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## Effect of Tramadol on the Development of Chick Embryos

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### Abstract:

Tramadol acts as a synthetic analgesic agent used to treat moderate to severe pain. The present study was designed to determinate the possible hazard effects of tramadol on the morphology of chick embryo and amniotic fluid biochemical changes. 240 Fertile chicken eggs were injected into the air sac with doses of 12.5,25,50 and 100 mg /kg egg wt, respectively, after 24 hr of incubation. Embryos were examined at 4 intervals on days 3, 6, 12, and 18 of incubation. Treated embryos showed an increase in mortality rates, growth retardation and many congenital malformations such as growth retardation, enlargement in the head region, and incomplete embryonic development on 3 days of incubation. Brain exencephaly, hematoma, acephaly, and ectopiacordis in 6 days of incubation. Undeveloped beak, reduced external auditory aperture and ectopia visceral were seen in 12-day treated embryos. While 18-day treated embryos exhibited oedema, anophthalmia, absence of feathers and limb deformities. Tramadol induced significant biochemical changes in the amniotic fluid including elevation in aspartate and alanine aminotransferases (AST and ALT), alkaline phosphatase (ALP), sodium (Na), creatinine and urea besides reduction in the total protein, potassium (K), calcium (Ca), phosphorus (Ph) and total antioxidant capacity (TAC) when compared to control groups. Tramadol has an embryotoxic effect, as shown by an increase in congenital abnormalities and a considerable modification of some amniotic fluid biochemical parameters Therefore, it is recommended that tramadol should be taken only with the prescription of doctor and self-medication of this medicine may be hazardous.

**Keywords:** Tramadol, Chick embryo, Congenital malformation, Biochemical studies.

### 1. Introduction

Opioid analgesics are considered the most common and effective pharmacologic agents used for the management and treatment of moderate to severe pain [1]. Mothers are among the most seriously impacted analgesic users, and the long-term repercussions of addiction will damage both them and the next generation [2,3]. As a result of the ease with which opioid analgesics can pass the placental barrier and reach the fetus during obstetric labour, attention and caution must be taken to reduce the risk of newborn depression [4]. Preterm birth, low

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birth weight, increased risk of spontaneous abortion, decreased head circumference, numerous limb deformities, sudden infant death, and infant neurobehavioral abnormalities are just a few of the negative pregnancy and infant outcomes that can result from widespread opioid use during pregnancy [5,6,7].

The world health organization (WHO) recommends tramadol as a step-2 analgesic or weak opioid and moderate pain medication for the treatment of cancer pain [8,9]. It also inhibits serotonin and norepinephrine reuptake [10]. Opioids are classified into old generation such as morphine and methadone and new generation pain killers such as tramadol. Many investigations demonstrated that older painkillers had teratogenic effects on human, rat, and chicken embryos. [11,12]. On the other hand, there are no sufficient studies to investigate the teratogenicity of newer opioids including tramadol. Tramadol's toxicity and teratogenicity must be determined using a preclinical model [13]. Due to its simplicity, low cost, high reproducibility of results, minimal ethical and legal implications, and the fact that the mother does not affect the drug's pharmacokinetics, the chick embryo serves as an appropriate model for in vivo assessment of the toxicity, biocompatibility, biodistribution, and pharmacokinetics of the drug [14]. Therefore, the purpose of this study was to evaluate the negative effects of tramadol on the development of chick embryos using morphological and biochemical investigations.

## **2. Materials and methods:**

### **2.1. Egg incubation**

240 Freshly fertile chicken eggs (*Gallus gallus domesticus*) weighing ( $50 \pm 5$  g.) were obtained from the Faculty of Agriculture Ain shams University breeder farm. Before incubation, eggs were cleaned with distilled water and 70% ethanol. After that, they were randomly positioned within a hatching incubator chamber (G.Q.F. Mfg. Co., Model 1502, USA) that was set to a temperature of  $38^{\circ}\text{C}$  while horizontal. Using a drip pan filled with sterile water, a constant humidity in the range of 60% to 70% was kept. Every four hours, the incubator's automated turner rotated the eggs to maintain equal environmental conditions and simulate the hens' normal nesting behavior [15]. After LD50 determination, different doses of tramadol were selected ranging from maximum tolerated dose (MTD) to the lowest dose with no observed adverse effect levels (NOAEL). The present study found that, the single Maximum Tolerated dose (MTD) was 100 mg/kg egg wt that can be administered to chick

embryos without causing severe toxicity or mortality [16]. Doses higher than MTD were highly embryo toxic. Therefore, doses 12.5, 25, 50 and 100 mg/kg egg wt of tramadol seemed to be the best doses for the induction of congenital malformation in the tested chick embryos.

The selected dose 50 mg /kg egg wt equivalent to the recommended human therapeutic dose [17]. Eggs were divided into six groups of 10 eggs each as the following: Eggs in Group (1) (G1) were not subjected to any injection and named as negative control group. Group (2) (G2) (Positive control group) was injected with 10 $\mu$ L of distilled water. Group (3) (G3) and Group (4) (G4) were administered 12.5 mg/egg kg wt and 25 mg / kg egg wt of tramadol (sub-therapeutic doses) and equivalent to 1/18 and 1/9 of the calculated LD50 respectively. Therapeutic dose of tramadol (50mg/kg egg wt) equivalent to 1/4 LD50 was administered to the eggs in Group (5) (G5) and 100 mg/kg egg wt of tramadol in suprathereapeutic dose and equivalent to 1/2 of LD50 was administered to the eggs in Group (6) (G6).

## 2.2. Drug:

Tramadol HCL, (225 mg tablets) was purchased from Indian Apple Company. Each Tablet (225 mg) of tramadol was grinded carefully to become powder and then was dissolved in 10 ml of sterile distilled water. So, every 1 ml of the solution contained 22.5 mg of the drug then diluted with distilled water to obtain the concentrated doses of tramadol equivalent to 12.5, 25, 50, and 100 mg/kg egg wt. Each calculated dose of tramadol was given as a single injection dissolved in 500 $\mu$ l distilled water by utilizing a 1 cc syringe with a very small, tiny needle (about 5 mm long) to create a 1 mm hole in each egg above the air chamber.

