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Effects of fasting and refeeding on intestinal cell proliferation and apoptosis in hammerhead shark (*Sphyrna lewini*)

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PEER REVIEW

Peer reviewer

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Comments

The effects of fasting and refeeding on intestinal cell proliferation and apoptosis in an opportunistic predator, hammerhead shark (*Sphyrna lewini*) of elasmobranch fishes were examined. This is the first study to indicate that the fasting and refeeding alter epithelial cell proliferation and apoptosis in the shark intestine. This paper, therefore, would seem worthy of publication in Journal of Coastal Life Medicine.
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ABSTRACT

Objective: To examine the effects of fasting and refeeding on intestinal cell proliferation and apoptosis in an opportunistic predator, hammerhead shark (*Sphyrna lewini*) of elasmobranch fishes which are among the earliest known extant groups of vertebrates to have the valvular intestine typical for the primitive species.

Methods: Animals were euthanized after 5−10 d of fasting or feeding, or after 10−day fasting and 5−day refeeding. Intestinal apoptosis and cell proliferation were assessed by using oligonucleotide detection assay, terminal deoxynucleotidyl transferase dUTP nick end labeling staining, and immunohistochemistry of proliferating cells nuclear antigen.

Results: Plasma levels of cholesterol and glucose were reduced by fasting. Intestinal apoptosis generally decreased during fasting. Numerous apoptotic cells were observed around the tips of the villi, primarily in the epithelium in the fed sharks, whereas fewer labeled nuclei were detected in the epithelium of fasted sharks. Refeeding returned intestinal apoptosis to the level in the fed sharks. Proliferating cells were observed in the epithelium around the troughs of the villi and greater in number in fed sharks, whereas fewer labeled nuclei were detected in fasted sharks.

Conclusions: The cell turnover is modified in both intestinal epithelia of the shark and the murines by fasting/feeding, but in opposite directions. The difference may reflect the feeding ecology of the elasmobranchs, primitive intermittent feeders.

KEYWORDS
Elasmobranch fishes, Intestine, Apoptosis, Cell proliferation, Fasting, Feeding

1. Introduction

Vertebrates possess multiple physiological and behavioral adaptations to feeding, and feeding/fasting has been shown to influence the physiological performance of the gastrointestinal tract. In the postabsorptive state, the gastrointestinal tract accounts for 20%−25% of the whole−body energy expenditure[1,2], even though these organs represent less than 10% of body weight. The disproportional energy requirement of the gastrointestinal tract is ascribed to the very rapid turnover of enterocytes as well as the continuous synthesis and degradation of mucous glycoproteins, which may serve to buffer amino−acid availability in the postabsorptive period[3,4]. Work has focused on the adaptive response of the murine intestine in maintaining its integrity during food deprivation, and has recently shown that the changes in cell turnover are highly significant[5,6]. However, far less is known of this intestinal response in intermittent feeders; such feeders typically consume large meals at irregular intervals in nature, which

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is accompanied by slow digestive processing. Further, the evolutionary significance of feeding/fasting is not well understood, especially with respect to primitive vertebrates.

Elasmobranch fishes are the predators that reside at or near the top of marine food webs, and mostly the intermittent feeders. Furthermore, they are among the earliest known extant groups of vertebrates to have the valvular intestine typical for the primitive species[7-9]. These make them highly suitable for investigating the above gastric response. For this reason, we evaluated the effects of fasting and refeeding on the intestinal cell proliferation and apoptosis in an intermittent feeder, hammerhead shark (*Sphyrna lewini*).

2. Materials and methods

2.1. Experimental animals

Juvenile scalloped hammerhead sharks (478-832 g) of both sexes were collected by hand-line fishing with baited hooks in Kaneohe Bay, Oahu, Hawaii, USA in August and September[10]. The captured sharks were immediately transferred to the Hawaii Institute of Marine Biology, and were held for at least 1 week prior to experimentation in circular outdoor tanks (3 m diameter) served with flow-through seawater. During this time, the animals fed actively and *ad libitum* on freshly thawed squid at single feeding each day. Sharks were assigned randomly to one of the five treatment groups. Group I fed continuously for 5 d; Group II fasted for 5 d; Group III fed continuously for 10 d; Group IV fasted for 10 d; Group V fasted for 10 d and then re-fed for 5 d.

