The Journal of Toxicological Sciences,
Vol.31, No.3, 191-195, 2006

EFFECTS OF FORMALIN-PRESERVATION ON ELEMENT CONCENTRATIONS IN ANIMAL TISSUES

Itaru SATO[1], Koichiro SERA[2], Tadahiko SUZUKI[1], Haruo KOBAYASHI[1] and Shuji TSUDA[1]

[1]*Department of Veterinary Medicine, Faculty of Agriculture, Iwate University*
3-18-8 Ueda, Morioka 020-8550, Japan
[2]*Cyclotron Research Center, Iwate Medical University*
348-58 Tomegamori, Takizawa 020-0173, Japan

(Received April 1, 2006; Accepted April 17, 2006)

ABSTRACT — Determination of the exposure level of environmental pollutants is essential in studies on environmental toxicology. If concentrations of exposed pollutants in tissues are not affected by formalin-preservation, a preserved specimen will provide not only histopathological information but also the exposure level of environmental pollutants. In the present study, concentrations of nine elements in the liver and kidney were compared between fresh and formalin- or neutral formalin-preserved specimens to validate the ultimate analysis of the preserved specimens. After one year of preservation, various elements had diffused from the specimens into the solutions. The concentrations of iron, copper, zinc (in the case of neutral formalin) and selenium in the central region of the specimens showed no alterations, suggesting that the diffusions of these elements were limited to the surface of the specimens. Therefore, preserved specimens may be available for the determination of these elements if the specimens are large enough for their surface to be removed. Concentrations of other elements in the preserved specimens were different from the original ones, because the diffusion or infiltration also occurred in the deep region of the specimens.

KEY WORDS: Formalin, Preservation, Ultimate analysis, Element

INTRODUCTION

Determination of the exposure level of environmental pollutants is essential in studies on environmental toxicology. Samples of air, water, plants and animals are assayed for this purpose. Animal samples, however, cannot always be obtained, especially in cases of wild animal research. Animal organs or tissues have occasionally been preserved in formalin solution. These specimens can give us significant histopathological information even after long-term preservation, because the microstructure of the tissues is fixed and kept permanently in the formalin solution. If the concentrations of exposed pollutants in tissues are also unchanged during the preservation period, the specimen will provide not only histopathological information but also the exposure level of environmental pollutants simultaneously.

There are a few studies on the validity of ultimate analysis using formalin-preserved tissues. Some investigators have reported that formalin-preservation has no effect on element concentrations in tissues, while others have concluded that formalin-preserved specimens are not available for ultimate analysis because the concentrations of all elements examined were significantly lower than those in the fresh tissues (Bush *et al.*, 1995; Koizumi *et al.*, 1994; Theron *et al.*, 1974). The inconsistency in the results of previous studies may be due to differences in experimental procedures such as preservation period and sample preparation. Therefore, we measured various elements in animal tissues before and after long-term preservation with formalin solutions in order to validate the ultimate analysis of the preserved specimens.

Correspondence: Itaru SATO (E-mail: satoital@iwate-u.ac.jp)

MATERIALS AND METHODS

Samples and treatment

The liver and kidney were used in this experiment, because they are commonly used for the ultimate analysis of animal tissues. A fresh swine liver was purchased from a market and a bovine kidney obtained from a slaughterhouse. To exclude the effect of individual differences, only one organ each was used. Twelve specimens (approx. 1 × 2 × 2 cm) were collected from each organ using a surgical knife. Four of them were analyzed immediately. The remaining specimens were soaked in 100 ml of 10% formalin or 10% neutral formalin (Wako Pure Chemical Industries, Tokyo, Japan), 4 specimens each. After one-year preservation, the surface (3 mm layer) of the preserved specimens was removed (except for the serous side of the kidney) prior to collection of assay samples.

Ultimate analysis

Assay samples (200-300 mg) were collected from each specimen using a surgical knife. The kidney samples were excised in a fan-shape to include the cortex and the medulla, because some elements are distributed unequally between these tissues (Casey *et al.*, 1982; Olsson and Oskarsson, 2001). The samples were put into a pressure-proof Teflon vessel containing the internal standard of indium (200 μg-In/g-tissue, standard solution for atomic absorption spectrophotometry, Wako Pure Chemical Industries) and 1 ml of nitric acid, and incinerated by heating in a microwave oven for one minute. Ultimate analysis was carried out by particle-induced X-ray emission (PIXE) at Nishina Memorial Cyclotron Center, Japan Radioisotope Association to determine the concentration of sodium, phosphorus, potassium, calcium, manganese, iron, copper, zinc and selenium. The formalin and neutral formalin solutions were also assayed by PIXE before and after tissue preservation in order to determine the elements contained in the solutions and the elements diffused from the specimens during preservation.

