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Original Article

Quantitative Analysis of DNA Degradation in the Dead Body

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Postmortem degradation of DNA was quantitatively estimated. Brain, liver, kidney and muscle samples were obtained from sacrificed rats left at 20℃ or 4℃. The quantity of DNA was measured by real-time PCR using a primer set for a sequence in the Rsrc 1 gene. When the quantity of amplified DNA using 10 ng Human Genomic DNA was defined as 100 RFU, the quantities in the brain, liver, kidney and skeletal muscle (each 2 μg of dry weight) on the day of sacrifice were 253 ± 11, 338 ± 22, 556 ± 14 and 531 ± 12 Relative Fluorescence Units (RFU), respectively (mean ± S.E., n = 5). The quantity of amplified DNA decreased to below 10 RFU in 1–3 weeks in the liver, kidney and skeletal muscle at 20℃, while that in the brain was more than 10 RFU for six weeks, demonstrating the usefulness of the brain as a sample for DNA analysis of decaying corpses. It was suggested that quantifying the amplified DNA in the brain at 20℃ and in the liver at 4℃ as well as the ratio of the quantity of amplified DNA in the liver to the brain at 4℃ might be useful for diagnosing time of death. This study provides the first quantitative analysis of the postmortem progress of DNA degradation in the corpse.

Key words: DNA degradation, postmortem interval, personal identification

In the last decade, DNA analyses such as typing of short tandem repeats (STRs), single nucleotide polymorphisms (SNPs) and mitochondrial DNA have become useful tools for personal identification in the field of forensic medicine and criminal science [1–4]. In particular, kits for analyzing STRs with a high exclusion chance are now available worldwide [5–7], and have became the routine method for that purpose. As this technology has become more readily available, DNA analysis for the personal identification of decomposed body has been frequently required. At the same time, however, difficulties with this application remain [8–11]. The most important of these difficulties is the degradation of template DNA (very shortly fragmented DNA) [12, 13], which is thought to be caused mainly by intracellular enzymes and decomposition through bacterial proliferation in the corpse [14, 15].

If DNA degradation is indeed caused by intracellular enzymes and bacterial proliferation, then its estimation may lead to a diagnosis of the degree of postmortem changes, including the time after death. And in fact, the relationship between the progress of DNA degradation and the postmortem interval in combination with conditions such as temperature has been investigated in some studies [15–18]. However, the relation has not been investigated in sufficient detail.

In this paper, the relation between changes of DNA degradation in various animal organs and the postmortem intervals was examined by quantitative analysis using real-time PCR in animal experiment, and the implications of the results were discussed.

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Materials and Methods

Animal experiment. Adult SD rats with about 300 grams of body weight were euthanized by carbon dioxide inhalation under etherization. Each rat was left at 20℃ or 4℃ in a separate airtight container to avoid desiccation/mummification.

Samples of the brain, kidneys, liver and skeletal muscle of the thigh were taken each week up to the sixth week after death. The former 2 organs were resected as whole organs. From the latter 2 a piece of more than 2 grams was obtained. Five rats were used for the trial with a postmortem interval of 1 month (0–4 weeks) and 3 animals were sacrificed for the longer postmortem period (5–6 weeks). These experiments met the standards and principles of animal care, and ethical approval was obtained from the Department of Animal Resources, Advanced Science Research Center, Okayama University.

Sample preparation and DNA extraction. The obtained organ was cut into minute pieces with scissors and frozen. After lyophilization it was stirred well so that it became homogenous powder.

A mixture containing 0.1 gram of the powdered sample, 75 μl of 10 mM phosphate buffered saline (pH 7.2), 75 μl of GM buffer (Applied Biosystems, Foster City, CA, USA), 15 μl of 10% sodium lauryl sulfate and 100 μg of proteinase K was shaken at 800 rpm and 65℃ for 24 h using a Thermomixer comfort (Eppendorf, Hamburg, Germany). During the digestion the same amount of proteinase K was added every 8 h.

After the digestion, DNA was extracted using a BIO ROBOT EZ1 Investigator Kit (Qiagen, Düsseldorf, Germany) and resuspended in 100 μl of the Tris-HCl EDTA buffer included in the EZ1 Investigator Kit.

