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Histopathological response of flounder, *Paralichthys olivaceus*, treated with formlin and neutral-formalin

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In this study, poikilocyte rate and histopathological observation of flounder, *Paralichthys olivaceus*, treated with formalin, neutral-formalin(0, 50, 100, 150, 200, 300 ppm) at two different temperatures (15 and 25℃) were conducted to determine the time need for recovery. In all test chamber, formalin was more toxic than neutral-formalin and moderate lesions were not observed at low concentration of formalin and neutral-formalin(50~100 ppm). As expectedly, time need for recovery of gill and kidney tissues was lengthening as increasing chemical concentration and temperature. Treatments caused edema, winding of secondary gill lamella, seperation of epithelial layer and thrombosis of secondary gill lamella in the gill; edema, hydropic and hyaline droplet degeneration in the kidney. Representatively, recovery period of fish gill that had been exposed to 300 ppm formalin and neutral-formalin was about 120 and 72 hr(25℃), 72 and 48 hr(15℃). Recovery period of kidney was about 72 and 48 hr(25℃), 48 and 48 hr(15℃) respectively. Maximum value of poikilocyte rate(27.84%) was shown in formalin 300 ppm treated fish at 25℃. Tendency of decreasing poikilocyte rate was similiar to recovery of tissues in treated fish.

Key words : Formalin, Neutral-formalin, Temperature, Flounder(*Paralichthys olivaceus*), Recovery, Poikilocyte, Histopathology

The intensified interest in flounder, *Paralichthys olivaceus*, as an economically important fish in korea has caused fish hatcheries and farms to place more emphasis on the production of flounder. Many studies about flounder has conducted by culturists, and farmers try to control the environmental condition, diseases and parasites in the field. Especially to culture flounder efficiently, dieases, parasites and pests must be controlled. And also, it must be consider that safty use of chemicals for treatments(Cruze and Pitogo, 1989). Among the many chemicals, formalin has been widely used in fish husbundary to control such external parasites. However, high concentration of formalin treatment can be expectedly toxic and substantial mortalities may occur(Wedemeyer and Yasutake, 1974 ; Reardon and Harrell, 1990). Commercial formalin, containing the paraformaldehyde, has been used directly in the field. But, it was reported that paraformaldehyde cause more

toxiside effects on fish and marine environment when suspending in the water(Rucker *et al.*, 1963). Park *et al.*, (1995), in a progress report, briefly described that the 1-, 2- and 4-hr median lethal concentration values(LC_{50}) of flounder were 2,520 ppm, 1,610 ppm and 868 ppm formalin, respectively. Toxicity is also quite species-dependent with flounder, Paralichthys olivaceus, being less sensitive than other fish. Generally, cultured fishes were exposed to formalin 50~300 ppm for 1 hour as control, but interval of treatment was irregular.

To our knowledge, no previously published report has described time of recovery on different formalin concentration and temperature. For that reason, the present experiment was designed to determine the time need for recovery by poikilocyte rate and histopathological observation associated with formalin, neutral-formalin treatments, at two different temperatures.

Material and Methods

Flounder, *Paralichthys olivaceus*, were obtained from hatchery in Tol-san, Yochun kun, Chunnam province. Fishes were acclimatized and rared in two circular-filteration tank which equiped with raring tank(105 ㎝×136 ㎝×150 ㎝) and filtering tank(79 ㎝×76 ㎝×59 ㎝) for two months at 15 and 25℃ temperatures. Water temperature was either continuously increased or decreased 0.5℃ /day until the 25 and 15℃ experimental temperatures were reached. We continuously controlled water temperatures to within ±0.5℃ by electronic thermometer. Experiments were conducted under stastic condition in black glass-closed system aquaria filled with 20 L of clean sea water. Dissolved oxygen level was above 6.0 ppm and salinity was 32.5 . Fishes were starved 24 hr prior to test.

