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Research Article

THE CAPABILITY OF BRINE SHRIMP TEST AS A TERATOGENICITY SCREENING SYSTEM

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ABSTRACT

Objectives: The goals of this study were to analyze the capability of brine shrimp test (BST) as a potent teratogenicity screening system on teratogenic agents (methotrexate, captopril, diclofenac, phenytoin, warfarin, and valproic acid).

Methods: Artemia cysts were hatched into 1st stage nauplii, then taken and put into seawater medium which contain test substance and kept alive until 2nd stage, 3rd stage, and 4th stage, and number of deaths, morphological abnormalities, body length, and retarded of development were observed for each stage.

Results: Hatch ability of cysts in methotrexate 0.015 mg/ml, captopril 0.25 mg/ml, diclofenac 0.075 mg/ml, phenytoin 1.56 mg/ml, and valproic acid 2.5 mg/ml were significantly different compared to control ($p<0.05$). Nauplii survival in methotrexate 0.015 mg/ml, captopril 0.25 mg/ml, diclofenac 0.075 mg/ml, phenytoin 1.56 mg/ml, and valproic acid 2.5 mg/ml were significantly different to control ($p<0.05$). The morphological abnormalities was found in methotrexate 0.015 mg/ml, captopril 0.25 mg/ml. Nauplii with retarded development were expressed in methotrexate 0.015 mg/ml, captopril 0.25 mg/ml, diclofenac 0.075 mg/ml, phenytoin 1.56 mg/ml, and valproic acid 2.5 mg/ml. Significant difference in body length was presented in captopril 0.25 mg/ml, and phenytoin 1.56 mg/ml compared to control ($p<0.05$).

Conclusion: BST can be used as an alternative method of the teratogenic screening test, although not as sensitive teratogenic tests on mammals. This screening method was not suitable for a compound which its chemical characteristic can change the tonicity of the medium.

Keywords: Brine shrimp test, Teratogenicity, Methotrexate, Captopril, Diclofenac, Phenytoin, Warfarin, Valproic acid.

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INTRODUCTION

Medicine for pregnant women must be safety for her and also her baby. Some of the medicines were contradictive for pregnant women because it can cause abnormality in the fetus development. The Word Health Organization predicted 303.000 newborns die within 4 weeks of birth every year, worldwide, due to congenital anomalies. Congenital anomalies correlated to long-term disability, which may have significant impacts on individuals, families, health-care systems, and societies [1].

The resolution of birth defects of the sixty-third World Health Assembly, promoting in primary prevention and improving in health of children with congenital anomalies were agreed by member states [1], by strengthening research and studies on etiology, diagnosing and preventing. One of the prevention studies is performing research regarding developmental toxicology or teratogenic test. Along with the development of science, some teratogenic testing had been developed.

Brine shrimps test is one of the teratogenic testing methods. This test using Crustaceans shrimp group, phylum Arthopoda, namely, *Artemia salina* L. *A. salina* L. cysts are more easily hatch into nauplii with rapidly growth. Every stage of its development can be observed, and relatively easy to maintain population in labotarorium condition, therefore, brine shrimp test (BST) is simple and effective to some biology and toxicology test [2].

The teratogen test system was revealed by Kerster based on disruption of elongation for 24 hrs and 48 hr after wetting Artemia cysts. The system is fast, inexpensive, requires little skill, and appropriated to test the teratogenicity of food additives, drugs, chemical formulations, and industrial wastes, but unsuited to test of gasses, particulates, very dilute wastes, or natural waters [3]. This study was later modified by Sleet dan Brendel [4], Artemia nauplii transcending instar I to later instars were assessed to observe their potential for indicating chemicals as potential developmental hazards and thus prioritizing them for more extensive in vivo testing. Some criterias were selected for evaluating the potential of the system for screening were the ability to collect homogeneous populations of instar I nauplii, characterize intermediate development by technically simple measurements and reveal development-related differentials in the vulnerability of nauplii. Artemia nauplii transcending instar I to IV useful for estimating chemicals that preferentially interact with developmental events [4].

