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Sodium fluoride induces hypercalcemia resulting from the upregulation of both osteoblastic and osteoclastic activities in goldfish, *Carassius auratus*

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ABSTRACT

The influence of sodium fluoride (NaF) on calcium metabolism was examined in goldfish (fresh water teleost). At 2 days after administration of NaF (500 ng/g body weight; 5μg/g body weight)(around 10^{-5} to 10^{-4} M in goldfish), we indicated that plasma calcium levels upregulated in both doses of NaF-treated goldfish. To examine the mechanism of hypercalcemia by NaF treatments, therefore, direct effects of NaF on osteoblasts and osteoclasts in goldfish were investigated by an original assay system using teleost scale which has osteoblasts, osteoclasts and bone matrix. Alkaline phosphatase activity in the scales increased with the treatment of NaF (10^{-6} and 10^{-5} M) during 6 hours of incubation. Also, tartrate-resistant acid phosphatase activity increased after exposure to NaF (10^{-5} M) at the 6 hours of incubation. To investigate the osteoclastic activation, the mRNA expression of osteoclastogenesis related factors were examined. The receptor activator of the nuclear factor-κB ligand (RANKL) which is known as a factor for osteoclastogenesis, increased in the NaF-treated scales after 6 hours of incubation. The ratio of RANKL/osteoprotegerin (osteoclastogenesis inhibitory factor) significantly increased after 6 hours of incubation. Resulting from the increase of RANKL mRNA level, the expression of transcription-regulating factors was significantly increased. Furthermore, the expression of functional genes, cathepsin K and matrix metalloproteinase-9 mRNA, was significantly increased. In our knowledge, this is the first report concerning the effects of NaF on osteoblasts and osteoclasts in teleosts. We concluded that NaF influences calcium metabolism *via* osteoclastic activation in goldfish.

Keywords: sodium fluoride; osteoblasts; osteoclasts; scales; calcium metabolism; RANKL; OPG; goldfish

Abbreviations: ALP, alkaline phosphatase; CTSK, cathepsin K; MMP-9, matrix metalloproteinase-9; NFATc1, nuclear factor of activated T-cells and cytoplasmic 1; TRAF6, TNF receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase; OPG, osteoprotegerin; RANK, receptor activator of the nuclear factor-κB ; RANKL, receptor activator of the nuclear factor-κB ligand

1. INTRODUCTION

Fluoride is abundant in environmental water. A major source of fluoride is drinking water, including fluoridated community drinking water and underground water contaminated by geological sources. An appropriate range of fluoride is thought to be safe and effective for caries reduction (Fordyce et al., 2007). In the case of aquatic animals, however, fluoride tends to accumulate in the exoskeleton of invertebrates and the bone tissue of fish (see a review, Camargo, 2003). These results indicated that fluoride affects calcium metabolism in fish. However, the direct effects of fluoride on osteoblasts and osteoclasts have not yet been elucidated in any fish, although there are several studies regarding the toxicity of fluoride in fish. Superior bioassay is strongly desired to analyze the effect of this chemical on both osteoclasts and osteoblasts in teleosts.

In all vertebrates, blood calcium levels are strictly kept at a constant concentration (around 2.5 mM) in spite of changing the internal milieu or external environment (Dacke, 1979). The scales of teleosts have an important role in regulating blood calcium because teleost scales, having both osteoblasts and osteoclasts, are known to function as potential internal calcium reservoirs similar to those in the endoskeletons of mammals, especially during increased calcium demand, such as sexual maturation or starvation (Yamada, 1961; Berg, 1968; Mugiya and Watabe, 1977; Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007; Ohira et al., 2007). We detected both cathepsin K and tartrate-resistant acid phosphatase (TRAP) mRNA expression in the osteoclasts of goldfish scales (Azuma et al., 2007). Several osteoblastic markers (such as osteocalcin, type 1 collagen, and osterix) were also detected in the scales of goldfish (Thamamongood et al., 2012). Therefore, we have developed

an *in vitro* assay system with goldfish scales and have analyzed the influence of calcemic hormones (calcitonin: Suzuki et al., 2000; Sekiguchi et al., 2009; parathyroid hormone: Suzuki et al., 2011a; prostaglandin E_2: Omori et al., 2012) and environmental pollutants (bisphenol-A: Suzuki and Hattori, 2003; heavy metal: Suzuki et al., 2004a; Suzuki et al., 2011b; Yachiguchi et al., 2014a; tributyltin: Suzuki et al., 2006; polychlorinated biphenyl: Yachiguchi et al., 2014b) on osteoblasts and osteoclasts. Therefore, we strongly believe that scale can be utilized as a model for bone, and that our *in vitro* scale assay system is effective for analyzing fluoride in fish bone metabolism.

In the present study, we first examined the effects of NaF on plasma calcium levels in an *in vivo* experiment with goldfish (fresh water teleosts). In the goldfish, thereafter, the detailed influence of NaF on bone metabolism was examined using the scale *in vitro* assay system.

2. Materials and methods

2.1. Animals

The Yamato strain of goldfish (*Carassius auratus*) that was purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) was artificially fertilized from a female and a male goldfish (20 – 30 g) in the Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology. Fish were fed a commercial pellet diet for puffer fish (Feed One Co., Ltd. Yokohama, Japan) every morning and were maintained in fresh water at 26°C. Growing fish were moved to Noto Marine Laboratory in Kanazawa University and used for the both *in vivo* and *in vitro* experiments.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

*2.2. Effects of NaF on plasma calcium level in the goldfish (*in vivo *experiment)*

Immature goldfish (4 - 6 g), which do not have developed gonads, were used for the *in vivo* study. In the experimental group, goldfish were anesthetized with 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) and then NaF (purity: 99.0%, Wako Pure Chemical Industries Ltd., Osaka, Japan) (low dose: 500 ng/g body weight; high dose: 5μg /g body weight) was injected intraperitoneally. The goldfish in the control group were injected with saline (0.9% NaCl) in the same manner as experimental goldfish. These goldfish were kept in the aquarium for 1 and 2 days (each n = 10). These experimental periods were adopted because hormonal and toxicological effects were influenced in goldfish during 2 days (Suzuki et al., 2004a; Suzuki et al., 2004b; Suzuki et al., 2011a; Omori et al., 2012; Yachiguchi et al., 2014b). During the experimental periods, these goldfish were fasted to exclude intestinal calcium uptake from diets. Each 1 and 2 day after injection, blood samples were collected from the caudal vessel using a heparinized syringe from individual, anesthetized goldfish (each n = 10). The collected blood was put into a 1.5 ml tube. Thereafter, the tube was centrifuged at 15,000 rpm for 3 min. Then, the separated plasma was immediately frozen and kept at –80 °C until use. The plasma total calcium level (mg/ 100 ml) was determined using an assay kit (Calcium E, Wako Pure Chemical Industries).

*2.3. Effects of NaF on scale osteoblastic and osteoclastic activities using the cultured scales of goldfish (*in vitro *experiment)*

Scales were collected from goldfish (n = 16) after anesthesia with 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich). Using these scales, we examined the influences of NaF on the osteoblasts and osteoclasts with ALP and TRAP as markers because, in mammals, the effects of hormones and some bioactive substances on osteoblasts and osteoclasts have been investigated using ALP and TRAP as respective markers (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999). These scales were incubated for 6 hours in Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., OH, USA) supplemented with NaF (10^{-7}, 10^{-6}, and 10^{-5} M). These concentrations of NaF *in vitro* experiment were including those *in vivo* experiment because NaF levels of whole body of goldfish *in vivo* experiment were around 10^{-5} to 10^{-4} M. This incubation time was adopted because biological responses were influenced in goldfish during 6 hours (Suzuki and Hattori, 2002; Suzuki and Hattori, 2003; Suzuki et al., 2004a; Suzuki et al., 2006; Suzuki et al., 2008; Suzuki et al., 2009a; Takahashi et al., 2008; Yachiguchi et al., 2014b). In one goldfish, 24 scales were taken from each side (left and right); one side was used for incubation with NaF, and the other side was used as a control. The 24 scales used in the present study were considered to use as follows: 1) 8 scales for ALP analysis by 10^{-7} M, 2) 8 scales for ALP analysis by 10^{-6} M, 3) 8 scales for ALP analysis by 10^{-5} M. The respective mean for ALP (obtained from 8 individual scales of one goldfish) was compared with those of right side (control group). After incubation, ALP activities were measured using the same methods described above (Suzuki et al., 2009b). The results are shown as means ± SEM (n = 8). In the case of TRAP, the same experiment as ALP was done repeatedly using 8 individual

goldfish.

