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Lactate dehydrogenase in fish spermatozoa and its role in sperm cell bioenergetics

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Lactate dehydrogenase (LDH) in spermatozoa of three fish species was studied to determine its role in sperm cell bioenergetics in normal and polluted conditions. The adenylate energy charge (AEC) has been used as the measure of energy storage for adenine nucleotide pool of living cells and tributyltin (TBT) as a model toxicant. It was demonstrated that carp *Cyprinus carpio* Linnaeus, 1758 spermatozoa exhibit higher LDH activity than catfish *Clarias gariepinus* (Burchell, 1822) and herring *Clupea harengus* Linnaeus, 1758 spermatozoa. Native electrophoretic pattern of LDH from spermatozoa compared to somatic tissues extract shows nine LDH isoenzymes in carp spermatozoa, two LDH isoenzymes in herring and one LDH isoenzyme in catfish spermatozoa. It was confirmed that lactate plus pyruvate, which are the substrates for LDH seems to maintain AEC value. It indicates ATP synthesis in tricarboxylic acid cycle prevails upon the ATP utilization in catfish spermatozoa. It was noticed that LDH-B_4 is more strongly inhibited by TBT in herring than the nine LDH isoenzymes in carp spermatozoa which confirms that herring spermatozoa may be more sensitive for pollution by decreasing their energetic state. LDH role in maintaining physiological bioenergetic state of spermatozoa is also discussed.

Keywords: Adenylate concentration, Carp, Catfish, *Cyprinus carpio*, *Clarias gariepinus*, *Clupea harengus*, Herring, Tributyltin

The duration of fish spermatozoa motility in the natural environment varies between fish species but in general it is limited to short periods. In spermatozoa of externally fertilizing fish, sperm motility is one of the most important viability parameter and it is the major energy utilizing process[1,2]. Spermatozoa metabolism of teleost fishes is based mainly on measurements of spermatozoal enzymes and metabolites in several fishes. The available data describe the main metabolic pathways e.g. tricarboxylic acid cycle, glycolysis or lipid metabolism[3,4]. All metabolic processes as glycolysis, mitochondrial respiration and oxidative phosphorylation have been confirmed in carp spermatozoa[5,6] and rainbow trout[7] by non-native methods such as electrophoresis, liquid chromatography and mass spectroscopy. In fish spermatozoa, glucose levels decreased during motility and during immotile storage. When respiratory activity is inhibited, the levels of lactate increase. Pyruvate is formed during glycolysis and catabolism of some amino acids. It has been shown that pyruvate and lactate could stimulate spermatozoa motility and viability when added as a substrate to the incubation medium[8-10].

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Very little is known about the native isoenzyme expression in fish spermatozoa as compared to their expression in somatic tissues. It was shown earlier that herring *Clupea harengus* spermatozoa possess high activity of cytosolic and mitochondrial creatine kinase, a different isoenzyme from skeletal muscle[11,12] and it had high activity of two molecular forms of malic enzyme - an NAD-preferring malic enzyme and an NADP-specific malic enzyme[13,14].

In most fish species, lactate dehydrogenase (LDH, EC 1.1.1.27) is a tetramer consisting of subunits coded by three independent loci. The enzyme catalyses the interconversion of pyruvate and lactate, using NAD as the coenzyme. Each LDH isoenzyme has different kinetic and physico-chemical properties and exhibits distinct tissue expression[15]. Two LDH isoenzymes in herring spermatozoa have been detected - LDH-A_2B_2 and LDH-B_4[16]. As a result of polyploidization in carp somatic tissues, up to 15 isoenzymes have been detected[17]. Carp *Cyprinus carpio* spermatozoa exhibit higher LDH activity than catfish *Clarias gariepinus*, brown trout *Salmo trutta*, salmon *Salmo salar* and herring spermatozoa[10].

ATP formed by glycolysis (substrate phosphorylation), tricarboxylic acid cycle and oxidative phosphorylation in mitochondria can transfer its terminal high energy phosphoryl group to

form chemical compounds of relatively similar high – energy character like creatine phosphate (CP)[18].

Adenylate energy charge (AEC) has been proposed as the measure of energy storage for the adenine nucleotide pool of living cells[19]. The synthesis of ATP and CP causes AEC increase and decrease, respectively.

