Behavioral Changes in Carbon Monoxide-Intoxicated Rats

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Behavioral Changes in Carbon Monoxide-Intoxicated Rats

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LIST OF ABBREVIATIONS

Abbreviation	Full name
ANOVA	One-way analysis of variance
ATPase	Adenosine Triphosphatase
Ca2+	Calcium
CO	Carbon monoxide
COX	Cytochrome c oxidase
DNS	Delayed neurological sequelae
ELISA	Enzyme-Linked Immunosorbent Assay
EPM	Elavated Plus Maze
FGF21	Fibroblast growth factor 21
GDF15	Growth differentiation factor 15
Hb	Hemoglobin
HBOT	Hyperbaric oxygen therapy
ISR	Integrated stress response
NaCl	Sodium chloride
OFT	Open field Test
Ols	Oligodendrocyte
OPCs	Oligodendrocyte precursor cells
PB	Phosphate buffer
PFA	Paraformaldehyde
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of mean

ABSTRACT

Behavioral Changes in Carbon Monoxide-Intoxicated Rats

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Directed by Professor Kyu-Sang Park

Carbon monoxide (CO) is a colorless and odorless toxic substance produced by incomplete combustion of carbon compounds. CO enters the bloodstream through the lungs and binds with hemoglobin (Hb) forming carboxyhemoglobin (COHb), reducing oxygen delivery throughout the body. A recurrence of delayed onset of neuropsychiatric symptoms after visible recovery from acute CO poisoning is delayed neurological sequelae (DNS). DNS are characterized with lethargy, emotional lability, mutism, cognitive impairment, personality changes, memory deficits, dementia, Parkinsonism, apraxia, aphasia, gait disturbance, and difficulty concentrating within 2 to 42 days after CO poisoning. Despite a high

prevalence of DNS, the prevention of it is currently not available. Organic changes of the brain and clinical manifestations do not show correlation. Thus, it is important to study behavioral changes as well as clinical manifestations to find the correlation of these two factors.

In this study, behavioral studies were done for 8 weeks to compare exploratory, locomotor, and anxiety between CO intoxicated rats and control rats. On immediate CO intoxication, the total distance was 5 times lower in the CO group compared to the control (69.08 ± 17.59 vs. 14.65 ± 8.36). The CO group showed no movement by sitting in the same spot where they were first placed, showing haziness and restrained movement in the open field test. However, there was no significant difference between groups after 3 days till 8 weeks, suggesting that exploratory behaviors being rapidly recovered. General activity in the plus maze between the CO group and control group showed significant difference from immediate CO poisoning. The resting time in the closed arm was 2 times higher in the CO group compared to the control (233.95 ± 166.68 s vs. 457.76 ± 148.11 s). The CO group stayed in the corner in one of the closed arm without movement, while the control group explored equally all arms. While the control group frequently explored with increased rearing and resting in open spaces, CO intoxicated rats preferred closed areas and moved 7 times less towards the open arms (128.20 ± 84.44 s vs. 19.46 ± 26.94 s) suggesting increased anxiety.

However, there was no significant difference between groups after 5 days till 8 weeks, suggesting fast recovery. In conclusion, behavioral studies suggest that acute behavioral changes occur with immediate CO poisoning; however, it was difficult to find long term behavioral changes due to fast recovery of rats. Thus, it was difficult to create a delayed neurological sequelae model. Furthermore, serum FGF21 and GDF15 levels representing oxidative stress and mitochondria damage caused by CO intoxication were increased on the day of CO intoxication indicating sustained and uncompensated mitochondrial stress due to CO intoxication.

Keywords: *Carbon monoxide (CO), Delayed Neurological Sequelae (DNS), Open field test (OFT), Plus maze, Behavior, Fibroblast Growth Factor 21 (FGF21), Growth Differentiation Factor 15 (GDF15)*

I. INTRODUCTION

1.1 Carbon monoxide poisoning

Carbon monoxide (CO) is produced by incomplete combustion of carbon compounds. CO is toxic, colorless, and odorless that usually is undetected before the exposure results injury and death. Inhalations cause serious injuries to humans even in even small quantities [1]. Common sources of CO exposure include stoves, grills, residential fires, malfunctioning heating systems, improperly ventilated motor vehicles, and generators. CO binds to ferrous heme-containing proteins with high affinity. Especially, the binding of CO to hemoglobin (Hb) is 240 times more than the binding to oxygen. CO reduces oxygen carrying capacity by competing with oxygen for binding to Hb. Also, CO binds to cytochromes throughout the body, leading to production of reactive oxygen species and peroxidates brain lipids [2]. Hypoxia that comes from CO intoxication cause variety of symptoms affecting organs and systems such as brain, heart, peripheral nerves, skin, skeletal muscle, and the kidney. In CO poisoning, the most common fatality relates to organs with high demands of oxygen such as brain and heart. Headache and dizziness are the most common symptoms of CO poisoning. Increased CO exposure produces severe neuropsychiatric manifestations, such as emotional disorders, dizziness, confusion, seizures, acute stroke-like syndromes,

paresthesias, convulsions, and coma [3,4].

1.2 Pathophysiological mechanism of CO poisoning

The mechanism of brain damage after CO poisoning remains poorly understood due to high complexity. Since the affinity of CO for Hb is highly stronger than that of oxygen, CO competitively binds to Hb reducing the oxygen carrying capacity via oxygen displacement. Current pathophysiological mechanism of CO intoxication is manifested by hypoxic stress. Since CO binds to heme-containing proteins, binding of CO to cytochrome c oxidase (COX; complex IV) inhibits mitochondrial respiration and oxidative phosphorylation. Thus, CO impairs the function of mitochondria, leading to reactive oxygen species (ROS) production that worsens tissue hypoxia. For instance, decreased ATP production and oxidative phosphorylation cause inactivation of plasma membrane Ca^{2+} ATPase, leading to increased calcium ions flowing into the cells causing calcium overload and brain damage. However, damaged oxygen transport to the cells is not fully explainable for complex cerebral injuries in CO poisoning. CO can directly affect the tissues in the brain, along with changes in cell structure and inflammatory responses involving pathophysiological cascade. Additional potential mechanisms are lipid peroxidation, degradation of unsaturated fatty acids, oxidative stress induced by ROS, free radicals, and neuronal nitric oxide.

