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Neurotoxic effect in lactating mice pups received oseltamivir phosphate (tamiflu) through milk from dosed nursing mothers during lactation period

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Summary

The present study was aimed to evaluate neurotoxic effects of oseltamivir phosphate in lactating pups of orally dosed mice mothers during lactation. Twelve recently parturited female albino mice were divided equally into three groups, one control and two treated groups, each group consists of 4 dosed dams and 8 chosen pups .The nursing dams of T1 and T2 dosed daily orally with 1mg/kg and 5mg/kg oseltamivir phosphate respectively representing the therapeutic dose and 5 fold dose of drug while control group dosed with distilled water. Lactating mice pups of all groups examined for the following parameters: First parameter was body weight changes and gain: In which T1group showed significant increase in mice pups body weight gain after 14 day of treatment in comparison with control group and T2. Second parameter was clinical symptoms observation /daily, all treatment groups that showed neurotoxic symptoms appeared from 1st dose and extended along the next few days of treatment to be gradually disappeared and completely lost within the last days of treatment in dose dependent manner.These neurotoxic symptoms were weakness, convulsions ,lay on back or side, extended body, incoordination ,extended limbs and limbs stiffness. Third parameter was gross and histopathological studies which demonstrate that the brain was the most affected organ beside extensive lesions in liver, kidney, stomach and small intestine of treated groups in dose dependent manner.

In conclusion of this study revealed that Oseltamivir phosphate produce neurotoxic effect in mice pups through indirect administration by nursing mothers dosing during lactation period and the level of toxicity was in dose dependent manner.

Key words: - oseltamivir phosphate, neurotoxic, lactation period, mice.

التأثيرات العصبية السمية للاوزيلتامفير فوسفيت (تاميفلو) في جراء الفئران المهقاءالراضعة لحليب امهات معالجه خلال فترة الرضاعة

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الخلاصة

اجريت الدراسه لتقييم التأثير العصبي السمي لعقار الأوزيلتامفير فوسفيت على جراء الفئران خلال فترة الرضاعه عن طريق المعامله الفمويه للأمهات المرضعه . تم تقسيم إثنا عشر من أمهات الفئران الوالده حديثآ إلى 3 مجاميع ,كل مجموعه مكونه من 8 جراء راضعه و4 أمهات معالجه مرضعه ,إشتملت مجاميع الدراسه على مجموعة سيطره واحده ومجموعتي علاج (T2,T1) جُرعت فموياً ويومياً 1ملغم/كلغم و5ملغم/كلغم من عقار الأوزيلتا مفيرفوسفيت لتمثل مجموعتي الجرعه العلاجيه و مجموعة خمسة أضعاف الجرعه العلاجيه على التوالي.

بينما جرعت مجموعة السيطره بالماء المقطر.خضعت جراء الفئران المقهاء لجميع المجاميع لفحوصات تسجيل المعاير التاليه :- ألمعيار الأول: وزن الجسم المتغير والمكتسب لجراء الفئران المهقاء:- في هذه الدراسه أظهرت مجموعة (T1) زياده في وزن الجسم المتغير والمكتسب بعد 14 يوم معالجه عند المقارنه مع مجموعة السيطره ومجموعة T2. ألمعيار الثاني :المراقبه اليوميه للعلامات السريريه لجراء الفئران المهقاء :- أظهرت جميع المجاميع العلاجيه بعض العلامات العصبيه منذ الجرعه الأولى تناسبت طرديا مع الجرع المعطاة وقد أمتدت العلامات العصبيه لتشمل الأيام اللاحقه من فترة التجريع حتىبدأت بالأختفاء و بشكل تدريجي حتى إختفت بشكل كامل خلال الأيام الأخيرة من فترة المعالجه.وقد أشتملت العلامات العصبيه السميه مايلي(وهن,تشنجات,,الإستلقاء الظهر او الجانب, إستطالة الجسم ,عدم التوازن,أطراف متصلبه وأطراف ممتده). المعيار الثالث: دراسة التغيرات العيانيه والمجهريه لمجاميع حيوانات التجربه :أظهرت الدراسه وجود آفات عيانيه ومجهريه كانت على أشدها في الدماغ حيث كان أشد الأعضاء تأثراً مع وجود آفات عيانيه ومجهريه في الكبد,الكلى,المعده والأمعاء الدقيقه.هذه الآفات كانت تتناسيب طرديآمع مقدار الجرعه المعطاة.

