2010;88:1719–26. <https://doi.org/10.1002/jnr.22336>
54. Huuskonen MT, Tuo Q-Z, Loppi S, Dhungana H, Korhonen P, McInnes LE., et al. The copper bis(thiosemicarbazone) complex cuii(atms) is protective against cerebral ischemia through modulation of the inflammatory milieu. *Neurotherapeutics: the Journal of the American Society For Experimental NeuroTherapeutics.* 2017;14:519–32. <https://doi.org/10.1007/s13311-016-0504-9>
55. Roberts BR, Lim NKH, McAllum EJ, Donnelly PS, Hare DJ, Doble PA., et al. Oral treatment with cuii(atms) increases mutant sod1 in vivo but protects motor neurons and improves the phenotype of a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosci.* 2014;34:8021–31. <https://doi.org/10.1523/JNEUROSCI.4196-13.2014>
56. Pohanka M. Copper and copper nanoparticles toxicity and their impact on basic functions in the body. *Bratislavské Lekárske Listy.* 2019;120:397–409. https://doi.org/10.4149/BLL_2019_065
57. Choi B-S, Zheng W. Copper transport to the brain by the blood-brain barrier and blood-csf barrier. *Brain Res.* 2009;1248:14–21. <https://doi.org/10.1016/j.brainres.2008.10.056>
58. Uriu-Adams JY, Keen CL. Copper, oxidative stress, and human health. *Mol Asp Med.* 2005;26:268–98. <https://doi.org/10.1016/j.mam.2005.07.015>
59. Chen Y, Saari JT, Kang YJ. Weak antioxidant defenses make the heart a target for damage in copper-deficient rats. *Free Radic Biol Med.* 1994;17:529–36. [https://doi.org/10.1016/0891-5849\(94\)90092-2](https://doi.org/10.1016/0891-5849(94)90092-2)
60. Pavlovic D, Pekic S, Stojanovic M, Popovic V. Traumatic brain injury: neuropathological, neurocognitive and neurobehavioral sequelae. *Pituitary.* 2019;22:270–82. <https://doi.org/10.1007/s11102-019-00957-9>
61. Isaev NK, Stelmashook EV, Genrikhs EE. Role of zinc and copper ions in the pathogenetic mechanisms of traumatic brain injury and alzheimer's disease. *Rev Neurosci.* 2020;31:233–43. <https://doi.org/10.1515/revneuro-2019-0052>
62. Sharma R, Kambhampati SP, Zhang Z, Sharma A, Chen S, Duh EL., et al. Dendrimer mediated targeted delivery of sinomenine for the treatment of acute neuroinflammation in traumatic brain injury. *Journal of Controlled Release: Official Journal of the Controlled Release Society.* 2020;323:361–75. <https://doi.org/10.1016/j.jconrel.2020.04.036>
63. Zhong C-C, Zhao T, Hogstrand C, Chen F, Song C-C, Luo Z. Copper (cu) induced changes of lipid metabolism through oxidative stress-mediated autophagy and nrf2/ppary pathways. *J Nutr Biochem.* 2022;100:108883. <https://doi.org/10.1016/j.jnutbio.2021.108883>
64. van Hameren G, Muradov J, Minarik A, Aboghazleh R, Orr S, Cort S., et al. Mitochondrial dysfunction underlies impaired neurovascular coupling following traumatic brain injury. *Neurobiol Dis.* 2023;186:106269. <https://doi.org/10.1016/j.nbd.2023.106269>
65. Donnelly PS, Xiao Z, Wedd AG. Copper and alzheimer's disease. *Curr Opin Chem Biol.* 2007;11:128–33. <https://doi.org/10.1016/j.cbpa.2007.01.678>

66. Singh SK, Balendra V, Obaid AA, Esposto J, Tikhonova MA, Gautam NK., *et al.* Copper-mediated β -amyloid toxicity and its chelation therapy in alzheimer's disease. *Metallomics: Integrated Biometal Science*. 2022;14:mfac018. <https://doi.org/10.1093/mto/mcs/mfac018>
67. Yang M, Wu X, Hu J, Wang Y, Wang Y, Zhang L., *et al.* Commd10 inhibits hif1 α /cp loop to enhance ferroptosis and radiosensitivity by disrupting cu-fe balance in hepatocellular carcinoma. *J Hepatol*. 2022;76:1138–50. <https://doi.org/10.1016/j.jhep.2022.01.009>