Ten fishes, average body weight 9.5±1 g(10±1 ㎝), were selected and placed in each test chamber. Experiments were run in 0 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 300 ppm formalin and neutral-formalin. Exposure time was one hour and each of these treatment was tested at two different water temperatures 15 and 25℃. Right after the exposure, fishes were immediately moved into the water tank filled with fresh sea water. Samples of each test group were randomly removed at desired time interval(0, 24, 48, 72, 96 and 120 hour). Caudal artery blood samples of fish was taken in blood sample bottle that contain the EDTA-K_2, these blood sample were stained with cyanide stain and examined microscopically to calculate poikliocyte rate. Gill and kidney tissues of each sample for histopathological observation were fixed with Bouin's solution for at least 24 hr. After the dehydration and clearfying series of alcohol-xylene, tissues were embaded in paraffin. Tissue sections were cut at 5~6 μm and stained with haematoxylin and eosin. Stained tissues were examined microscopically. Gill and kidney were qualitatively examined for general pathology, special categories of examination included edema of gill lamella, winding of the secondary gill lamella, seperation of epithelial layer in the gills ; edema of renal tubule, hydropic degeneration, hyaline droplet degeneration of renal tubule epithelium in the kidney.

Results and Discussion

In this study, mortality and moribund fishes were not observed during the experiment. Al-

though the intensity of cell damage increased with increasing chemical concentration, degree of lesions on treated fish gill and kidney were not noticiable compare to other fishes, since the flounder, *Paralichthys olivaceus*, has less sensitivity for formalin treatment than other fishes.

During the experiment periods, severe gill and kidney lesions were not observed in all test chamber. Also, moderate lesions were not observed at until formalin 150 ppm and neutral-formalin 200 ppm. Type of histological changes in the gill were qualitatively similar to those previously reported for formalin treated salmonid fish(Wedemeyer, 1971). The normal histology of the flounder gill filament is shown in Plate. 1-①. Following exposure to formalin and neutral-formalin, different histological structure were note, particularly in epithelium. Mild edema and winding of secondary gill lamella were occured in fish treated with 150 ppm neutral formalin, at 25℃(Plate. 1-②). Seperation of epithelial layer, hypertropy and scattered necrosis of the lamellar epithelial cell were observed in formalin(200 ppm) treated fish at 15℃(Plate. 1-③). Thrombosis of secondary gill lamella was noted in formalin 300 ppm treated fish at 25℃(Plate. 1-④). Such degenerative changes of gill were have been reported in fishes following exposure to potassium permanganate, un-ionized ammonia and aluminum(Shin *et al.*, 1993 ; Soderberg, 1985 ; Mueller *et al.*, 1991). And death of formalin-treated trout is due to severe gill pathology, resulting in their inability to maintain osmotic and acid-base balance(Smith and Piper, 1972).

After the treated fishes were moved into formalin-free seawater, recovery of fish gill by time passed, was shown in Table 1. As expectedly, time need for recovery of tissues was lengthening as increasing chemical concentration. Representitively, recovery period of gill that had been exposed to 150~300 ppm concentration of formalin and neutral-formalin for one hour was about 96 and 72 hr(25℃), 72 and 48 hr(15℃) in 150 ppm ; 120 and 72 hr(25℃), 72 and 48 hr(15℃) in 200 ppm ; 120 and 72 hr(25℃), 72 and 48 hr(15℃) in 300 ppm, respectively.

Plate. 2-① shows the normal structure of flounder kidney. Mild edema of renal tubule were observed in fish treated with formalin 150 ppm at 15℃(Plate. 2-②). Renal tubule epithelium showing hyaline droplet degeneration in formalin 300 ppm treated fish at 15℃(Plate. 2-③). Exposure of fishes to high levels of formalin (300 ppm) at 25℃, result in extensive hydropic degeneration and nuclear swelling of renal tubule epithelium (Plate. 2-④). Hyaline droplet degegeneration of renal tubule epithelium was shown in cutthroat trout fry exposed to ammonia and nitrite(Robert *et al.*, 1978). Cruze and Pitogo(1989) reported that tubular degeneration and deposition of pigments were observed occasionally in fish exposed to sublethal levels of formalin.

