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ORIGINAL ARTICLE

Molecular pathology of natriuretic peptides in the myocardium with special regard to fatal intoxication, hypothermia, and hyperthermia

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Abstract The present study investigated the molecular pathology of atrial and brain natriuretic peptides (ANP and BNP) in the myocardium to evaluate terminal cardiac function in routine forensic casework with particular regard to fatal drug intoxication (n=18; sedative–hypnotics, n=10; methamphetamine, n=8), hypothermia (cold exposure, n=13), and hyperthermia (heatstroke, n=10), compared with that in acute ischemic heart disease (AIHD, n=35) and congestive heart disease (CHD, n=11) as controls (total n=87; within 48 h postmortem). Quantitative analyses of myocardial ANP and BNP messenger RNA demonstrated that their expressions in bilateral atrial and ventricular walls were high in methamphetamine intoxication and hypothermia, comparable to those in AIHD and CHD, but were low in sedative–hypnotic intoxication and hyperthermia. In pericardial fluid, both ANP and BNP levels were increased in hypothermia, while CHD cases had an elevated BNP level, and ANP level showed a tendency to increase in hyperthermia; however, immunohistochemistry showed no evident differences in myocardial ANP and BNP among the causes of death. These findings suggest terminal high cardiac strain in methamphetamine intoxication, decreased cardiac strain in sedative–hypnotic intoxication and hyperthermia (heatstroke), and persistent congestion in hypothermia (cold exposure).

Keywords Forensic molecular pathology · Natriuretic peptide · Myocardium · Intoxication · Hypothermia · Hyperthermia

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Introduction

In forensic casework, difficulties remain in determining the causes of death involving functional deterioration without specific pathologies, which include fatal intoxication and thermal disorders, such as cold exposure and heat stroke. In such cases, investigation of characteristic functional changes of life-supporting organs, including the brain, heart, and lungs, may be helpful to reinforce pathological and toxicological findings, excluding the contribution of any other traumas and diseases to the death process. For this purpose, previous studies suggested possible application of postmortem molecular biological evaluation of the brain, heart, and lung [1–5]. As for markers of cardiac function, atrial and brain natriuretic peptides (ANP and BNP) in the myocardium rapidly respond to increased cardiac strain [6–9]; the application of molecular biological procedures to these markers may be useful to investigate terminal cardiac function, especially in "functional death," which presents with poor morphological findings [1].

The present study compared terminal cardiac function in fatal intoxication, hypothermia (cold exposure), and hyperthermia (heatstroke) to that of acute and chronic heart diseases, using molecular pathology of atrial and brain natriuretic peptides (ANP and BNP) in the myocardium as markers of cardiac strain.

Materials and methods

Materials

Forensic autopsy cases of fatal drug intoxication (n=18; sedative–hypnotics, n=10; and methamphetamine, n=8), hypothermia (cold exposure, n=13), and hyperthermia (heatstroke, n=10), as well as acute ischemic heart disease (AIHD, n=35) and congestive heart disease (CHD, n=11) as controls, were examined (total n=87; within 48 h postmortem). Case profiles are summarized in Table 1. For these groups, cases where the causes of death were established on the basis of complete autopsy and well-established circumstantial evidence were included, and those with significant complications were excluded. The inclusion criteria for hypothermia were typical pathologies including frost erythema and hemorrhagic gastric erosions (Wischnewski spots) as well as biochemical signs of elevated serum urea nitrogen and/or acetonemia [10–12], and those for hyperthermia were pathological and biochemical findings of multiple organ tissue damage [multiple organ dysfunction syndrome (MODS)] involving rhabdomyolysis [13–15], excluding those of drug abusers, chronic alcoholics, and death during bathing.

Pericardial fluid was collected aseptically using a syringe after opening the pericardial cavity at autopsy, centrifuged and stored at –20 °C until use. Routine heart tissue specimens were preserved in formalin for histopathology. Myocardial tissue specimens for messenger RNA (mRNA) measurements (about 50 mg) were taken from consistent sites of the bilateral atrial walls, anterior and posterior walls of the left ventricle, and right ventricular wall during autopsy, then submerged in 1 ml of RNA stabilization solution (RNAlaterTM, Ambion, Austin) and stored at 4 °C for <1 week until RNA extraction. The sample collections and analyses described below were performed within the framework of our routine casework, following the autopsy guidelines (2009) and ethics guidelines (1997 and 2003) of the Japanese Society of Legal Medicine, approved by our institutional ethics committee.

Methods

Measurements of ANP and BNP levels in pericardial fluids

The pericardial ANP and BNP concentrations were measured by chemiluminescent enzyme immunoassay using MIO_2 Shionogi ANP and MIO_2 Shionogi BNP assay kits (Shionogi Co. Ltd., Osaka), respectively. The ranges of measurement were <2,000 pg/ml for both ANP and BNP. Samples were diluted (×10 and ×100, respectively) to measure higher concentrations (>2,000 pg/ml), and measurements were performed in duplicate to exclude possible interference due to contaminants. The clinical serum reference ranges were 10–40 pg/ml for ANP and 2–20 pg/ml for BNP, and postmortem pericardial cut-off values were estimated to be 30 pg/ml for ANP and 150 pg/ml for BNP [16].

