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Letter

Maternal exposure to carbon black nanoparticle increases collagen type VIII expression in the kidney of offspring

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ABSTRACT — The potential health risks of inhaling nanomaterials are of great concern because of their high specific activity and their unique property of translocation. Earlier studies showed that exposure to nanoparticles through the airway affects both respiratory and extrapulmonary organs. When pregnant mice were exposed to nanoparticles, the respiratory system, the central nervous system and the reproductive system of their offspring were affected. The aim of this study was to assess the effect of maternal exposure to nanoparticles on the offspring, particularly on the kidney. Pregnant ICR mice were exposed to a total of 100 μg of carbon black nanoparticle on the fifth and the ninth days of pregnancy. Samples of blood and kidney tissue were collected from 3-week-old and 12-week-old male offspring mice. Collagen expression was examined by quantitative RT-PCR and immunohistochemistry. Serum levels of creatinine and blood urea nitrogen were examined. Exposure of pregnant ICR mice to carbon black resulted in increased expression of C*ollagen, type VIII, a1* (*Col8a1*) in the tubular cells in the kidney of 12-week-old offspring mice but not in 3-week-old ones. The levels of serum creatinine and blood urea nitrogen, indices of renal function, were not different between the groups. These observations were similar to those of tubulointerstitial fibrosis in diabetic nephropathy. These results suggest that maternal exposure to carbon black nanoparticle induces renal abnormalities similar to tubulointerstitial fibrosis in diabetic nephropathy are induced in the kidney of offspring.

Key words: Carbon black nanoparticle, Kidney, Collagen type VIII

INTRODUCTION

Exposure to airborne combustion-derived particulate matter has been implicated in adverse effects observed in epidemiological and clinical studies (Ostro *et al*., 2006; Pope *et al*., 2004). Experimental animal studies have also suggested that airborne particle matters exaggerate disease models in mice (Takano *et al*., 1997; Matsumoto *et al*., 2006; Sakai *et al*., 2009). It is well known that the core of combustion-derived particles is composed of carbon nanoparticles (BéruBé *et al*., 2007), which represent relevant surrogates for exhaust particles from modern diesel engines (Stoeger *et al*., 2009). Earlier studies showed that exposure to ultrafine carbon black through the airway affects the respiratory system (Donaldson *et al*., 2005; Tin-Tin-Win-Shwe *et al*., 2005; Erdely *et al*., 2009), the cardiovascular system (Donaldson *et al*., 2005; Erdely *et al*., 2009), the central nervous system (Donaldson *et al*., 2005; Tin-Tin-Win-Shwe *et al*., 2006) and other organs (Donaldson *et al*., 2005). However, it is not known how carbon black nanoparticle affects the fetus *in utero* and future offspring.

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The kidney receives a large proportion of cardiac output and has an important role in regulation of whole body blood circulation. It has been reported that inhaled particulate matters can translocate to kidney (Oberdörster *et al*., 2002, 2005; Gwinn and Vallyathan 2006), and that intravenous or intraperitoneal injection of nanoparticles induces nephrotoxicity in rodents (Lei *et al*., 2008; Chen *et al*., 2009). The findings suggest that the kidney is one of the significant target organs of nanoparticles. Therefore, we examined the effect of exposure to carbon black nanoparticle through the airway of pregnant mice on the kidney of the offspring. We studied collagen expression as the indicator for the effect of carbon black on kidney, since an increase in collagen expression followed by renal fibrosis is a harmful process independently of the primary renal disease which causes the original kidney injury (Gerth *et al*., 2007; van der Rest and Garrone 1991).

MATERIALS AND METHODS

Ultrafine Carbon Black

PRINTEX90 purchased from Degussa Ltd. (Frankfurt, Germany) was used as carbon black nanoparticle. The primary particle size and surface area are 14 nm and 300 m^2/g, respectively. It was suspended at 1 mg/ml in saline (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) with 0.05 % (v/v) Tween 80 and sonicated for more than 30 min just before administration. The carbon black can be used to examine the exclusive surface reactivity of carbon because it consists of 98% of pure carbon (Ganguly *et al*., 2009). The size distribution of carbon black nanoparticle in the suspension was analyzed by dynamic light-scattering using an FPAR-1000 particle analyser and the CONTIN program algorithm (Otsuka Electronics Co., Ltd., Osaka, Japan).