نستنتج من هذه الدراسة إن عقار الأوزيلتا مفيرفوسفيت يسبب تأثيراً سمياً عصبياً في جراء الفئران المهقاء المجرعه بشكل غير المباشر (عن طريق الأمهات المرضعة) بعقار الأوزيلتا مفيرفوسفيت خلال فترة الرضاعة وكانت هذه تأثيرات السميه العصبيه تتناسب طردياً مع مقدار الجرع المعطاة.

مفاتيح الكلمات:- للاوزيلتامفير فوسفيت , العصبية , الفئران.

Introduction

Oseltamivir phosphate (OP) is an orally administered anti-influenza agent of the neuraminidase inhibitor class. The ethyl ester prodrug oseltamivir is administrated orally as a phosphate salt and converted by hepatic esterases to the active metabolite oseltamivir carboxylate (OC) (1). OC specifically binds and inhibits the influenza virus neuraminidase enzyme that is essential for viral replication (2). 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 (3), while prophylactic administration prevents their onset (4). In recent years, abnormal or delirious behaviors have been reported with a low incidence in young individuals with influenza who were also receiving oseltamivir (5). 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 (6 and 7).

Nevertheless, health and regulatory authorities in Japan and elsewhere have amended the product label to include precautions on the use of oseltamivir in young persons. These actions, and the associated media coverage, have fostered renewed interest in the central nervous system (CNS) tolerability of oseltamivir(5). The aim of this study to evaluate neurotoxic effects of oseltamivir phosphate in lactating pups.

Materials and methods

Chemicals : Oseltamivir phosphate (flufly®)75 mg tablets provided by Julphar Ras Alkhima-UAE. The stock solution (1mg/ml) of oseltamivir phosphate were prepared by dissolving 1capsule (75)mg oseltamivir phosphate in 75 ml distilled water .(1)ml of stock was dilute with (9)ml of distilled water to prepare the concentration of 0.1mg/ml with dose volume of 0.1ml/10gm BW that used for dosing in therapeutic dose of 1mg/kg BW administrated toT1 .While 1ml of stock was diluted with 1ml of distilled water to prepare the concentration of 0.5mg/ml administrated with dose volume of 0.1ml/10 gm BW for 5 fold of therapeutic dose(5)mg/kg.BW administrated to T2. While control group administrated distilled water for the period of 14 day lactation. Total number of (12)pregnant female mice that kept after parturition with their of chosen (24) nursing pups ,weighed (3-4)gm that were divided equally into three groups, Two treatment group T1,T2 and one control group each consist of (4) nursing mother and(8) of their chosen lactating pups. The animals were raised and bred in the animal house of college of veterinary medicine/Baghdad University where the research was done. The animals were kept in cages of (20x15x15) cm^3 dimensions in average of one nursing mother with their

nursing pups in each cage, in optimum conditions of breeding at (22±3) ºC With (14/10) Hours (Light/Dark) cycle Standard pilliets and water Provided ad li-itum (8). When pups of treatment groups and control group reach 7 day of age, the mothers of control group was dosed orally, daily with distilled water for period of 14 day .While nursing mothers of treatment groups T1,T2 were dosed daily with therapeutic dose(1mg/kg).BW and fivefold the dose (5fd)5mg/kg.BW respectively for the rest period of lactation (14 day) to detect the neurotoxic effect of oseltamivir phosphate on mice pup during the lactation period and 7day after the termination of the nursing mothers treatment (at the end of lactation period). Pups of experimental groups (T1, T2and C) were tested for following parameters: Body weight gain/weekly1st and 14 day of treatment (at end of lactation). Clinical symptoms observation/daily. Gross and histopathological examination of organs and tissues /7 day after treatment termination. These organs involved brain, liver, kidney stomach and small intestine done according to (9).

Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of ($P<0.05$). Specific group differences were determined using least significant differences (LSD) as described by (10).

Results and Discussion

The result of body weight gain showed that at 14 day of treatment T1 recorded significant increase in body weight gain ($P\leq0.01$) which was 7.4 gm in comparison with control group and T2 in which body weight gain 5.6gm and 4.4 gm respectively. Table (1)

Table :(1) Body weight changes/grams of nursing pups received OP indirectly from daily dosed nursing mothers with different doses during lactation period.

