Short Communication

Improved fixation of the whole bodies of fish by a double-fixation method with formalin solution and Bouin's fluid or Davidson's fluid

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Abstract: To prevent fixation defects or artifacts in the whole bodies of fish caused by conventional fixatives, such as formalin solution, Bouin's fluid (BF), and Davidson's fluid (DF), the optimal fixatives and fixing method were examined. An improved method of fixing the whole bodies of fish was examined that makes use of a combination of 20% formalin and BF or DF. The fixatives were examined with four representative tissues, i.e., the gill, liver, intestinal tract, and kidney, to evaluate end points including the appearance of degraded tissues and artifacts caused by each fixative, overall morphological clarity of nuclei, staining intensity, and integrity of the other tissues. The best results were obtained when the fresh whole bodies were initially fixed in 20% formalin (primary fixation) at 4°C for 1 h and subsequently fixed in BF for 5 h at 4°C (secondary fixation). Therefore, the current findings led the authors to conclude that the combination of primary fixation with 20% formalin at 4°C for 1 h and secondary fixation with BF at 4°C for 5 h was suitable for fixation of the whole bodies of fish. (DOI: 10.1293/tox.2018-0001; J Toxicol Pathol 2018; 31: 201–206)

Key words: fish, fixative, double fixation, 20% formalin, Bouin's fluid, histopathology

There is increasing interest in histopathological investigation of fishes for evaluations of diseases in fish culturing, environmental monitoring, toxicity studies for environmental pollutants, screening studies for carcinogenicity or teratogenicity of chemicals, and so on[1–10]. Principally, conventional 10% formalin solution has been used as the histological fixative for fishes as in humans and other animals. Speilberg, *et al.*[11] compared five different fixatives, Bouin's fluid (BF), Davidson's fluid (DF), 10% buffered formalin, 10% formalin + 1% glutaraldehyde, and Karnovsky's fluid, for use in light microscopic examination of the liver morphology in Atlantic salmon and concluded that 10% buffered formalin and Davidson's fluid were most suitable for evaluation with hematoxylin and eosin (H&E)-stained sections. For large fishes, each organ or tissue is separately dissected and immersed into the fixative. On the other hand, in small fishes like himedaka or zebrafish, whole bodies of fish are immersed directly into the fixative. It is well known that fish tissues are rapidly degraded by autolysis after death, and hence, the key to good fixation is how quickly we can inactivate the endogenous enzymes that are responsible for self-digestion[11–13]. We have often experienced defective fixation of tissues such as the liver, intestine, and brain when whole bodies of fish have been immersed in conventional 10% formalin. Therefore, BF or DF, which are rapid permeable fixatives, has been recommended to use for the fixation of fish[11, 12]. It is, however, known that hemolysis or space formation by detachment of the epithelium is induced artifactually in the gills, intestine, or kidney of fish fixed with BF or DF[12]. On the other hand, many investigators have reported similar findings in organs of fish exposed to environmental pollutants or fish exposed to waterborne diseases. For example, epithelial lifting of gill lamellae (second gill lamellae) was observed in fish exposed to various metals, such as cadmium[5], aluminum[6], copper[7], and lead[13], and it was judged to be one of the prominent autolytic changes[13]. Also, hemolysis was observed in fish exposed to some pollutants, i.e., lead[13], cadmium[14], and nitrite[15]. Therefore, artifacts caused by fixatives could prevent evaluation of the effects of pollutants.

In this study, we tried to improve the fixing method for fish with a combination of formalin and BF or DF. The whole bodies of anesthetized fish were initially fixed in 20% formalin (primary fixation) at 4°C and then preserved in BF or DF (secondary fixation).

Fixatives

The formalin solution (FS) and DF were prepared by mixing each reagent listed in Table 1 before use. In general, neutral buffered formalin solution is often used as a fixative, but it is known that neutral buffered formalin solution is in-

ferior to unbuffered formalin solution in terms of tissue permeability[16], so unbuffered formalin solution was used here.

Fish

One-month-old himedaka (*Oryzias latipes*) with a body length of about 10 mm were bred at the Biosafety Research Center (BSRC). Five fish were used in each of the 6 groups described below. For fixation, fish were anesthetized with an agent, FA100 (Tamura-seiyaku Corp., Saitama, Japan), for fish or crustaceans. This study was reviewed and approved before initiation by the Institutional Animal Care and Use Committee (IACUC) of BSRC and was performed in accordance with the ethics criteria stated in the BSRC Guidance for Animal Testing (June 2, 2014).