Eggs were weighed (Scale 0.01 - 5000 gm, China) and given the determined dose of the drug following their weights. To prevent dehydration, the holes were closed using adhesive tape. The eggs were returned to the incubator after drug injection to allow embryonic development. The controls and treated eggs were opened after 3,6, 12, and 18 days of incubation for morphological and biochemical examination according to [18].

## 2.3. Morphological Investigation and measurements

The incubated eggs of the controls and treated groups were steadily checked for viability and mortality by using the candling technique. Dead embryos were delicately removed from the yolk sac, cleaned with saline, checked for any obvious malformations, and then classified in accordance with [19,20]. Dead embryos were determined by the absence of beating heart, no distinguishable vasculature, or the presence of blood spots. Viability and

mortality rates were then calculated. Embryo weights were recorded by using a sensitive balance (Scale 0.01 - 5000 gm, China). Furthermore, crown-rump length (CRL) was measured for each embryo by extending a thread from the base of the beak down the back to the tip of the coccyx and then measuring the length of the thread [21].

#### **2.4. Biochemical markers of the amniotic fluid sampling:**

The amniotic fluid was examined on day 12 of the incubation period [22]. After the eggshell and shell membranes were removed from the blunt end of the egg (n = 10 per group), An 18-gauge syringe was used to collect a sample of amniotic fluid about 5ml/embryo from each embryo. To remove cell debris, the samples were centrifuged at 3000 rpm for 10 min. The supernatant was then taken for biochemical analysis to measure the enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) using the kinetical method of [23]. Total protein activity was measured using the colourimetric method [24]. While Urea was determined by the method of [25] and creatinine was measured by the method of [26]. Minerals contents (Calcium, phosphorus, sodium and potassium) were measured using the colourimetric method according to [27]. Additionally, total antioxidant capacity (TAC) was measured using a colourimetric method according to [28]. All kits used in the present work were purchased from Biomed, Mostafa Kamel Axis - Mall 24 - New Cairo.

#### **2.5. Statistical analysis**

For statistical analysis, a computer program (SPSS 22 version) was employed. Means  $\pm$  standard error (SE) was used to express the results. For the comparison of the groups, data were analyzed using general linear models with one-way ANOVA techniques. According to Duncan's comparisons, differences between the groups were determined statistically significant when  $p < 0.05$ , highly significant when  $p < 0.01$  and extremely significant when  $p < 0.00$  or  $p < 0.000$ .

### **3. Results**

#### **3.1. Morphological investigations:**

##### **3.1.1. Mortality rate:**

Tramadol-treated groups had significantly higher overall mortality rates than the control groups. Which was correlated with the increasing dose concentration of tramadol.

Almost all the abnormally treated embryos were dead comparable to normal controls as shown in table (1).

Note: There is no significant difference between control –ve and control +ve.

Table (1): The percentage of mortality rates of control and treated chick embryos at different days of development (n=10 embryos).

Duration of incubation				
Groups	3days	6days	12days	18days
G1	0(0%)	0(0%)	0(0%)	0(0%)
G2	0(0%)	0(0%)	0(0%)	0(0%)
G3	0(0%)	2(20%)	1(10%)	1(10%)
G4	2(20%)	2(20%)	3(30%)	4(40%)
G5	3(30%)	3(30%)	4(40%)	5(50%)
G6	4(40%)	4(40%)	5(50%)	6(60%)

### 3.1.2. Body weight:

Administration of chick embryos with single doses of tramadol (25, 50, and 100 mg/kg egg wt) at days 3, 6, 12, and 18 of the incubation periods showed a significant ( $p < 0.05$ ,  $p < 0.0001$ ) decrease in the embryo's body weights. The reduction of the embryo's body weight was dose-dependent. The lowest dose (12.5mg /kg egg-wt) revealed non-significant ( $p > 0.05$ ) variation in the average body weights versus control groups as shown in table (2).

Table (2): The average body weight (grams) and percentages of change of control and treated chick embryos at different days of development.

Duration of incubation				
Groups	3days	6days	12days	18days
G1	0.43±0.011	0.82±0.006	4.57±0.066	31.23±0.51
G2	0.45±0.008	0.83±0.009	4.56±0.111	30.36±0.58
G3	0.44±0.014 (-2.32%)	0.77±0.017 (-6%)	4.17±0.117 (-8.7%)	28.37±0.50 (-9.1%)
G4	0.40±0.012 <sup>a*</sup> (-6.92%)	0.72±0.011 <sup>a*</sup> (-12%)	3.96±0.165 <sup>a*</sup> (-13.3%)	27.08±0.41 <sup>a*</sup> (-13.2%)
G5	0.36±0.019 <sup>a***</sup> (-16%)	0.70±0.019 <sup>a***</sup> (-14.6%)	3.32±0.181 <sup>a***</sup> (-27.3%)	21.09±3.50 <sup>a***</sup> (-32.4%)
G6	0.34±0.013 <sup>a***</sup> (-21%)	0.62±0.013 <sup>a***</sup> (-24.3%)	2.38±0.28 <sup>a***</sup> (-47.9%)	18.28±3.14 <sup>a***</sup> (-41.4%)

Values are represented as mean ±SE (n=10 embryos).

a: Statistically significant compared to the corresponding value in the control and treated groups. \*: (p<0.05), \*\*: (p< 0.01), and \*\*\*: (p<0.001) or (0.0001).

### 3.1.3. Crown rump length (CRL):

Tramadol administration at different doses (12.5, 25, 50, and 100 mg/kg egg wt) induced significant ( $p<0.01$ ,  $p<0.0001$ ) diminution of the embryo's CRL measurements at 3, 6, 12, and 18 days of incubation versus control groups. The CRL measurements of treated chick embryos were lower than those of the control groups, as shown in Table (3).