Lipid peroxidation can trigger delayed demyelination of white matter. In case of acute CO poisoning, vascular endothelium and platelet cells trigger dysregulated nitric oxide by forming oxygen free radicals such as peroxynitrite. This can lead to mitochondrial dysfunction, apoptosis, leukocyte sequestration, and capillary leakage.

Currently, the pathophysiological mechanism of demyelination after CO intoxication is not fully understood. However, it is believed that oligodendrocytes (OLs) dysfunction and white matter's failure of regeneration in the brain are key pathophysiological mechanisms leading to delayed brain injury via CO intoxication. It is suggested that oligodendrocyte precursor cells (OPCs) are prevented from generating oligodendrocytes due to CO-induced toxicity [5]

1.3 Delayed neurological sequelae

CO induced damage occurs to different degrees of severity depending on different levels and durations of CO exposure. The majority of CO poisoned patients recover from the acute stage of CO intoxication. However, some may exhibit a recurrence of a delayed onset of neuropsychiatric symptoms after visible recovery from acute CO poisoning referred as delayed neurological sequelae (DNS). DNS develop within 3 to 5 weeks in more than 40% of discharged patients with CO poisoning and there are no definite diagnostic criteria [2,6,7].

DNS are characterized as the recurrence of various symptoms such as emotional lability, lethargy, cognitive impairment, mutism, personality changes, memory deficits, dementia, Parkinsonism, apraxia, aphasia, gait disturbance, and difficulty concentrating within 2 to 42 days after CO poisoning [8-10]. Because there are no clear diagnostic criteria, the exact incidence is not known but ranges between 3 to 17% in children and 3 to 40% in adults [9]. CO-mediated delayed neuropathology is related to increased ROS, inflammatory response, depletion of antioxidant defense mechanism, enhancement of lipid peroxidation, and disruption of intracellular oxygen utilization of brain [8,11]. Main pathological features of DNS are demyelination and destruction of neurons in cerebral white matter and globus pallidus induced by CO poisoning [8].

Currently, the pathophysiology of DNS is undefined. The main purpose of the treatment in CO poisoning is to prevent DNS. For instance, hyperbaric oxygen therapy (HBOT) is widely used and is commonly preferred by physicians in order to accelerate the relief of the symptoms in CO poisoning. Therefore, it is important to identify risk factors and give appropriate treatment in preventing DNS. Despite the high prevalence of DNS, the prevention of it is currently not necessary. Organic changes of the brain and clinical manifestations do not show correlation. Thus, it is important to study the early behavioral changes as well as clinical manifestations to find the correlation of these two factors. By doing so,

we are able to establish a diagnosis and prognosis for DNS.

II. HYPOTHESIS

CO intoxication is a cause of neurological stress, so this would further lead to behavioral changes in CO exposed rats.

I have investigated to identify

1. The exploratory and locomotor changes between CO intoxicated rats and control using open field apparatus.
2. The anxiety levels CO intoxicated rats and control using plus maze apparatus
3. The changes in mitochondrial stress levels in serum.

III. MATERIALS AND METHODS

3.1 Reagents

Zoletil (Virbac, France), Rompun (xylazine injection) 2% (Bayer HealthCare, LLC, USA)

3.2 Ethnics of animal experiments

The animal experimental protocol used in this present study was approved by the Institutional Animal Care and Use Committee, Yonsei University Wonju College of Medicine. The approval number for CO intoxicated rats is YWC-190917-3.

3.3 Animals

Fifteen male Sprague Dawley rats weighing 200-230g (eight weeks old) were obtained from DBL (Eumseong, Korea). The rats were housed in plastic cages (2 animals per cage) with free access of food and water in a temperature-controlled room with constant temperature between 22 to 25℃ on a 12 h light and dark cycle. The animals were observed during the acclimatization process for a week after they were brought to the animal laboratory, and behavioral analyses were performed and recorded.

3.4 CO intoxication

The rats were caged in an airtight container with carbon monoxide (CO) gas, air, and oxygen available that is connected to the exhaust system. Firstly, 100% CO was injected until 3000ppm or more was confirmed by the detector inside the sealed container, and afterwards 1000ppm CO gas was circulated in the sealed container for 40 min. After 40 min, 3000ppm of CO gas was circulated in an airtight container for 20 min. Lastly, the sealed container was circulated with 20% oxygen and 80% nitrogen gas until the CO level dropped below 5ppm. After exposure to CO, blood gas analysis was performed once to measure the concentration of CO in blood.

3.5 Evaluation of neurologic and behavioral changes

In order to observe and analyze neurological and behavioral differences between control and CO intoxicated rats, open field and plus maze training was done two days prior to CO intoxication. The base line experiment was performed one day after maze training. After CO intoxication, open field and plus maze tests were each performed for day 0, 3, 5, 7, 14, 21, 28, 6 weeks, and 8 weeks. Open field and plus maze behaviors were tracked and analyzed using SMART video tracking system (Panlab/Harvard apparatus) (Figure 1A).

