Effect of Cold-CO₂ Anesthesia on Postmortem Levels of ATP-Related Compounds, pH, and Glycogen in Carp Muscle

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(Received July 16, 1993)

Postmortem changes in the levels of ATP-related compounds were investigated in the muscle of carp which were kept in a container for 10 h at 14°C and PCO₂=80 mmHg (cold-CO₂ group), at 14°C under bubbling pure O₂ gas (14°C-O₂ group), or at 23°C under bubbling pure O₂ gas (23°C-O₂ group), carp which were allowed to recover from cold-CO₂ anesthesia before death (CO₂-recovery group), and carp netted from a rearing tank and decapitated immediately (control group). The changes in glycogen and pH were also measured in the muscle of cold-CO₂, CO₂-recovery, and control groups. The initial ATP, pH, and glycogen levels were low, while the IMP level was high in the cold-CO₂ group compared with that in the control group. The carp anesthetized with cold-CO₂ seemed to be in a different metabolic state. The ATP degradation rate was rapid at an early stage of storage, while the freshness index (K value) was high in the cold-CO₂ group during storage compared with those in the control group. In the 14°C-O₂ and 23°C-O₂ groups, the postmortem levels of ATP-related compounds showed little difference from those in the control group. In the CO₂-recovery group, the postmortem levels of ATP-related compounds, pH, glycogen, and K value also showed little difference from those in the control group. The meat quality of the carp treated with cold-CO₂ anesthesia was unaffected when the carp was allowed to recover from anesthesia.

The anesthetic level of CO₂ is well-known for reducing the oxygen affinity and oxygen capacity of the blood, while also decreasing the blood^{1,2)} and tissue pH.³⁻⁶⁾ The lowering of the tissue pH is anticipated to reduce and/or disturb the tissue activity. In fact, a reduction of brain activity owing to a lowering of brain pH has been reported in mammals by several researchers.³⁻⁶⁾ A reduction of metabolism by a decrease in intracellular pH has also been reported in mammals,^{7,8)} lungfish,⁹⁾ land snail,¹⁰⁾ sea-urchin eggs,¹¹⁾ and brine shrimp embryos.^{12,13)} Moreover, Tappon¹⁴⁾ has observed that muscle PCr levels were virtually unaffected under conditions of 15% CO₂ exposure. Jackey and Shafer¹⁵⁾ have also reported that hypercapnia (15% CO₂, 12% O₂, balance N₂) was characterized by a conservation of high-energy phosphates. On the other hand, Itazawa and Takeda¹⁾ have reported an increase in oxygen debt in carp anesthetized with mixed bubbling of CO₂ and O₂ gas.

We have previously demonstrated the efficacy of cold-CO₂ anesthesia in longterm anesthesia of

adult carp in connection with the transportation of live fish.^{16,17)} The carp anesthetized with cold-CO₂ were safely kept in a favorable anesthetic state for 10 h. However, the metabolic state of the anesthetized carp is unclear. It is interesting to determine whether or not the anesthesia changes, reduces, and/or disturbs the energy state in the carp.

Stress including starvation and fatigue before death is known to change pre-rigor and/or rigor-mortis periods, and to cause postmortem biochemical changes in many fish species.¹⁸⁻²¹⁾ Similar findings have also been reported in the muscle of chicken treated with anesthetics.²²⁾ After the fish is killed, there is usually a time lag before the fish meat is consumed. Thus, it is important to determine the postmortem changes in the meat quality of the anesthetized carp.

In this study, postmortem changes in the contents of ATP-related compounds were examined in the muscle of carp of five treatment groups, cold-CO₂, 14°C-O₂, 23°C-O₂, CO₂-recovery, and control groups. The postmortem pH and glycogen

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The following abbreviations are used: HxR, inosine; Hx, hypoxanthine; PCr, creatine phosphate; Pi, inorganic phosphate.

levels were also examined in the muscle of cold- CO_2 , CO_2 -recovery, and control groups. The differences in physiological state between anesthetized and unanesthetized carp were discussed. The postmortem changes in freshness of carp muscle were also discussed in relation to meat quality.