Preparation of H&E slides

The fixatives and procedures used for the comparative investigations are summarized in Table 2. Since George, *et al.*[17] reported that refrigeration of tissue samples at 4°C was effective for delaying the progress of postmortem autolysis, in groups I and II, whole bodies of anesthetized fish were primarily immersed in 20% FS for 1 h at 4°C and then secondarily immersed in BF or DF for 5 h at 4°C. As a reference, the whole bodies of fish were immersed in each fixative at 4°C for 6 h in groups III to V. In order to confirm the effects of the primary fixation, group VI, in which the whole bodies of fish were fixed with 20% FS only at 4°C for 1 h, was also set as a reference. The fixatives were gently agitated with an automatic shaker during fixation periods.

After fixation, the fish fixed with the fixatives other than BF were stored in 80% ethanol overnight at room tem-

perature for degreasing. Then, they were decalcified in 10% formic acid formalin solution (10 mL of formic acid and 90 mL of 20% formalin solution were mixed before use) for 6 h at room temperature. After washing in running water, these fish were stored in 80% ethanol for 24 h for dehydration. The fish fixed with BF were stored in 80% ethanol directly for washing and dehydration. Then, they were dehydrated in a series of graded ethanol solutions, cleared in xylene, substituted, and embedded in paraffin. Sections were prepared using a gliding microtome (setting: 2 μm), cut longitudinally, and stained with H&E. Two slides were prepared per fish, with one slide mainly used for examination of the gill and the other one used for examination of the other tissues. For this evaluation, four tissues, i.e., the gill, liver, intestinal tract, and kidney, which were found in the slides of almost all groups and susceptible to the effects of autolysis or artifacts, were mainly examined. George, *et al.*[17] reported that these four tissues were sensitive to postmortem autolysis. The end points used in comparison of the fixatives included overall morphological clarity of nuclei, staining intensity, and integrity of the other tissues. Each end point was evaluated subjectively with a grade between 1 and 4 (absent, slight, moderate, and severe).

Morphology of the tissues

The histopathological findings observed in the gill, liver, intestinal tract, kidney, and other organs or tissues of fish fixed with conventional fixatives and improved fixatives are shown in Fig. 1–5. At first, fixation with 20% FS only for 6 h was conducted at room temperature. Gill filaments (first gill lamella) and gill lamellae (second gill lamella) with well-preserved structures were observed in the fish fixed with 20% FS. However, slight to moderate autolytic changes were observed in the liver and intestinal tract of all fish. Tissues with well-preserved structures were also observed in the other organs (data are not shown). Next, fixation with 20% FS only was conducted at 4°C for 6 h for prevention of the effect of postmortem autolysis. Defects of the liver and intestinal tract were diminished compared with those at room temperature. Therefore, the results presented below are restricted to those for tissues fixed at 4°C.

Gills

Gill filaments and gill lamellae with well-preserved structures were observed in fish fixed with 20% FS only (Fig. 1a). On the other hand, the epithelium was detached

Table 1. Composition of Each Fixative

Regents	Fixatives		
	20% formalin solution	Bouin's fluid[1]	Davidson's fluid
37% Formaldehyde	20 mL	25 mL	22 mL
Anhydrous ethanol	-	-	33 mL
Saturated picric acid	-	75 mL	-
Glacial acetic acid	-	5 mL	12 mL
Distilled water	80 mL[2]	-	33 mL

The volume of each reagent is shown as a total volume of 100 mL for each fixative. 1) This composition is quoted from the catalog of Wako Pure Chemical Industries. 2) Since 20% formalin solution was used for a short period of time, it was diluted with distilled water without using a buffer solution.