3.5.1 Open field

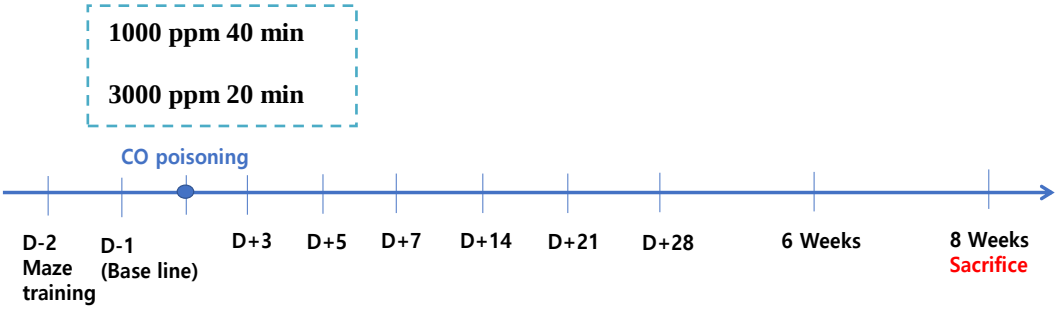
Rats were placed in the center of the box made of formax (80 x 80 x 40cm) in a brightly lit room (Figure 1B). They were allowed to explore the arena freely for total 10 min. Placing a rat in the open field, the amount of time it spends walking around the outer edge of the box vs. the center of the box was observed. Also, the total distance moved around the box, or the numbers of behaviors such as rearing, stretch attend postures, grooming, and freezing was observed. The movements were all tracked and analyzed using SMART video tracking system (Panlab/Harvard apparatus). After each test, the apparatus was cleaned with 70% ethanol to remove remaining odors.

3.5.2 Plus maze

The plus maze uses a plus-shaped apparatus consisting total four arms; two open and two enclosed arms. The apparatus used comprises two open arms (90 x 90 x (L) 700mm) across from each other and perpendicular to two closed arms (90 x 90 x (L) 700mm) with a center platform (Ø 1780 x (H) 300mm). The apparatus is made of acryl (black), and the closed arms have transparent covers on top (Figure 1C). Each rat was placed at the center of the maze with its head positioned toward a closed arm. The rats were allowed to explore all the arms freely for total

10 min. The movements were all tracked and analyzed using SMART video tracking system (Panlab/Harvard apparatus). After each test, the apparatus was cleaned with 70% ethanol to remove remaining odors.

A



B

C

Figure 1. Experimental design and schedule for behavioral tests. (A) Prior to CO intoxication, open field and plus maze training was done two days before. Base line experiment was done one day after maze training. After CO intoxication, open field and plus maze tests were done for day 0, 3, 5, 7, 14, 21, 28, 6 weeks, and 8 weeks 10 min each. At the end of the experiment, animals were sacrificed and blood samples were collected for determination of serum oxidative stress markers. (B) Open field apparatus. (C) Plus maze apparatus.

3.6 Blood sample collection

Blood samples (5ml) were collected from the rat heart in serum separating tubes (SST; Vacutainer SST II advance 8.5mL, cat no.367953, BD, Belliver Way, Roborough, UK). Serum was collected from the blood after centrifugation at 3000 rpm for 20 min at 4 ℃. Then, the serum samples were aliquot 200µl each into EP tubes and stored in -80℃ for future experiments.

3.7 ELISA

Fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) were measured with serum using Enzyme-Linked Immunosorbent Assay (ELISA). FGF21 quantikine ELISA kit (cat no. MF2100, R&D systems, 614 McKinley Place NE, Minneapolis, MN 55413, USA) and GDF15 quantikine ELISA kit (cat no. MGD150, R&D systems, 614 McKinley Place NE, Minneapolis, MN 55413, USA) was used to measure four experimental groups (day 0, day 3, day 7, 8 weeks, and control). All reagents such as the wash buffer, standard, calibrator diluents, assay diluents, substrate solution, and samples were brought to room temperature before use. In case of FGF21, serum samples required 2-fold dilutions and both samples and standard curve were duplicated when loaded onto the plate. First, 50ul of assay diluents were loaded, and 50ul of samples, control, and standard were loaded to each well. The plate was incubated

for 2 hours at room temperature. For GDF15, the plate required shaking using a microplate shaker set at 500 rpm. After aspiration and total of 4 washes, 100ul of conjugate was added and incubated in room temperature for 2 hours. Then, after the repeat of 4 washes 100ul of color substrate solution was added to each well and incubated in room temperature with the protection of light for 30 min. The optical density of each well was determined within 30 min using a microplate reader (SN. 17080220; BioTek Instruments, 100 Tigan street Highland park, Winooski, VT, USA) set to 450nm. The standard curve was created by reducing the data using computer software that generates a four parameter logistic curve fit. The average of duplicate readings was made for each standard, control, and sample generated by the standard curve.

3.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 8.0, GraphPad Software, San Diego, CA, USA). Statistical comparisons between two groups were made using two-tailed unpaired Student's *t*-test. Multiple comparisons were made using one-way ANOVA along with Tukey's multiple comparison tests. All data were presented as mean ± SEM, and P values less than 0.05 were considered as statistically significant for single and multiple comparisons.

IV. RESULTS

4.1 Exploratory behavior and general activity in open field test

Normally, rodents have natural aversion to brightly lit open areas. So, increased exploratory behavior represents decreased levels of anxiety. Less locomotion represents increased anxiety and preference to stay close to the walls of the field. When placing a rat in the open field test, the amount of time it spends walking around the outer edge of the box vs. the center of the box can be observed. Also, we can estimate the total distance moved around the box, as well as the numbers of behaviors such as rearing, stretch attend postures, grooming, and freezing [12].

The rats were placed in the center of the box in a brightly lit room. They were allowed to explore the arena freely for 10 min. Exploratory activities were tracked and analyzed after behavioral data were all compiled for total 8 weeks before and after CO poisoning. The total distance of rats in the open field was significantly different between the control group and CO intoxicated group on day 0 immediately after CO poisoning ($p \leq 0.001$). In case of the CO intoxicated group on day 0, there was barely any movement immediately after CO intoxication and throughout the behavior tracking. In other words, CO intoxicated rats preferred to

sit in the corner and showed no movement. According to my observations, stress response was shown as there was increased fecal material from the CO intoxicated group during behavior tracking. However, there was no significant difference between groups after three days till 8 weeks ($p>0.05$), suggesting that exploratory behaviors are recovered in CO intoxicated rats (Figure 2).