Recovery of fish kidney by time passed, was shown in Table 2. Recovery period of kidney that had been exposed to 150~300 ppm concentration of formalin and neutral-formalin was about 72 and 48 hr(25℃), 48 and 48 hr(15℃) in 150 ppm ; 72 and 48 hr(25℃), 48 and 48 hr(15℃) in 150 ppm ; 72 and 48 hr(25℃), 48 and 48 hr(15℃) in 300 ppm, respectively. In the kidney, according to treatment concentration, there was no different of recovery period significantly.

Gills of milkfish that had been exposed to various concentration of formalin for 96 hr and then

Table 1. Degree of gill degeneration of the flounder treated with various concentration of formalin and neutral-formalin at two different temperature(15 and 25℃)

Hours			0			24			48			72			96			120		
Chem.	Temp.(℃)	Conc.	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
F	25	0	−	−	−	±	−	−	−	±	−	−	±	−	−	−	−	−	−	−
		50	±	−	−	±	−	−	±	−	−	±	−	−	−	−	−	−	−	−
		100	±	±	−	±	±	−	±	−	−	±	−	−	±	−	−	−	−	−
		150	+	±	−	++	−	−	+	+	−	−	+	−	−	−	−	−	−	−
		200	++	+	−	++	+	−	+	+	−	+	+	−	+	−	−	−	−	−
		300	++	++	+	++	++	−	++	+	−	++	+	−	+	+	−	±	−	−
F	15	0	−	−	−	−	±	−	−	−	−	±	−	−	±	−	−	−	−	−
		50	±	−	−	−	−	−	±	−	−	±	−	−	−	−	−	±	−	−
		100	±	±	−	±	±	−	±	−	−	−	−	−	−	±	−	−	−	−
		150	++	−	−	++	−	−	+	±	−	−	−	−	±	−	−	±	−	−
		200	++	±	−	+	±	−	+	+	−	±	±	−	±	±	−	±	±	−
		300	++	+	−	++	+	−	+	+	−	±	±	−	±	−	−	±	−	−

Hours			0			24			48			72			96			120		
Chem.	Temp.(℃)	Conc.	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
N−F	25	0	−	−	−	±	−	−	−	−	−	−	±	−	−	−	−	−	±	−
		50	±	−	−	−	−	−	±	−	−	±	−	−	−	−	−	±	−	−
		100	±	±	−	±	−	−	±	−	−	−	±	−	±	−	−	−	−	−
		150	+	−	−	+	±	−	+	+	−	±	−	−	±	±	−	±	−	−
		200	+	+	−	++	+	−	+	+	−	±	±	−	±	−	−	±	−	−
		300	++	+	−	+	++	−	++	+	−	±	−	−	±	−	−	±	−	−
N−F	15	0	−	−	−	−	±	−	−	−	−	±	−	−	±	−	−	−	−	−
		50	±	±	−	±	−	−	±	−	−	−	±	−	±	−	−	−	−	−
		100	±	±	−	±	−	−	±	−	−	±	−	−	±	−	−	−	−	−
		150	+	+	−	+	+	−	±	±	−	−	±	−	−	±	−	±	−	−
		200	++	+	−	+	+	−	±	±	−	±	±	−	±	±	−	±	−	−
		300	++	+	−	+	+	−	±	±	−	±	−	±	−	±	−	±	±	−

*A : Edema of gill lamella, B : Winding of the secondary gill lamella, C : Seperation of epithelial layer, ± : slight, + : mild, ++ : moderate

Table 2. Degree of kidney degeneration of the flounder treated with various concentration of formalin and neutral-formalin at two different temperature(15 and 25℃)

