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Establishment of a New Rat Model of Alzheimer's Disease Using Copper Sulfate

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Abstract: ***Copper is of great importance for the normal brain. Excess of copper results in neurodegenerative diseases such as Alzheimer's disease (AD). The pathological hallmarks of AD are the amyloid-β (Aβ) peptides. Aβ peptides have been shown to bind copper with high affinity. We established a new rat model of AD by using copper sulfate ($CuSO_4$) solution with several doses and for different periods of time. The rats were randomly divided into normal control groups and AD groups. All rats underwent behavioral, histopathological and biochemical evaluations. The results of behavioral test revealed a significant improvement in the normal control groups. Conversely, AD groups showed impairments in the cognitive function as well as spatial memory with several degrees according to the duration and dose of $CuSO_4$ administration. The precise dose of $CuSO_4$ that showed the most cognitive deficits was 6 mg/L for eight weeks. Histochemistry staining revealed neuronal abnormalities in the brain sections of AD groups with several degrees. Biochemical analysis demonstrated that the level of γ-secretase was highest at a dose 6 mg/L $CuSO_4$ for eight weeks, conversely, the level of α-secretase was lowest at the same dose. We conclude that copper appeared to have a role in the formation of senile plaques, the hall mark of AD. According to these issues, we established a new rat model of AD using $CuSO_4$ solution.***

Keywords: Alzheimer's disease; Amyloid-β (Aβ) peptides, Object recognition test, Morris water maze (MWM) test, α-secretase, γ-secretase.

1. Introduction

AD is a progressive disease, it occurs over a long period before the onset of symptoms which are impaired memory, apathy, and depression [1]. The characteristics of AD consist in neurofibrillary tangles (intraneuronal aggregates of hyperphosphorylated tau proteins) and senile plaques [dense extraneuronal deposits composed of amyloid β (Aβ)]. The other features linked to these two core pathological hallmarks of AD are inflammation, oxidative stress, progressive synaptic, and neuronal loss. Although a myriad of evidence shows that the hippocampal volume decrease belongs to the AD earliest signs, as it is pointed out by the authors of the review paper presented in this issue, this element clearly could not be used as a diagnostic criterion [1].

Aβ peptides are generated by interplay of various secretases cleaving amyloid precursor protein (APP) [2]. Crucial steps in APP processing occur at the cell surface and in the trans-Golgi network TGN. From the TGN, APP can be transported to the cell surface or directly to an endosomal compartment. On the cell surface, APP can be proteolyzed directly by α-secretase and then γ-secretase, a process that does not generate Aβ, or reinternalized in clathrin-coated pits into another endosomal compartment containing the proteases β-secretase and γ-secretase. Although most APP must pass through the cell surface as part of its processing, this step is very rapid, as little APP is on the surface at any point in time. Why some surface APP is internalized into endosomes and some proteolyzed directly by α-secretase is unclear, although segregation of APP and β-secretase into lipid rafts may be a crucial element [3], [4].

Disbalance of some metal ions, especially zinc and copper, is thought to play an important role in the pathogenesis of many neurodegenerative diseases including multisystem atrophy, amyotrophic lateral sclerosis, Parkinson's, and Alzheimer's diseases [5]. The role of copper in the pathogenesis of AD is much less clear than the role of zinc; though it was revealed that copper ions as well as zinc ions are colocalized with Aβ plaques in the brain. In work an increased level of copper was shown in blood serum of patients with AD. Moreover it was found that copper level was significantly increased in cerebrospinal fluid [6].

It is interesting that all the molecules involved in amyloid plaque formation and neurofibrillary tangles bind copper [7]. Copper bound with Aβ becomes more toxic for neurons, and the cytotoxicity of copper Aβ peptides complexes directly correlates with the level of copper [8]. Indeed, binding of copper with Aβ is associated with the generation of oligomers capable of penetrating into cells. The toxicity of copper in AD is believed to be mediated through inhibition of cytochrome-c oxidase by Aβ bound copper ions and their ability to stimulate phosphorylation and aggregation of the tau protein [8].

