EFFECT OF COPPER ON TISSUE HYPOXIA IN ADULT RATS

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ABSTRACT

Hypoxic tissue injury is characterized by acute hypoxic and sustained hyperoxic tissue injury. In this study we aimed to evaluate the efficacy of a predefined treatment protocole in a rat model of hypoxic tissue damage. Treatment group consisted 6 rats -4 male, 2 female- and control group consisted 6 rats -4 male, 2 female-. Tails of all rats were squeezed by a cotton rope from the most proximal part under ketamine anesthesia. After visible hypoxic injury to tail, rats in treatment group were given parenteral copper ions, oral zinc sulphate, n-acetyl cysteine. Control rats were not treated in experiment day. Treatment decision was based on clinical status of rats in subsequent days. In treatment group, gangrene was restricted to distal part of the tail but in control group, gangrene was observed to be advancing to proximal parts of tail. In control rats, treatment given in subsequent days effectively prevented further tissue damage and exitus. In a male control rat, progressing tissue injury was treated successfully by repeating our treatment protocole 7 times. In conclusion tissue injury during reperfusion stage of hypoxic-ischemic insults can be diminished by copper ions, zinc sulphate and n-acetyl cysteine.

Key words: Copper, zinc sulphate, n-aetyl cysteine, hypoxia, reperfusion injury, vitamin C

INTRODUCTION

Hypoxia affects all tissues in the body and has more detrimental effects in perinatal period, neonatal central nervous system being the most vulnerable tissue –hypoxic-ischemic encephalopathy-.

Acute hypoxic-ischemic tissue injury is shown to be mediated by anoxic depolarisation, ATP depletion, free radical formation and nitric oxide production. After reestablishment of blood flow in ischemic tissue, increased free radical and nitric oxide synthesis, increased influx of calcium and increased translocation of polymorphonuclear leukocytes into the hypoxic tissue augment tissue damage –reperfusion injury- [1].
Intracellular calcium accumulation causes mitochondrial dysfunction which ends up with further depletion of ATP stores, overproduction of free radicals. At last cytoskeleton is damaged and cell loses its ability to maintain integrity of the cytoplasmic and intracellular membranes ultimately leading to cell death [1]. Copper was shown to be released from ischemic tissues which make a peak at the beginning of reperfusion stage [2]. Zinc is also shown to be released from metallothioneins to the extracellular space during ischemia [3]. But this efflux of copper and zinc ions is believed to increase hypoxic and reperfusion tissue injury [2 - 3].
We hypothesized that detrimental effects of hypoxic tissue injury should be mainly due to disruption of intracellular copper metabolism after mandatory efflux of copper from cells during hypoxic stress and if we can find an effective way to deliver extra copper to hypoxic tissue, this should protect against reperfusion injury. Although copper supplementation should restore aerobic respiration in mitochondira, increasing free copper in extra and intracellular fluids should cause devastating effects due to high redox potential of copper ions [4]. For this reason we planned to introduce zinc which is a strong reducing agent and cysteine which inreases nonprotein thiol binding capacity in 5-10 minutes after giving copper. Giving three agents from one route should cause redox reactions between them before absorption into cells. So we planned to give copper by parenteral, zinc and cysteine by enteral routes. Because copper salts should need to be metabolized in liver to become active and hypoxic metabolism should not perform this metabolic process, we decided to use copper ions formed by the corrosion of metallic copper in ¼ saline (%5 dextrose).
We thought that best way to study an hypoxic model should be through direct visualization. We planned to squeeze tail of the rats with cotton rope to induce tail hypoxia. By this way we should directly observe varying degrees of tail hypoxia and ischemia in treatment and control groups. To explore the effectiveness of our drug regimen we planned to administer our drug protocole soon after hypoxic stress in treatment group and withhold it in control group until signs of imminent exitus appear.

