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Kinetics and tissue distribution of bismuth, tin and lead after implantation of miniature shotgun alloy pellets in rats

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ABSTRACT

Introduction: Shotgun pellets containing bismuth (Bi) as substitute for lead (Pb) are increasingly being used due to environmental concerns. Information on toxicokinetics of Bi is lacking for the assessment of humans accidentally shot by Bi-containing shotgun alloy pellets.
Methods: Male Wistar rats were exposed to miniature alloy pellets containing Bi, tin (Sn) and minor amounts of Pb by implantation in muscle tissues of the hind legs.
Results: The concentrations of Bi in whole blood and urine increased up to 53 weeks after implantation. The highest concentrations of Sn in whole blood were observed three weeks after implantation, then declining to background levels 53 weeks after implantation. Lead in whole blood increased up to 13 weeks of exposure, and declined for the remaining observation period. Bismuth and Sn accumulated mainly in kidney, but also in liver, testicle and brain. Analytical field emission scanning electron microscopy of post-implant pellets showed depletion of Pb towards the pellet surface. Oxygen and chlorine accumulated in Sn rich lamellas in areas next to the pellet surface. The distribution of Bi remained visually unaffected as compared to pre-implant pellets.
Conclusion: The concentration of Bi increased during the whole observation period in blood, urine, kidney, brain, testicle and liver. The decline in the concentrations of Pb and Sn in blood and urine after reaching the peak concentration may be related to alterations in the chemical composition and element distribution of the implanted alloy pellets.

1. Introduction

We determined bismuth (Bi) in whole blood (B-Bi) and urine (U-Bi) in a hunter accidentally wounded by shotgun alloy pellets containing Bi, tin (Sn) and minor amounts of lead (Pb). Many fragments could not be removed by surgery. Measurements showed the highest B-Bi concentration of 15.7 μg/L three months after the accident declining slowly to 4 μg/L 36 months later. A median B-Bi concentration of 0.001 μg/L in non-occupationally exposed humans has been reported [1], suggesting a substantial systemic uptake of Bi in the hunter. As no scientific publications were found on Bi toxicokinetic and dose related effects in humans wounded by Bi-containing shotgun alloy pellets, clinical assessment of the patient was hampered.

Bismuth is mainly used in the production of chemicals, in alloys, as an additive in casting and in cosmetics [2]. It is also used for treatment of medical conditions, and in particular for gastrointestinal disorders [3]. The use of Bi as a substitute for Pb in shot shell ammunition is increasing because Pb shots have been banned in many countries. Bismuth compounds are considered to be slightly to moderately absorbed when inhaled or ingested. However, encephalopathy was reported in humans ingesting Bi subgallate and subnitrate for the treatment of various gastrointestinal disorders [4]. Toxic effects of Bi affecting kidneys, gastrointestinal tract and bone system have also been reported in humans [2].

There are, in contrast to missing data on kinetics, some animal data on Bi tissue distribution after implantation of shotgun pellets. In mice, Bi was detected in lung, kidney, spleen, and central nervous system four and nine weeks after intraperitoneal implantation [5], and in the kidney and neurons of the spine and brain six and 12 months, respectively, after implantation into rat muscles [6]. Mean Bi concentrations in kidney, liver, and gonads were 1.8, 0.19, and 0.46 μg/kg (wet weight) respectively, in mallards after commercial Bi alloy shots had been embedded in breast muscles, but no major histopathological abnormalities were observed [7]. Slight local inflammation was observed

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in rat muscle tissue up to 26 weeks after implantation of shotgun shells containing Pb and Bi [8].

Gastrointestinal and pulmonary absorption of inorganic Sn is low in humans [9]. This is one important reason for the general assumption of inorganic Sn having low systemic toxicity in humans [10]. However, increased amounts of Sn have been measured in different tissues in rats after gastrointestinal administration, e.g. bone, kidney, liver and brain [9]. Once inside the body, a number of toxic effects of inorganic Sn have been observed in animal studies, such as neurotoxicity, reduced bone calcium content, genotoxicity, immunotoxicity, and nephrotoxicity [9,11]. Biochemical testicular alterations was observed in rabbits exposed to stannous chloride [12]. The need for re-examination of the use of Bi as a substitute for Pb in shotgun pellets has been addressed, both for animal health reasons and to a lesser extent for human health concerns [13]. There is also need for more data on the release of Bi and associated uptake of the alloy elements into the blood, their distribution to critical tissues and organs, and subsequent elimination. The need for systematic monitoring of alloy elements during treatment of hunters accidentally wounded by shotgun pellets where Pb has been substituted has also been addressed [8].

The aims of this study were to investigate the kinetics of Bi, but also Sn and Pb, in blood and urine of rats after intramuscular implantation of simulated shotgun pellets, and to assess potential accumulation of these elements in the brain, liver, kidney and testicle. A further aim was to assess alterations in the implants by scanning electron microscopy (SEM).

2. Methods

2.1. Experimental design

Two experiments were carried out. Experiment 1 (Exp 1) was designed to assess concentrations of Bi, Pb and Sn in whole blood, urine and selected tissues at regular intervals up to 77 weeks after implantation of Bi-alloy miniature pellets in the muscles of the hind limbs of rats. In the subsequent eight weeks experiment (Exp 2), early element kinetics were explored by more frequent sampling of blood and urine using otherwise identical sampling strategy as in Exp 1. Tissue concentrations of Bi, Pb and Sn were determined in brain, liver, kidney and testicle. Possible organ toxicities and tissue reactions near the implanted pellets were assessed by light microscopy.

In accordance with the objective of the study and for animal welfare reasons, only one dose was applied in each experiment. The study was conducted in accordance with the laws and regulations controlling experiments on live animals in Norway and in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. Authorities according to the Norwegian Regulation on Animal Experimentation gave study approval (Id 306 # 07/21698).

2.2. Animals

Exp 1: Twenty male Wistar rats were purchased from Scanbur AB (Sollentuna, Sweden), acclimatized and assigned randomly to an experimental (N = 14) or a control (N = 6) group. Mean weights of the animals were 165 g in both groups (SD 8.8 and 6.5, respectively). Exp 2: Twelve male Wistar rats were purchased from and handled identically to the animals in Exp 1. Mean weights of the experimental (N = 8) and control animals (N = 4) were 132 (SD 6.5) and 125 g (SD 1.7), respectively.

