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Evaluation of teratogenic potency of colchicine by using *Biomphalaria alexandrina* snail embryo as an indicator

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ABSTRACT

The present study was planned to follow up the teratogenic potency of cholchicine on the developmental stages of *Biomphalaria alexandrina*. The results revealed that trochophore stage can be considered as a specific stage for colchicine at which the embryos are highly sensitive. The fractionation of proteins by SDS-PAGE electrophoresis showed that colchicine induced disappearance of the band with the molecular weight 158.5 kD and the increase in the intensity of some bands. The impact of colchicine against egg masses containing trochophore larvae induced many disturbances of organic substances such as a significant decrease in the total protein (58.96 % decreases), lipid (35.11 % decrease) and a glycogen levels (42.84 % decrease). The subsequent or co-treatment with recovery agents such as vitamin C, holothurin extract and folic acid were investigated. The teratogenic effect of colchicine on the morphogenesis of snail embryos is so intensive that the malformations couldn't be reversed by subsequent or co-treatment with vitamin C and holothurin. But, it is worth reporting that concomitant addition of folic acid to malformed embryos succeeded to produce significant amelioration of the teratogenic deformities induced by colchicine reached to 86.7 %. But, folic acid has a very slight recovery effect on the embryos post-treated with it. In conclusion the *B. alexandrina* snail embryo can be used as a bioindicator for studying the teratogenic effect of colchicine. Also using folic acid serves a good prophylactic agent against teratogenicity induced by colchicine.

Keywords

Freshwater snails, *Biomphalaria alexandrina*, embryogenesis, colchicine, teratogenecity, recovery.

Academic Discipline And Sub-Disciplines

Invertebrates

SUBJECT CLASSIFICATION

Zoology

TYPE (METHOD/APPROACH)

Microscopic study of the embryo before and after treatment, SDS-PAGE Electrophoresis and Biochemical assay.

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INTRODUCTION

The genus *Biomphalaria* is a pulmonate snail which shows wide geographical distribution [37]. It plays very important role in the hosting and transmission of some important parasitic diseases such as Schistosomiasis. Its direct development and the transparency of the egg mass and egg capsule membranes, allow easy observation of its embryonic development and behavior.

Embryological development is a very sensitive period for any species. It is highly influenced by the changes of ecological factors. Teratogenic effects on fresh water organisms are still poorly understood [31].

The drugs are among the non-biological environmental factors which may affect strongly on the embryo [64]. Perturbations to the events surrounding fertilization, early cleavage stages, and the morphogenetic movements leading to gastrulation and the establishment of three primary germ layers will frequently lead to arrested development and inviability of the developing organism.

Colchicine is a phytoalkaloid prepared from the dried corns and seeds of *Colchicum autumnale*, Autumn *crocus* or *Meadow saffron*. It was isolated in 1820 by Pelletier and Caventou. Colchicum is also present in *Gloriosa superba* [23, 45]. Although colchicine has been employed as an anti-inflammatory drug, it is a well-known mitotic inhibitor affecting microtubule assembly [59, 7]. It binds to tubulin and prevents its polymerization into microtubules, thereby blocking formation of the mitotic spindle and arresting nuclear division at metaphase [29]. It is quite toxic at doses slightly higher than therapeutic, resulting in wide ranging effects, including neuropathy, myopathy, and multi-organ failure [26]. Also, colchicine inhibits the DNA synthesis by inhibiting the induction of the key enzyme in DNA synthesis [63].

Since the numbers of chemical substances are used commonly in medical treatments, the necessity for protection from their negative-effects has increased. Nowadays, it is thought that the harm effect of these chemicals could be tolerated by using some vitamins, and several studies are being carried out for this aim [24]. Vitamin C (Ascorbic acid) is known to be potent antioxidant. In living organisms ascorbate act as an antioxidant by ameliorating the toxic effects of reactive species generated by chemical agents in biological systems [32, 65, 57, 5, 60]. Also, vitamin C could exert chemo protective effects without apparent toxicity at doses higher than the current recommended dietary allowance of 60mg/day [34]. Furthermore, vitamin C helps improve sperm count and motility and protects spermatozoa against endogenous oxidative DNA damage [21].

On the other hand, Folic acid have the capacity to resist (or to neutralize) the effects of free radicals [54]. Some information has been found in the literature regarding the preventive effects of folic acid in developmental defects of various living species. Perry and Miller [51] reported that folic acid is important for the metamorphosis and normal development of *Musca domestica*. Recent evidence linking folic acid to the prevention of neural tube defects [16, 10]. So, the use of supplemental folic acid by women pre- and post-conception diminishes the occurrence of neural tube defects in their offspring [15].

Holothurin (saponine), it is an important class of natural products first discovered in higher plants where they are widely spread [35]. It has also been isolated from marine organisms such as holothurians [47, 67], sea stars [38] and sponges [62]. It has been reported that holothurin is an antioxidant [8] and an antitumor agent [50].

The present work aims to use *Biomphalaria* sp. as a model species for an invertebrate embryo test to manage and control snail development and to evaluate the teratogenic potency of a drug and understand the mechanisms by which the drug operates in the embryo. Colchicine is used as a teratogen due to its availability, pharmaceutical importance, locally produced and low cost. Vitamin C, folic acid and holothurin were chosen to offer the possibility of using them as recovery agents against negative effects of colchicine and also to offer a new hope in using these agents in cancer therapeutics because of the great resemblances between the embryo and cancer cells.

Materials and Methods

Experimental snails

Adult snails of *Biomphalaria alexandrina* (8-10 mm) were purchased from laboratory bred colony in Medical Malacology Department, Theodor Bilharz Research Institute (TBRI). The snails were kept in four plastic containers (80 cm diameter and 20 cm depth) filled with dechlorinated tap water at 25 ± 3 °C with a 12/12 hours light/dark cycle. Snails were fed on fresh lettuce and water was renewed weekly.

Egg masses

The freshly egg masses of *Biomphalaria alexandrina*, laid on glass aquarium, were collected daily on polyethylene sheets floating on each aquarium or from the wall of the glass using a spatula and were then maintained in climatic chambers at 25 °C (±3 °C) until the end of the experiments.

Colchicine

Colchicine was purchased from El Nasr Pharmaceutical Chemistry Company, Cairo, Egypt. Colchicine is an alkaloid prepared from the dried corns and seeds of *Colchicum autumnale*, *Autumn crocus* or *Meadow saffron*. Its color is Pale yellow and darkens on exposure to light. The chemical structure of colchicines is (S)-N-(5, 6, 7, 9-Tetrahydro-1, 2, 3, 10-

tetramethoxy-9-oxobenzo [alpha] heptalen-7-yl) acetamide. Or N-(5, 5, 7, 9-Tetrahydro-1, 2, 3, 10-tetramethoxy-9 -oxobenzo [alpha] heptalen-7-yl) acetamide [13].