Immunostaining of ANP and BNP in the myocardium

Serial sections of 5-μm thickness were prepared from formalin-fixed, paraffin-embedded heart tissue specimens. Polyclonal rabbit anti-human ANP IgG (0780-0179; AbD

Table 1 Case profiles

Cause of death	n	Male/female	Age (year)		ST (h)		PMI (h)		Heart weight[a] (g)		Combined lung weight [b] (g)	
			Range	Median	Range	Median	Range	Median	Range	Median	Range	Median
Fatal drug intoxication												
Sedative-hypnotics	10	4/6	30–57	39	<3–36	6	15–37	28	200–545	333	760–1,670	1,213
Methamphetamine	8	8/0	33–62	43	<0.5–36	14	7–30	20	340–490	385	650–2,030	1,308
Hyperthermia	10	7/3	43–92	64	<3–72	6	15–35	27	295–515	338	495–1,965	1,050
Hypothermia	13	8/5	26–81	65	<6–24	6	15–37	27	175–440	355	370–1,465	735
AIHD												
AMI	16	14/2	44–85	65	<0.5–1	0.5	6–31	23	320–585	460	540–2,000	1,098
IHD	19	17/2	29–79	65	<0.3–0.5	0.5	5–35	18	235–640	400	525–2,505	1,000
CHD	11	5/6	29–88	59	unknown	–	11–45	23	260–650	390	715–1,665	1,190
Total	87	63/24	26–92	61	<0.3–unknown	3	5-45	22	175-650	380	370-2,505	1,070

ST estimated survival time from a fatal insult to death, *PMI* estimated interval from death to autopsy, *AIHD* acute ischemic heart disease, *AMI* acute myocardial infarction, *IHD* acute ischemic heart disease without apparent myocardial necrosis, *CHD* chronic congestive heart disease

[a] Heart weight was significantly higher in AIHD than in sedative–hypnotic intoxication by Steel–Dwass test

[b] Combined lung weight was significantly lower in hypothermia than in fatal drug intoxication (sedative-hypnotics and methamphetamine) and AIHD by Steel–Dwass test

Serotec, Oxford, UK; diluted 600-fold) and rabbit antihuman BNP IgG (16162; IBL, Takasaki, Japan; diluted 25-fold) as well as mouse monoclonal antihuman ANP IgG (0200-0648; AbD Serotec; diluted 50-fold) and antihuman BNP IgG (MCA2642; AbD Serotic; diluted 20-fold) were used. Following overnight incubation with the primary antibodies described above at room temperature, immunoreactions were visualized by the polymer method (ChemMate Envision; Dako Japan, Tokyo) and color was developed with 3,3′-diamino benzidine tetrahydrochloride (DAB liquid system, Dako Japan), according to the manufacturer's instructions (counterstaining with hematoxylin). Endogenous peroxide was inactivated by incubation with 3 % hydrogen peroxide for 10 min. For a control study to confirm the specificity of immunostaining, phosphate-buffered saline, rabbit IgG (Vector Laboratories, Burlingame, CA, USA) or mouse IgG (Vector Laboratories) was substituted for the primary antibody.

Quantification of mRNA in the myocardium

Total RNA was isolated with ISOGEN (Nippon Gene, Toyama) according to the manufacturer's instructions and stored at −80 °C until use. The extraction yield was quantified spectrophotometrically, and the quality (integrity) of total RNA was assessed by electrophoresis in agarose gels stained with ethidium bromide; 18S and 28S rRNA bands were visualized under UV illumination.

Reverse transcription PCR (RT-PCR) was performed using the TaqMan Gold RT-PCR Core Reagents kit on an ABI PRISM 7000 sequence Detection System (Applied Biosystems, Foster City, CA, USA). The contents of the amplification mix (20 μl/tube), including total mRNA (1.0 μl of 0.08–0.28 μg/100 μl solution), and the thermal cycling conditions were set according to the accessory protocols. Amplification of ANP and BNP mRNAs, together with the endogenous references described below, was performed. Primers and probes for these mRNAs were synthesized according to previous reports [2, 3, 17–23] and the GenBank nucleotide database, with probes spanning the junction of bordering exons. According to the manufacturer's instructions, the relative quantification of mRNA transcripts was carried out using the comparative threshold method. Each mRNA level was expressed as the ratio of the target normalized against endogenous references, for which potential quantitative references for normalizing real-time PCR data were generated for each sample using five common housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-microglobulin (β2M), β-actin, TATA box-binding protein (TBP), and cyclophilin A (CYCA) [19, 21]. The calibrator was obtained from a case of peracute death with accidental decapitation (ca. 24 h postmortem).

