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Original article

HISTOLOGICAL DISCRIMINATION OF FRESH FROM FROZEN/THAWED CARP (*CYPRINUS CARPIO*)

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Summary

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The aim of the study was to perform histological differentiation of dorsal and ventral musculature of fresh and frozen/thawed carps (*Cyprinus carpio*). Histological findings of muscle fibres (*Myofibra striata*) of fresh carps did not show any changes. Single freezing at –10 ºC resulted in extracellular gaps in the central part of some of fibres. After single freezing at –18 ºC, muscle fibres with cell destruction in the central part were identified while the periphery remained intact. Completely destructured and deformed areas of muscle fibres were demonstrated after single freezing at –27 ºC. Double freezing at –10 ºC resulted in shrinkage, extracellular gaps and fragmentation of fibres, while muscle fibres double-frozen at –18 ºC were impaired, degraded and with visible defects. The histological findings in carp muscle, double-frozen at –27 ºC comprised severely deformed muscle fibres with increased extracellular gaps from degraded muscle tissue. On the basis of findings, it could be concluded that double freezing of carps was not an appropriate method of storage and shelf-life extension.

Key words: carps, dorsal muscle, freezing, histological changes, skeletal muscle fibres, ventral muscle

INTRODUCTION

Fresh fish is defined in *Codex Alimentarius* as fish that has received no preserving treatment other than chilling (FAO/WHO, 2012). Shelf-life of fresh fish is 3 to 5 days depending on the time of chilling and storage (Ježek & Buchtová, 2012). Gokoglu & Yerlikaya (2015) determined the maximum shelf-life of fresh fish to 10–15 days at storage temperature between 0 and 2 °C.

Freezing is a commonly used method to prolong the shelf-life of fish as low temperatures create an unfavourable environment for bacterial flora development (Gandotra *et al.*, 2012). Freezing could not prevent the deterioration of fish quality. At temperature below –10 ºC, the bacteria do not develop, yet the chemical and physical processes still exert their adverse impact (FAO/WHO, 2012). Tolstorebrov

et al. (2016) also reported fish storage between –18 ºC and –30 ºC in order to increase the shelf-life of frozen fish.

Fish subjected to a freezing process sufficient to reduce the temperature of the whole product to a level low enough to preserve its inherent quality and maintained at this low temperature during transportation, storage and distribution up to the time of final sale is referred to as frozen (Anonymous, 2012).

Matsumoto (1980) affirmed that freezing is one of the most important methods for fish preservation, as it delays autolysis and putrefaction. During frozen storage, deterioration of the quality of fish could occur due to mechanical damage, denaturation of proteins and osmotic water removal (Benjakul & Bauer, 2001). The freezing rate and storage conditions, in the opinion of Shahidi & Botta (2012), had an effect on muscle structure. During frozen storage, the distance between muscle fibres increased, so the authors concluded that storage conditions had a greater influence than freezing rate. Gambuteanu *et al.* (2013) demonstrated severe damage of muscle fibres by formation of ice crystals among the fibres, resulting in their receding. Ice crystals formed inside the fibres are less deleterious for their integrity Ngapo *et al.* (1999) reported that during freezing of fresh meat, the intracellular fluid was the first to freeze. The frozen meat structure is evaluated by the size of formed gaps, corresponding to the size of ice crystals.

Earlier investigations in carps (*Cyprinus carpio*) showed the distribution of white and red muscle fibres (Dong *et al.*, 2017). The white muscle fibres are the principal and predominant group in carps. The size of red muscle fibres is smaller. They could be detected in the region of the lateral line as a thin subcutaneous layer (Mokhtar, 2017).

EC Regulations 1169/2011 and 1379/2013 (Anonymous, 2011; 2013) stipulate that the mandatory information about the state of food and its specific treatment should be correct and comprehensively described on the product label. If the fish has undergone previous freezing before sales and is sold after thawing, it should be designated at the label as defrosted.

The distinction of fresh from frozen/thawed fish only on the basis of organoleptic parameters is difficult. The marketing of frozen/thawed fish as fresh should be considered as fraud (Tinacci *et al.*, 2018). For this reason, various methods for detection of such fraudulent practices in fish sold in retail stores have been developed. A histology-based approach for distinction of fresh fish dorsal muscle from that of frozen/thawed was successfully used for the first time by Pavlov *et al.* (2008) in carps (*Cyprinus carpio*).

The aim of the study was to evaluate histological changes in dorsal and ventral muscles for discrimination of fresh common carps (*Cyprinus carpio*) from once or twice frozen ones at three temperature regimens (–10 ºC, –18 ºC and –27 ºC).