Preparation of colchicine

Stock solution of 1000 ppm colchicine was prepared on the basis of W/V using dechlorinated tap water (pH 7.0–7.5) by solubility of 1gm of colchicine per 1L of dechlorinated tap water. A series of concentrations (10, 20, 30, 40, 50, 100, 200 & 250 ppm) that would permit the computation of LC_{50} and LC_{90} values was prepared. Control embryos were maintained under the same experimental conditions in dechlorinated tap water. The effectiveness for this compound has been expressed in terms of LC_{50} and LC_{90} [36], the sub-lethal concentrations of colchicine used in this study were (1, 2, 3, 4 & 5 ppm).

Experimental design

Three experiments were undertaken during this study
Experiment 1: Study the ovicidal activity and the degree of toxicity of colchicine.
Experiment 2: Evaluation of teratogenic potency of colchicine by determination the specific stage of development, its protein profile and organic contents.
Experiment 3: study the effect of folic acid, vitamin C and holothurin as recovery agents against teratogenic effect of colchicine after or during exposure periods.

Experiment 1: Ovicidal activity and the degree of toxicity of colchicine

The toxicity of colchicine was screened as described by WHO [66]. Experiments with snail eggs were conducted in test container (petri-dishes) filled with 50 ml of a specific concentration of the testing chemical. Different developmental stages were exposed to series of concentrations of colchicine (10-200 ppm) to determine the LC_{50} and LC_{90}. The lethal concentrations were calculated using statistical probit analysis [20]. The developmental stages which are used in the ovicidal activity test were 2-cell stage, 4-cell stage, blastula stage, gastrula stage, trochophore larvae and veliger larvae. Three replicates were used for each test concentrations. For calculating the per cent mortality of eggs, non-motile, disintegrating embryonic forms or absence of movement and heartbeat of the embryos or embryos with an extended, unattached foot were tested for mortality by stimulating of the foot with the bristles of a fine brush. And when no movement of the embryo in the egg capsule was observed, it was considered as dead.

Experiment 2: Evaluation of teratogenic potency of colchicine by determination of the specific stage of development

The freshly laid egg masses containg fertilized eggs were exposed to different sub-lethal concentrations of colchicine. Control and treated samples were held at constant temperature at 25 °C (±3 °C). At least three concentrations with 3 replicates were used. Criteria for egg viability were based on the motility of the embryo within the egg. This could be distinguished by dissecting microscope examination. The effect of colchicine on different developmental stages of *B. alexandrina* were observed using digital camera microscope unit (Olympus microscope CX 31 ; Tokyo, Japan equipped with an image analyzing system) to detect the embryonic abnormalities and to determine the affected stage and the most effective concentration for using in biochemical assay. To determine the teratogenicity of colchicine, the treatment was initiated at each developmental stage (blastula, gastrula, trochophore and veliger stages) and observed after seven days. This teratogenic test of colchicine against developmental stages of *B. alexandrina* involved utilization of control groups exposed to dechlorinated tap water only. The experimental samples composed of 5 egg masses each with about 25 individuals. The sub-lethal concentrations used in this experiment was (1-5ppm) according to the LC50 investigation.

Preparation of samples for biochemical studies

To study the protein profile, total protein, total lipid and glycogen of both control and colchicine treated egg masses of *B. alexandrina*; numerous freshly laid egg masses were collected and divided into 2 groups. The first group is the control one which exposed to dechlorinated tap water until the formations of trochophore stage. The second group is the treated one which exposed to 5 ppm colchicine for three days until the formation of trochophore stage.

Electrophoretic study

Total protein contents were extracted from 0.02 g of either the exposed or the control trochophore embryo using 200 µl of 0.1 M Tris-buffer(pH= 7.5) containing EDTA (0.01 M), KCl (0.01 M) MgCl2 (0.1 M) and 4% polyvinyl pyrrolidine (PVP). Tissue homogenates were centrifuged at 8050g for 20 min at 4 °C (Sigma 3K18 cooling centrifuge). The protein extract was then transferred into a fresh eppendorff tube and used directly for electrophoresis or kept at -20 °C until use. SDS-PAGE was performed on 8% separating and 4% stacking gels according to the method of Laemmli [33]. The gels were stained with 2% Coomassie Brilliant Blue R-250. A similarity matrix was constructed on the basis of the presence / absence of bands from Dic similarity coefficient [17] using the formula: S= 2a/2a+b+c, where a = number of bands shared between samples 1 and 2, b= the number of bands present in 1 but not in 2 and c = number of bands present in 2 but not in one.

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Biochemical assay

Total protein

Total protein was determined using a commercial kit (Biomed Diagnostics, 30175 Hannover, Germany) and bovine serum albumin as a standard.

Total lipid

Total lipid was determined using a commercial kit purchased from Biodiagnostic (Egypt) which depends on the reaction of lipids with vanillin in a medium of sulphuric acid and phosphoric acid to form pink colored complex [68]. Olive oil ranging between 5 and 40 mg/dl were used for standard curves. Samples were homogenized in phosphate buffer (0.1 M Na2HPO4, pH 7).

Extraction and determination of glycogen

For extraction of glycogen, 200 mg of sample of embryos ground with 20 ml of 5% trichloro acetic acid (TCA) in a mortar or preferably in a homogenizer. The precipitate of proteins is filtered off and the clear filtrate submitted to analysis [52].

Experiment 3: Recovery

Determination of sub-lethal concentration of vitamin C, folic acid and holothurin

Vitamin C and folic acid were purchased from the local pharmacy. Holothurin extracted from the sea cucumber *Holothuria polii* was obtained as gift from Dr. Nahla Omran (associate professor of Invertebrates, Zoology Department, Faculty of Science, Tanta University). A series of concentrations of vitamin C, folic acid and holothurin has been prepared to calculate the mortality rate. The concentration at which no mortality was obtained is considered a sub-lethal concentration.

Recovery of malformed embryos post-treated with vitamin C, folic acid and holothurin

To examine the recovery role of vitamin C, folic acid and holothurin on colchicine teratogenicity, freshly laid egg masses of *B. alexandrina* were collected. These egg masses were divided into two groups, colchicine treated group (1ppm) and control group received no treatment. When the control egg masses reached trochophore stage, the malformed embryos from colchicine treated group were removed from it and washed through several changes of dechlorinated tab water and transferred for further development to three petri dishes. The first contains vitamin C (5ppm), the second contains folic acid (0.5 ppm) and the third contains holothurin (17 ppm). Control egg masses remained in dechlorinated tab water. When the control group reached pre-hatching stage, the post-treated embryos examined for growth and development. The percentage of recovered embryos was determined after 7 days.