Statistical analyses

Regression equation analysis was used to study the relationships between pairs of parameters. Steel–Dwass test was used for nonparametric multiple comparison among groups. In addition, comparisons between individual groups were performed using the nonparametric Mann–Whitney U test. A p value of <0.05 was considered statistically significant.

Results

ANP and BNP levels in pericardial fluids

Pericardial ANP level (cut-off value, 30 pg/ml) was elevated in most cases of hypothermia and hyperthermia, while BNP level (cut-off value, 150 pg/ml) was markedly increased in hypothermia and CHD (Table 2).

Immunostaining of ANP and BNP in the myocardium

Immunostaining demonstrated ANP and BNP in the cardiomyocytes (Fig. 1). The staining intensity was lower in bilateral ventricular walls than in atrial walls, showing a varied intensity and distribution by case; however, differences were not evident among the causes of death.

ANP and BNP mRNA expressions in the myocardium

Site difference in target and reference mRNA expression

When raw data of mRNA amplification were compared for all cases, C_T values for ANP mRNA were evidently lower in atrial walls (left, 16.2–32.0 with a median of 21.6; right, 16.0–33.7 with a median of 19.7) than in bilateral ventricles (19.2–37.9 with medians of 29.0–30.5; $p<0.05$); ANP mRNA expression was generally higher in the atria than in the ventricles. BNP mRNA expression was similar in the atria and ventricles (17.5–38.3 with medians of 23.3–30.2); however, C_T values were slightly lower in the left/right atrial wall (median, 25.9/23.3) than in the ventricular walls (median, 29.0–30.2), and lower in the right atrial wall than in the left ($p<0.05$), indicating higher expression in the atria, especially in the right. In bilateral atria, mRNA expression was higher for ANP than for BNP ($p<0.0001$). Among five housekeeping genes, C_T values of GAPDH (19.4–30.1 with medians of 22.7–23.9), β2M (19.2–30.3 with medians of 22.9–23.1), and CYCA (19.5–30.4 with medians of 22.3–23.7) were similar at each site, and those of β-actin were slightly higher (19.9–34.1 with medians of 23.0–25.2; insignificant), showing no site differences; however, C_T values of TBP (25.9–36.7 with medians of 28.9–29.5) were

Table 2 ANP and BNP levels in pericardial fluid

Cause of death	*n*	Pericardial ANP[a] (pg/ml)		Pericardial BNP[b] (pg/ml)	
		Range	Median	Range	Median
Fatal drug intoxication					
Sedative-hypnotics	10	0–50	9	3–662	25
Methamphetamine	8	6–50	22	40–6,580	148
Hyperthermia	10	7–217	41	23–4,370	265
Hypothermia	13	7–214	98	175–4,300	1,400
AIHD					
AMI	16	0–140	12	6–1,970	106
IHD	19	4–215	21	9–3,170	213
CHD	11	1–89	19	19–5,930	2,000

ANP atrial natriuretic peptide, *BNP* brain natriuretic peptide, *AIHD* acute ischemic heart disease, *AMI* acute myocardial infarction, *IHD* acute ischemic heart disease without apparent myocardial necrosis, *CHD* chronic congestive heart disease

[a] ANP showed no significant differences among the causes of death by Steel–Dwass test. The results of individual comparisons by nonparametric Mann–Whitney *U* test were as follows. Significantly higher: hypothermia vs. other groups except for hyperthermia; hyperthermia vs. sedative–hypnotic intoxication

[b] BNP was significantly higher in hypothermia than in sedative–hypnotic intoxication and AIHD by Steel–Dwass test. The results of individual comparisons by nonparametric Mann–Whitney *U* test were as follows. Significantly higher: hypothermia vs. other groups except for CHD; CHD vs. sedative–hypnotic intoxication and AIHD; hyperthermia vs. sedative–hypnotic intoxication

higher than those of others at each site ($p<0.05$), indicating lower mRNA expression.

Stability of relative mRNA quantification with regards to gender, age, survival period, postmortem interval, and endogenous reference genes

Simultaneous RT-PCR of mRNA of five housekeeping genes (GAPDH, β2M, β-actin, TBP, and CYCA) showed high correlations of C_T values of GAPDH to others at each site ($r=0.90$–0.96, $p<0.0001$), showing almost equivalent values. β2M and CYCA also showed high and almost equivalent correlations to others ($r=0.93$–0.95, $p<0.0001$). Correlations of β-actin or TBP to others were partly lower ($r=0.77$–0.96, $p<0.0001$) than those of others, and C_T values were not equivalent; β-actin and TBP showed higher C_T values, indicating lower mRNA expressions.

