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Immunohistochemical Investigation of Drug Related Renal Cell Changes

Akiko Ishigami [1], Brian Waters [2], Masayuki Kashiwagi [2], Aya Matsusue [2], Mio Takayama [2], Natsuki Ikematsu [3], Toshikazu Kondo[1], Shin-ichi Kubo[2*]

[1] Department of Forensic Medicine, Wakayama Medical University
[2] Department of Forensic Medicine, School of Medicine, Fukuoka University
[3] Department of Forensic Medicine, School of Medicine, University of the Ryukyus

Abstract

Background and Aim: A pathological evaluation of drug-induced renal impairment was investigated.

Materials and Methods: Kidney samples were collected from 44 forensic autopsy cases without any renal disease, and within 48 hours of postmortem interval (PMI). Cases with drugs detected from blood were treated as drug-related deaths, and cases without any drugs detected were classified as non-drug deaths. Immunohistochemically, antibody staining against vimentin, nestin, fibronectin, neutrophil gelatinase-associated lipocalin (NGAL), heme oxyganase-1 (HO-1), myoglobin, CD68, alpha-smooth muscle actin (α-SMA), and p-selectin was performed in the kidney. In the non-drug cases, statistical differences among age, gender, PMI and each immunoreactivity were investigated. Analysis of variance between drug- and non-drug group, and ratio of each immunoreactivity in each drug were examined. In all cases, the relationship between the immunoreactivity of each antibody was examined.

Results: In the non-drug cases, the immunoreactivity of vimentin, nestin, and NGAL in the glomerulus decreased according to age and PMI. Moreover, immunoreactivity of HO-1 and NGAL increased in traumatic shock and burning cases, respectively. In the comparison between drug- and non-drug cases, the ratio of immunoreactivity of fibronectin, CD68, and p-selectin increased in the drug-related group. A significant difference was seen with drugs such as

Address for correspondence: Shin-ichi Kubo*, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Department of Forensic Medicine, Fukuoka University, Japan. E-mail: kuboshin@fukuoka-u.ac.jp

Ketoprofen, Methylephedrin, and atypical antipsychotics, in each immunoreactivity.
Conclusion: Examining the immunoreactivity of these markers in the kidney of drug-related cases provided useful information about the antemortem renal function and diagnostic meaning of cause of death.

Keywords: immunohistochemistry, drug-related renal damage, forensic autopsy, vimentin, fibronectin, statistical analysis

INTRODUCTION

For evaluating the renal function in forensic autopsy cases, postmortem analyses of creatinine clearance and urea nitrogen are generally effective. However, these biochemical examinations might be not applicable if blood and urine cannot be collected. The purpose of this study is to evaluate antemortem renal function by investigating the kidney immunohistochemically, instead of those biochemical examinations.

In drug-induced nephropathy, vasomotor nephropathy and interstitial nephritis are major findings. Acute kidney injury by abuse of illicit drugs has been investigated (1). Previously, we reported the immunohistochemical significance of oxidative injury-related markers in the kidney of methamphetamine abusers (2, 3).

In this study, the kidneys of drug-related and non-drug forensic autopsy cases were investigated immunohistochemically by antibody-staining against proteins which appear in normal kidneys and renal damage markers. Then, an index of the pathological investigation of renal impairment was evaluated not only for forensic autopsy diagnosis, but also for the indicators of drug-induced renal cell injury.

MATERIALS AND METHODS

Sample/ Cases

According to the following requirements, kidney samples were collected from forensic autopsy cases at Fukuoka University: 1) Cases with no medical history of kidney disease; and 2) Cases within 48 hours of postmortem interval (PMI), to

Table 1. Summary of examined drug- and non-drug cases

Cases	Drug related	Non-drug	Total
(number)	25	19	44
Age (years)			
mean±SD	63.7±15.5	44.0±8.4	55.2±16.2
Range	36-92	29-60	29-92
Sex (number)			
male	17	12	29
female	8	7	15
PMI (hours)			
mean±SD	21.0±6.7	22.8±12.9	21.8±9.9
range	10-35	8.5-48	8.5-48
Cause of death			
intoxication	2	0	2
traumatic shock	2	1	3
bleeding	1	0	1
burning	3	2	5
infectious disease	2	0	2
prostration	1	0	1
asphyxia	2	2	4
drowning	4	1	5
natural sudden death	3	9	12
head injury	5	4	9
(number)	25	19	44

ensure the presence of an immune response. Cases in which medicine/drugs were detected from blood by toxicological screening analysis were defined as drug-related cases. Cases without medicine/drugs detected in blood were defined as drug non-drug cases.

