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Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* straus

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Abstract

Activity of lactate dehydrogenase (LDH) was used as an effect criterion in toxicity tests with *Daphnia magna*. In the first part of the work, the conditions for the use of LDH activity in toxicity tests with juveniles and adults of *D. magna*, were optimized. The influence of parameters such as the number of animals per sample, nutritional status, age and the presence of eggs in the brood chamber were investigated. In the second part of the study, both in vivo and in vitro tests based on the alteration of LDH activity of *D. magna* were developed and tested using zinc chloride as test substance. The results obtained indicate that LDH activity of *D. magna* may be used as an indicative parameter in aquatic toxicity tests. © 2001 Published by Elsevier Science Ltd.

Keywords: *Daphnia magna*; Lactate dehydrogenase; Biomarkers; Zinc

1. Introduction

In recent years, research has been undertaken into the development and validation of in vitro and in vivo tests based on biomarkers. These tests are able to detect toxic effects before alterations of important physiological functions, such as reproduction, growth or death occur (Janssen et al., 1993; Garle et al., 1994; Guilhermino et al., 1996; Blaise et al., 1997; Diamantino et al., 2000).

Lactate dehydrogenase (LDH) is a parameter widely used in toxicology and in clinical chemistry to diagnose cell, tissue and organ damage. However, the potential of this enzyme as an indicative criterion in invertebrate aquatic toxicity tests has been scarcely explored (Ribeiro et al., 1999). LDH is an important glycolytic enzyme being present in virtually all tissues (Kaplan and Pesce, 1996). Alterations of the normal LDH activity pattern were found after exposure to different concentrations of sodium bromide and 3,4-dichloroaniline (Guilhermino et al., 1994), copper gallium diselenide, copper indium diselenide and cadmium telluride (Morgan et al., 1995), cadmium (Hassoun and Stohs, 1996) or oxygen stress (Wu and Lam, 1997).

A number of procedures have been developed for measuring the total LDH activity in mammalian tissues. One of the most frequently used is the spectrophotometric method described by Vassault (1983) that quantifies the conversion of pyruvate to lactate. In this method, LDH activity is measured at 30°C as the

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amount of pyruvate consumed by continuously monitoring the decrease of absorbance due to the oxidation of NADH at 339 nm (Vassault, 1983). Two requirements of this technique severely limit its use in ecotoxicology: (i) a considerable volume of concentrate sample is needed and (ii) each sample takes about 4 min to be processed. In ecotoxicological studies, only a small amount of biological material is frequently available and a large number of samples should be analysed in a short period of time. In order to overcome these limitations, an adaptation to microplate technique of the Vassault method was developed and tested in our laboratory.

Daphnia magna is widely used as test organism in aquatic toxicology (Adema, 1978; Van der Meer et al., 1988; Soares et al., 1992). Toxicity tests with this species are required for the assessment of the potential impact of new chemicals on the aquatic environment (EEC, 1992). The objectives of this study were: (i) to optimize and standardize the conditions for the use of LDH activity as effect criterion in toxicity tests with the cladoceran *D. magna*, (ii) to develop both in vivo and in vitro LDH activity-based tests and (iii) to investigate the sensitivity of these tests. In order to achieve the first and second objectives, the influence of several parameters (e.g. number of animals per sample, nutritional status, age and presence/absence of eggs in the brood chamber) on LDH activity was investigated. In order to attain the third objective, the results from LDH tests were compared with those obtained in conventional acute toxicity tests, using zinc chloride as test substance.

2. Materials and methods

2.1. Parent animals

Parent animals were cultured in hard water (ASTM, 1980) supplemented with an organic additive (Baird et al., 1989), in groups of 10 animals per 1000 ml of medium, and fed with the algae *Chlorella vulgaris* (0.322 mg carbon/daphnid/day). The photoperiod was 16 hL:8 hD and the temperature was $20° \pm 1°C$. All the experiments were carried out with 3rd to 5th brood neonates from the clone A (sensu Baird et al., 1989) except when the effects of the presence of eggs in the brood chamber and the age of animals were tested.

