Trabajo Original **Toxicología Experimental**

Lower sensitivity to copper toxicity in female *Drosophila melanogaster.*

Arcaya JL, Salazar U, Morales L, Moncada Y, Bonilla E.

Sección de Neuroquímica Clínica. Instituto de Investigaciones Clínicas Dr. Américo Negrette. Facultad de Medicina. Universidad del Zulia. Maracaibo, Venezuela.

Corresponding autor: Arcaya JL.

e-mail: jlarcaya@yahoo.com

Abstract

In a previous study, we showed that copper induces mortality and behavioral disturbances in male *Drosophila melanogaster*. The aim of the present study was to evaluate the sensitivity of female flies to copper overload as compare to males. Flies of both sexes were supplemented with copper 31 and 47 μM in the feeding medium and their survival was assessed. In contrast to males, female´s lifespan was increased by copper. In addition, the resistance to starvation stress and spontaneous motor activity after 10 days of treatment with 31 μM copper was evaluated in flies of both sexes. A reduction of starvation resistance and spontaneous motor activity was detected in male intoxicated flies but not in females. Altogether, these results suggest a sexually dimorphic response since female *Drosophila melanogaster* flies have a higher capacity to withstand the deleterious effects of copper overload observed in male flies.

Key words: Copper toxicity, sex dimorphism, starvation resistance, *Drosophila*.

Resumen

Menor sensibilidad a la toxicidad por cobre en *Drosophila melanogaster* hembra

En un estudio previo, reportamos que el cobre induce mortalidad y alteraciones del comportamiento en *Drosophila melanogaster*. El objetivo del presente estudio fue evaluar la sensibilidad de de las moscas hembras a la sobrecarga con cobre en comparación con los machos. Moscas de ambos sexos fueron suplementadas con cobre 31 y 47 µM en el medio de cultivo y se evaluó la sobrevida. Además, se determinó la resistencia al estrés por inanición y la actividad motora espontánea luego de 10 días de tratamiento con cobre en moscas de ambos sexos. En contraste con los machos, la sobrevida de las moscas hembras fue aumentada en el grupo tratado. Además, se observó una disminución de la resistencia a la inanición y de la actividad motora espontánea en los machos, pero no en las hembras. En conjunto, estos resultados sugieren un dimorfismo sexual ya que las *Drosophila melanogaster* hembras tienen una mayor capacidad de soportar los efectos deletéreos de la sobrecarga con cobre en comparación con los machos.

Palabras clave: Toxicidad, Cobre, dimorfismo sexual, inanición, *Drosophila*.

Introduction

Copper is a heavy metal with a fundamental role on the function of diverse enzymatic systems (1). Copper dishomeostasis caused by the elevated ingestion or by genetic defects affecting its regulatory mechanisms causes toxicity and is related to several diseases. Previous studies have demonstrated the induction of mortality and alterations of dopaminergic cells in the brain of *Drosophila melanogaster* intoxicated with heavy metals (2,3). A growing body of evidence suggests the existence of gender specific differences in the susceptibility to several toxins (4,5). However, the question of what is the impact of toxic agents on both genders has not been sufficiently addressed. In fact, other authors have pointed out the necessity to evaluate the differences in disease pattern, response to drug therapy, and the exposure to environmental toxins (6,7).

In the present study we evaluated the sensitivity of female flies to copper intoxication. In contrast to males, the lifespan of female flies was increased by copper. In addition, the feeding of copper did not affect starvation resistance and spontaneous motor activity of female flies. These results suggest a lower susceptibility to copper toxicity in females compared to males. Further studies are needed to uncover the mechanisms of this sex specific effect.