The effects of tributyltin (TBT) on the energy metabolism and motility of carp and catfish spermatozoa caused a decrease in motility[20]. Among the enzymes investigated, only LDH was affected by TBT treatment with the reduction in activity for catfish and carp. Carp spermatozoa are less sensitive to TBT exposure than those of catfish[20]. Treatment of herring spermatozoa with TBT caused a time-dependent decrease of viability and release of creatine kinase into surrounding medium from damaged spermatozoa[11]. It was shown earlier that treatment of herring LDH-A_4 with TBT caused dose and time dependent decrease of enzyme activity[21].

To our knowledge, no studies on native lactate dehydrogenase isoenzymes expression of other fish spermatozoa including carp and catfish have been reported. Hence, in this study we demonstrated (i) LDH isoenzymes expression in spermatozoa of freshwater fish – carp and catfish with comparison to marine herring spermatozoa; (ii) the effect of some substrates in the storage medium on AEC value in catfish spermatozoa; and (iii) inhibition of herring spermatozoa LDH-B_4 and carp spermatozoa LDH activity by TBT, and also discussed the role of the enzyme LDH on maintaining of sperm cells bioenergetics.

Materials and Methods

Chemicals

Oxamate-Agarose, Na–pyruvate, phenasine methosulfate (PMS), NAD, NADH, Coomassie Brilliant Blue R and G were from Sigma, while acrylamide, N,N9-methylene-bisacrylamide, TEMED, riboflavin and ammonium peroxydisulfate were from Reanal (Hungary). 5'-AMP-Sepharose 4B, DEAE-Sepharose were from Pharmacia Fine Chemicals (Sweden) and Tris was from Serva (Germany). All other chemicals were obtained from P.O.CH. Gliwice (Poland).

Material collection

Spermatozoa of common carp *Cyprinus carpio* Linnaeus, 1758 and catfish *Clarias gariepinus* (Burchell, 1822) were kindly provided by the Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, Belgium. Sperm samples were prepared as described by Rurangwa *et al*.[20]. Specimens of herring *Clupea harengus* Linnaeus, 1758 from the Baltic Sea were purchased from local fishermen in the spawning season. Males were stripped and the semen was diluted 1:10 in 30 mM Tris-HCl buffer, pH 8.2 containing 80 mM NaCl, 40 mM KCl and 0.1 mM $CaCl_2$. Sperm was used immediately or frozen in liquid nitrogen until required. Somatic tissues were also used fresh or frozen as above.

Polyacrylamide gel electrophoresis

LDH isoenzymes from carp were analysed by slab polyacrylamide gel electrophoresis as described in details by Ziętara *et al*.[22]. The electrophoresis was run at 25 mA for 2.5 h in 5 mM Tris–37 mM glycine buffer, pH 8.3 using vertical slab gel apparatus Minigel–Twin G 42 (Biometra GmbH, Germany). Gels were stained for LDH activity in the dark at 37°C. The staining procedure was described by Ziętara & Skorkowski[23]. The staining mixture consisted of 120 mM Na–lactate, 0.25 mM NAD^+, 0.1 mg mL^{-1} *p*-nitro blue tetrazolium chloride and 0.1 mg mL^{-1} phenasine methosulfate in 0.1 M Tris–HCl buffer, pH 8.0.

Starch gel electrophoresis

LDH isoenzymes from catfish and herring were analysed by horizontal starch gel electrophoresis method. The method was described in details by Ziętara & Skorkowski[24]. The electrophoresis was run at 15 V cm^{-1} for 4 h in 15% starch gel. The electrode buffer consisted of 0.15 M Tris–citrate buffer, pH 7.0. Gels were stained for LDH activity in the dark at 37°C in following medium: 120 mM Na–lactate, 0.3 mg mL^{-1} NAD^+, 0.3 mg mL^{-1} *p*-nitro blue tetrazolium chloride, 0.3 mg mL^{-1} phenasine methosulfate and 1% agarose in 0.1 M Tris–HCl buffer, pH 8.0.

