Introduction

While some teleost fishes have no regeneration potency (Wagner & Misof 1992), the kidney regeneration through nephron neogenesis have been reported in several fishes such as *Oncorhynchus mykiss* (Cormier et al. 1995), *Ameiurus nebulosus* (Augusto et al. 1996), *Danio rerio* (Reimschuessel et al. 1996), *Oreochromis nilotica* (Reimschuessel 2001) and *Tetractenos hamiltoni* (Salice et al. 2001). Recently, the regeneration ability is reported in the caudal fin and the kidney of killifish, *Aphanius hormuzensis* (Zeinali & Motamedi 2017; Iranmanesh & Motamedi 2018).

Gentamicin is an aminoglycoside antibiotic, which causes iron release from renal mitochondria and forms an iron-gentamicin complex. This complex stimulates the appearance of free radicals and reactive oxygen species (ROS) (Kovacs 2012), and therefore causes nephritic damage (Reimschuessel et al. 1996; Reimschuessel 2001; Salice et al. 2001). Several studies demonstrated that the nephrotoxicity and ototoxicity are common side effects of gentamicin (Jeffrey et al. 1998; Wojciech et al. 2005; Selimoglu 2007; Huth et al. 2011). Moreover, vitamin C (ascorbic acid) is known for its antioxidant properties and associated with fish immunity (Roberts et al. 1995; Anbarasu & Chandran 2001) and improved resistance to stress and diseases (Durve & Lovell 1982; Navarré & Halver 1989; Verlhac et al. 1998; Montero et al.
Aphanius furcatus is an endemic killifish occurs in the extreme habitats characterized by low oxygen, hot sulphur-rich and high salt concentration in the Hormuzgan basin (Southern Iran) (Teimori et al. 2014). This species is often found sympatric with another endemic killifish A. hormuzensis in the Hormuzgan basin in this region (Teimori et al. 2018).

Recent studies indicated that regeneration of the caudal fin and kidney in the genus Aphanius occurs faster than the other traditional models such as zebrafish (Zeinali & Motamedi 2017; Iranmanesh & Motamedi 2018). Therefore, non-traditional model organisms such as members of the genus Aphanius can probably be suitable candidate to study regeneration phenomenon. To advance our knowledge on the regeneration ability in the genus Aphanius, we investigated the kidney regeneration process after renal damage induced by gentamicin in A. furcatus.

Drug-induced nephrotoxicity is an important cause of renal failure in mammals and the rate of renal dysfunction following amino-glycoside administration has been reported in previous studies (Baliga et al. 1997; Abdel Naim et al. 1999). In order to give the potential for the development of new therapeutic strategies in renal medicine, we investigated the kidney regeneration process after renal damage induced by gentamicin in A. furcatus. Besides this, the field of regenerative medicine is likely to benefit from understanding natural mechanisms of regeneration and the factors that influence the rate of regeneration. Therefore, we evaluated the possible role of vitamin C in toxicity reduction, and in the processes of kidney regeneration.

Materials and Methods

Laboratory experiments: The fish specimens were captured from the Shur River, Southern Iran and transferred to the laboratory, and kept in 60L aquariums for 20 days before the experiments for acclimatization. They fed three times a day with dry food that contains macronutrients, trace elements and vitamins. During the experiments, 20 percent of the aquariums water changed weekly. The fish specimens were maintained in freshwater at 25°C, pH 6.8-7, and 12:12 h (light: dark) photoperiod. Experiments were performed with the permission of the institutional ethics committee and approved by the Ethics Committee of the Biology Department of Shahid Bahonar University of Kerman (SBUK-139697).