MATERIALS AND METHODS

Experimental design

Twenty-one fresh carps *(Cyprinus carpio)* one year of age, weighing 500 g on the average were purchased from the retail market. Seven fish were used for investigation of histostructure of dorsal and ventral muscles in fresh fish, and the other 14 fish were frozen once or twice in conventional freezers at –10 ºC, –18 ºC and –27 ºC for 15 days. After that, frozen fish were thawed at 4 ºC for 24 h.

Sample collection and processing

After removal of the skin, samples with size of 1×1×0.5 cm were collected from each fish. The histological analysis was performed on 70 samples. The specimens were fixed immediately in 10% buffered formalin (pH 7.4), washed in running water, dehydrated in ascending alcohol series, cleared twice in xylene and embedded in paraffin. Cross sections of 5 µm were produced by means of YD-335A rotary microtome (China) and stained with haematoxylin/eosin. Histological evaluation was done on N-200 M biological microscope (Hangzhou Sumer Instrument Co., Ltd, China). Findings were photodocumented with digital camera Optikam B5 (Optika Srl, Ponteranica, Italy) and Optika Proview software (Optika Srl, Ponteranica, Italy).

RESULTS

Histological evaluation of skeletal muscle of fresh carp (Cyprinus carpio)

Histological findings in fresh fish muscle fibres in both anatomical regions did not exhibit any changes. The cross section of fresh muscle showed evenly arranged regularly formed muscle fibres and bundles and specific polygonal shape of muscle fibres. The shape was intact with pre-

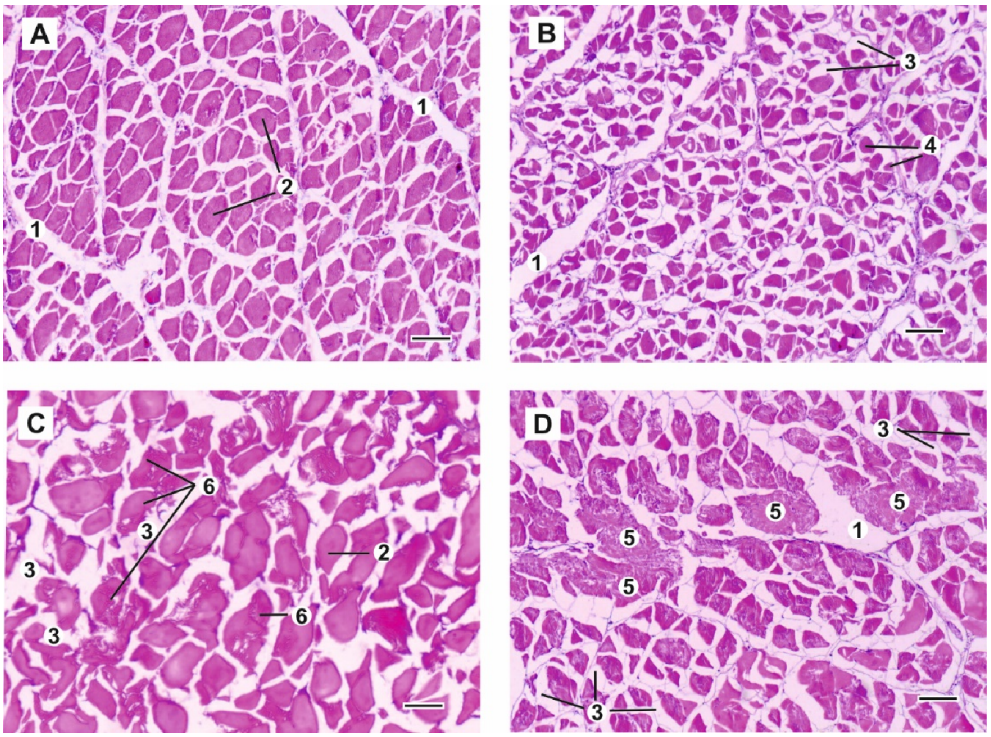

Fig. 1. Dorsal muscle of carp (*Cyprinus carpio*). A. fresh carp; H&E, bar=100 µm; B. once-frozen carp at –10 ºC, H&E, bar=100 µm; C. once-frozen carp at –18 ºC, H&E, bar=50 µm; D. once-frozen carp at –27 ºC, H&E, bar=100 µm. 1: perimysium internum; 2: skeletal muscle fibres with normal histostructure, 3: increased extracellular gaps; 4: shrunken skeletal muscle fibres, 5: fully destructured skeletal muscle fibres 6: skeletal muscle fibres with impaired central part and preserved peripheral part.

