J Toxicol Pathol 2022; 35: 237–246

Original Article

Failure to gulp surface air induces swim bladder adenomas in Japanese medaka (*Oryzias latipes*)

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Abstract: In order to elucidate the effects of swim bladder inflation failure on swim bladder carcinogenesis, we investigated the sequential histopathological changes of swim bladders at 13, 24, 35, and 53 days post-hatch (dph) in medakas with an uninflated swim bladder, which was experimentally induced by denying access to the air–water interface between 0 and 6 dph. The reactive oxygen species (ROS) levels were measured at 24 dph. An uninflated swim bladder was induced in 47.3% of the fish denied access to the air–water interface (the denied group). The total incidence of swim bladder adenoma was 54.1% in the denied group; however, these tumors were observed in all fish with an uninflated swim bladder. In fact, these tumors were observed from 13 dph and onwards. The TBARS levels of the juveniles showed a 2.6-fold increase in fish with an uninflated swim bladder in the denied group compared to that in the control group. It is speculated that swim bladder inflation failure has some effects on the gas gland to produce ROS, leading to DNA damage in the gas glandular epithelium, which develops into swim bladder adenomas. Consequently, it is concluded that denying access to the air-water interface between 0 and 6 dph in medaka is an easy method of inducing swim bladder tumors in a short-term period, and is a useful method for producing tumor-bearing fish. (DOI: 10.1293/tox.2022-0030; J Toxicol Pathol 2022; 35: 237–246)

Key words: adenoma, gulp surface air, gas gland, swim bladder inflation failure, tumor-bearing fish

Introduction

The swim bladder in fish, which is not seen in mammals, is an organ that controls whole-body density, buoyancy, and sound production[1]. Embryologically, the swim bladder is derived from the gut[1], and forms during early development as an evagination of the foregut. Anatomically, a connecting pneumatic duct runs between the digestive tract and the posterior end of the swim bladder[2]. The swim bladder is classified into two types based on the presence or absence of a pneumatic duct: the physostomous swim bladder (in families such as Salmoniformes, Cypriniformes, Clupeiformes) and physoclistous swim bladder (in families such as Myctophiformes, Perciformes, Gadiformes)[3], respectively. In physoclistous fish, including medaka, the larvae must access the air-water interface to gulp air during early development. Consequently, the swim bladder lumen fills up with gas via the pneumatic duct. The pneumatic duct regresses after swim bladder inflation, and thereafter, the volume of gas in the swim bladder lumen is controlled by oxygen secreted from the gas gland[4].

Tumors in the swim bladder can roughly be classified into two types based on their origin: mesenchymal cells and gas glandular epithelium. Spontaneous and chemically-induced tumors in the swim bladder are mainly swim bladder adenomas and/or adenocarcinomas originating from the gas glandular epithelium[5] (hereafter referred to as swim bladder tumors). Spontaneous swim bladder tumors are extremely rare in fish[6], with only a few described in a handful of physoclistous fish species: medaka[7], mullet[8], guppy[7, 9], seahorse[10], Nothobranchius fish[11], gilt-head bream[12], and Atlantic cod[13]. The incidence of spontaneous swim bladder tumors in medaka is known to be very low, at 0.02% (2/10,000)[7]. However, these tumors are more frequent in wavy or scoliotic medakas, according to our previous case reports[5, 14]. The common histopathological change accompanied by the tumors in these spinal deformity medakas was swim bladder inflation failure. Swim bladder tumors and hyperplasia in gilt-head bream and Atlantic cod are also reported to be accompanied by spinal deformities[12, 13]. In addition, swim bladder inflation failure is known to be closely involved in the occurrence of spinal deformities in cultured marine fish[15]. According to some studies, denying access to the air–water interface experimentally induces swim bladder inflation failure in medaka and zebrafish[16–18]. In particular, zebrafish with an uninflated swim bladder show spinal curvature[18]. Therefore, swim bladder carcinogenesis might be related to swim bladder inflation failure and/or spinal de-

Received: 28 February 2022, Accepted: 29 March 2022
Published online in J-STAGE: 21 April 2022
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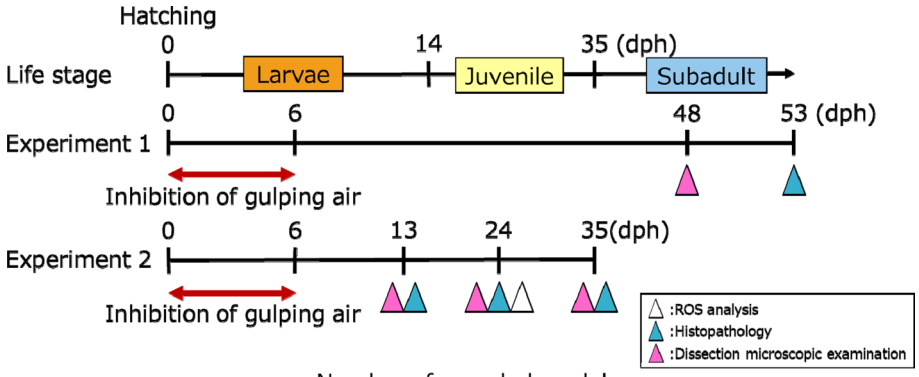