Table (3): The crown-rump length (cm) and percentages of change of control and treated chick embryos at different days of development.

Duration of incubation				
Groups	3days	6days	12days	18days
G1	0.51±0.005	3.73±0.053	8.20±0.07	16.06±0.092
G2	0.50±0.05	3.72±0.066	8.28±0.14	15.88±0.037
G3	0.44±0.55 (-13.7%)	3.34±0.060 <sup>a**</sup> (-10%)	7.94±0.24 <sup>a**</sup> (-3%)	15.62±0.17 <sup>a**</sup> (-2.7%)
G4	0.36±0.03 <sup>a*</sup> (-29.4%)	2.96±0.12 <sup>a**</sup> (-20%)	7.46±0.35 <sup>a**</sup> (-9%)	13.62±0.52 <sup>a**</sup> (-15%)
G5	0.29±0.033 <sup>a**</sup> (-43.13%)	2.20±0.10 <sup>a***</sup> (-41%)	6.26±0.25 <sup>a***</sup> (-23%)	12.38±0.86 <sup>a***</sup> (-22%)
G6	0.28±0.24 <sup>a**</sup> (-45%)	1.62±0.11 <sup>a***</sup> (-56%)	4.06±0.82 <sup>a***</sup> (-50%)	8.26±1.19 <sup>a***</sup> (-48%)

Values are represented as mean ±SE (n=10 embryos).

a: Statistically significant compared to the corresponding value in the control and treated groups. \*: ( $p < 0.05$ ), \*\*: ( $p < 0.01$ ) & \*\*\*: ( $p < 0.001$ ) or (0.0001).

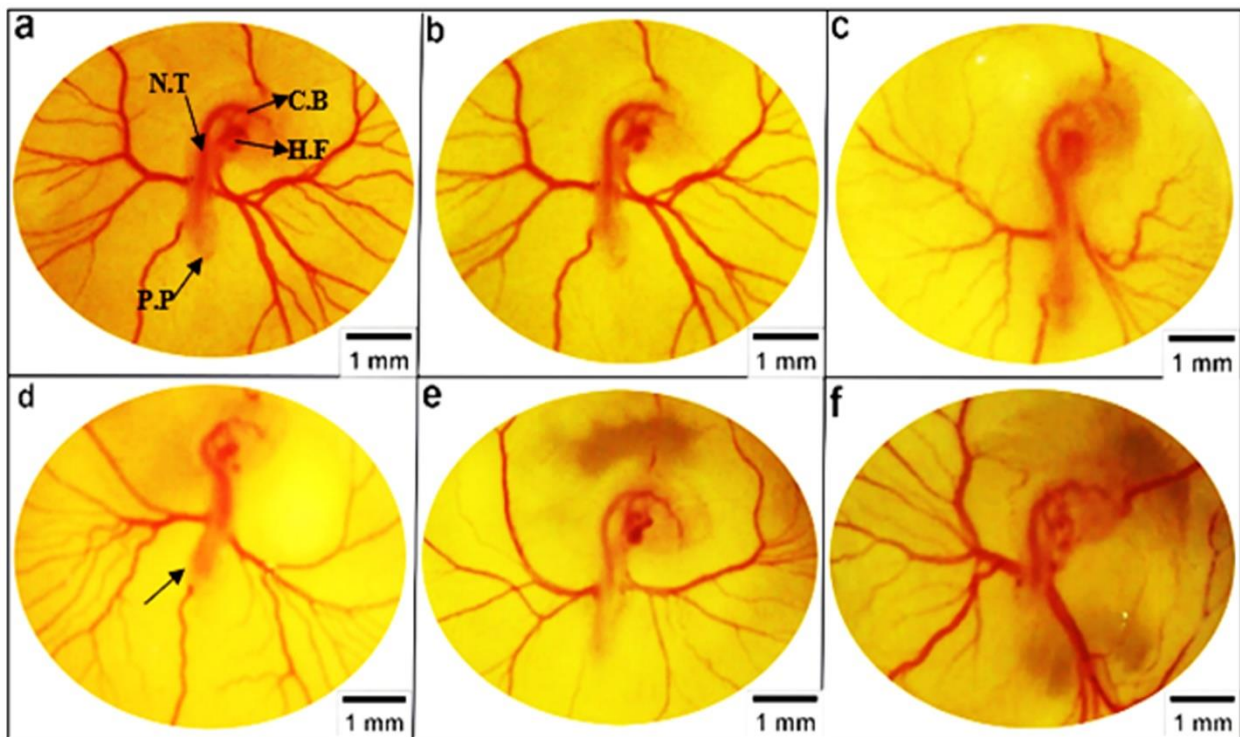
### 3.1.4. Gross evaluation of the external body features:

#### 3.1.4.1. Three –day- old chick embryos:

The external morphology of 3 day old chick embryos (HH stage 16) from the -ve control group (administered no treatment) and the +ve control group (given 10 µl distilled water) revealed that the embryo's flexure is now apparent and it is laying on its left side. blood circulation starting. The vitelline membrane covers the surface of the yolk. The head and tail can be discerned as well as the neural tube. The appearance of the cardiac structures which begin to beat (Figs.1a&b). When compared to the normal external morphology of the control



groups, three-day chick embryos treated with various doses of tramadol displayed signs of abnormalities in their external body features after 24 hours after injection. Tramadol-treated embryos at a low dose of 12.5mg/kg egg wt showed no signs of external abnormality as controls (Fig. 1c). Embryos treated with (25 mg/kg egg wt) tramadol displayed curved tail (Fig. 1d). Embryos treated with (50&100 mg/kg egg wt) tramadol exhibited incompletely embryonic development, growth retardation, enlargement in the head region and reduction in the tail bud



(Figs. 1e&f).