2.2. Preparation of homogenates

Homogenates were prepared in 1 ml of TRIS/NaCl buffer (TRIS 81.3 mmol l^{-1}; NaCl 203.3 mmol l^{-1}; pH 7.2) using a Ystral D-79282 Dottingen homogenizer and kept in ice during homogenisation. All the enzymatic assays were performed in the supernatant obtained by centrifugation of the homogenates for 5 min at 2916*g*. Samples were stored at $-20°C$ for a maximum of two weeks. For each experiment three replicates were used.

3. LDH determinations using a microplate technique

LDH activity was determined using an adaptation to microplate technique of the Vassault method (Vassault, 1983). Briefly, 250 μl of TRIS/NaCl/NADH solution, 40 μl of sample and 80 μl of TRIS/NaCl/pyruvate solution were successively introduced into each well of the microplate. After 30 s and during 5 min, the decrease of absorbance due to the oxidation of NADH was recorded each 20 s in a Labsystem Multiskan EX microplate reader at 340 nm. LDH activity was calculated from the slope of the absorbance curve. The enzymatic determinations were performed in triplicate or quadruplicate. The activity was expressed in Units (U) mg^{-1} protein (1 U = 1 μmol substrate hydrolysed per minute). The concentration of protein in the samples was determined in triplicate by the Bradford method (Bradford, 1976), adapted to microplate technique, using bovine γ-globulins as standard.

3.1. Number of animals per sample

In order to choose the suitable number of juveniles for sample preparation, the LDH activity determined in samples prepared with 35, 45 and 55 neonates per ml of buffer was compared. Three replicates per sample were used.

3.2. Effect of nutritional status

The effect of the nutritional status was studied by comparing LDH activity determined in homogenates of 45 juveniles (24 and 48 h old) cultured in the presence (0.322 mg carbon/*Daphnia*/day of *Chlorella vulgaris*) of food and 45 juveniles (48 h hold) cultured in the absence of food.

3.3. Presence of eggs in the brood chamber

In order to investigate the influence of the presence of eggs in the brood chamber on LDH measurements, the activity of the enzyme determined in homogenates prepared with 3, 5, 10 and 15 females with the first batch of eggs in the brood chamber was compared with the activity determined in homogenates prepared with the same number of females but without eggs. Furthermore, LDH activity in homogenates of the eggs of these females was also determined. Three replicates per sample were used.

3.4. Age of animals

The effect of the number and age of females on LDH activity was investigated by determining the enzymatic activity in homogenates prepared with three animals 7, 14 and 21 d old (without eggs). Three replicates per sample were used.

3.5. Conventional acute test

Conventional acute test with *D. magna* was carried out using hard water (ASTM, 1980) as test medium and initiated with third to fifth brood neonates (<24 h old). Twenty neonates per treatment, in groups of five per 100 ml of test solution were used. The measured effect was immobilization during 15 s after stimulation by a bright light. One control and 8 nominal concentrations of zinc (0.068, 0.137, 0.275, 0.550, 1.1, 2.2, 4.4 and 8.8 mg l^{-1}) were used. Temperature and photoperiod were as described above. Oxygen concentrations and pH levels were measured at time 0, 24 and 48 h.

3.6. In vivo LDH test

For the in vivo LDH test, third to fifth brood neonates (<24 h old) were cultured during 48 h without food in groups of 25 animals per 1000 ml of each test solution. Four nominal zinc concentrations (0.068, 0.137, 0.275 and 0.550 mg l^{-1}) and a control were tested. After 48 h, live organisms were used to prepare homogenates for enzymatic determinations (25 juveniles per 555 μl of buffer). Three replicates per treatment were used.

3.7. In vitro LDH test

Animals (<24 h) were cultured in ASTM during 48 h in groups of 25 animals per 1000 ml of medium. Homogenates, prepared with 25 juveniles per 555 μl of buffer, were incubated 30 min with different nominal concentrations of zinc (12.5, 25, 50, 75 and 100 mg l^{-1}). Three replicates per treatment were used. The activity of LDH was determined immediately after the incubation period and was expressed as U mg^{-1} of protein (1 U = 1 μmol substrate hydrolysed per minute).