Materials and methods

4.1.- Animals:

Male and female flies of the Oregon strain of *Drosophila melanogaster* were used. The basic culture medium was prepared as described earlier (3). In brief, 0.3 g of agar-agar, 5 g of corn flour, 1.5 g of bakers yeast, 5 ml of a saturated (50% w/v) of brown sugar and 0.065 g of methyl-parahydroxibenzoate diluted in 0.5 ml of absolute ethanol were mixed in 43.75 ml of distilled water. The preparation was heated to boiling point with constant agitation and then dispensed in glass vials and let solidify. Then, flies were separated by sex and transferred to the vials. Five flies were placed in each tube containing standard medium in the control group or medium supplemented with copper or other drugs at the concentrations specified below. Flies were maintained in a constant cycle of 12 hours light by 12 hours darkness. Room temperature was set at 25 °C, and humidity was not controlled.

4.2.- Intoxication with copper: Effect on lifespan

Our first goal was to determine if doses of copper that have been previously shown to induce mortality in male flies also decreased the lifespan of female flies. The control group was maintained in a standard corn flour medium while the treated groups were supplemented with 31 µM copper (CuSo4, Sigma-Aldrich). Copper was solved in the

medium just before dispensing in the culture vials. Survival of the flies was scored thrice a week when they were transferred to fresh medium.

4.3.- Copper intoxication and starvation resistance.

Since lifespan has been associated to stress resistance, we wanted to evaluate the response to starvation of both males and females treated with copper. In these experiments flies were fed with copper for ten days and then were transferred to empty glass tubes in which a piece of filter paper was placed and humidified with distilled water. Survivorship was scored every two hours until all flies were dead. Paper was humidified as needed so that the flies did not dehydrate. One hundred flies were used in each experiment and at least two replicates of each experiment were performed.

4.4.- Spontaneous motor activity under normal feeding conditions.

The spontaneous motor activity (SMA) was measured in flies that were maintained in standard medium or supplemented with a 31 μM concentration of copper in the feeding medium. Thirty flies per group were used in each reading and the observations were made at the start of treatment (day 2) and 10 days after the initiation of intoxication with copper (Figure 3A). Fly activity was recorded using the DAM 2 monitor (Trikinetics, Whaltham, MA). Flies were placed in glass vials containing food in one end and a cotton stopper in the other end. This device scores a movement when the fly crosses an infrared beam which bisects the vial. The score of SMA was performed in 60 min intervals for 24 h periods at the specified time points and the average activity was calculated as the average of movements scored during 24 hours.

4.5.- Spontaneous motor activity under starvation conditions.

In independent experiments, we also evaluated the spontaneous motor activity of flies of both sexes treated for 10 days and then subjected to starvation conditions. In this experiment we wanted to determine whether the behavioral response to starvation was different in female flies treated with copper compared with male flies. Flies of both sexes were divided in two groups. Control group was maintained in standard medium, whereas treated group was fed with copper (31 μM) supplemented medium for 10 days. Then, control and copper treated flies were transferred to activity tubes containing 0.5 % agar medium, which supplies hydration but not nutrients and the activity was scored for 24 hours in the same manner as in the previous experiment.

Data analysis.

Data were analyzed using the Graph pad Prism software version five. One way ANOVA and Kruskal-Wallis post test was used to evaluate the differences in mean survival between the copper treated and the control group. The difference among the groups was considered significant when $P < 0.05$. For the evaluation of differences of starvation resistance the mean survival (hours) of flies was evaluated by one way ANOVA followed by Dunn´s multiple Comparison test * $p<0.05$.

Results

Effect of copper on lifespan.

We evaluated the possibility that the susceptibility to toxicity of female flies was different from that previously observed in males (3). In contrast to males the feeding of copper to female flies did not decrease their lifespan. On the contrary, the lifespan of females was increased by treatment with the metal (figure 1A). The mean lifespan of the control group was 46.64 ± 1.98 days. The lifespan of this gender was significantly increased in the groups treated with 31 μM and 47 μM concentration of copper, 53.12 ± 1.49 days and 52.04 ± 1.49 is depicted in figure 1B.

Effect of copper treatment on starvation resistance.