Real-time PCR conditions and data analysis. A primer set was designed to amplify a 225-base-pair region of the Rsrc 1 gene on rat chromosome 2. The concrete sequence of the forward primer was 5'-ATGCCAATTAGGAGGTTGAGTA-3' and that of reverse one was 5'-CCTCCATTAATTGCAGGAAAA-3'.

Quantitation of this sequence was performed by real-time PCR using SYBER Green I. The reaction mixture consisted of 10 μl of 2 × AmpliTaq Gold Fast PCR Mix (Applied Biosystems), 0.1 μl of 1/100 SYBER Green I dilution (Molecular Probes/Invitrogen, Carlsbad, CA, USA), 0.3 μl of 0.02 mM ROX reference Dye (Roche Diagnostics, Basel, Switzerland), 6 μl of 2 M D-(+)-trehalose dehydrate (Sigma-Aldrich, St. Louis, MO, USA), the primers (200 nM each), 2 μl of sample DNA and DDW to 20 μl [19, 20].

The PCR was conducted by an initial hold at 95℃ for 5 min, followed by 40 cycles consisting of 95℃ for 15 sec, 58.5℃ for 30 sec and 72℃ for 60 sec. The melting curve analysis was performed as follows: 95℃ for 15 sec, 60℃ for 60 sec, and heating to 95℃ at a ramping rate of 0.3℃/sec.

Human Genomic DNA (Promega, Madison, WI, USA) was used as a standard. The strength of the fluorescent signal obtained from 5 ng/μl of the standard solution was defined as 100 RFU (Relative Fluorescence Units). A calibration curve was made using a stepwise diluted standard solution of 5 ng/μl. Tris-HCl buffer containing 1 mM EDTA (pH 8.0) was used as a negative control. Amplification, fluorescent signal detection and quantitation of the amplified fragment were performed using a StepOne Plus Real-Time PCR System (Applied Biosystems).

The analyzed data were collected using StepOne software and the ratios of the quantity of amplified DNA between organs were calculated. All data are presented as the means ± S.E. on the graph. Statistical analyses were performed using Student's *t*-test for comparisons of data between 2 successive weeks. Differences were considered significant at $p < 0.05$.

Results

The quantity of amplified DNA decreased postmortem in all investigated organs and conditions in general (Figs. 1 and 2). When the quantity of amplified DNA using 10 ng Human Genomic DNA was defined as 100 RFU, that in a 2 μg (dry weight) sample of the brain was 253 ± 11 RFU on the day of sacrifice (mean ± S.E., n = 5). In the liver, it was 338 ± 22 RFU (mean ± S.E., n = 5). The values in the kidney and skeletal muscle were higher, or 556 ± 14 and 531 ± 12 RFU, respectively (mean ± S.E., n = 5).

In the brains of sacrificed rats left at 20℃, the quantity of amplified DNA decreased almost linearly for 6 weeks, although it was maintained at approximately 100 RFU or more for 5 weeks and fell to

approximately 20 RFU on the sixth week. In the dead body left at 4℃, no obvious decrease in brain DNA was observed, and the quantity of DNA was always more than 200 RFU.

The quantity of amplified DNA in the liver decreased rapidly to below 10 RFU in the first week and this level was maintained until the sixth week, when the dead rats were kept at 20℃. In the rats left at 4℃ it decreased slowly along with the time after death, reaching a value below 100 RFU on the fifth week remaining above 10 RFU at the end of the experiment.

In both kidney and skeletal muscle, the quantity of amplified DNA decreased to less than 100 RFU in 2 weeks in the dead rat body left at 20℃. It then continued to decrease until the fourth week and reached a level less than 10 RFU. In the experiment at 4℃, the quantities of amplified DNA in these organs fell to 221 ± 12 and 259 ± 9 RFU in 2 weeks, respectively (mean $\pm$ S.E., n = 5). The level dropped to less than 100 RFU on the sixth week in the kidney, while that in the skeletal muscle was still approximately 200 RFU by the end of the experiment.