Control and treatment groups. In this study, 24 specimens (3.5-4.0cm in total length) were used and grouped as follow: Group-I contains two specimens and considered as control group with 7.5µl of normal phosphate-buffered saline-PBS injection sampled for 5 days post injection (dpi); Group-II contains 12 specimens with 10µg/g b.w gentamicin injection sampled for 10 dpi; Group-III includes two specimens with 60µg/g b.w vitamin C injection sampled for 5 dpi; and Group-IV includes 10 specimens with the injection of 10µg/g b.w gentamicin + 60µg/g b.w vitamin C sampled for 5 dpi.

Treatment process. For the treatment, we applied intraperitoneal injection using ultrafin extreme syringe (Pic solution-10µL syringe). After anesthetisation with 180 ppm of aqueous clove (Eugenia caryophyllata) solution (Zeinali & Motamedi 2017), 7.5µL of phosphate-buffered saline-PBS was injected for group-I as control, and 10µg/g body weight of gentamicin (Caspian Tamin-Pharmaceutical) was injected for group-II.

In addition, two specimens of group-III were treated only with 60µg/g body weight vitamin C. Group-IV is treated with 10µg/g body weight gentamicin. In addition, they received totally 60µg/g b.w of vitamin C in three different days within the experiments (20µg/g vitamin C injected on each day). They received vitamin C on a day before gentamicin injection and in the second and fourth days after gentamicin injection.

Histological procedure. In order to collect the kidney tissue of the experimental groups, the fish euthanized
by administering an overdose of clove oil dissolved in water. Thereafter, the ventral part of fish was dissected carefully with a stereomicroscope to collect the kidney tissues.

During the toxicity phase of renal system, the kidney tissues of group-I, III and IV were sampled within 10 hours post injection (hpi) to 5 days post injection (dpi) (Figs. 1A, 2). Also, the kidney tissues of group-II sampled daily for 10 dpi through the whole regeneration process (Fig. 1B-J).

The standard histological method described by Mochizuki et al. (2005) was used to prepare kidney tissue sections. In brief, tissue samples from kidney of both groups in the above mentioned days were fixed in 10% formaldehyde for 24 hours. Thereafter, the tissues were subjected to routine histological techniques; dehydration, clearing, infiltrations, and paraffin embedding (Behmer et al. 1976). The tissue sections (3μm width) were rapidly rehydrated in xylene for five minutes, and the decreasing concentration of ethanol (100, 90, 70, and 50%) were used for one minute in each solution. The tissue sections were stained with hematoxylin and eosin. Afterward, they were washed with water and dehydrated in increasing concentration of alcohols, cleared in xylene for five minutes and mounted. The Olympus CH2 microscope and Nikon DXM 1200 digital camera used to take photographs.

Results
Kidney regeneration in *Aphanius furcatus*: The normal histology of kidney in *A. furcatus* was evaluated in the specimens of group-I (Fig. 1A). No difference was observed between tissue sections of 1 dpi and 5 dpi. Our observation on the tissue sections of group-I indicates that the kidney nephrons, renal corpuscle (glomerulus within Bowman's capsule), proximal, distal tubules and collecting ducts are well-organized (Fig. 1A). Moreover, the fish kidney along with spleen contained hematopoietic tissue (Fig. 1A).

We also examined the kidney regeneration process of *A. furcatus* after damage induced by gentamicin in group-II (Fig. 1B-J). The nephritic damage at sub-lethal dose of gentamicin caused detachment of tubular epithelia from basement membrane in 10 hpi. Moreover, blood clots detected in the lumen of some damaged nephritic tubule (Fig. 1B). The severest damage in fish kidney tubes appeared within 2-4 dpi (Figs. 1C-E). Most of tubular epithelial cell detached from basement membrane and on 3-4 dpi several cysts were formed and cell debris was observed in the lumen of kidney tubes (Figs. 1E-F). We considered these times as a toxicity effect phase of gentamicin of *A. furcatus* kidney tissue (Fig. 1B-E). After this phase, the kidney tissue regeneration started and in the beginning of 5 dpi the mesenchymal cells aggregates were found in the lumen of proximal and distal tubules (Fig. 1F). This aggregation also detected in 6 dpi, which indicates the initiation of renal regeneration in *A. furcatus* (Fig. 1G). The formation of basophilic cellular aggregates which form a new nephron detected on 8 dpi (Fig. 1H). This process named as a nephron neogenesis (Fig. 1H). The kidney regeneration is completed within 9-10 dpi and our histological sections showed completely repaired kidney tissue in 10 dpi (Fig. 1I-J).