3.8. Chemicals

TRIS (99.8% pure), NaCl (99.5%–100.5% pure) and $ZnCl_2$ ($\geqslant$ 98% pure) were purchased from Merck. Pyruvate (>98% pure) and γ-bovine globulins (99% pure) were purchased from Sigma. All the solutions were prepared in nanopure water (conductivity < 5 μS cm^{-1}).

3.9. Data analysis

Data from experiments regarding the effects of the number of animals per sample, presence of eggs in the brood chamber, age of animals, in vivo and in vitro LDH tests were analysed using hierarchical (NESTED) analysis of variance followed by Tukey multiple comparison test (Zar, 1996). Data from nutritional condition and age of juveniles were analysed by *t*-test. Two-factor analysis of variance was used to compare samples with different number of females and with the presence/absence of eggs in the brood pouch. LC50, EC50 and IC50 were determined by Probit analysis (Finney, 1971). The significance level was 0.05.

4. Results

4.1. Number of animals per sample

No significant differences on LDH activity were found among samples prepared with 35, 45 and 55 juveniles and no differences in LDH determinations were found owing to the three groups of juveniles (NESTED ANOVA: $F = 1.5$; d.f. = 2, 6; $P > 0.05$ and $F = 1.0$; d.f. = 6, 18; $P > 0.05$, respectively).

4.2. Nutritional condition and age of juveniles

No significant differences in LDH activity were found between 24 h and 48 h old juveniles with food (*t*-Test: $t = 1.65$, $P = 0.11$) and no effects due to the presence of food were found (*t*-Test: $t = 2.00$, $P = 0.06$).

4.3. Presence of eggs in the brood chamber

Significant differences were found among homogenates prepared with 3, 5, 10 and 15 females with eggs in the brood chamber and no differences in LDH determinations were found owing to these four groups of females with eggs (NESTED ANOVA: $F = 53.6$; d.f. = 3, 12; $P < 0.05$ and $F = 0.1$; d.f. = 12, 32; $P > 0.05$, respectively). Results of three females were statistically different from 5, 10 and 15 females, as indicated by Tukey test. In addition, significant differences were found among homogenates prepared with 3, 5, 10 and 15 females without eggs but no differences in enzymatic determinations were found (NESTED ANOVA: $F = 136.68$; d.f. = 3, 12; $P < 0.05$ and $F = 0.7$; d.f. = 12, 32; $P > 0.05$, respectively). The activity measured in homogenates prepared with 3 and 5 females without eggs was significant different from that determined in homogenates of 10 and 15 females without eggs. Regarding LDH activity in eggs, no differences were found among all the groups of eggs and owing to these groups

(NESTED ANOVA: $F = 0.6$; d.f. = 3, 12; $P > 0.05$ and $F = 0.7$; d.f. = 12, 32, $P > 0.05$, respectively) (Fig. 1).

Significant differences in LDH activity were found between females with eggs and females without eggs and among the groups of samples prepared with 3, 5, 10 and 15 females (Two-way ANOVA: $F = 520.1$; d.f. = 1, 88; $P < 0.05$ and $F = 99.1$; d.f. = 3, 88; $P < 0.05$, respectively). The interaction between the two factors was also significant ($F = 80.1$; d.f. = 3, 88; $P < 0.05$).

4.4. Protein concentration in the homogenates

Significant differences in the concentration of protein were found between samples prepared from females with and without eggs in the brood chamber and among homogenates prepared with 3, 5, 10 and 15 females (Two-way ANOVA: $F = 424.8$; d.f. = 1, 64; $P < 0.05$ and $F = 166.6$; d.f. = 3, 64; $P < 0.05$). The interaction between the two factors was also significant ($F = 47.3$; d.f. = 3, 64; $P < 0.05$).