Statistical analysis

Statistical analysis was carried out by Student's *t*-test or Dunnett's test (Yoshimura, 1987).

RESULTS AND DISCUSSION

Fig. 1 shows the concentrations of nine elements in the formalin and the neutral formalin solutions. Before preservation of tissues, these solutions hardly contained these elements we examined except for sodium and phosphorus in the neutral formalin solution. This solution contained about 1,000 mg/l of sodium and phosphorus, which originated from sodium phosphate added to neutralize the solution.

After preservation of swine liver for one year, potassium, calcium, manganese, iron, copper and zinc concentrations were significantly elevated both in the formalin and in the neutral formalin solution. Sodium and phosphorus concentrations showed no alteration in the used neutral formalin solution, but increased in the formalin solution. Selenium was not detected in either solution even after tissue preservation. This result indicates that various elements diffused from the specimens into the solution during the preservation period.

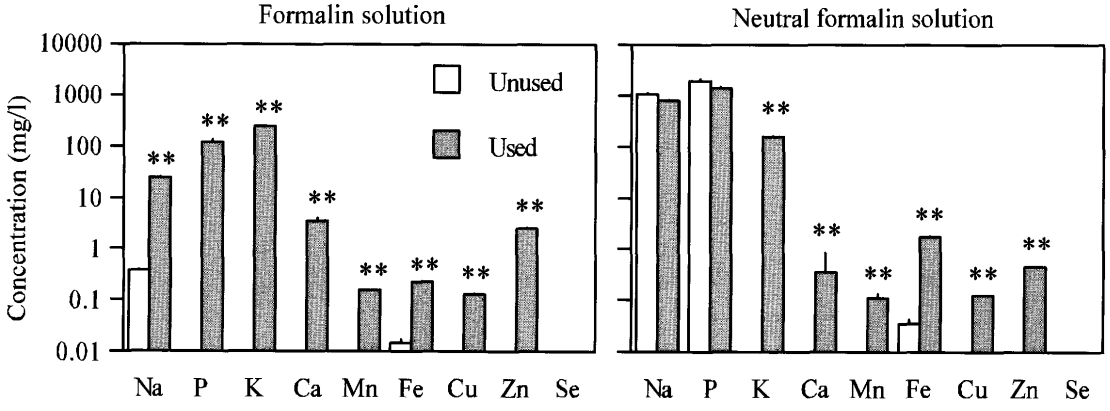

Fig. 1. Concentrations of various elements in formalin and neutral formalin solutions before and after preservation of swine liver. Data are expressed as mean ± SD (n=2). **: significantly different from unused solution at $p<0.01$.

However, sodium and phosphorus may not diffuse from the specimens when preserved in the neutral formalin solution, because this solution originally contains high concentrations of these elements. Conversely, these two elements may infiltrate into the specimens from neutral formalin solution if their concentrations in tissues are lower than those in the solution.

Fig. 2 shows the concentrations of the nine elements in the fresh, formalin-preserved and neutral formalin-preserved swine liver specimens. Concentrations of sodium, phosphorus, potassium, calcium, manganese and zinc were significantly reduced by formalin-preservation, but iron, copper and selenium remained at the same levels as those of fresh tissues. In the neutral formalin-preserved specimens, potassium, calcium and manganese concentrations were reduced, but sodium concentration was elevated substantially. There were no alterations in phosphorus, iron, copper, zinc and selenium concentrations of the neutral formalin-preserved specimens.

Fig. 3 shows the results from bovine kidney. Effects of formalin or neutral formalin on bovine kidney were almost the same as those observed in swine liver (Fig. 2). The decrease in manganese concentration in the preserved kidney was not statistically significant, probably due to the large variance in fresh tissue.

Increase of sodium in neutral formalin-preserved liver and kidney specimens was apparently caused by infiltration from the solution, which contained approximately 1,000 mg/l of sodium. After the preservation period of one year, sodium concentrations were about 800 mg/l in both the specimens and the solution. This indicates that sodium can reach an equilibrium concentration between the specimens and the solutions during the preservation period.