A noteworthy hypothesis has been proposed that explains a possible mechanism of the participation of copper ions in the pathogenesis of AD [9]. According to this hypothesis, an initially local dyshomeostasis of copper ions can trigger in the brain generation of the metal-induced Aβ aggregates, which can be transformed to amyloid fibrils. These early fibrils can initiate an autocatalytic fibrillation of Aβ that is manifested by a decrease in its concentration in the

cerebrospinal fluid where Aβ monomers can associate to fibrils and produce amyloid plaques. At this stage copper ions also can bind with fibrils and be incorporated into the plaques. With the further development of the process, redox-active copper cations can be captured by the plaques and thus become unavailable for sequestration by proteins binding metals [9].

According to these issues, in the present study, we established a new rat model of AD using copper sulfate solution in one of multi dispensary experiments.

2. Material and Methods

2.1. Animal

Six-week-old male Wistar rats (120 ± 20g) were supplied by the Experimental Animal Center of the Faculty of Medicine, Alex University, Egypt. The rats were housed, 5 per cage, at a controlled temperature of 22 ± 2 °C, relative humidity (50 ± 10%) and with a standard 12-h light/dark cycle. The animals were given food and water ad libitum. The study was approved by the Local Ethics Commission for the Animal Experimentation.

2.2. Grouping

Rats were randomly divided into normal control group and Alzheimer's disease groups. {1} Normal control group was maintained on plain water and was further divided into five sub groups termed N1, N2, N3, N4 and N5 (n=10 for each) that were scarified after 2, 4, 6, 8 and 10 weeks respectively; {2} Alzheimer's disease groups {AD groups} (A, B and C) were administrated copper sulfate ($CuSO_4$) solution in drinking water at a dose of 2, 4 and 6 mg/L respectively. AD groups were further divided into fifteen sub groups (n=10 for each) according to the duration of administrated $CuSO_4$ solution (time of sacrificing) as the following table:

Table 1: Classification of normal control and AD groups according to period spent and doses of $CuSO_4$.

Period spent (Week)	*Normal control groups*	*AD groups*		
	N (n=10 for each)	A (2 mg/L CuSO4)	B (4 mg/L CuSO4)	C (6 mg/L CuSO4)
2	N1	A1	B1	C1
4	N2	A2	B2	C2
6	N3	A3	B3	C3
8	N4	A4	B4	C4
10	N5	A5	B5	C5

2.3 Methods

2.3.1 Behavioral Procedures

2.3.1.1 Morris Water Maze Test

i. Training Trial

The rat was placed in a pool of water where it must use and remember visual cues located in the room to find a platform hidden underneath the surface of water. All rats were trained in the water maze for 3 consecutive days. The time required for reaching the hidden platform (latency) was measured manually by using a stopwatch [10].

ii. Probe trial

A probe trial (retention testing) consisted of 60 sec of a free swimming period for each rat with the hidden platform removed from the pool. The better the memory is consolidated the more time will the rat swim around the location of the platform. The time spent in the target quadrant (expressed as percent of the time spent in the pool) was calculated.

2.3.1.2 Object Recognition Test

In this task, a rat is placed in a square open-field with two identical (Familiar) objects on each side of the open-field. After a 10 minute exploration period, the rat is then put back into its home cage for 1 hour and then re-tested in the same open-field but this time one of the objects is replaced for another (New) object. The time spent by the animals in exploring each object was recorded manually by using a stopwatch. The reaction to a new object was measured by calculating the discrimination index (DI): time spent in exploring the new object over total exploration time. Consequently, a ratio of 0.5 reflects equal exploration of the familiar and the new object, indicating no learning retention [11].

2.3.2 Histopathological Observation

At end of the experiment, all rats were anesthetized with ether and sacrificed. The hippocampus tissue of the rat brains were removed and fixed with 10% paraformaldehyde in 0.1 mol/L phosphate buffer solution (pH 7.4) for 24h and embedded in paraffin for making hippocampal blocks [12]. Then, serial sections of the blocks were cut at 5 μm thick in a rotary microtome for hematoxylin & eosin (H&E) and modified Congo red staining.

2.3.2.1 Hematoxylin and Eosin Staining

In brief, after dewaxing the paraffin sections, hematoxylin staining was performed for 3 sec, followed by eosin staining for 3 min, and then the sections were dehydrated with alcohol, cleared with xylene, and sealed. The hippocampal histopathological abnormalities were investigated under a light microscope [13].