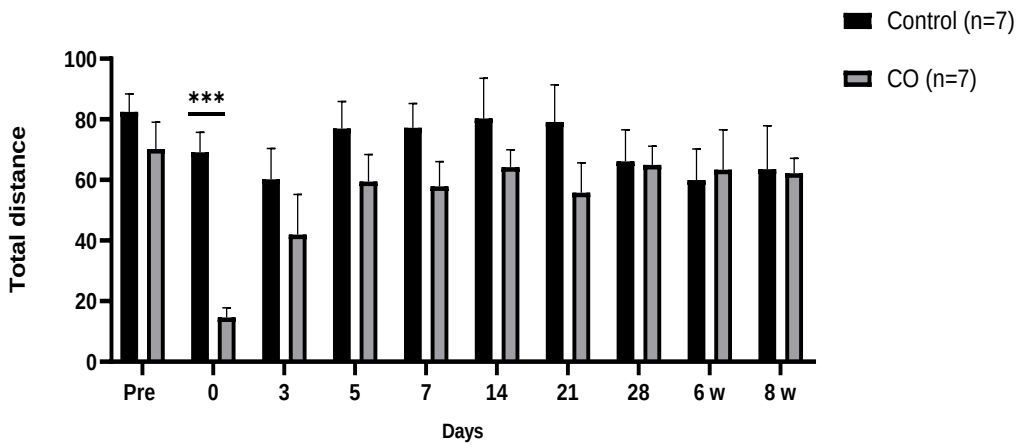


Figure 2. Total distance in open field test. The locomotor and exploratory activities were analyzed for total 8 weeks after CO poisoning. The locomotive distance of rats in open field was significantly different between the control group and CO intoxicated group on day 0 immediately after CO poisoning ($p \leq 0.001$). However, there was no significant difference between the groups after three days till 8 weeks ($p > 0.05$). Data was presented as mean ± SEM. Differences were analyzed using two-tailed unpaired *t*-test ***$p \leq 0.001$.n=7 for each group.

4.2 Anxiety-like behavior in plus maze test

The plus maze is plus-shaped, consisting total four arms; two open and two enclosed arms. This model uses the general aversion to open spaces leading to thigmotaxis of rats. Thigmotaxis is the tendency to remain close to the walls. In case of increased anxiety, rats limit their movement to the enclosed arms. Oppositely, reduction of anxiety is indicated by increased proportion of time spent in the open arms and an increase proportion of entries into the open arms.

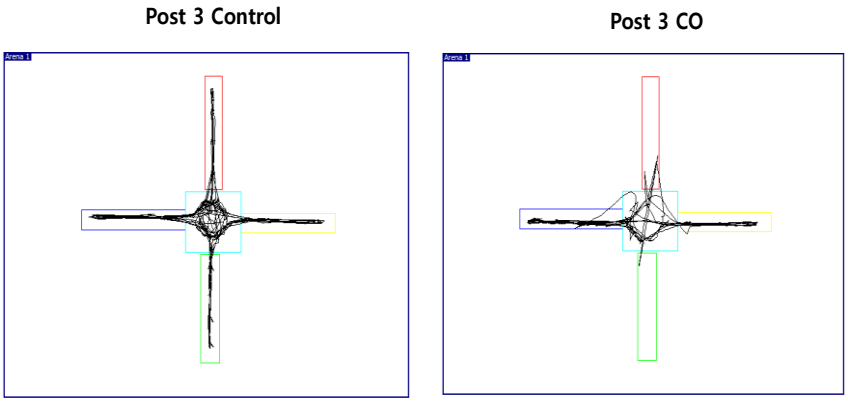
The rats explored all the arms freely for total 10 min. The movements were tracked and analyzed after behavioral data were all compiled for total 8 weeks before and after CO poisoning. In order to measure the levels of anxiety and depression, percent time in closed arm and resting time in closed arm was analyzed. Percent time in closed arm was significantly different between the control group and CO intoxicated group on day 0 immediately after CO poisoning ($p \leq 0.001$). However, there was no significant difference between groups after three days ($p > 0.05$) (Figure 3B). Similarly, resting time in closed arm was significantly different between the control group and CO intoxicated group on day 0 immediately after CO poisoning ($p \leq 0.01$). However, there was no significant difference between the groups after three days ($p > 0.05$) (Figure 3C).

In case of resting time in open arm, the control group tends to move more frequently and spends more time in the open arms. Whereas CO intoxicated rats

moved less towards the open arms. Thus, there was significant differences between the control group and CO intoxicated group on day 0 ($p \leq 0.05$) and day 5 after CO poisoning ($p \leq 0.01$) (Figure 3D).

According to the sample traces in the plus maze test, the pattern of movement between CO intoxicated group and control differs. While the control group equally goes through all four arms, both open and closed, CO intoxicated rats move back and forth among the closed arms. This would suggest increased anxiety or depression levels of CO intoxicated rats (Figure 3A).

A



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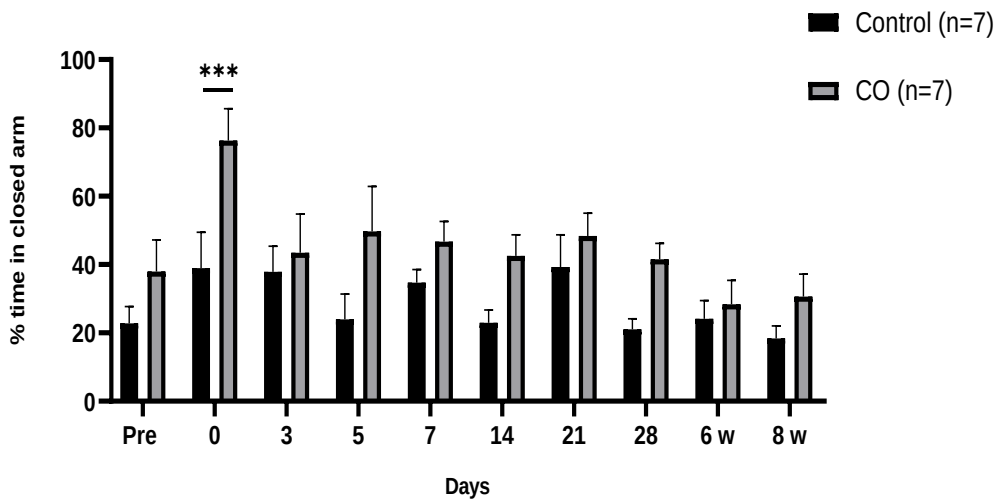