A total of 44 cases (25 drug-related; 19 non-drug cases) were examined. A summary of the examined cases is shown in Table 1.

Forty-two different kinds of drugs were detected in the 25 drug-related cases. Details of the detected drugs in each individual case are shown in Table 2.

Table 2. Drugs detected in each drug-related case.

The drug name of the abbreviated name and its drug class/action were summarized in the supplement table

Case No.	Detected Medicines and Drugs	
	Abbreviated name	Number
1	AcA/ AIAU	2
2	AcA/ KETO/ DHC/ MEPH/ LX	5
3	AcA/ MTCL	2
4	AcA/ MTCL/ REB/ LX	4
5	IBN/ EPH/ DXM/ MEPH/ ROSV	5
6	IPAs/ Atn/ MTCL	3
7	IPAs/ Tetr/ CPhM/ SULP/ EPH	5
8	KETO	1
9	NBZs/ AP/ AIAU/ AcA/ DTZM	5
10	SULP	1
11	SSRIs/ Sild	2
12	DPZ/ WF	2
13	TMB/ ADB	2
14	BARB/ FPZ/ BDZs/ HPD	4
15	BARB/ BDZs/ IBN/ PROP	4
16	AAPDs	1
17	BDZs	1
18	BDZs	1
19	BDZs/ DTZM	2
20	BDZs/ Tetr/ AMPH/ METH/ TZD	5
21	LHCl/ WF	2
22	AAPDs/ FPZ	2
23	AAPDs/ BDZs/ CBZ/ FURD/ FPHT	5
24	AAPDs/ BDZs/ SVA	3
25	AAPDs/ BDZs/ LPS/ DORAs	4

The most medicine/drugs detected in a single case was 5 in 6 cases (Cases 2, 5, 7, 9, 20, 23).

Table 3. Supplement table / Abbreviated names of detected drugs in drug-related cases

Abbreviated name	Drug Name	Drug Class / Action
AcA	Acetaminophen	antipyretics
ADB	Amlodipine	antihypertensive drug
AMPH	Amphetamine	central nervous system stimulant
AP	Antipyrine	antipyretics
AIAU	Apronalide	hypnotic/sedative drug
Atn	Atenolol	angina remedy
AAPDs	Atypical Antipsychotic	antipsychotic
BARB	Barbiturate	central nervous system depressant
BDZs	Benzodiazepine	sleep/ anxiolytics
CBZ	Carbamazepine	anticonvulsant
CPhM	Chlorpheniramine	anti-inflammatory
NBZs	Cyclopyrrolones	hypnotic agent
DXM	Dextromethorphan	antitussive
DHC	Dihydrocodeine	antitussive
DTZM	Diltiazem	angina remedy
DPZ	Donepezil	Alzheimer's disease drug
EPH	Ephedrine	bronchodilator
FURD	Furosemide	diuretic
HPD	Haloperidol	antipsychotic
IBN	Ibuprofen	anti-inflammatory drug
IPAs	Imidazopyridine	anxiolytic
KETO	Ketoprofen	anti-inflammatory drug
LHCl	Lidocaine	local anesthetic/ antiarrhythmic
LX	Loxoprofen	anti-inflammatory analgesic
LPS	Lubiprostone	constipation remedy
METH	Methamphetamine	central nervous system stimulant
MEPH	Methylephedrine	bronchodilator
MTCL	Metoclopramide	antiemetic
FPZ	Phenothiazine	tranquilizer
FPHT	Phenytoin	antiepileptic
PROP	Propofol	general anesthesia
REB	Rebamipide	anti-ulcer
ROSV	Rosvastatin	hypercholesterolemia drug
SSRI	Selective Serotonin Reuptake Inhibitor	antidepressant
Sild	Sildenafil	erectile dysfunction treatment
SVA	Sodium Valproate	antiepileptic drug
DORAs	Suvorexant	sleeping pills
SULP	Sulpiride	antipsychotic
Tetr	Tetracyclic Antidepressant	antidepressant
TZD	Trazodone	antidepressant
TMB	Trimebutine	gastrointestinal drug
WF	Warfarin	anticoagulant