Number of sampled medaka

	Day post hatching (dph)			
	13	24	35	48/53
Control group	10	10/10*	15	38
Denied group	10	10/10*	12	42

*: ROS analysis

Fig. 1. Experimental design.

formities. In the present study, we investigated the sequential histopathological changes of swim bladders at 13, 24, 35, and 53 days post-hatch (dph) in medakas with an uninflated swim bladder that was induced via denying access to the air–water interface between 0 and 6 dph, in order to elucidate the effects of swim bladder inflation failure on the carcinogenesis.

Materials and Methods

Fish breeding and embryo collection

Initial stocks of Japanese medaka (*Oryzias latipes*) of the NIES-R strain were purchased from the National Institute for Environmental Studies (Tsukuba, Japan). Stock medakas were bred in the Biological Research Laboratory, Nissan Chemical Corporation, and were maintained under the following conditions: 25 ± 1 °C water temperature, 16 h:8 h light:dark photoperiod, and two rounds of feeding with brine shrimp each day. After 7 days of breeding, medakas were crossbred, and their embryos were collected. Triangular flasks containing 100 mL of freshwater were prepared, and 10 of the collected embryos were bred per flask. Embryos were observed every day, with water conversion performed every three days. Freshwater was obtained from the local tap water that complies with the Japanese water quality criteria.

Experimental design and inhibition of gulping air

This study was conducted in two parts (Experiments 1 and 2), according to the Guidelines for Animal Experimentation in the Biological Research Laboratory, Nissan Chemical Corporation. The experimental design is shown in Fig. 1. Embryos obtained immediately before hatching were separated into two groups; the control group and the denied group. A total of 93 embryos (Experiment 1, 48 embryos; Experiment 2, 45 embryos) in the control group and a total of 98 embryos (Experiment 1, 52 embryos; Experiment 2, 46 embryos) in the denied group were housed between 0 and 6 dph in a 7 L glass aquarium filled with freshwater for each group in each experiment. In the denied group, the 7 L aquarium was covered with a glass board, and immersed in a 70 L glass aquarium with freshwater, to deny access to the air-water interface between 0 and 6 dph (Fig. 2). After 6 dph, the larvae were bred in a 7 L glass aquarium in the same manner as those in the control group. The embryos and fish in both groups were housed under the same conditions as the stock medakas. Inhibition of swim bladder inflation is known to be induced in medaka larvae that are denied access to the air-water interface for 5 days or longer. Further, their mortality rate does not significantly increase until 7 dph[16]. Therefore, the period for denying access to the air-water interface was set as 7 days. Fish in both groups were fed rotifers once per day until 6 dph. Thereafter, they were fed brine shrimp once per day. Observations regarding the presence or absence of a swim bladder lumen and spinal deformities in the fish were performed at 13, 24, 35,

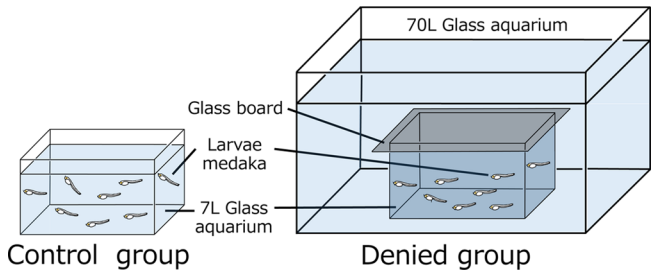

Fig. 2. Schematic of the environment for denying access to the air-water interface.

and 48 dph via dissection microscopy. Fish were sacrificed by overexposure to tricaine methanesulfonate (500 mg/L) at 13, 24, 35, and 53 dph. Dissolved oxygen was not measured in the present study.

Histopathological analysis

The sacrificed fish were fixed in Davidson's fixative overnight before being refixed in 10% neutral-buffered formalin. The fixed fish at 13 and 24 dph were embedded whole in paraffin, whereas those at 35 and 53 dph were separated into two sections via a mid-sagittal cut. Both sections were embedded in paraffin, serially sectioned at a thickness of 4 μm, and stained routinely with hematoxylin and eosin for histopathological examination.

