Degradation of Myocardiac Myosin and Creatine Kinase in Rats Given Alkaline Ionized Water

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ABSTRACT. Recently, the authors have shown that marked necrosis and fibrosis of myocardium were observed in rats given alkaline ionized water (AKW). To clarify the cause of myocardial lesions, the activities of myosin ATPase, actomyosin ATPase and creatine kinase (CK) in myocardium of rats given AKW at 15 weeks-old were compared with those in myocardium of rats given tap water (TPW). Furthermore, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of myocardiac myosin and isoelectric focusing (IEF) of myocardiac CK were performed which revealed a distinct difference between AKW and TPW groups. The activities of myosin ATPase and actomyosin ATPase in the AKW group were higher than those in the TPW group, and these elevated activities were caused by the degradation of myosin in the AKW group judging from the SDS-PAGE pattern of myosin. On the other hand, the activity of CK in the AKW group was lower than that in the TPW group, and the IEF pattern of CK showed leakage of myocardiac CK. These results indicate that increases in actomyosin ATPase activity and myosin ATPase activity, plus the decrease in CK activity caused the disorder of coupled reaction in male rats given AKW at 15 weeks-old. It is concluded that this disorder of coupled reaction may cause marked myocardiac necrosis and fibrosis in rats given AKW. — KEY WORDS: AKW, creatine kinase, myocardiac myosin.

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Alkaline ionized water (AKW) is used for the purpose of supplementing electrolytes and water lost by perspiration during exercise and for preserving health, because AKW is easily absorbed by the body and is excellent at regulating osmotic pressure *in vivo*. With regard to experimental animals given AKW, Watanabe and Shirai [19] reported that the body weight increment of the same litter at 3 weeks old borne by mother rats given AKW throughout their gestation and lactation periods was significantly larger than that of the control. Watanabe [20] also reported that AKW had substantial biological effects on postnatal growth, because intake of food and water in the mother rat given AKW was larger and the increment body weight of the offspring after being given AKW from the 14th day of suckling in males, and from the 21st day of weaning in males and females, was also larger than that of those given tap water (TPW), and in addition postnatal morphological development was accelerated. In cattle given AKW, Kuchida *et al.* [11] reported that the color of the meat was much brighter than that of the cattle given TPW, and the change of the color was inhibited by AKW intake. We reported that the body weight and metabolic activity in the rats given AKW increased, and marked necrosis and fibrosis of myocardium were conspicuously observed [21].

To clarify the cause of the myocardiac lesions, the activities of myosin ATPase, actomyosin ATPase and creatine kinase (CK) in rats at 15 weeks old given AKW were compared with those in rats given TPW. Furthermore, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of myosin and the isoelectric focusing (IEF) pattern of CK in the AKW group were also compared with those of the TPW group.

Sprague-Dawley rats (Jcl. SD, Clea Japan Inc., Tokyo, Japan) at 8 weeks of age were obtained and maintained at 23 ± 1°C, 40–60% humidity, 14-hr illumination, and given food (CE-2, Clea Japan Inc.) and water *ad libitum*. After 3 weeks of acclimation, animals without abnormal findings were used. Copulation was induced by placing an experienced male rat of the same strain in one cage made of aluminum with 10 female rats over 12 weeks-old with a regular estrous cycle which was confirmed by prior vaginal smears. The same male rat was used in all experiments. Smears were examined daily by microscope to confirm copulation. On the day sperm appeared on the smear, those females were separated from the male, and this day was designated as day zero of gestation. All pregnant rats were individually housed in a polycarbonated cage for delivery. AKW was subsequently given to gestational rats (test group, n=10). In the controls, the day that sperm appeared on the smear was defined as day zero of gestation, and TPW was given as before (control group, n=10). Copulated females were divided daily into approximately equal test and control sections. The female rats removed were replaced by new female rats so that there would always be 10 per 1 male rat. After weaning at 21 days after delivery, the dams were autopsied under anesthesia with ether, and various organs in the thoracic and abdominal regions were observed in gross, and the number of implantation sites were counted after extraction of the uterus.