Recovery of malformed embryos co-treated with vitamin C, folic acid and holothurin

Freshly laid egg masses of *B. alexandrina* were collected. These egg masses divided into three groups all of them treated with colchicine. At the same time the recovery agents (vitamin C, folic acid and holothurin) were added (one to each group). The concentrations of recovery agents as recorded in the previous experiment. The percentage of recovered embryos which complete its growth and development was determined after seven days from beginning of experiment.

Statistical analysis

To compare the malformation frequencies among the control and treated groups, abnormality values of colchicines were expressed as a mean ± S.D., and statistically analyzed by one-way ANOVA test. Biochemical analysis and egg laying capacity results were statistically analyzed using Student's *t*-test to determine significant differences between treated and control samples. Statistical analysis was performed with the aid of the SPSS computer program (version 17 windows).

Results

Colchicine LC_{50} and LC_{90} determination in different developmental stages of *B. alexandrina*

Death of embryos or absence of movement of the embryos was considered a good parameter for calculating the percent of mortality of developmental stages. The LC_{50} and LC_{90} values of colchicine against different developmental stages (fertilized eggs, cleavage, blastula, gastrula, trochophore and veliger stages) of *B. alexandrina* are summarized in Table 1. The exposure time is from the beginning of each stage. This analysis revealed relationship between sample mortality and colchicine concentration at tested exposure duration.

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Table 1. LC_{50} and L_{C90} of colchicine on different developmental stages of *B. alexandrina*. The recorded time is from the beginning of each stage.

Developmental stages	LC_{50}	LC_{90}
Fertilized egg after 1 hr	11	12.17
Fertilized egg after 2 hr	10.2	10.42
2-cell stag after 1 hr	3.5	3.81
4-cell stage after 1 hr	12.04	12.41
Blastula stage after 5 hrs	17.4	19.18
Blastula stage after 20 hrs	11.437	11.882
Gastrula stage after 5 hrs	11	11.95
Gastrula stage after 20 hrs	10.2	10.8
Trochophore stage after 10 hrs	29.3	30.5
Trochophore stage after 20 hrs	14.8	15.295
Veliger stage after 30 hrs	13.80	15.33

Ovicidal activity and the degree of toxicity of colchicines

As shown in Table 2, the mortality rate induced by colchicine treatment is concentration and time-dependent. All early embryonic stages (fertilized egg, 2-cell stage, 4-cell stage, blastula stage and gastrula stage) showed highest mortality rate at the exposure time recorded for each stage and at highest concentration (30 ppm). The results showed that there was no effect at the beginning of each stage. After sometime the effect became evident and increased gradually till the end of each stage. It is expected that colchicine penetrates slowly because of the resistance of capsular membrane. The same results were obtained in the case of late embryonic stages (trochophore and veliger stages) where the highest mortality rates were obtained after 20 hrs of exposure (for trochophore) and 30 hrs (for veliger) and at the highest concentrations (150 and 200 ppm) of colchicine Table 3.

Table 2. Mortality rate of *B. alexandrina* early developmental stages exposed to some concentrations of colchicine. The time recorded, for each stage, is from the beginning of it.

Developmental Stages	Time of Exposure	Mortality percentage		
		10 ppm	20 ppm	30 ppm
Fertilized egg	1hr	4.5	11.6	22.4
(2 hr)	2 hr	15.8	30.4	87.5
2-cell stage	1/2 hr	0	0	0
(2-3 hr)	1 hr	23	30.8	41.7
4-cell stage	1/2 hr	0	0	0
(3-4 hr)	1 hr	0	18.8	47.1
Blastula stage	1 hr	0	0	0
(10-30 hrs)	5 hrs	0	0	16.7
	10 hrs	0	0	16.7
	20 hrs	0	25	36.8
Gastrula stage	1 hr	0	0	0
(30-54 hrs)	5 hrs	4.5	11.6	22.2
	10 hrs	6.8	13.9	33.3
	20 hrs	6.97	14.1	33.3

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Table 3. Mortality rate of *B. alexandrina* late developmental stages exposed to some concentrations of colchicine. The time recorded, for each stage, is from the beginning of it.

Developmental Stages	Time of Exposure	Mortality percentage			
		50 ppm	100 ppm	150 ppm	200 ppm
Trochophore stage	1 hr	0	0	0	0
(54-78 hrs)	5 hrs	33.33	36	83.33	86.4
	10 hrs	35	36.2	83.5	86.6
	15 hrs	50	80	84	87.1
	20 hrs	50.5	80.43	100	100
Veliger stage	10 hrs	0	0	0	0
(78-126 hrs)	20 hrs	0	0	0	0
	30 hrs	21.4	25	28.57	38.9

The degree of teratogenicity of colchicines

The effect of sub-lethal concentration of colchicine on the fertilized egg till the end of developmental stages is shown in Figs. 1 and 2. The blastula and gastrula stages give lower percentages of malformation than those of trochophore and veliger larvae stages. Trochophore larva is the most malformed stage (Fig. 1). The percentage of malformation increased with the increasing of the concentration of colchicine. Fig. 2 shows that 5 ppm colchicine is the most effective concentration in which higher percentage of malformed trochophore larvae were obtained. At this concentration the development arrested at trochophore stage and its lyses begins after 10 days from oviposition.

The effect of sub-lethal concentrations of colchicine from the beginning of each developmental stage is shown in Fig. 3. The percentage of abnormal embryos depends on the dosage of colchicine and the time (stage) at which it is added to the medium. The treatment with low concentrations of colchicine (1-3 ppm) scarcely produced abnormal embryos especially when the treatment was initiated at blastulae and gastrulae stages. On the other hand 4 and 5 ppm of colchicine can induce high percentage of abnormalities. 100% of deformed embryos were obtained when embryos were exposed to colchicine at trochophore stage. So, this stage can be considered as a specific stage at which the embryos are highly sensitive to colchicine.