Supplementation with copper induced a dimorphic effect on the resistance to starvation evaluated ten days after the initiation of treatment. As shown in figure 2A, the feeding of copper decreased the resistance to starvation of male flies while this parameter was increased in females. The mean survivorship of male flies treated with copper was 41.90 ± 0.70 versus 47.41 ± 1.05 in untreated flies. On the contrary survival of female treated flies on starvation was significantly increased 55.21 ± 1.63 vs 48.94 ± 1.46 hours in the control group (figure 2B)

Spontaneous motor activity under normal feeding conditions.

As shown in table 1, spontaneous motor activity was evaluated before (day 2) and 10 days after initiation of copper treatment and in control flies. The study of motor activity revealed no differences between copper treated and control female flies. However, in male flies a significant was decrease of motor activity was observed after 10 days of treatment with copper.

Spontaneous motor activity under starvation conditions.

We evaluated spontaneous motor activity under starvation conditions in flies of both sexes treated or not with copper for ten days. No differences were observed in the pattern of activity of female flies fed copper with respect to control flies (figure 3A). Interestingly, the pattern of activity was increased throughout the day in male intoxicated flies (figure 3B). As shown in figure 3C, the total activity counts of female flies were not significantly different from that of untreated female flies while significant increase of activity counts was observed in male treated flies when compared to the control (figure 3C).

Discussion

Our results suggest the existence of a differential sensitivity to copper toxicity in *Drosophila melanogaster*. The observation of female flies not being sensitive to copper contrast with the effect of this metal in male flies. In our previous report we showed that similar doses of the metal shortened the lifespan and induced motor alterations of male flies (3).

Other authors have reported sex dimorphic effects of toxins in *Drosophila*. For example, it has been shown that male flies are more sensitive to the stimulatory

effect of ethanol compared to females. Conversely females are more sensitive to the sedative effect of the drug (8). Noteworthy, this differential sensitivity to ethanol in *Drosophila* resembles its sexually dimorphic effects in humans (9).

Our results contrast with those of other authors who recently showed the toxic effect of iron, manganese and copper on female flies (2). Such differences might be explained by the different dosage used in both studies. Bonilla-Ramirez et al used ten to twenty fold higher doses than the doses used in our study (0.5 mM and 1.0 mM versus 31µM and 47 µM). Another factor that could have an effect on the differences is that we provided the metal on standard corn fluor medium while they fed the flies with a 1% solution of glucose. In their study, Bonilla-Ramirez et al did not state the rationale to study the effect of those metals only on female flies.

Several observations suggest the association of lifespan and stress resistance. For example, stress resistance decreases with aging (10). Second, extension of lifespan by gene mutations or environmental manipulations is accompanied with an increased resistance to stress (11-15). However, other authors have reported the lack of association of lifespan and stress resistance under certain conditions (16).

We hypothesized that the lack of lifespan shortening effect of copper on female flies could be associated to a higher status of the defense mechanisms to general stress in this gender. To evaluate this, we assessed the resistance to starvation of male and female flies fed copper for ten days. Similarly to what we observed on the lifespan experiments, the supplementation with the metal affected the resistance to this form of stress in opposite directions on both genders.

Stress resistance can be interpreted as the capacity of the organism to withstand external or internal perturbations and return to equilibrium. Furthermore, a greater resistance to stress implies a healthier state of the organism. The observation that the feeding of copper decreased the resistance to starvation stress in males but not in females supports this notion.

Differences in sensitivity of male and female to the effects of toxins have been observed in diverse organisms. For example, (17) observed that exposure to mercury induced social behavior alterations in male prairie voles but not in females. Other authors

reported that methamphetamine has a greater neurotoxic effect on male mice compared to females (18). Such difference seems to be related to a greater activity of dopamine transporter and the vesicular monoamine transporter, which provides a more efficient protection for DA terminals (19-24). Recently, it was shown a differential response of male and female mice to methanphetamine treatment. Female mice were able to recover from the toxic insult while male animals entered a degenerative process. In addition, these authors show that the differences might be related to the activation of survival signaling pathways of AKT and ERK 1 / 2 in females but not in males (25).