At 3 weeks after delivery, the mother animal was weighed, and weaning was performed on the same day. Sex ratio (Male/Female) of weanlings and body weight of the offspring were estimated at 3 weeks after birth, after dividing a litter born from 10 mother animals in the test and control sections into males and females. The season why the body weight of the offspring was not weighed until 3 weeks of age is that the mother rat occasionally eats the offspring if the offspring are touched by hand immediately

after delivery and during lactation. Consequently, the body weights of the offspring were not weighed during the three weeks after delivery before weaning. Male and female F_1 rats were divided at random into groups of 15 animals each. Offspring were housed 5 rats to each plastic cage. For postnatal rats, AKW was given to the test group and TPW was given to the control group from 3 weeks after birth. Feeding was continued until 15 weeks after birth. The body weights of offspring in the litters born to 10 mother animals given AKW and TPW were measured at 3, 5, 7, 9, 11, 13, and 15 weeks. Consumption of food and water was measured weekly.

AKW was prepared from TPW by an electrolysis apparatus (Minekaru TBC-R 6103, Tokyo Seiden Co., Ltd., Tokyo, Japan). The principle was that of electrolysis of an electrolyte solution. Ions transferred varied with the amounts of the reacting substances, hydrogen ion concentration, and flow speed. The pH of AKW was 9.0 and the maximum flow-speed was 140 *l*/hr. AKW was produced daily at noon during the experimental period. Rats in the test group were given only AKW *ad libitum*. Acidic water produced by the flow of anions to the anode was discarded. For purification of AKW, TPW was electrolyzed without medicines.

The pH, alkalinity and ion concentrations in AKW and TPW were determined according to a guideline issued by the Japan Food Hygiene Association, a food-testing organization approved by the Minister of Health and Welfare in accordance with the Japan Food Sanitation Law and Japan Pharmaceutical Affairs Law. The methods and items of the tests are summarized in Table 1.

Whole blood was collected from the jugular vein of rats anesthetized with ethyl ether. Blood samples were examined to determine the number of erythrocytes and leukocytes, hematocrit value, hemoglobin and glucose concentrations. Numbers of erythrocytes and leukocytes and values of hematocrit were measured by the electronic counting method (Sysmex microcell counter CC-110, Toa Medical Electronics Inc., Kobe, Japan), by the capillary method (Toa Medical Electronics Inc.) and by the cyanmethemoglobin method [1]. Blood glucose was measured by the method of *o*-toluidine [10]. Serum was obtained by centrifugation at 500 × g for 5 min with blood coagulated at 4°C for 3 hr. Sodium and potassium concentrations in the serum were determined by a radiometer (KNA 1 sodium-potassium analyzer, Radiometer A/S Copenhagen, Copenhagen, Denmark) and chloride by a chloride meter (CL-7 Chloride Counter, Hiranuma Sangyo Co., Ltd., Mito, Japan) according to the instrument manual instructions. Calcium, magnesium and inorganic phosphorus concentrations in the serum were determined by the methods of *o*-cresolphthalein complexone [2], xylidyl blue [12] and molybdenum blue [18], respectively. Serum protein concentration was determined by a serum protein refractometer. Serum proteins were separated by cellulose acetate membrane electrophoresis (Separax-SP, Jookoo Co., Ltd., Tokyo, Japan) and the electrophoretograms were examined with a densitometer (Model PAN, Jookoo Co., Ltd., Tokyo Japan).