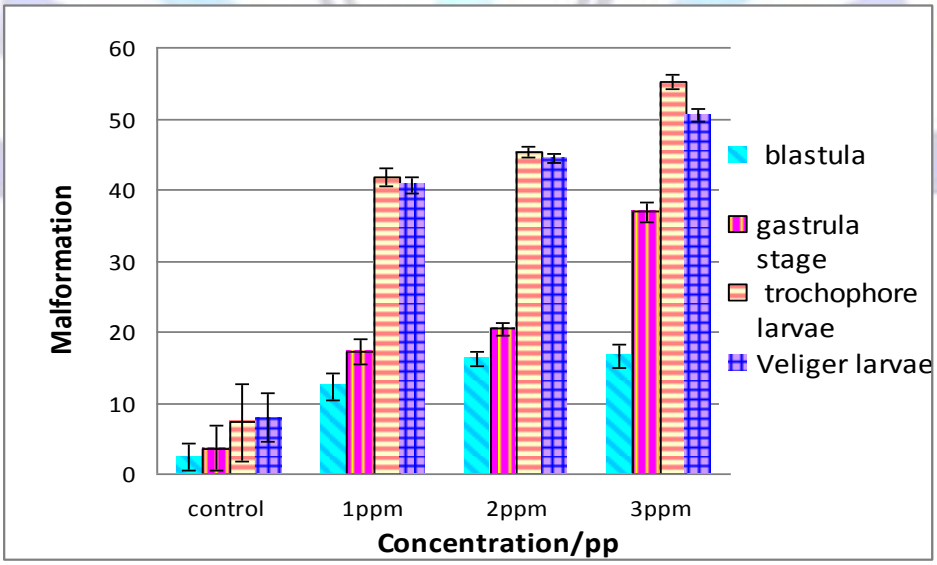

Fig 1: Percentage of malformed embryos (blastula, gastrula, trochophore and veliger) after treatment of fertilized egg with various concentrations of colchicine. *Significant P<0.05

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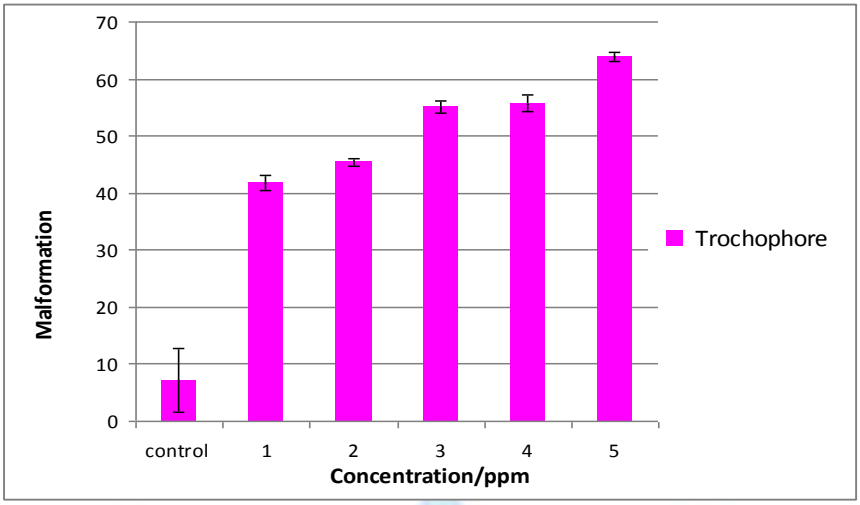

Fig 2: Percentage of malformed trochophore after transferring the fertilized egg to various concentrations of colchicine. *Significant P<0.05.

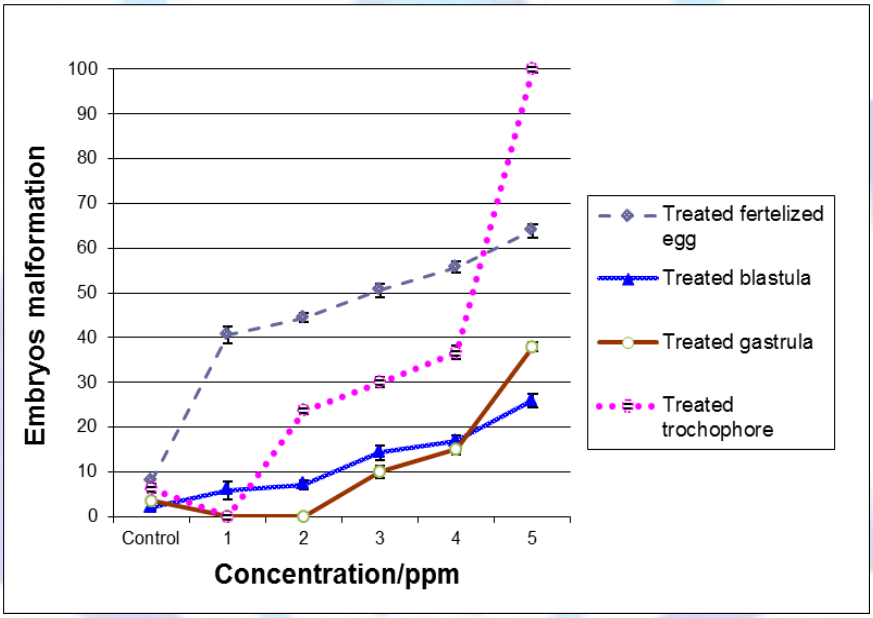

Fig 3: Relation between the stage of colchicine administration and the percentages of abnormal embryos after 7 days of treatment.

Morphological deformations

At exposure of egg masses to different sub-lethal concentrations of colchicine, the embryos in all developmental stages (cleavages, blastula, gastrula, trochophore, veliger, hippo-veliger and prehatching stages) revealed many deformations. The degree of deformations varying from slight to severe according to the exposure time and the concentration of colchicine.

The treated 16-cell stage was characterized with abnormal distribution and disintegration of embryonic cells (Figs. 4 B, C) and sometimes unknown large vesicles were appeared (Fig. 4 D) when compared with the control 16-cell stage (Fig.4 A).

At blastula stage (Fig. 4 E) treatment caused many kinds of deformations such as degeneration of ectodermal cells, formation of large blebs at the margin of the blastula and compaction blastocoel (Fig. 4 F). Treated gastrula stage (Figs. 5 B, C) showed degenerated ectodermal and endodermal cells on comparing with normal gastrula stage (Fig. 5 A). At the continuity of treatment, exogastrulation which reveal a vesicular embryo is occurred and the invagination of the archenteron is suppressed (Fig. 5 D).

At the trochophore larva stage (Fig 5 E), treatment caused abnormal changes (Fig. 5 F) like highly pigmentation and degeneration of its cells. The veliger larva, at the beginning of treatment, showed a fluid filled vesicle at one of its ends (Fig. 6 B). After sometime, it became very dark and lost its movement (Fig. 6 C) compared with the normal one (Fig.6 A). The abnormally developed hippo-veliger larva (Fig 6 E) showed reduced shell, deformation of the tentacles compared with the normal one (Fig. 6 D). The abnormally developed pre-hatcing larval stage showed enlarged head with enlarged eyes and tentacles, malformed shell and enlarged foot (Figs. 6 G) compared with the normal prehatching larval stage (Fig 6 F).