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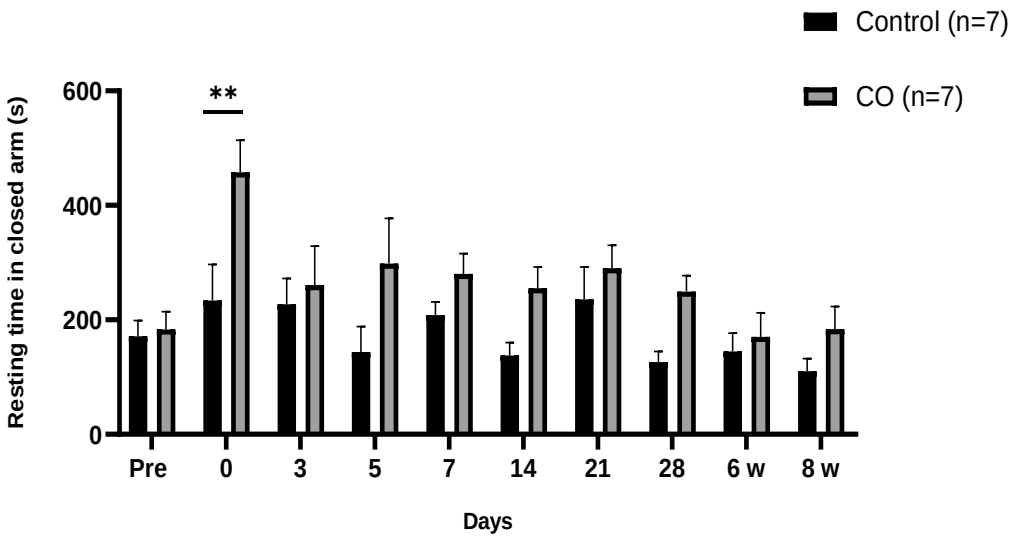

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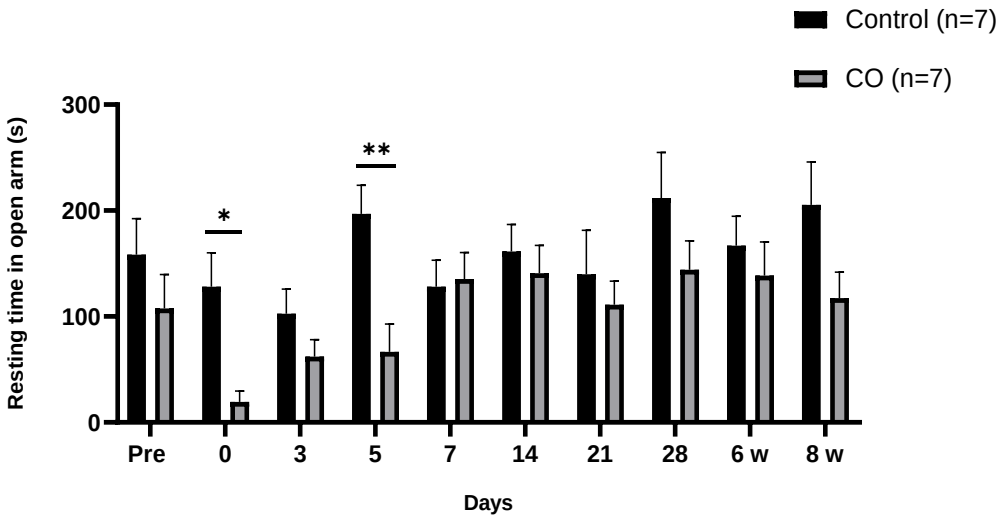

Figure 3. Percent time and resting time in arm entries using plus maze test. Anxiety and depression were analyzed for total 8 weeks after CO poisoning. (A) Sample traces in the plus maze test. (B) The percent time in closed arm of rats was significantly different between the control group and CO intoxicated group on day 0 immediately after CO poisoning ($p \leq 0.001$). However, there was no significant difference between groups after three days ($p > 0.05$). (C) Resting time in closed arm was significantly different between the control group and CO intoxicated group on day 0 immediately after CO poisoning ($p \leq 0.01$). However, there was no significant difference between groups after three days ($p > 0.05$). (D) Resting time in open arm was different between the control group and CO intoxicated group on day 0 ($p \leq 0.05$) and day 5 after CO poisoning ($p \leq 0.01$). Data was presented as mean ± SEM. Differences were analyzed using two-tailed unpaired *t*-test. ***$p \leq 0.001$, **$p \leq 0.01$. n=7 for each group.

4.3 Increased serum levels of FGF21 and GDF15 in CO intoxicated rats

Serum samples were collected and used to measure four experimental groups (day 0, day 3, day 7, 8 weeks, and control) with ELISA. Serum levels of FGF21 were increased on the day of CO intoxication ($p \leq 0.05$) and levels decreased sequentially till day 7. After day 7, FGF21 levels of CO intoxicated rats were near the level of the control group (Figure 4A). In case of GDF15 serum levels, there was a significant increase on the day of CO intoxication ($p \leq 0.001$), and levels became similar to the control group after day 3 (Figure 4B). Since serum levels of FGF21 and GDF15 were both increased on the day of CO intoxication, it could be an indication of sustained and uncompensated mitochondrial stress due to CO intoxication.