Animals and treatments

Pregnant ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and assigned randomly to treatment groups. The mice were housed under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and light (12-hr light/dark cycle) with access to food and water *ad libitum*. Mice in the exposure group (n = 13) were exposed to 50 µl of carbon black suspension (1 mg/ml) by intranasal instillation on gestational days 5 and 9. Mice in the control group (n = 12) were treated with 50 µl of vehicle. There was no difference in litter size between the groups: range 7-20 in the exposure group and 12-20 in the control group. Kidney and blood were collected from 3-week-old and 12-week-old male offspring mice. All experimental animals were handled in accordance with both institutional and national guidelines for the care and use of laboratory animals.

Real-time RT-PCR

Kidneys were collected from 3-week-old mice (n = 9 for exposure and control group) and from 12-week-old mice (exposure group, n = 16; control group, n = 15). RNA was isolated with Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) followed by treatment with Dnase (Promega Co., Madison, WI, USA), and was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative PCR was performed with SYBR Green Realtime PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan) and primers (Nippon EGT, Toyama, Japan) or Realtime PCR Master Mix (Toyobo) and TaqMan primer/probe sets (Applied Biosystems Japan, Tokyo, Japan) for the indicated genes. The primer and probe sequences are shown in Table 1. Statistical analysis was done with Student's t-test and the level of significance was set at $P < 0.05$.

Table 1. The primer and TaqMan probe sequences

Gene	Sequence (5' → 3')		Tm (°C)	Accession no.
Gapdh	F:	TGTGCAGTGCCAGCCTCGTC	60	NM_008084.2
	R:	GGATGCATTGCTGACAATCT		
Col1a1	F:	CTGGCGGTTCAGGTCCAAT	62	NM_007742.3
	R:	TTCCACGCAATCCACGAGC		
Col3a1	F:	ACGTAGATGAATTGGGATGCAG	62	NM_009930.2
	R:	GGGTTGGGGCAGTCTAGTG		
Col4a1	F:	ATGGGCTTGCCAGGTTCGCC	60	NM_009931.2
	R:	TCACCAGGCCGTCCCGGAAT		
Col8a1	F:	ACTCTGTCAGACTCATTCAGGC	60	NM_007739.2
	R:	CAAAGGCATGTGAGGGACTTG		

Histological analysis

Kidneys of 12-week-old mice (n = 5 per group) were fixed in phosphate-buffered fixative (4% (v/v) formaldehyde, 1% (v/v) glutaraldehyde). They were dehydrated in a graded series of ethanol and embedded in paraffin. Sections (5 mm) were stained with hematoxylin and eosin and analyzed by light microscopy.

Elastica van Gieson staining

The sections of paraffin-embedded kidneys (12-week-old mice) were also provided for elastica van gieson staining to detect deposition of collagen fibres. They were treated with 1% acid alcohol containing 1% HCl and 70% ethanol and stained by Weigert's Resorcin Fuchsin for 2 hr. After washing in water, they were treated with the 1% acid alcohol followed by staining with Weigert's iron hematoxylin for 5 min. After washing in water, they were treated with 0.5% hydrochloric acid followed by washing in running water, and then stained by Van Gieson's solution for 3 min. Stained sections were rinsed in water and 70% ethanol followed by dehydration in ethanol, and then analyzed by light microscopy.

Immunohistochemistry

Kidneys of 12-week-old mice (n = 5 per group) were perfusion-fixed in neutral buffered formaldehyde-glutaraldehyde fixative (4% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde), post-fixed in 10% (v/v) neutral buffered formalin for more than 72 hr, and then embedded in paraffin. Sections (4 μm thick) were cut for immunohistochemical analysis to visualize type VIII collagen. Immunohistochemical visualization was carried out on the sections using antibodies and avidin-biotin-peroxidase methods as previously described (Sugamata *et al*., 2006a). After blocking endogenous peroxidase and preincubation in 10% normal horse serum, sections were incubated in a primary rabbit polyclonal anti-mouse type VIII collagen antibody (LB-0883, LSL Co., Inc., Tokyo, Japan) diluted 1:500 in 0.1M PBS with 0.1% Triton X-100 for 16 hr at room temperature, and were incubated in a secondary biotinylated donkey anti-rabbit IgG (AP182B, Nihon Millipore K.K., Tokyo, Japan; 1:500) for 2 hr at room temperature, and finally treated with an avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA; 1:400) for 4 hr. Sections were then reacted for peroxidase activity in a solution of 0.02% 3,3'-diaminobenzidene (DAB) in 0.1 M Tris-HCl buffer (pH 7.6) and 0.01% H_2O_2 for 20 min. To obtain the data of negative control examination, the staining procedure omitting primary antibody was carried out.