The methods for measuring ALP and TRAP activities were as follows. An aliquot of 100 µl of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$) or an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) was added to each well. Then, the scales were put into their own well in a 96-well microplate. This microplate was frozen at -80°C immediately and then kept at -20°C until analysis. An aliquot of 100 µl of 20 mM para-nitrophenyl phosphate in an alkaline or acid buffer was then put into each well of the content of the defrosted plate, which had been stored previously at -20°C. This plate was incubated at 23°C for 60 min while being shaken. After incubation, the reaction was stopped by adding 50 µl of 3 N NaOH. One hundred fifty µl of a reacted solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP.

After measuring both ALP and TRAP activities, the size of the scales was measured with Image J. Afterward, ALP and TRAP activities were normalized to the surface area (mm^2) of each scale (Suzuki et al., 2009b).

*2.4. Analysis of gene expression in osteoblasts and osteoclasts of goldfish scales by NaF-treatment (*in vitro *experiment)*

Scales were collected from goldfish under anesthesia with 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich). To examine changes of genes expressed in osteoblasts and osteoclasts that responded to NaF (10^{-5} M), these scales were incubated for 6 hours in Leibovitz's L-15 medium (Invitrogen) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals). After incubation, the scales were frozen at -80 °C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (Qiagen GmbH). To examine osteoclastic activation of the scales, the involvement of receptor activator of the nuclear factor-κB (RANK)/ receptor activator of the nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) system (Kearns et al., 2008; Lacey et al., 2012) was investigated. Furthermore, transcription-regulating factors (TNF receptor-associated factor 6: TRAF6 ; nuclear factor of activated T-cells and cytoplasmic 1: NFATc1) and functional genes (cathepsin K: CTSK; matrix metalloproteinase-9: MMP-9) were studied. Gene-specific primers for RANK, RANKL, OPG, TRAF6, NFATc1, CTSK, and MMP-9 are indicated in Table 1. The amplification of Elongation factor 1α (EF1α) cDNA using a primer set (Table 1) was performed. The PCR amplification was analyzed by real-time PCR apparatus (Mx3000p, Agilent Technologies, CA, USA) (Suzuki et al. 2011a; Thamamongood et al., 2012). The annealing temperature of RANK, RANKL, OPG, TRAF6, NFATc1, CTSK, MMP-9, and EF1α was 60 °C. The RANK, RANKL, OPG, TRAF6, NFATc1, CTSK, and MMP9 mRNA levels were normalized to the EF1α mRNA level.

2.5. Statistical analysis

All results are expressed as the means ± SE (n = 8 or 10). The statistical significance between control and experimental groups was assessed by one-way ANOVA followed by Dunnett test (*in vivo* experiment) or paired *t*-test (*in vitro* cell activity experiment and *in vitro* mRNA expression experiment). In all cases, the selected significance level was $p < 0.05$.

3. RESULTS

*3.1. Effects of NaF on plasma calcium level in the goldfish (*in vivo *experiment)*

At 1 day after administration, plasma calcium levels increased significantly in high dose of NaF injected goldfish (Fig. 1). At 2 days after injection, thereafter, plasma calcium levels also significantly upregulated in both low and high dose of NaF-treated goldfish (Fig. 1).

*3.2. Effects of NaF on scale osteoblastic and osteoclastic activities using the cultured scales of goldfish (*in vitro *experiment)*

The NaF treatment induced upregulation of both ALP and TRAP activities at 6 hours of incubation. These results of ALP and TRAP are indicated in Figures 2 and 3, respectively. Namely, ALP activity in the scales of goldfish significantly increased by NaF (10^{-6} and 10^{-5} M) treatment although NaF (10^{-7} M) did not change ALP activity. Also, high concentration of NaF (10^{-5} M) induced a significant increase of TRAP activity.

*3.3. The mRNA expression related with osteoclastogenesis (*in vitro *experiment)*

The influence of NaF on the signaling of RANK/RANKL and OPG system was examined.

By NaF treatment, both RANK and RANKL mRNA expression increased significantly (Figs. 4A and 4B). RANKL/OPG was also upregulated because OPG expression did not change significantly (Figs. 4C and 4D).

*3.4. The mRNA expression of transcription-regulating factors and osteoclast functional genes (*in vitro *experiment)*

The changes in mRNA expression of transcription-regulating factors by NaF treatments were examined. The results are indicated in Figures 5A and 5B. The TRAF6 and NFATc1 mRNA expression in NaF-treated scales increased significantly compared with those in control scales.

Furthermore, the expression of functional genes, CTSK and MMP-9 mRNA, was significantly increased by NaF treatments (Figs 5C and 5D).

4. DISCUSSION

In the present study, we have indicated that NaF induces hypercalcemia in goldfish (fresh water teleosts). We previously reported that tributyltin acetate (Suzuki et al., 2006) and methylmercury (Suzuki et al., 2004a) induced hypercalcemia in goldfish. In addition, polychlorinated biphenyl (PCB) 118 induced hypercalcemia *via* the upregulation of RANKL in goldfish (Yachiguchi et al., 2014b). Therefore, several environmental pollutants affected fish calcium metabolism. It has been reported that excessive fluoride intake induces toxicity in hard tissues (teeth and skeleton) and soft tissues (kidneys, lungs, and brains) (Whitford et al., 1990; Barbier et al., 2010; Choi et al., 2012; DenBesten and Li, 2011) in mammals. In the case of aquatic animals, fluoride tends to accumulate in the exoskeleton of invertebrates and the bone tissue of fish (Camargo, 2003; Shi et al., 2009). In fact, the dietary fluoride derived from krill exoskeletons accumulates in the vertebral bones of yellowtail and inhibits the growth of yellowtail (Yoshitomi and Nagano, 2012). Thus, in the case of fluoride as well as other environmental pollutants, more attention should be given to the metabolism of fish calcium.

RANK/RANKL/OPG signaling is an important role of osteoclastic function (see a review by Lacey et al., 2012). RANK in osteoclasts binds the ligand (RANKL), resulting in osteoclast activation, whereby multinucleated osteoclasts (an active type of osteoclast) are formed. In

addition, OPG, a decoy receptor of RANKL, inhibits osteoclastogenesis by binding to RANKL. Therefore, the RANKL/OPG ratio has been used as an indicator of osteoclast activation (Kearns et al., 2008). In the present study, NaF upregulated both RANK and RANKL mRNA expression. Furthermore, OPG mRNA expression did not change significantly between NaF-treated and control scales. Therefore, the ratio of RANK/OPG in NaF-treated scales was significantly higher than that in control scales, and then NaF treatment activated osteoclasts.

Resulting from the increased RANKL mRNA level, the expression of transcription-regulating factors such as TRAF6 and NFATc1 (Kim and Kim, 2016) was significantly increased (Figs. 5A and 5B, respectively). Furthermore, the mRNA expression of functional genes CTSK and MMP-9 was significantly upregulated (Figs. 5C and 5D, respectively). We concluded that osteoclastogenesis is induced by NaF treatments in goldfish (see Fig. 6). Previously, we reported that parathyroid hormones first acted on osteoblasts at 6 hours of incubation and then activated osteoclasts at 18 hours of incubation *via* RANK/RANKL signaling (Suzuki et al., 2011a). Results similar to those of parathyroid hormones were obtained by prostaglandin E_2 (Omori et al., 2012). We strongly believe that NaF, as well as parathyroid hormone and prostaglandin E_2, acts first on osteoblasts and then activated osteoclasts. In an *in vitro* experiment, a detailed time course of osteoblastic and osteoclastic response will be performed.