2.3.2.2 Modified Congo Red Staining

Briefly, brain sections were stained with modified Congo red staining which was considered as an accepted histochemical marker for the β-pleated-sheet structure of amyloid. Sections were deparaffinized by embedding in xylene and alcohol and followed by tap water. Afterwards, slides were submerged for 20 min in a solution of 100% ethanol supersaturated with sodium chloride, then made alkaline with a final concentration of 0.01% sodium hydroxide [14].

Slides were then immersed for 60 min in a separate portion of alkaline alcoholic saturated sodium chloride containing 0.2% Congo red dye (solution filtered prior to use). Slides were then dehydrated with 8 dips in 95% ethanol, then 8 dips in two baths of 100% alcohol. The tissue was finally cleared in xylene and cover slipped with di-n-butylphthalate-polystyrene-xylene (DPX) [14].

2.3.3 Biochemical analysis

2.3.3.1 Tissue homogenate preparation

Hippocampus tissue was prepared for detection of γ-secretase and α-secretase. The tissue was homogenized in a 50 mM phosphate buffered saline (PBS) pH = 7.0. The homogenate was centrifuged for 10 min at 7000xg and the supernatant was then immediately frozen in -20 °C [15], [16].

2.3.3.2 Determination of γ-secretase and α-secretase in both rat serum and hippocampus tissue

γ-secretase and α-secretase were determined by rat γ-secretase ELISA kit (Glory Science Co., Ltd) using ELISA reader [15], [16].

Table 2: Performance of different groups during 3 days of training in Morris water maze task as regards the latency (sec) to reach the hidden platform.

Period spent (sacrificing time) (Week)	Normal control groups (n=10 for each)			AD groups (n=10 for each sub group)								
	Day 1	Day 2	Day 3	(A) (2 mg/L $CuSO_4$)			(B) (4 mg/L $CuSO_4$)			(C) (6 mg/L $CuSO_4$)		
				Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
2	36.08 ± 27.69	* 27.10 ± 22.46	*# 18.30 ± 12.51	38.50 ± 25.44	31.50 ± 17.67	*a 26.30 ± 16.73	39.60 ± 19.40	32.40 ± 12.69	*a 28.60 ± 15.15	42.20 ± 20.50	37.90 ± 15.79	*a 31.10 ± 13.87
4	34.30± 19.64	* 26.70 ± 14.05	*# 19.60 ± 10.88	a 44.10± 19.33	a 40.70 ± 13.47	*a 36.30 ± 10.75	a 47.50± 15.15	a 42.70 ± 15.49	*a 39.10± 17.24	a 50.80± 14.72	a 44.90 ± 13.68	*a 42.00± 12.07
6	32.60± 19.78	* 23.10 ± 11.37	*# 16.80 ± 12.29	a 47.30± 15.58	a 42.60 ± 14.27	*a 39.70 ± 16.68	a 50.40± 17.87	a 46.20± 15.14	*a 43.00± 12.07	a 52.50± 13.70	ab 49.60± 10.38	*ab 46.10± 10.81
8	34.10± 12.20	* 27.70 ± 7.95	*# 17.80 ± 7.41	a 51.00± 15.63	a 48.50 ± 13.93	a 46.20 ± 10.06	a 53.20± 14.23	a 50.50± 11.48	a 49.30± 13.92	a 57.20± 15.31	abc 58.00± 11.74	abc 58.90± 12.02
10	35.10± 11.09	* 24.20 ± 10.86	*# 16.90 ± 9.31	a 52.70± 9.52	*a 51.60 ± 7.84	a 51.10 ± 5.37	Died	Died	Died	Died	Died	Died

3. Results

3.1 Behavioral Observations

3.1.1 Morris Water Maze Test

i. Training Trial

The results of 3 days of training in the water maze task revealed that, in the normal control groups, there was a significant decrease in the mean time (latency) to reach the hidden platform (Table 2). These results demonstrate the baseline improvement in performance of the normal control groups. AD groups showed slightly, but not significant, improvement during each of the days of training when compared with the day before regarding the time spent to reach the hidden platform. However, AD sub group C4 (administrated 6 mg/L $CuSO_4$ for eight weeks) did not show any improvement during each of the days of training. Furthermore, AD groups spent a significantly more time to reach the hidden platform when compared with their corresponding normal control groups in each of the 3 days of training. (Table 2).

