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Some Enzymes of Tricarboxylic Acid Cycle and Metabolites of Carbohydrate Metabolism in Adult *Isoparorchis hypselobagri* (Digenea: Trematoda) During *in vitro* Starvation

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The presence of considerable amount of enzymes of TCA cycle isocitrate dehydrogenase (ICDH-NADP+, EC 1.1.1.42), α-ketogluterate dehydrogenase (α-KGD, EC 1.2.4.2) and malate dehydrogenase (MDH, EC1.1.1.37) in fresh control and *in vitro* starved adult *Isoparorchis hypselobagri* establish the functional TCA cycle in this fluke. The major metabolic end products are pyruvate, lactate, oxaloacetate and malate. The ratio of oxaloacetate/malate assess that oxaloacetate is reduced to malate and in this fluke the reverse TCA cycle is active. The pyruvate/lactate ratio shows pyruvate is reduced to lactate and the fluke is homolactate farmenters.

Key words: Adult *Isoparorchis hypselobagri*, *In vitro* starvation, TCA cycle enzymes, Metabolites, Pyruvate/lactate, Oxaloacetate/malate ratio

Introduction

The recent research on carbohydrate metabolism during *in vitro* starvation of fluke is lacking. However, earlier research works on carbohydrate metabolism in helminthes are available from different authors (Barrett, 1981; Marr and Mullar, 1995). Reports on tricarboxylic acid (TCA) cycle enzymes and metabolites during *in vitro* starvation period are also lacking. *Isoparorchis hypselobagri*, a piscine digenetic trematode harbours within the swim-bladder of *Wallago attu*, an oxygen rich habitat. The main objective of the present study is to find out the effect of *in vitro* starvation on three major TCA cycle enzymes, isocitrate dehydrogenase (ICDH-NADP$^+$, EC 1.1.1.42), α-ketogluterate dehydrogenase (α-KGD, EC 1.2.4.2), and malate dehydrogenase (MDH, EC 1.1.1.37) and metabolites pyruvate, lactate, malate and oxaloacetate in this fluke.

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Material and Methods

Live flukes collected from the swim bladder of *Wallago attu*, obtained from the local fish market as well as from the field at Kangsabati reservoir, Mukutmanipur, district Bankura, West Bengal, India during March 2004 to February 2005, were subjected to starvation for 6, 12, 18, 24, 36, 48, 60 and 72 hours in phosphate buffered saline (PBS, pH 7.0; Taylor and Baker, 1978) without glucose. The parasites were maintained alive under aerobic condition at room temperature (30±1°C) in PBS solution with 250 mg Streptomycin and 100000 Unit Penicillin per 100 ml (Srivastava and Gupta, 1977) to prevent bacterial contamination.

After taking weight in a semi-micro balance (ADA, 71/L) a single fluke was homogenized in 2 ml of ice-cold 0.1 M tris-HCl buffer (pH 7.4) using an all glass Potter-Elvehjem homogenizer at 4°C (Yusufi and Siddiqi, 1978). The samples were centrifuged at 10,000 r.p.m. at 0~4°C for 30 minutes to remove the cell debris. The supernatant thus obtained was used in the enzyme assay. The specific activity of the enzyme isocitrate dehydrogenase (ICDH-NADP$^+$, EC 1.1.1.42) was expressed in terms of NADPH produced per minute of incubation per mg of protein (Ochoa, 1955a), α-ketogluterate dehydrogenase (α-KGD, EC 1.2.4.2) was expressed in terms of NADH produced per minute of incubation per mg of protein (Kaufman, 1955) and malate dehydrogenase (MDH, EC 1.1.1.37) was expressed in terms of NADH oxidized per minute of incubation per mg of protein (Ochoa, 1955b) at 340 nm in UV-spectrophotometer.