Sample preparation for reactive oxygen species (ROS) analysis

Ten juveniles in each group were sacrificed at 24 dph via the method used for the ROS analysis. Five juveniles with an inflated swim bladder and five juveniles with an uninflated swim bladder were assigned to the denied group following confirmation of the presence or absence of a swim bladder lumen via dissection microscopy. These juveniles were wiped to remove body surface water, pooled for each group (the control group, the denied group/inflated swim bladder lumen, and the denied group/uninflated swim bladder lumen), and immediately frozen with liquid nitrogen. The resulting samples were stored at −80°C until analysis. The samples were weighed and then homogenized with 10 volumes of ice-cold 0.1 M sodium phosphate buffer (pH 7.4) using a tight-fitting Teflon-glass homogenizer. The homogenate was separated into two aliquots, which were centrifuged at 10,000×g for 15 min and 3,000 g for 10 min, respectively. The obtained supernatants were used for superoxide dismutase (SOD) and thiobarbituric acid-reactive substances (TBARS) assays, respectively.

SOD assay

SOD activity was determined using a SOD assay kit-WST (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. Briefly, 20 μL of the supernatant, 800 μL of water-soluble tetrazolium (WST) working solution, and 20 μL of working enzyme solution were mixed in a 96-well plate. After the plate was incubated at 37°C for 20 min, the absorbance at 450 nm was measured. SOD activity was estimated based on the WST reduction rate. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the WST reduction rate; SOD activity is expressed as units/mg of protein.

TBARS assay

TBARS levels were determined using a TBARS (TCA Method) Assay Kit (Cayman Chemical Company, MI). Briefly, 0.1 mL of the supernatant was mixed with 0.1 mL of 10% trichloroacetic acid and 0.8 mL of the color reagent (0.53% thiobarbituric acid solution) in microcentrifuge tubes with a locking cap. The mixtures were heated in a boiling water bath for 60 min and then centrifuged at 3,000 g for 10 min. The absorbance of the supernatants at 530 nm was then measured. Using malondialdehyde as a standard, the TBARS levels were calculated and expressed as nmol/mg protein.

Statistical analysis

Fisher's exact test was performed to assess the incidences of hatching, survival fish, fish with spinal deformities, uninflated swim bladder, hyperplasia, and adenoma (Pharmaco Basic, Scientist Press Co. Ltd., Tokyo, Japan). The level of significance was set at $p<0.05$ and $p<0.01$.

Results

Mortality and clinical findings

Denying access to the air-water interface did not have any effects on the incidences of hatching, mortality, or spinal deformity (Table 1). Fish in the denied group were smaller than those in the control group and were usually found at the bottom of the chambers. Some of these fish also required more effort to swim, shaking their tail fin during ascension to the water surface. The presence or absence of a swim bladder lumen was detectable in fish at 13 and 24 dph (Fig. 3, Table 2) but not at 35 or 48 dph via dissection microscopy.

Table 1. Effects of Denying Access to the Air–Water Interface on the Hatching, Mortality, and Spinal Deformities of Medaka

		Control group	Denied group
Experiment 1	Number of embryos	48	52
	Number of hatching	47	51
	Number of survival medaka	38	42
	Mortality of medaka (%)	20.8	19.2
	Number of medaka with spinal deformities	0	0
Experiment 2	Number of embryos	45	46
	Number of hatching	45	45
	Number of survival medaka	45	42
	Mortality of medaka (%)	0	8.7
	Number of medaka with spinal deformities	0	0

Swim bladder lesion prevalence

The histopathological findings of the swim bladder are summarized in Table 2. The number of fish with an uninflated swim bladder increased in the denied group, with a total incidence of 47.3%. The tumors had originated from the gas glandular epithelium and were accordingly diagnosed as swim bladder adenoma. These tumors were observed from 13 dph and onwards in the denied group. The total incidence of tumors was 54.1% in the denied group; however, the tumors were observed in all fish with an uninflated swim bladder. The total incidence of hyperplasia of the gas glandular epithelium was 9.5% in the denied group. Hyperplasia was observed in 17.9% of fish with an inflated swim bladder in the denied group but was not observed in those with an uninflated swim bladder.

Histopathology of the swim bladder

The histological development process of swim bladder tumors and hyperplasia in the denied group is shown in Fig. 4. The tumors were located posterior to the head kidney in the dorsal abdominal cavity, and grew rapidly over time. The tumors were non-invasive expansile encapsulated solid masses (Fig. 5a–c). They contained multiple various sized cysts and/or a focal aggregation of macrophages in the center of mass at 53 dph (Fig. 5c). In some of tumors, the tumor masses were connected to a rete mirabile that showed a hyperplastic capillary plexus (Fig. 5c). Tumor cells were arranged in cords, trabeculae, and solid patterns. These cells also had abundant eosinophilic cytoplasm and showed anisonucleosis and nuclear atypia with prominent nucleoli, despite the detection of few mitotic figures. Multinucleated cells were scattered in some tumors (Fig. 5d). Hyperplasia presented as an enlarged gas gland due to an increased amount of gas glandular epithelium and lacked a disrupted swim bladder architecture or cellular atypia (Fig. 5b).