Fluoride can directly interact with the bone mineral matrix. The *in vitro* fluoridation of bone with fluoride can lead to the conversion of carbonated hydroxyapatite to carbonated fluorapatite, which is more stable and resistant to acid dissolution than is hydroxyapatite (see a review, Everett, 2011). In mice, however, it has been reported that excessive fluoride can lead to abnormal bone biology (Yan et al., 2007). Recently, in ovariectomized rats, which are a model

animal for human osteoporosis, the administration of fluoride caused deterioration of the rat tibia with a coarse pattern of trabecular architecture, suggesting that fluoride exposure accelerates osteoporotic changes in postmenopausal women even at a low dose (Kakei et al., 2016). We must pay more attention to the treatment of fluoride in mammals as well as fish. In our next study, therefore, we will examine the influence of NaF on osteoclastogenesis with marine fish because fluoride also exists and actually affects fish in the food chain in the marine environment (Camargo, 2003; Shi et al., 2009). We will emphasize the toxicity of fluoride on fish bone metabolism.

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Figure legends

Fig. 1. Effects of NaF on plasma calcium level in the goldfish at 1 and 2 days after administration. NaF (low dose: 500 ng/g body weight; high dose: 5μg /g body weight) was injected intraperitoneally. Thereafter, the blood sampling was performed at 1 and 2 days. * and ** indicate statistically significant differences at $p < 0.05$ and $p < 0.01$, from the values in the control scales. n = 10 samples; one sample from one fish.

Fig. 2. Effects of NaF on ALP activity in cultured scales incubated for 6 hours
* indicates statistically significant differences at $p < 0.05$ from the values in the control scales. n = 8 samples; one sample from one fish.

Fig. 3. Effects of NaF on TRAP activity in cultured scales incubated for 6 hours
* indicates a statistically significant difference at $p < 0.05$ from the values in the control scales. n = 8 samples; one sample from one fish.

Fig. 4. Expression analysis of the receptor activator of the NF-κB (RANK) (A), the receptor activator of the NF-κB ligand (RANKL) (B), and osteoprotegerin (OPG) (C) mRNAs in scales treated with NaF incubated for 6 hours. RANK, RANKL, and OPG mRNA levels were normalized to the EF-1α mRNA level. The ratio of RANKL/ OPG was calculated and indicated in (D). The value of the ordinates indicates the relative ratios of RANK/ EF-1α (A), RANKL/ EF-1α (B), OPG/ EF-1α (C), and RANKL/ OPG (D), respectively. * and ** indicate

statistically significant differences at $p < 0.05$ and $p < 0.01$, from the values in the control scales. n = 8 samples; one sample from one fish.

Fig. 5. Expression analysis of transcription-regulating factors: TNF receptor-associated factor 6 (TRAF6) (A), nuclear factor of activated T-cells and cytoplasmic 1 (NFATc1) (B) and osteoclastic function genes: cathepsin K (CTSK) (C), Matrix Metalloproteinase-9 (MMP-9) (D) mRNAs in scales treated with NaF (10^{-5} M) incubated for 6 hours. TRAF6, NFATc-1, CTSK and MMP-9 mRNA levels were normalized to the EF-1α mRNA level. The value of the ordinates indicates the relative ratios of TRAF6/ EF-1α (A), NFATc-1/ EF-1α (B), CTSK/ EF-1α (C), and MMP-9/ EF-1α (D), respectively. *, **, and *** indicate statistically significant differences at $p < 0.05$ and $p < 0.01$, from the values in the control scales. n = 8 samples; one sample from one fish.

Fig. 6. Action of NaF in the scales of goldfish. NaF was upregulated both RANK and RANKL mRNA expression while OPG mRNA expression did not chang. As a result, the ratio of RANK/OPG was significant difference between NaF-treated and control scales. Therefore, NaF induced osteoclastic activation in goldfish scales *via* RANK/ RANKL/OPG system.

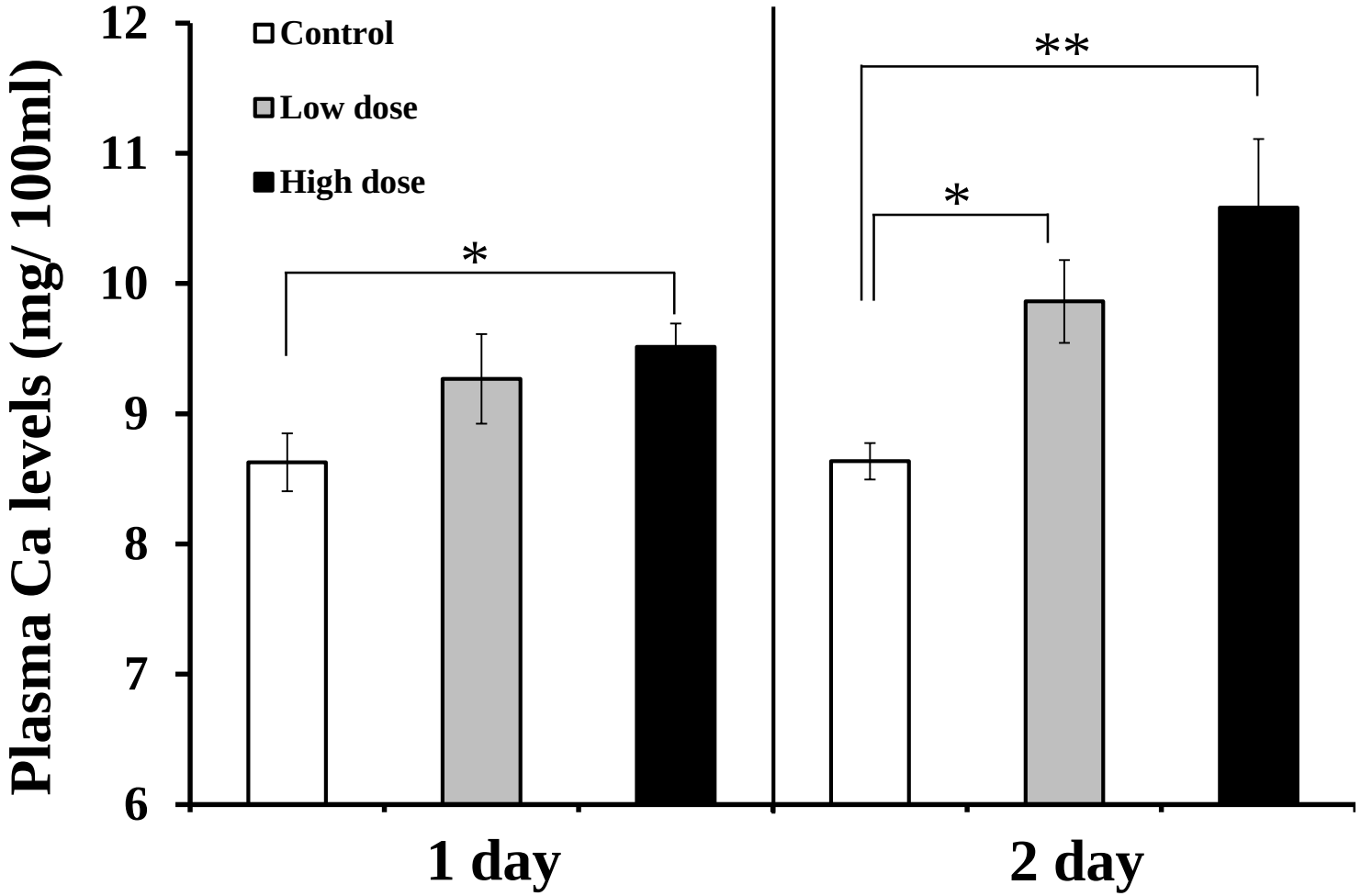


Figure 1 Sato et al.