2.3. Preparation of miniature pellets

Miniature Bi-alloy pellets simulating impact fragments were made from shotgun pellets obtained from commercial shotgun shells (The Bismuth Cartridge Company, marked “No-Tox”, Magnum Game, size No. 3, Dallas, Texas, USA). The mean weight of the original shotgun pellets was 159 mg (SD 11) (N = 10). After melting of single original pellets, flat flakes were formed under pressure from an iron stamp. The flakes were cut by scissors to form approximately 20 small identical sectors, which in turn were reheated under open flame to form spherical Bi-alloy miniature pellets. The miniature pellets were grouped by eight visually equal pellets with respect to shape and size. The average weight of the eight Bi-alloy miniature pellets implanted in each rat in Exp 1 was 56 mg (SD 0.3) (N = 14) and 56 mg (SD 0.3) (N = 8) in Exp 2. The element content of the original shotgun pellets was 93.5% Bi, 5.9% Sn and 0.5% Pb as determined by inductively coupled plasma optical emission spectrometry.

2.4. Implantation of pellets

In Exp 1 rats were either sham-operated (N = 6) or implanted (N = 14) with miniature pellets into muscle tissues in each flank of the hind limbs. General anaesthesia was induced by *i.p* injection of a blend of 50% midazolam (Dormicum™, Roche, Basel, Switzerland) and 50% fentanyl/fluanison (Hypnorm™, Janssen Animal Health, Wantage, UK) in equal volumes of water. Each rat was injected *i.p.* with 0.15 mL/100 g body weight. A small incision was made through the skin of the hind limbs of the rat and four nearby small grooves were made in each muscle by careful separation of the muscle fibres with a blunt dental tweezers. Four alcohol cleaned miniature pellets were implanted in the bottom of the grooves on both right and left sides, totaling eight pellets in each animal. The skin was sutured and sealed with surgical glue (Histoacryl™, Braun Medical Ltd., Sheffield, UK) to keep the pellets in position. The rats were given analgesia as a single bolus of 0.1 mg /kg *s.c.* buprenorphine hydrochloride (Temgesic™, Reckitt & Colman, Ltd, Hull, UK) and observed for signs of pain during the wakening phase after surgery. Identical procedures were followed for the sham-operated (N = 4) and implant-operated rats (N = 8) in Exp 2. No signs of pain or distress were observed in any of the animals after the procedure in any of the experiments.

2.5. Animal care and handling

Rats in the exposed and control group were caged two together and given free access to food (RM1, Special Diet Services, Essex, UK) and tap water after the surgical procedure. The room temperature was set at + 21 °C ± 1 in a 14 to 10 h light-dark cycle (light from 7 AM to 21 PM). The protocol included daily observations for well-being of the rats, cleaning of the cages twice a week and weekly recording of body weights.

2.6. Sampling of urine and whole blood

Exp 1: Heparinized whole blood (0.2–1.0 mL) from the right dorsolateral vein in the tail of the rats was collected in 1.5 mL easy-fit polypropylene micro-tubes (Trefflab, Degersheim, Switzerland, Cat. No. 96.07246.9.01) at weeks 0, 6, 13, 26, 53 and 72. At weeks 0, 6, 13, 26, 53 and 72 the rats were placed in metabolic cages overnight for 18 to 20 h with free access to food and water for the collection of urine in 25 mL self-standing centrifuge tubes (Sarstedt, Nümbrecht-Rommelsdorf, Germany). Exp 2: Sampling procedures for whole blood and urine at weeks 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 were identical to the procedures in Exp 1. All samples were frozen and stored at − 20 °C.

2.7. Autopsy procedure and collection of tissues

Exp 1: Rats were randomly selected for autopsy from the exposed (N = 3) and control (N = 2) groups at weeks 26, 59 and 77. Under anaesthesia with 5% pentobarbital (Pentobarbital 50 mg/mL, Ullevål Hospital Pharmacy, Oslo, Norway) 0.2 mL/100 g was administered *i.p.* After blood sampling, the animals were exsanguinated by cutting the

large abdominal vessels beneath the diaphragm, thus ensuring sacrifice. The rats were examined for gross pathological alterations. Tissue samples from liver, kidney, cerebrum, and testicle were immediately fixated in 4% phosphate buffered formalin (Formalin 4%, Ullevål Hospital Pharmacy,Oslo, Norway) and subsequently frozen and stored at −20 °C. Exp 2: At week 8, 7 exposed and 4 control rats were autopsied according to the protocol used for Exp 1. One exposed rat was not examined because it was prepared for whole body sectioning and elemental bio-imaging as the subject of a study of local reactions from the pellets [14].

2.8. Determination of elements in whole blood, urine and tissues

For determination of Bi, Sn and Pb in whole blood 2 mL of 65% ultrapure nitric acid and 100 µL of an internal standard solution containing 1 µg/mL of thallium and indium were added to whole blood aliquots (0.2–1.0 g) in 15 mL volume polypropylene (PP) tubes (Sarstedt AG & Co, Nümbrecht, Germany). After heating to 90 °C for 90 min in a laboratory oven, the samples were cooled to room temperature and diluted to 14 mL with 18 mΩ deionised water (DI).

One mL of 65% ultrapure nitric acid and 100 µL of the internal standard solution were added to 1 mL of urine in 15 mL volume PP tubes. After using the described heating procedure, the samples were diluted to 7 mL with DI water.

Tissue samples from kidney, testicle, brain and liver were pre-dried at 105 °C in a laboratory oven to constant weight, followed by transfer of 75–470 mg dry tissue into 15 mL volume PP tubes. After addition of 3 mL 65% ultrapure nitric acid and 100 µL of the internal standard solution, the tubes were heated to 95 °C for 3 h and subsequently diluted to 14 mL with DI water.

The samples were analysed for Bi, Sn and Pb by inductively coupled plasma sector-field mass spectrometry (ICP-SF-MS) using an Element 2 mass spectrometer (Thermo Electron, Bremen, Germany) calibrated with whole blood, urine and acid (for tissue samples) matrix matched standard solutions. Seronorm™ Trace Elements human whole blood and urine quality control materials (Sero Ltd., Asker, Norway) and bovine liver Standard Reference Material (SRM) 1577c (NIST, Gaithersburg, MD, USA) were used for quality assurance. The results obtained for Bi, Sn and Pb in Seronorm™ quality assurance materials and Pb in bovine liver SRM 1577c were within the producer's recommended reference ranges. There were no certified data for Bi and Sn in the latter material.