The pneumatic ducts in the control group were only detected until 13 dph in the rete mirabile (Fig. 5a). In contrast, the pneumatic ducts in some fish with tumors or hyperplasia were detected even at 53 dph (Table 3, Fig. 5b, 5c) and were differentiated into a gastrointestinal tract-like structure connected to the esophagus (Fig. 5b). Some fish with tumors or hyperplasia had inflated swim bladder; however, their lumen tended to be smaller than those in normal individuals (Figs. 4e, f). There were no histopathological findings of the gas gland or rete mirabile in the control group or in fish without tumors or hyperplasia in the denied group.

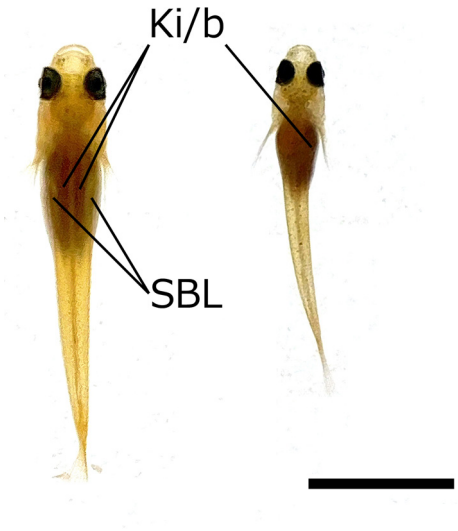

Fig. 3. Gross appearance of the juvenile at 24 dph.
Left, Detection of the swim bladder lumen as two white lines owing to the location of the body kidney on the dorsal side of the swim bladder. Control group; Right, No swim bladder lumen in the small-sized juvenile Denied group.
Ki/b: body kidney; SBL: swim bladder lumen. Bar=5 mm.

Table 2. Histopathological Findings of the Swim Bladder

Group	Autopsy (dph)	No. of medaka	Swim bladder lumen		Swim bladder findings (proliferative lesions)					
			Inflated	Uninflated	Normal		Hyperplasia		Adenoma	
Control	13	10	10[a)]	0[b)]	10	(10/0)	0	(0/0)	0	(0/0)
	24	10	10[a)]	0[b)]	10	(10/0)	0	(0/0)	0	(0/0)
	35	15	15	0	15	(15/0)	0	(0/0)	0	(0/0)
	53	38	38	0	38	(38/0)	0	(0/0)	0	(0/0)
	Total	73	73	0	73	(73/0)	0	(0/0)	0	(0/0)
Denied	13	10	5[a)]	5[b)] *	5	(5/0)	0	(0/0)	5 *	(0/5)
	24	10	5[a)]	5[b)] *	3	(3/0)	2	(2/0)	5 *	(0/5)
	35	12	9	3	4	(4/0)	2	(2/0)	6 **	(3/3)
	53	42	20	22 **	15	(15/0)	3	(3/0)	24 **	(2/22)
	Total	74	39	35 **	27	(27/0)	7 **	(7/0)	40 **	(5/35)

In parentheses: Number of fish with inflated lumen / Number of fish with uninflated lumen.
*, ** Significantly different from control at $p<0.05$, $p<0.01$, respectively (Fisher's exact test).
a: Identified presence of swim bladder lumen via dissection microscopy in live state.
b: Identified absence of swim bladder lumen via dissection microscopy in live state.

