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New Nephron Development in Goldfish (*Carassius auratus*) Kidneys Following Repeated Gentamicin-Induced Nephrotoxicosis

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Renal development in mammalian kidneys can only be studied in embryonic animals. Hence, research in this area is hampered by the need to maintain pregnant animals and by the small size of the embryonic kidney. Here, we describe a goldfish (*Carassius auratus*) model for studying renal repair and nephron development in an adult animal. Previous studies have indicated that chemically induced nephrotoxicosis in goldfish is followed by new nephron development. We tested the hypothesis that new nephron development is not a one-time only event and, thus, will occur after repeated nephrotoxic events. We used repeated injections of gentamicin (50 mg/kg of body weight), a nephrotoxic antibiotic, which has been used as a model nephrotoxicant to study renal repair. Fish were allowed either a recovery period of 9 or 24 weeks between injections. In both experiments, new nephrons developed after each injection of gentamicin, supporting our hypothesis. Nephron development occurring after a 9-week recovery period was similar to development observed after a 24-week recovery period; therefore, the shorter experimental paradigm appears sufficient and can save time and money. Future research using this fish nephrogenesis model may identify the genes responsible for nephron neogenesis. Such information is a prerequisite for developing alternative renal replacement therapies based on the induction of de novo nephrogenesis in diseased kidneys.

Kidney diseases affect more than three million people every year, with many suffering from end-stage renal disease (1). A clear understanding of the kidney's response to renal injury and its repair capacity is the first step to developing alternative therapies to renal failure. A number of mammalian models have been used to study renal injury, repair, and development (2-5). Mammalian models of nephron development require maintenance of pregnant animals and harvesting minute quantities of embryonic renal tissue. This is due to the fact that once nephrogenesis is complete in the mammalian embryo or neonate, the kidneys are incapable of developing new nephrons. Recent studies have indicated that fish can develop new nephrons after exposure to acute doses of nephrotoxic agents (6-9). This nephron neogenic response differs from the typical mammalian repair response (called renal regeneration) to nephrotoxicants and may offer unique opportunities to study kidney development and repair that are not afforded by mammalian systems.

Kidney development in fish and mammals is similar; both are characterized by formation of nephrogenic renal vesicles from a solid mass of cells in the intermediate mesoderm (10-13). As the vesicle grows and develops a lumen, it forms a C-shape, which bends and becomes S-shaped. One end of the S-shaped vesicle then differentiates to form the glomerulus while the other end elongates forming the primary tubule, which then fuses with a collecting duct to complete the nephron. Following injury induced by exposure to nephrotoxicants such as gentamicin, mammalian kidneys repair injured nephrons by re-population of the tubular epithelium. Surviving epithelial cells divide and migrate along the denuded basement membrane (14-16). Importantly, nephron repair can only occur if some epithelial cells survive and the basement membrane remains intact. In cases where injury is too severe and the nephron cannot repair itself, it is destroyed. If a large number of nephrons are damaged beyond repair, the animal succumbs to renal failure.

Similar to mammalian kidneys, goldfish kidneys can repair injured nephrons by re-population of the basement membrane with epithelial cells (7). In addition, goldfish kidneys are also capable of generating entirely new, distinct nephrons. Even under conditions of severe renal injury, where mammalian kidneys would be incapable of repair, goldfish can generate new nephrons. This phenomenon is unique to fish and has been documented in several species, including goldfish (6), trout (17), tilapia (8), and toadfish (9), in response to nephrotoxicants with differing mechanisms of toxicity.

The goldfish kidney, therefore, provides a utilitarian model for studying nephron development. This model allows examination of adult and developing nephrons in the same kidney. The kidneys are also substantially larger than those of embryonic animals, providing more tissue for analysis. Although studies have indicated that new nephrons develop following a single nephrotoxicant exposure, it is unknown whether this is a one-time-only response or a repeatable process. The former would perhaps indicate presence of embryonic remnants, whereas the latter would represent inducible nephron development. A laboratory model capable of inducible nephron development would provide a unique means of studying development and repair of nephrons. The purpose of the study reported here was to determine whether goldfish are capable of producing new nephrons following sequential nephrotoxic gentamicin injections.

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Materials and Methods

Animals: Goldfish (*Carassius auratus*), between 4 and 6 cm long of both sexes, were obtained from a local hatchery. Fish were maintained in flow-through 200-L polyethylene tanks with activated carbon filtered water. Water quality of the laboratory system water was routinely monitored for temperature(20 ± 2°C, pH (7.4 ± 0.2), salinity (< 0.1 g/L), nitrite (0 to 20 μg/L), and hardness (80 to 85 mg/L as $CaCO_3$). Lighting was maintained by use of a 16:8 light:dark photoperiod. Fish were fed trout grower diet (38% protein) (Ziegler Bros., Inc., Gardners, Pa.) three times per week except for the first seven days following the gentamicin or placebo injection. Results of previous experiments had indicated marked anorexia in gentamicin-injected fish for the first seven to ten days following injection (18); thus, food was withheld from experimental and control groups. Experimental fish were given gentamicin (50 mg/kg of body weight) as intraperitoneal (IP) injections. Control fish were given IP injections of an equal volume of sterilized water per weight.