The use of *A. salina* L. is alternative method which can replace the use of vertebrate animals at a higher level. This method is suitable strategy to streamline the testing on animals which is based on the principle of 3 R: Alternative methods can replace the use vertebrate animals are at higher level (replacement), reduce the number of animals used in the test (reduce), choose appropriate testing method to reduce stress and pain in animals (refined) [5].

Methotrexate, captopril, phenytoin, diclofenac, warfarin, and valproic acid are a drug that classified in the category X and D by FDA, the class of drug in an animal study and human experiments which gave evidence of fetal risk. Methotrexate cause skeletal defect, low birth weight, and disturbed neurulation. Fetal death, neonatal anuria, hypoplastic calvaria can be caused by captopril. Diclofenac cause premature closure of the ductus arteriosus, skeletal, and heart defect. Warfarin cause nasal hypoplasia, central nervous system defects, and neurological abnormalities. Some researches stated that phenytoin and valproic acid can cause spina bifida, anencephaly, and other neural tube

defects [6-8]. Although the benefits of this drug medication is required contraindication for pregnant women. In special condition is used only if benefits are higher than the risk. The aims of this study was to analyze the teratogenicity screening test using BST on teratogenic substances (methotrexate, captopril, diclofenac, phenytoin, warfarin, and valproic acid).

MATERIALS AND METHODS

Materials

Animals were *A. salina* L. cysts which were produced by Ocean Star International Inc., Utah, USA. The test substances were methotrexate, phenytoin, captopril, warfarin, diclofenac, and valproic acid were purchased from Sigma-Aldrich.

Hatching ability

As much as 10 cysts were hatched in a vial which contains 5 ml of seawater (with salinity 9 µg/ml) and test substance, at temperature of 25-28°C, aerated and illuminated. After 36 hrs, hatching ability of cysts in each group were analyzed and the replication was performed 5 times.

Death of nauplii, morphological abnormalities, retarded development, and body length

As much as 1 g cysts were hatched in a beaker which contain 400 ml of seawater (salinity 9 µg/ml) and test substance, at a temperature of 25-28°C, aerated and illuminated. After hatching the 1st stage of Artemia nauplii (1-2 hrs after hatching) were sampled out then tested in each group which contain seawater and test substance. In Group 1, nauplii were keep alive until 2nd stage; Group 2 keep alive until 3rd stage; Group 3 keep alive until 4th stage. Nauplii which were taken from each group then put into Bouin solution for fixating. The observation was carried out using a microscope with a magnification of 100 times, with 5 times replication, included the death of nauplii, morphological abnormalities, retarded development and body length of nauplii.

RESULTS

Hatching ability of cyst

A number of cysts were hatched in medium seawater which contains teratogenic substance with various concentration in each group. Hatching ability of cyst in methotrexate, captopril, diclofenac, phenytoin, warfarin, and valproic acid can be seen in Fig. 1.

Death of nauplii

Table 1 reveals all nauplii died since the second stage (19 hrs) in valproic acid group. The greatest average number of mortality were given by captopril group and phenytoin group which had significant different when compared to control ($p<0.005$).

Morphological abnormality

Evaluation in morphological abnormality was performed in control group and test substances group and 5 times replication were done (Fig. 2).

Retarded development

Evaluation in all test substances groups denoted that retarded development was given by all teratogenic test substances (Fig. 3).

Body length

As shown in Table 2 observation in body length in all groups teratogenic substances. Body length of nauplii in phenytoin group gave the shortest of body length.

DISCUSSION

Artemia is used in this study because it is relatively easy in handling in the laboratory and has high tolerance when exposured by toxic substances. A test substance which can cause death or inhibit the development of Artemia means that the substance is highly toxic and can cause developmental disorders in other animals that tolerance is lower than Artemia [9,10].