Probe Trial

In the probe trial, a probe test was conducted by removing the platform on the last day of MWM task. Results of memory retention testing revealed that normal control rats showed a significantly higher percent of the time spent in the target quadrant of the pool than those of AD groups. In particular, *AD sub group C4* showed a significantly lowest percent of the time spent in the target quadrant compared with its corresponding normal control group; i.e. *AD sub group C4* failed to remember the precise location of the platform. (Table 3).

Table 3: Performance of different groups during the 4th day (probe trial) of Morris water maze task as regards the percent of time spent in target quadrant

Period spent (sacrificing time) (Week)	Normal control groups (n=10 for each)	Copper groups (n=10 for each sub group)		
		(A) (2 mg/L $CuSO_4$)	(B) (4 mg/L $CuSO_4$)	(C) (6 mg/L $CuSO_4$)
2	44.10 ± 9.07	38.20^{a} ± 7.88	35.90^{a} ± 6.96	33.20^{ab} ± 6.79
4	43.90 ± 9.59	36.10^{a} ± 5.53	31.90^{ab} ± 6.43	28.30^{abc} ± 6.26
6	43.90 ± 8.72	33.10^{a} ± 6.21	28.10^{ab} ± 5.85	23.40^{abc} ± 6.20
8	43.10 ± 9.73	31.20^{a} ± 7.88	23.90^{ab} ± 6.96	16.80^{abc} ± 6.31
10	44.00 ± 6.67	26.80^{a} ± 7.99	Died	Died

Data are presented in mean ± S.D.
*: Significant at P value <0.05 between normal control group and AD groups
a: Significant at P value <0.05 between AD sub group (A) and AD sub groups (B,C)
b: Significant at P value <0.05 between AD sub group (B) and AD sub group (C)

3.1.2 Object Recognition Test

In the object recognition test (ORT), AD groups showed cognitive deficits, where the discrimination index was significantly lowered in AD groups compared with their corresponding normal control groups. The precise dose of $CuSO_4$ that showed the most cognitive deficits was 6 mg/L for eight weeks *(sub group C4)*, where the DI = 0.5, indicating no learning retention (Table 4).

Table 4: Performance of normal control and AD groups in object recognition test through calculation of discrimination index (DI)

Period spent (sacrificing time) (Week)	Normal control groups (n=10 for each)	Copper groups (n=10 for each sub group)		
		(A) (2 mg/L $CuSO_4$)	(B) (4 mg/L $CuSO_4$)	(C) (6 mg/L $CuSO_4$)
2	0.87 ± 0.03	0.78^{*} ± 0.01	0.72^{*a} ± 0.02	0.60^{*ab} ± 0.02
4	0.84 ± 0.03	0.76^{*} ± 0.01	0.68^{*a} ± 0.02	0.56^{*ab} ± 0.01
6	0.86 ± 0.03	0.73^{*} ± 0.01	0.64^{*a} ± 0.01	0.53^{*ab} ± 0.02
8	0.86 ± 0.03	0.72^{*} ± 0.02	0.61^{*a} ± 0.01	0.50^{*ab} ± 0.01
10	0.84 ± 0.02	0.59^{*a} ± 0.01	Died	Died

Data are presented in mean ± S.D.
*: Significant at P value <0.05 between normal control group and AD groups
a: Significant at P value <0.05 between AD sub group (A) and AD sub groups (B,C)
b: Significant at P value <0.05 between AD sub group (B) and AD sub group (C)

3.2 Histopathological Observations

3.2.1 Hematoxylin and eosin (H&E) staining

In normal control groups, the pyramidal cells in the CA1 region of hippocampus tissue were arranged neatly and tightly, and no cell loss was found. Additionally, cells were round and intact with nuclei stained blue (Figure 1 A-B). However, obvious hippocampal histopathological damage was observed in AD groups. The pyramidal layered structure was disintegrated, and neuronal loss was found in the CA1 region. Neurons with pyknotic nuclei and with shrunken cytoplasma. In addition, herano bodies as well as darkly stained cells as the beginning of tangle formation were observed (Figure 2A). Also, numerous plaques formation were seen (Figure 2B). These abnormalities were more intense in *AD sub group C4* over those of other AD sub groups. Where, herano bodies, plaques and granulovascular degenerations were seen in hippocampal section of *C4 sub group* (Figure 3).