At the time of sampling, sharks were caught by dip-net and anesthetized by immersion in aerated seawater containing 0.5% (v/v) 2-phenoxyethanol. The blood were then quickly sampled by caudal puncture with a #20 needle attached to a 10-mL syringe and centrifuged at 10 000 *g* for 5 min; the plasma was removed, and stored at -80 °C for later analyses. The sharks were killed by transection of the spinal cord and the body cavity was opened by a long mid-ventral incision. The slices of the anterior portion of the valvular intestine (excluding the duodenum), the posterior end (excluding the colon/rectum) and the remaining portion (middle segments) were dissected, placed into 2-mL microcentrifuge tubes, immediately frozen in liquid nitrogen, and stored at -80 °C for quantification of apoptosis. In addition, after the acclimation period, one tank continued to be fed and another tank was fasted for 10 d, at which time the intestine was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C overnight for histological analyses (n=4). Residual food was found in the stomach of all fed and re-fed groups as well as of those at the time of capture in the Bay, but not in those from the fasted fish. All experiments were carried out in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

2.2. Plasma analyses

Plasma glucose, cholesterol and urea concentrations were determined using the mutarotase-glucose oxidase method using a Wako Glucose C-test, the cholesterol oxidase method using a Wako Cholesterol E-test, and the urease-indophenol method using a Wako Urea NP-test (Wako Pure Chemical Industries, Osaka, Japan), respectively. Plasma osmolality and Cl^- concentration were measured using a vapor pressure osmometer (Wescor 5520; Logan, UT) and a chloride meter (Buchler Instruments Inc., Lenexa, KS).

2.3. Quantification of apoptosis

DNA internucleosomal fragmentation in the intestine was assessed using a cell death detection ELISA kit (Roche, Tokyo, Japan) as described for fishes by us and others[11,12]. This kit uses a quantitative sandwich ELISA that specifically measures the histone region (H1, H2A, H2B, H3, and H4) of mono- and oligonucleosomes that are released during apoptosis but not necrosis[13,14]. After a 10-minute reaction, color development ($A405_{nm}$-$A492_{nm}$) was quantified using an MTP-300 microplate reader (Corona, Ibaragi, Japan). To correct for differences in tissue content per homogenate, protein quantification was carried out by the bicinchoninic acid assay (Thermo Fisher Scientific Pierce biotechnology, Rockford, USA), using bovine serum albumin (BSA) (Sigma, Tokyo, Japan) as standard and the MTP-300 microplate reader.

2.4. In situ 3′-end labeling of DNA (TUNEL assay)

Nuclei of apoptotic cells were detected by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method using an *in situ* cell death detection kit (Roche, Tokyo, Japan) as validated for fishes[12,15,16]. Fixed tissue samples were dehydrated through graded alcohol concentrations and embedded in Paraplast. Sections were cut at 5 μm and attached to 3-aminopropyltriethoxysilane-coated slides. The sections were then treated with 20 μg/mL proteinase K (Roche, Tokyo, Japan) at 20 °C for 30 min, washed in PBS for 15 min, and immersed in 0.3% H_2O_2 in methanol at 20 °C for 30 min to inactivate endogenous peroxidase activity. After washing in PBS, the sections were incubated with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP at 37 °C for 1 h in a humidified chamber. The reaction was terminated by transferring the slides to PBS for 15 min. The sections were then incubated with peroxidase-labeled anti-fluorescein antibody at 37 °C for 30 min and then for 5 min with 3,3′-diaminobenzidine substrate solution (Roche, Tokyo, Japan). Omission of terminal deoxynucleotidyl transferase gave completely negative results.