Table 1. Results of testing qualities of alkaline ionized water (AKW) and tap water (TPW)

	Alkaline ionized water (AKW)	Tap water (TPW)
pH[a)e)]	8.7	7.3
Alkalinity (mg/l)[b)]	50	38
Calcium (mg/l)[c)]	20.1	17.5
Sodium (mg/l)[c)]	8.6	7.8
Potassium (mg/l)[c)]	2.1	1.7
Magnesium (mg/l)[c)]	4.4	4.1
Zinc (mg/l)[c)e)]	0.04	0.03
Iron (mg/l)[c)e)]	0.05 (less than)	0.05 (less than)
Chloridion (mg/l)[d)e)]	7.8	9.9

Note) Water qualities by the Japan Food Hygiene Association, as recommended by the Minister of Health and Welfare based on the Japan Food Sanitation Law and Japan Pharmaceutical Affairs Law. a) pH meter method. b) Sulfuric acid neutralization titrimetry method. c) Atomic absorption spectrophotometry method. d) Silver nitrate titrimetry method. e) Indicates use of the method for standardization of water quality based on the Japan Waterworks Act.

After whole blood was collected from the jugular vein of 15 weeks-old rats anesthetized with ethyl ether, their body weights were measured by an automatic balance for rat (Shin Maiko, Yamato Co., Tokyo, Japan), and their organs were removed for weighing with an electronic reading balance (Libror ED-200, Shimadzu Co., Tokyo, Japan).

CK and actomyosin were prepared by the method of Sugita *et al.* [17] with a slight modification. For comparison with the CK isozyme pattern of the myocardiac extract, the heart, male brain and male skeletal muscle (*m. biceps femoris*) of the TPW group (0.5–1.0 g, respectively) were minced with scissors in cold water, followed by homogenization for 2 min in 10 volumes of 20 mM $NaHCO_3$ with a homogenizer (Polytron PTA10-S, Kinematica Littau AG, Switzerland). The homogenate was centrifuged at 10,000 × g for 30 min, and the supernatant was used for CK determination. Actomyosin in the residue was extracted with 10 volumes of 0.66 M KCl and 50 mM Tris-maleate at pH 6.8 overnight, followed by dialyzation against 0.06 M KCl. After centrifugation at 10,000 × g for 30 min, the pellet was dissolved in a solution containing 0.66 M KCl and 50 mM Tris-maleate at pH 6.8. CK activity was determined by the method of Forster *et al.* [7]. Myosin ATPase was determined with actomyosin solution in 0.6 M KCl, 20 mM Tris-maleate at pH 6.8, 1 mM $CaCl_2$ and 1 mM ATP. Actomyosin ATPase was determined in 0.03 M KCl, 20 mM Tris-maleate at pH 6.8, 1 mM $MgCl_2$, 10 mM $CaCl_2$ and 1 mM ATP. Myosin and actomyosin ATPase activities were determined by the method of Sugita *et al.* [17], and the liberated inorganic phosphate was measured by the method of Taussky and Shorr [18].

Myosin and actin-component were partially purified from the pooled male actomyosin solution by the method of Yamaguchi *et al.* [22] and Hirata *et al.* [8], respectively. The relative activity (%) of male actomyosin ATPase was determined both in the presence of Ca^{2+} with no addition of

1 mM ethyleneglycol bis (2-amino ethyl ether) tetraacetic acid (EGTA) and in the absence of Ca^{2+} with addition of 1 mM EGTA.

SDS-PAGE with myosin and actin-component and IEF with CK were carried out with an electrophoresis device (PhastSystem, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the instrument manual. Myosin or actin-component containing 1 μg of protein was applied to SDS-PAGE with polyacrylamide gradient gels (PhastGel Gradient 8–25, Pharmacia LKB Biotechnology AB, Uppsala, Sweden), and detected by coomassie staining with a coomassie R 350 dye (PhastGel Blue R, Pharmacia LKB Biotechnology AB, Uppsala, Sweden). For CK, an 1 μl aliquot of 0.2 U/m*l* was isoelectrofocused with IEF gels (PhastGel IEF 3–9, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and detected by the standing method of Rosalki [16]. The stained bands were scanned using a laser densitometer (UltroScan XL Enhanced Laser Densitometer, LKB-Produkter AB, Bromma, Sweden) and then the scanning pattern was analyzed two-dimensionally with a computer (Personal Computer AT, IBM, U.S.A.) accompanied with software (2400 Gelscan XL Laser Densitometer Program, LKB-Produkter AB, Bromma, Sweden).