Period / Group n=8 pups	1 day of treatment M±SE	14 day of treatment M±SE	Body weight gain M±SE
C D.W	3.2±0.2 A b	8.8±0.37 AB a	5.6±0.24 B
T1(1mg/kg)	2.8±0.2 A b	10.4±0.4 A a	7.4±0.4 A
T2(5mg/kg)	3.2±0.2 A b	7.6±0.4 B a	4.4±0.24 B

T1= Pups of 4 nursing mothers, in which nursing mothers dosed with therapeutic dose (T.D) 1mg/kg.BW.
T2= Pups of 4 nursing mothers , in which nursing mothers dosed with 5 fold (5FD) therapeutic dose 5mg/kg.BW.
C=Pups in which nursing mothers dosed distilled water (D.W)
M±SE represent mean ± standard error
-Different small letters represent significant differences within groups ($P\leq0.01$)
-Different capital letters represent significant differences between groups ($P\leq0.01$).

This result may be due to effect of OP and/or OC on dopamine and/or other catecholamine. Its reported that dopamine-deficient mice grew normally up to around 10 day after birth, but at postnatal day (P10–14) they were distinguishable during subsequent development, they lost more body weight and gradually weakened, the phenotypic abnormalities of these mutant mice revealed that dopamine is essential for animal development and survival during the juvenile stage(11).Also this result may be

occur through effecting the appetite by alteration in dopamine levels .Its estimated that dopamine also play a major role in the regulation of appetite (12),also may be as a result of interference with growth hormone release .Other suggestion is due to the reported gross and histopathological effect of drug in our study on stomach and intestine of treated groups in dose dependent manner that may affect absorption of food or effect the appetite positively in low doses and negatively in high doses. Clinical observation of lactation study showed that T1 and T2 showed neurotoxic symptoms appeared 1.5-2 hours after lactation .These symptoms appeared from the first dose and extended along the next days of treatment in dose –depended manner .The intensity and number of affected animals reach to peck for T1 in the 3rd and 4th day of treatment and for T2 in the 3rd 4th ,5th and 6th day of treatment then gradually reduced to be completely disappeared in the last 4 days of treatment in T1 and in last 2 days of treatment in T2 .These clinical symptoms include weakness ,convulsions, Lay on back or side, irritability and itching ears by hind limbs ,extended body, incoordination, extended limbs and limbs stiffness. Table (2).

Table (2): clinical symptoms of mice pups dosed with two different doses by nursing mother of oseltamivir phosphate during lactation period.

GROUP n=8 pups	Day of treatment / Clinical symptoms	1st	3rd	5th	7th	9th	11th	14th
D.W Control	_	_	_	_	_	_	_	_
T1 Pups of 4 nursing mothers dosed with therapeutic dose (1mg/kg .BW) of OP	weakness	++3 +5	++5 +3	++6 +2	+8	+8	_8	_8
	Convulsion	_2 +6	+8	-6 +2	+8	_8	_8	_8
	Lay on back or side	4	4	-	-	-	-	-
	Itching ears by hind limbs	3	1	_	_	_	_	_
	Extended body	-8	3	-5	-5	-5	-5	-5
	Incoordination	+2 _6	_8	_8	_8	_8	_8	_8
	Extended limbs	+8	+3 _5	_8	_8	_8	_8	_8
	Limbs stiffness	+8	+3 _5	_8	_8	_8	_8	_8
T2 Pups of 4nursing mothers dosed with (5 fold) of therapeutic dose (5mg/kg.BW) of OP	Weakness	++3 +5	++6 +2	++6 +2	++3 +5	+3 _5	+2 _6	_8
	Convulsions	+8	+8	+2 _6	+2 _6	+8	_8	_8
	Lay on back or side	4	4	-	-	-	-	-
	Itching ears by hind limbs	_	4	3	1	_	_	_
	Extended body	2	5	_8	_8	_8	_8	_8
	Incoordination	+ 3	+2_6	+2 _6	+8	+8	8-	_8
	Extended limbs	+3 _5	+3 _5	+2 _6	_8	_8	_8	_8
	Limbs stiffnes	+3 _5	+2 _6	+2 _6	_8	-8	_8	-8

1-8=number of pups showing toxic symptoms.

