Nephropathy Effects of Intravenous Contrast, Iodixanol, on db/db and eNOS Knockout Mice

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ABSTRACT: Purpose To investigate, if Iodixnal, an intravenous contrast, causes Contrast Induced Nephropathy (CIN) in db/db and eNOS knockout mouse model, which mimics the patients with underline renal insufficiency. **Materials and Methods** Eight-week-old db/db mice and eNOS knockout mice were anesthetized. Iodixanol was retro-orbitally injected into the mice at doses 0.75 gI/kg and 2.75gI/kg, respectively in different treatment groups. Normal saline solution was injected into the mice in the control group (n=3). Three days and 7 days following Iodixanol administration, the animals were sacrificed, and their kidneys were harvested. Kidney injuries were evaluated using Hematoxylin and eosin stain (H&E stain) of paraffin embedded kidney sections. Ki67 mRNA levels were assessed by qRT-PCR. The Ki67 protein expression level was assessed by an immunohistochemistry (IHC) assay of paraffin embedded kidney sections. **Results** H&E staining shows no significant evidence of histopathological injuries caused by Iodixanol in both diseased mouse models. There is no increase of Ki67 expression in db/db and eNOS mice following Iodixanol administration, comparing with control group. **Conclusions** Iodixanol does not result in CIN even in diabetes mellitus and hypertension mice model based on histological and Ki67 examinations. To study the mechanisms of CIN, Iodixanol alone may not be toxic enough to create a research animal model.
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Key Words: contrast-induced nephropathy, iodixanol, db/db mice, eNOS knockout mice

INTRODUCTION

Intravenous contrast media (CM) is commonly used in clinical radiology studies such as angiography and computed tomography. CM can cause contrast induced nephropathy (CIN), especially in patients with pre-existing problems such as diabetes mellitus, hypertension, insufficient kidney function, etc[1].The mechanism of CIN is not fully understood, but the main hypotheses include direct toxicity of CM on tubular cells, renal vasoconstriction, renal medulla ischemia and reactive oxygen species[2-4] . Iodixanol (IDX), as a new non-ionic, iso-osmolar, dimmer contrast media, is believed to be safer than other contrast agents[5], but it may still cause CIN or other complications in certain patients[6,7]. In our current study, we used diabetic mice (db/db) and hypertension mice (eNOS -/-) to see the effects of Iodixanol on the kidneys with these two underline diseases. The mice were 8 weeks old which is equivalent to human in their 20s to minimize the renal function effects of aging and to investigate if CIN can be induced in these two diseased animal models. Histological changes and a sensitive kidney injury marker, Ki67, were used to evaluate the kidney damages following Iodixanol administration.

MATERIALS AND METHODS

Animals

Db/db mice, former name B6.BKS-Leprdb, JAX stock number 000697; and eNOS knockout mice, former name B6.129P2-Nos3tm1Unc, JAX stock number 002684 were utilized. These mice were mutants from background C57BL/6J mice. We ordered 7-week-old male mice and performed our experiments after one week accommodation. All animals were obtained from The Jackson Laboratory, and caged with free access to food and water. They were kept under conditions of 21 ± 1°C and 50% to 80% relative humidity at all times in an alternative 12 hours light-dark cycle in a central animal facility at Louisiana State University Health Science Center-Shreveport. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Animal Care and Use Committee at the Louisiana State University Health Sciences Center-Shreveport.

Iodixanol administration

Eight-week-old db/db mice and eNOS knockout mice were anesthetized with 150mg/kg Ketamine and 10 mg/kg Xylazine. Iodixanol was retro-orbitally injected into the mice at doses of 0.75 gI/kg and 2.75 gI/kg. Normal(0.9%) saline solution was given to the mice in the control group (n=3). Three days and 7 days after Iodixanol administration, the

animals were sacrificed. Their kidneys were harvested, cut sagittally, and placed into labeled cassettes and then fixed with 10% neutral formalin for 24 hours at room temperature. The kidney sections were then embedded into paraffin. The other half of the kidneys for RNA extraction were put into 10 volumes of RNAlater (Ambion) at 4°C overnight and then stored in an -80°C fridge.