All quantitative data were statistically analyzed by Student's *t(Welch)*-test.

Data of the body and organ weight, food and water consumption and hematological measurement have been abbreviated because of the similarities with a previous report [21].

Myocardiac myosin ATPase activity of male rats at 15 weeks-old was 0.210 ± 0.051 U/mg protein for the AKW group and 0.170 ± 0.041 U/mg protein for the TPW group, and a significant difference was observed between the two groups ($p<0.05$). However, those of female rats given AKW and TPW at 15 weeks-old were 0.228 ± 0.068 U/mg protein and 0.205 ± 0.065 U/mg protein, respectively, and there was no significant difference between the two groups ($p>0.4$) (Fig. 1 A). Furthermore, actomyosin ATPase activity of male rats given AKW at 15 weeks-old was 0.077 ± 0.024 U/mg protein which was significantly higher than that of the TPW group, 0.040 ± 0.013 U/mg protein ($p<0.001$), whereas those of female rats given AKW and TPW at 15 weeks-old were 0.061 ± 0.022 U/mg protein and 0.056 ± 0.019 U/mg protein, respectively, with no significant difference being observed between the two groups ($p>0.1$) (Fig. 1 B). The Ca^{2+} dependency of actomyosin ATPase activity of male rats at 15 weeks-old was examined in the presence and absence of Ca^{2+} (Fig. 1 C). In the absence of Ca^{2+} with addition of 1 mM EGTA, actomyosin ATPase activities of the AKW and TPW groups were decreased to 58.0 ± 7.9% and to 56.0 ± 7.5% ($p>0.5$) of that in the presence of Ca^{2+} with no addition of EGTA, respectively, suggesting that Ca^{2+} regulatory proteins in these actomyosins were not affected.

Myosin purified from actomyosin of male rats at 15 weeks-old was digested by myocardiac proteases. As a result of SDS-PAGE, heavy meromyosin (HMM) heavy chain (150 K) and many other digested components (35–78 K) were found in myosin which was composed of a heavy chain (210 K) and two light chains (17 and 25 K) (Fig. 2). When the degraded degree of AKW myosin was compared with that of TPW myosin, the concentration ratio of HMM heavy chain to myosin heavy chain (150 K/210 K) was 5.37 ± 0.66 for AKW myosin and 2.20 ± 0.75 for TPW myosin. The concentration of other digested components of AKW myosin was also higher than that of TPW myosin, and a significant difference was shown between AKW and TPW