Detection limits (DL) in Exp 1 were 0.49 (B-Sn), 0.15 (B-Pb), 0.027 (B-Bi), 0.45 (U-Sn), 0.11 (U-Pb) and 0.015 µg/L (U-Bi). The DLs in Exp 2 were 0.22 (B-Sn), 0.22 (B-Pb), 0.015 (B-Bi), 0.24 (U-Sn), 1.1 (U-Pb) and 0.17 µg/L (U-Bi). The percentage of concentrations < DL in control/exposed rats respectively, were 89/42 (B-Sn), 96/25 (B-Pb), 0/0 (B-Bi), 82/12 (U-Sn), 0/0 (U-Pb) and 56/20% (U-Bi) in Exp 1. The percentage of concentrations < DL in control/exposed rats respectively, were 15/5 (B-Sn), 75/8 (B-Pb), 3/4 (B-Bi), 78/1 (U-Sn), 0/0 (U-Pb) and 43/8% (U-Bi) in Exp 2. Data for urine and blood are not shown for week 72, because the data were few as four exposed rats had died unexpectedly due to failing to thrive.

2.9. Histopathological evaluation

Tissues were fixed for 24 h and dehydrated before being transferred to xylene and embedded in paraffin. Sections of 5 µm were cut, stained with haematoxylin and eosin and examined by light microscopy without knowledge of group assignment.

2.10. The distribution of Bi, Sn and Pb in alloy pellets

Two-dimensional distributions of selected elements were assessed by energy dispersive X-ray spectrometry (EDS) and field emission scanning electron microscopy (SEM). Two implanted pellets recovered at week 77, seven non-implanted pellets and five original shotgun pellets were embedded in conductive methyl methacrylate (Technovit® 5000, Heraeus Kulzer GmbH, Hanau, Germany). After hardening of the embedding material, the samples were first coarsely grinded with SiC particles followed by stepwise polishing to mirror-like finish using 0.25 µm SiC particles at the final step. The samples were mounted on a SEM sample holder with conductive, sticky carbon tape. As the embedding medium was made conductive with copper particles, no coating was used before observations in the SEM.

The specimens were studied with a SU 6600 FESEM instrument (Hitachi, Tokyo, Japan) equipped with a Quantax 200 (EDS) microanalysis system (Bruker-AXS Microanalysis GmbH, Berlin, Germany) with XFlash® 5010 silicon drift detector (SDD) with an energy resolution of 123 eV (Mn Kα). The elements detected were mapped on selected areas of the polished samples for several hours at an input rate of about 40 kcps. The obtained element maps were further treated with background subtraction and peak deconvolution before ZAF corrected standardless quantitative analysis.

2.11. Statistics

Concentrations < DL were substituted with ½ DL. Concentrations < DL were mostly observed in control rats. Grubb's test was used for the assessment of statistical outliers, resulting in the exclusion of one concentration each of B-Sn, B-Pb, B-Bi and Pb in kidney in Exp 1 and one concentration each of B-Pb and B-Sn in Exp 2. Group differences were assessed by the use of Mann-Whitney U-test. For the presentation of element concentrations in the exposed rats only the arithmetic mean (AM) and standard error (SE) were calculated. Least square regression analysis was used to assess univariate associations between element concentrations in blood and urine, yielding Pearson's correlation coefficients as the measure of association. A two-tailed significance level < 0.05 was considered to be of statistical significance. The Statistical Package for the Social Sciences (SPSS), version 24 (SPSS, USA), was used for the statistics.

3. Results

Exposed and control rats had a similar and steady increase in weights in both experiments (data not shown). The rats were heavier in Exp 1 than in Exp 2 (mean 165 vs 132 g) at baseline, but the mean weight of the administered miniature pellets were identical in Exp 1 and Exp 2 (mean 56 mg, SD 0.3). The element concentrations in blood and urine at baseline were comparable in Exp 1 and Exp 2.

3.1. Urine and blood concentrations

There were no statistically significant differences between exposed and control rats before exposure started for any of the elements in blood and urine in Exp 1 (Table 1). Concentrations of B-Bi (Fig. 1A) and U-Bi increased during the entire observation period. In contrast, B-Sn (Fig. 1B) and U-Sn peaked at week 6. The B-Sn concentrations returned to background levels while the concentrations of U-Sn were slightly higher than background at week 53. Concentrations of B-Pb (Fig. 1C) and U-Pb increased after start of exposure and peaked at week 13. U-Pb declined to background levels while B-Pb was slightly higher than background at week 53.

There were no statistically significant differences between the exposed and control rats before exposure started for any of the elements in blood and urine in Exp 2 (results not shown). The concentrations of all elements in blood and urine were statistically significantly higher in the exposed than in the control rats already at week 0.5. The mean B-Sn concentration peaked at week 3 (Fig. 2A), while U-Sn was similar between week 4 and week 8 (Fig. 2B). The mean concentration of B-Bi increased gradually during the eight week exposure (not shown), while U-Bi increased substantially up to week 2 (Fig. 2C).

Table 1
The median (and min-max) concentrations of Bi, Sn and Pb in whole blood (B) and urine (U) according to time of follow-up in 14 exposed (E) and 6 control (C) rats in Exp 1. The p-values refer to the difference between exposed and control rats at the respective time points.