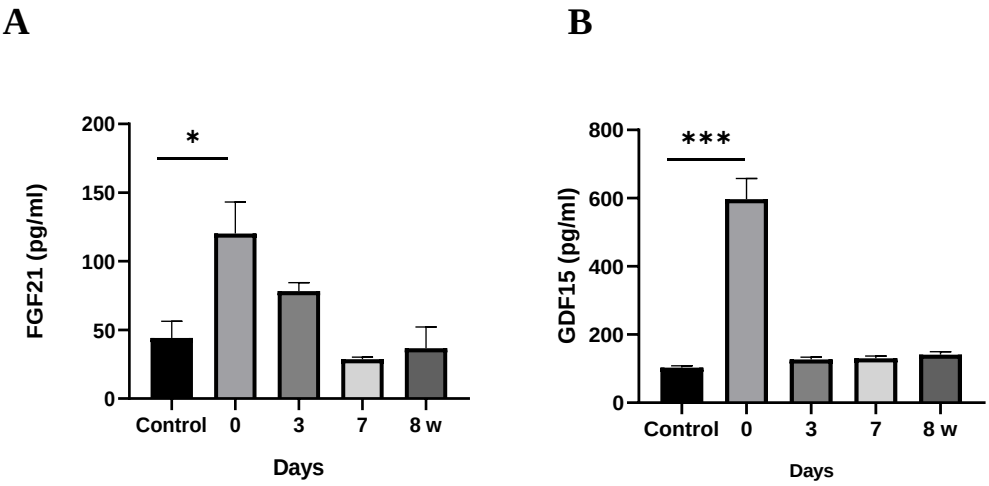

Figure 4. Serum concentration of FGF21 and GDF15 in control and CO intoxicated rats. (A) Serum levels of FGF21 were increased on the day of CO intoxication ($p \leq 0.05$) and levels decrease sequentially till day 7. (B) Serum levels of GDF15 were significantly increased on the day of CO intoxication ($p \leq 0.001$), and levels became similar to the control group after day 3. Data was presented as mean ± SEM. Differences were analyzed using one-way ANOVA (Tukey's post-hoc test). ***$p \leq 0.001$, *$p \leq 0.05$. n=3 for each group.

V. DISCUSSION

CO poisoning which is the most frequent intoxication in the world may cause many serious complications, especially acute and delayed brain damage. However, its definite pathogenesis is not yet clear. In recent years, studies mainly focused on the anoxic and metabolic pathophysiology about CO poisoning. However, this view incompletely explains some of the outcome and needs to be investigated. The effects of CO poisoning are difficult to diagnose because they are non-specific. Neuropsychological deficits occur immediately after CO exposure or delayed, presenting most symptoms within 20 days of CO exposure. Furthermore, psychiatric changes are shown such as anxiety, depression, delusions, hallucinations, and obsessive-compulsive behaviors. Although cognitive sequelae after primary exposure to CO are relatively well written in literature, the impact of CO poisoning on delayed neuropsychological functioning is still not clear [13].

The aim of this study was to develop a delayed neurological sequelae rat model caused by CO exposure, and determine long term neurologic and behavioral differences between CO intoxicated rats and healthy controlled rats. In addition, since oxidative stress contributes mainly in the pathogenesis of CO intoxication, mitochondrial stress-inducible humoral factors, such as FGF21 and

GDF15 was measured to compare mitochondrial stress levels between CO intoxicated rats and healthy controlled rats.

The open field test is known to observe anxiety-like behavior, locomotion, and exploratory behavior. This provides evaluation of drug related effects on various aspects of animal behavior. Rearing frequency and number of line crosses are usually used to measure locomotor activity. Currently, there are other factors that allow measures of exploration and anxiety [14]. Increased levels of such behaviors indicate a lower level of anxiety as well as increased exploration and locomotion. Generally, increased number of rears is associated with emotional changes and increased tendency to explore [15,16]. In addition, number, latency, and duration of central square entries are measures of anxiety and exploratory behavior. Since rodents mostly prefer staying in the peripheral zone, increased time spent in the center of the open field or showing decreased latency in central zone are indicators of anxiolysis [17]. Grooming behavior is referred to as a displacement response displayed in any unusual environment. Thus, grooming may represent anxiety in a stressful condition, having a role of as a behavioral marker [18]. Furthermore, defecation represents number of fecal boli that is also a measure of anxiety [19,20].

The elevated maze is a simple method to measure anxiety responses of rodents. This is used to analyze anxiety by the ratio of time spent on open and

closed arms. Other behavioral tests using anxiety responses normally rely on noxious stimuli such as food or water deprivation, exposure predator odors, electric shock, and loud noises that produce conditioned responses. On the other hand, elevated plus maze relies on rodent's approach toward dark and enclosed spaces as well as unconditioned fear of open spaces and height [21]. The behavior in the elevated plus maze are evaluated and quantified by an observer in which the rodents are typically recorded for 5 min being placed in the intersection of four arms. Studies have demonstrated that rats show the strongest avoidance in the first 5 min after being placed, and the behavior reflects a conflict between the preferences of closed arms and their intrinsic response to explore newer environments. Increased time spent in open arms or total number of open arm entries can determine anti-anxiety [22-24].

This study used two types of behavior apparatus; open field and plus maze to measure general activity and exploratory behavior by tracking and analyzing for 10 min, total 8 weeks before and after CO poisoning. In case of general activity, total distance of rats in open field was significantly different between the control group and CO intoxicated group on day 0 immediately after CO poisoning. This was because the CO intoxicated group showed no movement by sitting in the same spot where the rats were first placed. Thus, immediate CO intoxication led to haziness and restrained movement. However, there was no significant

difference between groups after 3 days till 8 weeks, suggesting that exploratory behaviors were rapidly recovered in CO intoxicated rats. In case of resting time in open arms and closed arms using the plus maze, general activity between the control group and CO intoxicated group showed significant difference on day 0 of immediate CO poisoning. CO intoxicated group stayed in the corner of one of the closed arm without movement, while the control group explored equally all arms for full 10 min. And in case of resting time in open arms, the control group frequently explored with increased rearing and resting in open spaces. However, CO intoxicated rats preferred closed areas and moved less towards the open arms suggesting increased anxiety. However, there was no significant difference between groups after 5 days till 8 weeks, suggesting that CO intoxicated rats were also fastly recovered in the plus maze. In conclusion, this behavioral study suggests that acute CO intoxication may lead to behavioral changes with immediate CO poisoning; however, it is difficult to find long term behavioral changes due to fast recovery of rats.

