Technical Report

Karnovsky's fixative prevents artifacts appearing as vacuolation derived from tissue processing in kidneys treated with antisense oligonucleotide

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Abstract: Antisense oligonucleotide (ASO) therapies have been identified as a new treatment modality for intractable diseases. In kidneys treated with ASOs, vacuoles, in addition to basophilic granules, are often observed in the proximal tubules. Some reports have described that these vacuoles are likely to be a secondary phenomenon resulting from the extraction of ASOs during tissue processing. In this study, we compared renal morphology after fixation with Karnovsky's fixative or 4% paraformaldehyde phosphate buffer (PFA) with that of 10% neutral-buffered formaldehyde solution (NBF). Female Sprague-Dawley rats, intravenously treated four times with 50 mg/kg locked nucleic acid containing antisense oligonucleotides (LNA-ASOs) for 1 or 2 weeks, were examined. Microscopically, vacuoles and basophilic granules in the proximal tubules were observed in the kidneys fixed with NBF. Basophilic granules are indicative of the accumulation of ASOs. Moreover, some of the vacuoles also contained faint basophilic granules, suggesting that the vacuoles were relevant to the accumulation of ASOs. Although moderate vacuolation was observed in the proximal tubules, the majority of the vacuolated epithelia were negative for kidney injury molecule-1 on immunohistochemical staining. Vacuoles in the proximal tubules were not observed in samples subjected to Karnovsky's fixation, although basophilic granules were observed. In samples subjected to PFA fixation, vacuoles and basophilic granules were observed in the proximal tubules, similar to those in samples subjected to NBF fixation. Overall, our findings demonstrated the possibility of overestimation of vacuolation due to artifacts during tissue processing when using conventional NBF fixation. Karnovsky's fixative is considered a useful alternative for distinguishing artificial vacuoles from true nephrotoxicity. (DOI: 10.1293/tox.2021-0007; J Toxicol Pathol 2021; 34: 367–371)

Key words: antisense oligonucleotide, artifact, Karnovsky's fixative, vacuolation, basophilic granule, kidney

Recently, antisense oligonucleotide (ASO) therapies have been established as new modalities for the treatment of intractable diseases[1, 2]. ASOs are short, synthetic, single-stranded oligodeoxynucleotides that bind to ribonucleic acid and suppress gene expression[3].

Toxicologically, ASO-induced toxicities are categorized into hybridization-dependent toxicities, which are related to the ASO sequence, and hybridization-independent toxicities, which are related to chemical modification. Hybridization-independent toxicities are induced by three main mechanisms: 1) ASO accumulation, 2) pro-inflammatory effects, and 3) aptameric binding effects[4]. The accumulation of ASOs is primarily observed in the liver and kidneys, and excessive accumulation leads to dysfunction of these organs[5]. The pro-inflammatory effect is induced by the stimulation of the innate immune system via binding to Toll-like receptors in rodents and monkeys or activation of the alternative complement pathway via inhibition of factor H by ASOs in monkeys[6]. The aptameric binding effect induces platelet activation and aggregation/thrombocytopenia to bind the platelet-specific receptor[7]. Moreover, aptameric binding to intracellular proteins in hepatocytes is considered one of the causes of hepatotoxicity[8–10]. To detect these toxicities, researchers have developed several nonclinical assays; among these, histopathological examination is one of the most sensitive tests to detect ASO accumulation.

Histopathologically, the accumulation of ASOs are commonly observed as basophilic granules in the cytoplasm of the Kupffer cells in the liver or the proximal tubules of the kidneys. The prominent accumulation of basophilic granules is correlated with an increased incidence or severity of degenerative changes, commonly observed as vacuolation or degeneration/necrosis of the proximal tubular epithelium in the kidneys[4]. Basophilic granules alone are not considered toxicologically significant[11]; therefore, the differentiation of basophilic granule accumulation from degenerative

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changes is important for evaluating ASO-related toxicities.

Vacuoles, which are often observed in the proximal tubules of the kidneys of cynomolgus monkeys treated with ASO, contain basophilic material and exhibit the same general distribution within the cells as basophilic granules[11, 12]. A few reports have suggested that the fixation process may influence the appearance of ASO accumulation within the phagolysosomal compartment[5, 11]. Additionally, Henry *et al.* and Engelhardt described that vacuoles in the proximal tubules are likely to be a secondary phenomenon resulting from the extraction of ASOs during processing because the vacuoles are absent in paraformaldehyde/glutaraldehyde perfusion-fixed kidneys[5, 11]. Vacuoles in the proximal tubules of cynomolgus monkeys treated with mipomersen were speculated to appear during the fixation process[13].