2.5. Proliferating cell counts

To label proliferating cells in the intestine, we used a mouse monoclonal (clone PC10) antibody (Sigma, Tokyo, Japan) against proliferating cell nuclear antigen (PCNA), as validated previously for fishes[12,15]. The slides were immersed in 0.3% H_2O_2 in methanol at 20 °C for 30 min to inactivate endogenous peroxidase activity. After a wash in PBS, the sections were placed in 5% normal goat serum in PBS at room temperature for 1 h to block non-specific binding. Sections were subsequently incubated at 4 °C overnight with the primary antibody diluted 1:1 600 in a solution containing 0.5% Triton X-100 and 1% BSA in PBS.

Sections were then washed three times in PBS, incubated with peroxidase-labeled goat anti-mouse secondary antibody (Sigma, Tokyo, Japan) diluted 1:70 in PBS containing 0.5% Triton X-100 and 1% BSA at room temperature for 1 h, and then developed for 5 min with 3,3′-diaminobenzidine substrate solution (Roche, Tokyo, Japan). Controls omitting the PCNA primary antibody were performed and yielded no immunoreactivity.

The number of PCNA-positive nuclei per several randomly-selected villi was counted. Around 10 sections per shark were examined, and at least 100 villi were analyzed. Proliferating indices were calculated as the numbers of positive cells per villous for each shark. From the cell counts, the average number of positive cells was computed for each shark group.

2.6. Statistical analyses

Quantitative data are expressed as means±SEM. The effects of nutritional state on the levels of intestinal apoptosis, plasma compositions and the plasma osmolality were tested with analysis of variance (ANOVA) followed by the appropriate post-hoc test for comparison between means. The effects of nutritional states on the intestinal cell proliferation were analyzed using student's *t*-test. The probability for establishing statistical significance was (P<0.05). All statistics were performed using Statview 5.0 (Abacus Concepts).

3. Results

Table 1 shows the effects of nutritional state on various plasma components in hammerhead shark. Plasma glucose concentration was lower at 10 d after fasting than that in the fed group. Plasma cholesterol concentration exhibited a more marked reduction after fasting and was significant after 10 d. Cholesterol levels in the re-fed group remained significantly reduced compared with levels observed in the fed fish. Levels of plasma urea, Cl^- and osmolality displayed inconsistent/minor fluctuations.

Figure 1 shows the effects on the intestinal apoptosis. Through the three portions of the valvular intestine, initial apoptotic levels were maintained in the fed sharks. The apoptotic levels in the anterior portion of the intestine tended to decrease in fasted sharks, but no significant differences were observed between fed and fasted sharks because of the large variation (Figure 1A). The similar changes were seen in the middle portion while the highest levels of apoptosis were observed in re-fed shark (Figure 1B). Fasting for 10 d significantly reduced the apoptotic levels in the posterior portion and these effects were reversed by refeeding (Figure 1C). When the data from three portions of valvular intestine were combined (Figure 1D), fasting significantly reduced the intestinal apoptotic levels while refeeding restored apoptosis to levels observed in fed sharks.