For all cases, an age-dependent increase was partly detected for β2M, β-actin, TBP, and CYCA mRNAs, especially in right atrial and ventricular walls ($r=0.32$–0.50, $p=0.10$–$p<0.01$; partly significant), but was insignificant for GAPDH. No gender-related difference was detected for each housekeeping gene expression. A slight survival time-dependent decrease was detected for CYCA mRNA in bilateral atrial walls ($r=0.38$, $p<0.05$ for the left; $r=0.50$, $p<0.01$ for the right). A tendency toward a postmortem decrease was significant for TBP and CYCA mRNA in left anterior and posterior ventricular walls, respectively, but was otherwise insignificant within 48 h postmortem.

For ANP mRNA, there was no age-dependent, gender-related difference, or survival time or postmortem time dependency. BNP mRNA showed a slight age-dependent increase in the right ventricle ($r=0.42$, $p<0.05$), and a survival time-dependent increase in right atrial and ventricular walls ($r=0.39$ and $r=0.40$, respectively, $p<0.05$). No gender-related difference or postmortem time dependency was detected. When neighboring portions of the myocardium at each site were compared ($n=29$), correlations of ANP/BNP mRNA measurements were high ($r=0.91/0.87$, $p<0.0001$), showing no significant differences, irrespective of the site; local differences did not affect the findings. The stability of ANP and BNP mRNA assays was partly confirmed by re-examination of the same RNA samples ($r=0.93$ and $r=0.99$, respectively, $p<0.0001$, $n=50$); the assay-to-assay deviation was insignificant and did not affect the findings

Relative quantification of ANP and BNP mRNA levels in the myocardium with regard to the cause of death

When ANP and BNP mRNA expressions were normalized against GAPDH, β2M, or CYCA mRNA, in consideration of the stabilities and equivalencies of the housekeeping genes mentioned above, similar findings were seen with regard to the cause of death, as follows. In fatal methamphetamine abuse and hypothermia cases, myocardial ANP and BNP mRNA levels in bilateral atrial and ventricular walls were as high as those in AIHD and CHD (medians for ANP/BNP: left ventricle, 1.75–25.28/2.40–21.56; right