Table 2. Fixation Procedures for Comparison of Different Fixatives

Group	Primary fixative	Fixation time	Temperature	Second fixative	Fixation time	Temperature
I	20% FS	1 h	4 °C	BF	5 h	4 °C
II	20% FS	1 h	4 °C	DF	5 h	4 °C
III	20% FS	6 h	4 °C	-	-	-
IV	BF	6 h	4 °C	-	-	-
V	DF	6 h	4 °C	-	-	-
VI	20% FS	1 h	4 °C	-	-	-

Abbreviations: FS, formalin solution; BF, Bouin's fluid; DF, Davidson's fluid.

from the capillary (it showed so-called epithelium lifting), and spaces formed artifactually in the gill lamellae in fish fixed with the conventional BF or DF only for 6 h (Fig. 1b). Notably, the gill filaments and lamellae fixed with DF only were poorly separated, and the gill structure was not so clear. In the fish fixed with BF for 5 h after 20% FS fixation for 1 h at 4°C, gill filaments and gill lamellae with almost well-preserved structures were observed (Fig. 1c). Though epithelium lifting was prevented in fish fixed with DF for 5 h after 20% FS fixation for 1 h at 4°C, the gill filaments and lamellae were still poorly separated.

Liver and intestine

Slight to moderate autolytic changes were observed in the liver and intestinal tract of all fish fixed with 20% FS only for 1 h and 6 h (Fig. 2a and 3a). The autolytic changes were limited in the region around the gall bladder. Autolytic changes similar to those in fish fixed with 20% FS were observed in fish fixed with BF and DF only (Fig. 2b). Though autolytic changes were not observed in intestinal tract, space formation was observed between the mucosal epithelium and submucosal tissue in fish fixed with BF and DF only (Fig. 3b). The livers of the fish fixed by double fixation with 20% FS and BF or DF exhibited well-preserved structures (Fig. 2c). Defectively fixed areas were also observed in the peripheral region around the gall bladder in fish fixed by double fixation. However, such artifactual changes were not observed in the intestinal tract in fish fixed by double fixation (Fig. 3c).
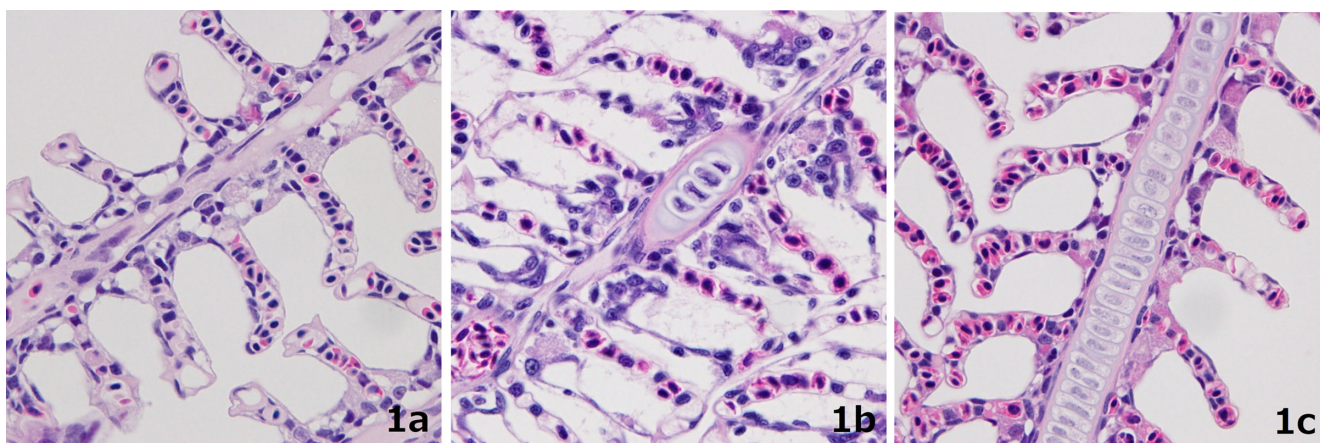