**Evaluation of vitamin C in toxicity reduction:** To evaluate the effect of vitamin C on kidney tissue histology, we established group-III and examined the kidney tissue sections in 1 dpi and 5 dpi (Fig. 2A). Our observation showed that kidney tissue sections have similar appearance to the tissue sections in group-I (Fig. 1A). The histopathological observation revealed that saline treated group (group-I) as well as the vitamin C treated group (group-III) has well organized nephrons in their kidney tissues.

In order to evaluate the effect of vitamin C on the reduction of gentamicin renal toxicity, we established group-IV and evaluated its effect within 10 hpi to 5 dpi; during the gentamicin toxicity phase and initiation of cell aggregation (Fig. 2B-F). Our results indicate that the damage signs of gentamicin are postponed in group-IV that received vitamin C (Fig. 2B-F). In compare to group-II (Fig. 1B-J), the tubular epithelia integrity is still preserved in the kidney of
group-IV in 10 hpi and 2 dpi (Fig. 2B-C). The histological sections in 3 dpi showed blood clot in the lumen of nephritic tubule which can be sign of gentamicin damage initiation (Fig. 2D). First detection of epithelial cell detachment from basement membrane happened in 4 dpi (Fig. 2E). A day after, the kidney regeneration started with migration of mesenchymal cell in the lumen of nephritic tubules (Fig. 2F), from this step, the kidney regeneration was the same as only-gentamicin treated
Motamedi et al. - Kidney regeneration in Aphanius furcatus

Discussion
Gentamicin is an aminoglycoside antibiotic that is widely used to manage the gram negative infections. However, its fraction is reabsorbed in the proximal convoluted tubule cells and causes damage site and leads to nephrotoxicity in human (Wiland & Szechcinski 2003). The mechanism by which the gentamicin leads to kidney damage is increasing of the reactive oxygen species (ROS) and free radicals production (Kovacs 2012; Kamel & Abdel Fadil 2015).

Gentamicin binds to the megalin receptor and accumulated in the epithelial cells of the renal tubular lumen which leads to the nephritic damage (Nagai et al. 2001; Nagai 2006). The previous studies on fishes such as goldfish, zebrafish and medaka indicated that gentamicin caused damage to renal nephrons, and then regeneration is done in the kidney by formation of new nephron (nephron neogenesis) (Cormier et al. 1995; Augusto et al. 1996; Reimschuessel et al. 1996; Reimschuessel 2001; Salice et al. 2001).

In this study, the role of ROS in gentamicin induced nephrotoxicity was assessed by administration of antioxidant vitamins C and further evaluation of histological changes, and provided additional new data on the kidney regeneration of another killifish A. furcatus. Our findings in this study showed that like some other teleosts, A. furcatus has conserve regeneration ability in kidney tissue and in severe renal tissue damage; it can produce new nephrons by formation of stem cell aggregates (nephron neogenesis). Considering our results, in A. furcatus the renal regeneration after damaging by gentamicin happened within 10 days as previous study showed for A. hormuzensis (Iranmanesh & Motamedi 2018). Therefore, it can be concluded that both species have chronologically the same period of renal regeneration.