Materials and Methods

The experiments were carried out on 50 carp *Cyprinus carpio*, weighing about 500 g (487 ± 39 g, mean $\times$ S.D.) each. Each group of 10 carp was reared in a 1000 l tank placed indoors, and acclimatized to $23 \pm 1^\circ\text{C}$ under a 14 h-light and 10 h-dark cycle for at least two months. They were fed a daily commercial diet, but were fasted one day prior to the experiment. In the case of cold- CO_2 treatment, ten carp were anesthetized at 4°C for 30 min and then placed in an experimental container maintained in advance at 14°C and $\text{PCO}_2 = 80$ mmHg by bubbling 11% CO_2 and 89% O_2 gas at 2.0 l/min for the following 9.5 h in the closed system as reported previously.^{16,17} Immediately after the total of 10-h cold- CO_2 anesthesia, they were decapitated (cold- CO_2 group). In the case of the 23°C - O_2 treatment, ten carp were anesthetized at 4°C for 30 min, and then placed in the container at 23°C for the following 9.5 h, with bubbling pure O_2 gas (2.0 l/min). Immediately after the treatment, they were decapitated (23°C - O_2 group). In the case of the 14°C - O_2 treatment, ten carp were anesthetized at 4°C for 30 min and then placed in the container at 14°C for the following 9.5 h, with bubbling pure O_2 gas (2.0 l/min). Immediately after the treatment, they were decapitated (14°C - O_2 group). In the case of the CO_2 -recovery treatment, ten carp were cold- CO_2 anesthetized for 10 h as described above and then returned to the rearing tank kept at 23°C for recovery. On the day after the 10-h cold- CO_2 treatment, they were gently netted and decapitated immediately (CO_2 -recovery group). As a control, ten carp were gently netted from the rearing tank and decapitated immediately (control group). White muscle was dissected from the dorsal part of the carp and held in a glass bottle with a ground stopper ($\phi 10 \times 10$ cm). The storage temperature was kept at 23°C . At each sampling, approximately 0.5 g of muscle from the anterior end of the muscle was cut and discarded. Subsequently, from the remaining muscle piece, a 1 g-portion of muscle was newly cut and was submitted for

extraction and determination of ATP-related compounds and glycogen.

Extraction and Determination of ATP-related Compounds

The extraction and determination of ATP-related compounds by HPLC was carried out in the same manner as reported previously.²³⁾

Calculation of K Value and Adenylate Energy Charge

The K value as a freshness index²⁴⁾ and the adenylate energy charge, which was proposed as a metabolic regulatory parameter by Atkinson,²⁵⁾ were calculated from the contents of ATP-related compounds as reported previously.²³⁾

Determination of Glycogen

After 0.5 g of muscle had been extracted in 5 ml of heated 30% potassium hydroxide, 5 ml of absolute ethanol was added to the solution and glycogen was precipitated. The obtained glycogen was hydrolyzed with 1 M sulfuric acid and the glucose formed was determined by a combination of mutarotase and glucose oxidase (Glucose C-Test, Wako).

Measurement of pH

Muscle pH was measured using a pH meter (TOA IM-40S), and a needle electrode (GST-5314C) was directly inserted into the muscle tissue.

Results and Discussion

Physiological State of Carp Muscle at the Time of Death

Table 1 shows the contents of ATP and IMP and energy charge values in the muscle of all five experimental groups at the time of death. The ATP content of the control group, about $7.3 \mu\text{mol/g}$, indicates that the carp was killed immediately. In the control group, the IMP content was low while the energy charge value was high. In the cold- CO_2 group, the ATP content was very low while the IMP content was very high compared with those in the control group. There were statistically significant differences in these values between the two groups ($p < 0.01$, Student's t-test). The energy charge value was also slightly but significantly reduced by the anesthesia ($p < 0.05$). The changes in these values in the cold- CO_2 group seemed to be caused mainly by the anesthetic

Table 1. Initial values of ATP and IMP content ($\mu\text{mol/g}$) and energy charge in each carp group.

Group	ATP	IMP	Energy charge
Control	7.3 ± 1.0	0.3 ± 0.2	0.93 ± 0.03
Cold-CO ₂	$4.5 \pm 1.5^*$	$1.8 \pm 1.0^*$	$0.88 \pm 0.04^*$
23°C-O ₂	6.6 ± 0.9	0.2 ± 0.3	0.94 ± 0.03
14°C-O ₂	7.0 ± 0.6	0.1 ± 0.1	0.94 ± 0.02
CO ₂ -recovery	6.7 ± 1.0	0.2 ± 0.2	0.94 ± 0.07

* Statistically significant at 5% level or better compared with control group. Values are expressed as mean $\pm$ S.D., $n=10$.

effect of CO₂, but not by cold temperature at 14°C, since the 14°C-O₂ group showed little difference in these values from the control group (Table 1). There was also little difference in ATP and IMP contents and in energy charge values between the control and 23°C-O₂ groups. An increase in erythrocyte ATP level has been reported by Timms and Mengel²⁶⁾ in rats exposed to hyperoxic conditions. However, the muscle ATP levels in the two O₂ groups were at the control level. The exact reason for this disagreement is not clear, but differences between species and/or tissues are factors to be considered.

