„Lactate dehydrogenase (LDH) isoenzymes in fish spermatozoa and tissues"

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Little is known about the function of lactate dehydrogenase isoenzymes (LDH, EC 1.1.1.27) in fish spermatozoa compared to their expression in somatic tissues. LDH is the terminal enzyme of the anaerobic phase of respiration (glycolysis), catalysing the reversible reaction to convert pyruvate to lactate, with nicotinamide adenine dinucleotide (NAD) as coenzyme. Pyruvate formed from the breakdown of glucose under aerobic conditions is completely oxidised to carbon dioxide and water in the mitochondria. Under anaerobic conditions, e.g. during intensive muscle work, pyruvate is reduced to lactate. The reaction is very important as it ensures the regeneration of NAD necessary to sustain glycolysis and ATP production.

In fish and other vertebrates, LDH is a tetramer with a molecular weight of approximately 140 kD. LDH subunits are encoded by three non-allelic genes (*Ldh-A*, *Ldh-B* and *Ldh-C*) whose products, after random aggregation, form homo- and heterotetrameric LDH iso- and allenzymes. The frequency of occurrence of the different forms in tissues depends on the activity of the genes controlling the synthesis of the subunits and their ability to aggregate. LDH isoenzymes differ in their kinetic and physicochemical properties and show distinct tissue expression.

Fish are exposed to changes in external environmental factors such as temperature, light, pressure, salinity and the amount of dissolved gases or various types of pollution. The presence of diverse forms of isoenzymes in fish creates greater catalytic capacity to cope with these changes, also influencing the intracellular environment and the enzymatic pathways operating within it. Spermatozoa are highly specialised cells that undergo a variety of metabolic states in their development, and are particularly vulnerable to environmental changes. During spermatogenesis, most of the energy is directed mainly into the process of biosynthesis. Mature spermatozoa switch the demand for ATP from the biosynthetic process to the movement that determines fertilisation. These two periods are separated by the resting period, which is characterised by low metabolic efficiency. The diversity of reproduction influences differences in the structure of the spermatozoa, as well as the expression of certain enzymes, particularly those involved in ATP homeostasis. In most fish, the sperm motility apparatus is blocked in the seminal fluid of the gonads. Only contact with the external environment (freshwater, sea water) unblocks their motility. In freshwater as

well as marine fish, sperm motility may be the result of non-specific hypo- or hyperosmotic changes, affecting changes in intracellular ion concentration. Among the many chemical contaminants to which aquatic organisms are exposed, tributyltin (TBT) is a persistent and toxic compound, stimulates endocrine disruption in fish, reduces sperm motility and viability and bioaccumulates. It was the most common organotin derivative used from around 1950 in antifouling paints for painting boat hulls, ships, pipes, cooling towers and water cooling systems, as a fungicide in textiles, paper mills, agriculture and also in breweries. Under the collective name TBT are several compounds that have in common the presence of the TBT cation in the structure. Examples include TBT hydride, chloride and hydroxide. Despite the ban on TBT, it is still found in the aquatic environment, with the highest concentrations reported in coastal waters, harbours, shipyards and bottom sediments. Spermatozoa are particularly vulnerable to environmental changes. They require an energy supply in the form of ATP to function adequately. ATP production can be supported by a reaction catalysed by LDH. Previous studies have shown that the maintenance of good ATP levels in fish sperm is closely linked to good motility. The adenylate energy charge (AEC) has been proposed as a measure of energy storage in the adenine nucleotide pool in living cells.

$$AEC=[ATP]+½[ADP]/[ATP]+[ADP]+[AMP]$$

Many previous studies have confirmed that the physiological value of the AEC in somatic cells ranges from 0.8 to 0.95.

In light of the above, the aim of my PhD research was to identify and isolate LDH isoenzymes from spermatozoa and other tissues of selected fish species, and to determine their physicochemical and catalytic properties, furthermore to determine the effect of TBT on LDH activity in spermatozoa compared to somatic tissue, and the effect of metabolites on sperm bioenergetics using a selected example.

The dissertation research started by determining the activity of LDH and several other enzymes in the spermatozoa of herring (*Clupea harrengus*), as a representative of saltwater species, and carp (*Cyprinus carpio)* and African catfish (*Clarias gariepinus*), representing freshwater environments. The results showed that LDH activity in herring spermatozoa was low, while it was about 10 and 14 times higher in catfish and carp spermatozoa, respectively. In contrast, the activities of other enzymes (CK, MDH, IDH, ME G-6-PDH) were higher in herring sperm than in carp and catfish sperm. This suggests a strategy of biochemical adaptation for metabolism under high oxygen