Preparations of enzymes used for experiments

LDH-B_4 was isolated from herring spermatozoa as described earlier[16]. Fractions containing LDH-B_4 isoenzyme were separated, concentrated and kept in 40% (v/v) glycerol in 0.1 M Na–phosphate buffer, pH 7.1, at –20°C. The enzyme was used for inhibition by TBT. To obtain LDH from carp, spermatozoa were homogenized in a 1:4 ratio (w:v) of cold 10 mM Tris –HCl + 2 mM EDTA, pH 8.2 and centrifuged at 22000 ×g for 20 min at 4°C. The supernatant was immediately used for TBT inhibition experiment.

Determination of adenylate content

Fifty microliters of catfish sperm were incubated for 168 h with the extender 1:10 (control) or experimental solutions containing different concentrations of added substrates: 50 mM lactate + 5 mM pyruvate (LP); 50 mM glucose (Glu); 50 mM glycine + 10 mM lactate + 1 mM pyruvate (GLP); 50 mM alanine (Ala); 50 mM serine (Ser); 50 mM glycine (Gly). Adenylates were extracted using 500 µL of ice cold 1.3 M $HClO_4$, followed by centrifugation at 14000 ×*g* for 3 min at 4°C and neutralization of the supernatants with 3 M K_3PO_4. After the next centrifugation step to remove precipitated $KClO_4$, supernatants were analysed using a high performance liquid chromatography (HPLC). The equipment used was a Merck-Hitachi System connected to Hewlett Packard 1050 diode array detector. The reversed-phase method used for determination of ATP, ADP and AMP has been described previously[25]. The identity of ATP, ADP and AMP peaks in the spermatozoa extracts were confirmed by similarity of UV spectra, using a diode array detector. The adenylate energy charge is calculated as follow AEC = [ATP] + 1/2 [ADP]/[ATP] + [ADP] + [AMP][19].

Inhibition of LDH by TBT

Several solutions of TBT concentrations (2 and 10 µM) were dissolved in dimethyl sulfoxide (DMSO) and added to the cuvette before activity estimation at zero time or were preincubated for appropriate time with LDH from herring or carp at 0, 0.5, 1, 2, 3, 4, 6 and 20 h. Final DMSO concentration in the cuvette equals always 10% and this DMSO concentration had no effect on LDH activity. The solutions were then mixed in the cuvette and TBT dissolved in DMSO was added before the activity estimation and the remaining activity was measured as described earlier[21].

Results

LDH isoenzyme expression differences in spermatozoa of carp, catfish and herring were compared. LDH isoenzymes were determined in four fish somatic tissues and spermatozoa. Electrophoretic studies of LDH in the carp revealed an isoenzyme pattern consisted of up to nine isoenzymes in most tissues. Number of LDH isoenzymes in carp spermatozoa resembled expression in carp eye. There were two major spots of LDH activity in carp skeletal muscle which indicated the predominant activity of *ldhA* locus (Fig. 1A). One LDH-B_4 isoenzyme present in catfish spermatozoa resembled expression in catfish liver and heart where *ldhB* locus activity is the highest and in the case of heart, five isoenzymes of the *ldhA* and *ldhB* products appeared (Fig. 1B). Two LDH isoenzymes present in herring spermatozoa were also expressed in herring heart and eye (Fig. 1C).

The influence of different energetic substrates on adenylate energy charge (AEC) value is showed in Fig. 2 for catfish spermatozoa. The data presented indicate that AEC value in catfish spermatozoa during storage at 4°C depends on energetic substrates present in the storage medium. The decrease in AEC value after 168 h was the lowest (0.7) in sperm stored in the presence of lactate plus pyruvate, which are substrates for LDH. Among the exogenous substrates studied (i.e. glycine, serine, alanine, glucose, glycine plus lactate plus pyruvate and lactate plus pyruvate) the glycine seems to be the least (0.09) favourable to maintain AEC value. Lower AEC values were also observed when lactate plus pyruvate was replaced by glycine plus lactate plus pyruvate, glucose, alanine and serine (0.46, 0.39, 0.31 and 0.26, respectively). Pyruvate and lactate, therefore, are significant metabolites for bioenergetics of catfish spermatozoa