4.5. Age of females

Significant differences in LDH activity were found among females 7, 14 and 21 d old and no significant differences among LDH determinations were found (NESTED ANOVA: $F = 128.6$; d.f. = 2, 9; $P < 0.05$; $F = 0.5$; d.f. = 9, 24; $P > 0.05$, respectively). LDH activity in 7 days old females was higher and significantly differs from the activity in 14 and 21 d old animals. Differences between females 14 and 21 d old were not significant.

4.6. Conventional acute toxicity test and in vivo LDH test

In both tests, oxygen levels were always above 7.0 mg l^{-1} and pH variation was always lower than 1 unit of pH. The 24 and 48-h LC50 values for zinc were 2.3 mg l^{-1} (95% CL: 2.1–2.5) and 0.8 mg l^{-1} (95% CL: 0.7–0.9), respectively.

In vivo exposure to zinc significantly inhibited LDH activity and no differences were found among LDH replicated determinations (NESTED ANOVA: $F = 4.5$; d.f. = 4, 15, $P < 0.05$ and $F = 0.43$; d.f. = 15, 40; $P > 0.05$, respectively). NOEC value for LDH depression was 0.275 mg l^{-1} and the LOEC was 0.550 mg l^{-1} (Fig. 2). EC50 was 100.9 mg l^{-1} (95% CL: 35.4–287.5).

4.7. In vitro LDH test

Zinc significantly inhibited LDH activity in vitro and no significant differences among LDH replicated determinations were found (NESTED ANOVA: $F = 25.1$; d.f. = 5, 18, $P < 0.05$ and $F = 0.86$; d.f. = 18, 48; $P > 0.05$, respectively). The concentration of 25 mg l^{-1} of zinc significantly increased LDH activity. NOEC was 50 mg l^{-1} and LOEC was 75 mg l^{-1} (Fig. 3). IC50 was 175.2 mg l^{-1} (95% CL: 174.8–175.7).

5. Discussion

LDH is involved in the production of energy, being particularly important when a considerable amount of

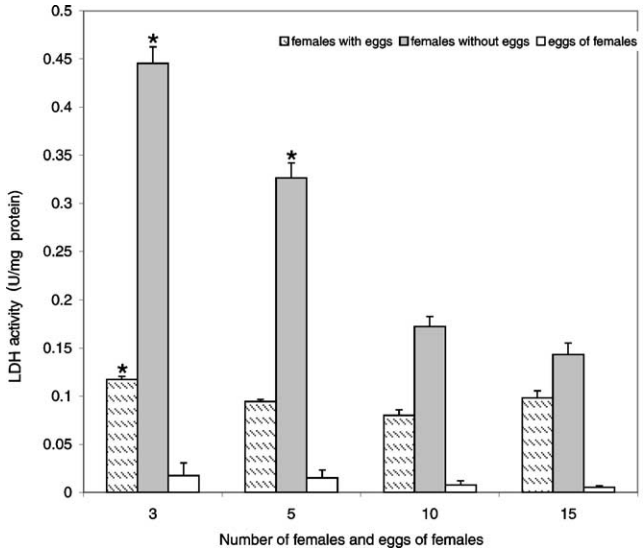

Fig. 1. LDH activity measured in homogenates of 3, 5, 10 and 15 females with and without the first batch of eggs in the brood chamber and in eggs produced by 3, 5, 10 and 15 females. Data are expressed as the mean ± SE of three samples (four measurements per sample) (* = significant differences at $P \leqslant 0.05$ between different number of females).