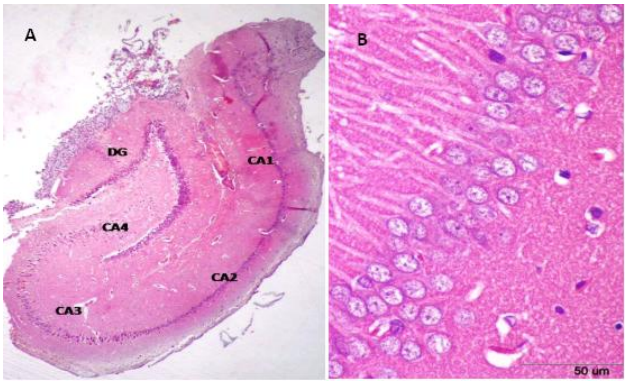

Figure 1: Morphologic structure of normal control rat hippocampus. (H&E X50)

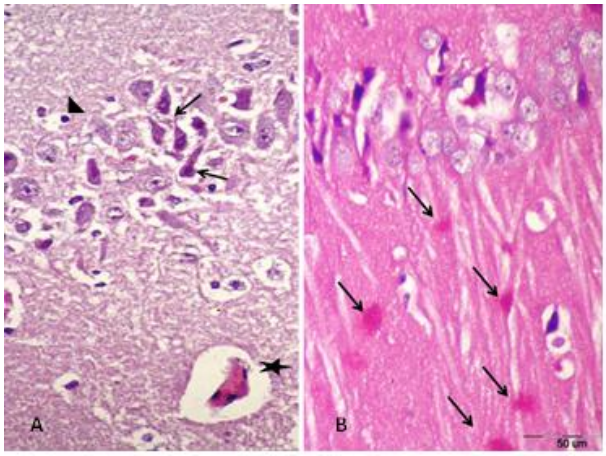

Figure 2: Hippocampal tissue sections of AD rat

(A) Cell degenerative changes, cytoplasmic shrinkage and dark pyknotic nuclei (arrowhead), also darkly stained cells as the beginning of tangle formation (arrows) and herano body (star) were seen. (H&E X50)
(B) Numerous plaques formation (arrows). (H&E X50)

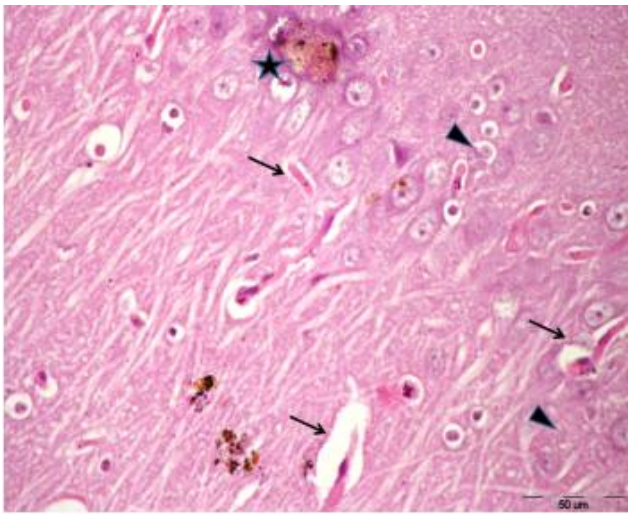

Figure 3: Hippocampal tissue section of AD sub group C4 rat showing: Intense neural destruction in the pyramidal layer of CA1 with the presence of plaque (star), herano bodies (arrows) and granulovacuolar degenerations (arrowhead). (H&E X50).

3.2.2 Modified Congo red staining

Histopathological slides of rat hippocampal sections were observed for the presence of Aβ deposits stained by Congo red as orange-red to brown deposits. The normal control rats did not show any Aβ deposits (Figure 4). However, AD groups showed Aβ deposits with various degrees of intensity. Forexample, rats administered 2 mg/L of $CuSO_4$ solution for 8 weeks showed little Aβ deposits (Figure 5A) and rats administered 4 mg/L of $CuSO_4$ solution for 8 weeks showed moderate Aβ deposits (Figure 5B), while rats administered 6 mg/L of $CuSO_4$ solution for 8 weeks showed strong Aβ deposits (Figure 5C). In addition, rats administered 4 and 6 mg/L of $CuSO_4$ solution for 10 weeks were died.