Hours			0			24			48			72			96			120		
Chem.	Temp.(℃)	Conc.	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
F	25	0	−	−	−	±	−	−	−	−	±	−	±	−	−	−	−	−	±	−
		50	±	±	−	±	−	−	−	−	−	±	−	−	−	−	−	±	−	−
		100	±	−	±	±	−	+	±	±	−	±	−	−	−	−	−	−	−	−
		150	++	−	−	++	−	+	+	−	−	±	−	±	±	−	−	−	−	−
		200	++	+	−	++	−	−	+	±	−	±	±	−	±	−	−	±	−	−
		300	++	+	+	++	++	−	+	+	−	±	−	−	±	±	−	−	−	−
F	15	0	−	−	±	−	±	−	−	−	−	±	−	−	±	−	−	−	−	−
		50	±	−	−	−	−	−	±	−	−	±	−	−	−	−	±	−	−	−
		100	±	−	±	±	−	−	±	−	−	±	−	±	−	±	−	±	−	−
		150	+	−	±	+	−	−	±	±	−	±	±	−	±	−	−	−	±	−
		200	+	+	−	++	−	±	±	±	−	±	−	−	±	−	±	−	−	−
		300	+	+	+	++	−	+	±	±	−	±	−	−	−	−	−	−	−	±

Hours			0			24			48			72			96			120		
Chem.	Temp.(℃)	Conc.	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
N−F	25	0	−	−	−	−	−	±	−	−	−	−	±	−	−	±	−	−	−	−
		50	−	−	−	±	−	−	±	−	−	±	−	−	−	−	−	±	−	−
		100	±	−	±	±	−	±	±	±	−	±	−	−	−	±	−	−	−	−
		150	+	−	+	+	−	−	±	−	−	±	−	±	−	−	−	−	−	−
		200	++	−	−	+	−	+	±	−	−	±	−	±	±	−	−	±	−	−
		300	+	+	−	++	−	−	±	−	±	−	−	−	±	−	−	−	−	−
N−F	15	0	−	−	−	−	−	−	−	−	−	±	−	−	±	−	−	−	−	−
		50	−	−	−	−	±	−	±	−	−	−	−	−	−	−	±	−	−	−
		100	−	−	−	±	−	−	±	−	±	±	−	−	−	−	−	±	−	−
		150	+	−	−	−	+	−	−	−	±	±	±	−	±	−	−	±	−	−
		200	+	−	+	+	+	−	±	±	−	±	−	−	±	−	±	−	±	−
		300	−	++	+	+	−	±	±	−	−	−	−	±	−	±	−	−	−	−

*A : Edema of renal tubule, B : Hydropic degeneration, C : Hyaline droplet degeneration of renal tubule epithelium, ± : slight, + : mild, ++ : moderate