Fig. 1. The histopathological findings observed in the gills of fish fixed with conventional fixatives and improved fixatives. 1a: Fixed with 20% formalin (FS) only for 6 h at 4°C. Well-preserved gill filaments are observed. 1b: Fixed with Bouin's fluid (BF) only for 6 h at 4°C. The epithelium detached artifactually from the capillary, resulting in formation of a space in the gill lamella. 1c: Fixed with BF for 5 h after 20% FS fixation for 1 h at 4°C. Almost well-preserved gill lamellae are observed.
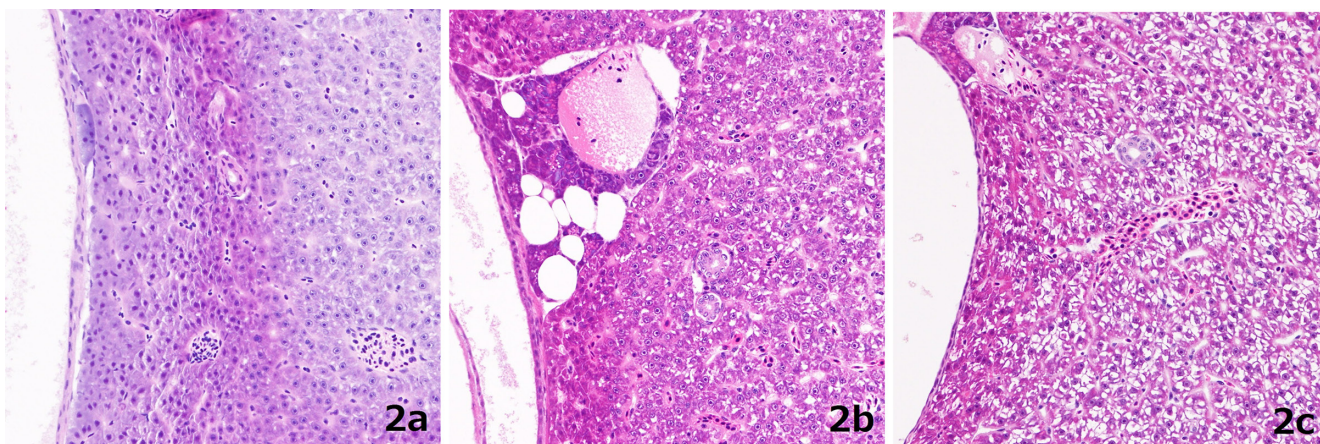

Fig. 2. The histopathological findings observed in the livers of fish fixed with conventional fixatives and improved fixatives. 2a: Fixed with 20% FS only for 1 h at 4°C. Though well-fixed liver is observed, a portion with an artifactual defect is apparent around the gallbladder. The area with the defect is rather wide in this sample. 2b: Fixed with BF only for 6 h at 4°C. A structure similar to that in 2a is observed. However, the area with the defect is smaller than that with 20% FS only fixation. 2c: Fixed with BF for 5 h after 20% FS fixation for 1 h at 4°C. An almost well-fixed liver is observed, and a portion with an artifactual defect is apparent around the gallbladder. However, the area with the defect is smaller than those with 20% FS only fixation.

Kidney

The structure of the kidney was well preserved in fish fixed with 20% FS only (Fig. 4a). The epithelium had shrunk and the artifactual spaces had formed around renal tubules in fish fixed with BF and DF only (Fig. 4b). Slight artifactual spaces were also formed around renal tubules fixed by double fixation (Fig. 4c).

Other tissues

Changes similar to those in the gill were observed in the skin. The structure of skin was well preserved in fish fixed with 20% FS only (Fig. 5a). The epithelium had lifted, and an artifactual space had formed under the epidermis and dermis in the skin fixed with BF and DF only (Fig. 5b). Slight artifactual spaces had also formed under the epidermis and dermis in the skin fixed by double fixation (Fig. 5c). Hemolysis was observed in fish fixed with all fixatives

(Fig. 5d–f). Rather shrunken erythrocytes were observed in fish fixed with BF and DF only and in fish fixed by double fixation (Fig. 5e and f).