The ratios of the quantity of amplified DNA in the liver, kidney and skeletal muscle to that in the brain (L/B, K/B and M/B) are shown in Figs. 3 and 4. On the day of sacrifice, they were 1.3 ± 0.04, 2.2 ± 0.06 and 2.2 ± 0.09, respectively (mean $\pm$ S.E., n = 5). L/B fell to less than 0.1 in the week after death at 20℃, while it decreased gradually at 4℃. The K/B values were 0.3 ± 0.03 and 1.3 ± 0.1 after 1 week at 20℃ and 4℃, respectively (mean $\pm$ S.E., n = 5). By the sixth week, the K/B value was 0.3 or less at 20℃. Under the lower-temperature condition of 4℃, K/B was at approximately 1.0 by the third week and then decreased. M/B fell to 0.7 ± 0.03 on the first week (mean $\pm$ S.E., n = 5) and to less than 0.1 on the third week or longer at 20℃. That at 4℃ was

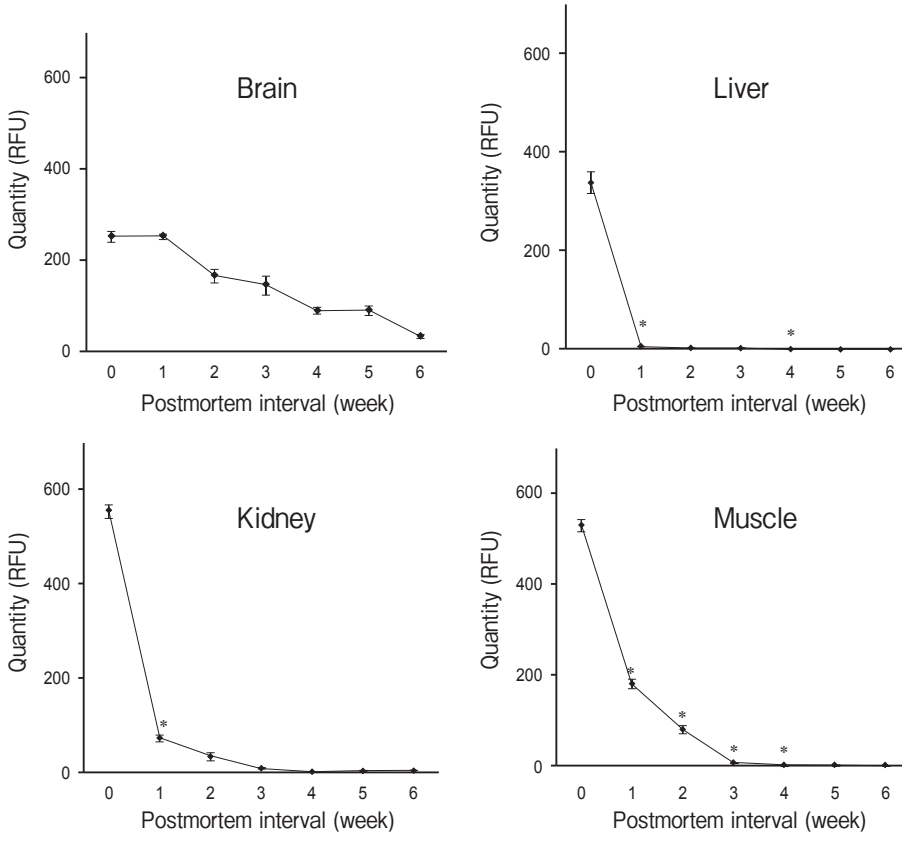

Fig. 1 Postmortem change of the DNA quantity in various organs obtained from the dead body of rats left at 20℃ (mean $\pm$ S.E.). The quantity amplified from 10 ng of Human Genomic DNA was defined as 100 RFU. The DNA was amplified using 2 μg (dry weight) of each organ. *Statistically significant difference versus the previous week at $p < 0.05$ by Student's *t*-test.