Currently, oxidative stress is known to be a major contributor of the pathogenesis of CO intoxication. CO can bind to mitochondrial cytochrome c oxidase, which inhibits electron transport and enhances superoxide generation. Oxidative stress can cause abnormalities in the function and mitochondrial structure due to the damage of mitochondrial components. Especially,

mitochondrial and ER stresses lead to vicious cycles involving CO intoxication's pathogenic mechanisms. In order to overcome cellular stresses and diminish pathologic progression, our body generates integrated stress response (ISR) allowing mitochondrial and metabolic flexibilities. For example, there is upregulation and secretion of mitochondrial stress induced humoral factors, such as fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15). However, uncompensated and sustained mitochondrial stresses lead to increased circulating FGF21 and GDF15. Thus, serum concentration of these factors is an indication of mitochondrial stress [25].

In this study, I measured serum FGF21 and GDF15 levels which represents oxidative stress and mitochondria damage caused by CO intoxication. Serum levels of FGF21 were increased on the day of CO intoxication and sequentially decreased till day 7. After day 7, FGF21 levels of CO intoxicated rats were near the level of the control group. GDF15 serum levels were significantly increased on the day of CO intoxication and levels became similar to the control group after day 3. Both serum levels of FGF21 and GDF15 were increased on the day of CO intoxication, it could be an indication of sustained and uncompensated mitochondrial stress due to CO intoxication.

VI. CONCLUSION

The total distance in open field was significantly different between the control group and CO intoxicated group immediately after CO poisoning. The CO intoxicated group showed no movement by sitting in the same spot where the rats were first placed showing haziness and restrained movement. However, there was no significant difference between groups after 3 days till 8 weeks, suggesting that exploratory behaviors were fastly recovered in CO intoxicated rats. General activity between the CO intoxicated group and control group showed significant difference from immediate CO poisoning. CO intoxicated group stayed in the corner of one of the closed arm without movement, while the control group explored equally all arms. While the control group frequently explored with increased rearing and resting in open spaces, CO intoxicated rats preferred closed areas and moved less towards the open arms suggesting increased anxiety. However, there was no significant difference between groups after 5 days till 8 weeks, suggesting rapid recovery of CO intoxicated rats.

In conclusion, behavioral studies suggest that acute behavioral changes occur with immediate CO poisoning. Serum FGF21 and GDF15 levels representing oxidative stress and mitochondria damage were also increased at the early period

of CO intoxication, indicating that there were sustained and uncompensated mitochondrial stresses due to CO toxicities. However, it was difficult to find long term behavioral changes due to fast recovery of rats, which implies that the condition used in this study is not appropriate for the development of delayed neurological sequelae model.

VII. FUTURE STUDY

1. In order to analyze specific behavior in detail using open field and plus maze, rearing and grooming behaviors as well as acceleration of movements should be added in the future.
2. Various behavioral tools should be added as parameters, such as passive-avoidance, sucrose preference, Morris water maze, and 8 arm radial maze.
3. Instead of using plus maze apparatus, it is recommended to use an elevated plus maze to increase the level of anxiety and fear response.
4. For elaborate explanation of this study, adding histological data using luxol fast blue staining and immunohistochemistry (IHC) is required (Iba1: expression of microglia and macrophages NeuN: identify mature neurons).

VIII. REFERENCES

1. Guzman JA. Carbon monoxide poisoning. Crit Care Clin 2012;28:537-48.

2. Rose JJ, Wang L, Xu Q, McTiernan CF, Shiva S, Tejero J, Gladwin MT. Carbon Monoxide Poisoning: Pathogenesis, Management, and Future Directions of Therapy. Am J Respir Crit Care Med 2017;195:596-606.

3. Katirci Y, Kandis H, Aslan S, Kirpinar I. Neuropsychiatric disorders and risk factors in carbon monoxide intoxication. Toxicol Ind Health 2011;27:397-406.

4. Yeh ZT, Tsai CF, Yip PK, Lo CY, Peng SM, Chen SY, Kung LY. Neuropsychological performance in patients with carbon monoxide poisoning. Appl Neuropsychol Adult 2014;21:278-87.

5. Guo D, Hu H, Pan S. Oligodendrocyte dysfunction and regeneration failure: A novel hypothesis of delayed encephalopathy after carbon monoxide poisoning. Med Hypotheses 2020;136:109522.

6. Betterman K, Patel S. Neurologic complications of carbon monoxide intoxication. Handb Clin Neurol 2014;120:971-9.

7. Oh S, Choi SC. Acute carbon monoxide poisoning and delayed neurological sequelae: a potential neuroprotection bundle therapy. Neural Regen Res 2015;10:36-8.

8. Liao SC, Mao YC, Yang KJ, Wang KC, Wu LY, Yang CC. Targeting optimal time for hyperbaric oxygen therapy following carbon monoxide poisoning for prevention of delayed neuropsychiatric sequelae: A retrospective study. J Neurol Sci 2019;396:187-92.

9. Sarı Doğan F, Güneysel Ö , Gökdağ E, Güneş M, Sümen SG. Demographic characteristics and delayed neurological sequelae risk factors in carbon monoxide poisoning. Am J Emerg Med 2020;38:2552-6.

10. Zhao N, Liang P, Zhuo X, Su C, Zong X, Guo B, Han D, Yan X, Hu S, Zhang Q, Tie X. After Treatment with Methylene Blue is Effective against Delayed Encephalopathy after Acute Carbon Monoxide Poisoning. Basic Clin Pharmacol Toxicol 2018;122:470-80.

11. Mazo J, Mukhtar E, Mazo Y, Nagaraj A, Mantello MT. Delayed brain injury post carbon monoxide poisoning. Radiol Case Rep 2020;15:1845-8.

12. Seibenhener ML, Wooten MC. Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. Journal of visualized experiments : JoVE 2015:e52434-e.

13. Watt S, Prado CE, Crowe SF. Immediate and Delayed Neuropsychological Effects of Carbon Monoxide Poisoning: A Meta-analysis. J Int Neuropsychol Soc 2018;24:405-15.