MATERIALS AND METHODS

A. Animal model

This experimental study was approved by the Experimental Animal Ethics Committee at Yüzüncü Yıl University (Van, Turkey), and the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education by the New York Academy of Sciences, Ad Hoc Animal Research Committee was followed. Rats were kept in a 12-hour dark/light cycle and fed a standard rat chow ad libitum before and after the experiment day.
Sixteen adult wistar albino rats –eight male, eight female- were divided into two groups: treatment and control groups consisted eight rats (four male, four female in each group).

B. Treatment protocole

Metallic copper is known to corrode in chloride solution and copper ions are released [5]. Outer plastic insulator was removed from copper electric cable and cut in 1 metre long. Each cut part is rolled. Rolled copper wire was washed by sterile saline and put into ¼ saline (%5 dextrose) in glass bottle under aseptic conditions. We took samples intermittently from this bottle for analysis of copper concentration by atomic absorption spectrophotometry (Solar AA Spectrometers®-Thermo Elk. Co./UK). By this analysis we constructed the following corrosion-time curve of metallic copper in ¼ saline (Figure 1). At 24th hour copper concentration in this solution was found to be 1.9mg/L. For a rat of 200g, 1cc of this solution included 10mcg/kg copper. In human adults dose of intravenous copper replacement is reported to be 30mcg/kg/day -2.4mg/70kg- [6]. So our copper dosage was approximately 1/3 of the human adult daily dose.

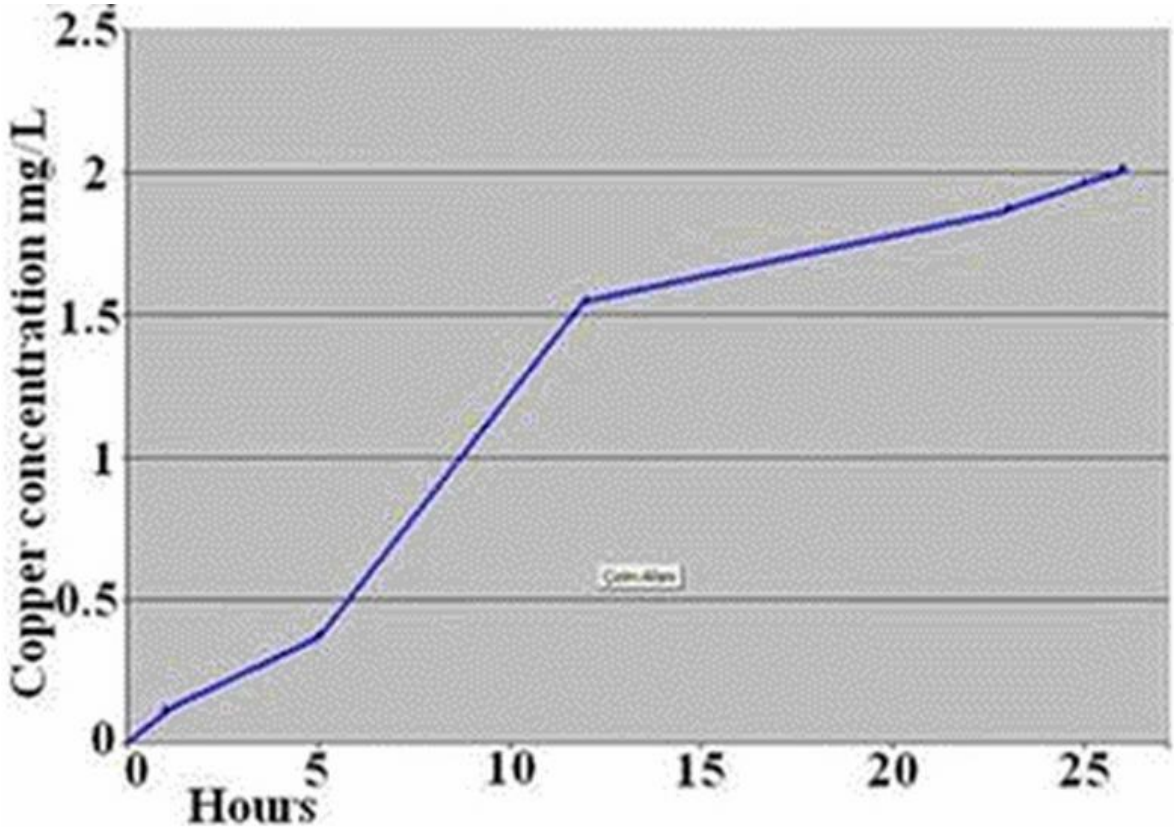


Figure 1. Corrosion-time curve of metallic copper in ¼ saline (%5 dextrose).