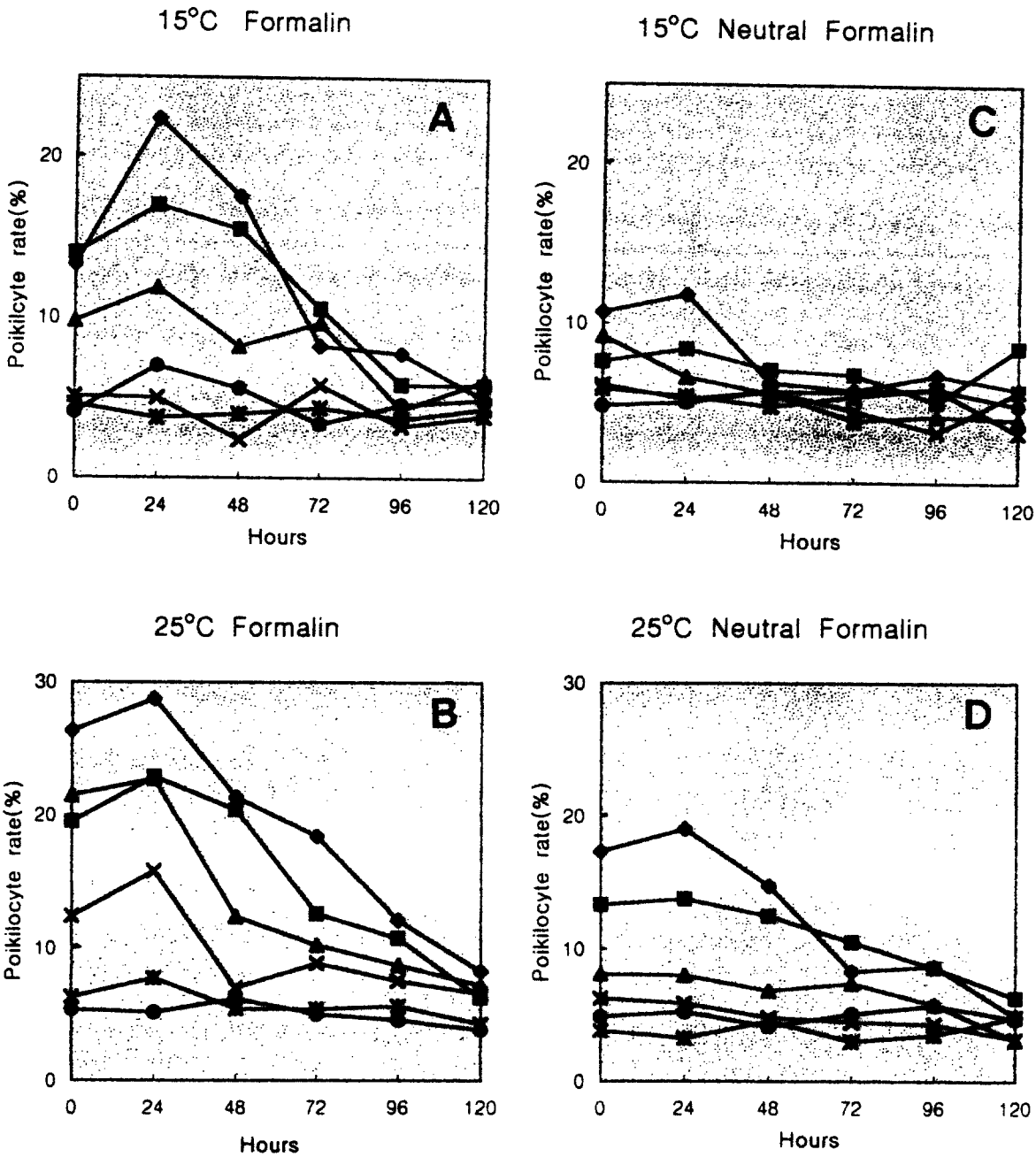

Fig. 1. Poikilocyte rate in formalin treated flounder at 15℃(A), 25℃(B), and in neutral-formalin treated fish at 15℃(C), 25℃(D). (-◆- 300ppm -■- 200ppm -▲- 150ppm -×- 100ppm -✳- 50ppm -●- 0ppm)

allowed to recover for 24 hr in formalin-free water were comparable to those of normal fish kidney tissue exposed to 300 ppm formalin for 96 hr and allowed to recover exhibited minimal histological chages(Cruze and Pitogo, 1989).

In the present study, although gill and kidney histological changes was seen, these degeneration was adequately recovered in a short period, since exposure time of formalin and neutral formalin (50~300 ppm) was just one hour. Wedmeyer and Yasutake(1974) indicated that the acid-base balance of two salmonids was adequately compensated and total plasma osmolity was not appreciable disturbed during a one hour exposure, although gill histopathological changes are seen.