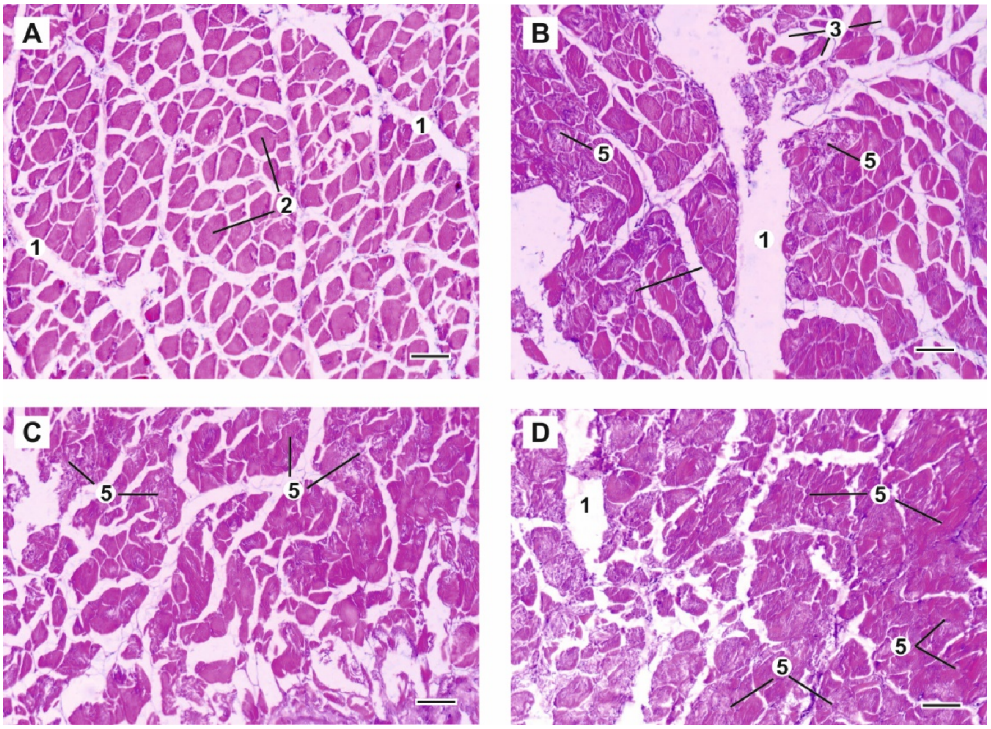

Fig. 2. Dorsal muscle of carp (*Cyprinus carpio*). A. fresh carp; H&E, bar=100 µm; B. double-frozen carp at –10 ºC, H&E, bar=100 µm; C. double-frozen carp at –18 ºC, H&E, bar=50 µm; D. double-frozen carp at –27 ºC, H&E, bar=100 µm. 1: perimysium internum; 2: skeletal muscle fibres with normal histostructure, 3: increased extracellular gaps; 5: fully destructured skeletal muscle fibres.

served periphery of fibres. The connective tissue (endomysium) surrounding each muscle fibre was intact and present in all fibres. Muscle bundles formed by muscle fibres' groups were separated one from the other by a variety of connective tissue termed perimysium internum. This connective tissue showed neither damage nor disruptions. No damaged and disrupted muscle fibres were observed in studied dorsal and ventral muscles. Light microscopy of fresh carp skeletal muscle did not identify any microstructural changes (Fig. 1A, 2A, 3A and 4A).

Histological evaluation of skeletal muscle of once-frozen/thawed carp (Cyprinus carpio)

The single freezing at–10 ºC resulted in structural changes in muscle fibres manifested as gaps in the central part of some of fibres. In others, gap-induced fragmentation of rhabdomyocytes was observed. muscle fibres were shrunken, but with distinct borders. Damaged skeletal muscle fibres were present in some observational fields. The perimysium internum was intact. Interstitial spaces tended to be enlarged. At –10 °, several muscle bundles were preserved with intact skeletal muscle fibre structure. The histological findings

of dorsal muscle frozen at –10 ºC were more similar to control samples (Fig. 1B and 3B).

After single freezing at –18 ºC, perimysium internum of muscle bundles was intact. The structure of few muscle fibres was impaired. A more common finding was muscle fibres in which destruction has affected their central part while their periphery was undamaged. The borders and specific polygonal shape of rhabdomyocytes were easily discernible due to the preserved integrity of the endomysium. Observational fields with indistinct borders of muscle fibres were rarely found out. Muscle fibres were shrunken by extracellular ice crystals. At –18 ºC, the integrity of skeletal muscle fibres was largely intact (Fig. 1C, 3C).