- (NON), + (SLIGHT), ++ (OBVIOUS), +++ (SEVER)

This result may be due to effect of OP and/or its metabolite OC on dopamine and/ or another catecholamine or may be other neurotransmitters of central nervous system like serotonin, GABA, glutamine and Ach. Primarily we think that OP and/or OC has ability to penetrate blood brain barrier(BBB).This penetration occur in time when BBB is still immature during lactation period and may be interfere with dopamine and /or another catacholamins and even other CNS neurotransmitters. It's reported that in developing mice BBB maturation is complete around the third postnatal week (13). Developing of elimination system of OP and/or OC beside gradual maturation of BBB may be were the main causes that reduced the observed clinical symptoms within last days of treatment. Its estimated that brain level of OP in pups were 1500 times that of adult animals exposed to the same dose(14).It's important to know that up to one fourth of OP is distributed via circulation and enters tissue though the BBB(15).We agree with recent study done by Yoshino(16) who used wibester rats in his study in which these rats received OP at dose 25 and 100 mg/kg.BW I/P the result indicate that increase extracellular dopamine with 156% and 223% of pre-administration level in prefrontal cortex (PFC) also was significantly greater than vehicle administrated rats and ataxia was observed. Also the neurotoxic symptoms observed in our study may be due to the extensive damage in brain of treated groups that involved cerebellum and also cerebrum. Weakness may be due to cerebellum damage .Studies shows that cerebellum dysfunction leading to delay initiation of movement and do not prevent it (17).This delay in movement may be appeared as weakness. Incoordination may be due to cerebellum and/or vestibular dysfunction caused by OP and/or OC effect .The cerebellum modulation and coordination of muscular activity are important in skilled voluntary movement as well as in the movement and posture equilibrium (18)while its damage may lead to incoordination and ataxia. Extended limbs may be due to effect of OP and/or OC on CNS neurotransmission, this cause that muscles receives impaired signals that lead to excessive muscle relaxation. Limbs stiffness may be attributed to imbalance between the two opposing muscles of limbs may be one of them more active than another. Extended body and back head noticed in T1 and T2.This result may be due to toxic effect of OP and/or OC on cerebellum causing more cerebellum dysfunction and more increase in stretch reflex that control muscle tone(19) . In recent study(2010) reported that When oseltamivir is administered in extremely high doses (500–1000 mg/kg) to young juvenile rats, central nervous system toxicity and death occurred in some animals. Mortality was not observed in older juvenile rats, suggesting a possible relationship between neurotoxicity and an immature blood-brain barrier. To assess potential neurologic adverse effects of oseltamivir use in infants, a retrospective chart review was performed in infants less than 12 months of age who received oseltamivir, amantadine, or rimantadine. The result revealed the occurrence of adverse neurologic events during therapy among subjects treated with oseltamivir versus those treated with the adamantanes but without significant difference .This is the largest report to date of oseltamivir use in children less than 12 months of age. Neurologic events were not more common with use of oseltamivir compared with that of the adamantanes (20).

The pathological changes study showed the presence of gross and histopathological lesions in dose dependent manner in all the examined organs, mainly were in the brain and to less extent in liver, kidney, stomach and small intestine. Brain of T1 macroscopically showed edema and slight congestion Fig (1) .while T2 showed severe congestion of meninges and hemorrhage. Microscopically brain of T1 showed Shrinkage of neurons with focal areas of hemorrhage Fig (2) in addition to congestion of blood vessels of cerebrum and meninges with focal gliosis. The cerebellum showed edema with degenerative changes and complete dissolution of purkinji cells. While T2 showed sever congestion of blood vessels of cerebral and cerebral meninges. Liver of T1 macroscopically showed enlargement and friability. Fig (3), while liver of T2 showed Hepatomegaly with rounded