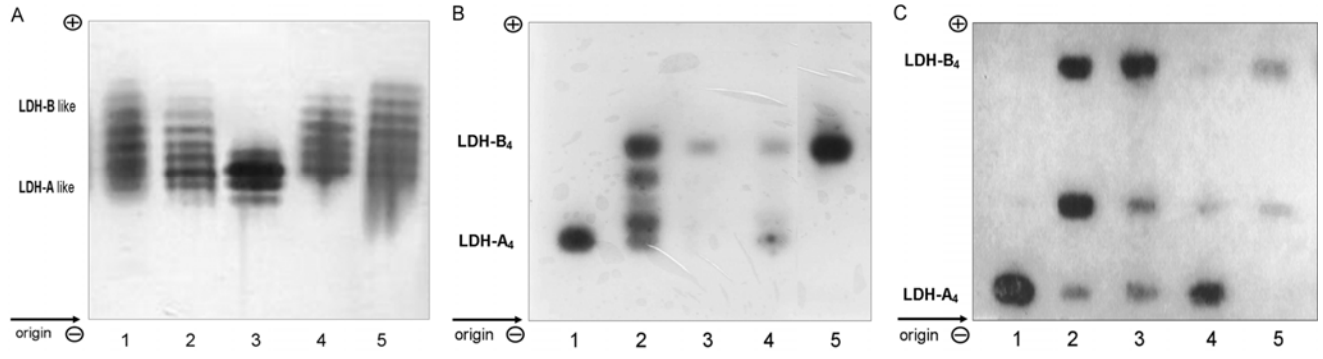

Fig. 1 — Native electrophoretic pattern of lactate dehydrogenase (LDH) isoenzymes from spermatozoa and somatic tissues extracts. (A) carp *Cyprinus carpio*: 1 – spermatozoa, 2 – eye, 3 – skeletal muscle, 4 – heart, 5 – liver; (B) catfish *Clarias gariepinus*: 1 – skeletal muscle, 2 – heart, 3 – spermatozoa, 4 – eye, 5 – liver; and (C) herring *Clupea harengus*: 1 – skeletal muscle, 2 – heart, 3 – eye, 4 – liver, 5 – spermatozoa.

and are responsible for keeping adenylate energy charge in good condition close to physiological values 0.78 after 120 h of incubation (Fig. 2).

The effect of TBT toxicity on LDH-B_4 activity from herring spermatozoa is presented in Fig. 3A. Time dependent inhibition of herring LDH-B_4 from spermatozoa by TBT showed that after 30 min. of pre-incubation only about 45% of its enzymatic activity remained in the presence of 10 µM TBT concentration. The effect of TBT inhibition of nine LDH isoenzymes from carp spermatozoa is demonstrated in Fig. 3B. Time dependent inhibition of nine LDH isoenzymes present in carp spermatozoa by TBT showed that after pre-incubation for 30 min. about 75% of its enzymatic activity remained in the presence of 10 µM TBT concentration.

Discussion

In fish spermatozoa during motility, LDH plays a crucial role. Pyruvate is also formed during glycolysis and from catabolism of some amino acid. It has been shown that pyruvate could stimulate spermatozoa motility and viability of Danube bleak *Alburnus chalcoides* and rainbow trout *Oncorhynchus mykiss* when added as substrate to the incubation medium[8]. In fish and other vertebrates, LDH is a tetramer consisting of subunits coded by three independent loci. The enzyme catalyses the interconversion of pyruvate and lactate. Each LDH isoenzyme has different kinetic and physicochemical properties and exhibits distinct tissue expression[24,26]. Electrophoretic studies of LDH in the carp revealed an isoenzyme pattern of multiple bands in most tissues up to 15 isoenzymes in carp somatic tissues[17,27]. This large number of isoenzyme is the consequence of polyploidization during the evolution of this species. In carp spermatozoa, three different genetic loci *ldhA*, B^1 and B^2 exist. The nine isoenzyme LDH pattern observed in our studies is the effect of the three loci expression with one $ldhB^1$ null allel and it is consistent to the phenotype $AB^2B^1{}_+/B^1{}_-$ described by Engel *et al.*[17]. It was shown earlier and confirmed in this study that there are only two LDH isoenzymes present in herring spermatozoa - LDH-B_4 and LDH-A_2B_2, three isoenzymes in heart – LDH A_4, LDH B_4 and LDH A_2B_2 and only one in skeletal muscle - LDH A_4[16]. Such difference of LDH izoenzymes expression in testis and muscle tissues was also reported from *Arctoscopus japonicas*[28]. The lack of expression of LDH-AB_3 LDH-A_3B isoenzymes in herring tissues was also reported by Markert & Faulhaber[29] and Odense *et al.*[30]. The presence of the LDH-B_4 and LDH-A_2B_2 isoforms which normally suite better for aerobic conditions agrees well with the Lahnsteiner *et al.*[8] findings. LDH activities in carp and catfish spermatozoa were about 14 and 10 times higher, than in herring spermatozoa respectively[16].