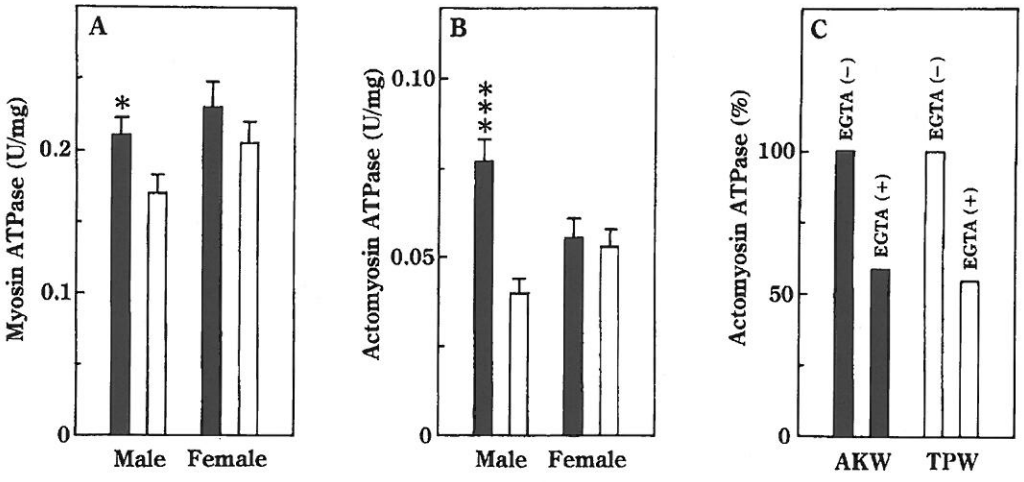

Fig. 1. Myocardiac myosin and actomyosin ATPase activities of males and females fed with alkaline ionized water (AKW) or tap water (TPW). AKW and TPW groups are denoted by a black bar and open bar, respectively. (A) Data are represented as the mean ± standard error, n= 15. *: $p<0.05$. (B) Data are represented as the mean ± standard error, n=15. ***: $p<0.005$. (C) The relative activity (%) of male actomyosin ATPase in the presence of Ca^{2+} added no 1 mM ethyleneglycol bis (2-amino ethyl ether) tetraacetic acid (EGTA (-)) and in the absence of Ca^{2+} added 1 mM EGTA (+).

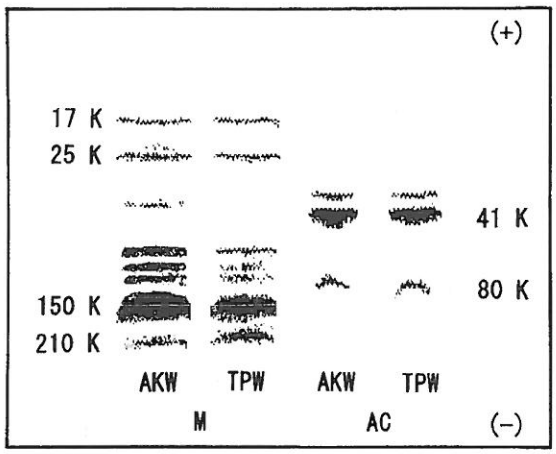

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of myocardiac myosin and actin-component of male groups given alkaline ionized water (AKW) or tap water (TPW). M: myosin and its electrophoretic bands. 150 K: heavy meromyosin heavy chain, 210 K: heavy chain, 17 K and 25 K: light chains. AC: actin-component and its electrophoretic bands. 80 K: troponin, 41 K: actin and tropomyosin.

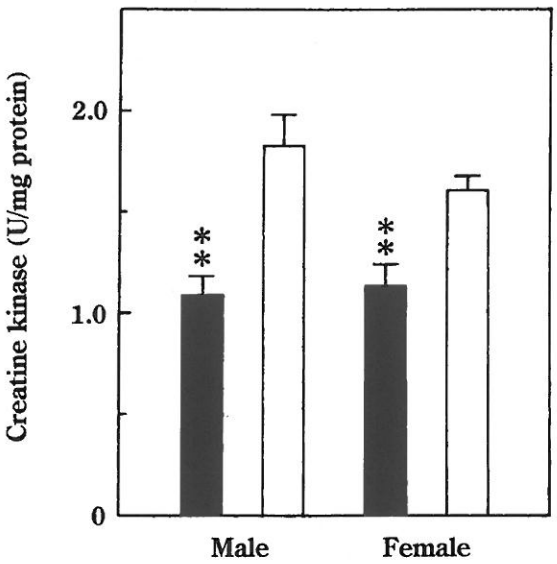

Fig. 3. Creatine kinase activity in the heart extracts of rats at 15 weeks-old given alkaline ionized water (AKW) and tap water (TPW). The CK activities of AKW and TPW groups are denoted by a black and open bar, respectively. Data are represented as the mean ± standard error, n=15. **: p<0.01.

myosin (p<0.001). On the other hand, actin-component purified from actomyosin of male rats was composed of 80 K protein (troponin), 41 K protein (actin and tropomyosin) and an unknown 33 K protein. The concentration ratio (%) was 17.9 ± 7.2, 62.9 ± 6.9 and 19.0 ± 4.9 for AKW actin-component and 18.0 ± 5.3, 64.0 ± 5.9 and 18.0 ± 3.7 for

