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Neurotoxic Effect of Oseltamivir Phosphate Directly Dosed To Mice Pups during Lactation Period

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Abstract
This study was performed to detect any behavioral or brain histological changes may be related to oseltamivir phosphate administration in mice pups.The neurotoxic effects of oseltamivir phosphate in mice pups was evaluated by the results of selected neurobehavioral tests and histopathological lesions that observed in the brains of treated animals by direct daily oral drug administration for the last fourteen days of lactation period ,the study began when pups reached 7 days of age. The experimental groups consist of three treated groups T1,T2,T3 were given the dose 2.5, 7.5 and 25 mg/kg B.W of oseltamivir phosphate orderly while control group treated with distilled water. The results of performed neurobehavioral test which including open field test, head pocking , cleft avoidance ,swimming rank, righting reflex and negative geotaxis tests indicated clearly presence of behavioral changes of treated animals and this results are supported by histological lesions of brain which observed a dose dependent manner effect, even at therapeutic dose.These results probably were attributed to the change of the level of CNS neurotransmitters or due to brain lesions may be caused by the drug which may affect the release of these neurotransmitters accordingly with oseltamivr phosphate dose.
Keywords:oseltamivir phosphate, neurobehavioral ,neurotransmitters, toxic effect

1. Introduction.

Influenza is a highly contagious, acute febrile respiratory infection caused by the influenza virus. Cases typically occur in a seasonal pattern, with localized epidemics during winter months. (1). Oseltamivir is an orally administered anti-influenza agent of the neuraminidase inhibitor class. The ethyl ester prodrug oseltamivir is delivered orally as a phosphate salt and converted by hepatic esterases to the active metabolite oseltamivir carboxylate (OC) (2).OC specifically binds and inhibits the influenza virus neuraminidase enzyme that is essential for viral replication (3).In this way, oseltamivir limits the spread of influenza virus subtypes A and B within the infected host .When used as treatment, oseltamivir reduces the severity and duration of symptoms (4),while prophylactic administration prevents their onset(5) . In recent years, abnormal or delirious behaviors have been reported with a low incidence in young individuals with influenza who were receiving oseltamivir (6). Cases arose most commonly in Japan but were also observed in Taiwan, Hong Kong, North America, Europe, and Australia. No causative association could be demonstrated, and similar events were also reported in the absence of oseltamivir (7). The relationship between abnormal behavior and oseltamivir medication remains an open question .Lactogenic study was performed to detect the neurotoxic effect of oseltamivir phosphate in mice pups suckling from nursing mice dosed with oseltamivir phosphate daily for fourteen days ,these suckling pups showed neurotoxic symptoms (8) .In this study we tried to explore the probable neurotoxic effects in 7 days aged mice pups directly dosed with oseltamivir phosphate in ordered to record for the first time such nurotoxic effects.

2.Materials and Methods

2.1.the drug

Oseltamivire phosphate (flufly) ® 75 mg produced by Julphar Pharmaceutical Industries,Ras AL - Khaimah,UAE.

2.2.Neurobehavioral Tests Apperatuses :Specific apperatus were used to performe neurobehavioral tests in isolated room .

2.3.Oeltamivir phosphate dosage and dose

Oseltamivir phosphate stock solution (1mg/ml) was obtained by dissolving 1capsule of 75mg of oseltamivir phosphate in 75ml distilled water. then 1 ml of stock was diluted with 7ml of distilled water to prepare the concentration of 0.125mg/ml .administrated with dose volume 0.1ml/5gm.BW in therapeutic dose of 2.5mg/kg.BW to T1,while 6 ml of stock solution was diluted with 10 ml distilled water to prepare the concentration 0.375mg/ml administrated toT2 in three folds of the therapeutic dose 7.5mg/kg.BW at dose volume 0.1ml/5 gm .BW ,the 10 folds dose was prepared by dissolving 1capsule 75 mg of oseltamivir phosphate in 60ml of distilled water to prepare the concentration of 1.25mg/ml with dose volume of 0.1ml/5gm.BW in 10 folds of therapeutic dose 25mg/kg BW. five pups of control group were given distilled water for 14 days. The mice pups were dosed daily by using specific plastic gevag needle.