It is important to mention that both 4 and 5 ppm colchicine arrested the development at the trochophore stage. A colchicine concentration higher than 5 ppm is fatal to the embryos at early developmental stages indicating that colchicine diffuses through the capsule within 10 hours.

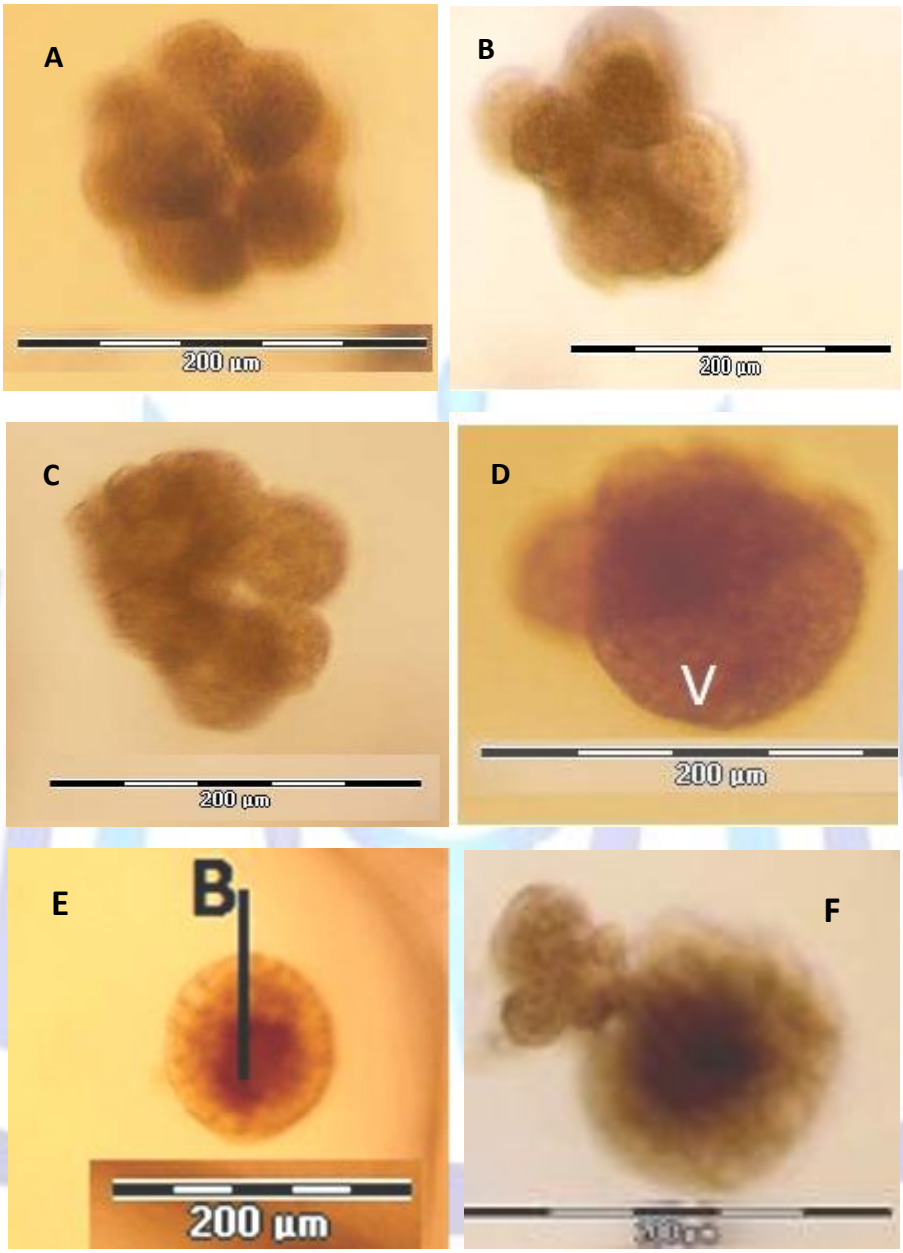

Fig 4: Early developmental stages before and after treatment with colchicines. A shows normal 16-cell stage; B, C show abnormal 16-cell stage; D is abnormal 16-cell stage showing large vesicle (v); E shows normal blastula with a blastocoels (B); F shows abnormal blastula with degenerated ectodermal cells, large blebs at the margin of the blastula and compaction blastocoels.

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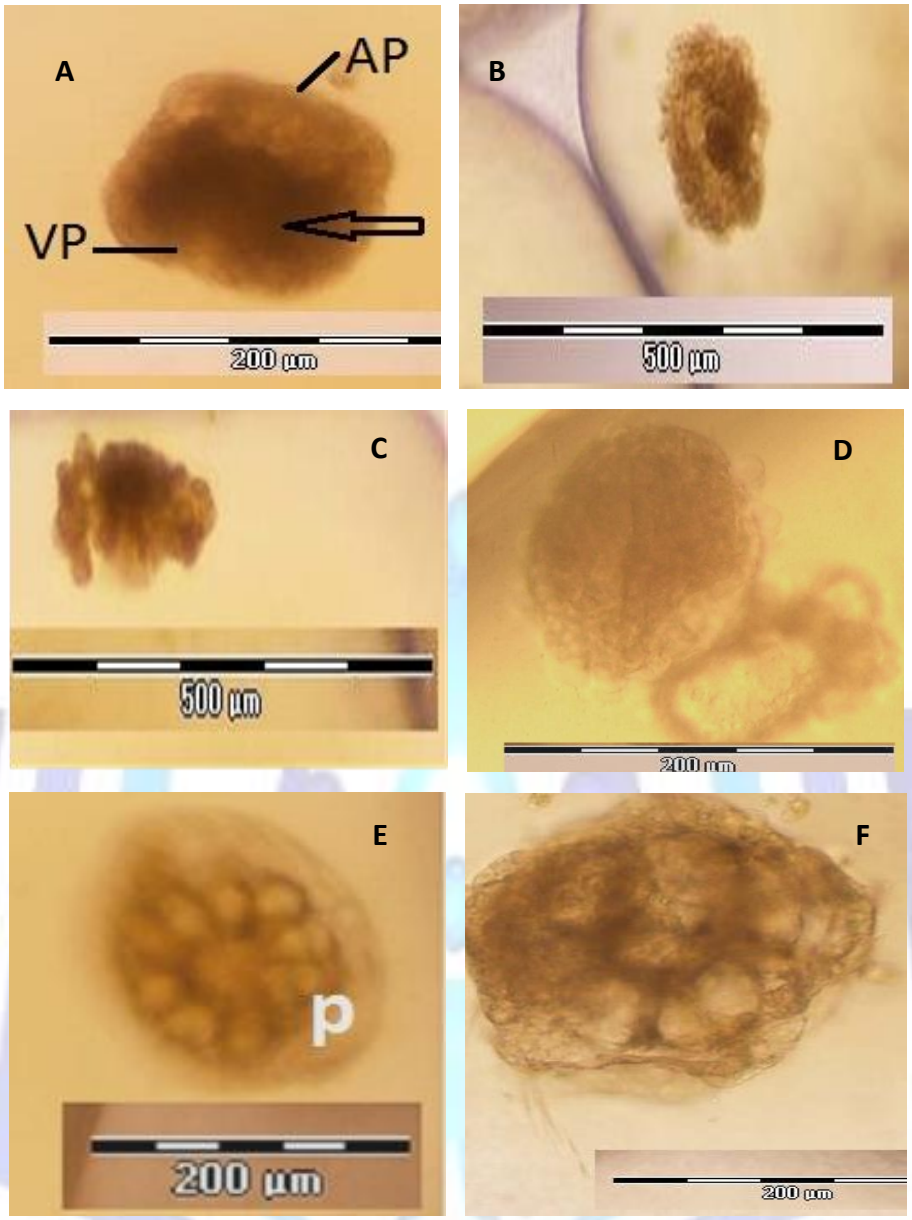

