789

学位論文

Hypothermia cannot ameliorate renal fibrosis after asphyxia in the newborn piglet

香川大学大学院医学系研究科
機能構築医学専攻
生殖・発育学部門
若林　誉幸

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Original Article

Title: Hypothermia cannot ameliorate renal fibrosis after asphyxia in the newborn piglet

Running Title:

Renal fibrosis in asphyxia

Takayuki Wakabayashi, MD[1], Shinji Nakamura, MD, DMSc[1], Yasuhiro Nakao, MD[1],Satoshi Yamato, MD, DMSc[1], Yinmon Htun, MD, DMSc[1], Tsutomu Mitsuie, CET[1], Aya Morimoto, MD[1], Makoto Arioka, MD[1], Kosuke Koyano, MD, DMSc[2],Yukihiko Konishi, MD, DMSc[1], Takanori Miki, MD, PhD[3], Masaki Ueno, MD, PhD[4], Takashi Kusaka, MD, DMSc[1]

[1]Department of Pediatrics, Faculty of Medicine, Kagawa University, Kagawa, Japan

[2]Maternal Perinatal Center, Faculty of Medicine, Kagawa University, Kagawa, Japan

[3]Department of Anatomy and Neurobiology, Faculty of Medicine, Kagawa University, Kagawa, Japan

[4]Department of Pathology and Host Defense, Faculty of Medicine, Kagawa University, Kagawa, Japan

Corresponding author:

Takashi Kusaka, MD, DMSci

Department of Pediatrics, Faculty of Medicine, Kagawa University

1750-1 Mikicho, Kitagun, Kagawa 761-0793, Japan

Email: kusaka@med.kagawa-u.ac.jp

Tel: +81-87-891-2171; Fax: +81-87-891-2172

Abstract (203 words)

Background: The effects of therapeutic hypothermia (TH) on renal function are not widely reported, especially in longer-term animal models. The hypothesis of this study was that TH of the kidneys of hypoxic–ischemic newborn piglets would reduce pathological renal fibrosis.

Methods: Twenty-five newborn piglets obtained within 24 h of birth were classified into a Control group ($n = 5$), hypoxic insult with normothermia (HI-NT) group ($n = 12$), and hypoxic insult with TH (HI-TH) group (33.5°C ± 0.5°C for 24 h; $n = 8$). Five days after the insult, all piglets were sacrificed under deep anesthesia by isoflurane inhalation. The kidneys were perfused with phosphate-buffered paraformaldehyde and immersed in formalin buffer. Territory fibrosis was studied and scored in the renal medulla using Azan staining.

Results: Fibrosis area scores (mean ± standard deviation) based on Azan staining were 1.00 ± 0.46 in the Control group, 2.85 ± 0.93 in the HI-NT group, and 3.58 ± 1.14 in the HI-TH group. The fibrosis area of the HI-NT and HI-TH groups was larger than that of the Control. The HI-NT and HI-TH groups were not statistically different.

Conclusion: Renal fibrosis is affected by perinatal asphyxia and cannot be prevented by TH, based on histopathological findings.

Keywords

Asphyxia, hypothermia, newborn, piglet, renal fibrosis.

histopathological changes in the kidney with or without TH on day 5 after HI insult. We hypothesized that TH would have the potential to reduce pathological renal injuries if TH showed neuroprotection in the Kagawa piglet model.

Methods

Ethical approval

The study protocol was approved by the Animal Care and Use Committee for Kagawa University, Kagawa, Japan (No. 15070-1) and in accordance with the Animal Research: Reporting In Vivo Experiments guidelines.

Animal model and experimental protocol

The animal preparation method for the HIE model was previously reported in detail (25). Briefly, 29 newborn piglets obtained within 24 h of birth were used in this study. The piglets were initially anesthetized with 1%–2% isoflurane in air using a face mask. Each piglet was then intubated and mechanically ventilated with an infant ventilator. The umbilical vein and artery were cannulated with a neonatal umbilical catheter for drip infusion, blood pressure monitoring, and blood sampling. After cannulation, the piglets were anesthetized with fentanyl citrate at an initial dose of 10 μg/kg, followed by a 5 μg/kg/h infusion. In addition, they were paralyzed with pancuronium bromide at an initial dose of 100 μg/kg, followed by a 100 μg/kg/h infusion. Maintenance solution was infused continuously at a rate of 4 ml/kg/h via the umbilical vein. Each piglet was placed under a radiant warmer to maintain rectal temperature at 38.0°C ± 1.0°C.