2.4.The Animals

Four recently parturated pups mice , each delivered 6-8 mice pups from each 5 pups were allocated to one treated group that dosed at age 7 days when the pups body weight 3-5 gm in order to study the neurobehavioral

effects of oseltamivire phosphate at different lactating and post treatment periods .The animals and their pups were procured from the animal house of the College of Veterinary Medicine /Baghdad University under ethical rules and huminity of the university.The animals were maintained in an air-conditioned room 22±3 ºC with about (14 /10) Hours(Light/Dark) cycle standard pilliets and water provided ad libitum (9).

2.5.Experimental Design

seven days after parturition total number of (20)pups of albino mice were divided equally into four experimental groups consist of one control group dosed daily with distilled water .While three treatment groups T1,T2 and T3 similarly divided and used to detect neurotoxic effect of oseltamivir phosphate through direct administration of oseltamivir phosphate to experimental pups during the last 14 days of lactation period when Blood Brain Barrier(BBB) is not fully mature 7-21 days of age ,and at 7th day after stoppage of treatment when BBB is fully mature after 28 day of age (10).

pups of experimental groups (T1,T2,T3and C) were tested for the following parameters:

1. Neurobehavioral tests to detect abnormal behavior in the 14thday of age (7 days of treatment) ,21th day of age (14 days of treatment) and 28th day of age (7th day post treatment) which included the following tests :- Open field test , Head pocking test, Negative geotaxis test, Righting reflex test, Cleft avoidance test, Swimming rank test.
2. Histopathological lesion of brain.

2.6.NEUROBEHAVIORAL TESTS:-

1. **Open field test/ 3 minutes** :- The test evaluates the general locomotor activity, exploration (squares crossed by four legs of animal forward & backward) , and also including frequency of defecation & urination (Autonomic nervous impressed) .Each mouse was placed in the center of arena of open field apparatus and the number of squares crossed was counted, rearing, fecal boluses and urine pools during (3) minutes . Arena was cleaned after each try (11). apparatus was used according to specification design in which box at diamensions 35x35x25 cm the arena divided into 25 equal squar 7x7cm² each as shown in. The arena used to perform open field tests for mice locomotor activity.
2. **Negative Geotaxis/ 60 seconds** : This test reflects vestibular function , neuromotor performance and coordination , the angle slope apparatus is 45º ,we placed mouse with the head was placed in down position on the inclined surface , Time that the mouse needed for completing 180º - turn was regestirated, the maximum time allowed was 60 seconds (12). Specific apparatus was used , it consist of a wooden slope with an angle 45°.
3. **Head Pocking Test/3minutes:** - This test determines the degree of cognitive function of animal exploration of environment by registration the frequency of head entrances into pores during (3) minutes (13). **:-** performed by using specific device with eight punchers in plastic Circular plate (30 cm diameter and 10 cm highness)

4.Righting Reflex Test : tested pups were kept laying on back . The time needed for adjusting posture recorded ; this test evaluates the motor activity of pups (14). Formica table with average height 9 cm , used to perform righting reflex test and also to perform cleft avoidance that reflects the proper reflex.

4. **Cleft Avoidance Test :** This was performed by putting pups close to cleft of table with highness more than (9) cm , the time needed by pups for turned of were recorded , this test is useful for detection of the proper reflexes (14).
5. **Swimming Rank Test/10 seconds** *:-* This test reflects the integration of brain function by monitoring each animal kept for (10) seconds for swimming in a pool containing warm water (30C º) and evaluated by grades of swimming rank as follows as shown in Fig(11)(15) and (16).

- Grade (0): When the nose is under the plane of water,Grade (1): The nose with plane or above water,Grade (2): The nose and crown with, or above the plane of water while the ears are under,Grade (3): As in grade (2) but the plane of water at the mid of ears,Grade (4): As in grade (3) but the plane of water under the ears

3.7.Statistical analysis: Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of (P<0.01). Specific group differences were determined using least significant differences (LSD) as described by (17).