14. Zimcikova E, Simko J, Karesova I, Kremlacek J, Malakova J. Behavioral effects of antiepileptic drugs in rats: Are the effects on mood and behavior detectable in open-field test? Seizure 2017;52:35-40.

15. Sturman O, Germain PL, Bohacek J. Exploratory rearing: a context- and stress-sensitive behavior recorded in the open-field test. Stress 2018;21:443-52.

16. Wang YL, Han QQ, Gong WQ, Pan DH, Wang LZ, Hu W, Yang M, Li B, Yu J, Liu Q. Microglial activation mediates chronic mild stress-induced depressive- and anxiety-like behavior in adult rats. J Neuroinflammation 2018;15:21.

17. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 2003;463:3-33.

18. Smolinsky AN, Bergner C, LaPorte J, Kalueff A. Analysis of Grooming Behavior and Its Utility in Studying Animal Stress, Anxiety, and Depression. 2009

19. Mareš P, Tichá K, Mikulecká A. Anticonvulsant and behavioral effects of GABAB receptor positive modulator CGP7930 in immature rats. Epilepsy & Behavior 2013;28:113-20.

20. Espejo EF. Effects of weekly or daily exposure to the elevated plus-maze in male mice. Behavioural Brain Research 1997;87:233-8.

21. Barnett SA: *The rat: A study in behavior*, Transaction Publishers, 2007

22. Montgomery K. The relation between fear induced by novel stimulation and exploratory drive. Journal of comparative and physiological psychology 1955;48:254.

23. Kim SS, Wang H, Li X-Y, Chen T, Mercaldo V, Descalzi G, Wu L-J, Zhuo M. Neurabin in the anterior cingulate cortex regulates anxiety-like behavior in adult mice. Molecular Brain 2011;4:6.

24. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nature Protocols 2007;2:322-8.

25. Chang JS, Chang E, Lee Y, Cha YS, Cha SK, Cho WG, Jeong Y, Kim H, Park KS. Hyperbaric Oxygen Exposure Attenuates Circulating Stress Biomarkers: A Pilot Interventional Study. Int J Environ Res Public Health 2020;17

IX. ABSTRACT IN KOREAN

일산화탄소 (CO)는 탄소 화합물의 불완전 연소로 생성되는 무색 무취의 독성 물질입니다. CO는 폐를 통해 혈류로 들어가 헤모글로빈 (Hb)과 결합하여 카르복시 헤모글로빈 (COHb)을 형성하여 몸 전체의 산소 전달을 감소시킵니다. 급성 CO 중독에서 가시적인 회복 후 신경정신과 증상의 발생이 지연되는 것은 지연된 신경 후유증(DNS)이라고 합니다. DNS는 CO 중독 후 2-42일 뒤에 무기력증, 정서적 불안정성, 인지 장애, 성격 변화, 기억력 결핍, 치매, 파킨슨증, 실어증, 보행 장애, 집중력 저하 등의 특징들로 나타납니다. DNS의 높은 발병률에도 불구하고 현재로써는 이를 방지 할 수 없습니다. 게다가, 뇌의 유기적 변화와 임상 증상은 상관 관계를 나타내지 않습니다. 따라서 이러한 두 요인의 상관 관계를 찾기 위해 행동 변화와 임상 증상을 연구하는 것이 중요합니다.

이 연구에서는 CO에 중독 된 쥐와 대조군 쥐 사이의 탐색 적, 운동성 및 불안을 비교하기 위해 8 주 동안 행동 연구를 수행 했습니다. Open field test에서 CO 그룹은 처음 배치 된 장소와 동일한 위치에 앉아 아무런 움직임도 보이지 않았으며, 제한된 움직임을 보여주었습니다. 그러나

3 일 후부터 8 주까지 그룹간에 유의 한 차이가 없었으며, 탐색 적 행동이 빠르게 회복되고 있음을 보여주었습니다. CO 그룹과 대조군 사이의 plus maze에서의 일반적인 활동은 즉각적인 CO 중독에서 상당한 차이를 보였습니다. CO 그룹은 움직이지 않고 closed arm 중 하나의 구석에 머물렀고, 대조군은 모든 arm을 동등하게 탐색했습니다. 대조군은 열린 공간에서 뒷다리로 서는 행동과 휴식을 증가시키는 것으로 탐색이 많았지만, CO에 중독 된 쥐는 닫힌 공간을 선호하고 불안이 증가했음을 암시하는 open arm 쪽으로 덜 움직였습니다. 그러나 5 일부터 8 주까지 그룹간에 유의 한 차이가 없어 빠른 회복을 보여주었습니다. 결론적으로 행동 연구는 급성 CO 중독에는 쥐들의 행동 변화가 즉각적으로 발생합니다. 그러나 쥐의 빠른 회복으로 인해 장기적인 행동 변화를 찾기가 어려웠습니다. 따라서 DNS 모델을 만드는 것에 한계가 있습니다. 또한, CO 중독으로 인한 산화 스트레스 및 미토콘드리아 손상을 나타내는 혈청 FGF21 및 GDF15는 CO 중독 당일에 증가하여 CO 중독으로 인한 미토콘드리아 스트레스를 잘 반영하였습니다.

X. PUBLICATION LIST

1. Jae Seung Chang, **Eunha Chang**, Yoonsuk Lee, Yong Sung Cha, Seung-Kuy Cha, Won Gil Cho, Yangsik Jeong, Hyun Kim, Kyu-Sang Park. Hyperbaric Oxygen Exposure Attenuates Circulating Stress Biomarkers: A Pilot Interventional Study. *Int J Environ Res Public Health* 2020;17

2. **Eunha Chang**, Jae Seung Chang, In Deok Kong, Soon Koo Baik, Moon Young Kim, Kyu-Sang Park. Multidimensional Biomarker Analysis Including Mitochondrial Stress Indicators for Non-alcoholic Fatty Liver Diseases. *Gut and Live*r Jun 2021 (accepted)