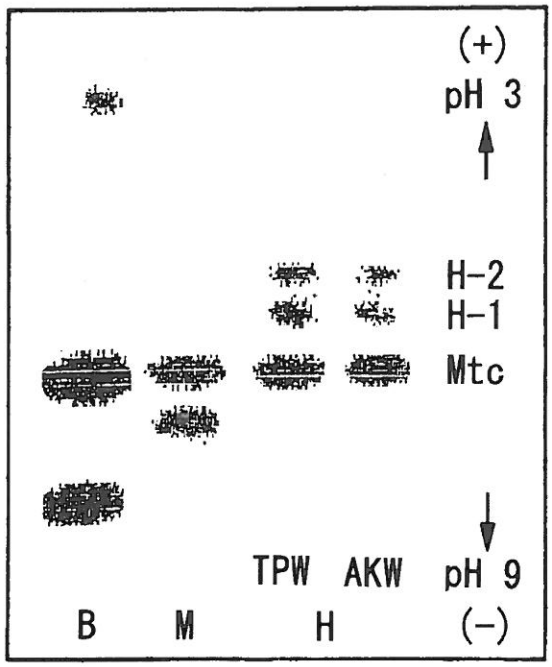

Fig. 4. Isoelectric focusing profile of creatine kinase isozyme of male rats at 15 weeks-old in the heart (H) extracts of alkaline ionized water (AKW) or tap water (TPW) groups. The profile was shown together with the muscle (M) and brain (B) extracts of TPW group for comparison. Mtc: mitochondria type, H-1: heart type-1, H-2: heart type-2, B: brain type, M: muscle type.

TPW actin-component, and no significant differences were observed between the two groups (p>0.9, p>0.6 and p>0.4, respectively).

CK activity in myocardiac extracts of male rats at 15 weeks-old was 1.09 ± 0.33 U/mg protein for the AKW group and 1.83 ± 0.59 U/mg protein for the TPW group, and the latter was significantly higher than the former (p<0.01). Also, CK activity of female rats at 15 weeks old given AKW and TPW was 1.14 ± 0.36 U/mg protein and 1.63 ± 0.18 U/mg protein, respectively, and a significant difference was observed between the two groups (p<0.01) (Fig. 3). When CK isozyme pattern in the myocardiac extract of male rats was compared with that of brain or skeletal muscle extracts, the myocardiac CK was composed of three isozymes, mitochondria type (Mtc), heart type-1 (H-1) and heart type-2 (H-2). All of the isozyme activities of the AKW group were lower than those of the TPW group (Fig. 4): the activities of Mtc, H-1 and H-2 were 0.68 ± 0.17 U/band, 0.30 ± 0.08 U/band and 0.11 ± 0.04 U/band, respectively, for the AKW group, and 1.15 ± 0.40 U/band, 0.51 ± 0.11 U/band and 0.17 ± 0.07 U/band, respectively, for the TPW group. There were significant decreases in Mct (p<0.001), H-1 (p<0.001) and H-2 (p<0.005), when compared with the control values.

The authors have shown in a previous paper that the body weight and erythrocyte hexokinase activity in rats

given AKW at 15 weeks-old increased more than those of rats given TPW [21]. Furthermore, though there was no significant difference between the heart weight of the two groups, histological findings revealed marked necrosis and fibrosis of myocardiac tissue. With regard to cardiac movement, the driving force is derived from the interaction among myocardiac myosin, actin and ATP [5], with the interaction between troponin and tropomyosin regulating the myocardiac contraction [4]. On the other hand, CK is essential to myocardiac contraction for supplement of ATP and is measured as a variable marker enzyme in myocardiac disorder [3, 9]. Taking these concepts into consideration, we compared the changes of activities on myocardiac contractile protein and CK between AKW and TPW groups.