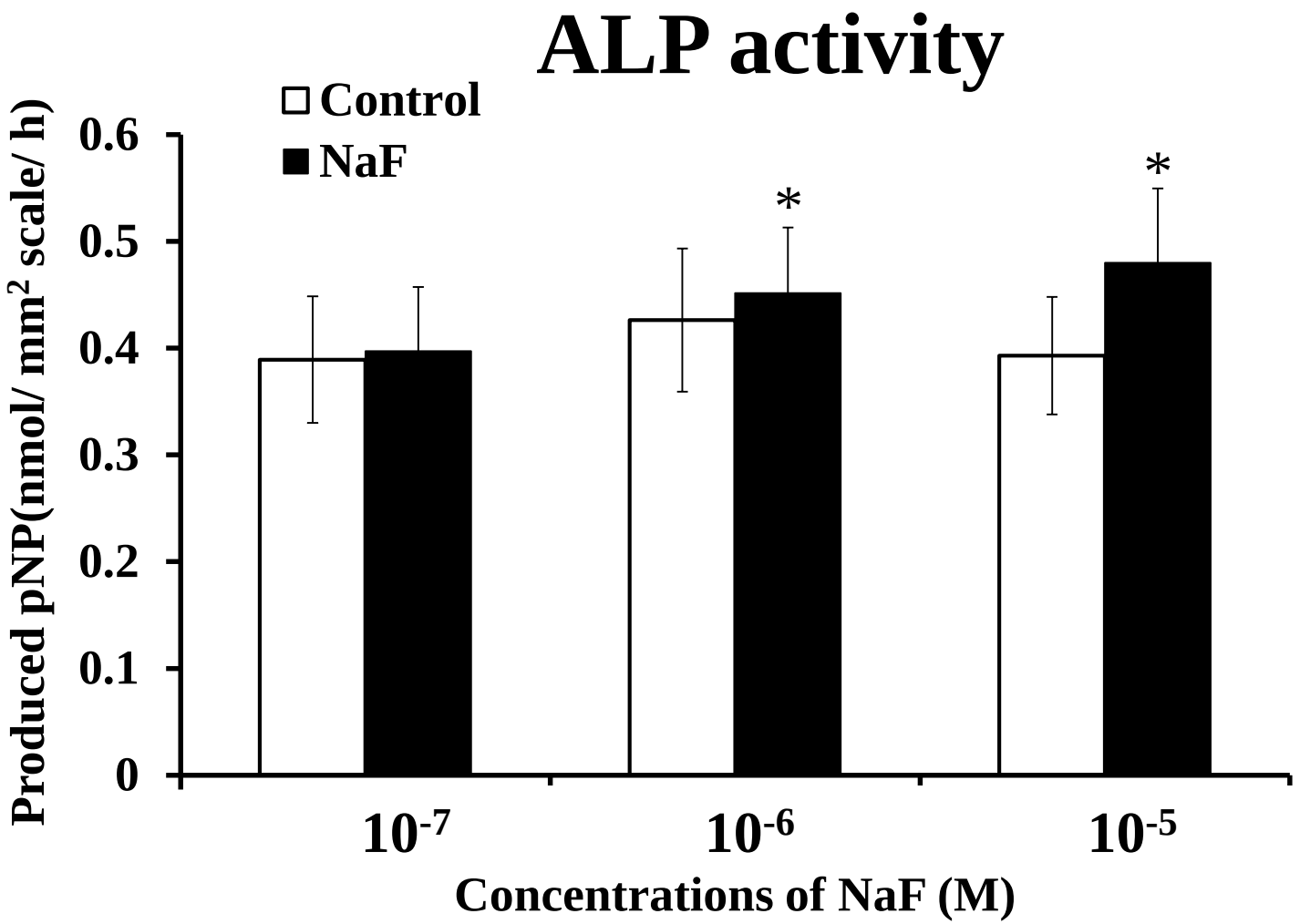
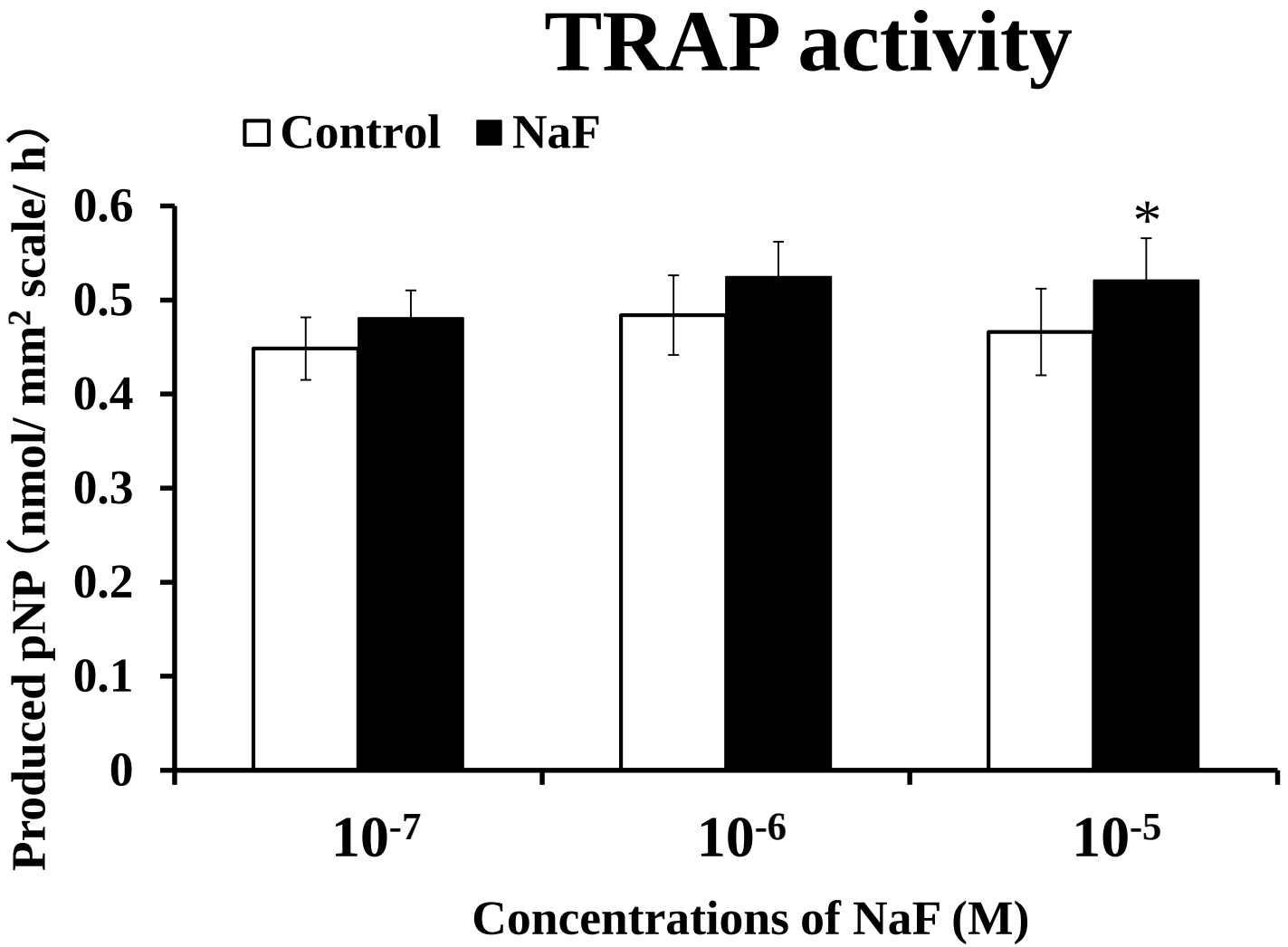


Figure 2 Sato et al.