			Week				
	Group		0	6	13	26	53[†]
B-Bi (ng/g)	E	Median	0.30[ns]	0.60[***]	0.74[***]	1.1[***]	1.3[**]
		Min-Max	0.23-0.85	0.37-0.90	0.39-2.0	0.57-2.6	0.98-3.4
	C	Median	0.31	0.18	0.28	0.31	0.22
		Min-Max	0.25-0.37	0.12-0.27	0.17-0.38	0.14-0.90	0.14-0.85
B-Sn (ng/g)	E	Median	< DL[ns]	3.5[***]	1.6[***]	0.88[ns]	< DL[ns]
		Min-Max	< DL-2.4	2.7-5.0	< DL-2.1	< DL-4.0	< DL-1.3
	C	Median	< DL	< DL	< DL	< DL	< DL
		Min-Max	< DL- < DL	< DL- < DL	< DL- < DL	< DL-1.9	< DL-0.53
B-Pb (ng/g)	E	Median	< DL[ns]	1.5[***]	3.2[***]	2.4[***]	1.3[*]
		Min-Max	< DL-0.83	< DL-2.1	2.4-4.1	< DL-4.3	< DL-1.6
	C	Median	< DL	< DL	< DL	< DL	< DL
		Min-Max	< DL- < DL	< DL- < DL	< DL- < DL	< DL- < DL	< DL-0.67
U-Bi (μg/L)	E	Median	< DL[ns]	21.1[***]	22.6[***]	30.4[***]	47.4[**]
		Min-Max	< DL-0.02	16.1-40.5	13.2-38.1	17.8-46.7	14.2-84.1
	C	Median	< DL	< DL	< DL	0.04	0.58
		Min-Max	< DL-0.02	< DL-0.13	< DL- < DL	< DL-1.1	0.45-1.2
U-Sn (μg/L)	E	Median	< DL[ns]	30.4[***]	24.6[***]	12.5[***]	4.2[**]
		Min-Max	< DL-1.12	28.3-46.1	16.6-36.5	5.6-19.7	2.1-11.0
	C	Median	< DL	< DL	< DL	< DL	< DL
		Min-Max	< DL-0.57	< DL- < DL	< DL-4.3	< DL-0.55	< DL- < DL
U-Pb (μg/L)	E	Median	3.2[ns]	5.1[ns]	6.2[*]	3.9[**]	2.5[ns]
		Min-Max	1.1-9.5	2.5-8.2	3.4-8.6	1.8-8.6	1.4-5.4
	C	Median	2.3	3.6	2.1	1.8	1.4
		Min-Max	0.76-3.8	1.9-6.3	1.8-7.0	0.78-4.0	0.63-2.7

[†] nine exposed and four control rats; [ns] not significant; [*] $p < 0.05$; [**] $p < 0.01$; [***] $p < 0.001$.

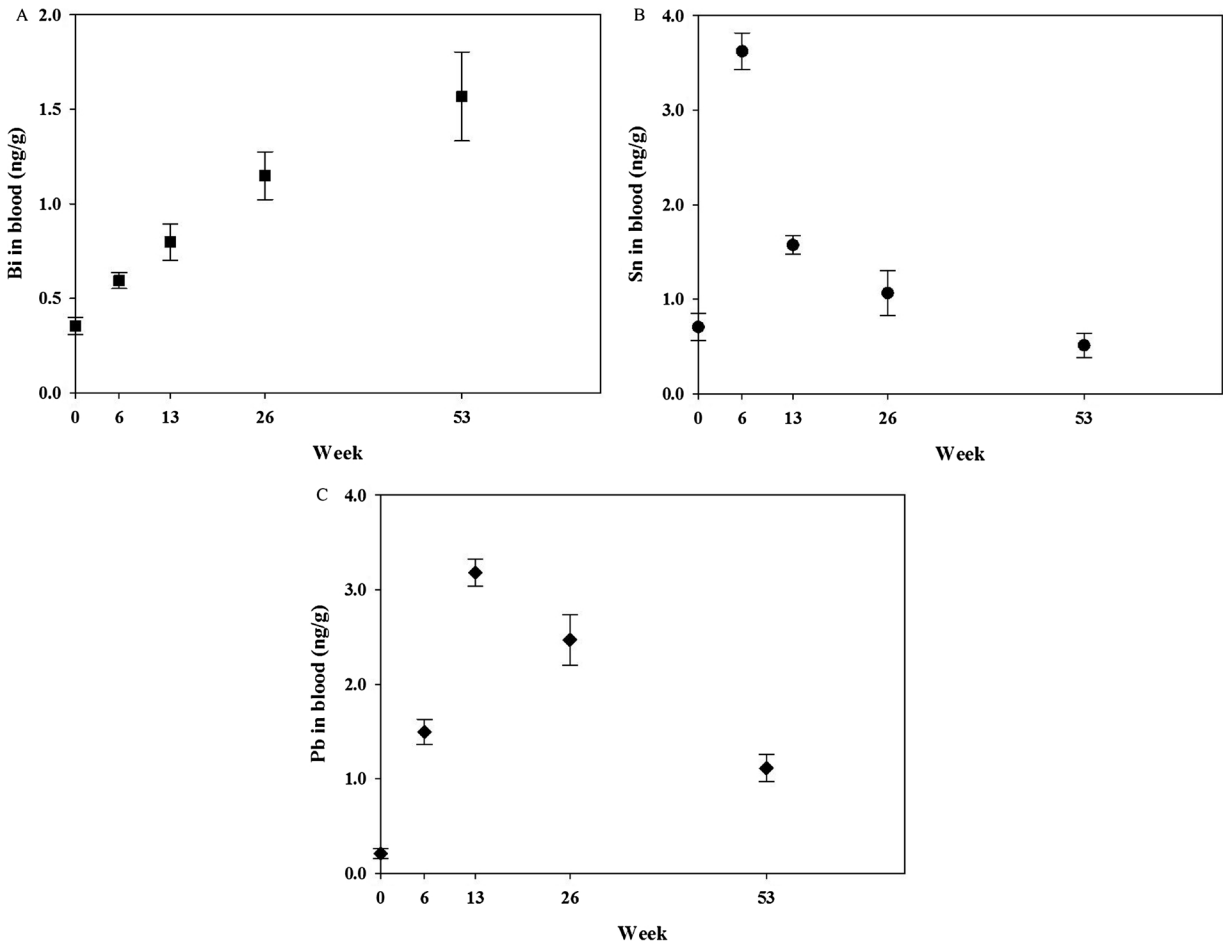

Fig. 1. A–C The mean (and standard error) concentrations of B-Bi (Fig. 1A), B-Sn (Fig. 1B) and B-Pb (Fig. 1C) in exposed rats according to time of follow-up in weeks.