RESULTS

3.1.NEUROBEHAVIORAL TESTS:

The results listed in table (1) revealed according to the tested behavior that T1 group pups at 7th day of treatment showed significant(P<0.01) increase in locomoter activity and exploration , defect in proper reflex (table 1.A and E),while T2 group showed a defect in motor function ,proper reflex and brain integration (table1.D,E,F), while T3group showed a decrease in locomotor activity and exploration table (1.A), defect in vestibular function interfere with short term memory , proper reflex , motor function table and brain integration (table1,A,C,E,D,F) .At the 14th day of treatment T1 showed an increase in autonomic nervous system activity (frequency of fecal bolus) and defect in motor function (table 1.A and D), while T2and T3 showed a decrease in autonomic nervous system activity (frequency of fecal bolus) ,defect in proper reflex ,motor function and brain

integration (table 1.A,E,D,F), but only T3 showed a decrease in locomotor activity and exploration , defect in degree of cognitive function of exploration (table1.A and B). At 7 day post treatment T1,T2and T3 showed defect in degree of cognitive function of exploration ,motor function (table 1.B and D), only T1showed a decrease in locomotor activity and exploration (table 1.A), while only T2 and T3 showed a decrease in autonomic nervous system activity (frequency of fecal bolus) and a defect in proper reflex (table 1.A and E).

Table (1)(A,B,C,D,E,F)Selected neurobehavioral tests of mice pups dosed directly and orally with three different doses of oseltamivir phosphate during lactation period.

(A) Open field/3 minutes			
Period / Group n=5 pups	**7 days of treatment** M±SE	**14 days of treatment** M±SE	**7 days after treatment termination** M±SE
1.Number of crossed squares/3 minutes			
C(D.W)	B 14±1.4 b	A 143.2±5.8 a	AB 125±14.1 a
T1(2.5mg/kg)	A18.8±1.0 c	A 145.60±4.044 a	B 103.20±7.6 b
T2(7.5mg/kg)	B13±1.3 c	A 144.4.± 8.0 a	A 133.60±0.1 b
T3(25mg/kg)	C 3.8±1.1 b	B 13±5.7 b	A 135.60±7.7 a
2.Frequency of fecal bolus/3minutes			
C(D.W)	A 0.2±0.1c	B 1.6±0.2 b	A 2.8±0.2 a
T1(2.5mg/kg)	A 0.2+±0.1b	A 3.6±0.5 a	A 4±0.3 a
T2(7.5mg/kg)	A 0.2±0.1 a	C 0.60±0.2 a	B 0.8±0.3 a
T3(25mg/kg)	A 0.2±0.1 a	C 0.2±0.1 a	B 0.2±0.1 a
3. Frequency of urination/3minutes			
C(D.W)	A0.2±0.1 a	A0.2±0.1 a	A 0.2±0.1 a
T1(2.5mg/kg)	A0.2±0.1 a	0.2±0.1 a	A 0.2±0.1 a
T2(7.5mg/kg)	A0.2±0.1 a	A0.2±0.1a	A 0.2±0.1 a
T3(25mg/kg)	A0.2±0.1a	A 0.2±0.1 a	A0.2±0.1 a

TESTs	**(B)Head pocking/3minute**		**(C)Negative geotaxis/60 seconds**			**(D) Righting Reflex test/seconds**		
Period / Group n=5pups	**14 days of treatment** M±SE	**7 days post treatment** M±SE	**7 days of treatment** M±SE	**14 days of treatment** M±SE	**7 days stop treatment** M±SE	**7 days of treatment M±SE**	**14 days of treatment M±SE**	**7 days after stop treatment M±SE**
C D.W	6.60±0.7 A a	11.6±1.3 A a	4.2±0.2 B a	3.4±0.4 A a	3.2±0.9 A a	21±0.6 B a	16.4±0.6 B b	15.6±0.2 B b
T1(2.5mg/kg)	6.80±0.7 A a	3.8±0.3 B b	4.60±0.6 B a	4.4±1.6 A a	5±0.31 A a	23.20±1.2 B a	23.20±0.7 A a	18.8±0.2 A b
T2(7.5mg/kg)	5.4±0.6 A a	3.80±0.4 B b	6.20±0.3 AB a	5.4±1.2 A ab	4.2±0.7 A b	100.4+±27.2 A a	23.40±0.6 A b	19.20±0.7 A b
(25mg/kg) T3	0.2±0.1 B a	1.6±1.1 C a	8.20±0.8 A a	5.4±0.7 A b	4.0±0.3 A b	101.80±19.8 A a	25±0.6 A b	20.66±1.2 A c