Study design: On the basis of results of a previous study (18) and preliminary experiments, we conducted two exposure/recovery experiments that differed in duration of recovery time before subsequent injections. Previous studies had indicated that the optimal time frame in which to identify developing nephrons is approximately one month following injection In a preliminary experiment, fish were given a second gentamicin injection 3 weeks after the first injection, during the period of active nephron neogenesis. The immature developing nephrons did not have signs of damage 1 week following the injection. If the second injection was given 6 weeks after the first, a moderate degree of necrosis was noted in the maturing new nephrons. Therefore, to allow ample time for the neogenic nephrons to develop into adult nephrons, we designed two experiments. The first experiment involved 9-week intervals between three separate injections. The second experiment had 24-week intervals between two injections. By 9 weeks following the first injection, the new nephrons appeared morphologically mature, but to be certain that we had definite, adult nephrons, we conducted an experiment that allowed a 24-week recovery period from the injection.

A total of 99 fish were used: experiment 1 (9 weeks) used 25 gentamicin-treated fish and 8 controls; experiment 2 (24 weeks) used 47 gentamicin-treated additional fish to verify necrosis and recovery. In all experiments, two fish were sampled prior to the first injection to determine baseline numbers of developing nephrons. Previous studies indicated that control fish usually have fewer than one developing nephron per square millimeter of kidney section. Further, two or three experimental fish were sampled three days following injection each (to verify necrosis), one day prior to each repeat injection (to verify that the kidneys had fully recovered), and one month after each repeat injection (to count the developing nephrons). For both experiments, controls included fish injected with sterile water only and fish injected first with gentamicin followed by sterile water injections for the repeat injections.

Pathologic examination: Sampled fish were anesthetized with MS222 (Crescent Research Chemicals, Phoenix, Ariz.) and decapitated. The kidneys were removed, cut into sections, and fixed in 10% phosphate buffered formalin. After 24 h fixation, kidney sections were embedded in paraffin, mounted on a slide, and stained with hematoxylin and eosin. Developing nephrons were counted by two pathologists, and the numbers were averaged. New nephrons in fish resemble mammalian developing nephrons and are characterized as small, basophilic cell clusters with a cavity, indicative of a newly forming lumen (Figs. 1 and 2). In addition, developing nephrons are associated with the presence of immature developing glomeruli. Alternatively, mature nephrons are eosinophilic, have a more pronounced lumen, and over time, become larger and more elongated (6).

Repair of the pre-existing injured nephrons is easily identified in mammalian and fish kidneys. This response is morphologically different from that of nephron neogenesis. The injured pre-existing nephrons are first identified in tissue sections by a denuded basement membrane with necrotic cells in the tubule lumen. During the first week following the injection, a flattened basophilic epithelium appears on the basement membrane. The tubule lumens appear dilated because the normal columnar epithelium has sloughed and is being replaced by the regenerating cells, which are squamous in appearance. These cells eventually become cuboidal and columnar. New nephrons, in the fish, arise de novo, and recapitulate the normal developmental stages seen in larval/embryonic nephron development. They arise from basophilic cell clusters located in the interstitium, which enlarge, form lumens, and eventually elongate (10-13, 19). These devel-

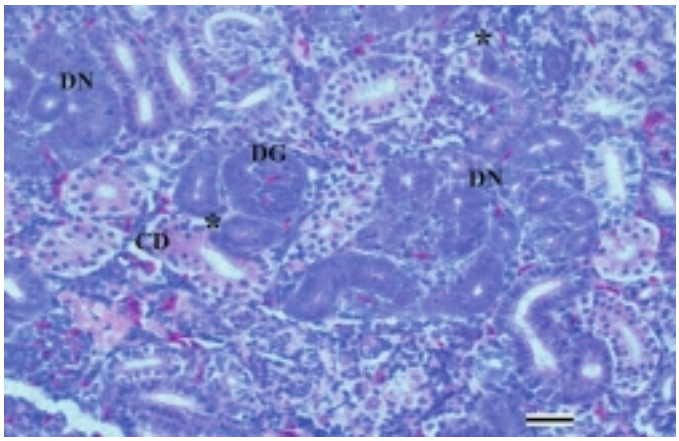

Figure 1. Photomicrograph of several developing nephrons (DN), which appear darker (basophilic) and denser, some with very little lumen showing. Mature nephrons are typically characterized by a larger lumen, distinct cells with nuclei and are generally lighter (eosinophilic) in appearance. A developing glomerulus (DG) is also evident. The developing nephrons penetrate (*) into the existing collecting ducts (CD). H&E stain; bar = 50 μm.