Pyruvate, lactate, oxaloacetate and malate content of the fluke extract was measured following the methods of

Table 1. Tricarboxylic acid cycle enzymes during *in vitro* starvation in adult *I. hypselobagri*

Study Group	ICDH-NADP$^+$ (nmol min^{-1} mg^{-1} protein)	α-KGD (nmol min^{-1} mg^{-1} protein)	MDH (nmol min^{-1} mg^{-1} protein)
Control (10)	572.2 ± 1.20	363.1 ± 1.28	674.1 ± 1.10
6 hours (10)	475.6 ± 1.23**	315.6 ± 1.26 **	565.2 ± 1.31 **
12 hours (10)	412.1 ± 1.37 **	265.8 ± 1.31 **	526.1 ± 1.28 **
18 hours (10)	365.3 ± 1.38**	226.5 ± 1.26 **	482.2 ± 1.13 **
24 hours (10)	253.2 ± 1.32 **	192.4 ± 1.26 **	466.9 ± 1.19 **
36 hours (10)	232.3 ± 1.33 **	181.2 ± 1.23 **	452.7 ± 1.16 **
48 hours (10)	203.2 ± 1.23 **	164.5 ± 1.15 **	480.6 ± 1.26 **
60 hours (10)	178.4 ± 1.31 **	147.7 ± 1.27 **	465.0 ± 1.15 **
72 hours (10)	151.8 ± 1.27 **	119.2 ± 1.25 **	462.7 ± 1.27 **

Results are expressed as Mean ± S.D.
Figure in the parentheses indicates the number of parasites studied
**P < 0.01 is highly significant

Table 2. Pyruvate, lactate, oxaloacetate and malate content during *in vitro* starvation in adult *I. hypselobagri*

Study Group	Pyruvate Content (μmol / mg protein)	Lactate Content (μmol / mg protein)	Oxaloacetate Content (μmol / mg protein)	Malate Content (μmol / mg protein)
Control (10)	11.46 ± 0.58	6.32 ± 0.16	5.27 ± 0.23	2.73 ± 0.18
6 Hours starvation (10)	11.96 ± 0.47 *	6.67 ± 0.19 **	5.46 ± 2.11 *	2.92 ± 0.17 **
12 Hours starvation (10)	12.27 ± 0.50 **	6.80 ± 0.15 **	5.60 ± 0.21 **	3.19 ± 0.16 **
18 Hours starvation (10)	12.47 ± 0.45 **	6.97 ± 0.17 **	5.70 ± 0.20 **	3.52 ± 0.19 **
24 Hours starvation (10)	12.87 ± 0.45 **	7.28 ± 0.17 **	5.92 ± 0.25 **	3.85 ± 0.20 **
36 Hours starvation (10)	13.32 ± 0.32 **	7.75 ± 0.18 **	6.31 ± 0.18 **	4.22 ± 0.22 **
48 Hours starvation (10)	13.87 ± 0.35 **	8.32 ± 0.20 **	6.95 ± 0.23 **	4.72 ± 0.21 **
60 Hours starvation (10)	14.35 ± 0.29 **	9.11 ± 0.23 **	7.43 ± 0.24 **	5.21 ± 0.19 **
72 Hours starvation (10)	15.34 ± 0.30 **	10.25 ± 0.28 **	8.12 ± 0.20 **	5.98 ± 0.20 **

Results are expressed as Mean ± S.D.
Figure in the parentheses indicates the number of parasites studied
*P < 0.05 is significant
**P < 0.01 is highly significant

Bucher *et al.*,1974; Gawehn and Bergmeyer,1974; Wahlefeld, 1974 and Hohorst, 1974 respectively. Protein was estimated following the method of Lowry *et al.*, 1951. Molar decadic absorption co-efficient ($1 \times mol^{-1} \times mm^{-1}$) for NADH and NADPH at temperature 25°C and 30°C are taken for practical use (NADH 6.3×10^2 at 340 nm) (Bergmeyer, 1974). In each experiment ten parasites were taken. Data collected on each specimen is recorded, categorical variables are analyzed and the results are expressed as Mean ± S.E.M (standard error of mean). Statistical probability is calculated using student t-test.