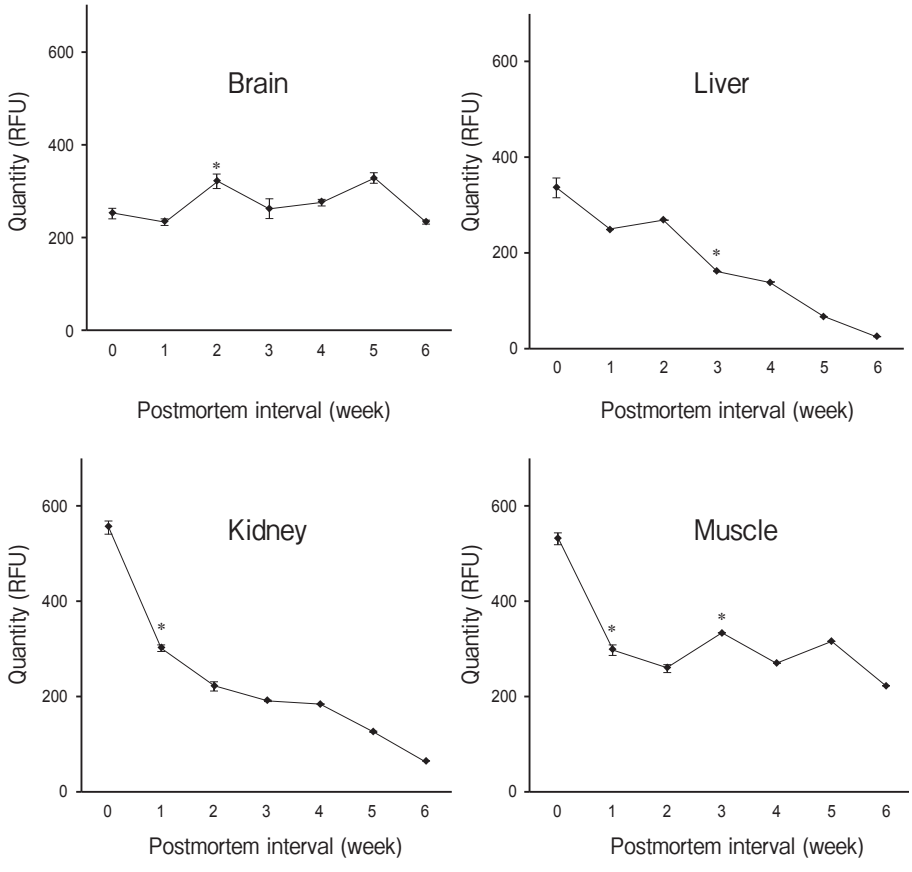

Fig. 2 Postmortem change of the DNA quantity in various organs obtained from the dead body of rats left at 4℃ (mean ± S.E.). The quantity amplified from 10 ng of Human Genomic DNA was defined as 100 RFU. The DNA was amplified using 2 μg (dry weight) of each organ. *Statistically significant difference versus the previous week at $p < 0.05$ by Student's *t*-test.

approximately 1.0 on the first week and remained at this level for the 6 weeks postmortem.

Discussion

There have been several reports investigating postmortem degradation of DNA in various organs. Bär *et al.* [15] analyzed the postmortem stability of DNA in 8 human organs and Johnson *et al.* [16] discussed the degradation of DNA in 5 porcine organs. However, only semi-quantitative evidences were shown in these reports. Niemcunowicz *et al.* [17] also referred to the postmortem change of DNA under various conditions, but in their study the DNA levels were estimated indirectly through the detection of STRs.

Although there was a previous study in which DNA was quantified and its degradation was directly estimated, the author did not investigate the changes according to the time after death [10]. Another study on the postmortem degradation of DNA by its quantification using real-time PCR was a typical *in vitro* experiment using Control Human Genomic DNA [18]. In the present study, we took these previously reported data into consideration and decided to estimate the postmortem degradation of DNA in organs in the dead body by quantitative analysis using real-time PCR, reasoning that the ongoing degradation of DNA in the organs of the dead body might cause a decrease in the template to be amplified. It was expected that the targeted sequence to be amplified had no variations/mutations which would cause a failure of amplification, and thus that all reductions in amplification could be attributed to the degradation of DNA. It was also expected that this sequence would be conserved among mammalian species, so that this method could