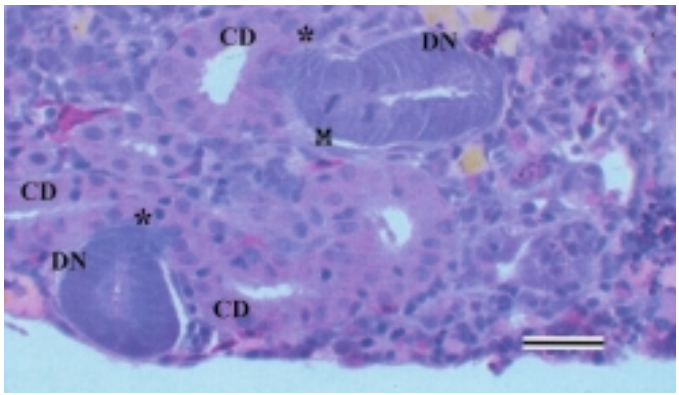

Figure 2. Higher magnification of DN penetrating (*) into collecting ducts (CD). Notice the mitotic figures in one of the DN (M). H&E stain; bar = 50 μm.

oping nephrons form small basophilic clumps of tiny tubules with small diameters. These basophilic clusters remain distinct for 2 to 4 weeks after the injection. As they mature about 5 to 6 weeks after the injection, the tubular epithelium becomes more eosinophilic, the tubule diameters enlarge, and the new nephrons' appearance is more like that of the fully mature nephron. Slight differences in tubule diameter and degree of eosinophilia can, however, still be recognized by the experienced pathologist.

The number of developing nephrons per kidney section, at 3 to 4 weeks after the injection, was normalized to kidney section area. Slides were digitally scanned and area was determined conducting image analysis, using NIH Image (20). A section of metric ruler was scanned along with each slide so that NIH Image could be accurately calibrated.

Analysis of data: Numbers of new nephrons/area were ranked on the basis of pathologic significance. Strong responders included kidneys with > 3 developing nephrons/mm^2, and were given a rank of 4; moderate responders, 1 to 2 developing nephrons/mm^2 were assigned a rank of 2; and non-responders and fewer than 0.99 developing nephrons/mm^2 were assigned a rank of 0. Resulting data were then analyzed using analysis of variance with alpha at 0.05.

The University of Maryland School of Medicine Institutional Animal Care and Use Committee approved all animal studies.

Results

Fish injected with gentamicin developed kidney tubule necrosis three days after injection of gentamicin. The nephrons had many dead cells in the lumen, with intact basement membranes (Fig. 1). Fish injected with gentamicin and sampled prior to the repeat injections had mature nephrons indicating complete recovery from the gentamicin-induced toxicosis. Fish kidneys sampled one month after the gentamicin injections (initial or repeated) contained numerous developing nephrons (Figs. 2 and 3). These nephrons had the same appearance as those described in previous studies (6, 17, 18).

Fish in the 9-week recovery experiment had the highest number of developing nephrons one month after the injection (Fig. 4). Fish given three injections and sampled one month after the third injection had significantly greater numbers of developing nephrons, compared with control fish that had received three injections of sterilized water.

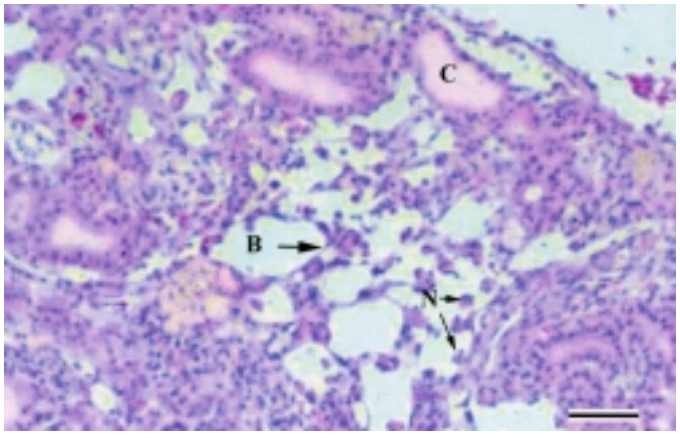

Figure 3. Photomicrograph of a section of injured nephrons from a goldfish three days after injection with 50 mg of gentamicin/kg of body weight. The denuded basement membrane (B) is intact. Some necrotic cells (N) are located within the tubule lumen. Casts (C) can be seen in the distal tubules. H&E stain; bar = 25 μm.