Thus, although vacuolation due to ASO treatment may be an artifact in some cases, this hypothesis has not been clearly proven, and no reports have identified the appropriate fixation method for toxicological evaluation. Accordingly, in this study, we compared renal morphology after fixation with Karnovsky's fixative or 4% paraformaldehyde phosphate buffer (PFA) with that of 10% neutral-buffered formaldehyde solution (NBF). Additionally, we demonstrated the utility of Karnovsky's fixative as an alternative fixative to prevent artifact-derived vacuolation.

Four-week-old female Sprague-Dawley rats were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan) and acclimatized to laboratory conditions. The animals were maintained under controlled conditions (12-hour light/dark cycle, temperature of $23 \pm 2°C$, relative humidity of $50 \pm 25\%$) and were allowed free access to a standard commercial diet (autoclaved CR-LPF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Twenty animals were allocated to the following four groups (with five animals each): the 1-week saline-treated group (Control-1W), 2-week saline-treated group (Control-2W), 1-week locked nucleic acid containing antisense oligonucleotide (LNA-ASO)-treated group (ASO-1W), and 2-week LNA-ASO-treated group (ASO-2W). The animals were 5 weeks old at the time of initiation of 50 mg/kg LNA-ASO administration. LNA-ASO was administered intravenously four times in total according to the following regimen: days 1, 4, 8, and 11 in the ASO-2W group; and days 1, 2, 3, and 4 in the ASO-1W group. The animals in the Control-1W and ASO-1W groups were sacrificed on day 7. The animals in the Control-2W and ASO-2W groups were sacrificed on day 14. At each terminal necropsy, blood samples for clinical pathology were collected from the abdominal vena cava under deep anesthesia with isoflurane. After blood sampling, all rats were euthanized by exsanguination by immediately cutting the abdominal aorta. The external body surface, thoracic and abdominal cavities, and viscera were macroscopically examined, and the left kidneys were removed and processed for microscopic examination. All procedures were performed in accordance with the Rules for Feeding and Storage of Experimental Animals and Animal Experiments of Mitsubishi Tanabe Pharma Corporation.

Serum samples were obtained by centrifugation of the blood samples at 4°C. Serum biochemical analysis was performed using a TBA-2000FR instrument (Canon Medical Systems Corporation, Tochigi, Japan) to assess the blood urea nitrogen (BUN) and creatinine (CRE) levels.

The kidneys were fixed using three fixative solutions as follows: After dividing the kidney transversely into three segments, the middle portion including the renal pelvis, was fixed in 10% NBF for 5 days; the cranial portion was fixed in Karnovsky's fixative (Muto Pure Chemicals Co., Ltd., Tokyo, Japan); and the caudal portion was fixed in 4% PFA for 17 hours at 4 °C and then placed in 10% NBF for 24 hours. Each fixed sample was dehydrated and embedded in paraffin. Tissue sections were prepared for hematoxylin and eosin (H&E) staining. Immunohistochemical staining was performed using antibodies targeting kidney injury molecule-1 (KIM-1; R&D Systems Inc., Minneapolis, MN, USA; 1:300 dilution) and lysosomal-associated membrane protein 2 (LAMP-2; Thermo Fisher Scientific Inc., Waltham, MA, USA; 1:1000 dilution) after antigen retrieval (citrate buffer, pH 6.0, heated for 10 min or heated under pressure for 20 min). Histofine Simple Stain Rat MAX PO (Nichirei Biosciences Inc., Tokyo, Japan) was used as the secondary antibody.

Macroscopically, no abnormal changes were observed. Additionally, in blood chemistry, renal parameters (i.e., BUN and CRE) showed no significant changes in any group (Table 1).

A summary of the histopathological findings in kidneys fixed with 10% NBF is shown in Table 2. Microscopically, basophilic granules and vacuoles in the proximal tubules, particularly in the S1 and S2 segments, were observed in both the ASO-1W and ASO-2W groups for kidneys fixed with 10% NBF (Fig. 1A). These changes were more severe in the ASO-2W group than in the ASO-1W group. The accumulation of basophilic granules was predominantly observed on the brush border of the proximal tubules as aggregates and/or punctate structures. As basophilic granules are reported to be present both within the endosomes or lysosomes and freely in the cytoplasm of the proximal tubular epithelium[14], these different forms may reflect their accumulated area; that is, the aggregates and punctuate structures may accumulate in lysosomes and the cytoplasm, respectively. The vacuoles in the proximal tubules were round and rough in shape, and most were fused with adjacent vacuoles. Moreover, some of the vacuoles contained faint basophilic granules (Fig. 1E), suggesting that the vacuoles were relevant to the accumulation of ASOs. Immunohistochemically, LAMP-2 was positive on the membrane surface of the vacuoles (Fig. 1G and 1H). The results indicated that the vacuolation was the dilatation of lysosomes. The difference in the severity of vacuolation between the ASO-1W and ASO-2W groups might indicate the difference in the amount of ASOs accumulated in lysosomes. We speculate that the amount of ASOs accumulated in lysosomes increases with increasing exposure time to ASOs. Consequently, lysosomal expansion appeared as vacuolation because of tissue processing.