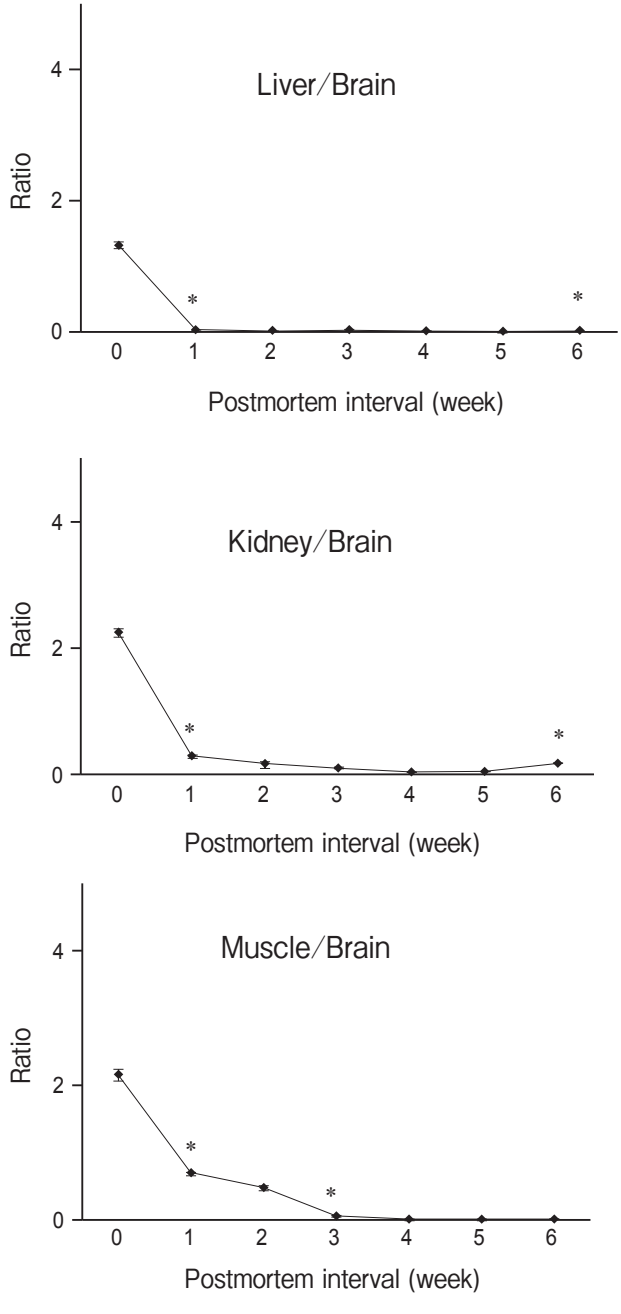

Fig. 3 Change of the ratio of DNA quantity in the liver, kidney and skeletal muscle to that in the brain of the rat conserved at 20℃ after death (mean ± S.E.). *Statistically significant difference versus the previous week at $p < 0.05$ by Student's *t*-test.