Toxicological Examination

To confirm drug intake, toxicological screening of right atrium blood and/or femoral vein blood was performed using gas chromatography-mass spectrometry (GC-MS) on a QP-2010Ultra (Shimadzu, Kyoto, Japan) and liquid chromatography-tandem mass spectroscopy (LC-MS/MS) on a Prominence UFLC (Shimadzu, Kyoto, Japan) coupled to a TSQ Quantum Access MAX MS/MS (Thermo Scientific, Waltham, MA, USA), according to our previous reports (4, 5).

Immunohistochemical analysis

The kidneys were fixed with a 10% formalin neutral buffer solution for 3 weeks, then paraffin-embedded, and sliced into 5-μm sections. After the tissue sections were deparaffinized, immunostaining was performed with the following antibodies: fibronectin as a marker of cell adhesion molecules (dilution: x 1000; manufacturer: Sigma-Aldrich, USA), vimentin as a marker of myofibroblasts (x 500; Santa Cruz Biotechnology, USA), heme oxyganase-1 (HO-1) as a marker of peroxidative injury (x 100; Sigma-Aldrich, USA), neutrophil gelatinase-associated lipocalin (NGAL) as a marker of ischemic nephrotoxic injury (x 400; Sigma-Aldrich, USA), nestin as a marker of intermediate filament of glomeruli (x 200; Covance, USA), P-selectin as a marker of cell adhesion molecules (x 200; Bio-Rad AbD Serotec, USA), CD68 as the marker of macrophage infiltration (x 50; Bioss, USA), alpha-smooth muscle actin (□-SMA) as a marker of myofibroblasts (x 200; Protein tech, USA), and myoglobin as a marker of skeletal muscle (x 200; Dako, USA). Next, the sections were incubated using VECTASTAIN® Universal Quick Kits (Vector Laboratories Inc., USA) following the manufacturer's instructions.

Hematoxylin-eosin (HE) and azan staining were applied for this study as conventional staining.

The immunoreactivity in each category was calculated as the positive rate (%: number of positive cases/number of total cases in the category x 100).

Statistical analysis

In the non-drug group, the correlation among the immunoreactivities in each antibody and the correlation between each immunoreactivity and age, sex, and PMI were evaluated according to the correlation coefficient and p value, respectively. We classified the cases as to the cause of death and the one-way analysis of variance and multiple analysis of variance were investigated and compared.

Table 4. Summary of drugs and individual case shown significant difference

	Non-drug group	Drug-related group shown significant differences					
	Immunopositive mean±SD (%)	Number of drugs	Name of drugs	*p* value	Number of cases	Case No.	*p* value
fibronectin PT	5.3± 22.9	5	**KETO**/ DHC/ **DXM**/ **MEPH**/ ROSV	< 0.01	4	Case **2**/ 5/ **8**/ 22	< 0.05
fibronectin IT	31.5± 47.8	1	BDZs	< 0.05	0	no cases	
vimentin PT	15.8± 37.5	1	**LX**	< 0.05	9	Case 17/18	< 0.01
						Case 2/4/6/13/14/23	< 0.05
vimentin GL	68.4± 47.8	0	no drugs		0	no cases	
HO-1	10.5± 31.5	11	**AAPDs**	< 0.01	8	Case 2/8/10/13/16/23/24/25	< 0.05
			ADB/ CBZ/ DORAs/ TMB/ SAV/ FPHT/ FURD/BDZs/ LPS/ REB	< 0.05			
NGAL	21.1± 41.9	0	no drugs		0	no cases	
Nestin	47.8± 20.7	0	no drugs		0	no cases	
P-selectin	0.0± 0.0	9	AMPH/ DHC/ DPZ/ TZD/ METH/ REB/**LX**	< 0.01	6	Case 2/4/12/16/20/22	< 0.01
			AcA/ AAPDs	< 0.05			
CD68	5.3± 22.9	27	AcA/ AIAU/ AP/ AMPH/ IBN/ EPD/ CBZ/ **KETO**/ NBZs/ DTZM/ SULP/ DXM/ DPZ/ TZD/ FPHT/ FURD/ PROP/ BDZs/ METH/MEPH/ MTCL/ Tetr/ REB/**LX**/ ROSV/ WF/ AAPDs	< 0.01	14	Case 3/4/5/8/9/10/12/15/16/20/22/23	< 0.01
						Case 17/18	< 0.05
α-SMA	36.8± 49.6	0	no drugs		0	no cases	
Myoglobin	21.1± 41.9	6	CBZ/ DORAs/ DPZ/ FPHT/ FURD/ LPS	< 0.05	0	no cases	