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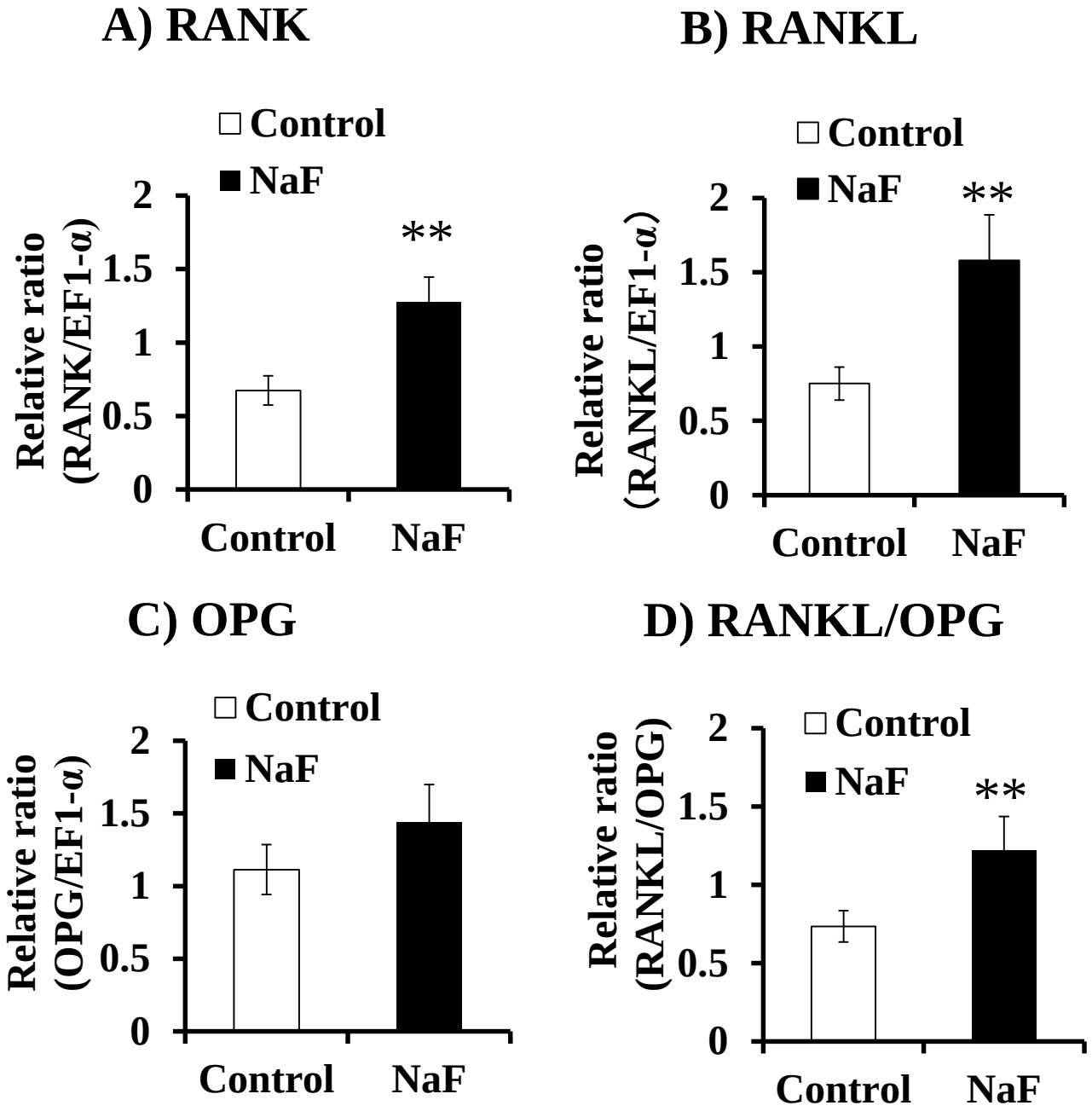

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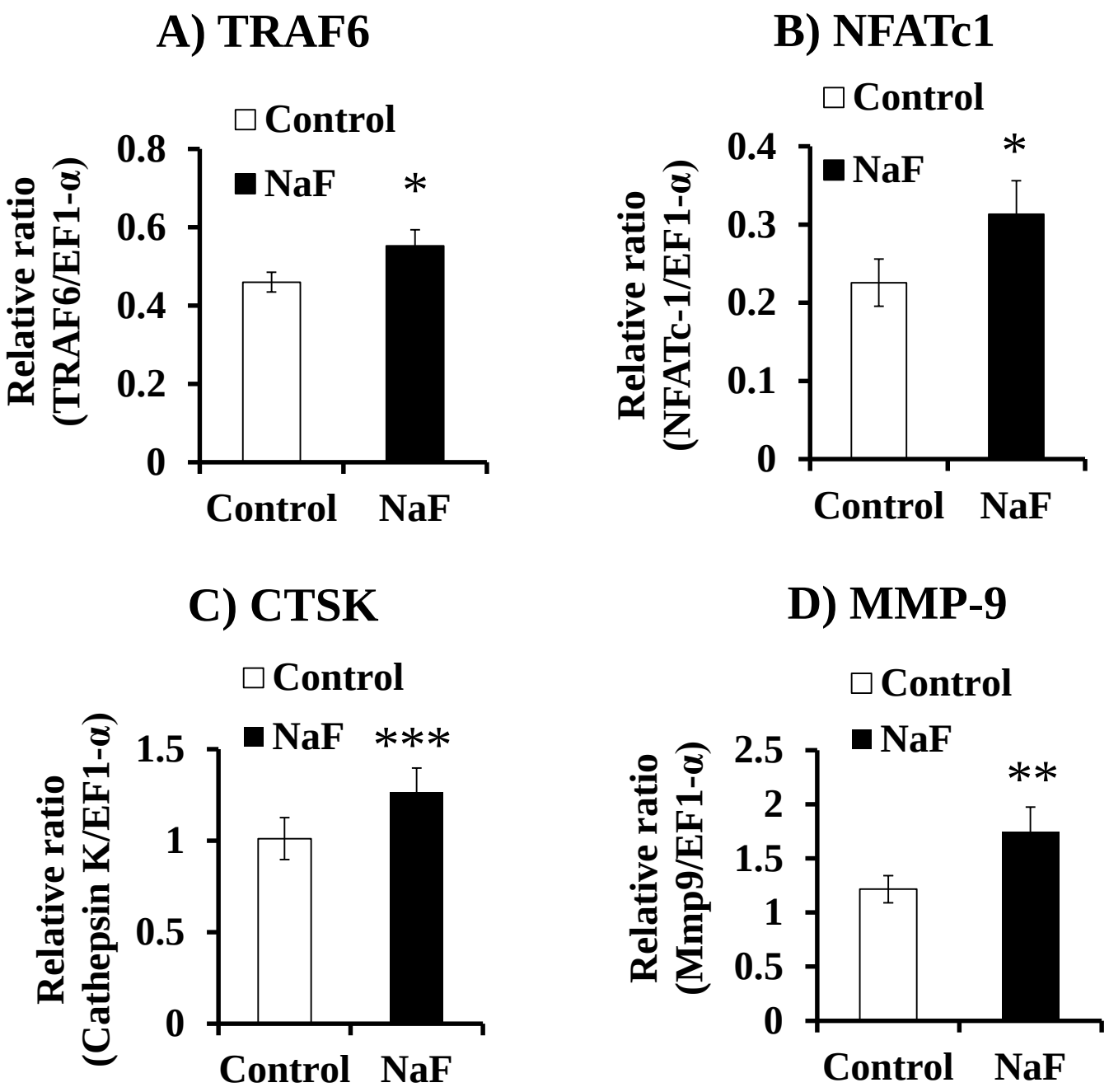


Figure 5 Sato et al.