Table 1. Blood Chemistry Data

Group	Control-1W	ASO-1W	Control-2W	ASO-2W
Number of Animals	5	5	5	5
BUN (mg/dL)	15.12 ± 1.15	16.06 ± 3.09	14.86 ± 2.01	14.66 ± 2.27
CRE (mg/dL)	0.12 ± 0.04	0.12 ± 0.04	0.20 ± 0.00	0.20 ± 0.00

Values are presented as mean ± standard deviation. BUN: blood urea nitrogen; CRE: creatinine; Control-1W: 1-week saline-treated group; Control-2W: 2-week saline-treated group; ASO-1W: 1-week locked nucleic acid (LNA) containing antisense oligonucleotide-treated group; ASO-2W: 2-week LNA containing antisense oligonucleotide-treated group.

Table 2. Histopathological Findings in the Kidneys Fixed with 10% NBF

Group		Control (1W and 2W)	ASO-1W	ASO-2W
Dose (mg/kg/occasion)		0	50	50
Number of Animals		10	5	5
Basophilic granules, proximal tubule	minimal	0	0	0
	mild	0	5	0
	moderate	0	0	5
Vacuolation, proximal tubule	minimal	0	2	0
	mild	0	3	3
	moderate	0	0	2

Control-1W: 1-week saline-treated group; Control-2W: 2-week saline-treated group; ASO-1W: 1-week locked nucleic acid (LNA) containing antisense oligonucleotide-treated group; ASO-2W: 2-week LNA containing antisense oligonucleotide-treated group.

Although moderate vacuolation was observed, proximal tubular degeneration was not evident, and the majority of the vacuolated epithelia were negative for KIM-1 in immunohistochemical staining (Fig. 1D). In contrast, for samples fixed in Karnovsky's fixative, vacuoles in the proximal tubules were absent in all individuals, whereas basophilic granules were frequently observed (Fig. 1B). The basophilic granules were more prominent than those in samples subjected to 10% NBF fixation because the cell structure was well preserved (Fig. 1F). In samples subjected to 4% PFA fixation, vacuoles and basophilic granules were observed; however, the severity of vacuolation was slightly lower than that in samples subjected to 10% NBF fixation (Fig. 1C).

Oligonucleotides are highly water-soluble molecules and are extracted from tissues during processing due to failure to completely crosslink the cellular components[5].

Based on these results, we concluded that vacuoles in proximal tubules were affected by the fixation method and that the morphological changes observed in tissues subjected to 10% NBF and 4% PFA fixation may be artifacts in the area of ASO accumulation during tissue processing. In other words, vacuolation may be toxicologically insignificant because no tubular degeneration, increased expression of KIM-1, or abnormal changes in renal parameters were observed. Therefore, we suggest using Karnovsky's fixative as the optional fixation method for accurate pathological examination of the kidney in ASO-treated animals.