The susceptibility to acetaminophen toxicity has been shown to be greater in male mice of the C57 strain by several authors (26-28). More recently, it was reported that female CD1 mice are also resistant to acetaminophen toxicity and that this resistance is related to a differential response of glutamate cysteine ligase, which restores hepatic gluthation levels and protects hepatic tissue from toxicity (29).

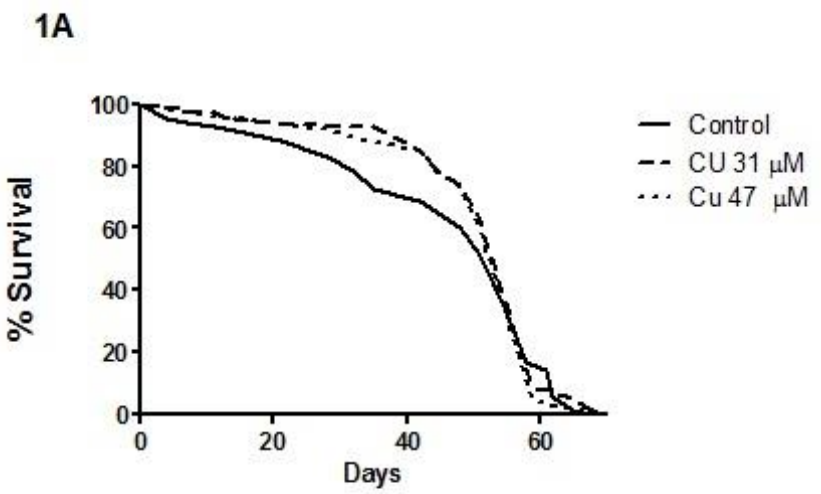
Previous studies have also demonstrated protective effects of ovarian hormones on in vitro toxicity models. For example, it has been shown that 17 beta-Estradiol protects astrocytes from neurochemical alterations induced by manganese (30, 31). More recently, estrogen receptor modulators have been shown to improve mitochondrial function and reduce oxidative damage in brain mitochondria of rats (32).

Differences in the absorption and metabolism of copper might also be related to this differential effect in both genders. For example, previous studies have related estrogens to absorption and metabolism of copper in humans. It has also been found that the plasma levels of copper and ceruloplasmin were higher in women when compared to men (33) and were further increased in premenopausal women taking oral contraceptives (34).

Performance on behavioral tests such as spontaneous motor activity may be related to physiological status. We observed a dimorphic behavioral response to treatment with copper since the spontaneous motor activity under normal feeding conditions was altered after 10 days of treatment in males but not in females. Other authors have shown that structural differences in higher brain areas are linked to some sex specific behaviors (35). Conversely, locomotor activity under starvation conditions was significantly increased only in males. Previous studies have shown that locomotor activity of flies is increased by starvation stress (36). We argue that the increased activity of male copper treated flies can be interpreted as a lower threshold of the organism to the disturbing situation. On the contrary, feeding of the metal does not impact the general homeostasis of female flies. Consequently, the response to stress is not affected in this gender. Other authors have observed differential responses to lead exposure in *Drosophila*. For example, Hirsch et al reported that the exposure to lead altered spontaneous motor activity in male but not in female flies (37).

In conclusion, copper supplementation decreases lifespan, stress resistance and locomotor activity of male but not female flies. Further studies are needed to uncover the mechanisms mediating these sexually dimorphic responses to copper intoxication in *Drosophila*.

Figure 1. Effect of the supplementation with copper on the lifespan. Flies of both sexes were maintained in culture medium supplemented with copper (31µM or 47 µM) from day 2 of age. The control group was kept in standard medium. Survival curves were generated with data from 3 replicas. Treatment with copper significantly increased the mean lifespan of flies ($p<0.05$).