The decrease in the energy charge and ATP levels in the cold-CO₂ group at the time of death indicated that the anesthetic level of CO₂ changed the energy metabolism in the carp muscle. On the other hand, Jacey and Schaefer²⁷⁾ have reported that arterial ATP, ADP, and AMP contents and the energy charge value were virtually unchanged in guinea pigs exposed to 15% CO₂ in 21% O₂ and balanced N₂. Similar findings were made by Granholm and Siesjö²⁸⁾ in the brains of cats acutely exposed to various concentrations of CO₂ up to a level which produced arterial Pco₂ values of 100 mmHg. They observed no changes in tissue ATP or ADP levels or in PCr content. However, these findings were on mammals and the dose of CO₂ was below the anesthetic level. The decrease in ATP content and energy charge value, and the increase in IMP content in the cold-CO₂ group may partially be explained by the finding of Itazawa and Takeda¹⁾ that there was an increase of oxygen debt in carp during CO₂-anesthesia. The anesthetic level of CO₂ is well-known for reducing the oxygen affinity and oxygen capacity of blood. The carp in the cold-CO₂ group seemed to be in a substantial hypoxic condition despite the high level of partial pressure of O₂ in water (Po₂=650 mmHg).^{16,17)} In a preliminary experiment,²⁹⁾ we examined the PCr content in the carp anesthetized with CO₂ and reported that

Table 2. Initial values of pH and glycogen content (mg/g) in the carp muscle.

Group	pH	Glycogen
Control	6.9 ± 0.1	10.5 ± 1.1
Cold-CO ₂	$6.6 \pm 0.1^*$	$7.1 \pm 0.9^*$
CO ₂ -recovery	6.9 ± 0.1	9.4 ± 1.4

* Statistically significant at 5% level or better compared with control group. Values are expressed as mean $\pm$ S.D., $n=10$.

the PCr content was considerably reduced by a high level of CO₂ compared with that of unstressed fish. Van den Thillart *et al.*³⁰⁾ have reported that anoxia causes a rapid decrease in PCr in carp, and that after the PCr store had been exhausted by more than 85%, the ATP level fell, whereas IMP accumulated markedly. These findings support the above assumption. However, when the carp recovered from cold-CO₂ anesthesia, their energy state returned to the control levels (Table 1).

Table 2 shows the pH and glycogen contents in the muscle of control, cold-CO₂, and CO₂-recovery groups. Significant differences were found in these values between the control and cold-CO₂ groups. An aerobic metabolism in fish is usually based on glycolysis,³¹⁾ and the rate-limiting steps of glycolysis are those catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase. Normally, the glycolytic rate is mainly determined by the rates of hexokinase and phosphofructokinase while the pyruvate kinase keeps its step. Since phosphofructokinase activity has been shown to be pH-dependent,³²⁾ its activity should be affected by acute hypercapnia with its profound acidosis and accompanying inhibition of glycolysis. Concerning this phenomenon, Jacey and Schaefer²⁷⁾ have reported that the blood phosphofructokinase activity in guinea pigs was inhibited by 55% when the blood pH was lowered by 0.4 as a result of hypercapnia (15% CO₂, 22% O₂, and N₂ balance). In this study, the glycogen still remained at 6.6 mg/g in the muscle of the cold-CO₂ group. The muscle pH in the cold-CO₂ group was lower by 0.3 than that in the control group. The carp in the cold-CO₂ group seemed to lack a supply of high-energy phosphates by glycolysis. However, the changes in pH and glycogen content in carp muscle by cold-CO₂ anesthesia were reversible. There were no significant differences in these values between the control and CO₂-recovery groups (Table 2).

Postmortem Changes of Carp Muscle

Figure 1a shows postmortem changes in the

Fig. 1. Changes in average content of ATP-related compounds in carp muscle ($n=10$) during storage at 23°C.

a, Control group; b, cold- CO_2 group; c, 23°C- O_2 group; d, 14°C- O_2 group; and e, CO_2 -recovery group.

○-○, ATP; △-△, ADP; □-□, AMP; ●-●, IMP; ▲-▲, HxR; ■-■, Hx; and ■-■, Total.

content of ATP-related compounds in the control group. The ATP content gradually decreased during storage. The ATP content observed at 1 h and 3 h postmortem was 0.4 and 1.2 $\mu\text{mol/g}$ lower, respectively, than that at 0 h. The contents of IMP and HxR seemed to be directly related to the extent of ATP breakdown. IMP and HxR thus showed a gradual increase during storage. ADP, AMP, and Hx were at low levels of less than 0.8 $\mu\text{mol/g}$.