Fig 5: Late developmental stages before and after treatment with colchicines. A shows normal gastrula stage with, animal pole (AP), vegetal pole (VP) and yolk rich cells (black arrow); B and C show degenerated gastrula; D shows exogastrulation; E is the trochophore stage with prototroch (p); F is abnormal trochophore stage.

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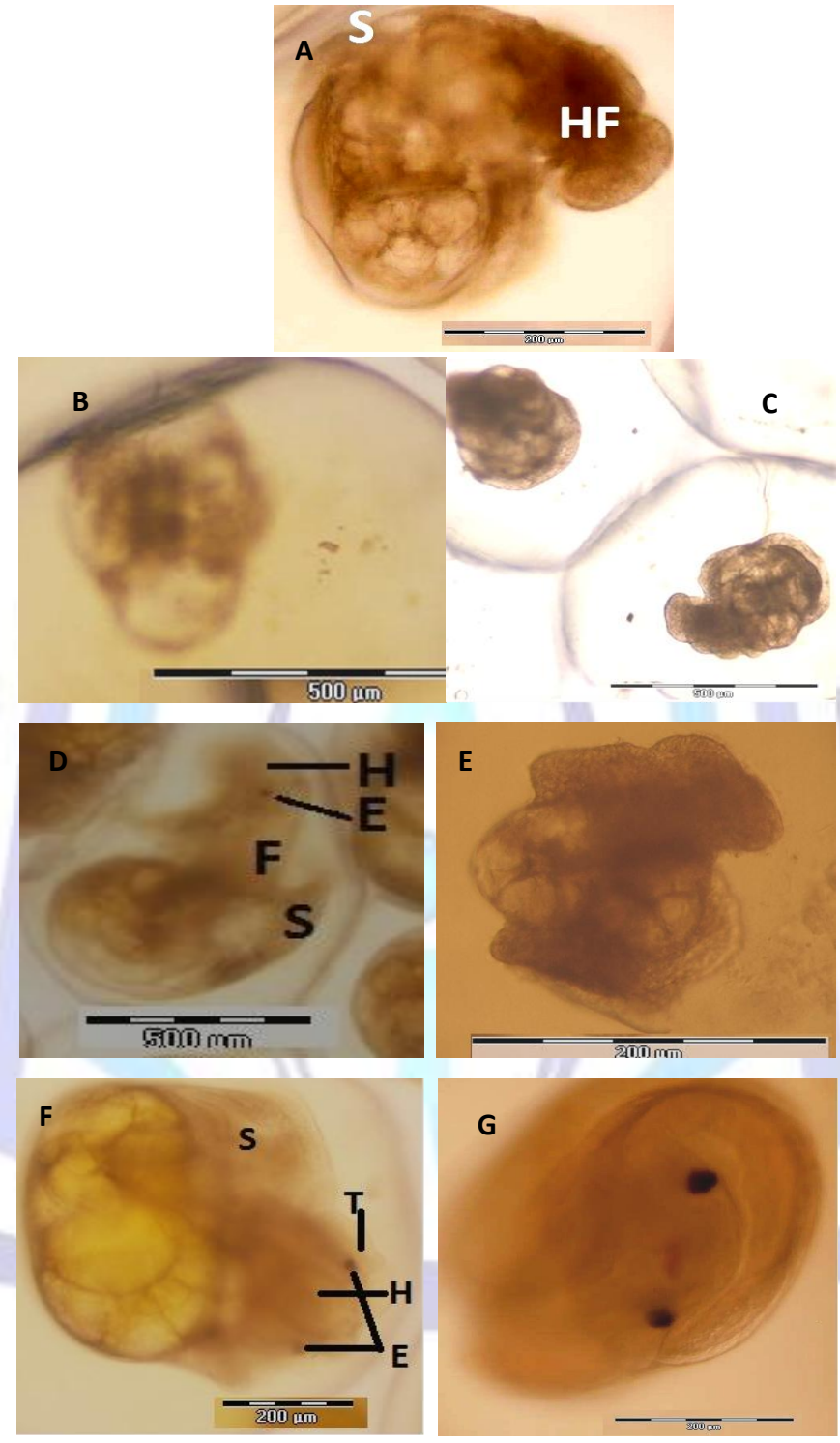

Fig 6: A is a veliger stage showing shell (S) and head foot (HF). B is abnormal veliger stage with fluid filled vesicle at one of its ends; C shows dark gastrula; D shows Hippo-veliger stage showing shell (S), head (H), foot (F) and eye (E); E shows abnormal hippo-veliger stage with reduced shell; F is mature prehatching stage showing shell (S), head (H) with tentacle (T)) and eye (E); G shows malformed prehatching stage with enlarged head, enlarged eyes and malformed shell and enlarged foot.