Different small letters represent significant differences within groups(P≤0.01)
-Different capital letters represent significant differences between groups (P≤0.01)
M±SE=mean+ standard error

Histopathological lesions 3.2.

- **Brain**

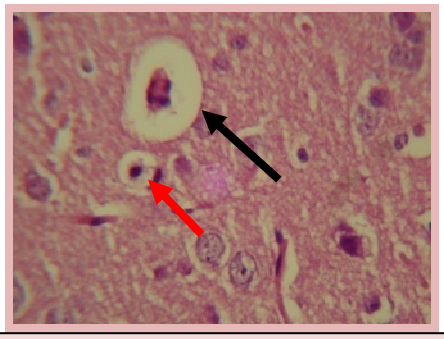
1.Control group:- Organs of the control group showed no histopathological changes (Fig.1).

2.Therapeutic dose (group T1):-Cerebrum :Congestion of cerebral blood vessels with perivascular and perineuronal edema and shrinkage of neurons (Fig.2). Cerebellum:showed edema between molecular and granular layer with degeneration of many purkinji cells and complete dissolution of the others (Fig.3).

3.Three folds dose (group T2):-In addition to histopathological changes seen in therapeutic dose there is severe hemorrhage in meninges (Fig.4) with mild hemorrhage in the cerebellum (Fig.5) .

Test	(E) Cleft avoidance/minutes			(F) Swimming rank test		
Period / Group n=5 pup	7 days of treatment M±SE	14 days of treatment M±SE	7 days after stop treatment M±SE	7 days of treatment M±SE	14 days of treatment M±SE	7 days after stop treatment M±SE
C D.W	B1.4±0.244 a	B 0.8±0.2 a	B 0.8±0.2 a	A 3.8±0.2 a	A 3.8±0.2 a	A 3.8±0.2 a
T1(2.5mg/kg)	A 2.60±0.244 a	AB 1.4±0.244 b	AB1.4±0.244 b	A 3.20±0.2 a	A 3.8±0.2 a	A 3.8±0.2 a
T2(7.5mg/kg)	A 3.2±.0.734 a	A 1.6±0.244 b	A 1.6±0.244 b	B 2.2±0.2 c	B 2.8±0.2 b	A 3.8±0.2 a
T3(25mg/kg)	A 3.4±0.244 a	A 1.8±0.374 b	A 1.8±0.48 b	B 1.8±0.2 c	B 2.8±0.2 b	A 3.2±0.2 a

4.Ten folds dose group(T3) :-The cerebrum showed multiple areas of focal gliosis (Fig.6) .Other areas showed spongiosis (Fig.7).In addition to cerebral vascular inflammation and necrosis (Fig.8).



Fig(2) :Histipathological section of cerebrum of brain of mouse pup of (T1) treated with 2.5 mg/kg BW/day for 14 day shows perivascular( ) and perinural ( ) odema with shrinking of neurons(H&Ex400) .

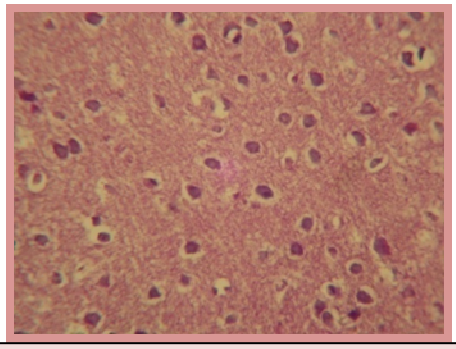


Fig (1): Histopathological section of brain of mouse pup of (control group) treated with distilled water for 14 day shows normal histological structure (H&Ex100)