edges and pale color with multiple areas of necrosis (gray-white) in color. Microscopically liver of T1 showed dilatation and congestion of blood vessels .Massive necrosis of hepatic parenchyma and increase in apoptosis, severe hemorrhage with infiltration of large numbers of neutrophils, Focal aggregation of neutrophils and lymphocytes in hepatic parenchyma with infiltration of neutrophils in sinusoids were also seen .The portal blood vessels and sinusoids contain serum protein, while T2 showed extensive areas of necrosis and heamorrhage. Neutrophils aggregation in parenchyma ,dilatation and congestion of blood vessels and sinusoid containing neutrophils in their Lumina with infiltration of mononuclear cells in portal areas and formation of granulomas beside blood vessels Fig (4). Kidney of T1 macroscopically showed slight congestion and atrophy, while T2 showed Atrophied kidney. Fig(5).Microscopically T1 showed Perivascular cuffing, congestion of blood vessels ,shrinkage of glomerular tuft .multiple areas of severe cortical hemorrhages with mononuclear cells infiltration in the interstitial tissue and around glomeruli with slight periglomerular fibrosis and proliferation of parietal layer of capsule with vacuolation of glomerular tuft ,also there was necrosis and apoptosis Fig(6 and7),while T2 showed Degeneration and necrosis of epithelial cells forming epithelial casts, congestion of blood vessels .severe destruction of renal tissue with hemorrhage. Stomach of T1 and T2, macroscopically, .showed thickened, corrugated mucosa Fig (8). While Microscopically stomach of T1 and T2 showed that in the non glandular regions: There was papillary proliferation of epithelial lining with marked hyperkeratosis Fig (9).The glandular region showed severe congestion of blood vessels of mucosa. Macroscopically small intestine of T1 showed edema Fig(10),while small intestine of T2 showed congestion of serosa surface ,with areas of hemorrhage, microscopically small intestine of T1 showed marked hyperplasia of lymphoid tissue with increase in cellularity of mucosa while T2 showed increase in numbers of goblet cells with increase in mucin secretion ,also infiltration of inflammatory cells in the lamina propria of mucosa Fig(11). All pathological changes were in dose dependent manner.

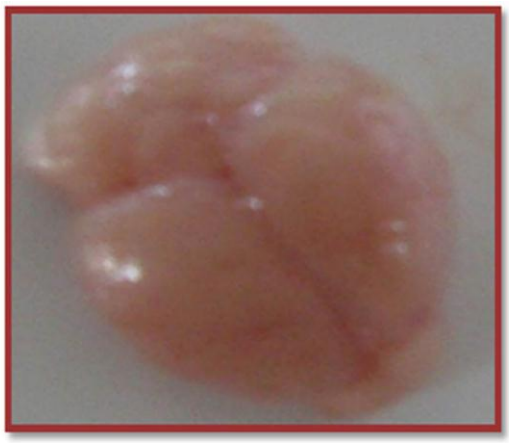
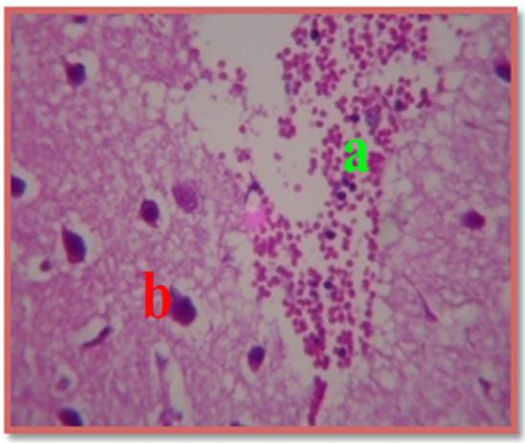
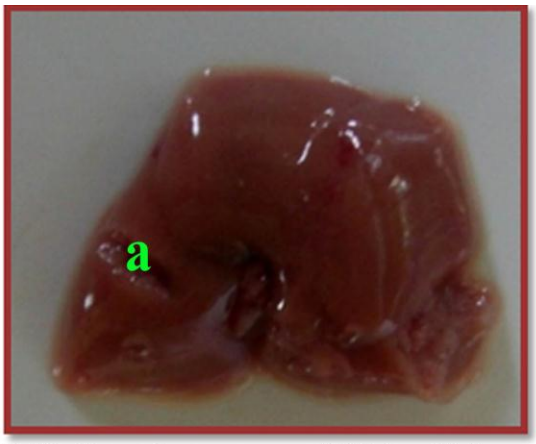


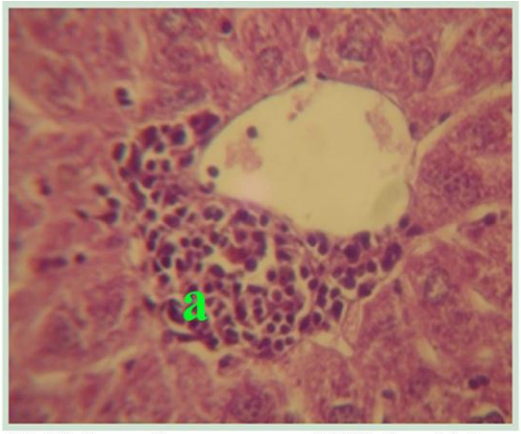
Fig (1) Macroscopical section of brain of mouse pup of T1 in which nursing mother treated with 1mg/kg.BW/dayof oseltamivir phosphate for 14 day of lactation period. Shows edema and slight congestion.