Smith and Piper(1972), in a progress report, briefly described that poikilocyte value was elevated in formalin treated rainbow trout. Also, in the present study, increasing of poikilocyte rate was observed in treated fishes. It may suggest that response to hypoxia resulting from damage to gill respiratory epithelium. Poikilocyte rate value in this study indicated that formalin was more toxic than neutral-formalin, and values were little bit higher in 25℃ than 15℃. Similarly, in the previous study, the elimination of formaldehyde in treated korean rockfish was temperature-dependent, and residue of formaldehyde in formalin treated fish was showed little bit higher than in neutral-formalin treated fish(Cho and Yang, 1996). Maximum value of poikilocyte(28.74%) was shown in formalin 300 ppm treated fish at 25℃, 24 hr after moved into fresh-sea water. Tendency of decreasing poikilocyte rate was similiar to recovery of tissues in treated fish. Thus, we suggest that use of neutral-formalin would better than formalin when treat fish for health and since it is difficult to control the parasite after infected fish, it will must control the external parasites, as scutica, before infected fish in the field. Also, it would probably be wise to avoid and fish-cultural operation which would impose additional stress during this recovery period, these would include pond cleaning and handling or stocking immediately following formalin treatment.

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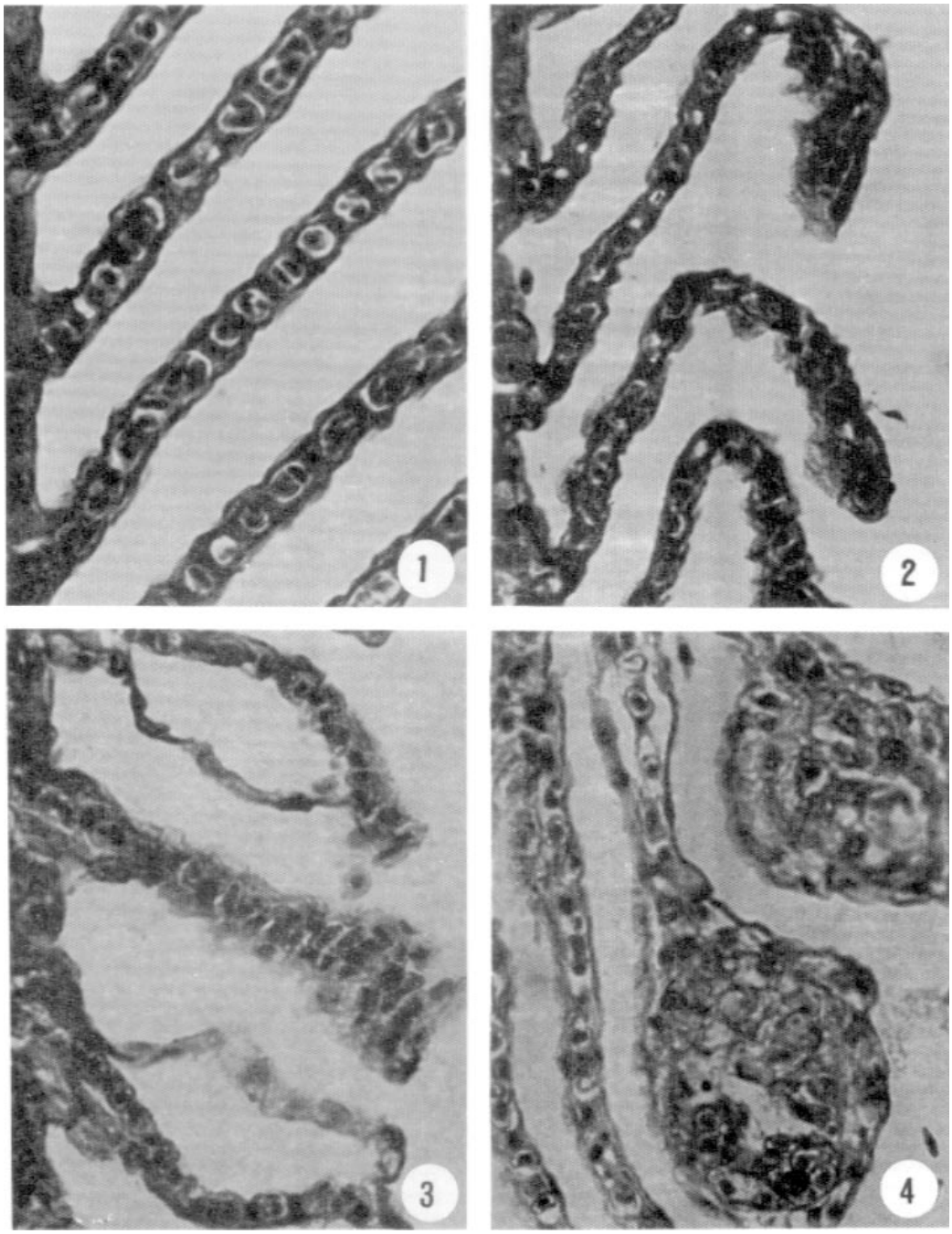