The parameters for evaluation

The scores of eight parameters are shown in Table 3. The results obtained in this study indicated that fixation with 20% FS only and double fixation with 20% FS and BF gave relatively high scores for each parameter. Though fixation with 20% FS only gave relatively high scores by preventing the effects of postmortem autolysis at 4°C, the autolytic changes in the liver and intestinal tract with 20% FS only were more severe, and the intensity of H&E staining was inferior to that with BF fixation for 5 h after 20% FS fixation for 1 h. Furthermore, considering the field study at ambient temperature, fixation at 4°C is considered to be difficult, so the difference between fixation with 20% FS only
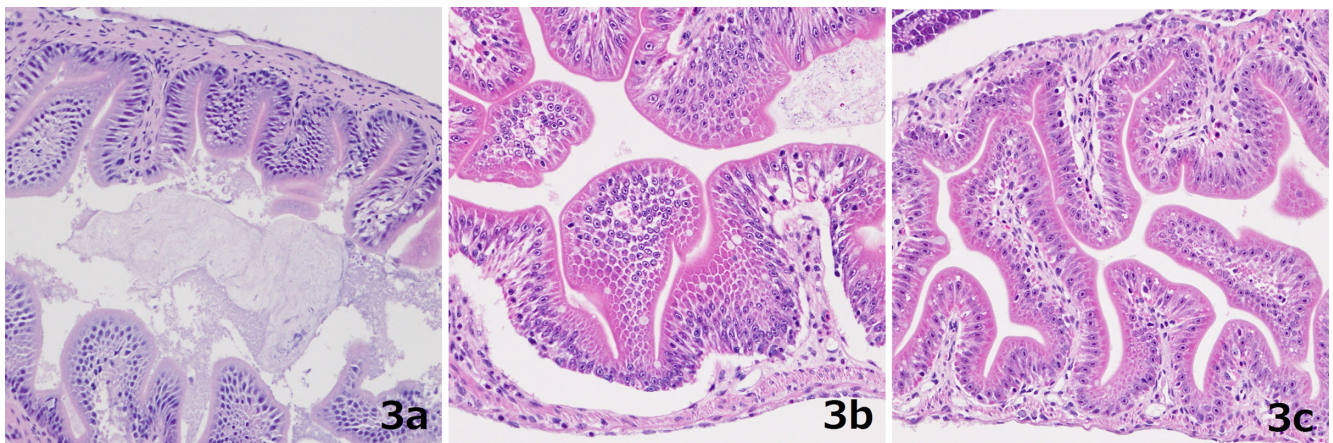

Fig. 3. The histopathological findings observed in the intestinal tracts of fish fixed with conventional fixatives and improved fixatives. 3a: Fixed with 20% FS only for 1 h at 4°C. A defectively fixed region caused by autolysis is observed. 3b: Fixed with BF only for 6 h at 4°C. Though a well-fixed intestinal tract is observed, space formation is prominent as a result of lifting of the mucosal epithelium. 3c: Fixed with BF for 5 h after 20% FS fixation for 1 h at 4°C. A well-fixed intestinal tract is observed.
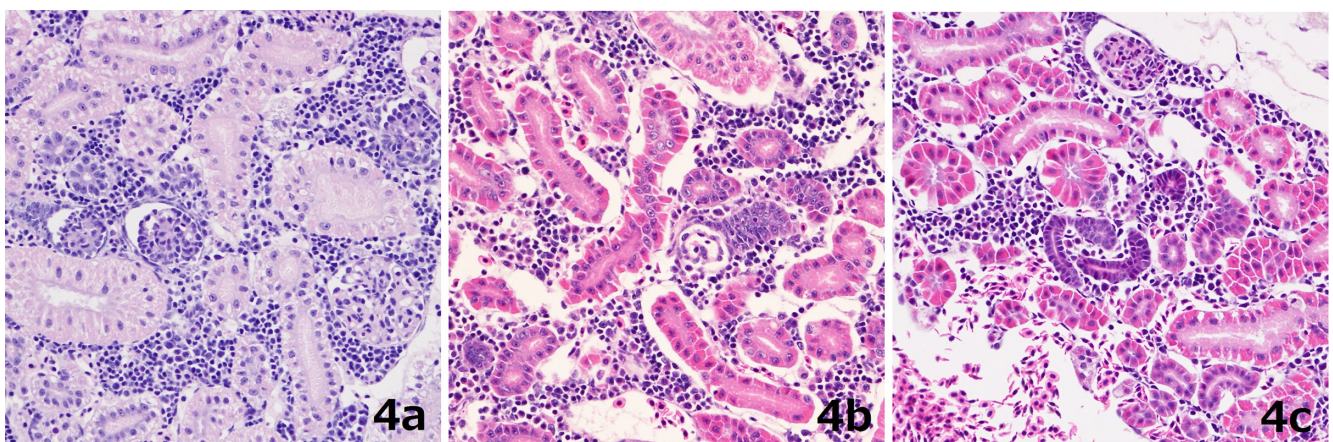