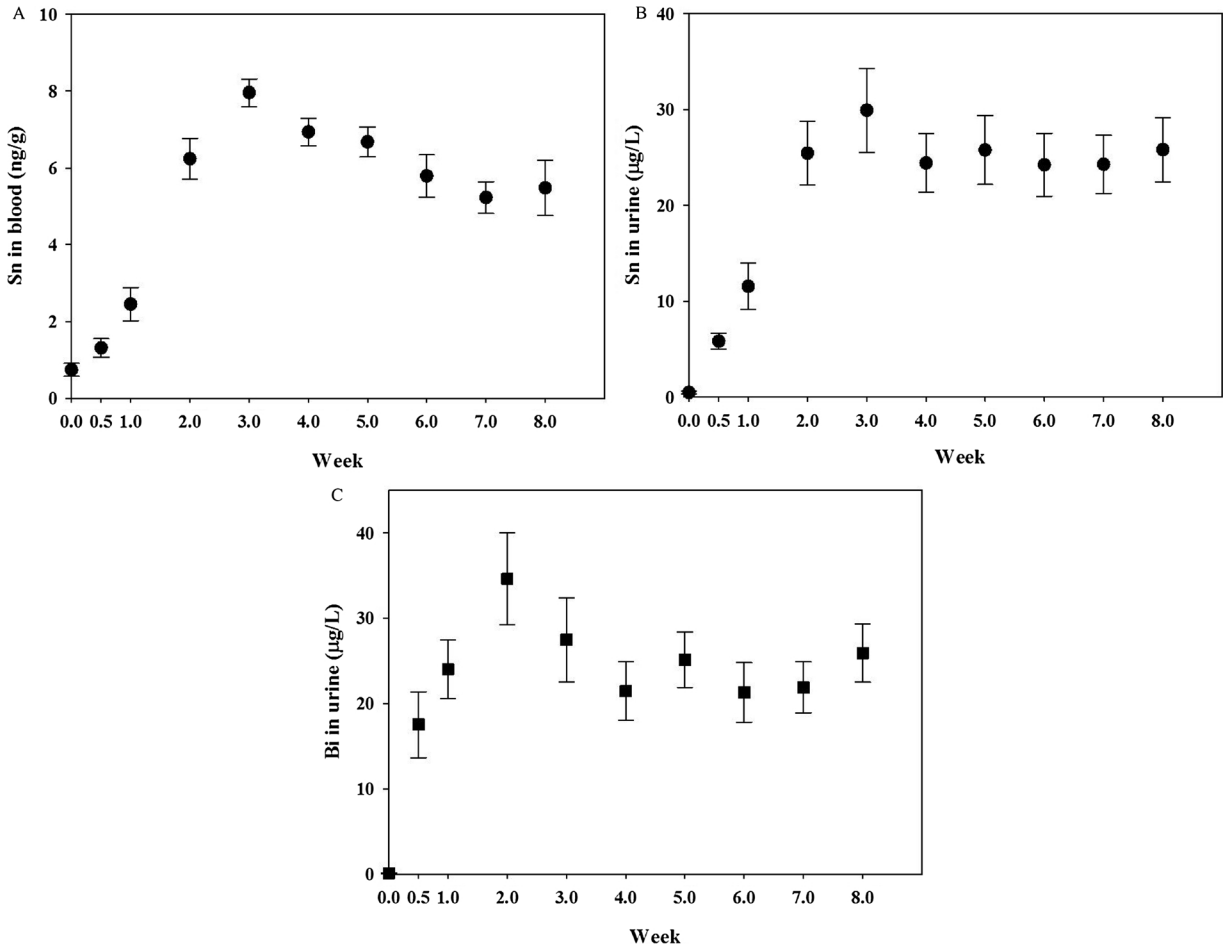

Fig. 2. A–C The mean (and standard error) concentrations of B-Sn (Fig. 2A), U-Sn (Fig. 2B) and U-Bi (Fig. 2C) in exposed rats according to time of follow-up in weeks.

There were high correlations between Bi and Sn concentrations measured in urine and blood in the exposed animals in Exp 1. The Pearson's correlation coefficients were $r_{Bi} = 0.60$ ($p < 0.001$, N = 68), $r_{Sn} = 0.77$ ($p < 0.001$, N = 68) and $r_{Pb} = 0.32$ ($p = 0.007$, N = 67), and correspondingly $r_{Bi} = 0.17$ ($p > 0.05$, N = 84); $r_{Sn} = 0.83$ ($p < 0.001$, N = 84) and $r_{Pb} = -0.04$ ($p > 0.72$, N = 84) (not tabulated) in Exp 2.

3.2. Tissue element concentrations

Tissue samples from exposed rats in Exp 1 were collected at weeks 26, 59 and 77, and at week 8 in Exp 2 after implantation. Table 2 shows the tissue concentration according to sampling week. Week 0 represents the concentrations in the control rats of Exp 1 and Exp 2 combined. The Bi concentrations were higher in exposed compared to control rats in all examined tissues, and the concentration increased by increasing exposure duration. The highest Bi concentrations were measured in kidney (Fig. 3A), but a substantial accumulation was also observed in brain (Fig. 3B) and testicle. The Sn concentrations were also significantly higher in exposed rats. Testicular Sn concentrations increased during the whole observation period (Fig. 3C). In contrast, the pattern observed in kidney (Fig. 3D) with the highest Sn concentrations at week 8 is similar to the pattern observed for Sn concentrations in blood and urine.

3.3. SEM-examinations of pellets

A similar distribution of Sn and Pb located in numerous oars or lamellae within a dominant Bi matrix was observed by SEM of polished cut surfaces of the original shotgun alloy pellets and pre-implant Bi-alloy miniature pellets (Fig. 4A). Some Sn-rich structures with Pb-rich spots apparently always as neighbors to Sn, had appearances suggesting connections to the boundary of the cut surface, i.e. the pellet surface.

The distribution of Bi did not visually change in the miniature pellets recovered at week 77. In contrast, Pb was no longer observed in the oars at a distance of less than about 200 µm from the pellet surface (Fig. 4B). At these sites, increased concentrations of oxygen (O) within the Sn-rich oars near the boundaries of the recovered pellets were detected. Also chlorine (Cl) was detected at some of the Sn-rich sites (Fig. 3C). Point EDS analysis within the Sn rich oars of the miniature pellets recovered at week 77 gave O/Sn ratios (At %) of 1.80 (N = 9, SD = 0.31) and 0.26 (N = 9, SD = 0.05) in outer and central areas of the pellets, respectively. The corresponding values for pre-implant miniature pellets were 0.09 (N = 9, SD = 0.01) near the surface and 0.09 (N = 9, SD = 0.01) in the central area, while the values for the original shotgun pellets were 0.10 (N = 9, SD = 0.01) near the surface and 0.09 (N = 9, SD = 0.01) in the central area.