conditions in the case of herring[**1**]. To identify LDH isoenzymes in herring, carp and catfish in spermatozoa, and four somatic tissues (skeletal muscle, heart, eye and liver), horizontal electrophoresis in a 14 % starch gel conducted under native conditions was used. In herring, it showed the presence of two isoenzymes LDH-B_4 and LDH-A_2B_2 in the spermatozoa, heart, eye and liver and the LDH-A_4 isoenzyme in skeletal muscle, liver and little activity of this form in the heart and eye [**1**] [**2**]. In carp, the electrophoretic pattern of LDH in most tissues consisted of a maximum of nine isoenzymes. The number of LDH isoenzymes in spermatozoa resembled the expression in the carp eye. Two main sites of LDH activity were observed in the skeletal muscle of the carp as a result of the predominant activity of the *Ldh-A* locus. The large number of isoenzymes is a consequence of polyploidisation during the evolution of this species. In contrast, the single LDH-B_4 isoenzyme present in catfish spermatozoa resembled expression in the liver and heart, where the activity of the *Ldh-B* locus is the highest, and in the case of the heart, five isoenzymes appeared - products of *Ldh-A* and *Ldh-B* [**2**]. To obtain pure forms of both LDH isoenzymes from herring spermatozoa, 5'-AMP-Sepharose 4B followed by DEAE-Sepharose was used. Fractions containing the LDH-B_4 isoenzymes and LDH-A_2B_2 were combined separately, concentrated in a vacuum bag and 40% glycerol in 0.1 M Na-phosphate buffer, pH 7.1. They were stored at - 20 ^oC. LDH-A_4 isoenzyme from herring skeletal muscle was obtained after purification on DEAE-Sepharose, 5'-AMP-Sepharose 4B, Oxamate-agarose [**1**] It was concentrated and stored in the same way as isoenzymes from spermatozoa. The purity was analysed by polyacrylamide gel electrophoresis (PAGE), which showed the presence of single bands in all purified forms of isoenzymes [**1**]. Several kinetic properties were determined for the obtained isoenzymes performed at the predetermined pH optimum for the pyruvate reduction reaction. The study showed the greatest similarity between the isoenzymes LDH- A_2B_2 and LDH-B_4. The LDH-A_2B_2 isoenzyme showed the highest affinity for pyruvate, LDH-B_4 intermediate, and the LDH-A_4 isoenzyme the lowest. Similarly, the inhibition of the reaction by high concentrations of pyruvate is higher for LDH- A_2B_2, and the lowest for the LDH-A_4 isoenzyme. The thermostability results carried out for the individual isoenzymes, indicated the LDH-A_4 isoenzyme as the most thermolabile, while the LDH-B_4 isoenzyme achieved the highest thermostability by losing activity above 70 ^oC [**1**].

The pollution to which aquatic organisms are exposed negatively affects their physical state and this is reflected in the poor functioning of enzymes in various

enzymatic pathways. Experiments, were performed using a purified form of LDH-A_4 from skeletal muscle and LDH-B_4 from herring sperm. The results showed a dependence of LDH activity on the concentration of TBT in the incubation medium [**2**, **3**]. Immediately after the addition of 10 μM TBT, the activity of LDH A_4 decreased to 65%, and within 30 minutes to 30% [**3**]. Inhibition of LDH activity was observed with increasing TBT concentration and incubation time. A higher activity (45%) under the same conditions was retained by the LDH-B_4 isoenzyme from herring spermatozoa. In contrast, nine LDH isoenzymes from carp spermatozoa retained 75% of the enzymatic activity [**2**]. To protect against the negative effect of TBT on lactate dehydrogenase activity, bovine serum albumin (BSA) was tested by adding it to the incubation medium. The results indicated that the addition of BSA at a concentration of 10 μg ml^{-1} was able to protect LDH-A_4 activity up to a concentration of 30 μM TBT. At a concentration of 100 μM TBT in the presence of 10 μg ml^{-1} BSA, 90% of the enzyme activity was observed [**3**].

Spermatozoa undergoing various metabolic states need good levels of ATP, which is measured by the energy charge value of adenylates. In this study, we investigated the effect of several exogenous substrates (i.e. glycine, serine, alanine, glucose, glycine plus lactate plus pyruvate, lactate plus pyruvate) on the adenylate energy charge (AEC) value for catfish spermatozoa. The data obtained proved that pyruvate and lactate are important metabolites for the bioenergetics of catfish spermatozoa, as they maintain the AEC at a good 0.78 after 120 h of incubation, close to the physiological state. Among the exogenous substrates tested, glycine appears to be the least favourable (0.09) for maintaining AEC values [**2**].

The results discussed in the work included in the dissertation shed more light for a better understanding of the function of LDH in sperm and fish tissues and its role in cell metabolism:

- LDH-A_2B_2 and LDH-B_4 in herring spermatozoa, LDH-B_4 in catfish spermatozoa confirm better adaptation to aerobic metabolic conditions.
- LDH-A_4 from herring muscle is the most inhibited isoenzyme by TBT.
- Both substrates of the LDH reaction (pyruvate and lactate) maintain the adenylate energy charge (AEC) in catfish spermatozoa within the range of physiological values.

Publications included in the dissertation

1. **Gronczewska J**, Ziętara MS, Biegniewska A, Skorkowski EF. 2003. Enzyme activities in fish spermatozoa with focus on lactate dehydrogenase isoenzymes from herring *Clupea harengus*. *Comparative Biochemistry and Physiology Part B* 134, 399-406

2. **Gronczewska J**, Skorkowski EF. 2020. lactate dehydrogenase in fish spermatozoa and its role in sperm cell bioenergetics. *Indian Journal of Experimental Biology*, 58, 7-13

3. **Gronczewska J**, Biegniewska A, Ziętara MS, Skorkowski EF. 2004. Inhibition by tributyltin of herring skeletal muscle lactate dehydrogenase activity. *Comparative Biochemistry and Physiology Part C* 137, 307-311