The piglets were classified into three groups. The Control group (n = 5) was maintained under a 0.21 fraction of inspiratory oxygen (FiO_2) for 24 h and extubated. The HIE animals

Five days after the extubation, all piglets were sacrificed under deep anesthesia by isoflurane inhalation. Blood samples were collected before the insult, at the end of the insult, and at 1 h, 3 h, 6 h, 24 h, and 5 days after the insult. Systemic organs, including the kidney, were perfused with phosphate-buffered paraformaldehyde and immersed in buffered formalin.

Histopathological analysis

Kidneys were embedded in paraffin and sectioned. The sections (4-μm-thick) were stained with hematoxylin & eosin (H&E) and periodic acid-Schiff (PAS) staining to investigate inflammatory cell invasion and examine the histological changes in the cytoplasm with the brush border of tubular epithelial cells and with Azan staining to identify glomerulosclerosis and to enable estimation of the extent of renal medulla fibrosis. To objectively estimate the extent of renal medulla fibrosis, 10 random images of the renal medulla of the kidney tissue were obtained from an Azan-stained slide from each piglet. We measured the surface area of the fibrosis, which was stained blue by the Azan stain, using image processing software (ImageJ; US National Institutes of Health, Bethesda, MD). The mean surface area was calculated for each piglet.

To assess cytoplasmic injury in tubular epithelial cells, we examined the percentage of tubules with an invisible lumen. We identified 20 tubules per field of view in the cortex near the cortical-medullary border using H&E-stained specimens. Ten of these tubules were randomly selected and examined in each renal tissue specimen, and the mean percentages were compared among the three groups.

Statistical analysis

Four HI-TH piglets were excluded from the present study because the piglets did not survive 5 days after the insult. In total, 25 piglets were used in the analysis: 5 in the Control group, 12 in

In contrast, base excess was lower in the HI-TH group than in the HI-NT group ($p < 0.05$, at 3 h after HI insult) and lactate tended to be higher in the HI-TH group than in the HI-NT group (Table 1).

Histopathology

Based on the H&E and PAS staining (Fig. 1A–F and J–O), the HI-NT and HI-TH groups showed no renal injury, such as infarction, focal hemorrhagic necrosis, or cellular casts, 5 days after the HI insult. However, in the renal cortex, H&E and PAS staining revealed the presence of tubules with an invisible lumen in the HI-NT group, presumably due to cytoplasmic swelling of the renal tubular epithelial cells (Fig. 1). Some tubular lumens in the HI-TH group were also invisible, whereas others were visible. The mean (SD) percentages of tubules with an invisible tubular lumen were 6.4% (4.7%) for Control, 15.7% (9.4%) for HI-NT, and 14.3% (8.5%) for HI-HT. The brush border of tubular epithelial cells was seen in both HI-NT and HI-TH groups (Fig. 1E and F). Azan staining revealed no clear differences in fibrotic findings among the three groups (Fig. 1G–I). In the renal medulla, H&E and PAS staining showed that there were no differences in histological findings among the three groups (Fig. 1J–O). However, Azan staining revealed that fibrosis was evident, especially in the HI-NT and HI-HT groups (Fig. 1P–R); the fibrotic area scores were significantly higher in both the HI-NT and HI-TH groups than in the Control group, although there was no significant difference between the HI-NT and HI-TH groups in the mean (standard deviation) fibrosis area (%): Control, 1.00 (0.46); HI-NT, 2.85 (0.94); and HI-TH, 3.58 (1.14) (Fig. 2).