The activities of myocardiac myosin ATPase and actomyosin ATPase in male rats given AKW at 15 weeks-old were significantly higher than those given TPW (Figs. 1 A and B). Both myosin and actomyosin ATPase activities in female rats given AKW at 15 weeks-old were also higher than those given TPW, but no significant differences were observed. Actomyosin ATPase is not controlled by Ca^{2+}, but its susceptibility to Ca^{2+} could be activated by a tropomyosin-troponin complex of myosin B which is an important protein essential in myocardiac contraction and relaxation. Therefore, an experiment was conducted for checking the dependence of actomyosin ATPase on Ca^{2+} in order to confirm the grade of the myocardiac necrosis. No change was observed in susceptibility to the calcium ion of actomyosins both in the AKW and TPW groups, and these results suggest that regulatory proteins in actomyosin of AKW are not affected (Fig. 1C).

SDS-PAGE on myocardiac contractile proteins revealed that myosins of the AKW and TPW groups were degraded into HMM , but not actin-component (Fig.2). The degree of degradation of AKW myosin into HMM was larger than that of TPW myosin. Since HMM ATPase activity is 1.2–1.5 times higher than that of myosin, and acto-HMM ATPase activity is also higher than that of actomyosin ATPase [6, 14], the increase in HMM resulting from degradation of myosin of the AKW group may elevate myosin and actomyosin ATPase activities more than those of the TPW group (Fig. 1). Murakami and Uchida [15] reported that myosin-cleaving protease activity, which was high in rat cardiac myofibrils, was capable of degrading myosin and actomyosin. This result supports the view that the necrosis and fibrosis of rat myocardiac muscle occurs naturally, especially in male rats and rabbits [13], and this myocardiac degeneration develops further in the AKW group. Furthermore, the myocardiac CK activity of the male AKW group was significantly lower than that of the TPW group (Fig. 3), and the decrease appeared to result from the leakage of Mtc, H-1 and H-2, judging from the IEF profile of the CK isozyme in the extract (Fig. 4).

From the present results, it may be concluded that the increase of myosin and actomyosin ATPase activities and the decrease of CK activity in rats given AKW cause the disorder of coupled reaction. The resulting unbalance between myocardiac contraction and relaxation may increase myosin-cleaving protease activity, inducing myocardiac necrosis and fibrosis.

The enzyme activity of the myocardium significantly increased in the male rats but only slightly in the female rats when compared with the control ones. The factor directly causing the difference is unknown. In addition, the body weight in 11 to 13-week-old male rats given AKW was about 2 times that of female rats also given AKW. Therefore, it is possible that the marked increase of the body weight in the male rats might be one of the factors causing the enzyme activity change in myocardium and tissues, creating the difference with the females. The body weights of the offspring born to mother rats given AKW during their gestation and lactation showed a significant increase in both males and females compared with those of offspring born to mother rats given TPW. One possible explanation is as follows. AKW is produced by the movement of cations in TPW towards the cathode through electrolysis. The amount of ions transferred depends on the quantity of the electrolytes, pH, and flow rate. Since only cations are transferred to AKW, the total amount of the cations in AKW would accordingly be increased. The alkalinity of AKW being higher than that of TPW may support such a hypothesis. Consequently, AKW contains a higher concentration of cations which agrees with the actual measurement of electrolytes in AKW and TPW.

Since most of the cations transferred to AKW are hydrated with water molecules through electrolysis, they are rapidly absorbed and readily employed in an active form. The increase of the body weight in the offspring might therefore occur due to the water-hydrated cations acting as a nutritional supplement transferred to fetus through the placenta or milk. It is still unclear whether the increase of the body weight in the offspring was caused by the increase of the milk secretion and the improvement of milk quality in response to the supply of AKW with a high concentration of cations, or induced by the supply of AKW to the mother rats during the gestation and lactation. The abnormal increase of the body weight in the offspring deserves further study. In addition, the significant increase of both food and water intakes in the group given AKW is another question to answer.

The increased body weight in the offspring is a phenomenon important enough for further study. Moreover, the causes of myocardiac necrosis remain to be determined. It is possible that the necrosis might be caused either by AKW or by some unknown material dissolved in AKW from the electrode of the electrolytic water ionizer used in this study.

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