Fig(2) Histopathological section of brain cerebrum of mouse pup of (T1) in which nursing mother treated with 1mg/kg BW/day of oseltamivir phosphate for 14 day during lactation period shows shrinkage of neurons (a) with focal areas of severe hemorrhages (b) (H&Ex400).

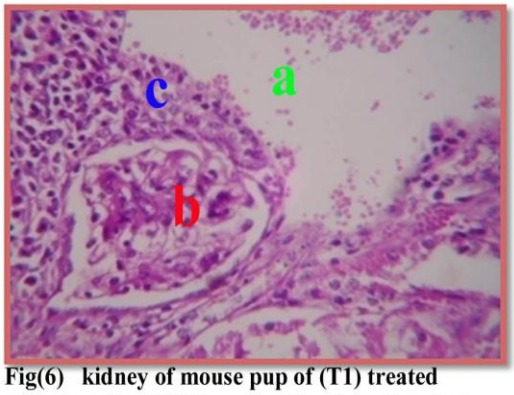


Fig(3) liver of mouse pup of T1 in which nursing mother treated with 1mg/kg.BW /day of oseltamivir phosphate for 14 day of lactation period shows enlargement and friablity.

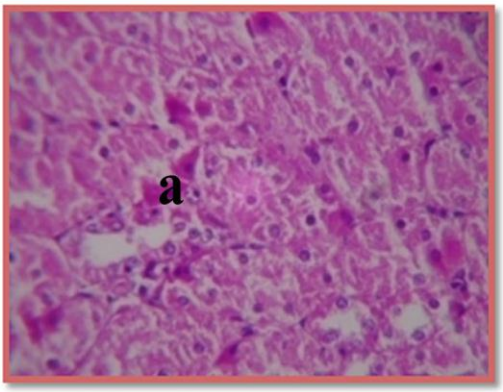


Fig(4) liver of mouse pup of (T2) in which nursing mother treated with 5mg/kg .BW/day of oseltamivirphosphate during lactation period for 14 day shows formation of granuloma beside the congested blood vessels (H&Ex400).

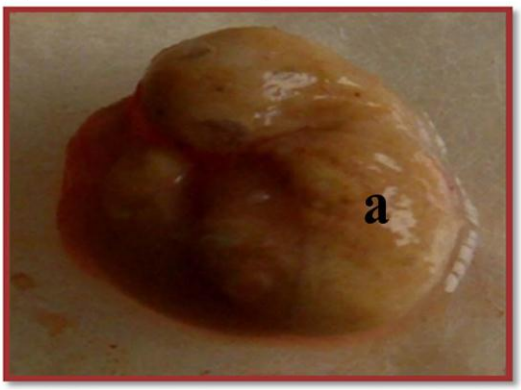
Fig (5) kidneys of mouse pup of T1 in which nursing mother treated with 1 mg/kg .BW /day of oseltamivir phosphate for 14 day Shows atrophy , with focal areas of necrosis.



Fig(6) kidney of mouse pup of (T1) treated with 1mg/kg .BW/day of oseltamivir phosphate for 14 day during lactation period shows severe cortical hemorrhage (a) with mononuclear cells infiltration in interstitial tissue, around glomeruli (b)and slight periglomerular fibrosis and poliferation of parietal layer of capsule (c)with vacuolation of glomerular tuft(H&E40X).



Fig(7) kidney of mouse pup of (T1) in which nursing mother treated with 1mg/kg.BW/day of oseltamivir phosphate for 14 day during lactation period shows severe necrosis and apoptosis(a) (H&Ex400).



Fig(8) Stomach of mouse pup of T1 in which nursing mothertreated with 1 mg/kg .BW /day of oseltamivir phosphate for 14 day shows thic kened ,corugated mucosa(a) .