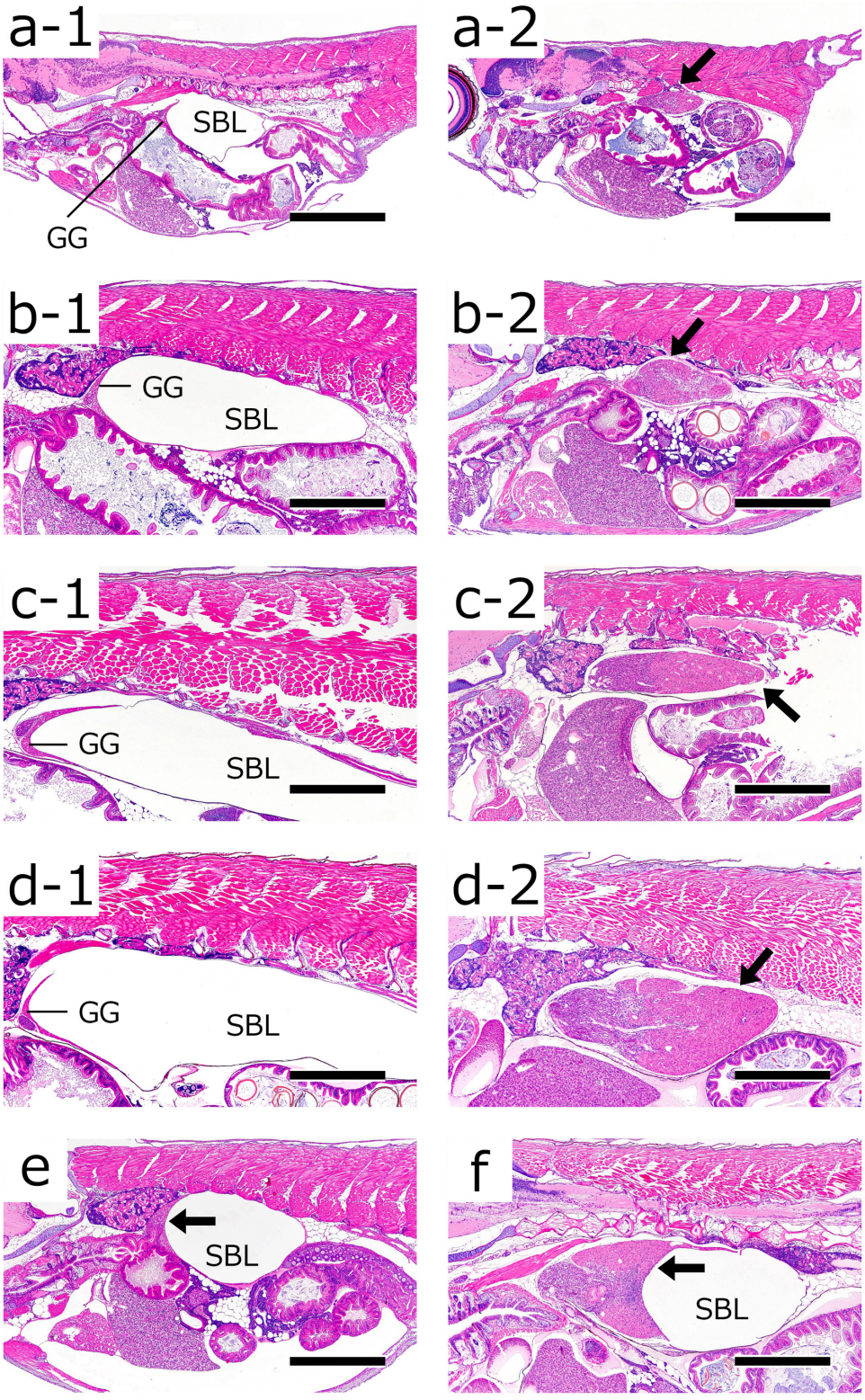

Fig. 4. Histological development process of swim bladder adenoma and hyperplasia.
a–d) Loupe image of adenoma with the uninflated swim bladder.
Tumor mass (→). a) 13 dph. b) 24 dph. c) 35 dph. d) 53 dph. 1) Control group. 2) Denied group.
e) Loupe image of hyperplasia with the inflated swim bladder.
Hyperplasia (→) with insufficient expanded swim bladder lumen. 24 dph. Denied group.
f) Loupe image of adenoma with the inflated swim bladder.
Tumor mass (→) with insufficient expanded swim bladder lumen. 53 dph. Denied group.
Bar=600 μm. Hematoxylin and eosin stain.
GG: gas gland; SBL: swim bladder lumen.

Oxidative stress

There were no differences in SOD activity between the control and denied groups (data not shown). In contrast, the TBARS levels in juveniles with inflated and uninflated swim bladders in the denied group showed a 1.5- and 2.6-fold increase, respectively, compared to levels in the control group (Fig. 6).