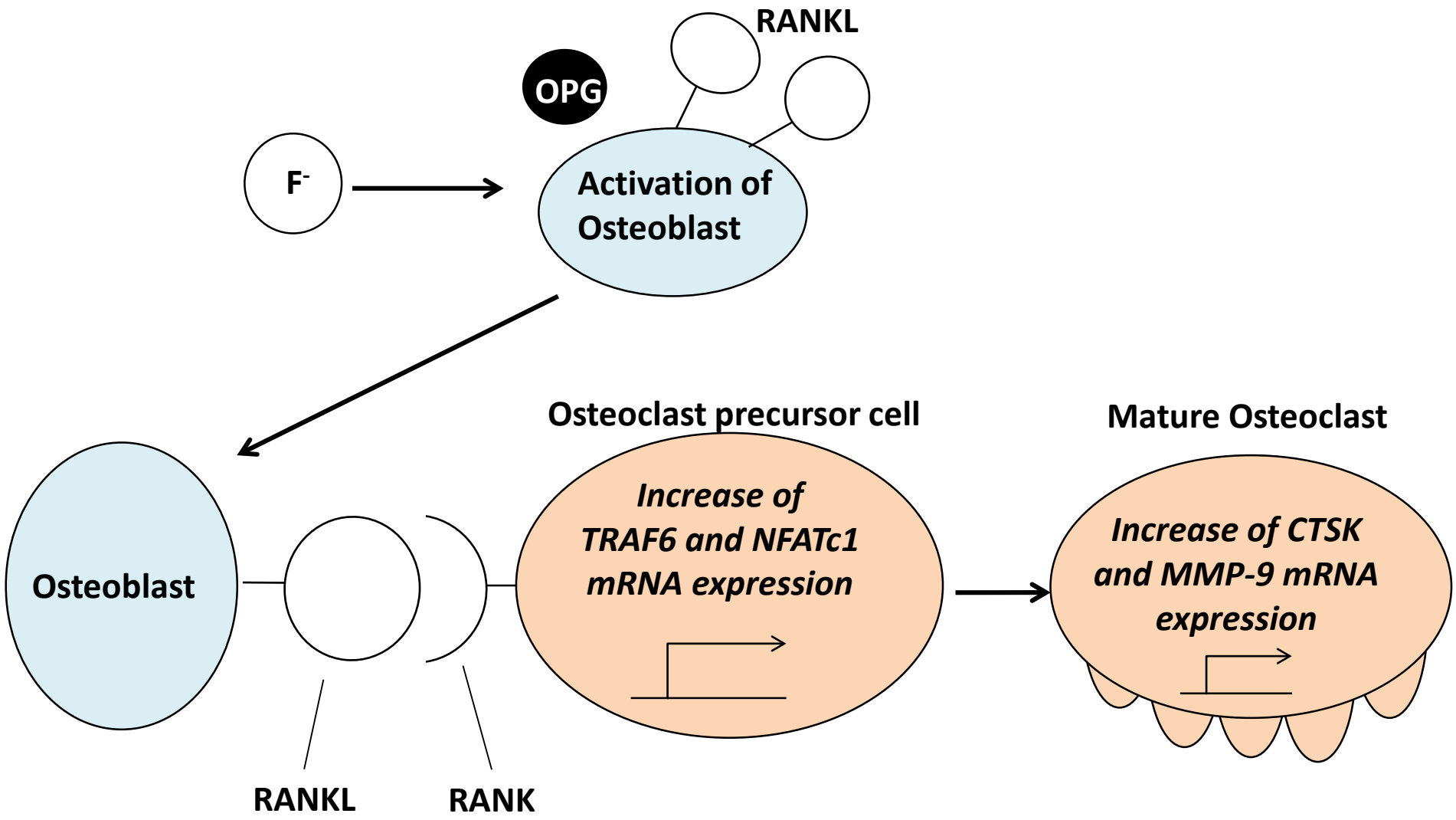


Figure 6 Sato et al.

Table 1. Primer sequences for real-time quantitive PCR

Name	Forward primer	Reverse primer	Accession No.
RANK	GGGAGATGCTGCGAAAAATG	TTTAGGGTTGTGTGGACGAGTG	AB894121
RANKL	CGAGTGTGGCGATTTTGTTG	ATGGGCGTCTTGATTGGAAG	AB894120
TRAF6	TCTGATGGGTCTTCGCTCGGCT	ACTGGACATTTCTGCCCCGTGT	LC149878
NFATc1	CTGTGGCTTTGCTTGTGGATGTC	GATGCTGGTGTTTTGGCTGTAACC	AB685221
MMP-9	GCTTCTGCCCCAGTGAGCTT	GTGGAGCACCAGCGATACCC	AB889498
CTSK	TGGGAGGGCTGGAAACTCAC	CATGAGCCGCATGAACCTTG	AB236969
OPG	CGTGAACACGGTGTGCGAGTGT	CCTCTGCGCAGGCCTCACA	AB970727
EF1α	ATTGTTGCTGGTGGTGTTGG	GGCACTGACTTCCTTGGTGA	AB979720

RANK: Receptor activator of nuclear factor-κB, RANKL: Receptor activator of nuclear factor-κB ligand, TRAF6: TNF receptor associated factor 6, NFATc1: Nuclear factor of activated T-cells cytoplasmic 1, MMP-9: Matrix metallopeptidase-9, CTSK: Cathepsin K, OPG: Osteoprotegerin, EF1α: Elongation factor 1α

Table 1 PAH and NPAH concentrations in the seawater of both Alexanderia and Suez Canal sites.

		Alexanderia site	Suez Canal site
Ring no.	**Name of PAH**	(ng/l)	(ng/l)
2-ring	**Naphthalene**	420.0	303.91
3-ring	**Acenaphthene**	369.7	277.32
	Fluorene	133.2	105.51
	Phenanthrene	218.4	162.90
	Anthracene	16.2	4.86
4-ring	**Fluoranthene**	22.0	16.14
	Pyrene	37.8	39.42
	Benz[*a*]anthracene	21.4	6.75
	Chrysene	13.6	7.36
5-ring	**Benzo[*b*]fluoranthene**	18.7	12.11
	Benzo[*k*]fluoranthene	10.1	4.34
	Benzo[*a*]pyrene	49.3	26.41
	Dibenz[*a,h*]anthracene	0	0
6-ring	**Benzo[*ghi*]perylene**	33.5	25.53
	Indeno[*1,2,3-cd*]pyrene	0.69	0
	Total	**1364.59**	**992.56**
	Name of NPAH		
3-ring	**2-Nitrofluorene**	3.30	1.19
	2-Nitroanthracene	2.10	0.07
	9-Nitroanthracene	0.11	0.11
4-ring	**1-Nitropyrene**	2.10	0.21
	3-Nitrobenzanthrone	3.30	1.81
	6-Nitrochrysene	0.13	0.47
	7-Nitrobenz[*a*]anthracene	1.50	0.04
5-ring	**6-Nitrobenzo[*a*]pyrene**	0.209	0.014
	Total	**12.749**	**3.914**

Table 2 Effect of PAHs (6 ng/l) and NPAHs (40 pg/l) on ALP activity (produced pNP nmol/ mm^2 scale/ h) in cultured scales incubated for 6 hrs

	Naphthalene	Acenaphthene	2-Nitrofluorene	3-Nitrobenzanthrone
Control group	0.477± 0.055	0.445 ±0.045	0.493± 0.056	0.480±0.049
Experimental group	0.463± 0.052	0.402 ±0.034*	0.476± 0.050	0.469±0.042

Data as mean ± SE

Statistical analysis: paired t-test

*: significantly difference ($P < 0.05$)