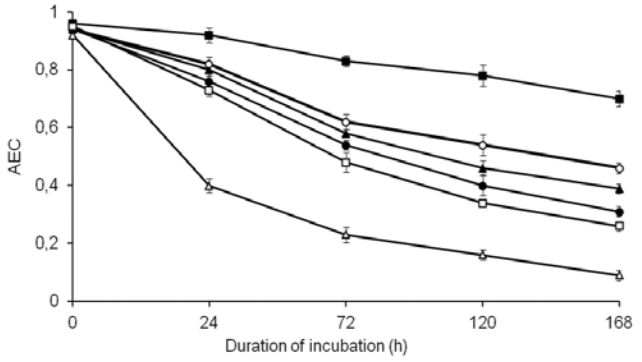

Fig. 2 — Effect of substrates on the adenylate energy charge (AEC) of catfish *Clarias gariepinus* spermatozoa during seven days storage. Adenylate content (nmol/1 µL sperm). LP: 50 mM lactate + 5 mM pyruvate (■); Glu: 50 mM glucose (▲); GLP: 50 mM glycine + 10 mM lactate + 1 mM pyruvate (○); Ala: 50 mM alanine (●); Ser: 50 mM serine (□); Gly: 50 mM glycine (△). Each point represents the means of the six measurements.

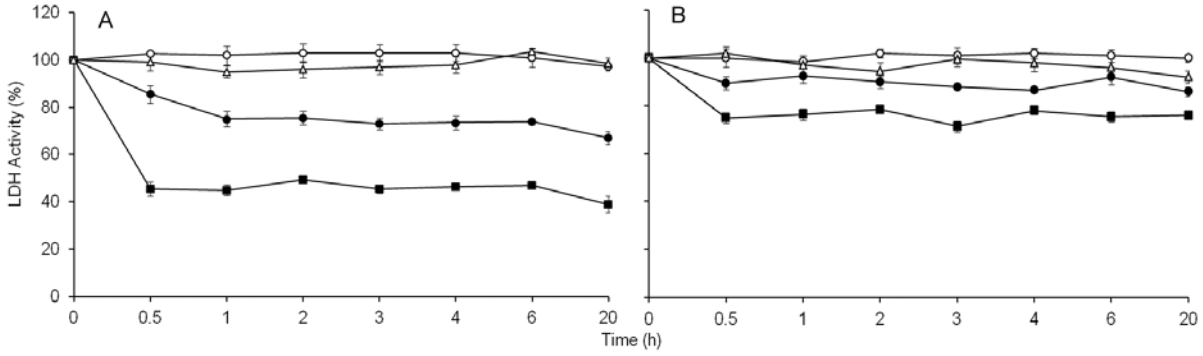

Fig. 3 — Time-dependent inhibition of lactate dehydrogenase (LDH) by tributyltin (TBT). (A) herring *Clupea harengus* spermatozoa LDH-B_4; and (B) carp *Cyprinus carpio* spermatozoa LDH. LDH activity were measured in cuvette of 1 mL volume at final concentration: control (○); 10% DMSO (△); 2 µM TBT (●) and 10 µM TBT (■). Each point represents the means of the six measurements.