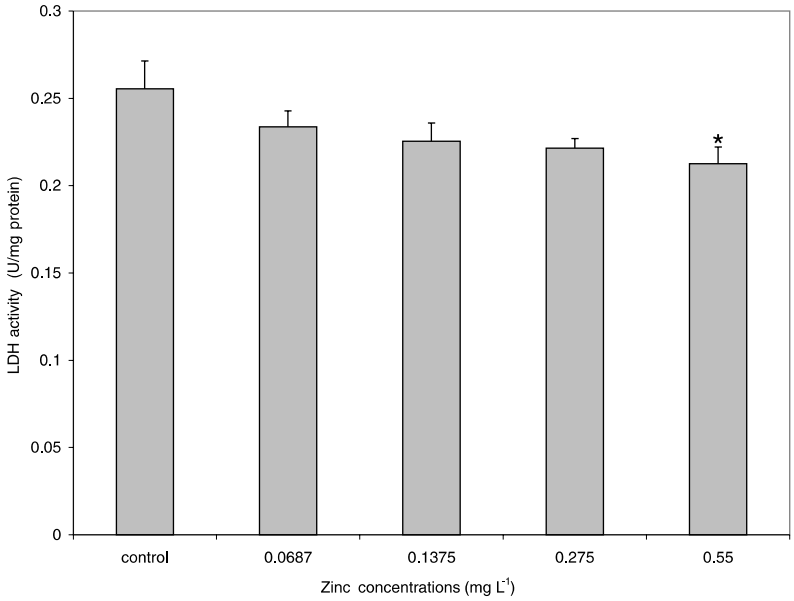

Fig. 2. In vivo effects of zinc (Zn^{2+}) on LDH activity. Data are expressed as the mean of three samples ± SE (Four measurements per sample) (* = significantly differences at $P \leqslant 0.05$).

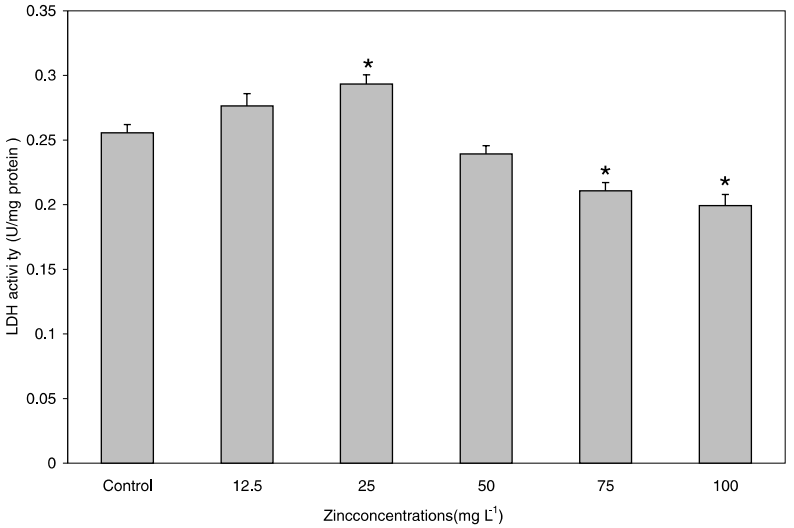

Fig. 3. In vitro effects of zinc on LDH activity. Data are expressed as the mean of three samples ± SE (Four measurements per sample) (* = significantly differences at $P \leqslant 0.05$).

additional energy is rapidly required. A negative correlation between LDH activity and ambient oxygen levels for some aquatic organisms, suggesting a possible biochemical adjustment in response to the lowered oxygen levels (Wu and Lam, 1997). This probably occurs also in situations of chemical stress. Therefore, this enzyme may be a sensitive criterion both in laboratory and in biomonitoring studies with invertebrates.

The first objective of this study was to optimize and standardize the conditions for the use of LDH activity as effect criterion in toxicity tests with the cladoceran *D. magna*. In order to achieve this goal, the influence of several parameters on LDH activity was investigated. The first parameter investigated was the influence of the number of individuals used per sample. This number should be a compromise between the sensitivity of

enzymatic determinations and costs (in money, time and human effort) needed to culture the animals required to prepare homogenates (Guilhermino et al., 1996). The results of this study suggest that the use of 35 juveniles per ml of buffer allow the obtention of a homogenate suitable for LDH determinations. However, in some experimental conditions clutches of a large number of small juveniles are produced (Baird et al., 1988; Naylor et al., 1992). Since homogenates prepared with 35 small juveniles may have lower LDH levels than those prepared with the same number of large organisms, this may be a source of error in tests based on this enzyme. For this reason, 45 juveniles per ml of buffer are recommended for homogenate preparation. Thus, all further experiments with juveniles were performed using this proportion.