Discussion

This is the first report on the presence of renal fibrosis 5 days after HI insult in the newborn piglet, with no elevation of the serum creatinine level, which reflects AKI, and no

nitric oxide and several profibrotic and proinflammatory cytokines and growth factors, and induce the subsequent production of deleterious reactive oxygen species. Each of these factors, as well as others, further potentiates the tissue damage and dysfunction and initiates a repair process that can be adaptive or maladaptive. After apparently recovered ischemia-reperfusion injury, there is a long-term reduction in peritubular capillary density that precedes the development of visible fibrosis (32-34). We did not find any inflammatory cell invasion in the H&E- and PAS-stained sections. Renal hypoxia is pathophysiologically involved in the AKI-to-chronic kidney disease (CKD) transition. After AKI occurs, capillary rarefaction, which is probably induced by decreased expression of vascular factors, such as vascular endothelial growth factor in tubular cells, or is associated with pericyte detachment, causes renal hypoxia. Hypoxia damages tubular epithelial cells, activates fibroblasts, and induces inflammatory reactions, all of which lead to tubulointerstitial fibrosis. Tubulointerstitial fibrosis, in turn, aggravates hypoxia. Thus, hypoxia and tubulointerstitial fibrosis form a vicious cycle, resulting in progression to CKD (30). In addition to immune factors, a wide range of non-immune factors, including reactive oxygen species, advanced glycation end-products, and conditions such as hypertension, hypoxia, hyperglycemia, and proteinuria have been implicated in driving renal fibrosis (35). This indicates that fibrosis does not usually occur after inflammation. Another possibility may be that the inflammation in the renal medulla in this model was very weak and had disappeared/improved by 5 days after the insult.

Against our hypothesis, we found that TH was unable to reduce the renal fibrosis. The efficacy of TH has been controversial in the literature. TH has been widely used after HI brain injury. In this context, it is important to determine the effects of hypothermia on other organs, including the kidney. Several studies have reported the ability of mild hypothermia to preserve kidney function for transplantation. However, very few studies have assessed the influence of mild and moderate hypothermia on the kidney (21-24). These studies, examining the effects of

should consider a new adjuvant therapy together with TH, such as erythropoietin, to prevent the renal fibrosis after birth asphyxia.

In conclusion, these findings in our newborn piglet model demonstrate that renal fibrosis due to AKI can develop without elevation in the serum creatinine level after HI insult and, furthermore, that this fibrosis after HI insult cannot be ameliorated by TH. Accordingly, HIE neonates with or without TH should be considered to be at high risk of developing renal fibrosis and at a much higher risk of developing CKD later in life. It is therefore of major clinical importance to confirm if CKD has a perinatal origin in order to prevent the loss of renal function in adult life.

References

1. Durkan AM, Alexander RT. Acute kidney injury post neonatal asphyxia. J. Pediatr. 2011; 158 (2 Suppl): e29-33.

2. Aggarwal A, Kumar P, Chowdhary G, Majumdar S, Narang A. Evaluation of renal functions in asphyxiated newborns. J. Trop. Pediatr. 2005; 51: 295-299.

3. Kaur S, Jain S, Saha A, et al. Evaluation of glomerular and tubular renal function in neonates with birth asphyxia. Ann. Trop. Paediatr. 2011; 31: 129–134.

4. Sweetman DU, Riordan M, Molloy EJ. Management of renal dysfunction following term perinatal hypoxia-ischaemia. Acta Paediatr. 2013; 102: 233-41.

5. Selewski DT, Jordan BK, Askenazi DJ, Dechert RE, Sarkar S. Acute kidney injury in asphyxiated newborns treated with therapeutic hypothermia. J. Pediatr. 2013; 162: 725-729.

6. Perlman JM, Tack ED. Renal injury in the asphyxiated newborn infant: relationship to neurologic outcome. J. Pediatr. 1988; 113: 875-879.

7. Martín-Ancel A, García-Alix A, Gayá F, Cabañas F, Burgueros M, Quero J. Multiple organ involvement in perinatal asphyxia. J. Pediatr. 127:786-793.

8. Sarkar S, Askenazi DJ, Jordan BK, et al. Relationship between acute kidney injury and brain MRI findings in asphyxiated newborns after therapeutic hypothermia. Pediatr. Res. 2014; 75: 431-435.