Results

The mean specific activity of the enzymes of TCA cycle in fresh control and *in vitro* starved adult *I. hypselobagri* are shown (Table 1). The specific activities of ICDH-NADP$^+$ and MDH are comparatively high in fresh control fluke than α-KGD. The specific activity of ICDH-NADP$^+$, α-KGD, and MDH in fresh control fluke varies between 570.2 to 574 nmol min^{-1} mg^{-1} protein, 360 to 364 nmol min^{-1} mg^{-1} protein and 673 to 676 nmol min^{-1} mg^{-1} protein respectively. All the enzymatic activities are significantly ($p < 0.01$) reduced during *in vitro* starvation period. The specific activity of α-KGD is relatively low than ICDH-NADP$^+$ and MDH in fresh control fluke.

The carbohydrate metabolite contents in fresh control and *in vitro* starved adult *I.hypselobagri* are shown (Table 2). The pyruvate content by far show maximum value than lactate, malate and oxaloacetate. All the metabolites increases significantly during *in vitro* starvation time period. The ratio of pyruvate/lactate and oxaloacetate/malate are shown (Table 3).

Table 3. Oxaloacetate/malate and pyruvate/lactate ratio during *in vitro* starvation in adult *I. hypselobagri*

Study Group	Oxaloacetate/ Malate Ratio	Pyruvate/ Lactate Ratio
Control (10)	1.93	1.81
6 Hours starvation (10)	1.87	1.80
12 Hours starvation (10)	1.75	1.80
18 Hours starvation (10)	1.62	1.80
24 Hours starvation (10)	1.53	1.76
36 Hours starvation (10)	1.50	1.72
48 Hours starvation (10)	1.47	1.66
60 Hours starvation (10)	1.42	1.57
72 Hours starvation (10)	1.36	1.49

Figure in the parentheses indicates the number of parasites studied

Discussion

The TCA cycle is of great importance to many aerobic organisms because its energy yield is much greater than that resulting from glycolysis. In the present investigation three major functionally linked TCA cycle enzymes of ICDH-NADP+, α-KGD and MDH in adult *I.hypselobagri* is indicative of functional TCA cycle is operative in this fluke.

Isocitrate dehydrogenase (ICDH) is one of the key enzyme of TCA cycle which catalyzes the oxidation of isocitrate. In the present study, ICDH is NADP linked and quite high activity in fresh control fluke provide a better clue for the operation of TCA cycle, since this is considered to be a rate limiting step of the pathway. The mean specific activity of ICDH-NADP+ in fresh control *I.hypselobagri* is 572.2 ± 1.2 nmol min^{-1} mg^{-1} protein which is comparable to the report in *Gastrothylax crumenifer* and *Srivastavaia indica* as 292.72 ± 6.30 μmol min^{-1} mg^{-1} protein and 94.29 ± 4.5 μmol min^{-1} mg^{-1} protein respectively (Yusufi and Siddiqi, 1978) showing higher activity than the present fluke. Barrett (1981) considered ICDH-$NADP^+$ is low in parasitic helminthes. However, differences of enzyme activities in various helminthes rule out the influence the habitat (Yusufi and Siddiqi, 1978). During *in vitro* starvation the specific activity of ICDH-$NADP^+$ declines (Table 1) significantly due to decline of oxygen consumption (Nizami and Siddiqi, 1975) during *in vitro* starvation.

The α-Ketogluterate dehydrogenase (α-KGD) is also considered as the enzyme for rate limiting steps of TCA cycle. It is a multienzyme system irreversibly catalyzes the decarboxylation of α-KGD. The mean α-KGD activity in fresh control fluke, 363.1 ± 1.28 nmol min^{-1} mg^{-1} protein, is lower than that of other two enzymes described (Table 1). The low activity of α-KGD is also reported in *Ligula intestinalis* as 420 nmol min^{-1} mg^{-1} protein (McManus, 1975), in *Schistocephalus solidus* as 22 nmol min^{-1} mg^{-1} protein (Korting and Barrett, 1977), in *Echinococcus multilocularis* and *E. grnulosus* are 46 and 20 nmol min^{-1} mg^{-1} protein respectively (McManus and Smyth, 1982). In the present investigation α-KGD activity in *in vitro* starved fluke also declines (Table 1) significantly, due to to decline of oxygen consumption (Nizami and Siddiqi, 1975) during *in vitro* starvation.