Fig. 4. The histopathological findings observed in the kidney of fish fixed with conventional fixatives and improved fixatives. 4a: Fixed with 20% FS only for 1 h at 4°C. A well-fixed structure is observed. 4b: Fixed with BF for 6 h at 4°C. Shrunken epithelia of urinary tubules and space formation around the renal tubules are observed. 4c: Fixed with BF for 5 h after 20% FS fixation for 1 h at 4°C. The similar figure is also observed; however, it looks rather slight.
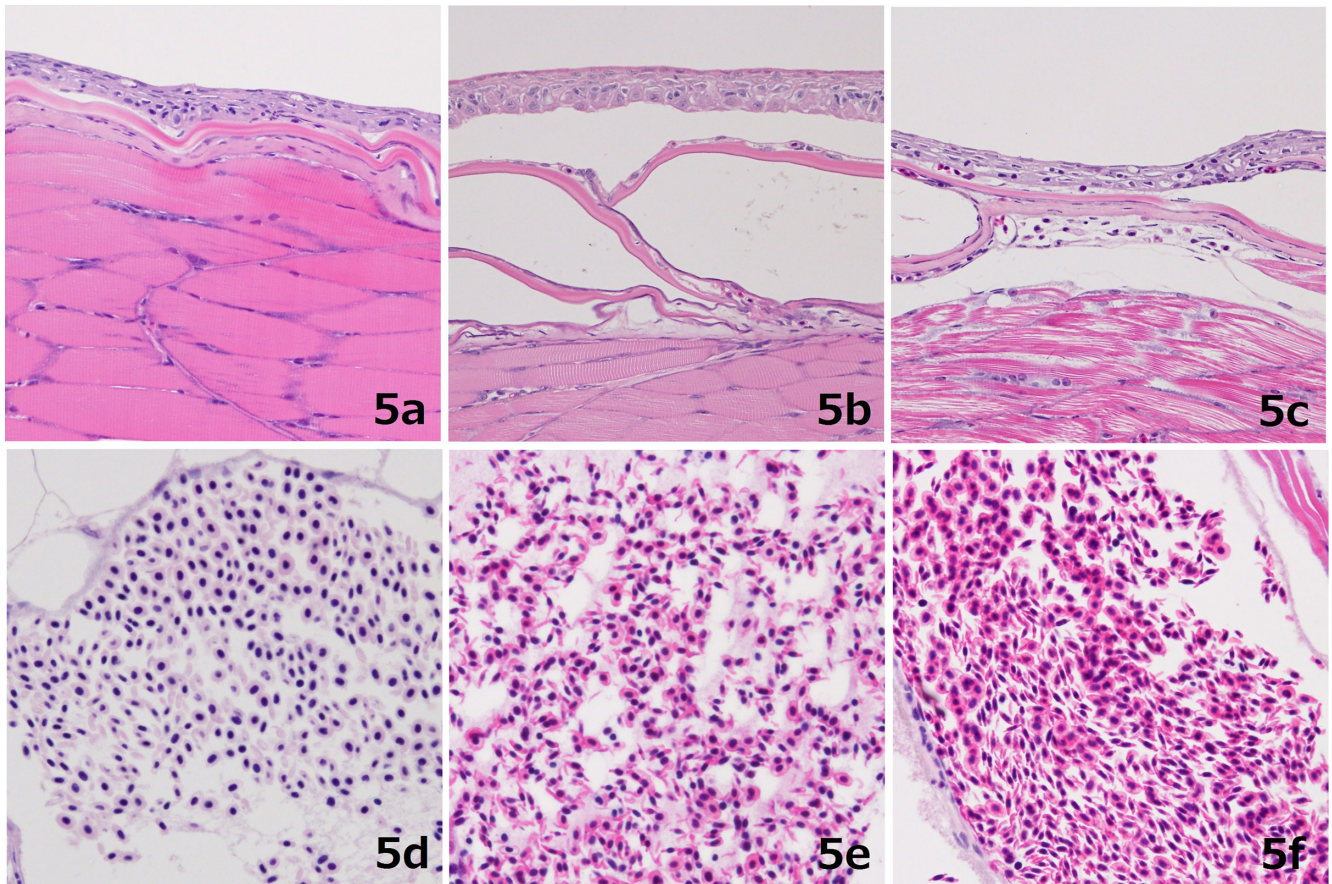
Fig. 5. The histopathological findings observed in the other organs or tissue of fish fixed with conventional fixatives and improved fixatives. 5a: Skin fixed with 20% FS only for 1 h at 4°C. A well-fixed structure of the skin is observed. 5b: Skin fixed with DF for 6 h at 4°C. Lifting of the epithelium is observed, resulting in space formation under the epidermis or dermis. 5c: Skin fixed with BF for 5 h after 20% FS fixation for 1 h at 4°C. Artifactual space formation similar to that in 5b is also observed. 5d: Vessels fixed with 20% FS only for 1 h at 4°C. Though hemolysis is observed, shrunken erythrocytes are not prominent. 5e: Vessels fixed with BF only for 6 h at 4°C. Hemolytic and shrunken erythrocytes are prominent. 5f: Vessels fixed with BF for 5 h after 20% FS fixation for 1 h at 4°C. Shrunken erythrocytes are prominent.