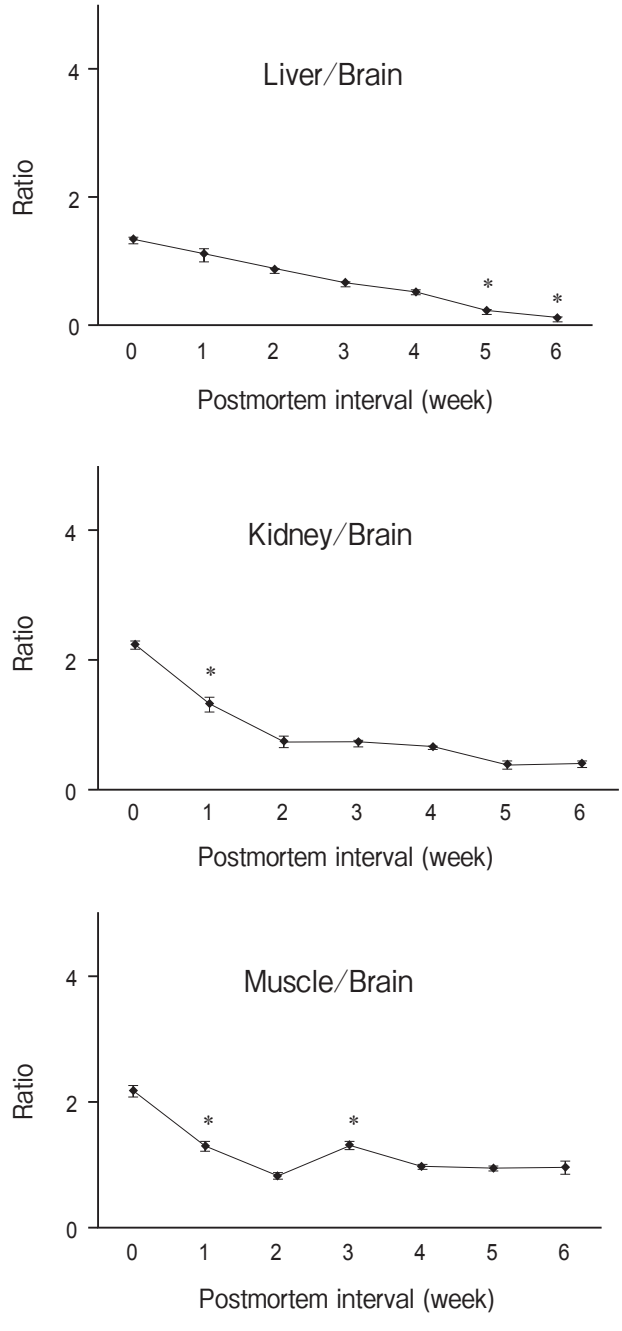

Fig. 4 Change of the ratio of DNA quantity in liver, kidney and skeletal muscle to that in the brain of the rat conserved at 4℃ after death (mean ± S.E.). *Statistically significant difference versus the previous week at $p < 0.05$ by Student's *t*-test.

be practically applied to forensic cases. Therefore we took a note on human chromosome 3 in which many genes were well conserved among mammals [21], and the concrete sequence amplified was found in a region of Rsrc 1 gene, for which no variation/mutation had been reported in rat by UCSC Genome Bioinformatics <http://genome.ucsc.edu/cgi-bin/hgTracks?hgsid=188022 807&db=rn4&position=chr2:156944410-

156944635&hgPcrResult=pack. accessed on March 3, 2011>.

It was thought that the quantities of extracted DNA to be analyzed were inconsistent among the specimens, when a piece of organ was taken at random. This was because the organs contained regions of different cellular densities, and it was not possible to obtain a sample with a constant ratio of these parts —*e.g.*, the parenchyma and interstitial area, cortex and medulla, gray matter and white matter, muscular cells and fascia/tendons. Therefore we lyophilized and powdered the entirety of each obtained specimen, then stirred it well to obtain samples with a consistent cellular density for the amplification.

According to our investigation, the degradation of DNA progressed along with the time passed after death (Figs. 1 and 2). Although this fact has already been reported by previous authors [10, 15–18] and observed in actual criminal investigations, the present study is the first to provide quantitative evidence of the phenomenon.

On the day of sacrifice, the quantity of amplified DNA in the liver was approximately half of that in the kidney/skeletal muscle, and that in the brain was the least among the investigated organs. The reasons for this seemed to be the higher activity of hydrolysis enzymes in the liver and the low cellular density in the brain [15, 16].

Although the quantity of amplified DNA became less than 10 RFU at 1–3 weeks in the liver, kidney and skeletal muscle at 20℃, that in the brain remained more than approximately 100 RFU by the fifth week postmortem and more than 10 RFU in the sixth week. This change was almost linear, and thus the quantification of DNA in the brain was thought to be useful for estimation of the postmortem interval. The quantity of DNA remaining in the brain also means that this organ may be a recommended specimen for the personal identification of a putrefied corpse. In the previously reported cases, bone has usually been used for this purpose [8, 9, 13, 22], but the extraction of DNA from bone is a very time-consuming process. However, brain is the organ that is likely to remain in the cranial cavity, if the body is highly putrefied.

Practically we experienced 2 cases in which the brain was a useful material for DNA analysis. The victims were unidentified and highly putrefied/mummified. The postmortem intervals of these cases were estimated to be 1–2 months, but the muddy brain remained in the cranial cavity (Fig. 5). In the examination of ABO blood group genotype [23] using the brain, the obtained electrophoretogram was satisfactory to judge the type in both cases (Fig. 6-A, B). It was revealed that the brain was a usable material for DNA analysis, even if its appearance had changed to an extraordinary degree.