The enzyme MDH catalyzes the oxidation of malate to oxaloacetate. In the present study the mean specific activity of MDH in fresh control fluke is 674.1 ± 1.10 nmol min^{-1} mg^{-1} protein which is higher than other two enzymes described (Table 1) and facilitate reduction of oxaloacetate to malate. The significant amount of MDH is also reported in *Gastrothylax crumenifer* and *Srivastavaia indica* as 120.78 ± 0.87 and 4461 ± 43 μmol min^{-1} mg^{-1} protein respectively (Yusufi and Siddiqi, 1978). High levels of MDH activity is indicative of the successful operation of TCA cycle in this fluke. However, different levels of activity of this enzyme may be due to species differences as suggested by Yusufi and Siddiqi (1978). The decrease of MDH activity (Table 1) during *in vitro* starvation period suggest the decreased formation of malate from oxaloacetate, also found in oxaloacetate/malate ratio (Table 3). This trend of decline in the starved fluke indicates the redox potential in the cell being affected and thereby affecting the electron transport system.

The study of metabolites as well as end products of carbohydrate metabolism under *in vitro* starvation can often give useful information as to the pathways operating within a tissue or parasitic organisms. Pyruvate is the key substrate of the carbohydrate metabolism and occupies a very important position in the energy metabolism of the fluke. It was also regarded as the end product of glycolysis formed by the action of PK or malic enzyme (Barrett, 1981; Smyth and Halton, 1983). It is not only formed by glucose but also formed by the transamination of certain amino acids like alanine, glutamine, cysteine, threonine and serine. Even the OAA can reversibly form pyruvic acid and hence it can be considered to some extent as a central point for the energy metabolism.

The pyruvate content of the fresh control fluke varies between 10.46 to 12.08 mmol/mg proteins with an average value of 11.46 ± 0.58 mmol/mg protein is increased significantly during *in vitro* starvation. The significant amount of pyruvate is reported in *Neokrimia singhia*, 40.702 ± 1.783 mg of pyruvate/g wet wt. (Siva Sai Kumari and Ratnamala Rao, 1993), *Ligula intestinalis*, 76 ± 13 nmol/g fresh wt. (Mc Manus and Sterry, 1982) and *Echinococcus granulosus*, 0.0328 mg/g fresh wt. (Barrett 1981).

The increasing trend of pyruvate content in *I. hypselo-*

bagri in *in vitro* starvation period, may be due to the fact that pyruvate is either produced from PEP by PK or from malate by malic enzyme which is reported to be present in *Gastrothylax crumenifer* and *Srivastavaia indica* (Yusufi and Siddiqi, 1978) or may be formed by the transamination of certain amino acids like alanine, glutamine, cysteine, threonine and serine (Smyth and Halton, 1983; Barrett, 1981; Siva Sai Kumari and Ratnamala Rao, 1993). Thus, the pyruvate content determined in *I. hypselobagri* is found to be in significant quantity which also increase in significant level may be of considerable importance in the energy metabolism and in the biosynthesis of amino acids, carbohydrates and in view of the fecundity and fast growth.