The freezing at –27 ºC induced more severe lesions compared to previously described temperature regiments. In most areas, shrinkage of muscle fibres was more intensive by reason of large extracellular ice crystals. Completely destructured and deformed muscle tissue areas in which fibre borders could not be identified, were also observed (Fig. 1D, 3D).

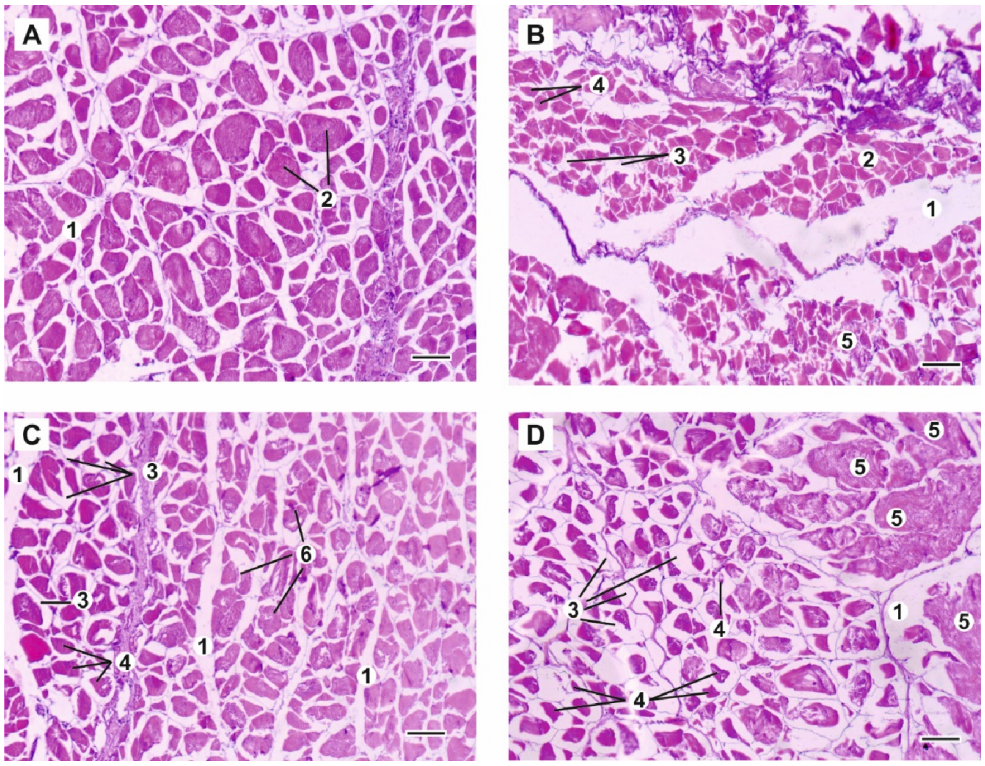

Fig. 3. Ventral muscle of carp (*Cyprinus carpio*). A. fresh carp; H&E, bar=100 µm; B. once-frozen carp at –10 ºC, H&E, bar=100 µm; C. once-frozen carp at –18 ºC, H&E, bar=100 µm; D. once-frozen carp at –27 ºC, H&E, bar=100 µm. 1: perimysium internum; 2: skeletal muscle fibres with normal histostructure, 3: increased extracellular gaps; 4: shrunken skeletal muscle fibres, 5: fully destructured skeletal muscle fibres 6: skeletal muscle fibres with impaired central part and preserved peripheral part.