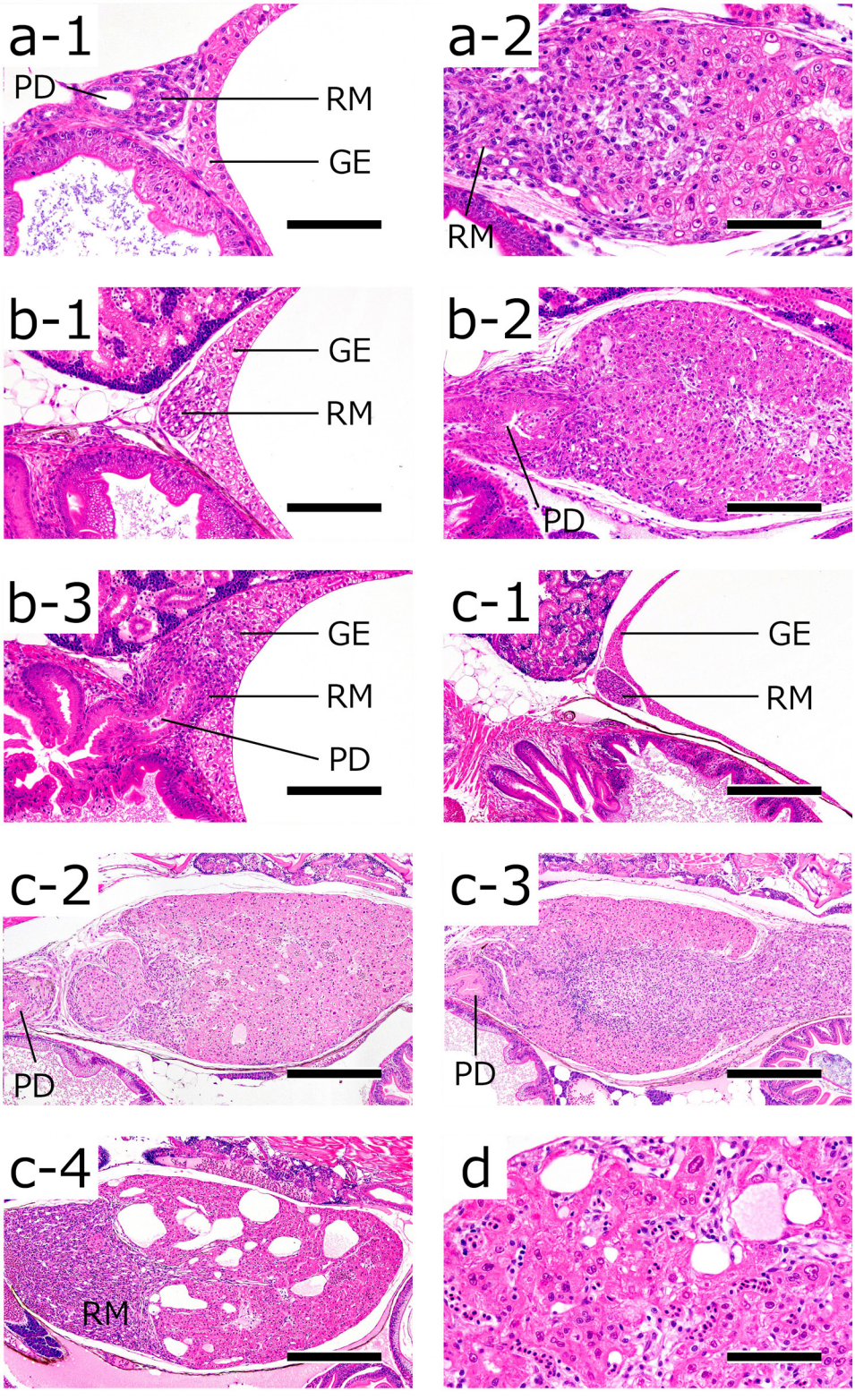

Fig. 5.

Discussion

Tumors in the swim bladder are reported to be induced in fish via exposure to environmental contaminants and carcinogens[19, 20]. In medaka, these tumors are experimentally induced by exposure to N-methyl-N′-nitro-N-nitrosoguanidine (incidence, 0.47%)[21], 4-chloroaniline (incidence, 17.1%)[22], and aniline (incidence, 36.7%)[22]. In the present study, the incidence of swim bladder tumors induced by denying access to the air–water interface was 54.1%, which was higher than that obtained for the chemically-induced tumors in the swim bladder. In particular, all medakas with an uninflated swim bladder had swim bladder tumors. Thus, it was revealed that swim bladder tumors occurred as a consequence of swim bladder inflation failure in medaka. In contrast, spinal deformities were not conclusively involved in swim bladder carcinogenesis in medaka. Experimentally, swim bladder inflation failure in medaka is induced by exposure to N-nitroso-N-methylurea[23], cypermethrin[24], 4-tert-octylphenol[25], selenomethionine[26], bitumen[27], 17α-ethinylestradiol[16], levonorgestrel[16], and diclofenac[16] during the embryo-larval life stage. However, the

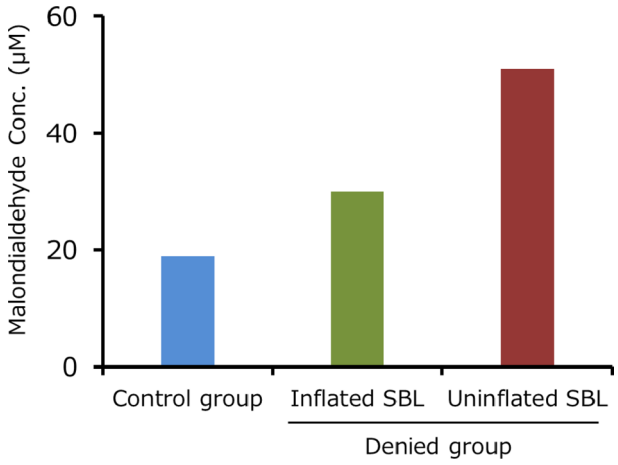

Fig. 6. TBARS level in juveniles at 24 dph.
A 1.5- and 2.6-fold increase in TBARS level in pooled juveniles with inflated and uninflated swim bladders in the denied group, respectively, compared to that in the control group.
Abbreviations: Inflated SBL, juveniles with inflated swim bladder lumen; Uninflated SBL, juveniles with uninflated swim bladder lumen.