The quantity of amplified DNA decreased relatively gradually or showed almost no change in all organs left at 4℃ postmortem, compared with those left at 20℃. This was thought to attributable to the temperature-dependence of the activity of hydrolysis enzymes [10, 17]. The concrete quantity of amplified DNA in the kidney was more than approximately 100 RFU, even if the time after death passed 5 weeks. That in the skeletal muscle was approximately 200 RFU after 2 weeks postmortem or later. Though the degradation of DNA progressed most rapidly in the liver also at 4℃, the quantity of amplified DNA decreased almost linearly, and it was more than 10 RFU even on the sixth week after death. In the brain the degradation of DNA almost did not progress at

Fig. 5 Appearance of the brain in the cranial cavity changing to a mud-like consistency, with which ABO blood group genotypes were examined.

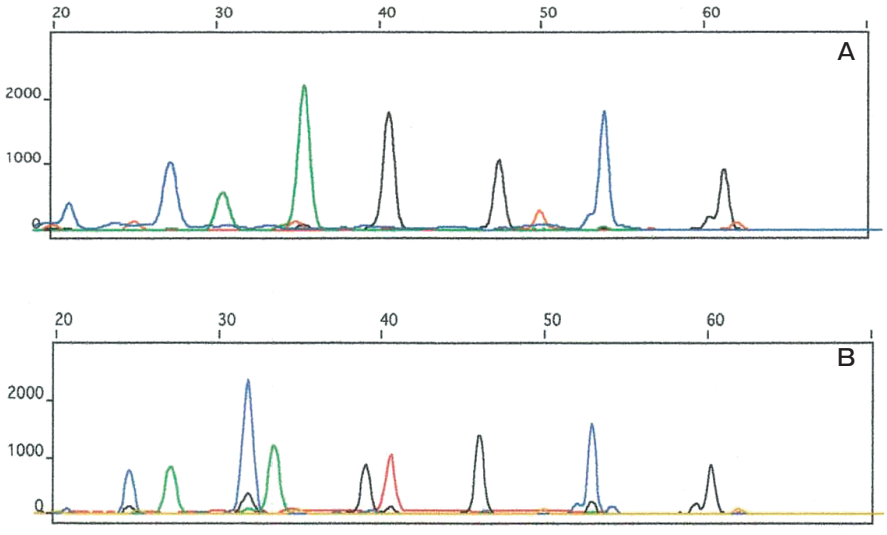

Fig. 6 Electrophoretogram of ABO blood group genotyping using the brain of 2 highly decomposed bodies. Six SNPs characterizing the ABO genotype were clearly detected from the highly putrefied and mud-like brain by multiplex single-base primer extension reaction using fluorescence-labeled ddNTPs in both cases and the type was determined as AO^A in 1 case (**A**) and AO^G in the other case (**B**) according to the method reported by Doi *et al*. [23]. The X-axis indicates the length of the DNA fragment. The Y-axis indicates relative fluorescence units (RFU).

4℃. According to an additional experiment, the quantity of amplified DNA in this organ was kept to more than 100 RFU for 20 weeks postmortem. Considering the above-mentioned changes, the liver seemed to be the most useful of the investigated organs for estimating the postmortem interval by quantifying the DNA, when the corpse was left at the cooler temperature.

It is well known in forensic practice that the progress of putrefaction sometimes varies considerably among dead bodies. In an attempt to reduce this effect, we calculated the ratios of the quantity of amplified DNA in the liver, kidney and skeletal muscle to that in the brain in the same individual and investigated their significance.

The ratio of the liver to the brain (L/B) was 1.3 ± 0.04 on the day of sacrifice (mean ± S.E., $n = 5$), and it decreased to less than 0.1 by the end of the first week postmortem at 20℃. The ratios of kidney and skeletal muscle to brain (K/B and M/B) declined more rapidly than the L/B, falling from 2.2 ± 0.06 to 0.3 ± 0.03 and from 2.2 ± 0.09 to 0.7 ± 0.03, respectively (mean ± S.E., $n = 5$).

When the dead body of the rat was conserved at 4℃, the ratios of L/B, K/B and M/B all decreased to approximately 1.0 within 1 week postmortem. For the K/B and M/B ratios, this level was maintained until at least the third week, and in the case of M/B it was maintained until the end of the experiment. In contrast, L/B decreased almost linearly over the 4 weeks after death. Therefore, quantifying the amplified DNA in the liver and brain as the same time may become a useful tool for the forensic diagnosis of time of death in the cold season.

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