We planned to give 200mg/kg/dose n-acetyl cysteine orally which is the initial dosage of n-acetyl cycteine in paracetamol intoxication. We planned to give 8mg/kg/dose elemental zinc which is twice the recommended dietary allowance dose for infants -4mg/kg/day-. But very high doses of zinc sulphate is reported to be quite safe [7]. Instead of pure zinc sulphate we choosed a tablet containing 15mg elemental zinc and 30mg vitamin C due to its better solubility in water than pure zinc sulphate.

In conclusion we planned to give 1.55mcg copper intravenously and 200mg/kg n-acetyl cysteine and 8mg/kg zinc sulphate via orogastric catheter 5-10 minutes after copper administration.

C. Preliminary work for an experiment day

24 hours before each experiment, copper solution described earlier was prepared. In each experiment day, two rats with the same sex –one from treatment, one from control group- were choosen. A syringe containing 1cc (1.55mcg) of copper solution was prepared. After weighing the experiment rats, 1 tablet of NAC 600mg effervescent tablet (Basel İlaç, İstanbul) was dissolved in 15cc tap water and a syringe containing 200mg/kg of n-acetyl cysteine was prepared -1cc for a rat weighing 200g-. 1 tablet of Zinco-C 15mg tablet (Berko İlaç, İstanbul) was dissolved in 10cc of tap water and a syringe containing 8mg/kg elemental zinc was prepared -1cc for a rat weighing 200g-. We also prepared multipl syringes containing 75mg/kg ketamine and a set of syringes containing half doses of copper, zinc sulphate and n-acetyl cysteine.

D. Experiment day: Induction of tail hypoxia

Two experiment rats were anesthesized by intraperitoneal ketamine. When rat was observed to unresponsive to painfull stimuli -by skin pinching-, we squeezed tail of the rat from the most proximal part. Squeezing was kept tight enough to decrease tail perfusion as much as possible. If signs of reduction in depth of anesthesia were observed during tail hypoxia induction phase, intraperitoneal ketamine was repeated.

E. Experiment day: Treatment

Tail squeezing was continued until there was visible hypoxia –esp. cutis marmorata- or gangrene at the tip of the tail or there were signs of exitus –esp. wheezing and grunting-. After induction of adequate tail hypoxia, control rat was taken to routine care. Treatment rat was given 1cc copper solution intraperitoneally. After 5-10 minutes of this intervention, zinc and n-acetyl cysteine solutions were given via an orogastric catheter in upright position to prevent aspiration. If exitus was still a possibility in the following hours, half doses of each drug were given in the same manner just described.

F. Follow-up

After the experiment day if exitus risk was observed in any rat in any group, treatment protocole was repeated. At the 67th day of beginning the experiment, all rats were euthanised under ketamine anesthesia.

G. Recording

Every visit to the rats and every intervention to them were recorded by a video recorder. Almost all results of this study were based on these video records.

H. Alterations in study design

We planned to take serial blood gas analyses during tail induction and treatment interventions but we observed that blood gas analysis did not change much before and after the treatment but we could observe a marked clinical improvement after our treatment protocole in each rat. So we followed all rats on clinical grounds after 2nd experiment day. Due to problems in venous catheterisation we began to give copper solution through intraperitoneal route after the 3rd experiment day without visible side effects.