PT: proximal urinary tubule; GL: glomerulus; IT: interstitial tissue

HO-1: heme oxganase-1; NGAL: neutrophil gelatinase-associated lipocalin; □-SMA: □-smooth muscle actin

Bold shows significant difference in both drugs and cases

Immunoreactivity in non-drug cases

In cases in the non-drug group, significant differences in immunoreactivity were observed in all categories except sex.

Age and PMI: A negative relationship between age and the immunoreactivities of vimentinGL ($p < 0.01$), NGAL ($p < 0.05$), and nestin ($p < 0.01$) were observed. Vimentin is a peculiar intermediate filament to mesenchymal cells (6), and nestin appears in the podocytes of the glomeruli (7). It seems that vimentin and nestin decrease with age because these proteins express in a normal glomerular cell. As for NGAL, it had been reported that the phagocytic activity of the neutrophil reduces with ageing (8). It was suggested that the secretion of NGAL decreases by reduced activity of neutrophils. A negative relationship between PMI and the immunoreactivity of vimentinGL, NGAL, and nestin was also observed ($p < 0.05$). It was considered that the decrease of immunoreactivity might be resolved by autolysis. It might be necessary to consider the effects of age and PMI in evaluating the immunoreactivity of each antibody.

Cause of death: In the immunoreactivity of HO-1, traumatic shock deaths showed a significant difference with several other causes of death (Fig. 1). Xanthine oxidase produces oxygen free radicals in PT of the ischemic kidney (9). In traumatic shock cases, it was considered that the oxidative damage occurred to the ischemia due to massive bleeding. Thus, immunoreactivity of HO-1 might be an indicator for oxidative damage.