The second factor investigated was the influence of food on LDH activity. Since no differences in LDH activity were found between 24 and 48 h old animals cultured in the presence or absence of food in the culture medium, LDH may be used as an effect criterion in tests with juveniles until an age of 48 h with no food supply. This will eliminate potential problems resulting from food-toxicant interactions in acute toxicity tests.

No statistical differences were found between LDH activities in eggs. However, significant differences were found in both LDH activity and protein levels in homogenates prepared from females with and without eggs. These findings suggest that the use of females with eggs to produce homogenates may lead to an over- or under-estimation of the enzyme activity. Therefore, it is important to define the presence/absence of eggs in homogenates of females and, if eggs are present, it is important to take their number into consideration. This may be particularly important if LDH is used as an effect criterion in tests with adults.

The results obtained in this indicate that LDH activity shows variations with the age of the daphnids. The highest LDH activity was obtained with 7 d old females. Similar results were observed for acetylcholinesterase activity (Guilhermino et al., 1996).

The second objective of this study was to develop both in vitro and in vivo acute toxicity tests using the activity of LDH as an effect criterion. In order to attain this objective, the conditions of exposure, homogenate preparation and enzymatic determinations were optimized and standardized. For in vivo tests, animals may be exposed to several concentrations of the test substance, in groups of 25 per 1000 ml of test medium. Since no differences in LDH activity were found between 24 and 48 h old juveniles, the period of test may be 48 h. This period of time seems to be sufficient to detect effects on the enzyme activity resultants from the exposure to the toxicant (data not shown). Since 48 h of fasting does not interfere with LDH determinations, the use of food should be avoided in order to prevent possible interactions between food and toxicant. This criterion may also be used in tests with adults. However, care should be taken in order to avoid possible errors due to the presence of eggs in the brood chamber.

In order to attain the third objective of this study, the results from LDH inhibition tests were compared with results obtained in conventional acute toxicity tests using zinc chloride as test substance. LC50 for zinc determined at 48 h in the present study is compared with the corresponding values from the literature (Attar and Maly, 1982; Khangarot et al., 1987; Baird et al., 1991; Guilhermino et al., 1997).

A significant in vivo inhibition of LDH was observed at concentrations under the LC50 determined (Fig. 2). In order to access the sensivity of in vivo LDH test, the 48 h EC50 value for LDH inhibition should be compared with 48 h LC50 value. However, the wide 95% CL associated with the EC50 determined, make impracticable such comparison. This may be a problem in in vivo LDH tests since severe depressions of LDH activity may cause the death of the animals before the end of the test. This may be overcome by using the index proposed by Guilhermino et al. (1997) which greatly reduces the importance of 95% CL. In our opinion, the use of sublethal parameters shows clear advantages relative to death in toxicity tests and should be incremented.

Low concentrations of zinc chloride (less or equal to 25 mg l^{-1}) seem to stimulate the activity of LDH in vitro and high concentrations seem to have the opposite effect. This pattern conforms to the hormetic curve form described by Stebbing (1982) and is frequently observed in ecotoxicological studies. Zinc significantly inhibited LDH activity in vitro with an NOEC of 50 mg l^{-1}, LOEC of 75 mg l^{-1} and IC50 of 175.2 mg l^{-1}. These concentrations are in general difficult to find in the environment. Furthermore, it is well known that in vitro assays do not take into consideration the integrated processes that occur in live organisms. Despite their low ecological relevance, they may be of great value in the study of specific effects and in the identification of toxicity mechanisms. Furthermore, their use as screening tests will allow the reduction of costs, time and human effort required for toxicity evaluation.

In summary, our results indicate that LDH activity of *D. magna* may be used as sub-lethal indicative parameter in aquatic toxicity tests with *D. magna*, particularly when the microplate technique is used since it allows the processing of a great number of samples in a short period of time and may be performed with a small amount of biological material.

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