Fig. 1: Photographs of 3day-old- chick embryos of control and tramadol-treated groups. (a) -ve Control (received no treatment) and (b) +ve control (administered 10  $\mu$ l distilled water) embryos showed normal embryonic development of the cephalic bud (C.B), heart formation (H.F), neural tube (N.T), (P.P) posterior body part with usual external features. (c) Embryo treated with (12.5mg/kg egg wt) tramadol showed no signs of external abnormality as controls. (d) Embryo administrated with (25 mg/kg egg wt) tramadol displayed curved tail (arrow). (e) Embryo administrated with (50 mg/kg egg wt) tramadol exhibited enlargement in the head region. (f) High- dose tramadol-treated embryo (100 mg/kg egg wt) revealed growth

retardation, incomplete embryonic development, enlargement in the head region and reduction in the tail bud.

### 3.1.4.2. Six-day-old chick embryos:

The external morphology of the control group of 6-day-old chick embryos (HH stage 29) showed that the head was larger in diameter than the body. Large, symmetrical eyes with a round shape were present. The egg's tooth was missing, but the beak appeared prominent. The skin covered the viscera located in the thoracic and abdominal chambers, including the heart.

Both the upper and lower limbs were paddle-shaped, but there was no sign of digit development (Figs. 2a&b). 6-day-old chick embryos treated with different doses of tramadol showed signs of abnormality in their external body features after 24 hours of injection in a dose-dependent manner compared to the normal control groups. Tramadol-treated embryos at a low dose (12.5mg/kg egg wt) showed mild reduction in body weight and length (Fig. 2c). Embryos treated with (25 mg/kg egg wt) tramadol displayed an absence of beak and lower limb (Fig. 2d) whereas embryos treated with (50 mg/kg egg wt) tramadol exhibited protruding brain (exencephaly), subcutaneous haemorrhage (hematoma) in the head region and absence of upper and lower limbs (Fig. 2e). Additionally, high dose tramadol-treated embryos (100 mg/kg egg wt) revealed overall growth retardation with the absence of brain (acephaly), absence of two eyes (anophthalmia), and the heart situated outside the thoracic cavity (ectopiacordis) (Fig. 2f).

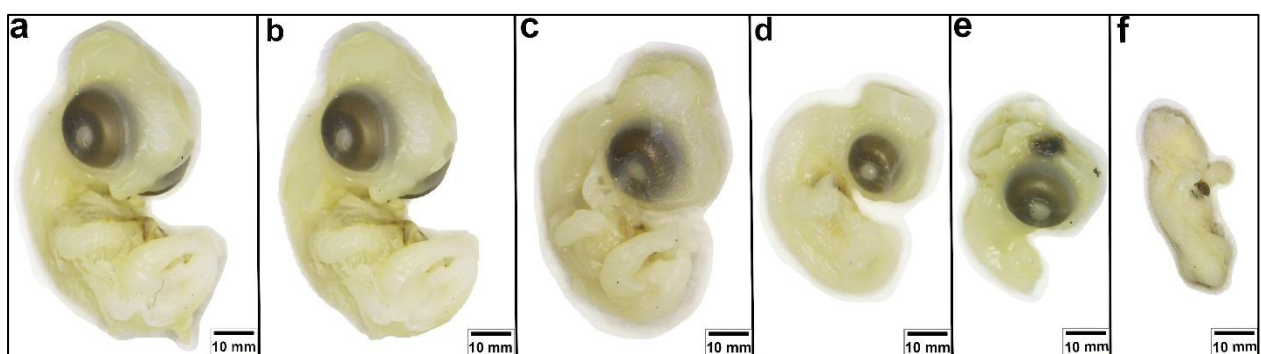


Fig .2: Photographs of 6-day-old chick embryos of control and tramadol-treated groups. (a) -ve Control and (b) +ve Control embryos showed normal external features. (c) Tramadol (12.5mg/kg egg wt) treated embryo showed mild reduction in body weight and length. (d) Embryo administered with (25 mg/kg egg wt) tramadol

displayed absence of beak and lower limb. (e) Embryo administrated with (50 mg/kg egg wt) tramadol exhibited exencephaly, subcutaneous haemorrhage in the head region and absence of upper and lower limb. (f) High dose tramadol-treated embryo (100 mg/kg egg wt) revealed overall growth retardation with acephaly, anophthalmia, and ectopiacordis.

### 3.1.4.3. Twelve- day-old chick embryos:

The embryonic body parts of 12-day chick embryos (HH stage 38) in the control groups had developed short, sparse down feathers at this point, and the top beaks had grown larger and harder with white scales covering the tip. The external auditory apertures which were situated at the right angle about 2 mm behind the eyes and in the shape of thin spherical openings with elevated edges, were situated between the nostrils, which were narrow slits. The eyes had well-developed eyelids and were significantly larger than the size of the head. The embryo's leg lacked scales and its wing displayed the hen's typical wing parts. Their digits were comprised of distinct phalanges that terminated in tiny claws. Normal control 12-day-old chick embryos showed normal development of the brain, eye, upper limb, lower limb, beak, and closure of thoracic and abdominal walls (Figs. 3a&b). Embryos treated with (12.5mg/kg egg wt) tramadol showed no signs of external abnormality as controls (Fig. 3c). On the other hand, embryos treated with (25 mg/kg egg wt tramadol) exhibited an absence of lower limbs and undeveloped beak (Fig. 3d). Embryos received (50mg/kg egg wt) tramadol showed exencephaly with oedema of the eye, undeveloped beak, narrow neck, limb defects and reduced external auditory aperture (Fig. 3e) whereas embryo received (100 mg/kg egg wt) tramadol suffered from external body malformations and growth retardation. Internal organs were abnormally exposed to ectopia visceral and atrophy of the legs (Fig. 3f).