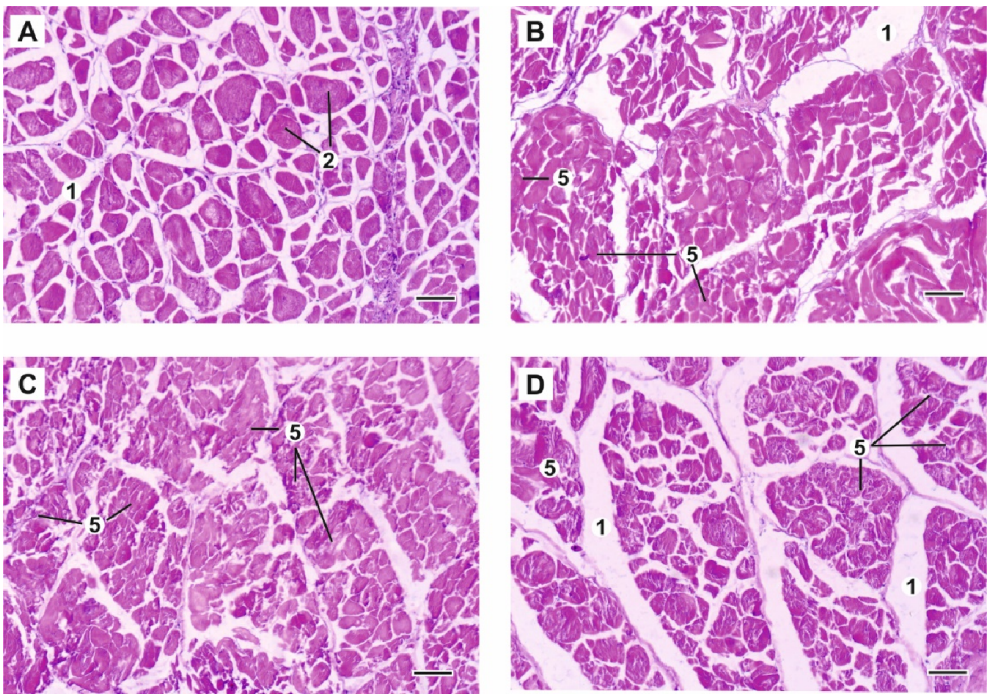

Fig. 4. Ventral muscle of carp (*Cyprinus carpio*). A. fresh carp; H&E, bar=100 µm; B. double-frozen carp at –10 ºC, H&E, bar=100 µm; C. double-frozen carp at –18 ºC, H&E, bar=50 µm; D. double-frozen carp at –27 ºC, H&E, bar=100 µm. 1: perimysium internum; 2: skeletal muscle fibres with normal histostructure, 5: fully destructured skeletal muscle fibres.

Histological evaluation of skeletal muscle of double-frozen/thawed carp (Cyprinus carpio)

Double freezing at –10 ºC induced shrinkage of muscle fibres. In some of them, central gaps were observed, while others were fragmented. Damage was influenced more strongly by shrinkage of fibres – specific large gaps among muscle fibres and indistinctly outlined muscle fibres and bundles. Skeletal muscle was largely destructured due to degraded sarcolemma and skeletal muscular endomysium (Fig. 2B, 4B).

After double freezing at –18 ºC, the extent of muscle fibres' shrinkage was greater. The damage of rhabdomyocytes was extensive. Muscle fibres were impaired, degraded, with visible defects. Fibre borders were indistinct due to their shrinkage; borders of muscle bundles were also unclear (Fig. 2C, 4C).

The histological findings of muscle fibres double-frozen at –27 ºC demonstrated severe deformation due to ice crystals sublimation. Increased extracellular spaces due to muscle tissue degradation were observed. Muscle tissue was substantially damaged, neither muscle fibres nor bundles could be identified (Fig. 2D, 4D).

DISCUSSION

Tinacci *et al.* (2018) reported white muscle fibres as structural elements of dorsal muscles. Dong *et al.* (2017) outlined that

ventral muscles also comprised white muscle fibres. In our study, two anatomical regions were investigated. The results confirmed the position of white muscle fibres in studied parts of carp body.

The histological alterations under various temperature regiments were followed out to establish optimum conditions for frozen storage of carp (*Cyprinus carpio*). Observed changes were morphological. In this study, slow freezing in a conventional freezer was used, and tissue destruction was the main parameter of muscle organisation. Popelka *et al.* (2014) found out deformations in muscle fibres of frozen and total degradation of muscle fibres of double-frozen trout (*Oncorhynchus mykiss*) at –35 ºC. Tinacci *et al.* (2018) described partial or total muscle destruction in European hake (*Merluccius merluccius*) after frozen storage in a conventional freezer at –20 ºC for 15 days or in freezing tunnel at –35 ºC to –45 ºC for 1–3 h. Chevalier *et al.* (2000) reported that slow freezing resulted in formation of large ice crystals. This was confirmed by our study as well, where large ice crystals formed in the extracellular space resulted to muscle fibres' shrinkage and dehydration (reduced cross section). In this connection, Alizadeh *et al.* (2007) affirmed that traditional freezing approaches led to greater morphological alterations of muscle tissue, confirmed also by out findings. Tinacci *et al.* (2018) described also the presence of interstitial protein matter and specifically shaped vacuoles. Our results agreed with those of Popelka *et al.* (2014) yet they were dissimilar with data of Tinacci *et al.* (2018). A possible reason was the young age and weight of fish.

CONCLUSION

On the basis of findings, it could be concluded that double freezing of carps was not an appropriate method of storage and shelf-life extension. The structure of double-frozen muscle tissue was damaged at a significant extent, which influenced fish quality. Histological approach is reliable for detection of fraudulent practices related to marketing frozen/thawed fish as fresh.

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