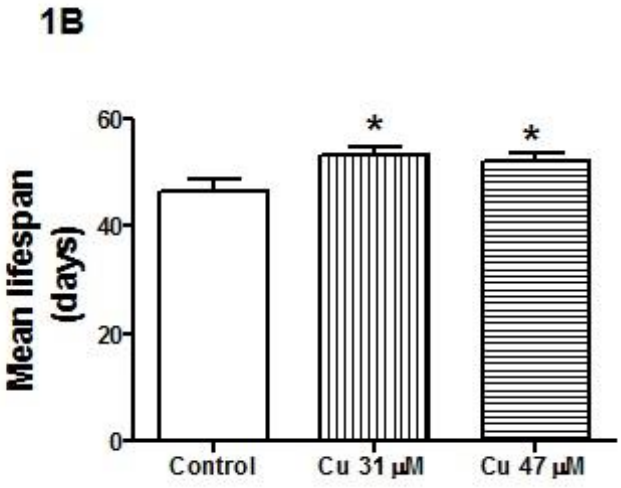


Figure 2. Effect of copper on starvation resistance. Copper treatment has opposite effects on starvation resistance in male and female flies 2A. Mean survival (hours) 2B of flies was evaluated by one way ANOVA followed by Dunn´s multiple Comparison test. * p<0.05.