Table 3. Remaining Pneumatic Duct in the Swim Bladder

Group	Autopsy (dph)	No. of medaka	Normal			Hyperplasia/Adenoma		
			Non-remaining	Remaining pneumatic duct		Non-remaining	Remaining pneumatic duct	
				Normal	Differentiated		Normal	Differentiated
Control	13	10	7	3	0	-	-	-
	24	10	10	0	0	-	-	-
	35	15	15	0	0	-	-	-
	53	38	38	0	0	-	-	-
Denied	13	10	5	0	0	0	0	5
	24	10	3	0	0	0	0	7
	35	12	4	0	0	5	0	3
	53	42	15	0	0	14	0	13

-: Not present.

Fig. 5. Histopathology of swim bladder adenoma and hyperplasia.
a) Swim bladder adenoma at 13 dph.
1) Normal. Control group. 2) Swim bladder adenoma. Non-invasive encapsulated solid mass connected to the hyperplastic rete mirabile. Denied group. Bar=60 μm.
b) Swim bladder adenoma and hyperplasia at 24 dph.
1) Normal. Control group. 2) Swim bladder adenoma. Non-invasive expansile encapsulated solid mass with a pneumatic duct differentiated into a gastrointestinal tract-like structure. 3) Swim bladder hyperplasia. Enlarged gas gland due to an increased number of gas glandular epithelia without disrupted swim bladder architecture or cellular atypia. Differentiated pneumatic duct connected to the esophagus. Denied group. Bar=120 μm.
c) Swim bladder adenoma at 53 dph. 1) Normal. Control group. 2) Swim bladder adenoma. Tumor cell proliferation in cords, trabeculae, and solid patterns. Denied group. 3) Swim bladder adenoma. Focal aggregation of macrophages in the center of mass with a pneumatic duct differentiated into the gastrointestinal tract-like structure. Denied group. 4) Swim bladder adenoma. Multiple various sized cysts. Marked hyperplastic capillary plexus in the rete mirabile. Denied group. Bar=240 μm.
d) High magnification of tumor cells at 53 dph. Tumor cells showing anisonucleosis and nuclear atypia with prominent nucleoli. Scattered multinucleated cells. Denied group. Bar=60 μm.
Hematoxylin and eosin stain.
GE: gas glandular epithelium; PD: pneumatic duct; RM: rete mirabile.

induction of swim bladder tumors in these larvae is unclear, as histopathological examinations were not performed in these studies or were performed on the 4-hour post-hatch larvae. In addition, swim bladder tumors are not observed in larvae with uninflated swim bladders for cultured physoclistous fish species, such as striped trumpeter[28], tilapia[29], striped bass[29], and walleye[30]. Consequently, to our knowledge, this is the first study to prove that swim bladder inflation failure induces swim bladder tumors in fish. In addition, medaka with swim bladder tumors can be selected by observing the presence or absence of a swim bladder lumen via dissection microscopy, which can be identified in the live state until 24 dph. Thus, it is considered that denying access to the air–water interface between 0 and 6 dph in medaka is an easy method of inducing swim bladder tumors in an extremely short-term period, and a useful method for producing tumor-bearing fish. However, it is unclear whether swim bladder carcinogenesis induced by denying access to the air–water interface is a specific change in medaka or a non-specific change that occurs in other physoclistous fish. In contrast, in physostomous fish, the gas gland and the rete mirabile are often poorly developed or absent[31]. Further, no spontaneous case reports of swim bladder tumors have been published in them. However, in the rainbow trout, swim bladder tumors have been induced by exposure to methylazoxymethanol[19] and dimethylnitrosomorpholine[32]. Additionally, it is possible that the gas glandular epithelium-like cells are scattered throughout the swim bladder wall in zebrafish[33]. Therefore, the application of this carcinogenic mechanism in the swim bladder in both physoclistous and physostomous fish species must be confirmed.

In the present study, a notable increase in the TBARS level was found in juveniles with an uninflated swim bladder in the denied group; however, no corresponding increase in SOD activity was found. Although the reasons for the absence of increased SOD activity in the present study are unknown, it has been reported that increased oxidative stress is not necessarily accompanied by increased SOD activity in rats[34, 35] and freshwater fish[36]. Therefore, the increased TBARS level indicated an overproduction of ROS in juveniles with an uninflated swim bladder, and the oxidative stress production might have contributed to the swim bladder carcinogenesis. In addition, TBARS level was increased in juveniles with an inflated swim bladder in the denied group, despite a weaker degree of increase than that found in juveniles with an uninflated swim bladder. Hyperplasia was detected histopathologically in a few juveniles with an inflated swim bladder at the same sampling time in the denied group. Therefore, a few juveniles with swim bladder hyperplasia are assumed to be included in the samples for the ROS analysis and to contribute to the increased TBARS level. However, the TBARS assay was performed using pooled juveniles at a single point in the present study; a detailed investigation with TBARS measurement at multiple points is needed to further elucidate the carcinogenesis.