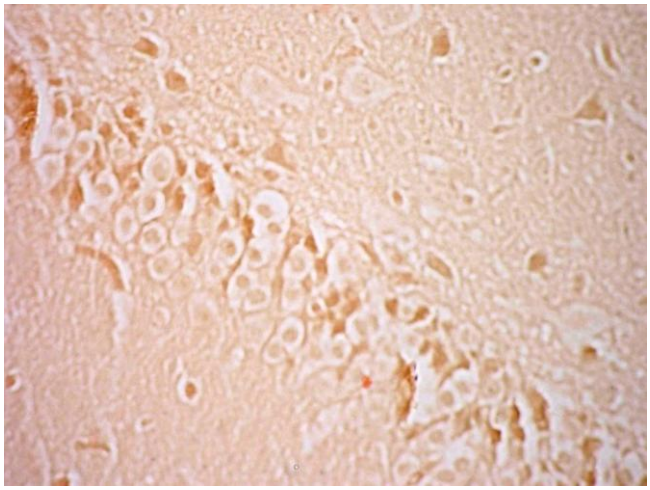

Figure 4: Section of normal control rat hippocampus, showing: no amyloid depositions (Congo red x50)

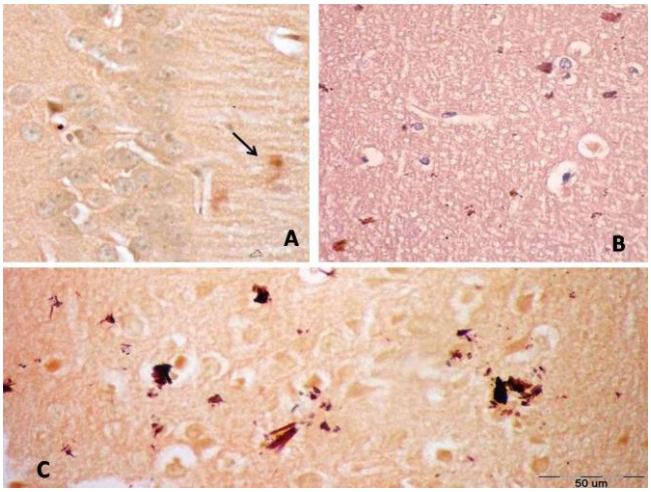

Figure 5: Sections of hippocampus of AD groups showing:
(A) Weak Congo red stain indicated little amyloid deposits, eight weeks after administration of 2 mg/L $CuSO_4$ solution (AD sub group A4).
(B) Moderate amyloid deposits, eight weeks after administration of 4 mg/L CuSO4 solution (AD sub group B4).
(C) Strong amyloid deposits, eight weeks after administration of 6 mg/L CuSO4 solution (AD sub group C4)

3.3 Biochemical Observations

3.3.1 The level of γ-secretase in serum and hippocampus tissue

As shown in the table (5); compared to normal control groups, the levels of γ-secretase (in both serum and hippocampus tissue) were increased in AD groups as the duration of experiment proceed and dose of $CuSO_4$ increased with the highest level at a dose *6 mg/L for 8 weeks*.

Table 5: The level of γ-secretase in both serum (pg/ml) and hippocampus tissue (pg/gm tissue) among different studied groups. Data are presented as mean ± S.E

Period spent (sacrificing time) (Week)	Normal control groups (n=10 for each)		Copper groups (n=10 for each sub group)					
			(A) (2 mg/L $CuSO_4$)		(B) (4 mg/L $CuSO_4$)		(C) (6 mg/L $CuSO_4$)	
	Hippocampus (pg/gm tissue)	Serum (pg/ml)	Hippocampus (pg/gm tissue)	Serum (pg/ml)	Hippocampus (pg/gm tissue)	Serum (pg/ml)	Hippocampus (pg/gm tissue)	Serum (pg/ml)
2	3061 ± 103.35	441 ± 5.07	3066 ± 105.21	441 ± 5.02	3074 ± 110.28	449 ± 6.49	4102 ± 112.67	469 ± 6.13
4	3058 ± 96.99	438 ± 4.71	3068 ± 104.26	445 ± 5.81	3085 ± 118.38	453 ± 6.41	4113 ± 115.61	474 ± 6.58
6	3049 ± 98.52	429 ± 4.24	3073 ± 106.71	447 ± 5.11	3089 ± 116.56	462 ± 6.08	4122 ± 122.34	486 ± 7.03
8	3060 ± 105.38	440 ± 5.1	3076 ± 109.25	451 ± 6.07	4096 ± 116.72	467 ± 5.98	4139 ± 118.5	497 ± 7.12
10	3053 ± 102.4	433 ± 5.12	3080 ± 107.46	456 ± 5.19	Died	Died	Died	Died