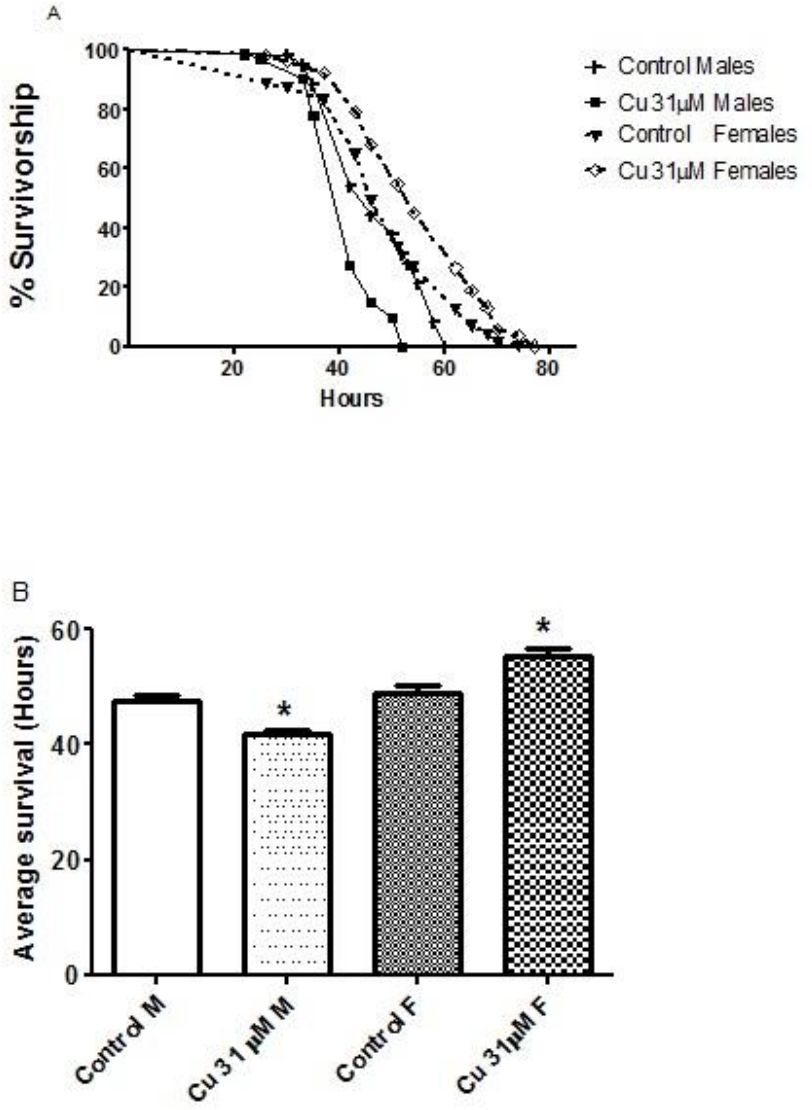
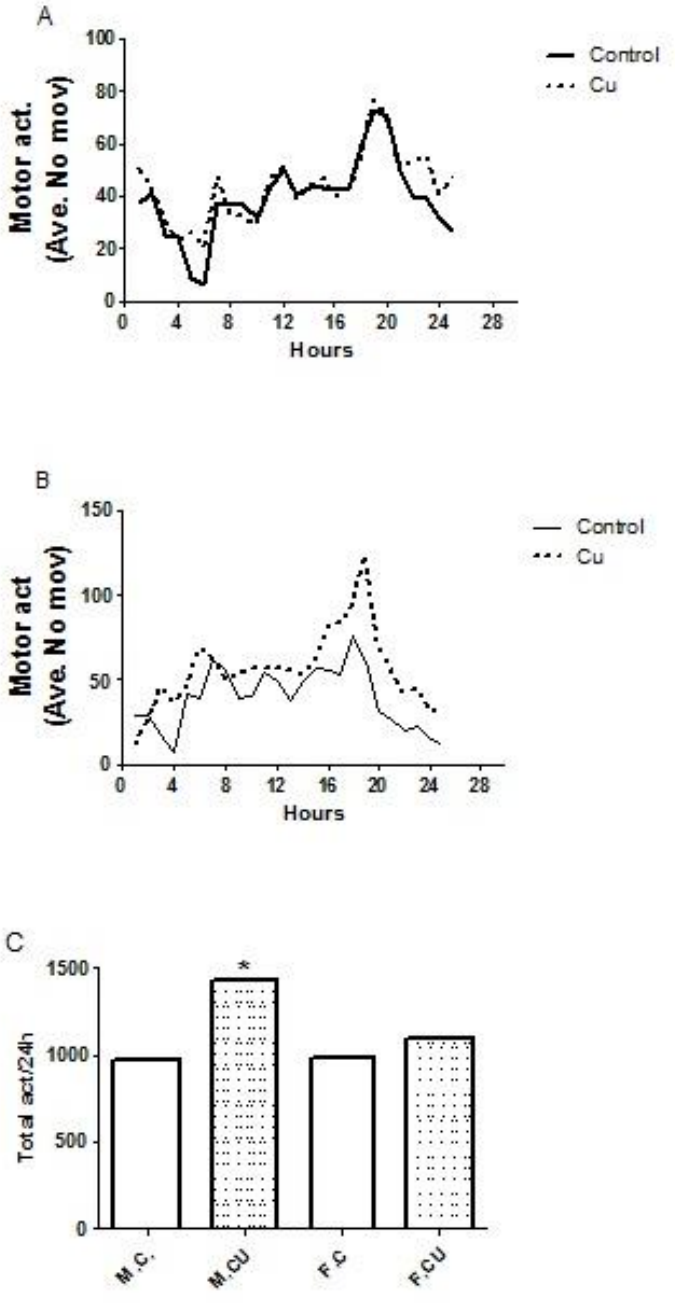


Figure 3. Spontaneous motor activity under starvation conditions. Twenty-four hour pattern of activity of female (A) and male (B) flies after 10 days of copper treatment (MC, males control, MCU, males treated with copper; FC, Females control; FCU, Females treated with copper). Mean values of activity counts on the 24 hours period. * p<0.05 versus control group.