ATP level in spermatozoa of different fish have been positively correlated with viability[2,31]. It means that good ATP level (meaning AEC = [ATP] + 1/2 [ADP]/[ATP] + [ADP] + [AMP] value) is strictly related to good motility and finally to better fertilising potential. Carp spermatozoa have about 50% more ATP per one cell than catfish spermatozoa[20]. This would explain why catfish spermatozoa display lower motility than carp spermatozoa. However, other possibilities could not be excluded eg. activity of expressed lactate dehydrogenase. The adenylate energy charge (AEC) has been proposed as the measure of energy storage in the adenine nucleotide pool of living cells[1]. Many studies have confirmed that the physiological AEC of somatic cells ranges from 0.8 to 0.95. Thus we treated of stored catfish spermatozoa with nutrients which support metabolic activities to keep AEC close to physiological values as long as possible. When conversion of lactate to pyruvate is inhibited by TBT, the redox state of a cell is compromised, and potentially marks the cell for accelerated cell death. Pyruvate is a very significant metabolite for bioenergetics of fish spermatozoa and is responsible for keeping AEC in good condition close to physiological values. Pyruvate is a substrate for cytosol lactate dehydrogenase and mitochondrial malic enzyme and pyruvate dehydrogenase. The results discussed in this paper give more light for better understanding of LDH in fish spermatozoa and its role in cell metabolism.

Tributyltin (TBT) is known to exert toxic effects in a variety of aquatic animals and cell cultures[11,32-34]. TBT is the most common organotin derivative used in antifouling paints for water pipes and vessels. Despite the prohibition of its use it has already accumulated in sediments and can be picked up by some animals. The effect of TBT on the energy metabolism and motility of catfish and carp spermatozoa but in the presence of glycine only was shown earlier[20]. Catfish spermatozoa are more sensitive to TBT than those of carp[20]. It can be speculated that in the case of carp spermatozoa, not all nine LDH isoenzymes products of three loci are inhibited in the same manner by TBT. In the case of herring LDH-B_4 from spermatozoa, TBT inhibition showed that after 30 min. of pre-incubation only about 45% of its enzymatic activity remained. In carp spermatozoa, TBT inhibited only 25% of LDH activity which can by explain partly by high presence of LDH–B like subunits within isoenzymes studied in our phenotype. The effect of TBT on the enzyme activities showed that among the six enzymes studied, only LDH was affected by TBT[20].

The toxic effect of TBT on herring spermatozoa was also observed. It can be tested by using a specific sperm viability kit enabling the observation of live and dead sperm cells[11]. Treatment of herring spermatozoa with TBT caused a time dependent decrease of viability and motility[20]. Creatine kinase (CK) released from damaged spermatozoa into the surrounding medium was positively correlated with TBT concentration[11], but creatine kinase from fish spermatozoa was not inhibited by TBT[20]. Cytotoxic effect of toxicants is usually assessed by release of LDH from the intoxicated cells into surrounding medium. When LDH is inhibited by TBT, the CK which is not affected by TBT could be a better biomarker of integrity of fish sperm cell membrane. In our study, the well-known LDH system was chosen to better understand the biological mechanisms underlying the TBT toxicity. Pure LDH-A_4 from herring skeletal muscle showed TBT dose-dependent loss of enzymatic activity within 30 min. and only about 30% of its enzymatic activity remained in the presence of 10 μM TBT concentration[21]. To compare to our previous results of herring LDH-A_4 inhibition by TBT[21], it is visible that LDH-B_4 is less inhibited. Catfish spermatozoa possess only one LDH-B_4 izoenzyme and carp spermatozoa have nine LDH isoenzyme with predominance of LDH-B subunit that are more stable against TBT intoxication. Reduced activity of LDH could be due to a direct interaction of TBT with the enzyme sulfhydryl group, because the addition of albumin (BSA)[21] or glutathione (GSH) were able to protect enzyme activity from the toxicant[35]. The influence of different energetics substrates on AEC showed that substrates for LDH are the best for keeping sperm cell in good conditions contrary to observation when catfish spermatozoa are supported only with glycine as shown earlier[13]. When conversion of lactate to pyruvate by LDH is inhibited by TBT, the redox state of a cell is compromised, and potentially marks the cell for accelerated cell death.

Conclusion

Lactate dehydrogenase (LDH) from spermatozoa is coded by one, two or three genes. Its native electrophoretic pattern shows one LDH isoenzyme in catfish, two LDH isoenzymes in herring and nine LDH isoenzymes in carp spermatozoa. Pyruvate and lactate are significant metabolites for bioenergetics of

catfish spermatozoa and are responsible for keeping adenylate energy charge (AEC) at physiological values. Reduced activity of LDH caused by tributyltin (TBT) could be due to a direct interaction with the enzyme sulfhydryl group.

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Conflict of interest

The authors declare no conflict of interest.

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