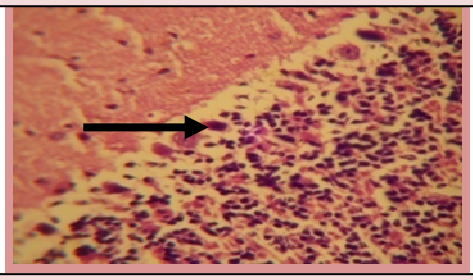
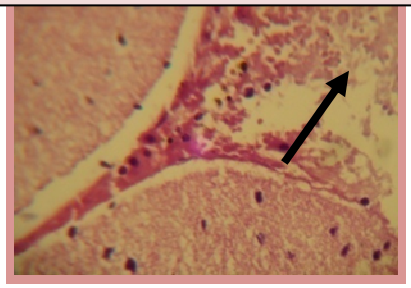
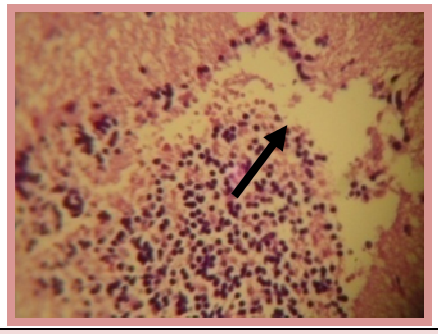


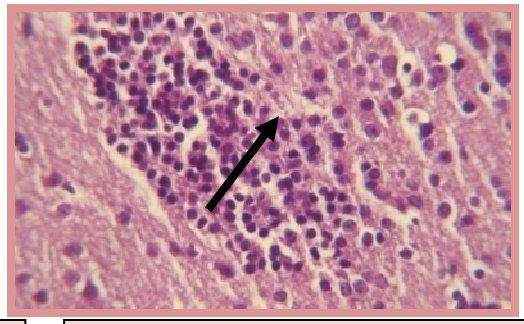
Fig (3): Histopathological section of cerebellum of brain o f mouse pup of(T1) treated with 2.5 mg/kg BW/day of oseltamivir phosphate for 14 day shows edema between molecular and granular layer with degeneration of many purkinji cells and complete dissolution of the others( ) (H&Ex400) .



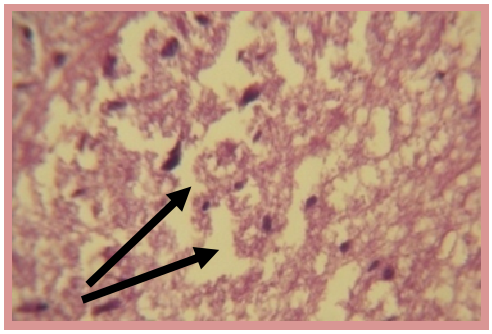
Fig(4): Histopathological section of brain of mouse pup of (T2) treated with 7.5 mg/kg BW/day of oseltamivir phosphate for 14 day shows severe hemorrhage in meninges ( ) (H&Ex400)



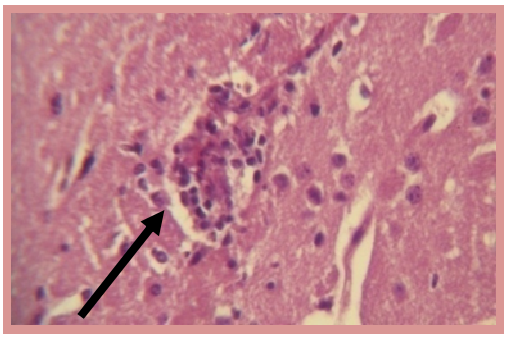
Fig(5): Histopathological section of cerebellum of mouse pup of (T2) treated with 7.5 mg/kg BW/day of oseltamivir phosphate for 14 day shows mild hemorrhage( ) (H&Ex400).



Fig(6): Histopathological section of cerebrum of mouse pup of (T3) treated with 25 mg/kg BW/day of oseltamivir phosphate for 14 day shows focal gliosis ( ) (H&Ex400)



Fig(7): Histopathological section of cerebrum of mouse pups of (T3) treated with25 mg/kg BW/day of oseltamivir phosphate for 14 day shows spongiosis ( ) (H&Ex400)



Fig(8):Histopathological section of cerebrum of mouse pup of (T3) treated with 25 mg/kg BW/day of oseltamivir phosphate for 14 day shows cerebral vascular vasculitis and necrosis ( ) (H&Ex400).