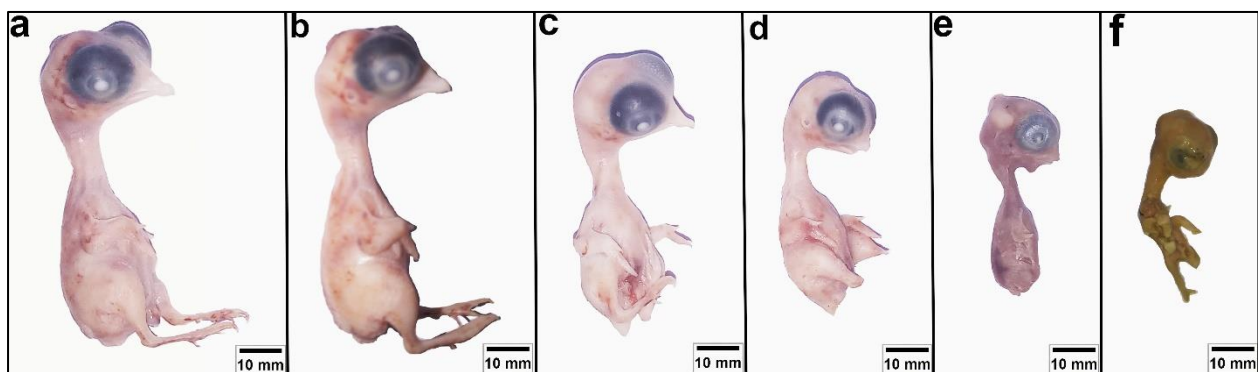


Fig.3: Photographs of 12-day-old chick embryos of control and tramadol-treated groups. (a) -ve Control and (b) +ve Control embryos showed normal external features. (c) Embryo treated with (12.5mg/kg egg wt) tramadol showed a reduction in weight and length. (d) Embryo treated with (25 mg/kg egg wt tramadol) exhibited absence of lower limbs and undeveloped beak. (e) Embryo received (50mg/kg egg wt) tramadol showed exencephaly with oedema of the eye, narrow neck, undeveloped beak and limb defects. (f) Embryo received (100 mg/kg egg wt) tramadol suffered from external body malformations, growth retardation, ectopia visceral and atrophy of the legs.

#### 3.1.4.4. Eighteen- day-old chick embryos:

The external morphology of control group 18-day chick embryos (HH stage 44). The beak's translucent peridermal layer is beginning to proximally peel off. The head became under the right wing. The results showed normal external morphology of all control groups (Figs. 4a&b). Embryos treated with (12.5mg/kg egg wt) tramadol showed a mild decrease in weight and length (Fig. 4c). Tramadol (25mg/kg egg wt) treated embryos showed crossed beak, absence of one eye (anophthalmia), a hematoma on the head and absence of feathers (Fig. 4d). Embryos administered (50mg/kg egg wt) of tramadol revealed body hematomas, swelling and oedema of the eye, and disappearing of the external auditory aperture (Fig. 4e). High dose (100 mg/kg egg wt) tramadol -treated embryos exhibited a symmetrical wing (phocomelia), weakness and paralysis in the hind limb, short beak, haemorrhage inside eye, growth retardation and exencephaly (Fig. 4f).

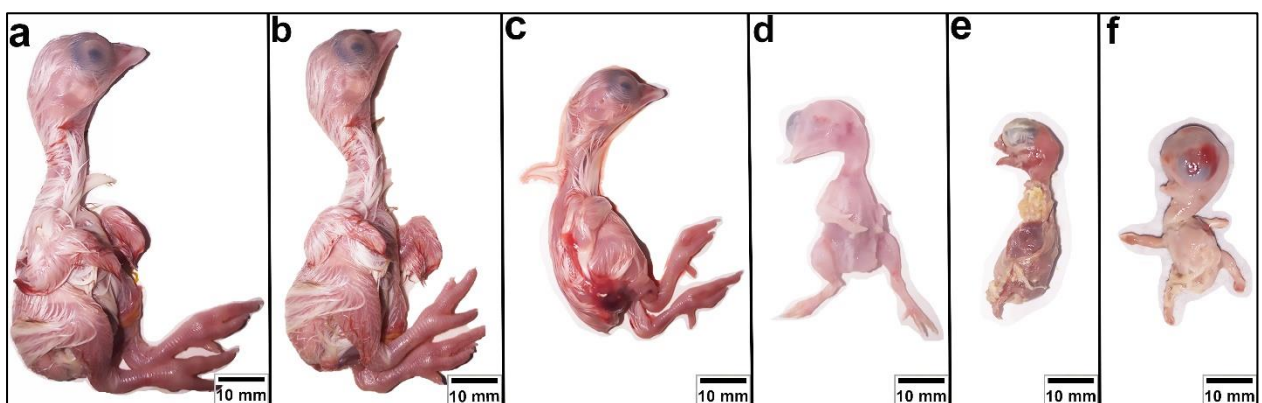


Fig.4: Photographs of 18-day-old chick embryos of control and tramadol-treated groups. (a) -ve Control and (b) +ve Control embryos showed normal external

features. (c) Embryo treated with (12.5mg/kg egg wt) tramadol showed mild decrease in weight and length. (d) Tramadol (25mg/kg egg wt) treated embryo displayed crossed beak anophthalmia, ahematoma on the head and absence of feathers. (e) Embryos administered (50mg/kg egg wt) of tramadol revealed body hematomas, swelling and oedema of the eye, and disappearing of the external auditory aperture. (f) High dose (100 mg/kg egg wt) tramadol treated embryo exhibited a symmetrical wing (phocomelia), weakness and paralysis in the hind limb, short beak, haemorrhage inside the eye, growth retardation and exencephaly.

### 3.2 Biochemical studies:

#### 3. 2.1. Liver functions

Detecting activities of liver enzymes in the amniotic fluid of chick embryos at day 12 of incubation revealed a significant increase ( $p < 0.01$ ,  $P < 0.0001$ ) in aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzymes activities with the administration of 12.5, 25, and 50 and 100 mg/ kg egg wt tramadol, respectively versus controls. In addition to, a significant ( $P < 0.05$ ) elevated level of alanine aminotransferase (ALT) has been found in chick embryos receiving tramadol at high doses of 50 mg/ kg egg wt and 100 mg/ kg egg wt whereas the levels of ALT were not significantly ( $P > 0.05$ ) changed with 12.5 and 25mg/ kg egg wt doses of the drug compared to control groups (Table 4). On the other hand, total protein content concentrations revealed a significant ( $p < 0.01$ ) reduction at doses of 12.5, 25 and 50 mg/kg egg wt and a highly significant ( $p < 0.0001$ ) decline at 100 mg/ kg egg wt of the tramadol treatment compared to controls as shown table (4).