3.3.2 The level of α-secretase in serum and hippocampus tissue

The levels of α-secretase, a neuroprotective enzyme, (in both serum and hippocampus tissue) were decreased in AD groups compared to normal control groups as the duration of experiment proceed and dose of $CuSO_4$ increased with the lowest level at a dose *6 mg/L for 8 weeks.*

Table 6: The level of α-secretase in both serum (pg/ml) and hippocampus tissue (pg/gm tissue) among different studied groups. Data are presented as mean ± S.E.

Period spent (sacrificing time) (Week)	Normal control groups (n=10 for each)		Copper groups (n=10 for each sub group)					
			(A) (2 mg/L $CuSO_4$)		(B) (4 mg/L $CuSO_4$)		(C) (6 mg/L $CuSO_4$)	
	Hippocampus (pg/gm tissue)	Serum (pg/ml)	Hippocampus (pg/gm tissue)	Serum (pg/ml)	Hippocampus (pg/gm tissue)	Serum (pg/ml)	Hippocampus (pg/gm tissue)	Serum (pg/ml)
2	4864 ± 148.51	445 ± 14.72	4856 ± 146.39	437 ± 13.82	4833 ± 151.35	417 ± 15.73	4817 ± 156.42	339 ± 15.83
4	4860 ± 147.32	441 ± 14.15	4850 ± 152.24	432 ± 14.39	4829 ± 147.58	408 ± 14.62	4810 ± 147.38	387 ± 16.75
6	4871 ± 148.24	449 ± 14.80	4843 ± 148.32	425 ± 14.71	4822 ± 155.64	402 ± 16.07	4802 ± 158.49	383 ± 17.03
8	4867 ± 146.53	446 ± 13.25	4837 ± 150.63	421 ± 15.06	4816 ± 152.71	396 ± 15.43	4797 ± 156.25	374 ± 16.92
10	4862 ± 150.28	441 ± 14.31	4830 ± 149.71	413 ± 14.27	Died	Died	Died	Died

4. Discussion

It is well known that copper (Cu) is an essential transition metal for all living organisms, functioning as cofactor for many enzymes [17]. However, in *vivo* and in *vitro* studies demonstrated that excess inorganic Cu produces increased levels of reactive oxygen species (ROS) and damage to biomolecules, ultimately promoting cell death [18]. Humans are continuously at risk from excess Cu due to involuntary exposure to pollution (contaminated water, food), professional activities, ingestion of dietary supplements, and prolonged use of intrauterine devices [19].

Elevated Cu plasma levels have been associated with neurodegenerative damage. There has been a considerable increase in the number of published papers relating Cu to the neurodegenerative process. In line with this, Brewer hypothesized that ingestion of inorganic Cu from different sources is at least a partial cause of Alzheimer disease (AD) in developed countries [20].

In the present study we developed an extensive multidisciplinary analysis of the CNS effects of $CuSO_4$ administration with different concentrations and different periods of time in a mouse model. We used behavioral,

histological and biochemical approaches to examine the impact of $CuSO_4$ administration in *vivo* on a hall marker of AD-related pathology (Aβ plaques). In the current work, we demonstrated that rats which administrated $CuSO_4$ at a dose of 6 mg/L for eight weeks (*AD sub group C4*) exhibited obviously spatial learning and memory impairments assessed by behavioral, histological and biochemical approaches to examine the impact of $CuSO_4$ administration on the hall marker of AD-related pathology (Aβ plaques).

5. Conclusion

In conclusion, inorganic Cu causes neurodegenerative changes particularly in brain hippocampus, and thus Cu can be used to establish a new rat model of Alzheimer's disease (AD). This in *vivo* study reveals that the precise dose of $CuSO_4$ that showed strong Aβ deposits was 6 mg/L for eight weeks, so we have created a new animal model for AD. In view of the abundant evidence of disturbed Cu homeostasis in AD, we strongly recommend more in-depth studies on the mechanisms responsible for the pro-neurodegenerative effects of the association between Cu and Aβ. Furthermore, it is recommended that the present experimental evidence be used to promote the investigation of the emerging biomarkers to be applied in peripheral plasma as predictive tools in high-risk populations.

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