The anti-mouse type VIII collagen antibody was purchased from LSL Co. The data obtained from LSL indicated that this anti-type VIII collagen (mouse, rat, bovine, human) does not cross-react with collagen of type I, II, III, IV, V and VI, and with laminin and fibronectin.

Markers of renal function in blood

Blood was obtained from 3-week-old mice (n = 9 per group) and from 12-week-old mice (n = 12 per group). Serum samples purified from two or three mice were pooled because the amount of blood obtained from each mouse was limited. Serum creatinine and blood urea nitrogen (BUN) levels were examined by SRL Inc. (Tokyo, Japan).

RESULTS

Characterization of the ultrafine carbon black in suspension

Suspended carbon black consisted of a mixture of agglomerated and individual particles in saline with 0.05% (v/v) Tween 80 (Fig. 1A). The carbon black aggregates had mass median aerodynamic diameters of 104.0 (± 45.9) nm at a concentration of 1 mg/ml (Fig. 1B).

Renal collagen expression and histology

Quantitative RT-PCR analysis showed an increase in *Collagen, type VIII, α1* (*Col8a1*) and a decrease in *Collagen, type I, α1* (*Col1a1*) among several types of collagen in 12-week-old mice in the exposure group (Fig. 2B). There was no significant difference between the groups in mRNA expression of collagens in the kidney of 3-week-old mice (Fig. 2A). Immunohistochemical analysis showed that expression of type VIII collagen was increased in the tubular cells in the renal cortex of 12-week-old mice in the exposure group (Fig. 3). Elastica van Gieson staining showed a deposition of collagen fibres in the renal interstitium in the exposure group (12 weeks old) (Fig. 4). These observations are similar to that in human diabetic nephropathy (Gerth *et al*., 2007).

Markers of renal function in blood

Maternal exposure to carbon black did not affect serum creatinine or BUN levels in the offspring at 3-week-old or 12-week-old (Fig. 5).

DISCUSSION

This study was prompted by reports of abnormality of various organs, including the central nervous system, the respiratory system and the reproductive system, in the offspring of pregnant mice exposed to particulate

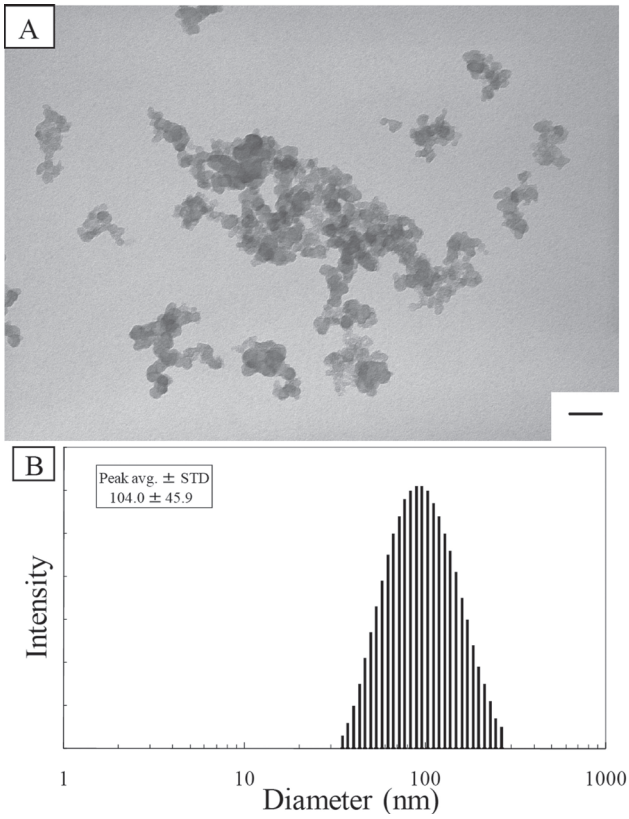

Fig. 1. Characterization of ultrafine carbon black in suspension. (A) TEM images of carbon black nanoparticle. The scale bar represents 50 nm. (B) Particle diameter distribution of carbon black in suspension examined by dynamic light-scattering.