Developmental toxicology test using the BST has been conducted by number of researchers to test the effects of several substances in development stages with different parameters, such as:

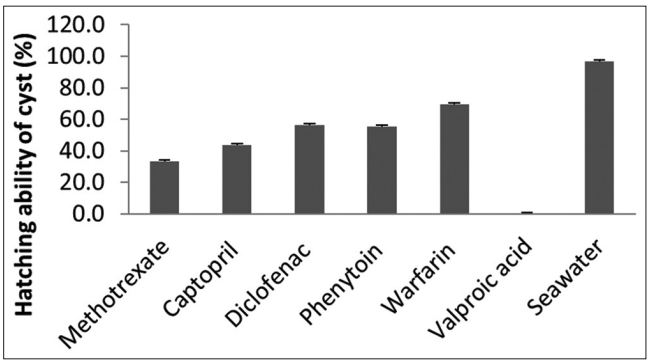

Fig. 1: Hatching ability of cyst in teratogenic substances

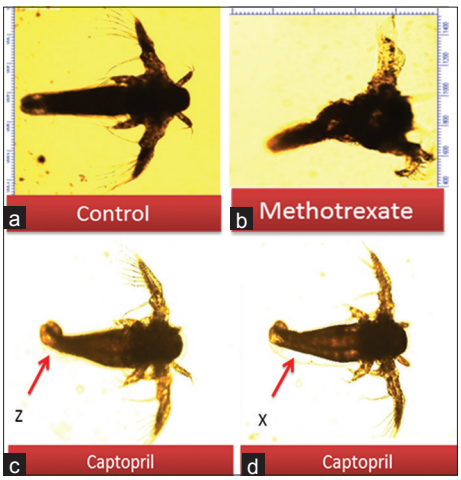

Fig. 2: Morphological abnormality of nauplii, x, z: Arch at the tail, (a) Control, (b) Methotrexate, (c) Captopril 1st replication, (d) Captopril 2nd replication

Table 1: Death of nauplii in teratogenic substances

Test	Death of nauplii (%)		
Substances	**2nd stage**	**3rd stage**	**4th stage**
Seawater	0.00±0.00	0.00±0.00	46.70±2.31
Methotrexate 15 µg/ml	0.00±0.00	0.00±0.00	100.00±0.00*
Captopril 250 µg/ml	20.00±1.00*	60.00±3.06*	100.00±0.00*
Diclofenac 75 µg/ml	0.00±0.00	23.30±2.08	100.00±0.00*
Phenytoin 1562 µg/ml	13.30±0.58*	30.00±1.73	100.00±0.00*
Warfarin 5 µg/ml	0.00±0.00	0.00±0.00	30.00±1.73
Valproic acid 2500 µg/ml	100.00±0.00*	100.00±0.00*	100.00±0.00*

Replication is performed 5 times, n=50, * = significantly different compared to control ($p<0.05$)

Table 2: Body length of nauplii in teratogenic substances

Test	Body length (µm)		
Substances	**2nd stage**	**3rd stage**	**4th stage**
Seawater	896.70±90.86	1074.00±70.27	1505.64±90.06
Methotrexate 15 µg/ml	872.76±78.73	1019.84±38.15	-
Captopril 250 µg/ml	742.08±13.05*	906.15±3.82*	-
Diclofenac 75 µg/ml	805.03±66.52	1011.56±33.37	-
Phenytoin 1562 µg/ml	736.50±22.92*	861.54±63.30*	-
Warfarin 5 µg/ml	936.63±17.09	1092.19±14.21	1448.83±37.44
Valproic acid 2500 µg/ml	-	-	-

Replication is performed 5 times, n=50, * = significantly different compared to control (p<0.05)

Butyrylcholinesterase in the early development of the brine shrimp larvae [11]; effects of copper, cadmium, and zinc on the hatching success of brine shrimp [12]; effects of cupric sulfate, lead nitrate, zinc sulfate and nickel sulfate on early life stages of the brine shrimp [13]; development-related differentials in naupliiar vulnerability for cadmium sulfate, mercuric chloride, and sodium azide (NaN_3) [4].

This study used five parameters of the test that is hatching cysts, death nauplii, abnormal morphology on nauplii, retarded development nauplii at each stage of development, and a body length nauplii at each stage of development. All of five parameters are determined based on Wilson's theory, which stated that there were four manifestations of developmental disorders of the fetus at death, morphological abnormalities, growth retardation, and functional disorder. Each parameter is adjusted by the manifestation, except for functional disorders, because it is difficult to measure the functional disorders of the brine shrimp nauplii [14].