The neutral formalin solution also contained considerable amounts of phosphorus, but its concentration in the specimens was not elevated significantly. As the phosphorus concentration in liver and kidney was comparable to that in the neutral formalin solution, changes in phosphorus concentration were not enough to be detected statistically.

Although the diffusion of various elements from the tissues into the solution had been expected, preserved specimens would be available for ultimate analysis if the diffusion is limited to the surface of the specimens. We thus removed the surface of the preserved specimens before collecting assay samples. Although the diffusion of iron and copper was observed in both solutions, their concentrations in the specimens were not affected by preservation. This suggests that the diffusion of iron and copper is limited to the surface of the specimen. The behavior of selenium may be the same as iron and copper, although the diffusion of this element was not observed in either solution (Fig. 1). Even though diffusion of selenium occurred, it would not be

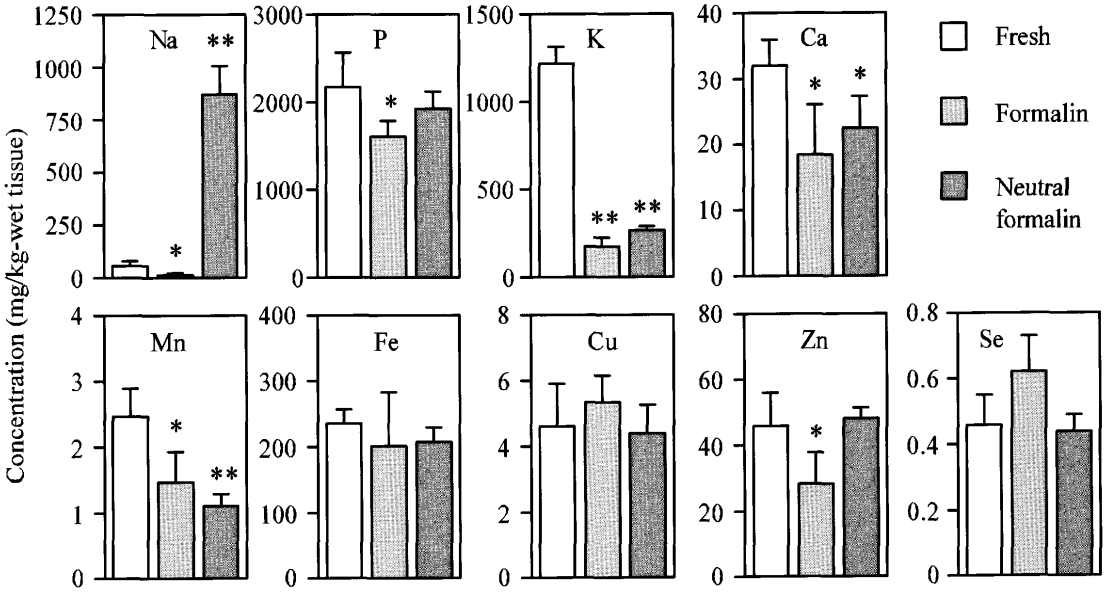

Fig. 2. Effect of formalin-preservation on concentrations of various elements in swine liver. Data are expressed as mean ± SD (n=4). * and **: significantly different from fresh tissue at $p<0.05$ and $p<0.01$, respectively.

detected in the solution because of its very low concentration in swine liver.

Zinc concentration decreased in the formalin-preserved specimens, but not in the neutral formalin-preserved specimens. As the formalin solution is acid (pH 3.6), zinc mobility may be dependent on the pH of the solution. The decrease of potassium, calcium and manganese in the specimens indicates that these elements can diffuse not only from the surface but also from the deeper region of the specimens.

The difference in the mobility of elements may be explained to some extent by the state of each element in tissues. Mobile elements such as sodium and potassium are mostly in the ionic state, while immobile elements are firmly bound to proteins such as ferritin and hemoproteins for iron, superoxide dismutase and metallothionein for copper and zinc, and glutathione peroxidase for selenium (Mertz, 1987).