Fig. 2. Histological staining of Aphanius furcatus kidney regeneration after vitamin C and gentamicin treatment: (A) group-III: (B-F) group-IV. (A) 10 hours post injection with no sign of damage; (B) 2 dpi, cell derbies observed in the lumen of nephritic tubule; (C) 3 dpi, blood clot detected in the lumen of nephritic tubule; (D) 4 dpi, arrows indicate detached epithelial cells from basement membrane; Hematopoietic tissue (H), nephritic tubules (Nt), Cell debris (Cd), Blood clot (Bc), Tubular epithelium (Te), Me (Mesenchyme cell) (Scale bar=25μm).
In our further examination, we evaluated the preventing possibility of gentamicin-induced oxidative stress by supplementing the antioxidant therapy. We selected vitamin C for this purpose because it has already been documented that vitamin C has antioxidant properties and correlate with fish resistance to stress and diseases immunity (Roberts et al. 1995; Anbarasu & Chandran 2001; Durve & Lovell 1982; Navarre & Halver 1989; Montero et al. 1999). Besides this, vitamin C is the cheapest water-soluble antioxidant and act as scavenger of peroxyl radicals and can trap notorious hydroxyl radical (Wayner et al. 1986).

Considering the histological results in gentamicin-induced oxidative stress (group-II), we could state that the most severe damage in the kidney tissue of *A. furcatus* occurred within 1-4 dpi. However, in the *A. furcatus* specimens that antioxidant therapy supplied with vitamin C (group-IV), damage effect of gentamicin has remarkably repressed (Fig. 2 B-E).

Based on our findings, it can be stated that administration of vitamin C is effective in reduction of gentamicin-induced nephrotoxicity in the studied spices in genus *Aphanius*. However, it cannot affect the needed time to complete regeneration procedure. Therefore, antioxidants therapy for prevention/reduction of gentamicin is useful. It should be noted that more extensive studies such as antioxidant enzyme levels measurement require for substantiating the benefits of antioxidants in these situations.

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مقاله پژوهشی

درمان کلیه در کپورماهی دندان بدون فلس (Aphanius furcatus) ماهیان استخوانی عالی: کپورماهیان دندان دار پس از آسیب الکه شده با جنتامایسین و ارزیابی ویتامین C در کاهش سمیت

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چکیده: جنتامایسین به عنوان یک ماده اکسیدزاز فعال اکسیژن از طریق استرس اکسیداتیو منجر به آسیب کلیوی در برخی از گونه‌های ماهی‌دار می‌گردد. بررسی ترمیم کلیه در کپورماهی دندان‌دار با نفوذ جنتامایسین و ارزیابی ویتامین C با همبستگی با آنتی‌اکسیدان در کاهش سمیت و آسیب کلیوی انجام شد. تعداد 24 نمونه (با طول استاندارد 5/3-4 سانتی‌متر) به چهار گروه تقسیم شدند. در گروه اول دو عدد ماهی با 5/7 میلیلیتر آب نمک معمولی تیمار شدند. در گروه دوم 10 نمونه ماهی به میزان 75/10 میکروگرم آنتی‌اکسیدان گرفتند. در گروه سوم دو نمونه ماهی به میزان 60 میکروگرم ویتامین C تیمار شدند. و در گروه چهارم ده نمونه ماهی به میزان 10 میکروگرم جنتامایسین و 60 میکروگرم ویتامین C را با هم دریافت کرده و چهار ماه در طول دوره آسیب نفروی نمونه‌برداری شدند. نتایج نشان داد که جنتامایسین با تثبیت سطح ویتامین C در مدت زمان 100 ساعت بهبودی محور داشته و ویتامین C اثر غالب در کاهش سمیت جنتامایسین داشت. نتایج نشان داد که افزایش قدرت آنتی‌اکسیدانی جنتامایسین در روان داده و افزایش ترمیم کلیه ماهی با استفاده از ویتامین C کاهش سمیت جنتامایسین را بهبود بخشیده و در نهایت می‌تواند به افزایش مقاومت ویتامین C برابر با آنتی‌اکسیدان و کاهش سمیت جنتامایسین کمک کند.

کلمات کلیدی: نفوذ جنتامایسین، آنتی‌اکسیدان، سمیت کلیوی