Table (4): liver function tests and percentages of change in the amniotic fluid of 12 days old chick embryo following tramadol treatment.

groups parameters	G1	G2	G3	G4	G5	G6
AST (IU/L)	1.65± 7.29	1.74± 2.94	3.10±7.56 <sup>a**</sup> (87%)	4.18±1.19 <sup>a**</sup> (150%)	5.65±2.07 <sup>a**</sup> (242%)	5.82±3.87 <sup>a***</sup> (250%)

ALP (IU/L)	32.06 ±0.70	31.98 ±0.89	39.42±0.54 <sup>a**</sup> (22%)	76.38±3.05 <sup>a**</sup> (138%)	87.56±4.38 <sup>a**</sup> (170%)	104±5.43 <sup>a***</sup> (224%)
ALT (IU/L)	0.91± 0.07	1.39± 0.28	1.16±0.10 (27%)	1.49±0.09 (63%)	2.16±0.18 <sup>a*</sup> (137%)	4.15±0.42 <sup>a*</sup> (350%)
Total protein (mg/dl)	2.96± 0.16	2.96± 0.25	2.42±0.23 <sup>a**</sup> (-18%)	1.64±0.05 <sup>a**</sup> (-44%)	1.60±0.18 <sup>a**</sup> (-45%)	1.46±0.12 <sup>a***</sup> (-50%)

Values are represented as mean ±SE (n=10).

a: Statistically significant compared to the corresponding value in the control and treated groups. \*: ( $p < 0.05$ ), \*\*: ( $p < 0.01$ ) & \*\*\*: ( $p < 0.001$ ) or (0.0001).

### 3. 2.2. Kidney functions

Data of urea and creatinine concentrations in the amniotic fluid of chick embryos at day 12 of incubation are represented in table (5). The data showed that urea levels at doses (50 mg/ kg egg wt and 100 mg/ kg egg wt) were significantly ( $p < 0.01$ ) higher than control groups, whereas (12.5 and 25mg/ kg egg wt) were non-significantly ( $p > 0.05$ ) elevated from the control groups. Furthermore, creatinine values at all doses were significantly ( $p < 0.01$ ) higher than control except the lowest dose (12.5 mg/ kg egg wt) showed a non-significant ( $P > 0.05$ ) change in the level of creatinine value from control groups.

Table (5): kidney function tests and percentages of change in the amniotic fluid of 12 days old chick embryo following tramadol treatment.

groups parameters	G1	G2	G3	G4	G5	G6
Urea (mg/dL)	15.30± 0.24	15.13± 0.29	16.71±0.45 (9%)	17.12±0.52 (11%)	22.88±1.47 <sup>a*</sup> (49%)	24.25±1.17 <sup>a**</sup> (58%)

Creatinine (mg/dl)	0.30± 0.04	0.26± 0.04	0.44±0.07 (31%)	1.14±0.08 <sup>a**</sup> (280%)	1.50±0.07 <sup>a**</sup> (400%)	1.6±0.08 <sup>a**</sup> (430%)
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Values are represented as mean ±SE. (n= 10)

a: Statistically significant compared to the corresponding value in the control and treated groups. \*: ( $p < 0.05$ ), \*\*: ( $p < 0.01$ ) & \*\*\*: ( $p < 0.001$ ) or (0.0001).

### 3. 2.3. Mineral contents

Sodium concentration levels in the amniotic fluid of chick embryos at day 12 of incubation significantly ( $p < 0.001$ ) increased after a single injection with tramadol at doses (25, 50 and 100 mg/kg egg wt) compared to controls. whereas (12.5 mg/ kg egg-wt) of the drug showed non-significant ( $p > 0.05$ ) variations versus controls. In contrast, at doses 25, 50, and 100 mg/kg egg wt, respectively, potassium, calcium, and phosphorus concentrations showed a significant ( $p < 0.05$  to  $p < 0.0001$ ) reduction when compared to control groups. Treatment with (12.5 mg/kg egg wt) showed non-significant ( $p > 0.05$ ) changes according to the corresponding controls as shown in table (6).

Table (6): Concentrations of mineral contents and percentages of change in the amniotic fluid of 12 days old chick embryo after tramadol treatment.

Groups Parameters	G1	G2	G3	G4	G5	G6
Sodium (mmoI/L)	1.05± 2.16	1.08± 2.41	1.09±2.3 5 (3%)	1.24±1.03 <sup>a***</sup> (18%)	1.29±1.12 <sup>a***</sup> (22%)	1.33±2.28 <sup>a***</sup> (26%)
Potassium (mmoI/L)	6.82± 0.46	6.76± 0.41	5.16±0.2 8 (-24%)	4.97±0.26 <sup>a**</sup> (-27%)	3.88±0.38 <sup>a***</sup> (-43%)	3.76±0.91 <sup>a***</sup> (-44%)

Calcium (mg/dL)	0.77± 0.05	0.71± 0.06	0.76±0.0 6 (-1.2%)	0.65±0.02 <sup>a*</sup> (-9%)	0.47±0.02 <sup>a***</sup> (-38%)	0.40±0.09 <sup>a***</sup> (-48%)
Phosphorus (mg/dL)	7.38± 0.38	7.12± 0.35	7.24±0.4 4 (-1.8%)	6.34±0.26 <sup>a*</sup> (-14%)	4.71±0.47 <sup>a***</sup> (-36%)	4.03±0.74 <sup>a***</sup> (-45%)

Values are represented as mean ±SE (n= 10).

a: Statistically significant compared to the corresponding value in the control and treated groups. \*: ( $p < 0.05$ ), \*\*: ( $p < 0.01$ ) & \*\*\*: ( $p < 0.001$ ) or (0.0001).