9. Sweetman DU, Onwuneme C, Watson WR, O'Neill A, Murphy JF, Molloy EJ. Renal function and novel urinary biomarkers in infants with neonatal encephalopathy. Acta Paediatr. 2016; 105: e513-e519.

10. Gupta C, Massaro AN, Ray PE. A new approach to define acute kidney injury in term newborns with hypoxic ischemic encephalopathy. Pediatr Nephrol. 2016; 31: 1167-78.

11. van Wincoop M, de Bijl-Marcus K, Lilien M, van den Hoogen A, Groenendaal F. Effect of therapeutic hypothermia on renal and myocardial function in asphyxiated (near) term

21. Amess PN, Penrice J, Howard S, et al. Organ pathology following mild hypothermia used as neural rescue therapy in newborn piglets. Biol. Neonate. 1998; 73: 40-6.

22. Satas S, Løberg EM, Porter H, Whitelaw A, Steen PA, Thoresen M. Effect of global hypoxia-ischaemia followed by 24 h of mild hypothermia on organ pathology and biochemistry in a newborn pig survival model. Biol. Neonate 2003; 83: 146-156.

23. Stojanović V, Vučković N, Spasojević S, Barišić N, Doronjski A, Zikić D. The influence of EPO and hypothermia on the kidneys of rats after perinatal asphyxia. Pediatr. Nephrol. 2012; 27:139-44.

24. Stojanovic VD, Vesna D Stojanović VD, Vučković NM, Barišić NA, Srdić B, Doronjski AD, Peco Antić AE. Early biomarkers of renal injury and protective effect of erythropoietin on kidneys of asphyxiated newborn rats. Pediatr. Res. 2014; 76: 11-16.

25.Nakamura S, Kusaka T, Yasuda S, et al. Cerebral blood volume combined with amplitude-integrated EEG can be a suitable guide to control hypoxic/ischemic insult in a piglet model. Brain Dev. 2013; 35: 614-625.

26. Nakamura S, Kusaka T, Koyano K, et al. Relationship between early changes in cerebral blood volume and electrocortical activity after hypoxic-ischemic insult in newborn piglets. Brain. Dev. 2014; 36: 563-571.

27. Nakamura M, Jinnai W, Hamano S, et al. Cerebral blood volume measurement using near-infrared time-resolved spectroscopy and histopathological evaluation after hypoxic-ischemic insult in newborn piglets. Int. J. Dev. Neurosci. 2015; 42: 1-9.

28 Jinnai W, Nakamura S, Koyano K, et al. Relationship between prolonged neural suppression and cerebral hemodynamic dysfunction during hypothermia in asphyxiated piglets. Brain Dev. 2018; 40: 649-661.

29 Kubo H, Shimono R, Nakamura S, et al. Hypoxic-ischemic encephalopathy-associated liver fatty degeneration and the effects of therapeutic hypothermia in newborn piglets.

Figure legends

Fig. 1. Representative images of H&E (A–C), PAS (D–F), and Azan (G–I) staining in the renal cortex and those of H&E (J–L), PAS (M–O), and Azan (P–R) staining in the renal medulla in the Control (A, D, G, J, M, P), HI-NT (B, E, H, K, N, Q), and HI-TH (C, F, I, L, O, R) groups. Arrows indicate the brush border of tubular epithelial cells in E and F. Fibrosis is dyed blue by Azan staining in P–R. Scale bar, 100 μm.

Fig. 2. Percentage of area stained with Azan in the renal medulla in the three piglet groups (Control, $n = 5$; HI-NT, $n = 12$; HI-TH, $n = 8$). The percentage of area stained with Azan was significantly larger in the HI-NT and HI-TH groups than in the Control group. There were no significant differences between the HI-NT and HI-TH groups.

Table 1. Physiological parameters, arterial blood gas, and lactate before, at the end of, and at 1, 3, and 6 h after HI insult in the Control, HI-NT, and HI-TH groups.