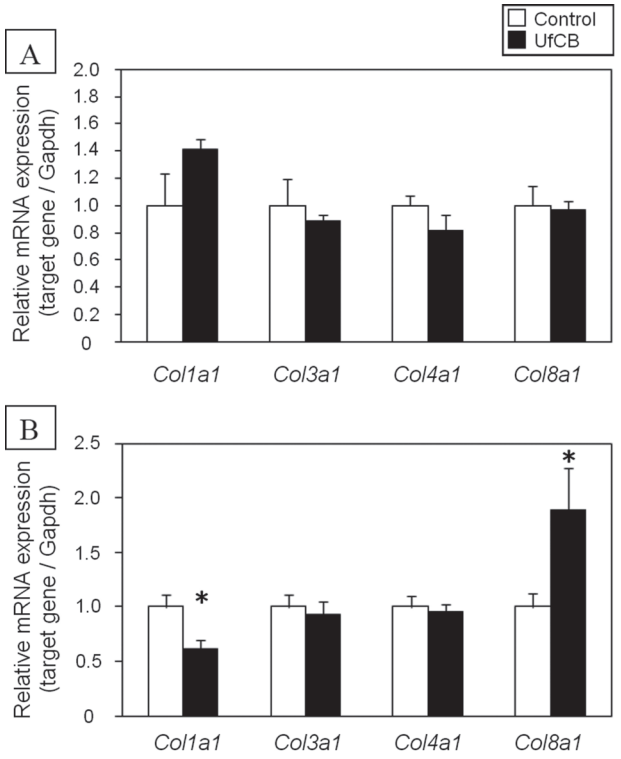

Fig. 2. Expression of mRNAs of collagen in kidney. Expression levels of *Col1a1*, *Col3a1*, *Col4a1*, and *Co8a1 mRNAs* in the kidney of mice of (A) 3-week-old and (B) 12-week-old were examined by quantitative RT-PCR. Data are shown as mean ± S.E.M. Statistical significance was set at $*P < 0.05$. Abbreviation: UfCB, ultrafine carbon black.

air pollutants, including nanoparticles (Fedulov *et al*., 2008; Xu *et al*., 2009; Takeda *et al*., 2009; Shimizu *et al*., 2009; Takahashi *et al*., 2010). An *in vitro* study using a human placental perfusion model showed that nanoparticles (< 240 nm, polystyrene beads) can cross the placental barrier (Wick *et al*., 2010). It was reported that diesel exhaust particle-like substances (Sugamata *et al*., 2006b) and titanium dioxide nanoparticles (Takeda *et al*., 2009) were transferred from pregnant mice to the brains of their offspring.

In the present study, we examined four types of collagen associated with renal fibrosis (Gerth *et al*., 2007; van der Rest and Garrone 1991). As a result, an increase in expression of type VIII collagen and a decrease in type I collagen mRNA in the exposure group were shown in the kidney of 12-week-old mice. Type I collagen is a fibrillar collagen that is found mostly in connective tissues, whereas type VIII collagen is non-fibrillar (van der Rest and Garrone 1991). There are few reports describing an increase in renal type VIII collagen; however, it has been reported that expression of type VIII collagen was increased in the kidney of diabetic nephropathy in humans (Gerth *et al*., 2007). The observation of increased expression of type VIII collagen in the tubular cells was similar to that in our study. Gerth *et al*. (2007) indicated the possibility that the promoter of COL8A1 contains a specific glucose-sensible regulatory element. However, we have preliminary evidence that blood glucose level is not altered in mice exposed maternally to nanoparticles. The study of type VIII collagen also indicated that the other transcript of the collagen, COL8A2, was similarly increased in diabetic nephropathy in humans (Gerth *et al*., 2007). Although there is no data of Col8A2 in the present study, it may be increased concomitantly with an increase in Col8a1. Type VIII collagen also facilitates movement of endothelial cells and is implicated in angiogenesis,