3.4. Histopathological evaluations

\Many of the Bi-alloy pellets recovered from the implantation sites in Exp 1 were encapsulated by thin scar tissue. Slight accumulations of mononuclear inflammatory cells and some collagen fibrosis were observed at week 8 in Exp 2 in tissues adjacent to the implantations sites. In Exp 1, such accumulations were more prominent at week 26, but to a lesser extent up to weeks 59 and 77. No exposure-related histopathological abnormalities were observed by light microscopy in the organs examined in Exp 1 or Exp 2. Four exposed rats were euthanized due to

Table 2
The concentrations (in μg/kg) of Bi, Sn and Pb in brain, liver, kidney and testicle in exposed rats according to week of autopsy. Control rats represent all non-exposed rats in Exp 1 and Exp 2 combined.

	Non-exposed (N = 10)		Week 8 (N = 7)		Week 26 (N = 4)		Week 59 (N = 4)		Week 77 (N = 3)		
	GM	Min-Max	GM	Min-Max	GM	Min-Max	GM	Min-Max	GM	Min-Max	p_{ANOVA}
Brain											
Bi[bcd]	1.4	0.4-4.2	2.5	1.4-5.3	3.4	2.1-4.1	16	8.8-49	14	12-15	< 0.001
Pb[c]	3.5	1.2-6.8	3.6	2.3-6.3	6.2	5.1-6.7	7.7	4.2-24	7.0	3.8-12	0.04
Sn[acd]	0.8	0.3-3.6	1.9	1.1-13	1.8	1.2-3.6	8.9	3.9-55	7.7	4.0-22	< 0.001
Liver											
Bi[abcd]	0.8	0.2-4.0	4.6	3.1-7.1	9.4	6.4-12	15	11-22	30	21-46	< 0.001
Pb[abcd]	3.4	1.2-7.1	9.7	6.9-13	16	12-29	6.2	4.0-8.5	7.6	5.2-14	< 0.001
Sn[abcd]	1.4	0.9-2.3	18	15-20	21	19-22	9.2	6.1-12	13	11-14	< 0.001
Kidney											
Bi[abcd]	1.0	0.2-6.9	455	291-617	970	469-1497	1936	1196-2614	3167	2724-4186	< 0.001
Pb[abcd]	14	7.9-30	125	115-142	161	126-207	62	40-86	88	62-106	< 0.001
Sn[abcd]	3.7	2.1-9.7	331	231-536	199	180-223	97	86-116	109	96-127	< 0.001
Testicle											
Bi[abcd]	0.7	0.1-11	2.8	1.5-4.6	7.2	4.3-9.3	16	7.5-26	24	22-25	< 0.001
Pb	2.6	0.7-9.7	1.4	0.7-2.4	3.8	2.8-5.2	5.1	3.6-7.2	5.6	3.9-11	0.02
Sn[abcd]	0.7	0.3-2.3	8.7	6.0-11	21	20-24	27	21-29	28	22-32	< 0.001

[a] $p < 0.05$ between Week 0 and Week 8; [b] $p < 0.05$ between Week 0 and Week 26; [c] $p < 0.05$ between Week 0 and Week 59.[d] $p < 0.05$ between Week 0 and Week 77.

failing to thrive in Exp 1, but no signs of exposure-related pathology was observed. No overt clinical effects were observed in any of the rats during the experiments.

4. Discussion

The preparation of smaller Bi-alloy pellets from an original shotgun pellet and the subsequent implantation in muscles of rats was done to simulate the internal exposure to multiple brittle fragments of Bi shotgun pellets which could be caused by a shooting accident. There