Histopathology and histology scoring

Histopathology evaluation was performed as described by Hoffmann et al[8]. Briefly, the embedded tissues were cut into 5 μm sections and stained with Hematoxylin and eosin stain (H&E stain). The histological damages of the sections were evaluated under a light microscope by a pathologist, who was blind to the study, and a severity grading scale of 0 to 3 was used to grade pathological injuries: 0, no to minimal; 1, mild acute tubular injury manifested by mild tubular distension, a low epithelial lining, and nonspecific degenerative changes of the epithelial cells; 2, moderate acute tubular injury, with frequent single cell necrosis within the epithelial layer; and 3, severe acute tubular injury demonstrated by the presence of wide spread epithelial cell necrosis, low epithelial lining, and accumulation of cellular debris in the tubule lumens.

Quantitative real-time PCR

Pieces of kidney specimen in the RNAlater were cut off from the samples and weighted, and then total RNA of each piece was isolated using the RNeasy mini kit (QIAGEN, Valencia, CA). The leftover samples were restored back into the RNAlater in an -80°C fridge. First-strand cDNAs were synthesized from total RNA by reverse transcription (1 μg total RNA in 20 μl of reaction volume) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) primed with oligodT and random hexamer primers. SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA) was used for dye-based detection. Reactions were conducted in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Fluorescence was monitored during every PCR cycle at the annealing step. The primers for Ki67 were: 5'-AGA AGT CCA GGT CTA CAG-3', 5'-TAG GCA TCG TAG TAG TTG-3' GADPH: 5'-GAG TCA ACG GAT TTG GTC GT- 3', 5'-TTG ATT TTG GAG GGA TCT CG-3'. PCR conditions were as follows: 95 ℃, 30 sec; (95 ℃, 2 sec; 55 ℃, 2 s) × 40 cycles; 60 ℃, 1 min, Melt curve: 65 ℃-95 ℃ in 0.5 ℃ interval, 2 sec/step. Results were analyzed with a Bio-Rad CFX manager. Results for all experiments represent triplicate determinations. Results are represented as means ± SD.

Immunohistochemical (IHC) procedures

Paraffin embedded tissues were cut into 5 μm sections. The slides were deparaffinized and dehydrated by immersing into vessels containing the following chemicals for an indicated time: xylene 10 minutes, xylene 10 minutes, 100% ethanol 4 minutes, 100% ethanol 4 minutes, 95% ethanol 4 minutes, 70% ethanol 4 minutes, 50% ethanol 4 minutes, distilled water 4 minutes. Then antigen retrieve was performed as follows. Slides were put into a vessel containing sodium citrate buffer (10mM sodium citrate, 0.05% tween 20, pH 6.0) and were preheated approaching 100°C for about 20 minutes.The slides were then cooled for 20 minutes at room temperature. After the slides were washed in distilled water, endogenous peroxidase was inhibited with 0.3% peroxide in 50% methanol (in PBS) for 30 minutes at room temperature. The slides were washed with PBS three times, and then blocked for 20 minutes with 2.5% normal goat serum. Following this, the primary antibody, rabbit anti mouse Ki67 antibody (Abcam, #ab16667), was diluted 100 times in 0.1% normal goat serum PBS solution and put onto the slides and incubated for 1 hour at room temperature. Slides were washed with PBS 5 times, and then the secondary antibody, goat anti rabbit IgG-HRP (Abcam, #ab6721), was diluted 500 times in 0.1% normal goat serum PBS solution and added onto the slides and incubated for 1 hour at room temperature. The slides were washed again with PBS 3 times, and chromogen 3,3'–Diaminobenzidine (DAB) (Abcam) was added onto the slides before they were incubated for another 7 minutes. After a PBS rinse, the slides were counter stained with hematoxylin (Sigma) for 2 minutes and rinsed under tap water for 5 minutes. Slides were dehydrated by immersion into vessels containing the following chemicals for indicated time: distilled water 4 minutes, 50% ethanol 4 minutes, 70% ethanol 4 minutes, 95% ethanol 4 minutes, 100% ethanol 4 minutes, 100% ethanol 4 minutes, xylene 10 minutes, xylene 10 minutes. Finally cover slips were mounted onto the slides with permount.