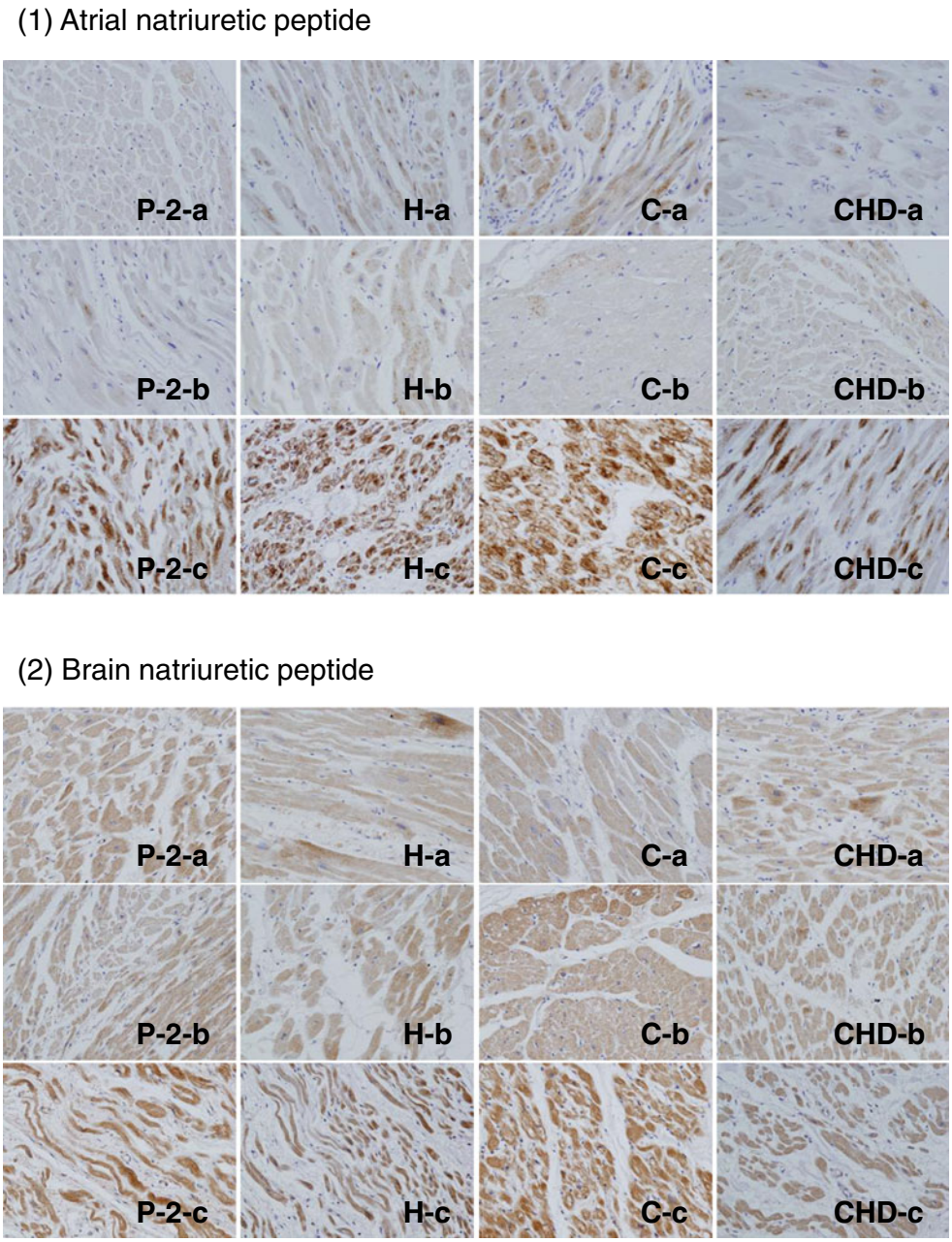

Fig. 1 Immunostaining of ANP (*1*) and BNP (*2*) in the cardiomyocytes in cases of methamphetamine intoxication (*P-2*), hypothermia (*H*), hyperthermia (*C*), and congestive heart disease (*CHD*), using monoclonal anti-human ANP and BNP. The atrial walls were more intensely positive than bilateral ventricular walls, showing varied intensity and distribution by case; however, differences were not evident among the causes of death. The findings were similar when polyclonal reagents were used. *a* Anterior wall of left ventricle; *b* right ventricular wall; c right atrial wall. *P-2* (62-year-old man; survival time, about 15 h; about 26 h postmortem); *H* (64-year-old man; survival time, about 3 h; about 28 h postmortem); *C* (64-year-old woman; survival time, about 6 h; about 30 h postmortem); *CHD* (52-year-old woman, survival time unknown; about 36 h postmortem)

ventricle, 0.33–1.23/0.46–23.05; bilateral atria, 786.88–2,336.28/30.19–337.79) (Fig. 2). Overall, these markers were low in sedative–hypnotic intoxication (medians for ANP/BNP: left anterior and posterior ventricular wall, 0.23 and 0.20/0.13 and 0.10; right ventricle, 0.14/0.30; left and right atria, 452.86 and 560.33/12.04 and 21.54) and hyperthermia (medians for ANP/BNP: left anterior and posterior ventricular wall, 0.35 and 0.19/0.29 and 0.23; right ventricle, 0.09/0.04; left and right atria, 63.70 and 412.50/1.10 and 4.18), compared with those in other groups; significant differences were detected for left ventricular ANP and BNP mRNA in sedative–hypnotic intoxication, and bilateral atrial and posterior left ventricular BNP in hyperthermia.

Expression of ANP mRNA was evidently higher in bilateral atria than in the ventricles, and a similar tendency was detected for BNP. Site-to-site correlations depended on the cause of death and were significant: in sedative–hypnotic intoxication, for ANP mRNA between right and left anterior ventricular walls as well as for BNP mRNA between bilateral atria, and between right and left posterior ventricular walls (r=0.85–0.99, p<0.01–0.0001); in fatal methamphetamine abuse, for ANP mRNA between bilateral atria as well as for BNP mRNA between bilateral atria, between anterior and posterior walls of the left ventricle, and between right and left anterior/posterior ventricular walls (r=0.91–0.99, p <0.01–0.0001); in hyperthermia, for ANP mRNA between