n = 9 samples; one sample from one fish

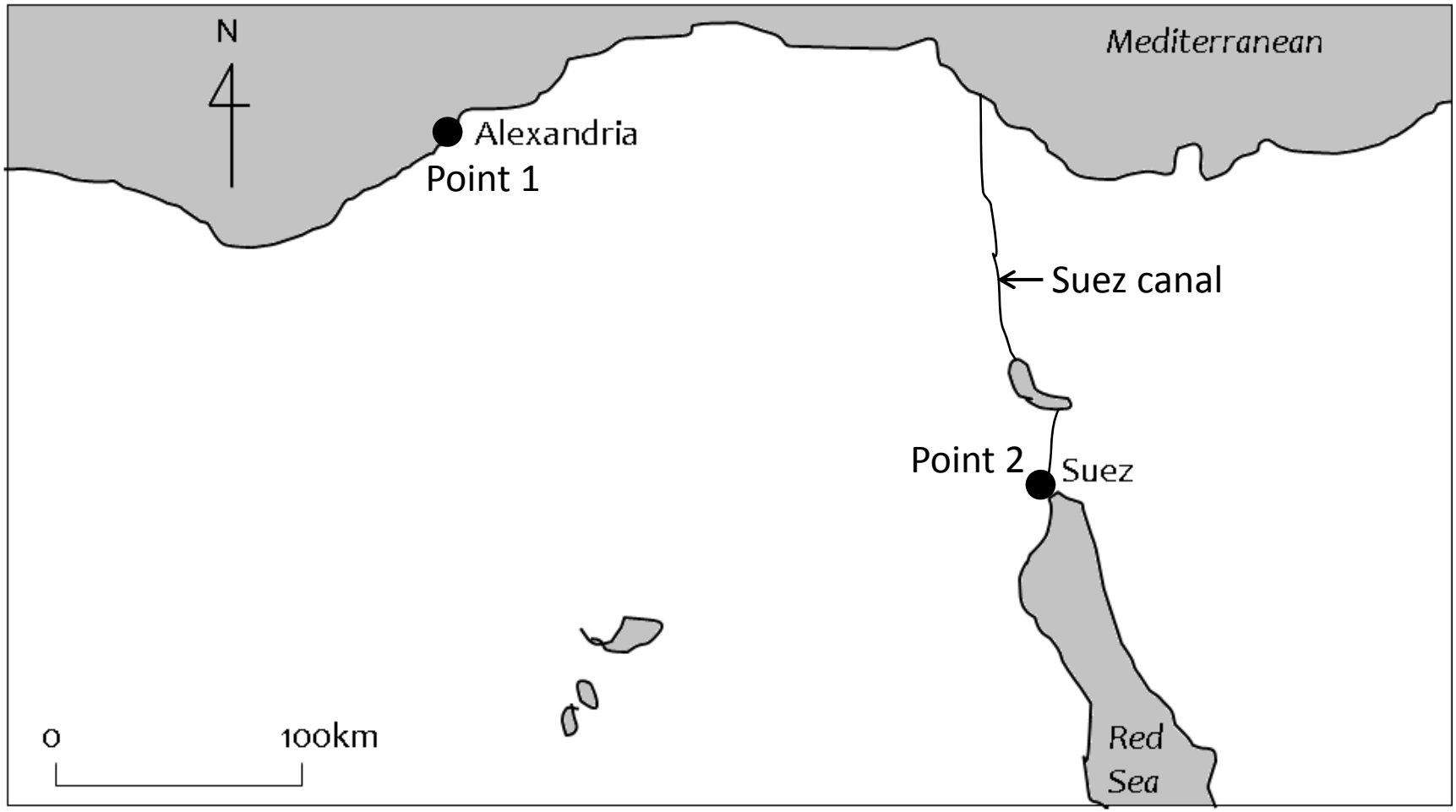
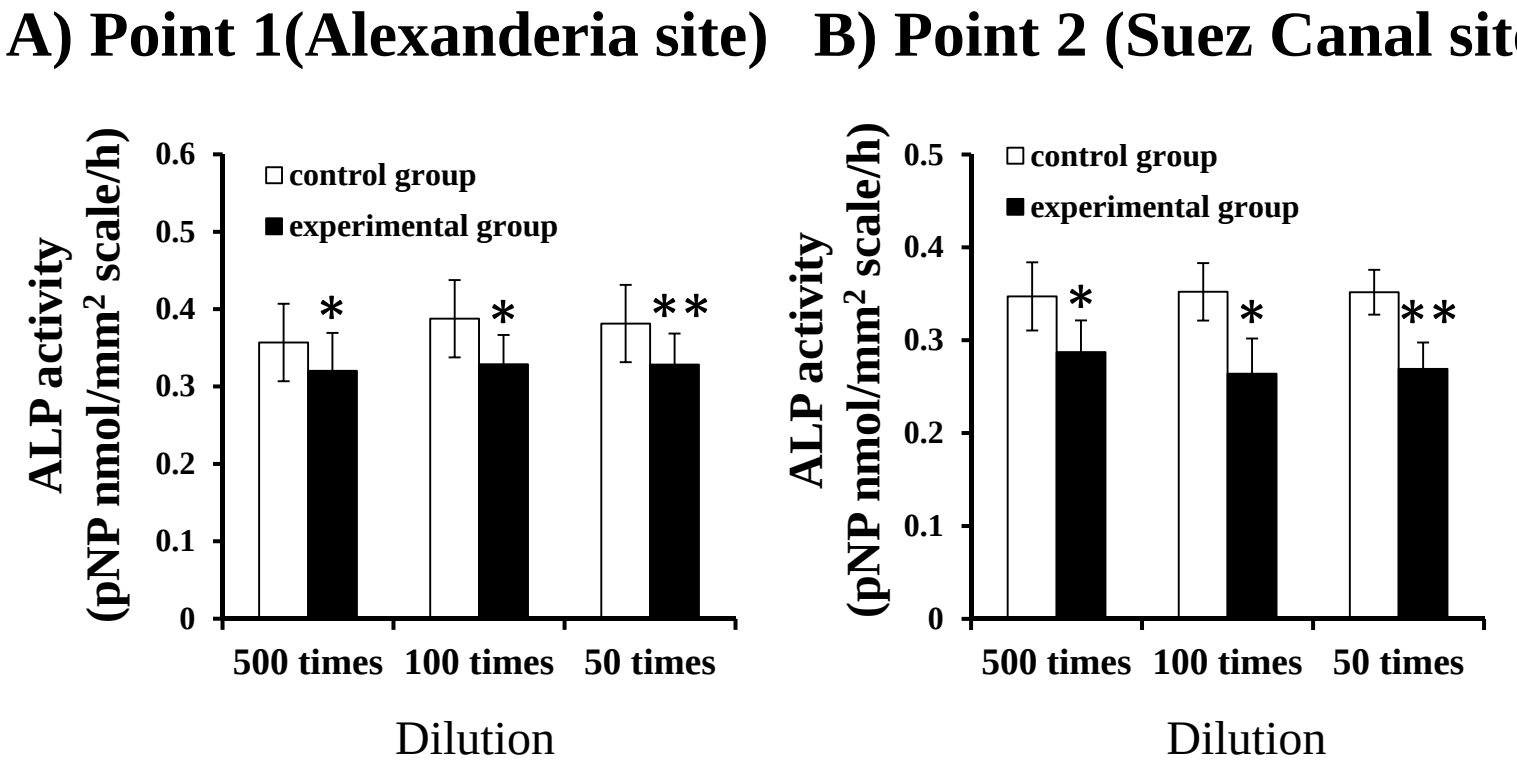


Figure 1 Suzuki et al.