Statistical methods

Statistical analysis of changes in Ki67 RNA and protein expression were performed with Student's*t*-test using Prism software (GraphPad). A P value of 0.05 or less was considered statistically significant. Data are expressed as means ±SD.

RESULTS

Histopathology injuries after Iodixanol administration in both diseased mouse models.

H&E staining showed no significant histopathological damages after Iodixanol administration in both diseased mouse models. Our results of H&E staining (Figure1) and histology scoring demonstrated that all the db/db and eNOS mice had no to minimal injury, with score 0. There was no significant difference between control groups of db/db and eNOS mice and the treatment groups of the mice treated with Iodixanol. There was no evidence of CIN

in db/db and eNOS mice.

Ki67 expression after Iodixanol administration in both diseased models.

Our biochemistry results (Figure2, Figure3) revealed that Iodixanol did not add any further damage to the kidneys as there was no increase of Ki67 expression after Iodixanol administration, although Ki67 in db/db and eNOS mice had a higher background level as compared to normal C57BL/6J mice.

DISCUSSION

Although there are reports of clinical studies indicating that diabetes mellitus and hypertension are risk factors for CIN[9-11] , the causal association between diabetes mellitus/hypertension and contrast media and CIN remains unclear. Most patients with diabetes mellitus and/or hypertension, who develop CIN following intravenous contrast administration, may also have the other problems such as nephritis, dehydration, aging, etc., which may contribute to CIN as well. To investigate the contributions of diabetes mellitus and hypertension as risk factors for CIN development, we use db/db and eNOS knockout mice, which are accepted as diabetes mellitus and hypertension animals models without any other underline diseases.

Severed and pronounced histopathology alterations of kidneys were observed in animal models that developed CIN[12,13], but our *in vivo* studies failed to demonstrate histological evidence for any kidney injuries induced by Iodixanol at different doses in db/db mice or eNOS knockout mice at the ages of 8 weeks. A more sensitive parameter, Ki67 was also investigated in this study. Ki67 is a cell nuclear protein and a cell proliferation marker. Since kidney is a non-proliferative organ, Ki67 positive cell level is very low in healthy kidneys. Renal proliferation increases when kidney is subjected to damages or diseases. Therefore, Ki67 is considered a sensitive marker of kidney injuries.

Assessment of the Ki67 expression level has become a routine tool to evaluate kidney injuries or diseases[14]. In our study, although increased Ki67 was observed in db/db and hypertension mice as a baseline injury, additional Ki67 expression was detected following Iodixanol injection.

Our study was based on strictly designed animal assays. The conditions of the animals were controlled. There were no any other diseases, except diabetes mellitus and hypertension, in studied animals, and we used youngmice to minimize the renal effects of aging. Our data showed no significant injury caused by Iodixanol even in the animal with underline diabetes or hypertension. To interpret the results, we think the possible explanations are: 1. Iodixanol, as a contrast agent with lower renal toxicity, may not cause CIN in young animal models; 2. Mouse may be resistant to CIN even if diabetes or hypertension is pre-existed.

Further investigations need to be performed. Our preliminary results indicate that young db/db and eNOS mice are not good animal models for CIN studies. To investigate whether Iodixnaol is safer than other contrast agents, it is necessary to compare Iodixnaol with other contrast agents.

CONCLUSIONS

Iodixanol failed to induce CIN in diabetes mellitus and hypertension mice modelsbased on histological and Ki67 examinations. To study the mechanisms of CIN, Iodixanol alone may not be toxic enough to create a research animal model. Young db/db and eNOS mice may not be ideal animals for the studies on CIN.