Discussion

Primarily in this study it was thought that OP and/or its metabolite oseltamivir carboxylate(OC) has the ability to penetrate blood brain barrier(BBB) just like many drugs ,at time when BBB is not fully mature in early neonate life during lactation period. There is growing interest in the penetration of oseltamivir and its active form into the brain(18) .It was reported that in developing mice BBB maturation is complete around the third postnatal week.(10). Because of this probable penetration that may be occured during lactation period may be interfere with CNS neurotransmitters . Neurotransmitters are chemical signals in by which communications between nerve cells –nerve cells and effector organs are occurs .CNS neurotransmitters divided in to 3 groups (biogenic amines , neuropaptide and amino acids)a. biogenic amines including Ach which is excitatory responsible for (arousal ,short term memory ,learning and movements),b .norepinehprin which is excitatory involved in (arousal ,wakefulness, mood and cardiovascular regulation), c.dopamin which is excitatory involved in emotion reward system and motor function)d. serotonin which is excitatory /inhibitory involved in feeding behavior ,control of body temperature modulation of sensory pathways including nociception (stimulation of pain nerve sensor)regulation of mood and emotion and sleep /wakefulness)(19) ,so any defect in production ,storage and release of these neurotransmitters cause defect in the functions that these neutronsmitters are involoved with.It was reported during a study performed on wibester rats , when these rats received OP at dose 25 and 100 mg/kg.BW I/P the result indicated that an increase extracellular dopamine significantly from pre-administration level in prefrontal cortex (PFC) (20).While in this current study the results of open field test indicated that the significant increase in movement(crossed squares /3minutes)and (frequency of fecal bolus/3 minutes)recorded in T1 pups group was possibly due to marked CNS and autonomic cholinergic effect caused by drug at therapeutic dose, while their significant decrease in higher doses (T2,T3) was inhibitory CNS either due to opposite effect (dopaminergic or other CNS neurotransmitters like serotonin) or may be due to brain damage that more noticed histologicaly in higher doses of OP or may be due to that OP and/or its metabolite that may be peripherally have muscarinic receptors which change gut motility that affect fecal frequency.The phenotype of mutant mice with impairments in dopamine or norepinephrine biosynthesis exhibit severe motor dysfunctions characterized by a reduction in spontaneous locomotion and cataleptic behavior, and defects in drug-induced hyperactivity at the juvenile stage (21). It was showed that activation or inhibition of neurotransmitters cause significant alteration in performance of human and laboratory animals on variety of

tasks of spatial attention (22) and working memory(23). Based largely on the main pharmacological properties of serotonin and noradrenalin uptake inhibitors used for the treatment of depression, deficient extracellular levels of these monoamines have long been hypothesized to play a key role in the development of the core symptoms of this disorder (24),also an indirect evidence from cat, rat and mouse preparations suggests that glutamenergic projections contribute to the initiation of locomotion since application of glutamatergic antagonists at the spinal cord level block locomotor activity under certain conditions (25).The probable change in the levels of neurotransmitters might explain the results of head pocking test,cleft avoidance test,righting reflex test,negative geotaxis test and swimming rank test. While histopathological lesions of brain may be due probable penetration of OP and/or OC cross BBB and cause damage to multiple areas of brain.The histopathological lesions reported in present study were positively proportional to the OP doses even in the therapeutic dose may explain the proposed changes in CNS neurotransmitters levels and their consequence effect on the results of neurobehavioral tests that done on pups directly dosed with OP during lactating period that explain the response effect according to increasing doses. The present neurotoxic effects of neonates in dosed groups might considered early report to explain neurotoxic symptoms noticed in human neonates and children after administration of the drug.

Conclusion The prophylactic dose of 2.5mg/kg.BW for infants ,should be reviewed since it induces neurotoxic effect in lactating pups at age (7-21 days postnatal) .

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