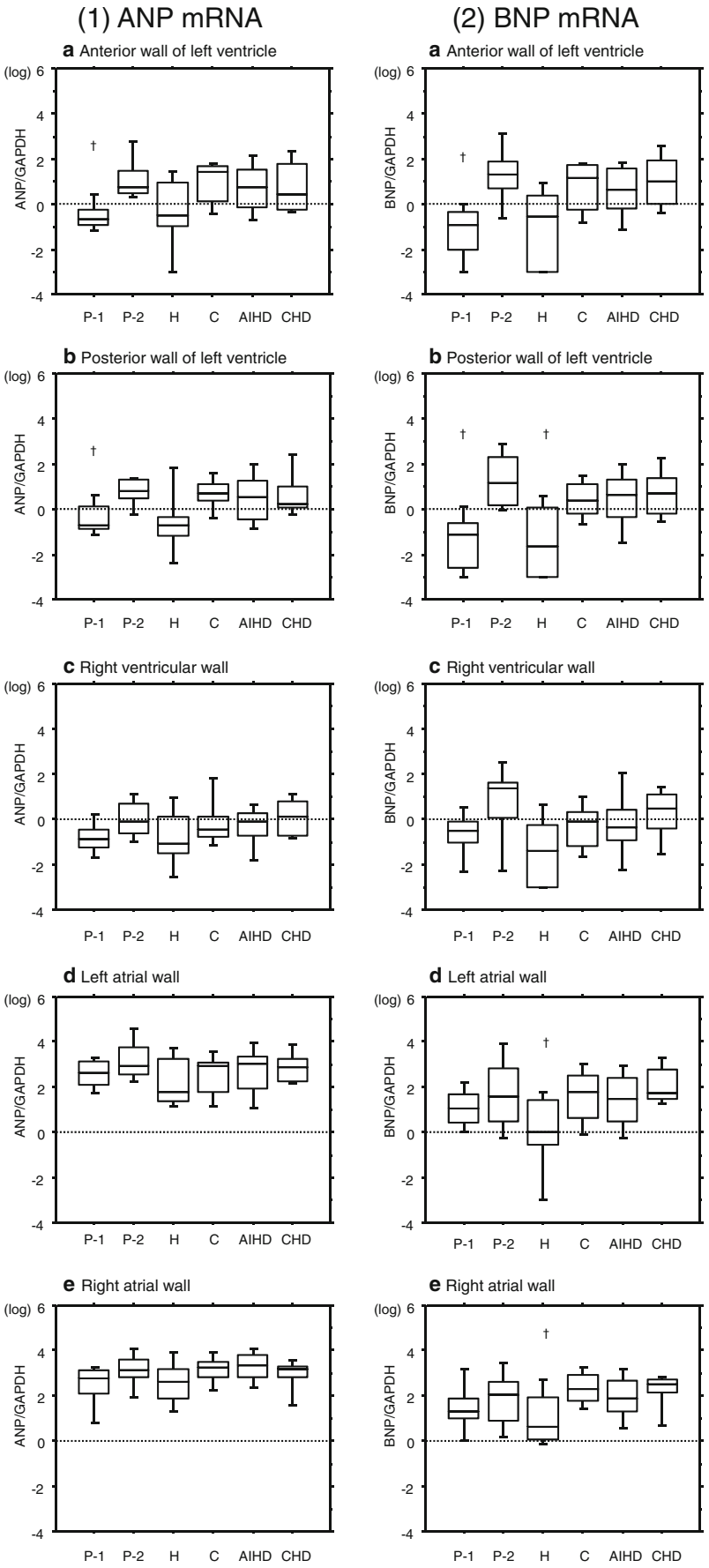
(1) ANP mRNA
(2) BNP mRNA
a Anterior wall of left ventricle
b Posterior wall of left ventricle
c Right ventricular wall
d Left atrial wall
e Right atrial wall
ANP/GAPDH
BNP/GAPDH
(log) 6
4
2
0
-2
-4
P-1 P-2 H C AIHD CHD
†

Fig. 2 ANP and BNP mRNA quantification on the logarithmic scale with regard to the cause of death. *ANP* atrial natriuretic peptide, *BNP* brain natriuretic peptide, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *P-1* sedative–hypnotic intoxication, *P-2* methamphetamine intoxication, *H* hyperthermia, *C* hypothermia, *AIHD* acute ischemic heart disease (including acute myocardial infarction and acute ischemic heart disease without apparent myocardial necrosis), *CHD* chronic congestive heart disease. *1* ANP mRNA quantification, normalized against GAPDH. *Dagger* Significantly lower: **a** anterior wall of left ventricle, sedative–hypnotic intoxication (*P-1*) vs. other groups except for hyperthermia (*H*) ($p<0.05$); **b** posterior wall of left ventricle, sedative–hypnotic intoxication (*P-1*) vs. methamphetamine intoxication (*P-2*) and hypothermia (*C*) ($p<0.05$); by Steel–Dwass test. The results of individual comparisons by nonparametric Mann–Whitney *U* test were as follows. Significantly lower: **a** anterior wall of left ventricle, P-1 vs. other groups except for H ($p<0.005$); H vs. other groups except for P-1 ($p<0.005$); **b** posterior wall of left ventricle, P-1 vs. other groups except for H ($p<0.01$-$p<0.005$); H vs. other groups except for P-1 and P-2 ($p<0.05$-$p<0.005$); **c**) right ventricular wall, P-1 vs. AIHD and CHD ($p<0.05$); **e** right atrial wall, P-1 vs. AIHD ($p<0.05$) and C ($p<0.01$); H vs. AIHD ($p<0.05$). These findings were similar when normalized against β2-microglobulin (*β2M*) or cyclophilin A (*CYCA*). *2* BNP mRNA quantification, normalized against GAPDH. *Dagger* Significantly lower: **a** anterior wall of left ventricle, sedative–hypnotic intoxication (*P-1*) vs. other groups except for hyperthermia (*H*) ($p<0.05$); **b** posterior wall of left ventricle, sedative–hypnotic intoxication (*P-1*) vs. other groups except for hyperthermia (*H*) ($p<0.05$), and hyperthermia (*H*) vs. methamphetamine intoxication (*P-2*) and acute ischemic heart disease (AIHD) ($p<0.05$); **d** left atrial wall, hyperthermia (*H*) vs. chronic congestive heart disease (*CHD*) ($p<0.05$); **e** right atrial wall, hyperthermia (*H*) vs. hypothermia (*C*) ($p<0.05$) by Steel–Dwass test. The results of individual comparisons by nonparametric Mann–Whitney *U* test were as follows. Significantly lower: **a** anterior wall of left ventricle, P-1 vs. other groups except for H ($p<0.005$-$p<0.001$); H vs. P-2, AIHD and CHD ($p<0.05$); **b** posterior wall of left ventricle, P-1 vs. other groups except for H ($p<0.005$-$p<0.001$); H vs. other groups except for P-1 ($p<0.05$-$p<0.005$); **c** right ventricular wall, P-1 vs. P-2 ($p<0.05$); H vs. P-2 and CHD ($p<0.05$); **d** left atrial wall, P-1 vs. CHD ($p<0.05$); H vs. C, AIHD and CHD ($p<0.05$-$p<0.005$); **e** right atrial wall, P-1 vs. P-2 ($p<0.05$); H vs. C, AIHD and CHD ($p<0.05$-$p<0.005$). These findings were similar when normalized against β2-microglobulin (*β2M*) or cyclophilin A (*CYCA*)