References

1. Tapiero H, Townsend DM, Tew KD. Trace elements in human physiology and pathology. Copper. Biomed Pharmacother; 2003. 57(9):386-98.
2. Bonilla-Ramirez L, Jimenez-Del-Rio M, Velez-Pardo C. Acute and chronic metal exposure impairs locomotion activity in *Drosophila melanogaster:* a model to study parkinsonism.Biometals. 2011. 24:1045-1057.
3. Arcaya JL, Tejeda CM, Salazar U, Silva EJ, Urdaneta K, Varela K. Copper intoxication decreases lifespan and induces neurologic alterations in *Drosophila melanogaster*. Invest Clin; 2013. 54(1):47-57.
4. Weiss B. Same sex, no sex, and unaware sex in neurotoxicology. Neurotoxicology; 2011. 32(5):509-517
5. Kong B, Csanaky IL, Aleksunes LM, Patni M, Chen Q, Ma X, Jaeschke H, Weir S, Broward M, Klaassen CD, Guo GL. Gender-specific reduction of hepatic Mrp2 expression by high-fat diet protects female mice from ANIT toxicity. Toxicol Appl Pharmacol. 2012; 261(2):189-195.
6. Wizermann TM, Pardue M. The Future of Research on Biological Sex Differences: Challenges and Opportunities. Exploring the biological contributions to human health. Does sex matter?. 2001. National Academy Press. Washington DC.
7. Schmetzer O, Florcken A. Sex differences in the drug therapy for oncologic diseases. Nandb. Exp Pharmacol. 2012. 214: 411-442.
8. Devineni AV, Heberlein U. Acute ethanol responses in *Drosophila* are sexually dimorphic. Proc Natl Acad Sci U S A; 2012. 109(51):21087-21092.
9. Miller MA, Weafer J, Fillmore MT. Gender differences in alcohol impairment of simulated driving performance and driving-related skills. Alcohol Alcohol; 2009. 44(6):586-93.
10. Minois N and Le Bourg E. Resistance to stress as a function of age in *Drosophila melanogaster* living in hypergravity. Mech Ageing Dev; 1999. 109(1):53-64
11. Larsen PL. Aging and resistance to oxidative damage in *Caenorhabditis elegans*. Proc Natl Acad Sci U.S.A; 1993. 90(19):8905-8909
12. Lin YJ, Seroude L and Benzer S. Extended life-span and stress resistance in the *Drosophila* mutant Methuselah. Science; 1998. (5390):943-946
13. Honda Y and Honda S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. FASEB J; 1999. (11):1385-1393
14. Johnson TE, Henderson S, Murakami S, de Castro E, de Castro SH, Cypser J, Rikke B, Tedesco P, Link C. Longevity genes in the nematode Caenorhabditis elegans also mediate increased resistance to stress and prevent disease. J Inherit Metab Dis; 2002. 25(3):197-206

15. Wang HD, Kazemi-Esfarjani P, and Benzer S. Multiple-stress analysis for isolation of *Drosophila* longevity genes. Proc Natl Acad Sci U S A; 2004. 101(34):12610-12615.

16. Mockett RJ, Bayne AC, Kwong LK, Orr WC, and Sohal RS. Ectopic expression of catalase in *Drosophila* mitochondria increases stress resistance but not longevity. Free Radic Biol Med; 2003. 34(2):207-217.

17. Curtis JT, Hood AN, Chen Y, Cobb GP, Wallace DR. Chronic metals ingestion by prairie voles produces sex-specific deficits in social behavior: an animal model of autism. Behav Brain Res; 2010. 213(1):42-49.

18. Bourque M, Liu B, Dluzen DE, Di Paolo T. Sex differences in methamphetamine toxicity in mice: effect on brain dopamine signaling pathways. Psychoneuroendocrinology; 2011. 36(7):955-69.

19. Bhatt SD, Dluzen DE. Dopamine transporter function differences between male and female CD-1 mice. Brain Res; 2005. 1035(2):188-95.

20. Dluzen DE, Bhatt S, McDermott JL. Differences in reserpine-induced striatal dopamine output and content between female and male mice: implications for sex differences in vesicular monoamine transporter 2 function. Neuroscience; 2008. 154(4):1488-1496

21. Ji J, McDermott JL, Dluzen DE. Sex differences in K+-evoked striatal dopamine output from superfused striatal tissue fragments of reserpine-treated CD-1 mice. J Neuroendocrinol; 2007. 19(9):725-731.