		Baseline	End of insult	1 h after HI insult	3 h after HI insult	6 h after HI insult
HR (beats/min)	Control	246.8 ± 33.5	243.8 ± 25.6	245.2 ± 33.1	235.2 ± 32.8	228.8 ± 28.7
	HI-NT	220.1 ± 29.3	145.0 ± 21.2***	232.8 ± 22.7	261.1 ± 18.9	226.2 ± 14.7
	HI-TH	223.6 ± 29.7	160.8 ± 27.1**	211.4 ± 34.4	212.3 ± 10.7##	213.6 ± 7.0
MABP (mmHg)	Control	81.8 ± 8.0	79.4 ± 10.2	80.4 ± 7.0	69.0 ± 7.0	66.0 ± 12.6
	HI-NT	79.9 ± 9.7	47.6 ± 10.5***	67.9 ± 7.9	67.5 ± 7.7	68.8 ± 7.0
	HI-TH	82.6 ± 12.0	47.4 ± 8.1***	71.9 ± 10.2	74.3 ± 8.7	70.8 ± 7.1
BT (°C)	Control	38.0 ± 0.5	38.2 ± 0.5	38.4 ± 1.0	38.2 ± 0.8	38.5 ± 0.9
	HI-NT	37.6 ± 0.6	37.1 ± 0.7	37.8 ± 0.8	37.9 ± 0.6	38.1 ± 0.6
	HI-TH	37.8 ± 1.0	37.3 ± 0.4	34.0 ± 0.9***###	33.8 ± 0.6***###	34.0 ± 0.3***###
pH	Control	7.50 ± 0.03	7.47 ± 0.07	7.49 ± 0.05	7.47 ± 0.06	7.50 ± 0.06
	HI-NT	7.42 ± 0.09	6.91 ± 0.11***	7.30 ± 0.05**	7.50 ± 0.04	7.48 ± 0.08
	HI-TH	7.41 ± 0.10	6.81 ± 0.10***	7.22 ± 0.10**	7.44 ± 0.08	7.45 ± 0.05
$PaCO_2$ (mmHg)	Control	35.3 ± 3.0	38.7 ± 3.5	37.4 ± 5.6	38.0 ± 5.3	34.7 ± 7.7
	HI-NT	46.0 ± 9.54	29.5 ± 7.7	39.6 ± 7.4	39.4 ± 3.1	43.4 ± 11.0
	HI-TH	45.0 ± 10.4	38.4 ± 14.8	43.4 ± 8.5	38.8 ± 3.6	42.8 ± 6.7
PaO_2 (mmHg)	Control	81.8 ± 15.1	79.8 ± 16.3	81.9 ± 14.8	78.5 ± 19.3	81.0 ± 22.4
	HI-NT	108.8 ± 27.4	16.4 ± 5.4***	104.3 ± 34.6	91.8 ± 21.7	103.2 ± 27.1
	HI-TH	98.1 ± 14.2	19.7 ± 6.2***	107.7 ± 19.7	85.2 ± 20.8	88.2 ± 21.1
BE (mmol/ml)	Control	4.4 ± 2.8	4.2 ± 3.1	4.6 ± 3.6	4.0 ± 3.6	3.2 ± 3.5
	HI-NT	5.3 ± 2.2	−25.1 ± 3.7***	−6.5 ± 2.6***	7.2 ± 2.7	7.6 ± 2.5*
	HI-TH	3.2 ± 3.0	−27.4 ± 2.4***	−9.5 ± 4.0***	2.0 ± 3.8 #	4.8 ± 2.5
Lactate (mg/dl)	Control	27.8 ± 9.2	27.8 ± 10.8	31.2 ± 19.4	33.4 ± 21.0	41.4 ± 21.8
	HI-NT	16.3 ± 5.4*	215.9 ± 27.7***	113.3 ± 28.6***	31.9 ± 13.2	24.5 ± 9.0
	HI-TH	20.5 ± 8.8	215.1 ± 21.7***	125.3 ± 26.3***	66.6 ± 36.3†	38.6 ± 23.9

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control; ##$p < 0.01$, ###$p < 0.001$ vs. HI-NT.