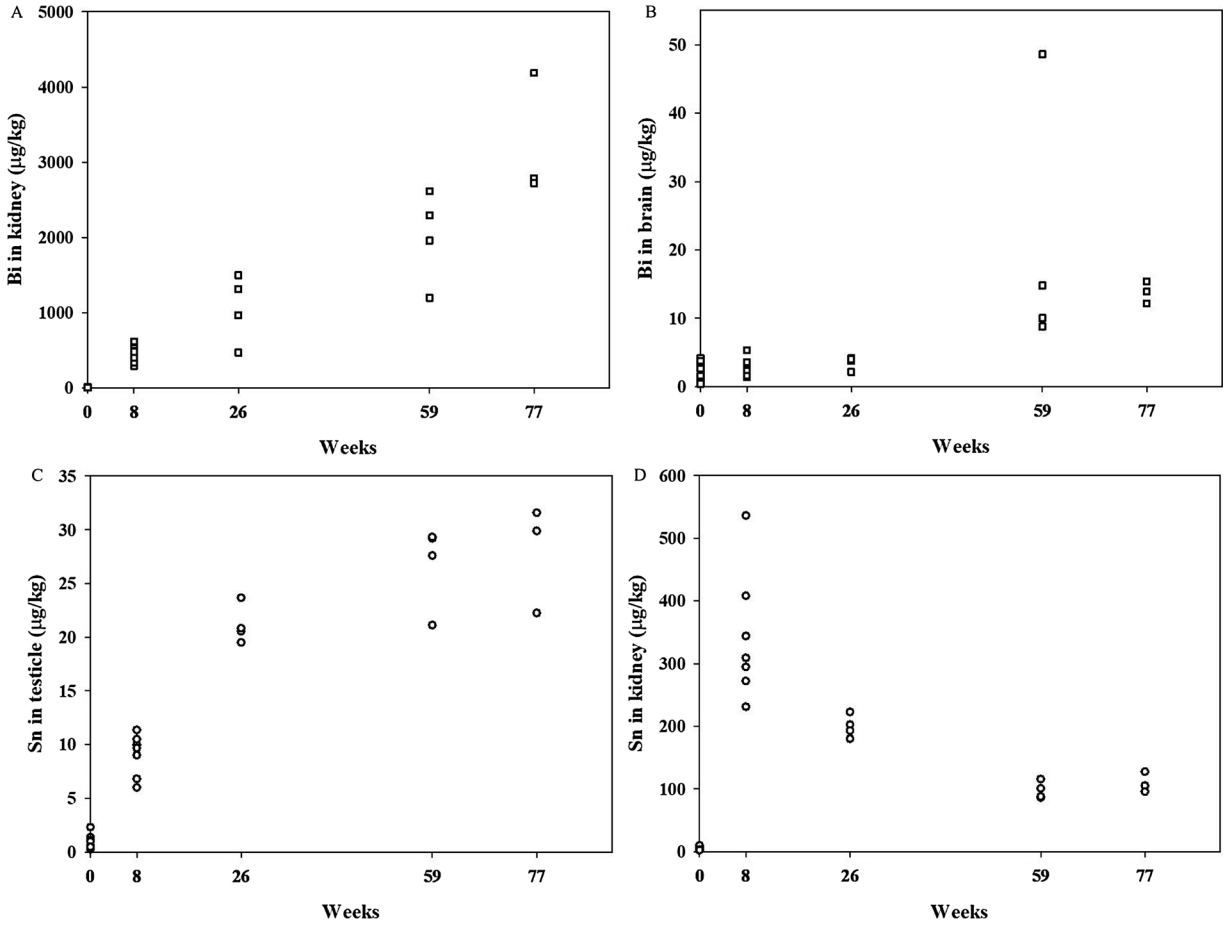

Fig. 3. A–D The concentrations of Bi in kidney (Fig. 3A), Bi in brain (Fig. 3B), Sn in testicle (Fig. 3C) and Sn in kidney (Fig. 3D) of rats according to number of weeks exposed.

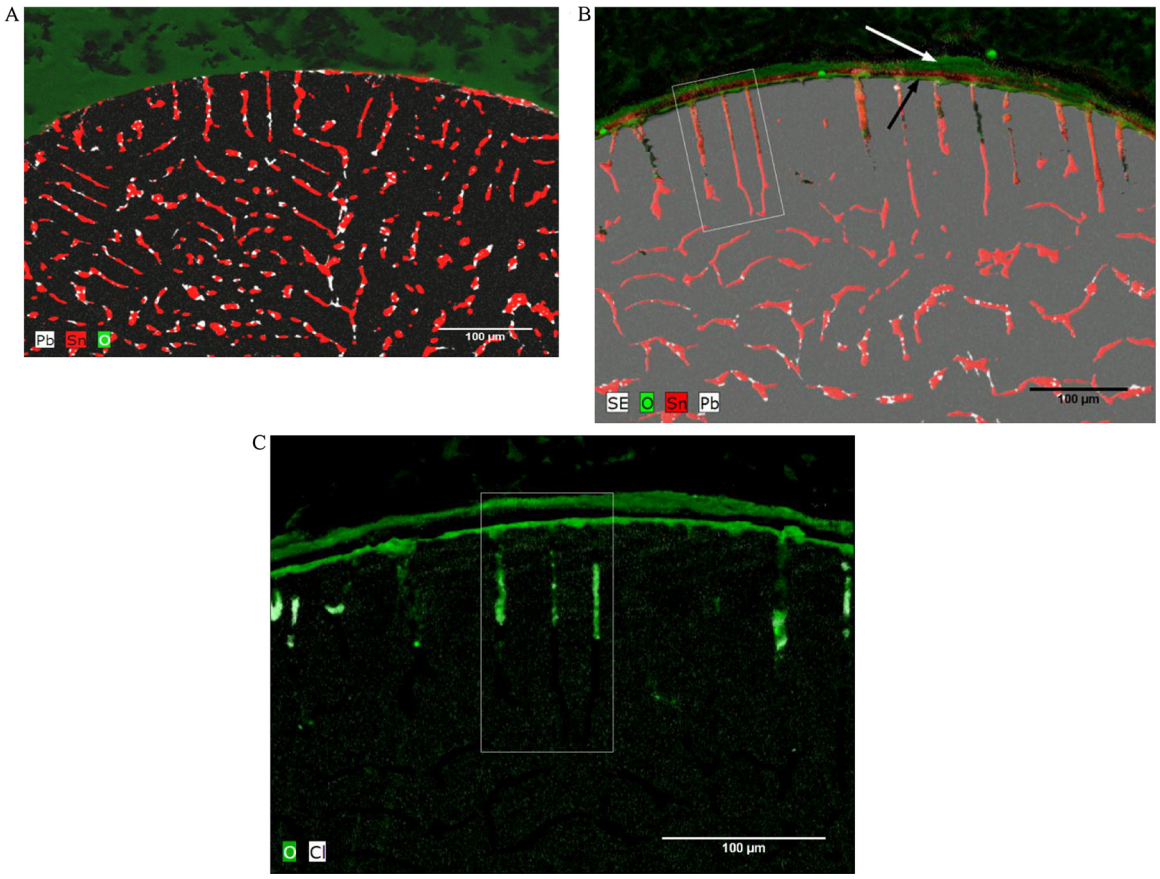

Fig. 4. A–C Distribution of alloy elements in pellets before and 77 weeks after implantation (energy dispersive (EDS) element maps from cross-sections of Bi-alloy pellets). Fig. 4A Preimplant miniature pellet: Islands of Pb (white) associated with Sn (red) are evenly distributed in the Bi matrix (black) across the section. Fig. 4B EDS map of O (green), Sn (red) and Pb (white) overlaying secondary electron image (SE) in post-implant pellets recovered at week 77. Pb is located adjacent to Sn at distance from the surface of the pellet, whereas an 200 µm wide peripheral area is almost devoid of Pb. Radiating Sn lamellae show presence of O (green) near the pellet surface which is covered by a thin near continuous O rich layer (black arrow) layer. An O rich rim covering the pellet represents organic materials in adherent scar tissue (white arrow). Rectangles show identical areas of the cross section as in Fig. 4C. Fig. 4C EDS map; O (green) and Cl (white) superimposed of post-implant pellets recovered at week 77. Rectangles show identical areas of the cross section as in Fig. 4B. Fig. 1A.

were no obvious structural differences between the original shotgun pellets and the pre-implant pellets, suggesting that the preparation of the pre-implant pellets had not caused structural alterations, as shown by analytical SEM examination. The exposure conditions of the rats may thus be similar to Bi-alloy shotgun pellet exposures in humans. This study adds new information on kinetics and bioaccumulation of Bi and Sn, and chemical alterations of implanted pellets, following deposition in rat muscle tissues.

The mean concentration of B-Bi and U-Bi increased during the entire observation period in Exp 1, indicative of Bi bioaccumulation under these exposure conditions. This has previously not been reported. Data indicate that absorbed Bi is mainly excreted in urine [2]. Elimination of Bi after exposure to inorganic Bi salts from blood follows a multi-compartment model, the shortest biological half-life in humans being 3.5 min, and the longest 17 to 22 years [15].