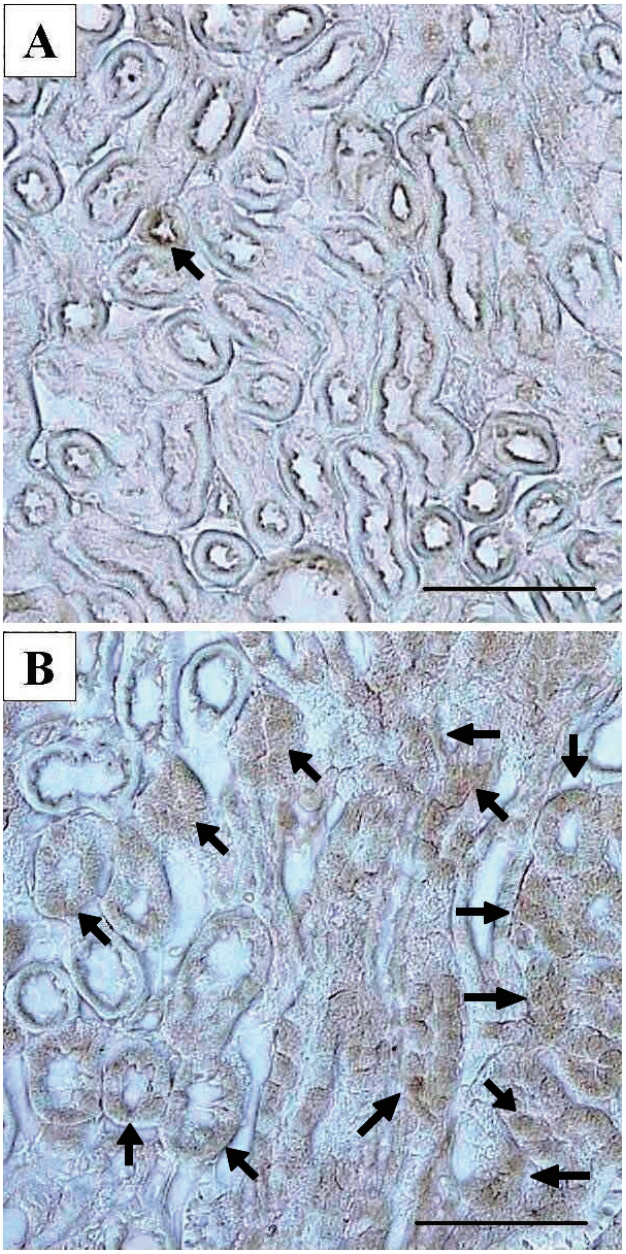

Fig. 3. Immunohistochemical analysis of the expression of type VIII collagen in the kidney. Photomicrographs of slices of the kidney (12-week-old mice) labeled for Col8a1 are shown: (A) control group; (B) maternal carbon black exposure group. Arrows indicate tubular cells positive for Col8a1. The scale bars represent 50 μm.

which supports the growth and differentiation of endothelial cells (Shuttleworth, 1997). An increase in glomerular capillary length that leads to glomerular hypertrophy has been observed in mice with diabetic nephropathy (Guo *et al*., 2005). It has been reported that vascular smooth muscle cells expressing type VIII collagen can modify the microenvironment and can spread and migrate on the tissue without type I collagen (Adiguzel *et al*., 2006). There is no report of the downregulation of type I collagen during the formation of fibrosis; therefore, the meaning of the downregulation of *Col1a1* remains unclear. The levels of serum creatinine and BUN, which are markers of renal function, were not altered in the exposure group (data not shown). This observation is similar to that made in diabetic nephropathy (Gerth *et al*., 2007). In the present study, the alteration of collagen expression in the kidney was observed in 12-week-old mice but not in 3-week-old mice. Mice aged 4-17 weeks have been used to examine any alteration in the kidney induced by change of the prenatal environment (Singh *et al*., 2007). The results were consistent with a hypothesis of "early developmental origins of adult disease", which indicates the environment that the fetus senses indirectly through the mother can be linked to other diseases in adulthood (Xu *et al*., 2009). Histological analysis showed an increase in deposition of collagen fibres in renal interstitium of the exposure group; however, this observation is not severe. The data suggest that maternal UfCB exposure may be a risk of some renal abnormalities rather than a significant cause of renal fibrosis.