The effect of colchicine on trochophore protein profile

The pattern of protein profile identified by SDS-PAGE electrophoresis for the control and treated egg masses containing trochophore larva of *B. alexandrina*, is shown in Table (4) and Fig. (7). The results indicated that the protein pattern has a total number of 11 and 10 bands for control and treated egg masses respectively and their molecular weight ranged from 15.1 to 158.5 kDa. It was noticed that there were 10 shared bands between control and treated egg masses. A protein

band with the molecular weight 158.5 kDa was found in normal embryos, but was not detectable at the treated ones. The bands number 2, 3, 4, 9 and 10 of treated egg masses, have great intensity rather than those of control ones.
The similarity coefficient ″S″ was based on the number of protein bands separated by SDS-PAGE (Table 5). The results obtained revealed that the similarity between control and 5 ppm colchicine treated trochophore was (0.95).

Table 4. SDS-PAGE of proteins of control and treated trochophore larvae with 5 ppm colchicine. The numbers between brackets show rf; the other numbers show the molecular weight (kDa).

Number of bands	control trochophore	5 ppm colchicine treated trochophore
1	158.5 (0.2)	----
2	114.8 (1.2)	114.8 (1.2)
3	87.1 (2)	87.1 (2)
4	75.9 (2.5)	75.9 (2.5)
5	66.1 (3)	66.1 (3)
6	50.1 (4)	50.1 (4)
7	45.7 (4.2)	45.9 (4.2)
8	37.2 (4.9)	37.2 (4.8)
9	19.1 (7.1)	19.1 (7.1)
10	17.4 (7.4)	17.4 (7.4)
11	15.1 (7.9)	15.1 (7.9)

Table 5. Matrix of similarity index (SD) of protein subunits in trochophore larvae.

Number	Lan	1	2
1	Control	1	
2	Treated	0.95	1

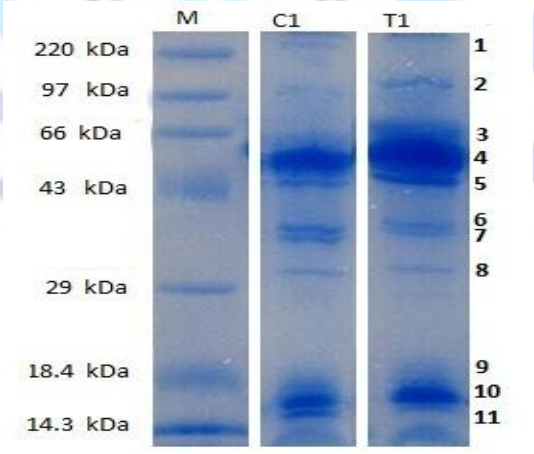

Fig 7: Protein fractions of control trochophore larva of *B. alexandrina.* C1 = control trochophore, T1=5 ppm colchicine treated trochophore larvae and M= (Marker) /kDa.

Effect of colchicine on the organic content of trochophore larvae:

The impact of colchicine against egg masses containing trochophore larvae induced many disturbances of organic substances. As shown in Table (6) there is a significant decrease in the total protein level in the egg masses containing

trochophore larvae (0.872 ± 0.174g/dl), with a ratio of decrease reaching 58.96% (F= 0.069, df = 4 and P = 0.001). In the case of lipid, the results revealed a significant decrease in the total lipid (3.16 ± 0.42mg/dl), with a ratio of decrease reaching 35.11% (F = 3.2, df = 4 and P = 0.04). Also, there is a decrease but insignificant in the glycogen level (5.322667 ± 1.5279942 mg/100g of tissue), with a ratio of decrease reaching 42.844% (F = 1.962, df = 4 and P = 0.1).

Table 6. The effect of sublethal concentration (5ppm) of colchicines on biochemical parameters of egg mass containing trochophore larvae. * Significant at P < 0.05

Groups	Total protein g/dl	Percentage of change	Total lipid mg/dl	Percentage of change	Glycogen mg/100g of tissue	Percentage of change
Control trochophore	2.12 ± 0.14		4.87 ± 0.84		9.3126 ± 2.9	
Treated trochophore	0.86 ± 0.17 *	58.96% ↓	3.16 ± 0.42 *	35.11↓	5.323 ± 1.53	42.844 ↓

Use of folic acid, vitamin C and holothurin as recovery agents against teratogenic effect of colchicine after or during exposure periods.

The results of protective effect of some recovery agents (vitamin C, folic acid and holothurin) are presented in Fig. 8.
The results revealed that the toxic effect of colchicine on the morphogenesis of snail embryos was so intensive that the malformations couldn't be reversed by subsequent or co-treatment with recovery agents, vitamin C and holothurin. It is worth reporting that concomitant addition of folic acid to malformed embryos succeeded to produce significant amelioration of the teratogenic deformities induced by colchicine. It can recover 86.7 % of malformed embryo. But, folic acid had a very slight recovery effect on the embryos post-treated with it. It can recover only 6.1% of malformed embryos.

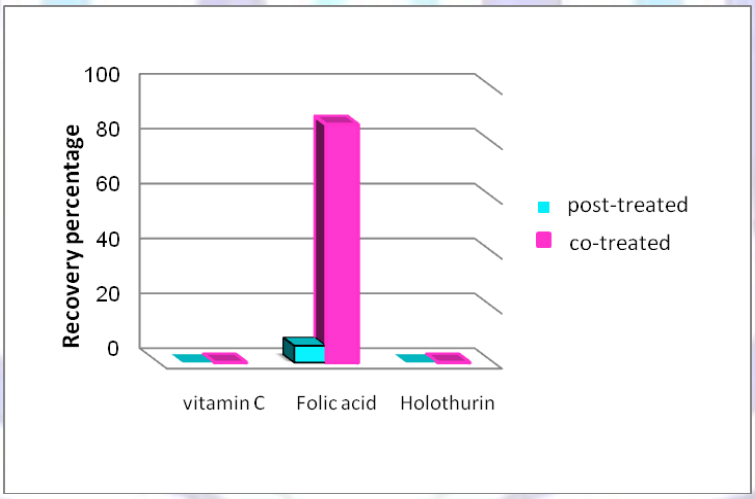

Fig 8: Percentage of recovered malformed embryos induced by colchicine.