Table 3. Scores on Comparison of Different Fixatives

Effects	Organ	Grade[1]	Group[2]					
			I	II	III	IV	V	VI
Fixation defects by autolysis	Liver	a	3	3	3	3	3	2
	Intestine	a	3	3	3	4	4	3
Space formation	Gill	a	3	3	4	1	1	3
	Kidney	a	3	3	4	3	3	4
	Intestine	a	4	3	4	3	3	4
Poor separation of gill filament	Gill	a	3	2	3	3	2	3
Hemolysis	Vessel	a	3	3	3	3	3	3
Integrity of the other tissues	-	b	3	3	3	3	3	3
Nuclear morphological clarity	-	b	3	4	3	3	4	3
Intensity of H&E stain	-	b	4	3	2	4	4	2
Ease to cut a specimen	-	c	4	3	3	4	3	2
Total score			36	33	35	34	33	32

Group: I, BF (5 h) and 20% FS (1 h) double fixation at 4°C; II, DF (5 h) and 20% FS (1 h) double fixation at 4°C; III, 20% FS (6 h) fixation at 4°C; IV, BF (6 h) fixation at 4°C; V, DF (6 h) fixation at 4°C; and VI, 20% FS (1 h) fixation at 4°C. Scores of grading: a) 4, absent; 3, slight; 2, moderate; 1, severe. b) 4, normal; 3, slightly inferior; 2, moderately inferior; 1, severely inferior. c) 4, easy without decalcification (DCAL); 3, easy with DCAL; 2, slightly difficult with DCAL; 1, difficult with DCAL.

and double fixation with 20% FS and BF is considered to be larger.

Fixatives are used to prevent autolysis by inactivating lysosomal enzymes and to stabilize the fine structures both inside and between cells by making macromolecules resistant to dissolving in water and other liquids. The most commonly used fixative for histopathology is a 10% FS because it includes inorganic salts that maintain a near-neutral pH and an osmotic pressure close to that of mammalian extracellular fluid. It is known that despite rapid penetration into tissues, FS has the characteristic of slowly reacting with tissue proteins, especially in the formation of methylene bridges. Therefore, in this examination, it was also considered that FS causes defective preservation of some tissues in intact fish bodies. On the other hand, BF or DF is designed to rapidly penetrate into tissues by mixing with formalin. However, BF and DF have a defect in that they cause artifactual space formation through the shrinking effect of picric acid and ethanol. They have another defect in that they cause hemolysis through the lytic effect of acetic acid. So, we tried to improve the fixing procedures to prevent these artifacts. The double fixation method with glutaraldehyde and osmium, which is a well-known method for biological electron microscopy examinations, was applied. In this improved method, 20% FS was used as the primary fixative, and subsequently BF or DF was used as the secondary fixative. We intended the semi-fixation effect caused by immersion into the primary fixative prevented the artifacts caused by the secondary fixative. We set the temperature and duration for the primary fixation at 4°C and 1 h, respectively, and then we examined both the most suitable fixative and better fixing conditions for the secondary fixation. The results showed that BF is most suitable as the secondary fixative for fixation at 4°C for 5 h for whole bodies of juvenile fish. However, since artifactual space formation in the skin or kidney could not be prevented completely, it may be necessary to use other fixatives if artifactual space formation is obvious in target organs.

Conclusion

The results of the present study showed that the double fixation method with 20% FS and BF is most suitable for fixation of whole bodies of juvenile fish at 4°C. We also examined the whole body-fixation procedures for adult fish (body length: approximately 20 to 25 mm), and we found that fish must be dissected along the ventral midline from the anus to just below the gills and that the abdominal cavity should be opened so that it can be filled with the fixatives.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflict of interest.

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