These results suggest that maternal exposure to carbon nanomaterials promotes the aggregation of collagen fibre, which is associated with the development of renal fibrosis. These observations were made in the exposure group of mice after growth. Some limitations of our study merit discussion. First, we used a single bolus dose of particles via intranasal instillation of pregnant mice. Intranasal instillation is not a realistic exposure route compared with inhalation exposure; however, it is a simple and appropriate way to study the effects of exposure to chemical substances through the airway. The total dose of carbon black nanoparticle in this study was 2 mg/kg, which was lower than the dose used in many earlier studies (Chen *et al*., 2009; Fedulov *et al*., 2008; Tin-Tin-Win-Shwe *et al*., 2005, 2006; Takeda *et al*., 2009; Shimizu *et al*., 2009; Takahashi *et al*., 2010) but a little higher than that (1.6 mg/kg) used by Elderly *et al*., (2009). Further investigation using aerosol exposure and dose-response analysis is required to understand the effect of maternal exposure to nanoparticles. Second, it is possible that it is a parental alteration that can cause abnormality in the offspring. Further investigation of the parental factors causing the renal abnormality seen in the offspring is needed. Finally, the mechanisms underlying an increase in collagen type VIII

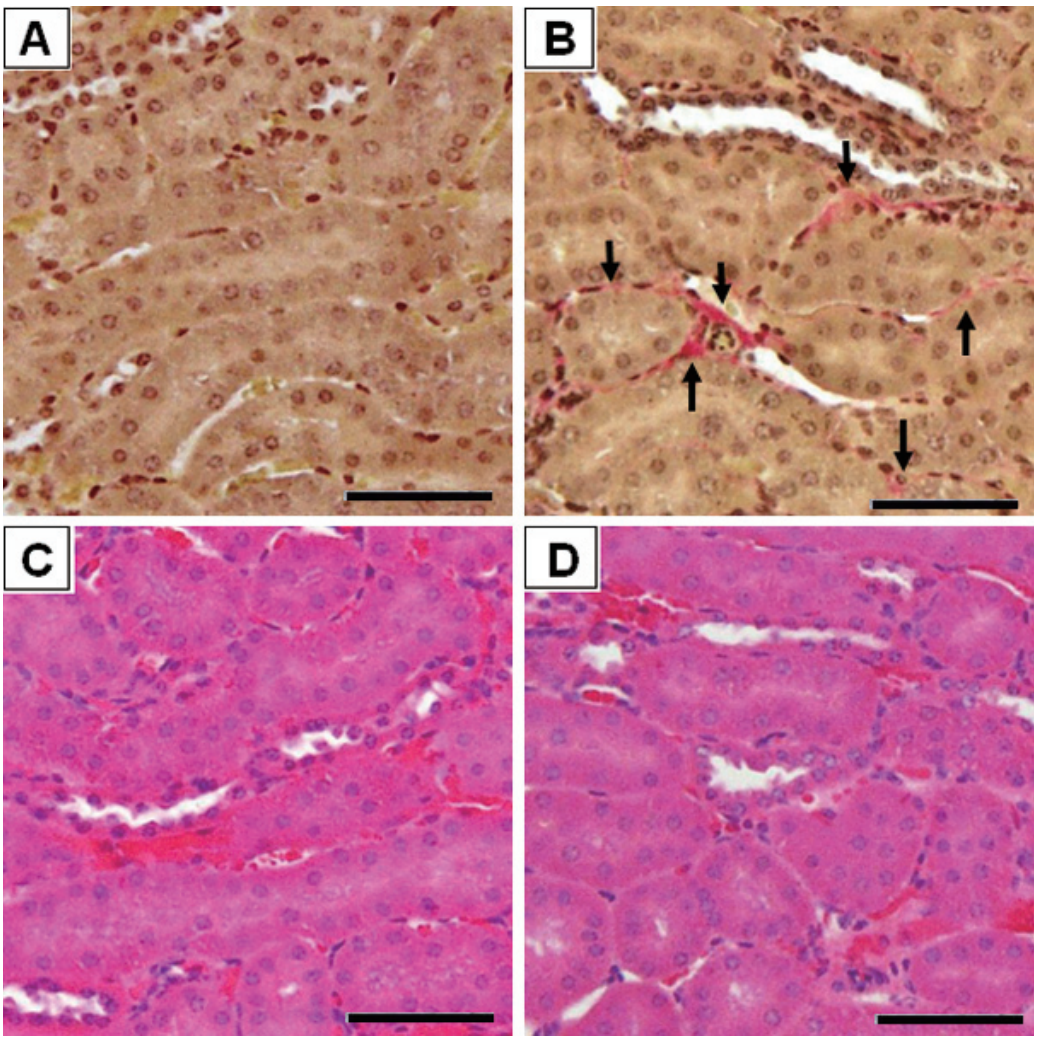

Fig. 4. Images of the tubular cells and interstitium in the kidney stained with an Elastica-van Gieson method and Hematoxylin-and-Eosin. Photomicrographs of slices of the kidney (12-week-old mice) stained with (A, B) a Elastica-van-Gieson method and (C, D) Hematoxylin and Eosin are shown: (A, C) control group; (B, D) maternal carbon black exposure group. Arrows indicate collagen fibres deposited in the renal interstitium. The scale bars represent 50 μm.