22. Morissette M, Di Paolo T. Sex and estrous cycle variations of rat striatal dopamine uptake sites. Neuroendocrinology; 1993. 58(1):16-22.

23. Morissette M, Di Paolo T. Effect of chronic estradiol and progesterone treatments of ovariectomized rats on brain dopamine uptake sites. J Neurochem; 1993. 60(5):1876-1883.

24. Walker QD, Rooney MB, Wightman RM, Kuhn CM. Dopamine release and uptake are greater in female than male rat striatum as measured by fast cyclic voltammetry. Neuroscience; 2000. 95(4):1061-1070.

25. Bourque M, Dluzen DE, Di Paolo T. Sex and temporally-dependent effects of methamphetamine toxicity on dopamine markers and signaling pathways. Neuropharmacology. 2012 Jun;62(7):2363-72.

26. Botta D, Shi S, White CC, Dabrowski MJ, Keener CL, Srinouanprachanh SL, Farin FM, Ware CB, Ladiges WC, Pierce RH, Fausto N, Kavanagh TJ. Acetaminophen-induced liver injury is attenuated in male glutamate-cysteine ligase transgenic mice. J Biol Chem; 2006. 281(39):28865-75

27. Dai G, He L, Chou N, Wan YJ. Acetaminophen metabolism does not contribute to gender difference in its hepatotoxicity in mouse. Toxicol Sci; 2006. 92(1):33-41

28. McConnachie LA, Mohar I, Hudson FN, Ware CB, Ladiges WC, Fernandez C, Chatterton-Kirchmeier S, White CC, Pierce RH, Kavanagh TJ. Glutamate cysteine

ligase modifier subunit deficiency and gender as determinants of acetaminophen-induced hepatotoxicity in mice. Toxicol Sci; 2007. 99(2):628-36

29. Masubuchi Y, Nakayama J, Watanabe Y. Sex difference in susceptibility to acetaminophen hepatotoxicity is reversed by buthionine sulfoximine. Toxicology; 2011. 287(1-3):54-60.

30. Lee ES, Yin Z, Milatovic D, Jiang H, Aschner M. Estrogen and tamoxifen protect against Mn-induced toxicity in rat cortical primary cultures of neurons and astrocytes. Toxicol Sci; 2009. 110(1):156-67.

31. Lee ES, Sidoryk M, Jiang H, Yin Z, Aschner M. Estrogen and tamoxifen reverse manganese-induced glutamate transporter impairment in astrocytes. J Neurochem; 2009. 110(2):530-544.

32. Irwin RW, Yao J, To J, Hamilton RT, Cadenas E, Brinton RD. Selective oestrogen receptor modulators differentially potentiate brain mitochondrial function. J Neuroendocrinol; 2012. 24(1):236-48.

33. Mason KE. A conspectus of research on copper metabolism and requirements of man. J Nutr; 1979. 109(11):1979-2066

34. Milne DB, Johnson PE. Assessment of copper status: effect of age and gender on reference ranges in healthy adults. Clinical Chemistry; 1993. 39(5):883-887

35. Cachero S, Ostrovsky AD, Yu JY, Dickson BJ, Jefferis GS. Sexual dimorphism in the fly brain. Curr Biol; 2010; 20(18) 1589-1601.

36. Lee G, Park JH. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. Genetics; 2004. 167(1):311-323.

37. Hirsch HV, Mercer J, Sambaziotis H, Huber M, Stark DT, Torno-Morley T, Hollocher K, Ghiradella H, Ruden DM. Behavioral effects of chronic exposure to low levels of lead in *Drosophila melanogaster*. Neurotoxicology; 2003. 24(3):435-442.

Recibida: 23/02/14

Aprobada: 27/02/14