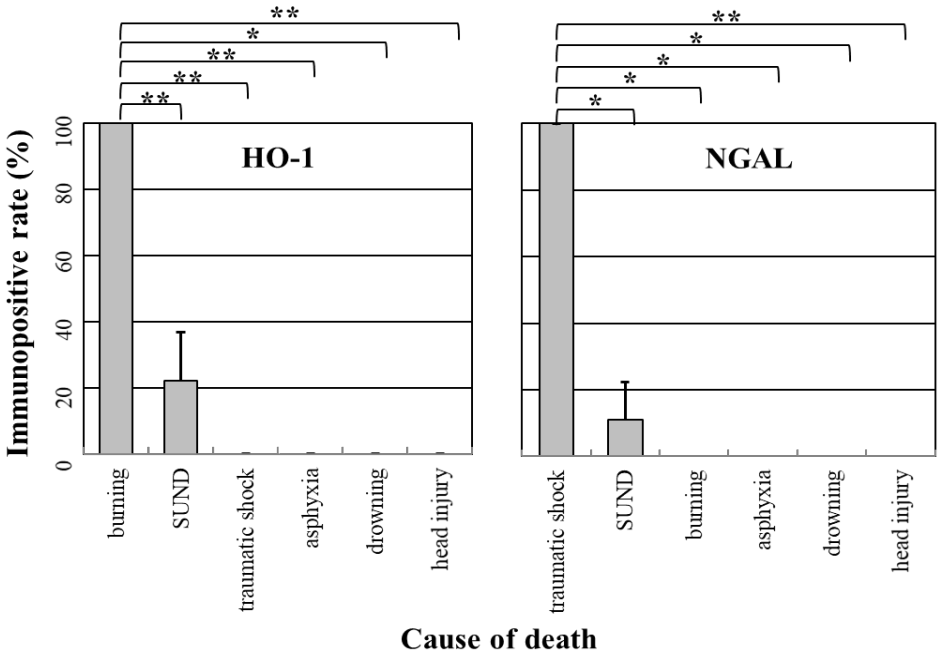

Figure 1. Immunopositive rate among causes of death

SUND: sudden unexpected natural death
**: $p < 0.05$, **: $p < 0.01$*

Moreover, a significant difference was seen in burning deaths in the immunoreactivity of NGAL (Fig. 2). It was reported that acute carbon monoxide (CO) poisoning causes intravascular neutrophil activation by interactions with platelets (10). Since NGAL is stored in specific granules of the neutrophil (11), it was thought that CO increases the expression of NGAL.

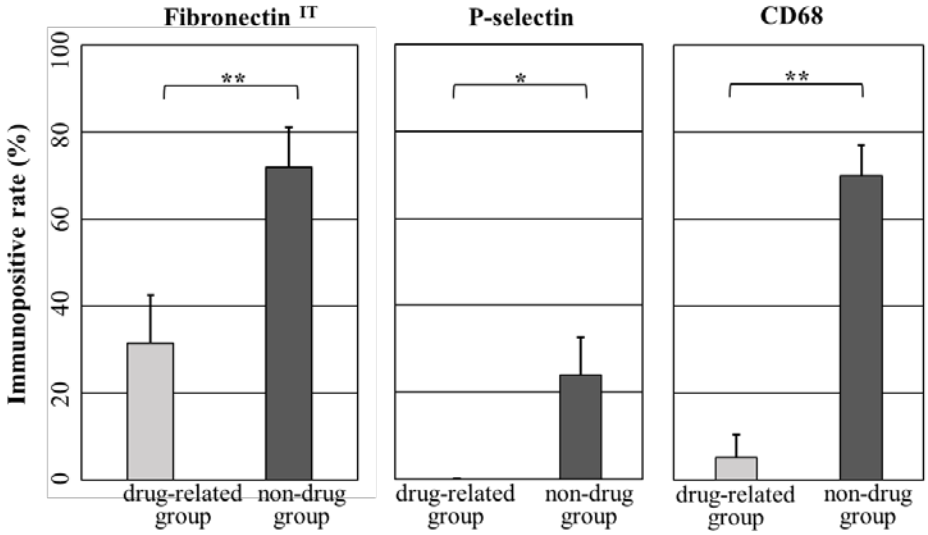

Figure 2. Immunopositive rate between drug- and non-drug group

IT: interstitial tissue
**: $p < 0.05$, **: $p < 0.01$*

Relationship among antibodies: In each immunoreactivity, the correlation coefficient was analyzed. A positive correlation was seen with vimentin^{GL} and nestin ($p < 0.01$). This positive correlation was observed because both vimentin and nestin appear in the normal podocyte (6, 7, 12). A positive correlation was also seen in the immunoreactivity of P-selectin and CD68 ($p < 0.01$). P-selectin plays a role in recruitment of leukocytes to injury sites (13). CD68 is known as a macrophage infiltration marker. It is reported that P-selectin partially mediates glomerular infiltration of macrophage in experimental crescentic glomerulonephritis (14). It was suggested that P-selectin and macrophages might cooperatively affect other types of kidney injuries.

Moreover, a positive correlation was seen in the immunoreactivity of HO-1 and CD68 ($p < 0.05$). HO-1 is an inducible enzyme with potent antioxidant, anti-inflammatory, and antiapoptotic properties, and HO-1 is known to suppress acute kidney injury (15, 16). This might be related to the macrophage production of free radicals and the induction of oxidative stress (17).

A positive correlation was seen between fibronectin^{IT} and α-SMA ($p < 0.01$), between vimentin^{PT} and fibronectin^{IT} ($p < 0.05$), between vimentin^{PT} and α-SMA ($p < 0.05$), and between vimentin^{PT} and nestin ($p < 0.05$). Fibronectin is important in cell adhesion, migration, growth and differentiation (18). In the kidney, fibronectin was stained in mesangium, glomerular capillary wall, vessels and interstitium. An increase of immunoreactivity was also observed in most types of the glomerulopathy (19). α-SMA is a

mesenchymal cytoskeletal protein and expressed in various renal diseases (20, 21). It is considered that fibronectin and α-SMA are expressed from myofibroblasts by the process of wound healing after renal injury. Vimentin does not express in normal urinary tubules, but does express in damaged kidneys and shows regenerating and proliferating activity of tubular lesions (22). In this study, it was thought that vimentin^{PT} might repair proximal urinary tubules with fibronectin and α-SMA.