bilateral atria, between anterior and posterior walls of the left ventricle, and between the left atrium and left anterior/posterior ventricular walls as well as for BNP mRNA between right and left posterior ventricular walls ($r=0.66$–0.95, $p<0.05$–0.0001); in hypothermia, for ANP/BNP mRNA between bilateral atria ($r=0.85$, $p<0.001$/$r=0.87$, $p<0.0001$); in AIHD, for ANP mRNA between bilateral atria, between the left atrium and left posterior ventricular wall, and between right and left anterior ventricular walls ($r=0.35$–0.90, $p<0.05$–0.0001) as well as for BNP mRNA between bilateral atria, between anterior and posterior walls of the left ventricle, between the right atrium and ventricle, and between the left atrium and left posterior ventricular wall ($r=0.35$–0.90, $p<0.05$–0.0001); and in CHD, for ANP mRNA between bilateral atria, between anterior and posterior walls of the left ventricle, and between right and left anterior ventricular walls ($r=0.64$–0.96, $p<0.05$–0.0001).

The heart weight was relatively large in AIHD and small in sedative–hypnotic intoxication (significantly different between these groups), compared with those in other groups (insignificant), and combined lung weight was increased in most cases other than hypothermia (Table 1). In fatal methamphetamine abuse, anterior left ventricular ANP mRNA level correlated with the heart weight ($r=0.73$, $p<0.05$), and CHD showed correlations of right ventricular ANP, and left and right atrial BNP mRNA levels to the heart weight ($r=0.769$, $p<0.01$, $r=0.697$, $p<0.02$, and $r=0.670$, $p<0.05$, respectively). Correlations with the combined lung weight were detected for anterior left ventricular ANP mRNA in AIHD and CHD. Otherwise, there was no correlation of ANP or BNP mRNA to the heart or lung weight.

Discussion

Various ancillary procedures have been published for determining deaths due to hypothermia (cold exposure) and hyperthermia (heat stroke), including immunohistochemical and biochemical markers related to stress responses, metabolic deterioration, and systemic tissue damage [11, 13, 14, 24–31]. These markers can demonstrate metabolic deterioration and persistent heart failure without substantial damage to life-supporting organ tissues, predispositions and complications in death from cold exposure, and dehydration and/or advanced multiple organ tissue damage (MODS) in death from heat stroke, as well as different stress responses in these causes of death [11, 13, 25–29]. These procedures are also useful for investigating systemic dysfunction in fatal intoxication [14, 30–34]. In addition, previous studies have suggested that relative mRNA quantification can be used for postmortem investigation of molecular biological alterations in the death process [1–5, 17, 18, 20, 22, 23].

In the present study, the stability of relative mRNA quantification using RT-PCR for autopsied myocardium specimens was established for target and reference genes; endogenous reference markers (GAPDH, β2M, β-actin, TATA, and CYCA) showed high correlations in simultaneous assays. Among these reference markers, however, expressions of GAPDH, β2M, and CYCA mRNAs showed high correlations and equivalencies without evident age-dependent or gender-related difference, and their expression levels were similar to those of target mRNAs (ANP and BNP). Using these housekeeping genes, relative mRNA expression levels of individual target genes (ANP and BNP), normalized against each endogenous reference, showed no significant deviation between neighboring sites or in re-examination, representing mRNA expressions at the site of sampling; target mRNA expression levels could be successfully evaluated within 2 days postmortem.