Plate. 1. ① : Normal gill tissue from control. ×450. ② : Mild edema and winding of secondary gill lamella, neutral-formalin 150 ppm(25℃). ×450. ③ : Seperation of epithelial layer, hypertropy and scattered necrosis of lamella epithelial cell, formalin 200 ppm(15℃). ×450. ④ : Thrombosis of secondary gill lamella, formalin 300 ppm(25℃). ×450.

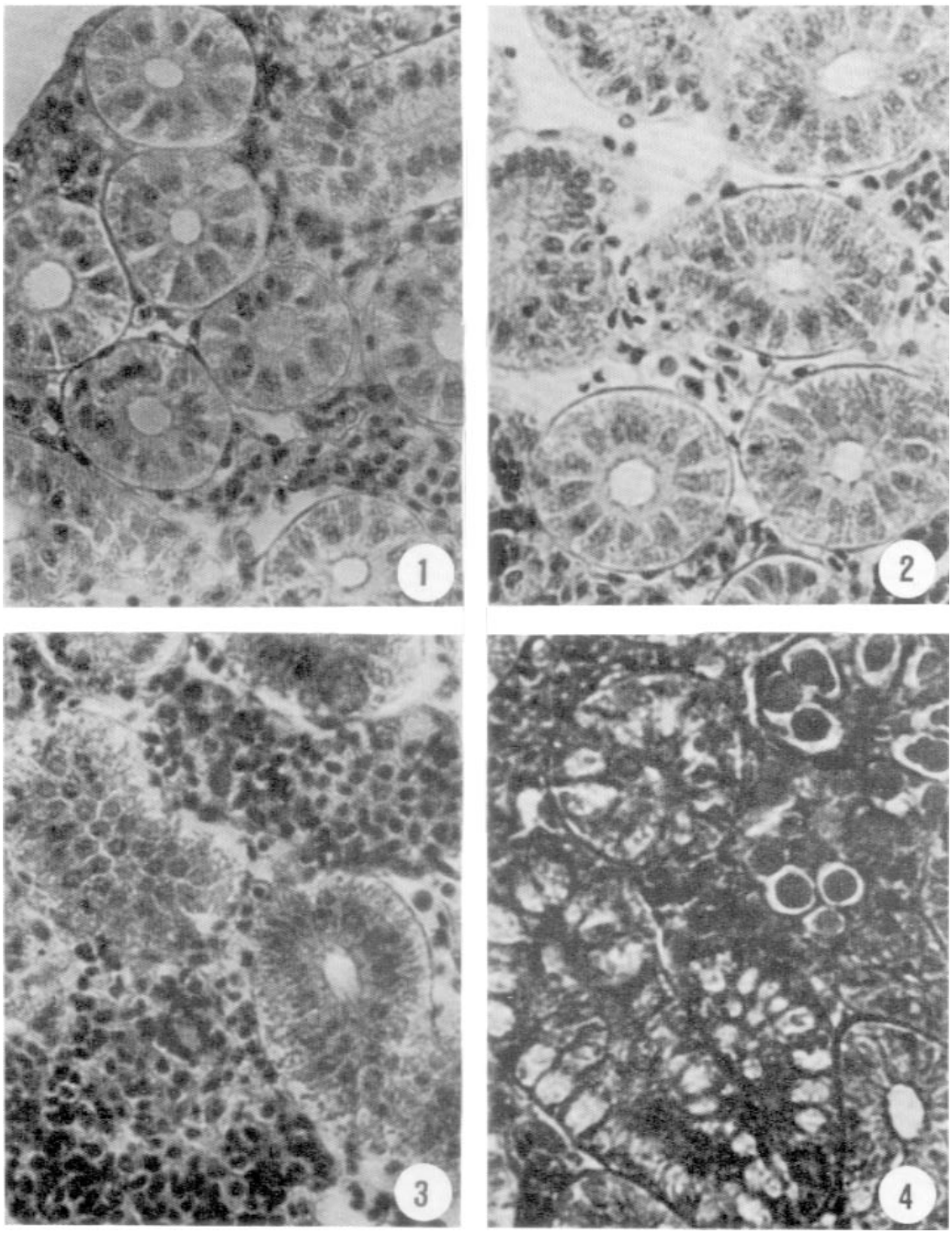

Plate. 2. ① : Normal kidney tissue from control. ×450. ② : Mild edema of renal tubule, formalin 150 ppm(15℃). ×450. ③ : Hyaline droplet degeneration of renal tubule epithelium, formalin 300 ppm(15℃). ×450. ④ : Extensive hydropic degeneration and nuclear swelling of renal tubule, formalin 300 ppm(25℃). ×450.

포르말린 및 중성 포르말린 약욕 넙치(*Paralichthys olivaceus*)의 병리조직학적 반응