BE, base excess; BT, body temperature; HR, heart rate; $PaCO_2$, partial pressure of carbon dioxide.

Fig. 1

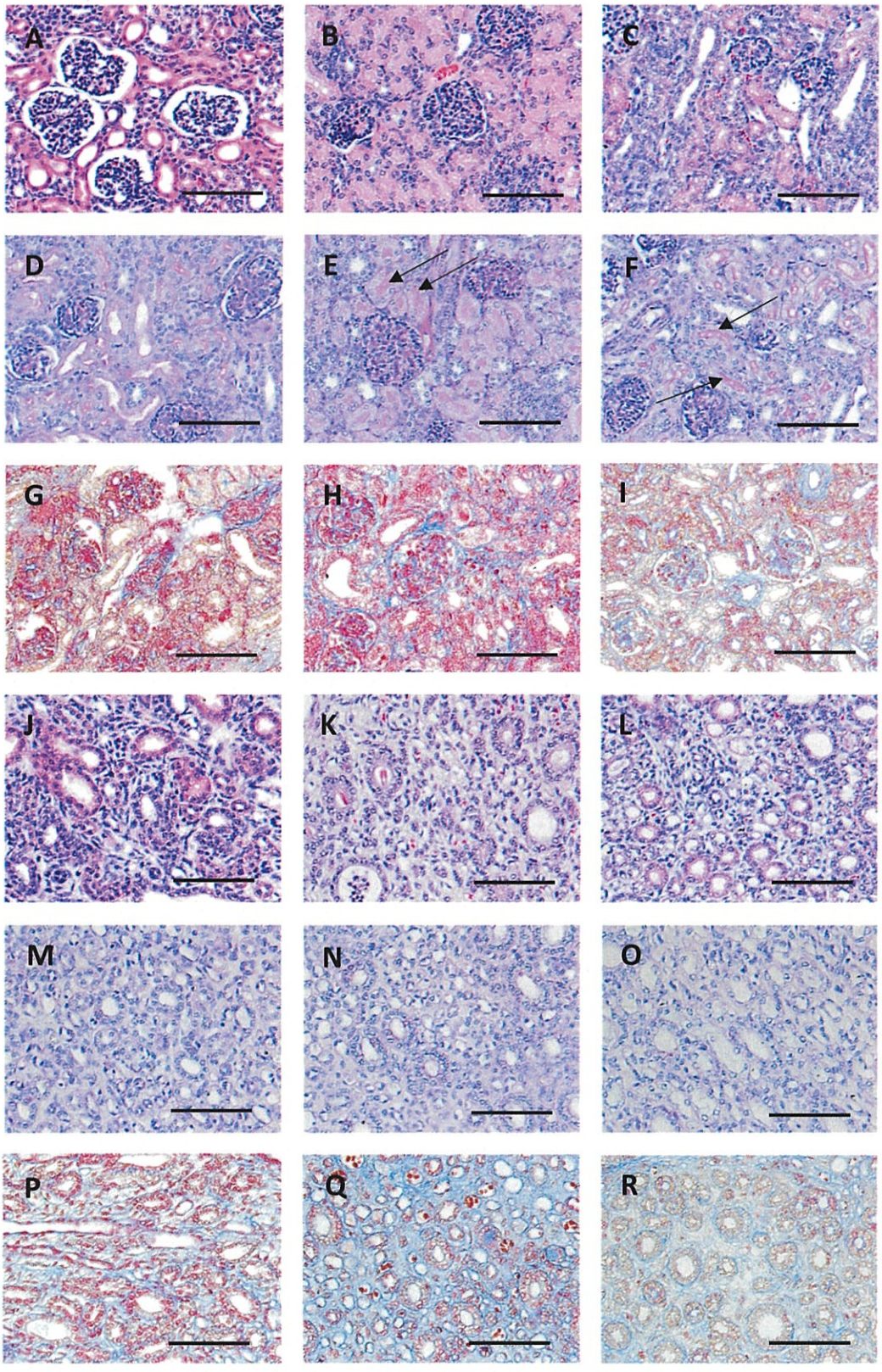

Fig. 2