Rat no:5 was a male control rat and experienced massive tissue damage in following days of the experiment day. We had to repeat our treatment protocole 7 times and had to add oral sodium bicarbonate to his drug regimen to prevent exitus and induce tissue healing in 1 month period.

We planned to euthanise all rats at 10th day of the experiment day but due to unexpected tissue damage in rat no:5, we planned to prolong observation of other rats after the experiment day. After recovery of rat no:5, we euthanised all rats in 67th day after beginning our experiment. Nevertheless we did not observed any tissue damage after 10 days in all rats apart from rat no:5. They were healthy but with shorter tails.

RESULTS

Four female rats were excluded from the results due to inadequate follow-up visits that should show their clinical status. 5th male control rat was near the electric heater during experiment day. He developed massive tissue injury in the following days –probably due to hypoxic+thermal stresses-. His tissue damage was treated by our treatment protocole successfully. Study was finished with 8 male and 4 female rats. No rats died during 67 days of study period. In 2 male rats and 4 female rats variable degrees of gangrene in tails could be induced.

Male rats were observed to resist hypoxia by hyperventilation. We could induce tail gangrene after a minimum of 4 hours of tail squeezing in male rats. But female rats developed cutis marmorata in their tails after 10-15 minutes and tail gangrene could be induced after a minimum of 100 minutes of squeezing.

After adequate tail hypoxia we could differentiate a distal avascular, gangrenous part, a middle part with partial perfusion defect –cutis marmorata was evident in this part- and a proximal well perfused part. We marked these demarcation lines by a pen in some rats.

After variable period of tail squeezing rats developed a coma state and need for ketamine anesthesia diminished after the development of this coma state. Hyperventilation was a persistent finding in all rats at this stage.

After administation of copper solution rats were observed to relax, respirations became regular but coma state and cyanosis were not reversed. After administration of zinc sulphate and n-acetyl cysteine, movements on the face and eyes were observed and hyperventilation and cyanosis subsided in minutes. There were sudden head movements –resembling a kind of startle reflex-.

Imminent exitus risk was reversed in every rat at any time with our protocole. Gangrene in tails of rats in treatment group was restricted only to the most distal avascular part. The border between gangrene and well perfused tissues was sharp in the 2nd day (Figure 2).

In rats of control group, gangrene in tails also included the partially perfused proximal tail tissue (Figure 3). Tails of treatment rats were longer than their controls of the same experiment day (Figure 4).