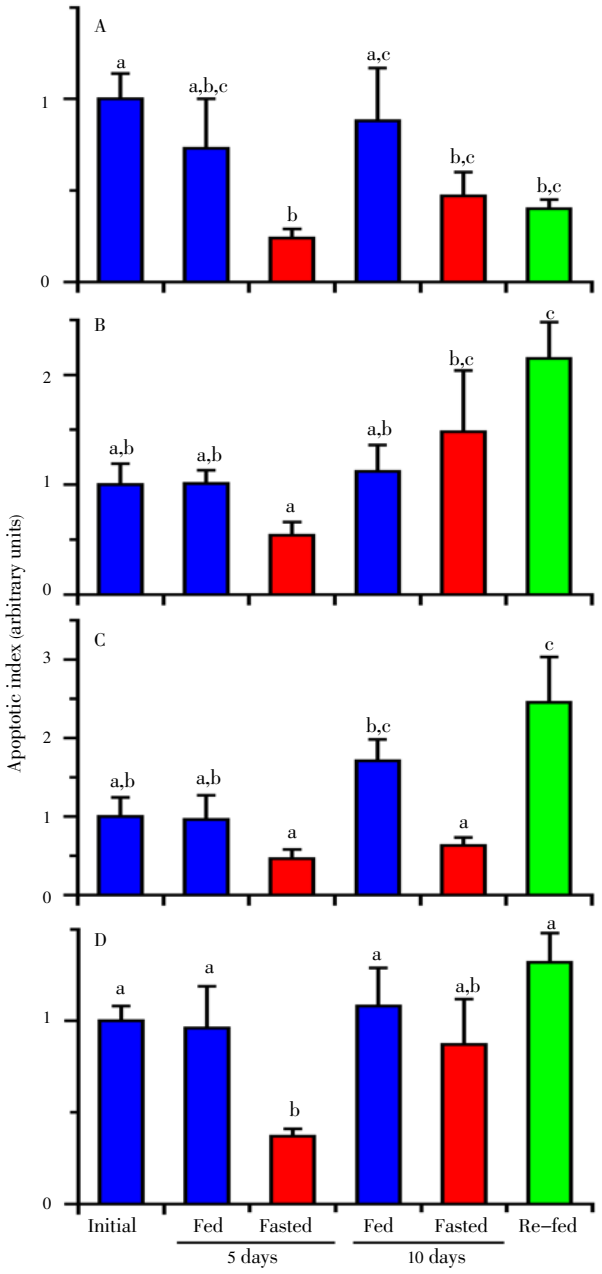

Figure 1. Effects of nutritional status on the apoptosis in anterior of hammerhead shark.
A: middle; B: posterior; C: intestine; D: Combined data on these three parts of the intestine. Initial: the intestines were sampled at the time of capture in Kaneohe Bay. Data (mean±SEM, n=7-12 fish) are normalized to the protein contents, and expressed relative to average value of the intestine from initial group for ease of comparison. Groups with different letters in each intestinal part are significantly (P<0.05) different.

Table1

Effects of nutritional state on the levels of plasma compositions and osmolality.

Parameters	Initial*	Fed (5 d)	Fasted (5 d)	Fed (10 d)	Fasted (10 d)	Re-fed
Glucose (mmol/L)	11.5±0.7^{ab}	12.3±0.7^{a}	11.9±1.0^{ab}	12.4±0.4^{a}	9.6±1.3^{b}	11.8±0.7^{ab}
Cholesterol (mmol/L)	2.2±0.1^{a}	2.3±0.4^{a}	1.9±0.4^{ad}	3.3±0.2^{b}	0.9±0.3^{c}	1.2±0.2^{cd}
Urea (mmol/L)	396.3±12.7^{ab}	392.9±8.5^{ab}	386.4±9.8^{ab}	374.6±8.7^{a}	411.8±14.0^{b}	396.8±11.5^{ab}
Cl (mEq/L)	269.1±1.1^{ab}	267.2±5.0^{a}	280.8±8.7^{b}	265.1±2.6^{a}	273.8±2.9^{ab}	268.1±2.5^{a}
Osmolaity (mOsm/kg)	1 037.2±2.8^{ab}	1 042.6±2.7^{a}	1 041.5±3.7^{a}	1 039.8±4.2^{ab}	1 034.7±3.5^{ab}	1 030.8±2.1^{b}

Values are mean±SEM (n=6-8 fish). *: The plasma samples were collected at the time of capture in Kaneohe Bay. Groups with different letters in each parameter are significantly (P<0.05) different.

In as much as the combined data were essentially consistent with the most obvious effects that were observed in the posterior portion, the apoptotic cell localization and cell proliferation were examined in these representative portions of the shark fed or fasted for 10 d. In the intestine of fed sharks, large number of apoptotic cell nuclei labeled by TUNEL for DNA breaks were detected mostly in the tips of villous, primarily in the epithelium (Figure 2A). On the other hand, in fasted sharks, the number of the apoptotic cells was much reduced and localized to the epithelium (Figure 2B). In fed sharks, labeling with the antibody against PCNA revealed many proliferating cells in the epithelium around the troughs of the intestinal fold (Figure 3A), whereas proliferating cells were few and limited to the troughs of the intestinal folds in fasted sharks (Figure 3B). In the fasted sharks, the number of proliferating cell nuclei was significantly reduced compared with levels in fed sharks (Figure 3C).