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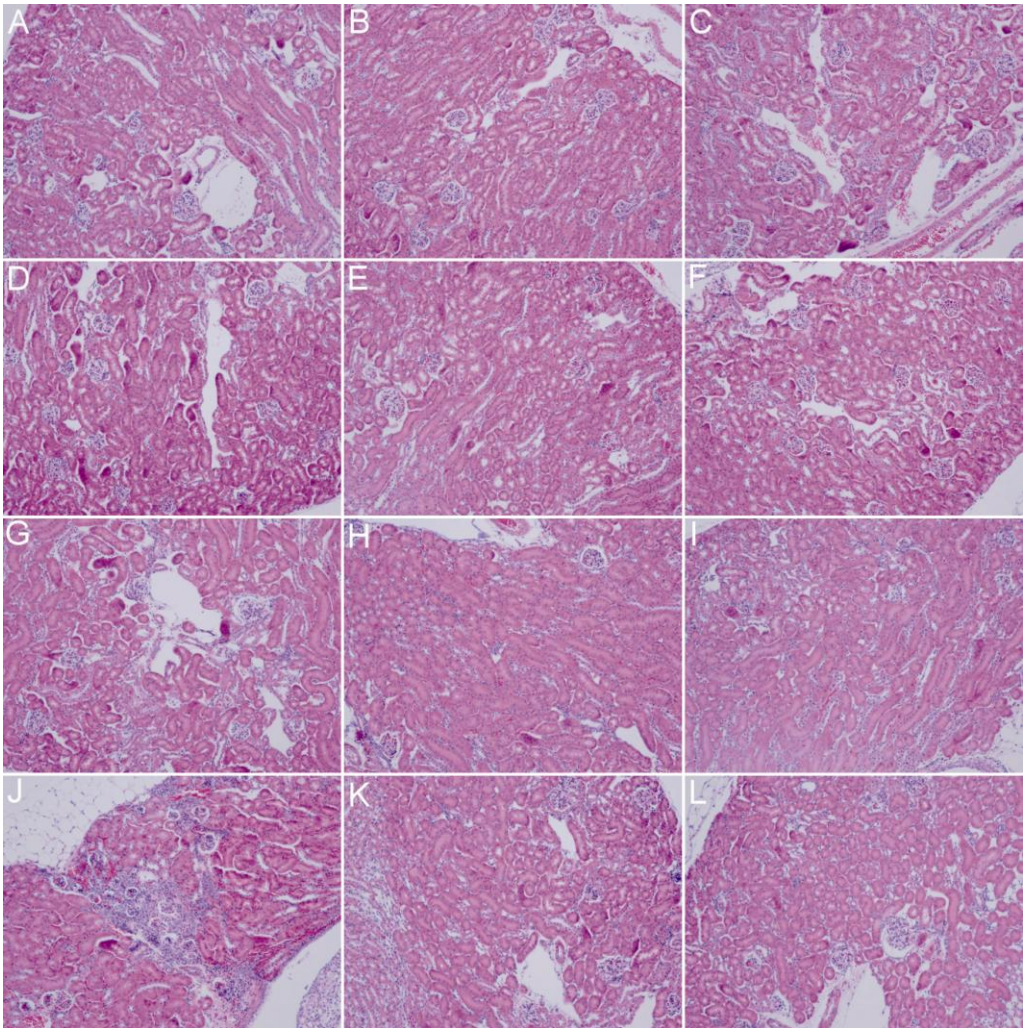

Figure 1.Representative images of formalin-fixed, H&E-stained kidney histology sections of PBS-treated versus Iodixanol treated mice (n=3). Db/db mice: A, PBS control, day3. B, 0.75 gI/kg Iodixnaol, day3. C,2.75 gI/kg

Iodixnaol, day3. D, PBS control, day 7. E, 0.75 gI/kg Iodixnaol, day7. F, 2.75 gI/kg Iodixnaol, day7.eNOS knockout mice: G, PBS control, day3. H, 0.75 gI/kg Iodixnaol, day3. I,2.75 gI/kg Iodixnaol, day3. J, PBS control, day 7. K, 0.75 gI/kg Iodixnaol, day7. L, 2.75 gI/kg Iodixnaol, day7.