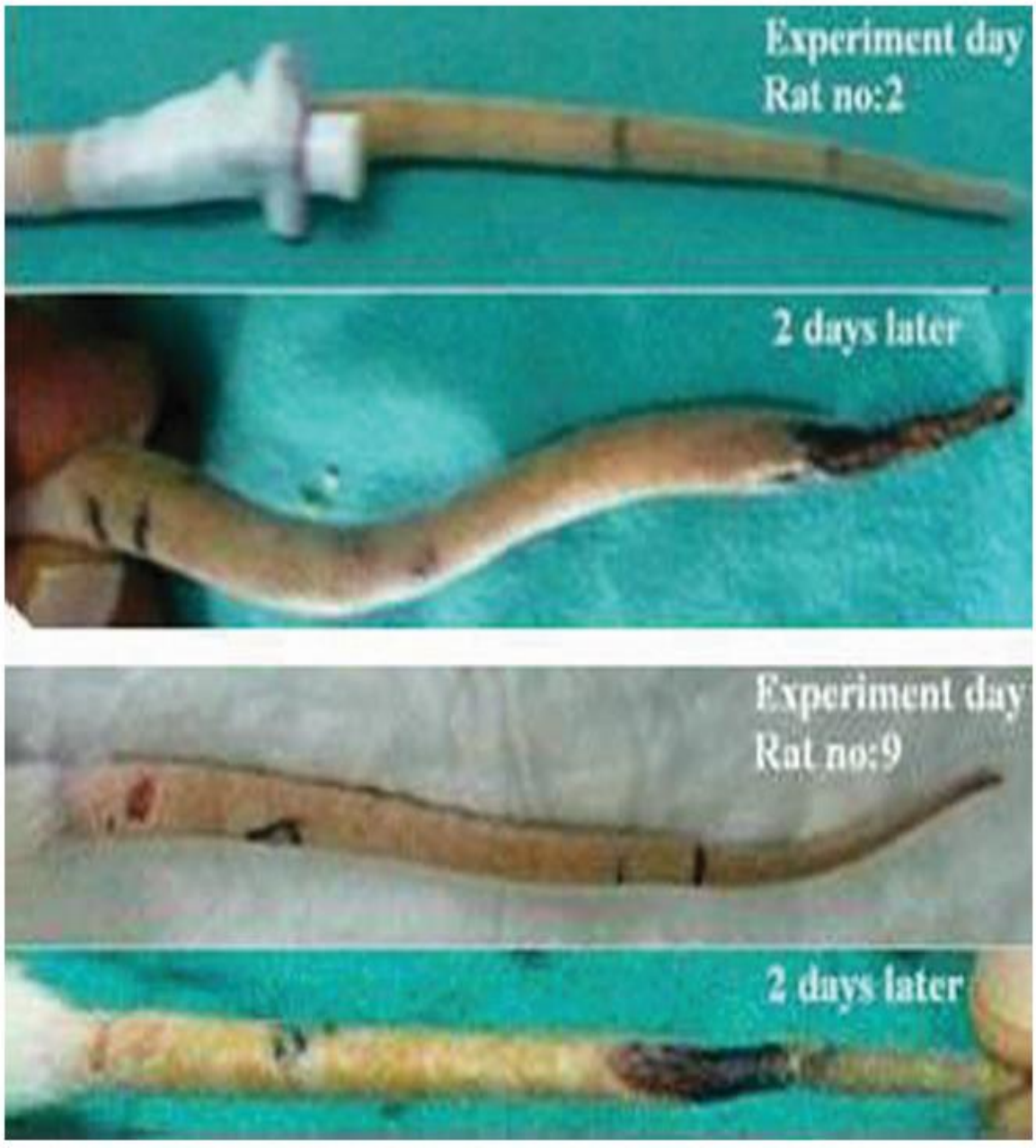


Figure 2. Photographs of tails of two rats in treatment group: rat no:2 was a male, rat no:9 was a female. Please note the restriction of gangrene to poorly perfused, distal tail tissue.



Figure 3. Photographs of rat no:12, a female in control group. Note that although pretreatment condition of her tail was not good, without treatment in experiment day she almost completely lost her tail. We had to administer treatment protocole to her in the following days to prevent exitus.