There were significant difference in hatching ability of cyst in methotrexate, captopril, diclofenac, phenytoin, warfarin and valproic acid compared to control group (p<0.05). The method which was used in this study was adopted from sargelous [15] with minor modification. Artemia cysts were hatching at 15-35 ppm of salinity within 24-36 hrs [13]. Even though in this study the salinity seawater was 9 ppm, a significant decrease in hatching ability of cyst in a medium which contain teratogenic substances indicated that the substances may affect the development of Artemia cysts.

Survivability of nauplii at every stage was evaluated in this study. With the same background, increasing in mortality can be caused by increasing in density, turbidity, or acidity. Research by Stappen [16] demonstrated that density, turbidity, and acidity might change the tonisity of medium, which can directly affect to the survival of nauplii.

Table 1 shows that nauplii in valproic acid group could not survive, all of nauplii died since the second stage (19 hrs). At the second stage nauplii in captopril group had a greatest average number of mortality (20%). Mortalities in captopril group and phenytoin group (13.30%) at the second stage were significantly different compared to control (p<0.05). At the third stage, nauplii in captopril group still had a greatest number of mortality (60%), whereas at the fourth stage only nauplii in control group and warfarin group can still survive, while in the others test substances (methotrexate, captopril, diclofenac, phenytoin, valproic acid) the death of nauplii were 100%.

Observations have been performed in all groups to see abnormalities of morphology. The test was carried out 5 times of replication. The result showed that from 5 times of replication was found three defects. The morphological abnormalities were found in this study at the second stage which were two nauplii with morphological abnormalities were found in methotrexate group and captopril group, nauplii with amorphous form in methotrexate group, nauplii with arch in the tail in captopril group (Fig. 3). In body of adult Artemia, there are 11 pairs of torakopoda, the torakopoda serves as a means of motion, gills, and foragers [15].

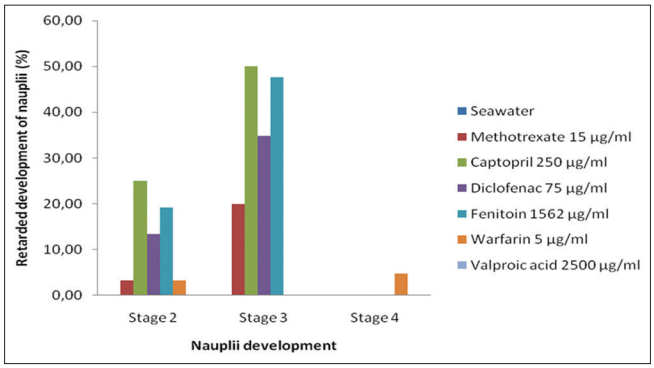

Fig. 3: Percentage of retarded development of nauplii

Nauplii with abnormalities in any part of the body that would later become torakopoda, may cause disturbances in the respiratory system, transport, digestion, and reproduction in adult Artemia.

Nauplii different development at each stage can be observed on the growth and changes in anatomy [10,15]. In this study, the development of nauplii was observed only until the fourth stage because a lot of nauplii could survive until the fourth stage. All groups of teratogenic substances showed influence on nauplii development both at the second stage and the third stage, and only captopril and phenytoin groups at the third group were significantly different compared to control (Fig. 3). Sleet and Brendel [4] denoted that nauplii of Artemia at the first stage to the fourth stage can be used to observe the effect of chemical substances on the development process of Artemia nauplii [3]. Some tested potent teratogenic substances showed their influence on nauplii development although was not statistically significant. Most of the retarded development of nauplii which should have reached at an advanced stage showed anatomical characteristic at below stage. The retarded development may be caused the differences of hatching time of each cyst, therefore difficult to ascertain the retarded development was caused by the effect of the test substance or variety of cyst hatching time.

Body length of all teratogenic substances groups had been evaluated. Body length of nauplii in captopril and phenytoin groups at the second stage and the third stage were significantly different to control (p<0.05), and body length in phenytoin had the shortest body length. Nauplii of Artemia has a different body length at each stage of its development [15], body length nauplii is then measured to determine their growth disturbance in nauplii caused by some chemicals, differences body length in the test group may indicate a growth disorder in nauplii [2,3], but it can also be attributed to differences in hatching time.

CONCLUSION

BST can be used as an alternative method of teratogenic screening test, although not as sensitive teratogenic tests on mammals. This screening method was not suitable for a tested compound with chemical characteristic can change the tonicity of the medium.

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