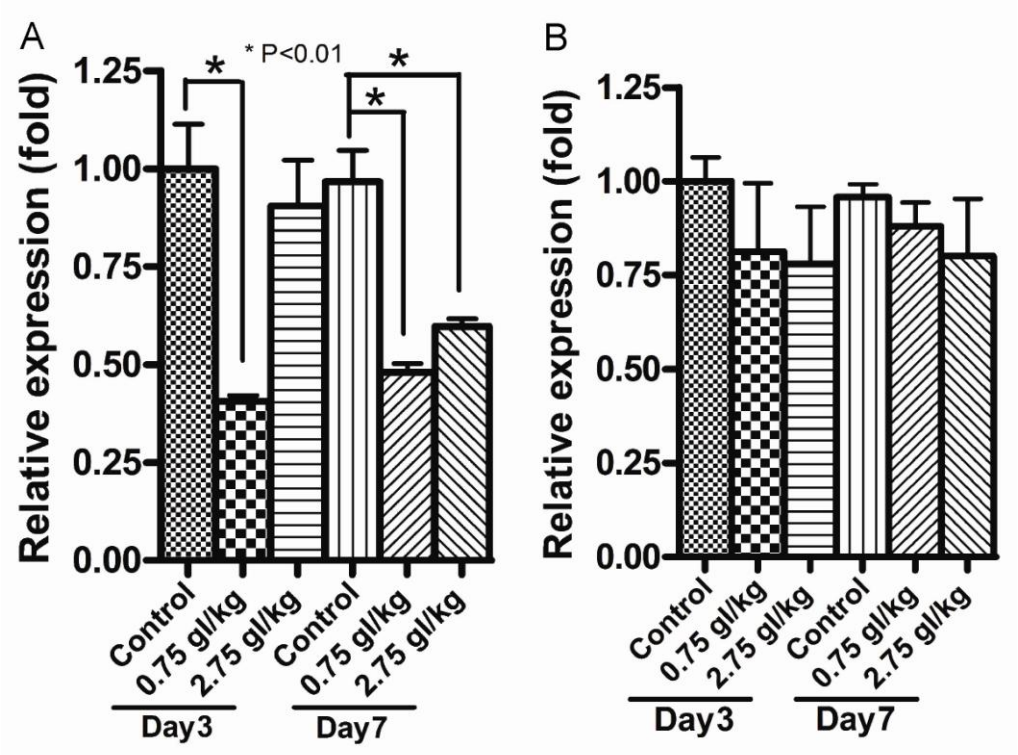

Figure 2. Ki67 mRNA level in PBS-treated versus Iodixanol treated mice (n=3). A, db/db mice. B, eNOS knockout mice.