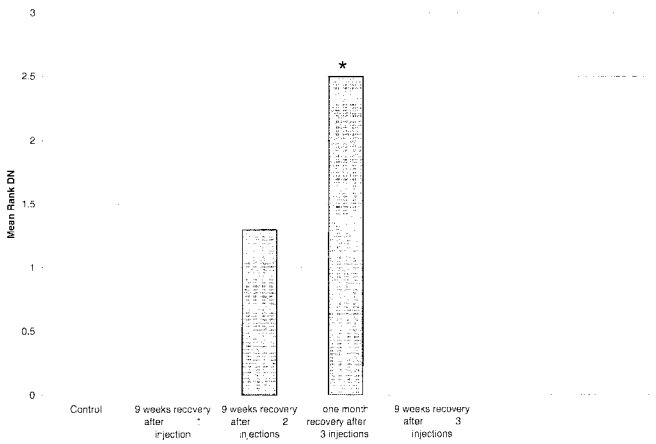

Figure 4. Developing nephrons after repeated gentamicin-induced nephrotoxicosis with nine week recovery periods between injections. Star (*) indicates significantly different from control on the basis of results of analysis of variance (α = 0.05) of ranked new nephrons.

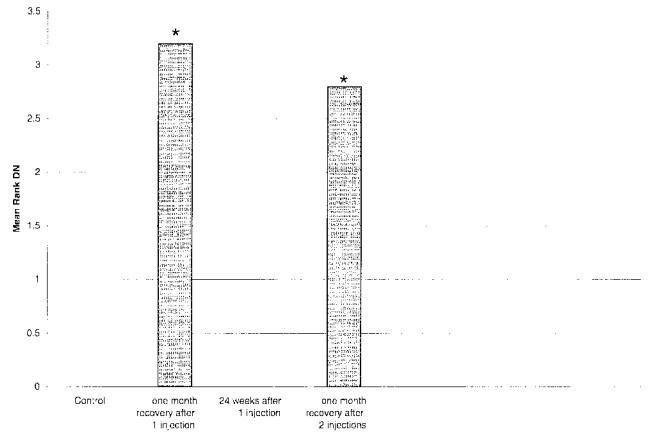

Figure 5. Developing nephron after repeated gentamicin-induced nephrotoxicosis with 24-week recovery periods between injections. Star (*) indicates significantly different from control on the basis of results of analysis of variance (α = 0.05) of ranked new nephrons.

The results from the 24-week recovery experiment indicated significantly more developing nephrons one month after the first and one month after the second injection, compared with values for all controls (Fig. 5).

Discussion

The results indicate that goldfish kidneys are capable of developing new nephrons after repeated gentamicin-induced nephrotoxicosis. The data suggest that goldfish given a 9-week recovery between injections respond similarly to fish with a 24-week recovery period. Future experiments could be conducted with 9-week recovery periods, which would be more cost effective and less labor intensive. Results of this study indicate that nephron neogenesis in goldfish is a repeatable process and, therefore, most probably is an inducible event.

Although the regenerative capacity of goldfish nephrons has implications for use in human health and renal biology, identification of developing nephrons in naturally occurring teleosts may also be a valuable biomarker of environmental nephrotoxicants.

Cormier and co-workers (21) reported that tomcod (*Microgadus tomcod*) from the Hudson River had increased numbers of developing nephrons and basophilic clusters relative to those in samples from the less contaminated Saco and Roval Rivers. They found a similar relationship for bullheads (*Ictalurus nebulosus*) from the Cuyahoga River, compared with the less-contaminated Old Women Creek and Toussaint River. These results suggest that the presence of new nephrons in adult fish may serve to help identify habitats polluted by nephrotoxic agents. Data of this type can be used to identify and assess human health and ecological risks associated with contaminated habitats.

The repeat nature of nephron development suggests that genes responsible for nephron development remain inducible throughout the life of the fish. Unlike humans, fish have indeterminate growth whereby somatic growth continues until the individual dies. Under these circumstances it is likely that inductive mechanisms, such as those required for new nephron development, are in place and functional. Since goldfish continue to grow, inductive mechanisms would be called on to synthesize new nephrons or other structures. Preliminary studies using the differential display technique have identified differences in mRNA expression between fish treated with gentamicin and untreated fish (22). Future research using this fish nephrogenesis model may eventually identify the genetic mechanisms involved in nephrogenesis and the molecular basis for the lack of a neogenic response in mammalian kidneys. Such information will ultimately be useful in developing alternative renal replacement therapies.

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