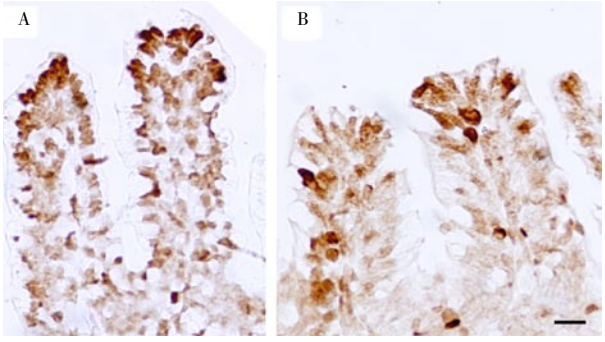

Figure 2. Detection of apoptotic cells (dark nuclei) by TUNEL in the shark intestine.
A: In shark fed for 10 d, a large number of TUNEL-positive nuclei are observed around the tips of the villi, primarily in the epithelium. B: In shark fasted for 10 d, fewer labeled nuclei are detected. Scale bar=40 μm.

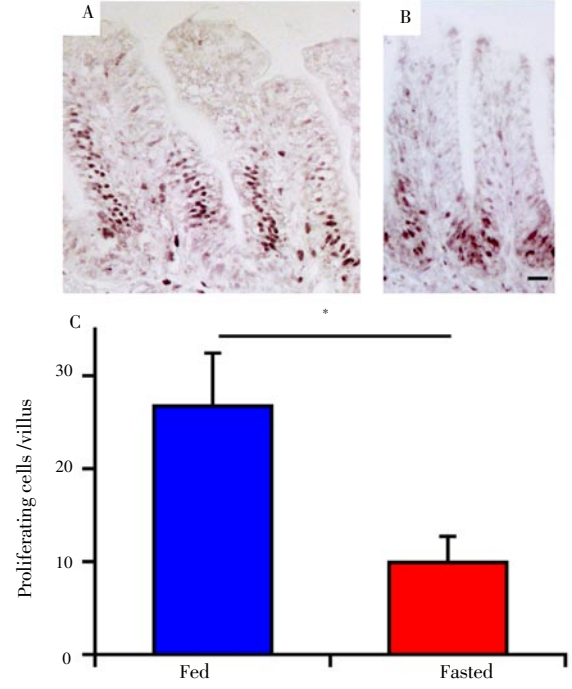

Figure 3. Proliferation cells assessed by PCNA immunocytochemistry (dark nuclei) in the shark posterior intestine.
A: In shark fed for 10 d, many PCNA-positive nuclei were appeared in the epithelium around the troughs of the villi. B: In shark fasted for 10 d, fewer labeled nuclei are detected. Scale bar=40 μm. C: Proliferating index (PCNA-positive cells/villus) in the posterior intestine of fed and fasted shark. Around 10 sections per shark and at least 100 villi were examined. Values are means± SEM (n=4). *: P<0.05.

4. Discussion

In the present study of the hammerhead shark, plasma levels of cholesterol and glucose were reduced by fasting, which is in agreement with the findings of most studies on teleost fishes[17-19]. In these teleosts, plasma levels of cholesterol and glucose returned closely, but not completely to pre-fasted levels after a few days of refeeding[17,19]. In the shark, plasma glucose and cholesterol did not completely recover with re-feeding. The differences in these finding may derive from a variation of the digestive-absorptive process, and/or from a difference in the experimental diets (natural bait or artificial food).

The present study of the primitive valvular intestine in shark showed that apoptotic cells were mostly observed in the tips of villous epithelia and proliferating cells located the troughs of the intestine. In the advanced tubular intestine of tetrapods (*e.g.*, mammals, chicken and reptiles)[20-26], similar localizations of apoptotic or proliferating cells in the epithelia have been reported, whereas proliferating cells in fresh water and apoptotic cells in seawater were observed throughout the intestinal epithelia of euryhaline teleosts[15,27]. Furthermore, the degree of cell proliferation and apoptosis were modified in both intestinal epithelia of the shark and the murines by fasting/feeding, but in opposite directions[5,6,28,29]. Whereas a reversible decrease in the epithelial apoptosis and cell proliferation are observed in the shark during fasting, epithelial cell turnover is induced in the murine intestinal mucosa after fasting[6,28,29]. This difference may reflect the differing feeding ecology of sharks most of which, unlike murines, tend to eat large meals at irregular intervals[30,31]. Similar studies in other intermittent feeders, such as reptiles and teleosts, should be intriguing.