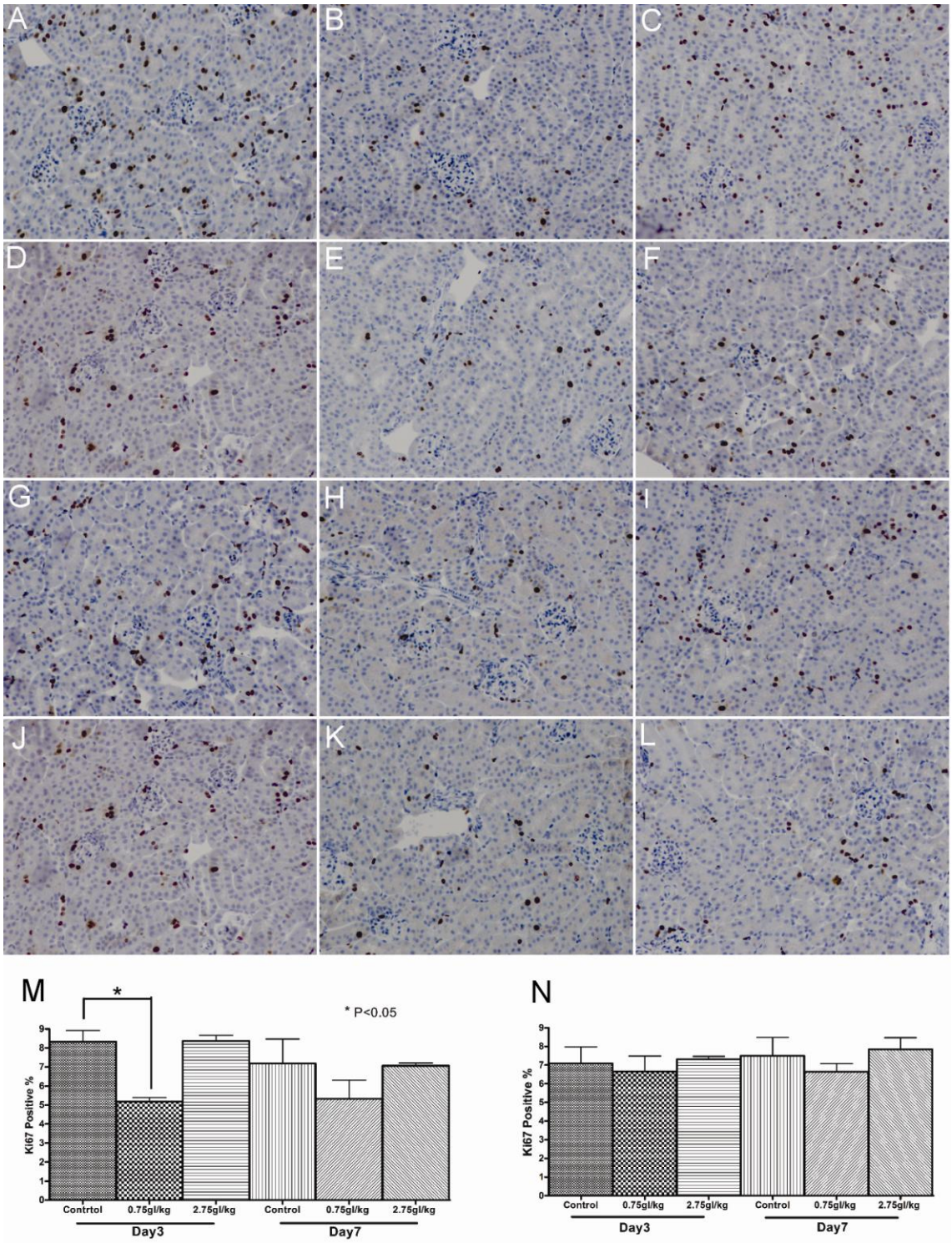

Figure 3. Representative images of formalin-fixed, Ki67 IHC results of kidney histology sections in PBS-treated versus Iodixanol treated mice (n=3). Db/db mice: A, PBS control, day3. B, 0.75 gI/kg Iodixnaol, day3. C,2.75 gI/kg Iodixnaol, day3. D, PBS control, day 7. E, 0.75 gI/kg Iodixnaol, day7. F, 2.75 gI/kg Iodixnaol, day7. eNOS knockout mice: G, PBS control, day3. H, 0.75 gI/kg Iodixnaol, day3. I,2.75 gI/kg Iodixnaol, day3. J, PBS control, day 7. K, 0.75 gI/kg Iodixnaol, day7. L, 2.75 gI/kg Iodixnaol, day7. M, db/db mice Ki67 expression level calculated according to Ki67 IHC results. N, eNOS knockout mice Ki67 expression level calculated according to Ki67 IHC results.