BNP is secreted by both cardiac atria and ventricles in response to persistent cardiac strain, although the main source is the ventricle, whereas ANP is secreted from the atrium in a physiological state, and the induction of ANP mRNA is seen in a pathological state involving heart failure [35–38]. In the present study, immunostaining of the myocardium detected no differences among the examined causes of death; however, biochemical and molecular pathological analyses demonstrated significant differences among these causes of death, as described below. Overall expression of BNP mRNA and higher ANP mRNA expression in bilateral atria than in the ventricles was consistent with the major site of physiological secretion and induction in response to increased cardiac strain, described above [35–38]. Generally higher ANP mRNA expressions in bilateral atria may represent increased central venous (right atrial) and pulmonary capillary (left atrial) pressure as a sign of terminal heart failure, thus showing minor differences among the causes of death.

In intoxication, high myocardial ANP and BNP mRNA expressions in the whole heart in fatal methamphetamine abuse, which were similar to those of AIHD, suggested high cardiac strain as a sign of cardiac dysfunction accompanied by intense pulmonary congestion [39]. The correlation of anterior left ventricular ANP mRNA with the heart weight suggested the contribution of pre-existing left ventricular strain related to cardiac hypertrophy, whereas complication of advanced hyperthermia may reduce these mRNA expressions, as described below [40, 41]. In sedative–hypnotic intoxication, however, overall lower ANP and BNP mRNA expressions, especially in the left ventricle, in sedative–hypnotic intoxication indicated decreased left ventricular strain, which can be the consequence of reduced left ventricular filling due to advanced pulmonary edema, accompanied by diffuse myocardial damage [42]. Meanwhile, no significant increase in pericardial ANP or BNP level was detected in these intoxication cases. These findings were consistent with pathological findings of increased lung weight in methamphetamine and sedative–hypnotic intoxication, mainly accompanied by acute congestion and edema, respectively, showing differences in these drug toxicities. Determination of fatal intoxication depends on toxicological data; however, blood drug levels varied by case and were often below lethal levels, especially in cases of a longer survival combined with drug abuse. With respect to this, previous studies using biochemical and immunohistochemical markers have indicated systemic deterioration of nervous systems accompanied by myocardial and skeletal muscle damage, suggesting toxic or adverse effects of drugs, including methamphetamine and sedative–hypnotics [26, 30, 43]. The present study demonstrated a difference in terminal cardiac function between methamphetamine and sedative–hypnotic intoxication, which may be used to interpret major drug effects in combined drug abuse; the molecular pathology of myocardial natriuretic peptides can provide additional findings for evaluating terminal cardiac function related to fatal intoxication.

Hypothermia (cold exposure) presented with characteristic findings, involving higher expressions of myocardial ANP and BNP mRNA in the whole heart, accompanied by elevated pericardial ANP and BNP levels, indicating cardiac dysfunction accompanied by persistent heart failure without substantial damage to the myocardium [16, 44], ready for recovery by adequate medical management [44–46]. In hyperthermia (heatstroke) cases, however, especially lower expressions of posterior left ventricular and bilateral atrial BNP mRNA, accompanied by an increase in pericardial ANP may represent the terminal cardiac status as a consequence of reduced circulatory blood volume due to generalized vasodilatation and dehydration, accompanied by increased venous return and high-output cardiac failure, as well as diffuse myocardial damage involved in multiple organ tissue damage (multiple organ dysfunction syndrome), followed by circulatory collapse [47–49]. These observations indicate a difference in terminal cardiac function between hypothermia and hyperthermia as well as between hypothermia and sedative–hypnotic intoxication and also between hyperthermia and methamphetamine intoxication, which can contribute to analysis of terminal cardiac dysfunction on a case-by-case basis in individual fatalities due to extreme environmental temperatures under possible influence of drugs.

In relative mRNA quantification, the amount of tissue sample or the site of sampling does not immediately affect the assay. In the present study, site-to-site correlations were detected depending on the cause of death, suggesting parallel responses to bilateral atrial and ventricular strain in fatal methamphetamine abuse and AIHD as well as to bilateral atrial strain in sedative–hypnotic intoxication and hypothermia (cold exposure), and the ANP-dominant responses to cardiac strain in the death process of hyperthermia (heatstroke) and CHD. Interindividual differences in ANP and BNP mRNA expressions in each cause of death, showing partial overlap with other groups, may represent the severity of terminal cardiac dysfunction. Because of microanalysis, however, the findings may depend on the status of tissue at the site of sampling; thus, at least histological evaluation of the sampling site is needed. Double sampling at each site, as well as site-to-site comparisons of ANP and BNP mRNAs, may be helpful to establish the findings in individual case studies.

In conclusion, the present study demonstrated terminal high cardiac strain in methamphetamine intoxication, decreased cardiac strain in sedative–hypnotic intoxication and hyperthermia, and persistent congestion in hypothermia, suggesting the possible application of postmortem molecular biological analysis of myocardial ANP and BNP to demonstrate terminal cardiac dysfunction as part of systemic functional changes of life-supporting organs related to fatal

intoxication, hypothermia (cold exposure), and hyperthermia (heatstroke), which may be helpful to reinforce pathological and toxicological findings.

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