Discussion

The snail embryo appears to be an appropriate in vitro screening test for predicting the potential teratogenicity. This model also proves to be multi-informative, because it permits the investigation of the toxicity of the molecules tested, throughout embryogenesis. The present work showed that teratogenicity could be recognized as developmental abnormalities up to larval stages which could be related, in some cases, to a delay in the first cleavage or hatchability.
In this study, the molluscicidal effect of colchicine against embryonic development of *B. alexandrina* snails was tested. The results of mortality data of colchicine against all developmental stages of herein species refer that 30 ppm colchicine is the highest concentration that cause the highest LC90 values for all early developmental stages. So, the application of colchicine at 30 ppm in ponds may lead to a significant reduction in snail embryos population.
The present work attempted to determine more accurately the critical point for cleavage inhibition by external application of colchicine solution at different times and to determine the critical amount of colchicine for cleavage inhibition. It was clearly demonstrated that colchicine influences embryogenesis of *B. alexandrina*. Although exposure to colchicine at concentrations between 10-30 ppm caused death among all early embryonic stages (fertilized egg, 2-cell stage, 4-cell stage, blastula stage and gastrula stage), concentration dependent differences between the test groups were noted. Concentrations of 10 ppm resulted in deceleration of development and death at the trochophore stage, whereas the two higher colchicine concentrations 20 and 30 ppm caused blastula and gastrula death. Also, exposure to colchicine at

concentrations between 50-200 ppm caused death among late embryonic stages (trochophore and veliger stages). So colchicine proved to be toxic against embryogenesis of *B. alexandrina*.

The teratogenic effect of colchicine on snail embryos at varying stages and varying doses, reported that gross congenital malformations were found in a significant number of embryos especially at trochophore stage. This finding is of special interest, because of a general belief among teratologists that malformations are produced particularly during the organogenetic period [55].

Colchicine had an inhibitory effect on protein synthesis, but it differs from other inhibitors of protein synthesis in that it specially inhibits synthesis of a particular protein, tubulin, which is indispensable for karyokinesis [61]. So, colchicine is believed to inhibit cell division by preventing the formation of spindle and asters or destroying them after their formation [25]. According to these previous studies, the present results suggest that the abnormality of cleavage in early developmental stages is due to destroying the mitotic apparatus which acts as a passive guide for the furrow. This suggestion is also supported by the finding of Borisy and Taylor [7] who concluded that the binding of colchicine with protein in sea urchin eggs prevents assembly of the subunit into a microtubule and consequently prevents the normal cleavage.

However, the possibility that the colchicine may cause embryo toxicity by interactions with DNA shouldn't be ignored. Holmes et al. [28] and Sinclair [56] mentioned that DNA damage in germ cells may lead to malformations and/or genetic defects in the offspring.

Other investigators Brachet [11] and Mulherkar et al. [44] suggested that SH groups containing proteins are important in the embryonic development, particularly during morphogenetic movements. Diwan [18, 19], assumed that colchicine seems to react with –SH groups causing its inhibition. This reasoning is supported by the fact that cysteine (-SH) can reverse all the effects produced by colchicine [19].

Colchicine has also been found to exert effects on other cellular processes, including disruption of membrane ion transport [9], osmoregulation [6], and nucleoside uptake [41]. Although it is possible that the colchicine caused abnormalities (colchicine syndrome) may arise from the inhibition of mitotic activity, it would be risky to conclude that mitotic inhibition can be a direct or only cause of abnormal development. However, the present study can refer the effect of colchicine on development to its action on microtubules and some other cellular processes.

It was documented that the protein as a physiological "sign" illustrates the disturbed state of any organism [30]. So, its fractionation by SDS-PAGE electrophoresis was undertaken to assess accurately the effect of colchicine on its synthesis. It was found that colchicine decreased number of bands and increased the intensity of some bands. This decrease may be to the fact that colchicine forms a complex with a special protein, tubulin and this link resulting in the reduction of protein synthesis [7, 40, 61].

To the best of our knowledge, no studies conducted on the physiological effects of colchicine on embryos of fresh water snail *B. alexandrina*, total protein, total lipid and glycogen, so that they have been measured in this study. The sub-lethal concentration of colchicine (5 ppm) resulted in a significant decrease in the total protein and total lipid and an insignificant decrease in the glycogen levels in the egg masses containing trochophore larvae. The reduction of total protein agree with Al-Sharkawy [1] and Mantawy [39] who found that molluscicids *Ammi majus*, *Allium cepa* and *A. sativum*, caused reduction in protein and referred this reduction to direct interference of active component of the extract with the protein synthesis.

Glycogen is the primary source for serum glucose, known to be the most important anaerobic energy source in anoxic-tolerant mollusks [22]. The decrease in glycogen may be due to increased glycogenolysis [43]. According to Bridges [12], amphibians spend extra energy to decrease the toxic effects of chemicals and regain their destroyed physiological balance. These previous findings are in accordance with our results. Lipids are also the storage form of energy like glycogen. So, the decrease in its level is expected in response to the stressful condition induced by molluscicide.

The cell cycle is the universal process by which cells reproduce, and which underlies the growth and development of all living organisms. Because of the universality of the cell cycle regulators from yeast to human [48, 46, 49], the writer postulate that snail model will provide clues to the molecular mechanism of the consequences of colchicine to health as well as the mechanism for cellular protection against the negative effects of this drug.

The results of herein study indicated that colchicine can be considered as a molluscicide and teratogen because it possesses developmental toxicity potential indicated by induction of snail embryo abnormalities (teratogenesis). The widespread use of this drug for pharmaceutical (medical) purposes by human has stimulated research for recovery agents against their negative effects. Also the great similarity between embryo cell and cancer cell [2] provoke these recovery trials.

There is often a period of vulnerability to the effects of toxic chemicals during fetal development. This vulnerability occur during the period of development of various organ systems (period of organogenesis); where permanent structural birth defects or permanent functional changes may occur [3]. The present study coincides well with these findings and indicated that the period of vulnerability (specific-stage) is the trochophore stage at which a high percentage of abnormalities are yielded. So the recovery experiments were carried out on these stages.

According to the findings obtained from the present study, teratogenic effects caused by colchicine can be removed by using folic acid. In agreement with these findings, Spiegelstein, et al. [58] concluded that folic acid blocks occurrence of malformations and has a positive effect on the offspring. Similar results supported that folic acid has protective effect against arsenic, which contaminates drinking water easily like phenol in mouse embryo [53]. Also, it was recorded that folic acid is essential for development and demands for folic acid increase during pregnancy [14]. Protective effects of folic acid against murine fetal deformities caused by hyperthermia have been studied by [27]. They found that folic acid can restore embryos damaged by hyperthermia. It is also well known that folic acid is necessary for DNA methylation, protein methylation, DNA synthesis, and maintenance of the overall integrity of DNA [42]. Aşkin et al. [4] suggests that folic acid could effectively inhibit phenol-induced abnormalities in developmental stages of *Drosophila melanogaster*.

In conclusion, the present study showed that colchicine has a molluscicidal effect through its effect as a teratogen. On the other side snail embryo serves an easy tool to study the teratogenic toxicity of colchicine. Also the present study elucidated the therapeutic effects of folic acid as a recovery agent as it caused significant improvements in teratogenicity and minimize the embryo toxicity.

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