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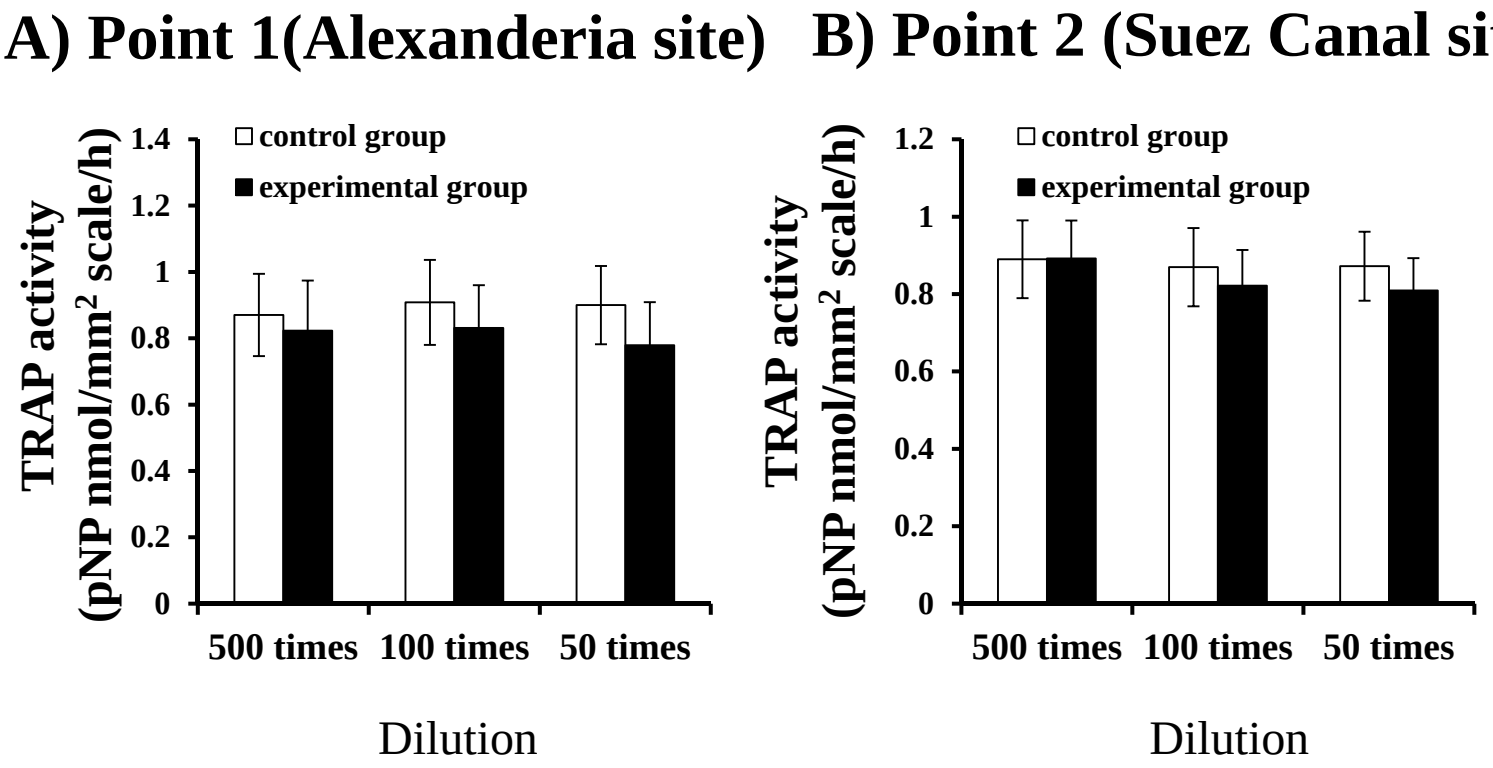
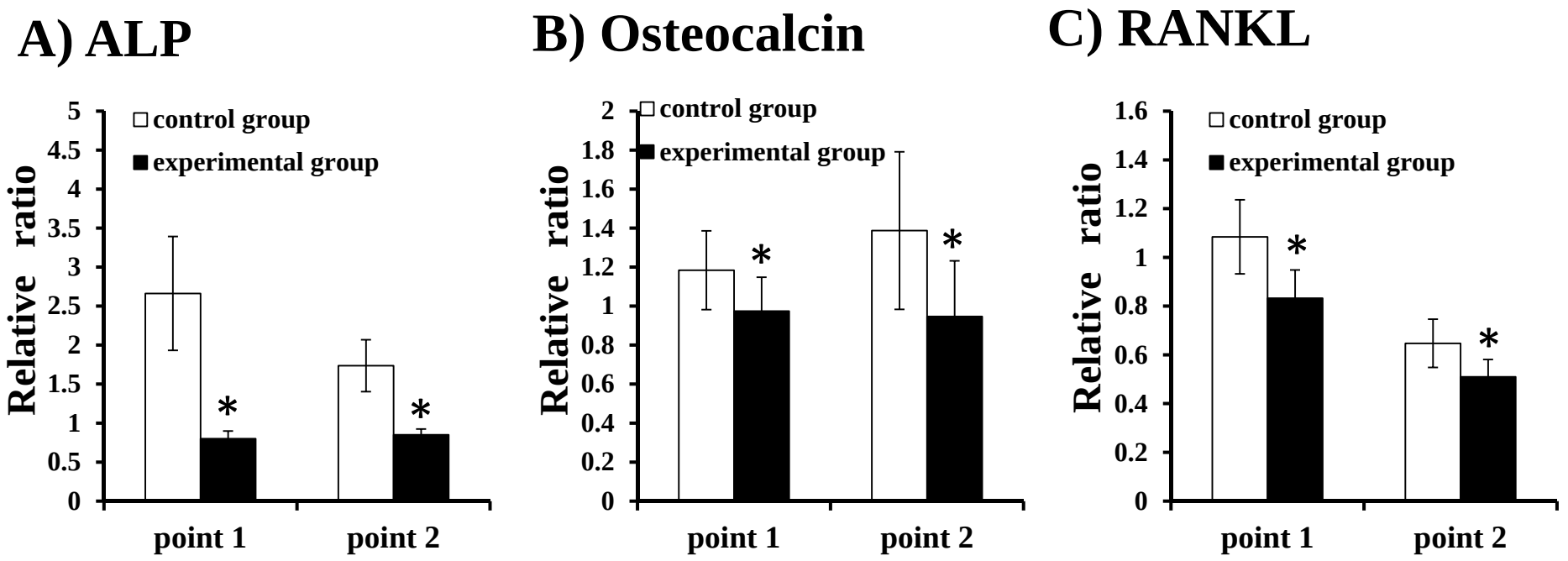


Figure 3 Suzuki et al.



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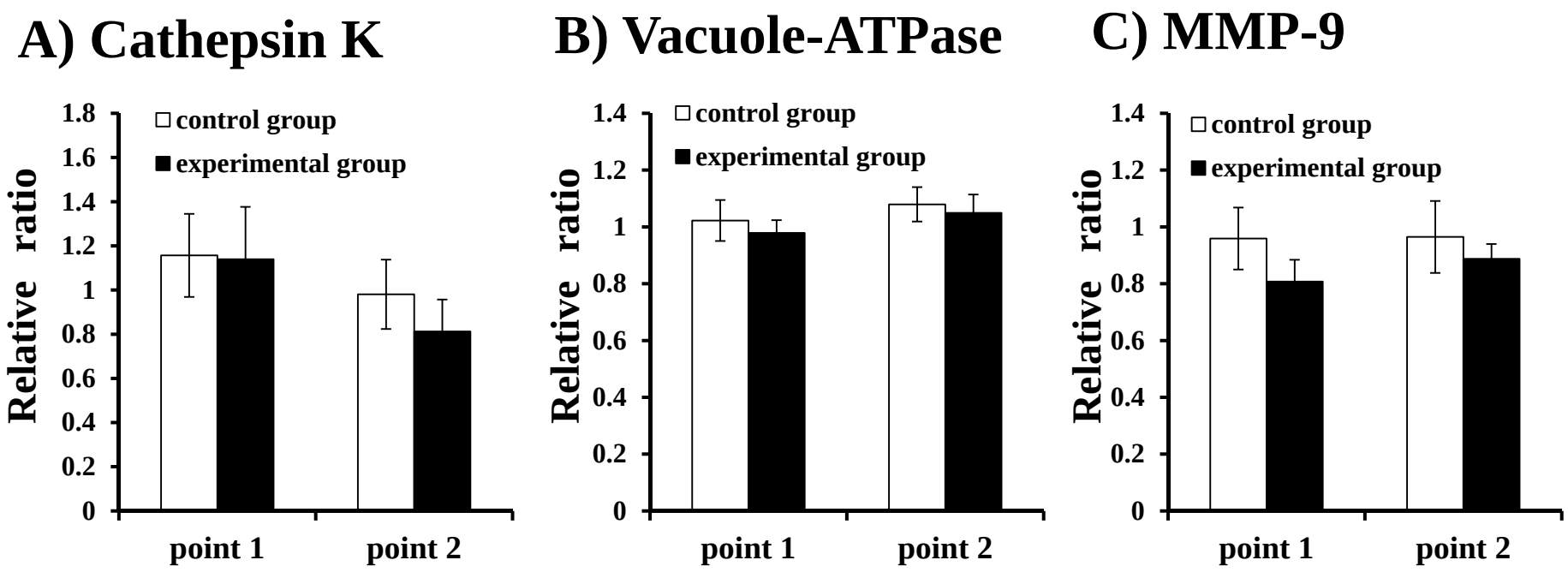

Figure 5 Suzuki et al.