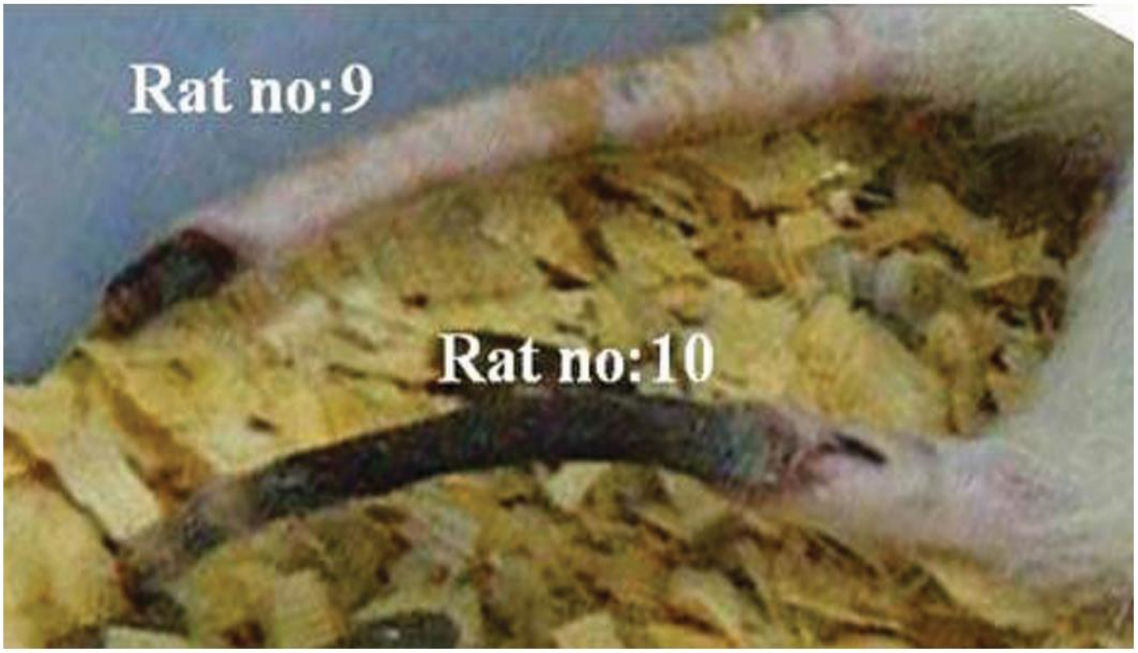


Figure 4. Photograph of tails of rats no:9 –treatment- and no:10 –control- at 14th day of their experiment day.

Rat no:5 was a male control rat. Tail squeezing was performed for 6 hours to this rat. He developed massive tissue damage following experiment day. He was hardly able to walk due to edema in his lower extremities in the 3rd day. He was given treatment protocole in 3rd, 6th and 9th days of experiment day. At 10th day he was able to walk and edema dissapeared. We assumed that we gave enough therapy at this stage but at 23rd day, skin over his left tigh was lost and left testis was visible outside. There was edema in his lower extremities and he was again unable to walk. We administered treatment protocole for 4 additional times in every 2 days. We also added oral sodium bicarbonate to our treatment protocole. In 25th day edema dissappeared again and perfusion of his open wound seemed to increase and surrounding skin was covering the wound. At 31th day wound was almost completely closed by his skin (Figure 5). At the day of euthenasia -57th day-, the defect was completely closed by his skin but skin color was dark pink –instead of bright pink color seen in other rats- and his hairs could be easily pulled out.



Figure 5. Clinical progression of disease in rat no:5. 10th, 24th, 57th days of experiment day.

DISCUSSION

Cellular injury during acute hypoxic stage would be untreatable. During the experiment we observed that our treatment protocole greatly reduces tissue injury during reperfusion – unlike acute hypoxic tissue injury-. By help of this protocole metabolism was able to confine the gangrene to the most distal part of the tail where tissue perfusion was observed to stop during tail hypoxia induction phase, otherwise gangrene was observed to advance proximally (Figure 2 and 3).

We could observe the effectiveness of our treatment protocole in acute stage. The depth of anesthesia reversed within minutes after administration of our protocole. We could also prevent death at any time of the experiment by our treatment protocole. Rat no:5 made us more confident that we could repeatedly reverse chronic tissue injury by our protocole.

Administration of our protocole as an on demand basis would have increased its effectiveness and be with less adverse effects than a fixed interval course therapy because reagents in our protocole should be members of host defense against various type of stresses, indeed they are not unfamiliar chemicals to the metabolism.

We could not find a study in medical literature that is similar to our experiment in the aspects of study design and results. Liability of female rats to hypoxic stress – just similar to human newborns- have let us think that our animal model should be a good representative of neonatal hypoxic-ischemic tissue injury. Nevertheless our approach should be valid for all age groups.

Vitamin C - known to have beneficial effects in hypoxic tissue stress [8]- was added to treatment protocole in an unintentional manner. The role of this vitamin in our results should be delineated by further research. Results of our interventions to rat no:5 showed us that copper ions should give harm to rapidly proliferating tissues esp. skin. New studies should focus on the pathophysiology of this tissue damage.

CONCLUSION

Tissue injury during reperfusion stage of hypoxic-ischemic insults can be diminished by copper ions, zinc sulphate and n-acetyl cysteine.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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