Bush *et al.* (1995) measured the concentrations of 10 elements in fresh and neutral formalin-preserved specimens collected from 30 human subjects by autopsy, and concluded that formalin-fixation and preservation has little effect on most element concentrations in tissues. However, their preservation period was only one week except for an analysis of aluminum and manganese. As concentrations of aluminum and manganese varied following prolonged preservation of up to one year, their results do not exclude the possibility that long-term preservation affects various elements.

Koizumi *et al.* (1994) compared iron, copper, zinc and cadmium concentrations between fresh and formalin-preserved tissues (liver and kidney), and reported that the concentrations of these elements in preserved tissues were significantly lower than in fresh ones. In our present study, zinc concentration was decreased by preservation with formalin, but iron and copper concentrations remained unchanged. This inconsistency may be due to the difference in sample preparation, although their procedure was not detailed in their paper. We did not use the surface of the preserved specimens, from which various elements diffused into the solution, as shown in Fig. 1. If the whole specimen were used, iron and copper concentrations would also have decreased in our study.

Cadmium and lead are important toxic elements to which human and animals are occasionally exposed. However, the effect of formalin-preservation on these two elements could not be estimated in this study, because their concentrations in swine liver and bovine kidney were too low to be determined by PIXE. In general, cadmium and lead concentrations in domestic animals are very low (Doganoc, 1996; Jorhem *et al.*, 1991). Thus, experimentally exposed animal tissues or more sensitive analytical methods should be used to evaluate the effects on these elements.

In the present study, it was confirmed that various elements diffuse from the tissues into the formalin

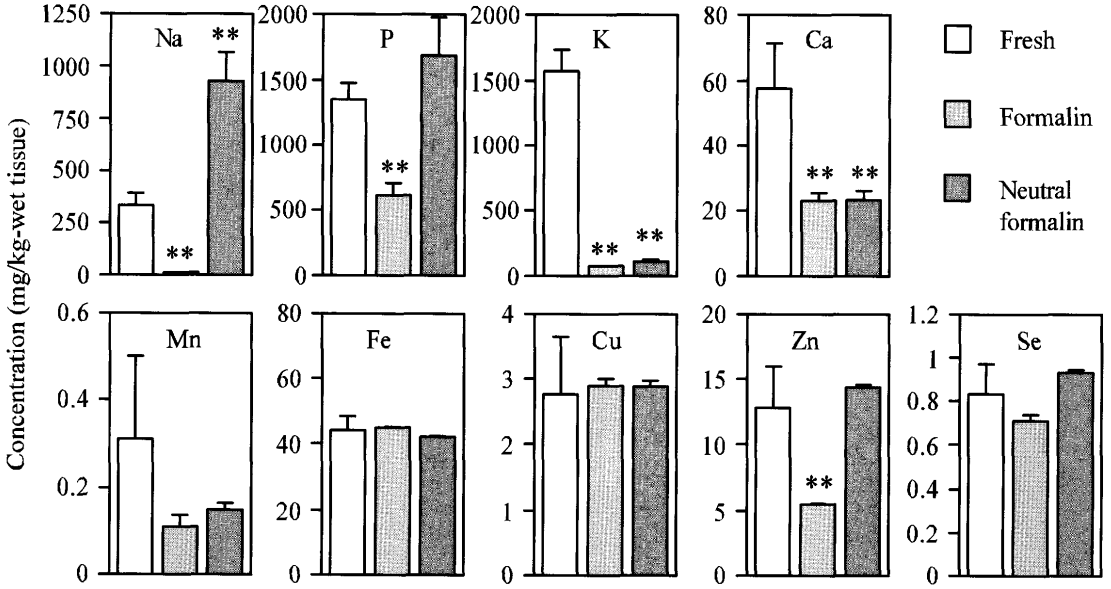

Fig. 3. Effect of formalin-preservation on concentrations of various elements in bovine kidney. Data are expressed as mean ± SD (n=4). **: significantly different from fresh tissue at $p<0.01$.

solution during the preservation period. However, preserved specimens may be available for determination of iron, copper, zinc (in the case of neutral formalin) and selenium if the specimens are large enough to remove their surface, because the diffusions of these elements were limited to the surface of the specimens. Original concentrations of other elements cannot be determined with preserved specimens, because their diffusion or infiltration also occurs in the deep region of the specimens.

ACKNOWLEDGMENT

This study was carried out as one of the common-use studies of Nishina Memorial Cyclotron Center, Japan Radioisotope Association.

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