The kidney accumulates Bi more readily than any of the other examined organs as shown in this study and also by others after implantation of Bi-alloy shot pellets in rodents and waterfowls [5–7]. This is compatible with the strong renal metallothionein inducing effect of Bi [16]. Bismuth also accumulated in the testicle, perhaps in testicular macrophages, as previously reported [17]. Reduced serum testosterone levels were observed after injecting Bi subnitrate into rats [18]. Accumulation of Bi in the central nervous system of mice after intraperitoneal implantation [5], in neurons of spine and brain after implantation into rat muscles [6], and in liver and gonads of mallards exposed to Bi-alloy shots embedded in breast muscles [7] have been shown previously. As high Bi exposure has caused encephalopathy in humans [4], the potential consequences of Bi brain accumulation demonstrated in this study should be further elucidated.

Tin was more easily mobilized into blood than Bi. The mean concentration of B-Sn and U-Sn increased rapidly and reached its peak three weeks after implantation. The higher B-Sn concentration at week 6 in Exp 2 compared to Exp 1 may be related to the lower body weight of the rats in Exp 2 compared to Exp 1 by similar exposure. The concentration of B-Sn had returned to background level at 53 weeks of exposure, while U-Sn was slightly higher than background. Thus, a slight mobilization of Sn occurred during the whole observation period. This pattern of decline is also partly reflected in the tissue concentrations of Sn, which were lower for the kidney in the long term Exp 1 as compared to Exp 2. In contrast, the highest testicular concentrations of any of the elements were observed for Sn, suggesting higher accessibility of Sn than Bi into the testicles and that Sn remains longer in the testicles than in the other examined organs. Little data are available on toxic effects of Sn in testicles, but biochemical testicular alterations were observed in rabbits exposed to stannous chloride [12]. Also tributyl tin exposure, although not quite comparable, caused reduced sperm count in mice [19]. Whether effects of Bi on the testicles may enhance effects caused by Sn remains to be elucidated.

Gastrointestinal absorption of inorganic Sn is low in rats [20]. Gastrointestinal absorption and pulmonary uptake of inorganic Sn is also low in humans [9]. This is important for the general assumption that inorganic Sn has low systemic toxicity in humans. However,

potential toxic consequences of the mobilization of Sn from Bi-alloy pellets into tissues like the brain and testicle should be examined further. Once inside the body, a number of toxic manifestations of Sn have been observed in animal studies, such as neurotoxicity, genotoxicity and nephrotoxicity [11]. Rats sacrificed at week 77 in this study had approximately 10-fold higher brain Sn concentrations than the control rats. To what extent this may have e.g. behavioural consequences remains to be elucidated.

Although the pellets contained only 0.54% Pb, the concentration of B-Pb was significantly higher in the exposed animals of Exp 1 compared to controls six weeks after implantation. The mean B-Pb and U-Pb concentrations peaked at week 13 and declined gradually until week 53. A similar pattern was observed in man wounded in a shotgun accident [21]. An initial increase of B-Pb followed by decreased B-Pb was also observed in mallards, and the authors suggested that the slight to moderate fibrous tissue encapsulation of the implants could explain the decrease [7]. However, the fibrosis observed in the present study may not explain the decrease of B-Pb, because B-Bi increased during the entire observation period.

Blood concentrations of Sn and Pb were higher than Bi during the first weeks after implantation, suggesting a higher mobilization from the miniature pellets. Metallic Pb and Sn are almost insoluble in water, but Pb may in the presence of oxygen form more leachable oxidic surface layer components in aqueous environments. Lead mobilization from pellets in soil at outdoor shooting ranges is stimulated by the presence of carbonate, sulfate and phosphates [22]. We have not found any information on tissue mobilization mechanisms of Pb in the literature, but it is reasonable to assume that similar surface reactions may be involved in the release of Pb from the alloy pellet implants. Tissue mobilization mechanisms for Sn and Bi are unknown. The mean concentrations of B-Sn and B-Pb peaked early, at week 3 and week 13, respectively. The concentrations then declined substantially in contrast to B-Bi that increased during the entire observation period.

In order to study reasons for the decline in B-Pb and B-Sn, SEM was used to examine the implants. Lead was observed only in the depth of the oars at a distance of at least about 200 μm from the surface in pellets recovered at week 77 in contrast to the pre-implant pellets, indicating that Pb located near the pellet surface had been mobilized and depleted. The disappearance of Pb from the surface location within the pellets may mechanistically be related to the decrease of the B-Pb concentrations after week 13. The distribution of Sn was essentially the same in pre- and post-implant pellets. However, higher amounts of oxygen and chlorine, not observed in the pre-implant pellets, were also detected in the elongated structures and oars at the Sn locations adjacent and connected to the pellet outer surface. In Sn containing solder alloys, leaching of Sn and formation of insoluble surface corrosion products such as oxides, oxide hydroxides or oxychlorides may occur in NaCl solutions [13,23,24]. It is therefore possible that the formation of low solubility Sn-compounds may inhibit dissolution and release of Sn from the pellets, which may explain gradual reduction of Sn release into the blood.

The recovered pellets were found to be encapsulated in small amounts of scar tissue. Slight local inflammation and fibrosis at the implantation site is in agreement with previous results from this study [14]. Mild local inflammatory tissue reactions at the implantation sites were also seen two weeks after implantation of a Bi-Sn alloy in the muscle of rats, but the inflammatory score was much lower than the score for nickel-plated steel shotgun pellets [8]. Bi-Sn shots embedded in the tissues are also generally more resistant to corrosion as opposed to steel shots [25]. Scar tissue observed on the pellet surface in the present study was apparently also less abundant than previously observed for Pb implants in muscle tissue [7].

Accumulation of Bi, Sn and Pb in blood and organs is an unwanted property for a substitute of Pb in shotgun shells. This is to our knowledge the first study to demonstrate kinetics and tissue accumulation of Sn released from Bi-alloy shotgun pellets located in muscle tissues. Potential toxic consequences of Bi and Sn released from alloy pellets in accidental wounds warrants further investigations. Also more thorough examination and follow-up of wounded hunters with measurements of the alloy elements in body fluids are recommended for better human risk assessment and handling of such patients.

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