In conclusion, our current findings revealed the possible overestimation of vacuolation when using the conventional NBF fixation method. Karnovsky's fixative may be a useful tool for distinguishing artificial vacuoles from true nephrotoxicity.
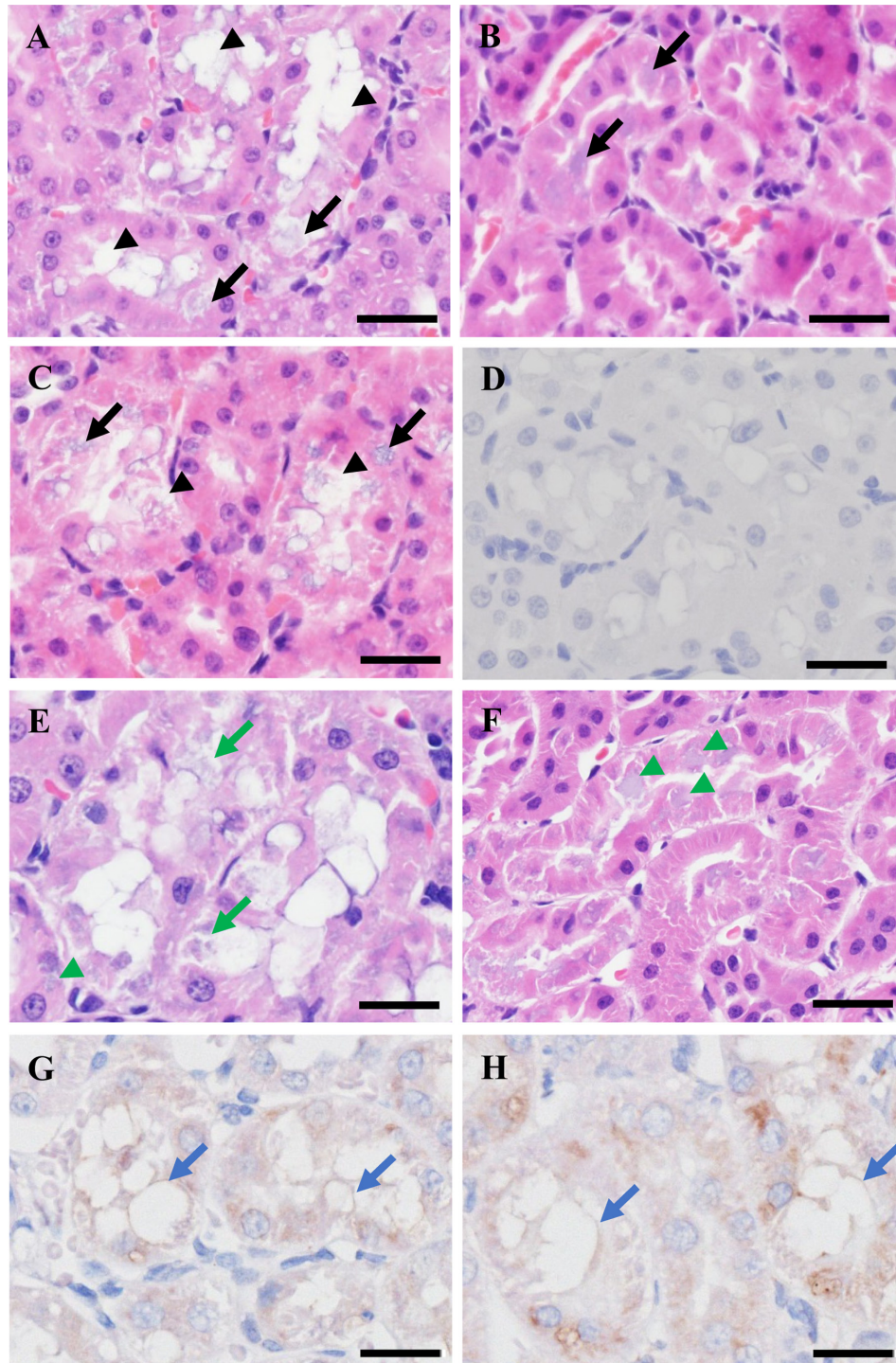
Fig. 1. Representative photomicrographs of the kidneys treated with antisense oligonucleotide (ASO). Histopathological changes in the 2-week locked nucleic acid containing antisense oligonucleotide (LNA-ASO)-treated group (ASO-2W) for kidneys fixed with (A) 10% neutral-buffered formaldehyde solution (NBF), (B) Karnovsky's fixative, or (C) 4% paraformaldehyde phosphate buffer (PFA). (D) Immunohistochemical staining for kidney injury molecule-1 (KIM-1) of ASO-2W for kidneys fixed with 10% NBF. Histopathological changes in the 1-week LNA-ASO-treated group (ASO-1W) for kidneys fixed with (E) 10% NBF and (F) Karnovsky's fixative. Immunohistochemical staining for lysosomal-associated membrane protein 2 (LAMP-2) of (G) ASO-1W and (H) ASO-2W for kidneys fixed with 10% NBF. (A) Basophilic granules (black arrows) and vacuoles (black arrowheads) are observed in the proximal tubules. Hematoxylin and eosin (H&E) staining. Bar=20 μm. (B) Vacuoles are not observed, but basophilic granules (black arrows) are observed. H&E staining. Bar=20 μm. (C) Basophilic granules (black arrows) and vacuoles (black arrowheads) are observed. However, the severity of vacuolation was lower than that in samples subjected to 10% NBF fixation. H&E staining. Bar=20 μm. (D) The majority of the vacuolated proximal tubules are negative for KIM-1. Bar=20 μm. (E, F) Some of the vacuoles observed in proximal tubules contained basophilic granules faintly (green arrows). The basophilic granules are clearly observed, and the cell structure is preserved (green arrowheads). H&E staining. Bars=20 μm. (G, H) The membrane surface of the vacuoles are positive for LAMP-2 (blue arrows). Bars=20 mm.

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