The changes in content of ATP-related compounds in the cold- CO_2 group were markedly different from those in the control group shown in Fig. 1b. The ATP content at 1 h and 3 h postmortem was 0.7 and 1.5 $\mu\text{mol/g}$ lower, respectively, than that at 0 h. There were significant differences ($p<0.05$) in the decrease rate of ATP contents observed during 1 h and 3 h post-

mortem between the control and cold- CO_2 groups. The faster ATP-breakdown in the cold- CO_2 group may be partially explained by the findings that muscle PCr content was low in the CO_2 group compared with that in the control group,²⁹⁾ since the PCr acts as an energy reservoir for maintaining ATP levels.³⁰⁾ Nakayama *et al.*²¹⁾ reported that the muscle of carp which struggles severely prior to death is characterized by a lower ATP level at the time of death and is followed by a more rapid decrease in the ATP level compared with those of unstressed carp. Their findings coincide with our findings on ATP level and its postmortem breakdown. IMP gradually increased from 1.8 to 3.2 $\mu\text{mol/g}$ during storage, and then decreased. HxR increased gradually during storage, although the corresponding content at each storage time was higher in the cold- CO_2 group than that in the

control group.

The changes in the contents of ATP-related compounds in the 23°C-O₂ and 14°C-O₂ groups were nearly the same as those observed in the control group (Figs. 1c and 1d). The ATP content was high and the breakdown proceeded gradually. IMP and HxR increased gradually just as in the control group (Fig. 1a). These findings indicate that the carp in the container at 23°C or 14°C under bubbling pure O₂ gas were kept in an unstressed state. The contents and changes of ATP-related compounds in the CO₂-recovery group were the same as those observed in the control group (Fig. 1e).

A rapid decrease in glycogen content was observed during 1 h postmortem in the control group (Fig. 2). Thereafter, it decreased gradually. On the other hand, the decrease in the glycogen content in the cold-CO₂ group during 1 h postmortem was much slower than that in the control group. The rate of the glycolysis seemed to be faster in the control group than in the cold-CO₂ group during 1 h postmortem. This may be one of the reasons for the difference in ATP degradation rate between the control and cold-CO₂ groups (Figs. 1a and 1b). From the faster rate of glycogen breakdown in the control group than in the cold-CO₂ group, we expected the rate of pH decline to be faster in the control group than in the cold-CO₂ group during 1 h postmortem. However, the rate of pH decline hardly showed any statistical difference between the control and cold-CO₂ groups during 1 h postmortem (Fig. 3). The exact reason for this phenomenon was not clear, but the buffering capacity of the muscle may be different in the two groups. The changes in pH and glycogen content in the CO₂-recovery group showed little difference compared with the control group (Figs. 2 and 3). These findings on pH and glycogen along with the results on ATP-related compounds indicate that the carp in the CO₂-recovery group had recovered their energy state and muscle metabolism to the levels of the control group. There would be little difference in the onset of rigor mortis and its development between the control and the CO₂-recovery groups, since there was little difference in the levels or changes in the ATP content and pH between the two groups (Figs. 1a, 1e, and 3).³³⁾

Figure 4 shows the changes in K value of carp muscle during storage. The specimens of both the control and the cold-CO₂ groups hept for 24 h emitted an unpleasant odor. This indicates that the decrease in glycogen observed in the muscle

Fig. 2. Changes in average glycogen content of carp muscle ($n=10$) during storage. ▲-▲, Control group; ●-●, cold-CO₂ group; ○-○, and CO₂-recovery group.

Fig. 3. Changes in average pH value of carp muscle ($n=10$) during storage. ▲-▲, Control group; ●-●, cold-CO₂ group; ○-○, and CO₂-recovery group.

Fig. 4. Changes in average K value of carp muscle ($n=10$) during storage. ▲-▲, Control group; ○-○, cold-CO₂ group; □-□, 23°C-O₂ group; ■-■, 14°C-O₂ group; and ●-●, CO₂-recovery group.

from 8 h to 24 h postmortem resulted from bacterial action. There were statistically significant differences ($p<0.01$) in the corresponding values between the control and cold-CO₂ groups. However, the levels and changes in the K value in the 14°C-O₂, 23°C-O₂, and CO₂-recovery groups showed little difference from the control group. As a result, the change in freshness was the same in the carp that had recovered from anesthesia as in the control group. Additionally, the levels and

changes in the biochemical parameters in the CO₂-recovery group indicate that the onset of rigor mortis and its development in carp recovering from anesthesia would be the same as those in the control group. Therefore, the meat quality of carp anesthetized with cold-CO₂ anesthesia would be unaffected, if the carp is allowed to recover from the anesthesia before death.

Acknowledgments

We wish to thank Professor M. Sakaguchi of the Faculty of Agriculture, Kyoto University, for his valuable comments on this manuscript. We also wish to thank Miss. M. Tanioka for skilled technical assistance.

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