Immunoreactivity in drug-related cases

Significant differences were also observed between the drug- and non-drug groups, in fibronectin^{IT}, P-selectin, and CD68 (Fig. 2). It is considered that they might become markers of drug-induced renal damage.

Relationships between antibodies and medicines/drugs: In the non-drug group, 42 different drugs were detected in the 25 drug-related cases. Only five cases (Cases 8, 10, 16, 17, and 18) detected a single drug. In addition, only four drugs were detected independently (KETO, SULP, AAPDs, and BDZs). Therefore, it was considered difficult to clarify immunohistochemical renal changes induced by a single drug. Therefore, we statistically analyzed the detected drugs and cases for each immunohistochemically observed finding, and examined the relationship between drugs and immunoreactivity. A summary of the drugs and individual cases that showed significant differences is shown in Table 3.

As for the immunoreaction of fibronectin^{PT}, a significant difference was seen in 5 drugs and 4 individual cases. Ketoprofen (KETO), dextromethorphan (DXM), and methylephedrine (MEPH) also showed a significant difference in 2 cases (Case 2 and 8). As for KETO, a significant difference was also seen in the immunoreaction of CD68. It is known that nonsteroidal anti-inflammatory drugs (NSAIDs) might increase the risk for chronic kidney disease (23). And it was reported that topical KETO treatment induced acute renal failure (24). Renal injury caused by KETO could be diagnosed by postmortem immunohistochemical study. DXM is used in many cough and cold medicines for its antitussive effects. Overdose of DXM induces complications such as hypertension, seizures, tachycardia, psychosis, and rhabdomyolysis (25), but it has not been reported that therapeutic use of DXM causes renal injury. In our case where DXM was detected, IBM, EPH, METH, and POSV were also detected. Similarly, MEPH is considered to have adrenergic effects; however, it has milder side effects (26). We need to consider the possibility of damage by the combined use of multiple drugs.

As for the immunoreactivity of vimentin^{PT}, a significant difference was observed only in LX and 8 individual cases. Moreover, LX showed significant differences in the immunoreactivity of P-selectin and CD68. It is thought that these significant differences reflected renal damage caused by ischemia induced constriction of the renal artery, because prostaglandin production is

suppressed by the COX inhibitory effect of LX (27).

A significant difference in immunoreactivity of vimentin^{PT} was also observed in one case of using both an SSRI and Sild. It is reported that Sild is useful for treating SSRI-induced sexual dysfunction (28), but this combination might cause microscopic renal injury as a side effect.

In the stainability of HO-1, AAPDs caused significant differences in 11 drugs and 8 individual cases. AAPDs are used as the first alternative in the treatment for schizophrenia. As side effects of AAPDs, diabetes due to weight gain and glucose tolerance become problems (29). Moreover, it is reported that hyperglycemia stimulates the production of mitochondria-derived superoxide from the vascular endothelial cell (30). It was thought that this superoxide might cause oxidative damage in the kidney.

A significant difference of the stainability of myoglobin was observed with CBZ. CBZ used with antipsychotics is known to cause neuroleptic malignant syndrome. Malignant syndrome causes myoglobinuria and renal dysfunction. It is considered that using CBZ with AAPDs causes rhabdomyolysis and damages the kidney.

CONCLUSIONS

Vimentin^{GL}, nestin, and NGAL might be useful in the determination of the cause of death, age, and PMI, respectively, in non-drug cases. Moreover, the present study showed the possibility that certain combinations of drugs which had not been previously reported causes renal damage. The combination of immunostaining for renal damage markers could be prove renal dysfunction induced by therapeutic drugs. Furthermore, immunostaining might give some information to help diagnose the cause of death and aid in personal identification.

Conflicts of interest: The authors have no conflicts of interest to declare.

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