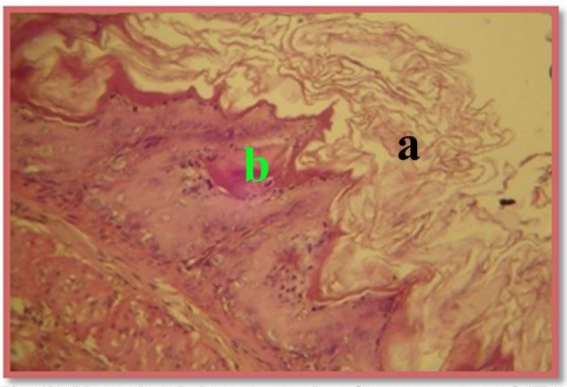
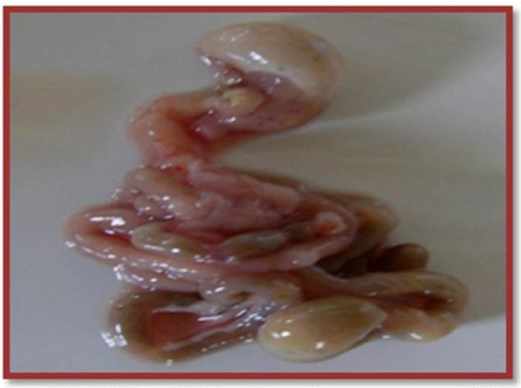


Fig (9) Non-glandular stomach of mouse pup of (T1) in which nursing mother treated with 1mg/kg .BW/day of oseltamivir phosphate for 14 day during lactation period shows papillary proliferation of epithelial lining (a) with marked hyperkeratosis(b) (H&E40X) .



Fig(10)Small Intestine of mouse pup of T1in which nursing mother treated with 1mg/kg. BW/day of oseltamivir phosphate for 14 day of lactation period shows edema .

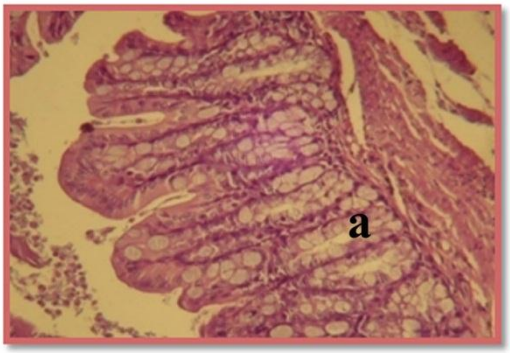


Fig (11) Intestine of mouse pup of (T2) in which nursing mother treated with 5mg/kg .BW/day of oseltamivir phosphate for 14 day during lactation period shows increase in numbers of goblet cells (a) with increase mucin secretion (H&E40X) .

In our study the gross and histopathological lesions of examined organs may be due to excessive exposure to OP and/or its metabolite OC. Gross and histopathological lesions of brain may due to penetration of OP and/or OC cross BBB and cause damage to multiple areas of brain ,we thought that OP may be more implicated in damage of brain structures and may be effected other organs. Its estimated that brain level of OP in pups were 1500 times that of adult animals exposed to the same dose (14). Because OP contain phosphate salt that may cause extensive damage and toxicity in the brain, when the BBB is still immature during the first days of study, as reported by James Farrelly from center of drug evaluation and research in (2000) who performed studies using rats and marmosets dosed orally for one month and he found tha t lesions were chronic progressive nephropathy,corticomedullary mineralization,tubular minerli-zation,tubular vacuolation, basophilic tubules and focal nephropathy,he suggest that the lesion of kidney were due to excessive exposure to more toxic prodrug OP where rats could not hydrolyze the OP sufficiently to its metabolite that will cause accumulate excessive amount of phosphate this would negatively affect the dietary calcium/phosphate ratio in species known to be sensitive to this type of change ,this consequencely would lead to mineralization of kidney, he showed no histopathological changes in liver of treated animals with OP .When he used marmosets as a labratory animals in his studies he showed that no histopathological changes seen in liver,kidney and bones except in GI in which OP was extremely irritant in primate more than of rodents ,in marmosets the lesions exist only in GI represented by severe gastric mucosal

inflammation ,atrophy ,hemorrhage ,erosion and ulceration are associated with 1000mg/kg. the ratio of OP:OC was 1:3 while in marmosets was 1:15 in urine analysis showed may be rodent could not hydrolyze OP efficiently like primates, OP is pro-ester drug we expect that its easily penetrate BBB and cause damage in brain in dose dependent manner. This not exclude the effect of OC .We thought that nursing mothers may be play an important role in transporting excessive amounts of OC to lactating pups within lactation period ,when metabolism system of adult mice more developed than of neonates.

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