### 3. 2.4. Total antioxidant capacity (TAC) level

The total antioxidant capacity (TAC) recorded a highly significant ( $p < 0.0001$ ) decrease at all doses of tramadol administration when compared with the control groups in the amniotic fluid of chick embryos at day 12 of incubation as shown in table (7).

Table (7): Total antioxidant capacity levels and percentages of change in the amniotic fluid of 12 days old chick embryo following tramadol treatment.

Groups parameters	G1	G2	G3	G4	G5	G6
Total antioxidant capacity (mM/L)	7.87± 0.29	7.42± 0.12	4.50±0.059 <sup>a***</sup> (-42.8%)	2.61±0.21 <sup>a***</sup> (-66.8%)	1.6±0.14 <sup>a***</sup> (-79.6%)	1.11±0.24 <sup>a***</sup> (- 85.8%)

Values are represented as mean ±SE (n= 10).

a: Statistically significant compared to the corresponding value in control and treated groups. \*: ( $p < 0.05$ ), \*\*: ( $p < 0.01$ ) & \*\*\*: ( $p < 0.001$ ) or (0.0001).

## 4. Discussion

Embryogenesis is a complicated process, which can be affected by genetic and environmental factors as well as drugs that cause birth defects. Opioids are one of the factors having a significant impact on the developmental process and raising the risk of congenital



abnormalities. [29]. In Egypt and other Middle Eastern nations, tramadol misuse has been progressively rising over the past few years. The cheap price, wide availability, and illegal importation of tramadol may be to blame for its high prevalence in these counties [30].

Overdosing on tramadol has been linked to fights, automobile accidents, and inadvertent self-inflicted injury. Tramadol has been reported to cross the placental barrier [31]. Long-term opioid painkiller use by pregnant mothers can cause fetal death [32].

The current study revealed that tramadol-treated embryo groups had significantly greater mortality rates than the control groups. Factors that have been implicated in the mortality include the strength and duration of opioid administration, control of temperature, humidity, and turning of the eggs 10-12 times a day during the incubation period [19,33]. Our findings were in agreement with those of [34,35] who established that morphine administration (0.01 mg/ml and 20 mg/kg) in rat embryos and nalufin injection during the organogenesis phase of chick embryos resulted in higher mortality rates when compared to control and sham groups [36]. The present work confirmed that the increased mortality rate is linked to increasing drug concentrations.

The present study elucidated a significant ( $p < 0.05$ ,  $p < 0.0001$ ) decrease in the body weights and crown-rump lengths of chick embryos that administrated tramadol compared to the controls. These were in agreement with [36,37] who found the same results after nalufin (20 mg/egg, single dose) and tramadol (1,3,9,27,50 and 100 mg/kg) injections. Reduction in weight and length may be a sensitive and accurate indicator of growth retardation. This is due to the decrease in energy supply and blood flow as evidenced by the altered properties of macromolecules of the yolk sac and the decrease in the nutrient transfer which is responsible for embryonic growth [38].

Similarly, [39] reported that morphine injection at different doses in either pregnant rats or mice resulted in decreased length and weight of the fetuses. The authors explained that it is suggested that growth retardation results from morphine increasing dopamine and xanthine oxidation, which can increase reactive oxygen species (ROS) [40] which, in turn, cause an overall loss of body mass [35].

Tramadol appears to cause vasoconstriction, which then inhibits blood flow [41]. Tramadol use during pregnancy can have an immediate influence on fetal growth, decrease the number of cells in various organs, and negatively impact cell division, normal growth, and

development [42]. These findings could be the result of free radical production, which led to oxidative stress and, in turn, metabolic dysfunction [43].

In the current study, injection of tramadol triggered various morphological abnormalities in chick embryos such as an increased percentage of acephaly, exencephaly, limb deformities, anophthalmia, ectopia visceral, hematoma, and short beak. This is in agreement with [10] who found that using opioids during pregnancy leads to similar congenital malformations in embryos due to the toxic effects of the opioid's metabolic products in the gastrointestinal tract. Moreover, [44] found that injection of methadone hydrochloride at a dose of 20 and 40 mg per kg body weight resulted in malformation in mouse, rabbit, and chick embryos such as exencephaly which was suggested to occur due to extensive dilatation of blood vessels in the averted brain hemispheres. Furthermore, [36] verified that injection of nalufin showed morphological abnormalities in chick embryos as an increased percentage of omphalocele, exencephaly, limb deformities, scanty feathers, head enlargement, and short beaks. The authors explained that these anomalies might result from irregular angiogenesis, which is a significant factor in the pathogenesis of anencephaly, and from the failure of primordial cerebral vessels to integrate into the body's circulatory system.

The amniotic fluid that surrounds and monitors the embryo has a very complex dynamic property. It is frequently used in clinical diagnostics and is considered an indicator of the developmental stage of the embryo. Results from the present study indicated that tramadol has a toxic effect on liver tissue and the amniotic fluid of chick embryo at day 12 of incubation that was recognized by significant elevation ( $p < 0.01$   $p < 0.0001$ ) in enzymatic liver markers (ALT, AST, and ALP) *via* significant ( $p < 0.01$ ) reduction of the amniotic protein concentrations compared to controls.

The intracellular enzymes ALP, ALT, and AST are found in the cells of many different avian organs, including bone, kidneys, intestine, and liver, and their rise is usually linked to tissue damage and the potential for these enzymes to enter the amniotic fluid in birds [45]. In addition, the teratogenic effect of the agents may also elevate such enzymes in the amniotic fluid [46].

In the same line, [47,48,49] explained the significant increase in the liver enzymes (ALT, AST) to the impairment of hepatocytes, high cell membrane permeability, and hepatocellular necrosis in albino rats treated with tramadol (60 mg/kg/day) for 90 days in comparison with the control group.