In the mammalian intestine, several hormones involving nutritional status and/or fuel metabolism, such as growth hormone, insulin-like growth factor-I, leptin and ghrelin, are reported to play important roles in regulating enterocyte turnover[32-34]. In our previous studies on teleost fishes, cortisol, prolactin and growth hormone were found to directly regulate cell turnover in the gastrointestinal tract[12,15,35]. The cell turnover system in the shark intestine also appears to be under endocrine regulation and we are currently identifying these hormones as well as other gastrointestinal peptides that may be active (*e.g.*, ghrelin and motilin)[36]. Some differential regulation of apoptosis observed among the intestinal portions may depend on the distributions of these hormonal receptors. As suggested for the similar regional difference in the regulation of apoptosis in the rat small intestine[6], parasympathetic regulation of apoptosis may also be involved.

In conclusion, our results suggest that the fasting and refeeding alter epithelial cell proliferation and apoptosis in the shark intestine. Until now, such study of intestinal cell turnover has been limited to the murine tubular intestine, and little is known about the mechanism controlling cell turnover in the primitive valvular intestine of elasmobranches, intermittent feeders. The juvenile hammerhead shark appears to be a valuable model for further investigation, because of its relative abundance. In Kaneohe Bay, over 7000 hammerhead sharks are born each year, but the juvenile attrition has been estimated to be 85%-93% in the first year of life as a fraction of neonate population size. Moreover, data on weight loss strongly suggest that the observed very high natural mortality is caused by prolonged

starvation[37]. Thus, the research on digestive physiology may contribute to the understanding of ecophysiology of this elasmobranch species.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was supported in part by grants to T.S. (Grants-in-Aid for Scientific Research Nos. 17570049 and 19570057 from JSPS), to H.T. (research fellowships for young scientists Nos. 192156 and 214892 from JSPS), and to S.H. (the Japan-USA Research Cooperative Program No. 07033011-000122 from JSPS).

Comments

Background

Vertebrates possess multiple physiological and behavioral adaptations to feeding, and feeding/fasting has been shown to influence the physiological performance of the gastrointestinal tract. However, the evolutionary significance of feeding/fasting is not well understood, especially with respect to primitive vertebrates.

Research frontiers

Until now, the study of intestinal cell turnover has been limited to the murine tubular intestine, and little is known about the mechanism controlling cell turnover in the primitive valvular intestine of elasmobranches, intermittent feeders. Therefore, the research on digestive physiology may contribute to the understanding of ecophysiology of this elasmobranch species.

Related reports

In the present study of the hammerhead shark, plasma levels of cholesterol and glucose were reduced by fasting, which is in agreement with the findings of most studies on teleost fishes [Pérez-Jiménez *et al.* (2007), Figueiredo-Garutti *et al.* (2002) and Power *et al.* (2000)]. In these teleosts, plasma levels of cholesterol and glucose returned closely, but not completely to pre-fasted levels after a few days of refeeding. In the shark, plasma glucose and cholesterol did not completely recover with re-feeding. The differences in these finding may derive from a variation of the digestive-absorptive process, and/or from a difference in the experimental diets (natural bait or artificial food).

Innovations and breakthroughs

Intestinal apoptosis and cell proliferation were assessed by using oligonucleotide detection assay, TUNEL staining, and immunohistochemistry of proliferating cells nuclear antigen (PCNA).

Applications

In Kaneohe Bay, over 7 000 hammerhead sharks are born each year, but the juvenile attrition has been estimated to be 85%-93% in the first year of life as a fraction of neonate population size. Moreover, data on weight loss strongly suggest that the observed very high natural mortality is caused by prolonged starvation. Thus, the research on digestive physiology may contribute to the understanding of ecophysiology of this elasmobranch species.

Peer review

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