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본 실험에서는 두 온도 조건(15, 25℃)하에서 포르말린과 중성 포르말린(0, 50, 100, 150, 200, 300 ppm)으로 약욕한 넙치(*Paralichthys olivaceus*)의 회복에 필요한 시간을 알아보기 위해 아가미와 신장의 병리 조직학적인 관찰과 혈액내 변형 적혈구율이 조사되었다. 모든 실험구에서 포르말린이 중성 포르말린보다 독성이 강하게 나타났고, 낮은 농도(50~100 ppm)에서는 병변이 아중증까지 진행되지 않았다. 예상대로, 약욕 농도와 온도가 증가할수록 회복시간이 길어졌다. 약욕에 의한 아가미의 병변은 부종, 2차 새변의 뒤틀림, 상피세포의 박리, 혈전 증상이 나타나고 신장에서는 부종, 수증성 퇴행성 병변, 초자적 변성이 일어났다. 대표적으로, 포르말린과 중성 포르말린 300 ppm에 침지된 어류의 아가미 조직 회복시간은 25℃조건의 포르말린에서는 약 120시간, 중성 포르말린에서는 72시간과 15℃조건의 포르말린에서는 72시간, 중성 포르말린에서는 48시간 정도였다. 신장 조직의 회복은 25℃조건 포르말린에서는 72시간, 중성 포르말린에서는 48시간 정도였고 15℃조건의 포르말린과 중성 포르말린에서는 다같이 48시간 정도였다. 혈액내 변형적혈구율의 최대 수치는 25℃ 수온조건, 포르말린 300 ppm 구에서 27.84%로 나타났다. 변형적혈구율의 감소 추세는 조직의 회복과 비슷한 양상을 보였다.

Key words : Formalin, Neutral-formalin, Temperature, Flounder(*Paralichthys olivaceus*), Recovery, Poikilocyte, Histopathology