Moreover, [50,51] declared that the significant increase in ALT, AST, and ALP in rats treated with (12.5, 25, 50 mg/kg/day) tramadol hydrochloride for two weeks along with reduction of protein content in amniotic fluid might be due to the effect of tramadol on normal structure of hepatocytes that led to increased use of various amino acids in the generation of antibodies in response to tramadol administration and decreased albumen and globulin synthesis in the liver tissue [52, 53].

The current study suggested that significant ( $p < 0.01$ ) higher levels of urea and creatinine in the amniotic fluid of the experimental groups of chick embryos to the negative impacts of the tramadol on the kidney functionality compared to control groups. [54, 55] showed that the assessment of creatinine is highly important to determine kidney function and the glomerular filtration rate in birds. The results of the present study were consistent with those of [56,57,58], who showed that tramadol-treated groups of mice had significantly higher levels of creatinine and urea than the control group due to pronounced kidney function impairment, and its retention was thought to be an indicator of glomerular insufficiency [52, 59, 60, 61].

In the present investigation, sodium concentration levels in the amniotic fluid at day 12 of incubation chick embryos significantly ( $p < 0.001$ ) increased after a single injection with tramadol. On the other hand, potassium, calcium, and phosphorus concentrations have been shown a significant ( $p < 0.05$  to  $p < 0.0001$ ) decrease in the drug-treated groups compared to the control groups. [62] elucidated that elevation in sodium is suggested due to tissue injuries besides possible transfer of its ions and edema into the amniotic fluid.

[52] stated that the fluctuations of sodium, potassium, and phosphorus concentrations in amniotic fluid might reflect its effect on blood circulation during embryonic development. [60] demonstrated that the decreased calcium level during pregnancy disrupts skeletal development and embryonic growth. These findings also revealed that the levels of electrolytes: ( $\text{Na}^+$ ), ( $\text{K}^+$ ), ( $\text{Ca}^{2+}$ ), and ( $\text{Po}_4^{3+}$ ) may be impacted by tissue damage due to hypoxia and asthmatic medication in response to tramadol administration as shown in rabbits.

In the current study, the total antioxidant capacity (TAC) recorded a highly significant ( $p < 0.0001$ ) decrease at all doses of tramadol administration when compared with the control groups in the amniotic fluid at 12 days of incubation. This significant decrease in TAC may be attributed to the enhanced lipid peroxidation leading to tissue damage and failure of the antioxidant defence mechanism [63]. The decrease in TAC might be due to the fact that

antioxidant enzymes were found to contain a transition metal as a cofactor and tramadol may interact with these enzymes' metal substrates, which would inhibit TAC [49].

Measurement of the antioxidant capacity of biological fluids represents an indicator of the overall ability of the body to counteract ROS, prevent oxidative damage, and counter oxidative stress-related diseases [64]. To combat ROS and reduce their damage, living organisms have evolved complex antioxidant systems. The TAC of the system is equal to the amount of endogenous antioxidants [65, 66].

Tramadol and its metabolites resulted in the excessive production of ROS that causes DNA damage and triggers cellular apoptosis in rats. Additionally, many studies have indicated a connection between prolonged opioid use and increased reactive oxygen species generation (ROS) [67, 68].

## 5. Conclusion

Our results indicate that tramadol has an embryotoxic effect *via* induced significant reduction of total antioxidant capacity and failure of antioxidant defenses to neutralize the increased production of ROS from opioid administration that in turn led to internal tissue injury, malformation, increasing mortality rate, growth retardation, reduction of protein levels and some minerals along with alteration of liver and kidney enzymes in amniotic fluid. Therefore, it is essential to make people aware of the effects of tramadol, especially for the pregnant women who should avoid consumption altogether.

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## الملخص العربي

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قسم علم الحيوان - كلية البنات للعلوم والأداب والتربية - جامعة عين شمس-القاهرة -مصر

#### الملخص العربي:

الترامادول يعمل كمسكن اصطناعي لعلاج الآلام المتوسطة والشديدة. صممت الدراسة الحالية لتحديد الآثار الخطرة المحتملة للترامادول على جنين الفرخ والتغيرات الكيميائية الحيوية في السائل الأمنيوسي. في هذا البحث تم حقن 240 من بيض الدجاج المخصب مرة واحدة بجرعات 12.5، 25، 50 و 100 ملجم / كجم من وزن البيض ، على التوالي في الغرفة الهوائية بعد 24 ساعة من التفريخ. تم فحص الأجنة على اربعة فترات ، أيام 3 و 6 و 12 و 18 من التفريخ. أظهرت الأجنة المعاملة زيادة في معدلات النفوق في النمو والعديد من التشوهات الخلقية مثل تأخر النمو وتضخم منطقة الرأس وعدم اكتمال نمو الجنين عند 3 أيام من التفريخ. خروج للدماغ ، ورم دموي ، وغياب الدماغ، وخروج القلب خارج الجسم عند 6 أيام مقارنة بالمجموعة الضابطة. شوهد منقار غير مكتمل، واختفاء فتحة الأذن الخارجية ، وخروج الاحشاء خارجيا للمعاملة لمدة 12 يوماً. بينما أظهرت الأجنة المعاملة لمدة 18 يوماً غياب العين ، وغياب الريش وتشوهات في الأطراف. أظهرت النتائج أن الترامادول أحدث تغييرات كيميائية حيوية كبيرة في السائل الأمنيوسي بما في ذلك الارتفاع في مستويات انزيمات الكبد والصوديوم والكرياتينين واليوريا بالإضافة إلى انخفاض البروتين الكلي والبوتاسيوم والكالسيوم والفسفور وإجمالي قدرة مضادات الأكسدة عند مقارنتها بالمجموعات الضابطة. في النهاية اوضحت هذه الدراسة أن الترامادول له تأثيرات سامة علي أجنة الدجاج وذلك من خلال زياده حدوث التشوهات الخلقية والتغيرات الكبيرة في بعض الخصائص الكيميائية الحيوية للسائل الأمنيوسي. لذلك يجب الحد من استخدام هذا الدواء خلال فترة النمو الجنيني.