The lactate or lactic acid is the end product of the carbohydrate metabolism in parasitic helminthes, which are facultative aerobes. The lactate produced in the anaerobic metabolism is not oxidized to pyruvate as in the free-living organism, but it is either stored in the cell or excreted out. In the present work, lactate content of the fresh control fluke varies between 6.15 to 6.66 μmol/mg protein with an average value of 6.32 ± 0.16 μmol/mg protein is comparable to the reports of *Echinococcus granulosus*, 0.4388 mg/g fresh wt. (Barrett, 1981) and *Ligula intestinalis*, 1536 ± 251 nmol/ g fresh wt. (McManus and Sterry, 1982). In *I. hypselobagri* lactate is found to increase during *in vitro* starvation where oxygen consumption of the fluke is decreased (Nizami and Siddiqi, 1975). Tielens *et al*. (1989) reported that glycogen is degraded to lactate and is the main end product of carbohydrate metabolism in different species and strains of schistosomes. In *S. japonicum* lactate is produce in *in vitro* both aerobically and anaerobically which suggests active partial reversed TCA cycle (Kawanaka *et al*., 1989). So, the lactate is produced in this fluke either aerobically or anaerobically due to the metabolism of glucose or glycogen. The pyruvate/ lactate ratio (Table 3) also shows that the pyruvate is reduced to lactate and lactate is the main end product of carbohydrate metabolism in this fluke.

The oxaloacetate is TCA cycle intermediate and metabolites in parasitic helminthes. In parasitic helminths, CO_2 fixation pathway can take place into PEP via PEPCK which results in the formation of oxaloacetate. The oxaloacetate is then reduced to malate by cytoplasmic MDH. The oxaloacetate content of the fresh control fluke varies between 4.95 to 5.62 μmol/mg protein whereas the average value as 5.27 ± 0.23 μmol/mg protein is comparable to that in *E. granulosus* and *E. multiloculoris* as 8 and 10 nmol/g fresh wt. respectively, *Ligula intestinalis* as 80 nmol/g fresh wt. (McManus and Sterry, 1982) and *Schistocephalus solidus* as 50 nmol/g fresh wt. (Beis and Barrett, 1979).

In the present work, the oxaloacetate content is found to increase significantly, may be due to the fact that during *in vitro* starvation period PEPCK also increased to produce OAA from PEP (Bera and Manna, 2007) or may be that widely distributed malic enzyme in parasitic helminth carboxylate the pyruvate to give oxaloacetate (Barrett, 1981).

The malate, another TCA cycle intermediate in helminths and in some cases is the end product of carbohydrate metabolic pathways (Barrett, 1981). The malate is either oxidized to pyruvate via malic enzyme or to fumarate via fumarase, which is reduced to succinate via a fumarate reductase complex. During carbon dioxide fixation PEP proceed to OAA which is reduced to malate. Malate also acts as an inhibitor of PK reported in *Moniezia expansa* and is also a modulator in *Dicrocoelium dendriticum* and *Haemonchus contortus*, where malate have a dual role in activating PEPCK and inhibiting PK (Smyth and Halton, 1983).

The malate content of the fresh control fluke varies between 2.49 to 3.10 μmol/mg proteins with an average value of 2.73 ± 0.18 μmol/mg proteins in the present fluke. Malate is reported as end product in *Echinococcus granulosus* and *Echinococcus multilocularis*, 786 nmol/g fresh wt. and 846 nmol/g wt. (McManus and Symth, 1982) respectively, *Schistocephalus solidus*, 134 nmol/g fresh wt. (Beis and Barrett, 1979), and *Ligula inteslinalis*, 80 nmol/g fresh wt. (McManus and Sterry, 1982; Barrett, 1981). In the present work, the malate content increased in *in vitro* starved *I. hypselobagri* as OAA is reduced to malate by a cytoplasmic malate dehydrogenase and malate show lower values than OAA may be subjected to a redox dismutation.

Thus, in the present investigation the intense activity of dehydrogenases of TCA cycle provides circumstantial evidence for the presence of successful operation of TCA cycle and may therefore play an important role in the energy metabolism *in vivo* as well as *in vitro* starvation. The ratio of oxaloacetate/malate is greater than 1 and oxaloacetate is reduced to malate during *in vitro* starvation depicts that reverse TCA cycle is also operative in this fluke. Among the metabolites pyruvate content is greater and pyruvate/lactate ratio is also greater than 1 and pyruvate is reduced to lactate during *in vitro* starvation. The lactate is produced from pyruvate as end product and the fluke is homolactate farmenters.

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