expression in the kidney of offspring. Addition to direct effects of freely translocated particles, the particles taken up by macrophages may act on systemic or local inflammatory response. The critical factor for the effects on offspring remains unresolved question. The presence of carbon black nanoparticle or particle-containing phagocytes in the kidney was not observed in the offspring of pregnant mice exposed to carbon black. Further investigation is needed to clarify how much carbon black was translocated to the kidney of the offspring mice.

In conclusion, this article showed that maternal exposure to carbon nanomaterials increases the expression of collagen, especially type VIII collagen, in the tubular cells of the renal cortex in the offspring, which can lead to the development of fibrosis (Gerth *et al*., 2007). The method used in this study provides a valuable mouse model for analysis of environmental exposure during pregnancy and the effect on various organs of offspring. Clarifying the effects of exposure to nanomaterials on humans and their offspring and the details of the underlying mechanism will lead to safe and profitable nanotechnology.

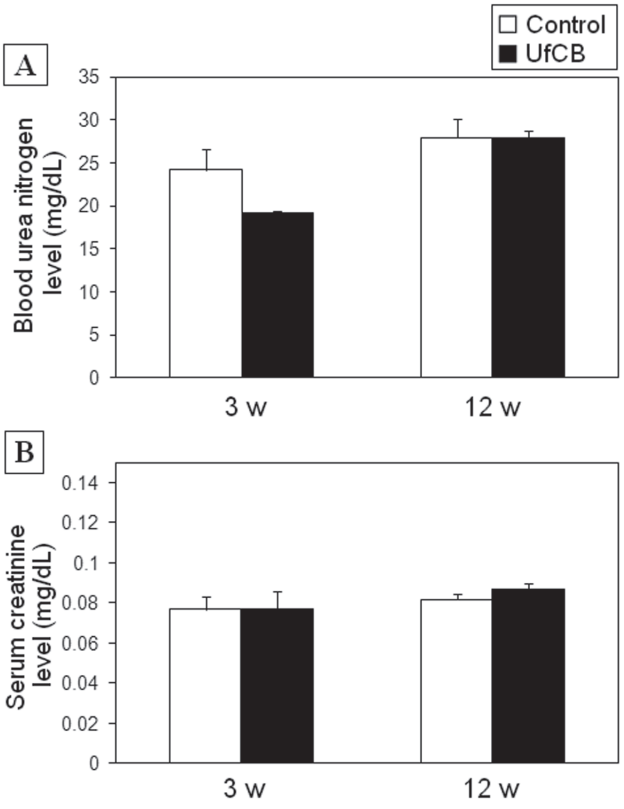

Fig. 5. Levels of renal function markers in blood. The levels of (A) blood urea nitrogen and (B) serum creatinine are shown as mean ± S.E.M. Abbreviation: UfCB, ultrafine carbon black.

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