In physoclistous fish and some types of physostomous fish, the filling and emptying of the gas bladder are respectively performed by a secretory and resorbing section[14, 37]. In physoclistous fish, the secretory section is anatomically composed of the rete mirabile and the gas gland: the rete mirabile is a dense bundle of parallel arterial and venous capillaries arranged side by side, whereas the gas gland is composed of folded cuboidal or columnar epithelium. The counter-current system in the rete mirabile and the anaerobic metabolism of glucose to lactate in the gas glandular epithelium lead to a significant increase in the blood oxygen partial pressure in the capillaries in the gas gland than that in the swim bladder lumen, allowing oxygen to diffuse from the blood into the swim bladder lumen[38]. Accordingly, swim bladder inflation failure may have some effects on the gas gland, leading to ROS production during the oxygen secretion process and the induction of DNA damage in the gas glandular epithelium. These initiated cells are assumed to proliferate via the promotion activity caused by swim bladder growth during the larval life stage and develop rapidly into swim bladder adenomas.

Assays for carcinogenicity evaluation using small fish species have been investigated since the 1980s, owing to their usefulness as an alternative to carcinogenicity testing. Although the utility of these assays is limited for screening unknown chemicals for potential carcinogenicity, they serve as a shorter-term assay[39] and provide a more sensitive evaluation for hepatocarcinogens than assays with rodents[40]. In particular, the alkylating agents, diethylnitrosamine[41] and methylazoxymethaol[42], induce liver tumors within just 2–3 months in medaka. The limited ability to repair DNA adducts in the medaka liver might be an important factor contributing to the high sensitivity for alkylating agent-induced hepatocarcinogenicity[42, 43]. In the present study, as the swim bladder tumors were observed from 13 dph and onwards, it was revealed that tumors in medaka developed in an extremely short-term period, not only in the liver but also in the swim bladder. A further study on DNA repair functions in the gas gland is required to identify the factors associated with the swim bladder carcinogenesis achieved over a short-term period in medaka.

Larvae must access the air-water interface to gulp air bubbles during a narrow, crucial time window in early development to achieve swim bladder inflation before the pneumatic duct undergoes stenosis, atrophy, and degeneration[29]. The ability to conduct initial swim bladder inflation lasts only a few days in most species[44]. The crucial time window for swim bladder inflation by gulping air bubbles at the air-water interface differs across fish species, as follows: striped trumpeter, 9–10 dph[28]; longtooth grouper, 6–10 dph[44]; red spotted grouper, 5–8 dph[44]; and, Australian bass, 6–11 dph[45]. In addition, it has been reported that the pneumatic duct is retained, when the swim bladder fails to inflate the lumen in the larvae[28, 30]. In medaka, the crucial time window for swim bladder inflation is actually considered to be 1–5 dph[16], although the swim bladder generally inflates within 24 h of hatching under normal conditions[46]. Nevertheless, half of the fish in the denied group formed a normal inflated swim bladder in the present study. The

pneumatic ducts in normal fish were detected at 13 dph. Further, these ducts in some fish with tumors or hyperplasia remained even at 53 dph and displayed a gastrointestinal tract-like structure. Therefore, the incidence of swim bladder tumors could be increased by further extension of the denied period. However, extending the denied period to ≥7 days increases the mortality rate of medaka juveniles[16]. The optimal period for denying access to the air–water interface must be determined for each fish species to increase the incidence of tumors without adversely affecting the fish. In addition, the remaining pneumatic duct differentiates into a gastrointestinal tract, according to the embryological origin of the swim bladder, and the regression of the pneumatic duct is considered to be inhibited by swim bladder inflation failure as a compensatory reaction.

Medaka larvae with an uninflated swim bladder are reported to require more oxygen than normal larvae[17] and higher energy for swimming and maintaining position. These larvae also face difficulty in capturing food, resulting in reduced growth and increased mortality[15, 28]. In the present study, fish with an uninflated swim bladder displayed labored swimming during ascension to the water surface and tended to be small compared to normal individuals, although fish weight data were not collected. These results support the abovementioned reports. Further, the inadequate growth of these fish is assumed to be attributed to the increased energy demands rather than the secondary effects of the swim bladder tumor.

In conclusion, denying access to the air–water interface between 0 and 6 dph induces swim bladder inflation failure and leads to swim bladder adenoma in medaka from 13 dph and onwards. The swim bladder carcinogenesis is closely related to the overproduction of ROS. This is an easy method of inducing swim bladder tumors in an extremely short-term period, and a useful method for producing tumor-bearing fish.

Disclosure of Potential Conflicts of Interest: The authors declare that there is no conflict of interest.

Acknowledgments: The authors would like to thank Dr. Jeffrey C. Wolf at the Experimental Pathology Laboratories Inc. for his valuable suggestions, as well as Ms. Yukiko Sudo, Ms. Yoshie Nagashima, Mr. Atsushi Funakoshi, and Ms. Kaori Maejima at Nissan Chemical Co. for their excellent technical assistance.

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