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Apoptosis demonstration of the renal tubules after ischemia and reperfusion by acridine orange technique

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توصيف موت الخلية المبرمج في النبيبات الكلوية بعد الفاقة واعادة التروية

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الخلاصة

إن الفاقة وأعاده التروية ظاهره معقدة تؤدي إلى ضرر الخلية من خلال عمليه ثنائيه الطور حيث إن الفاقة تحدث جرح الانسجة بسبب نقصان أو فقدان كلي للطاقة الضرورية للمحافظة على التوازن البدني.

ان اعادة التروية تزيد من الضرر الخلوي عن طريق مختلف العمليات المحتملة مثل التفاعل الالتهابي وتحرير جذور الاوكسيجين الحرة.

إن ضرر الانسجة الناتج من الفاقة وأعاده التروية يؤدي إلى موت الخلية المرضي (التنكرز) وموت الخلية المبرمج في النماذج البشرية والنماذج التجريبية للفاقة الكلوية.

اقيمت هذه الدراسة لاظهار موت الخلية المرضي (التنكرز) وموت الخلية المبرمج في الانابيب الكلوية تحت تأثير الفاقه واعادة التروية باستخدام صبغة الاكردين المضيئة.

اخذ خمسة وعشرون نموذجا من ذكر الجرذ وقسمت إلى ستة مجاميع هي : مجموعة السيطرة، ومجموعة الفاقه لمدة 40 دقيقة،و مجاميع التروية (بعد ساعة، ثلاث ساعات و ست ساعات). حضرت النماذج للدراسة النسيجية وصبغت المقاطع بصبغة الاكريدين المضيئة لدراسة موت الخلية المبرمج وفحصت بواسطة المجهر المتأين.

النتائج اظهرت التغيير الطفيف بمجموعة الفاقه بعد 40 دقيقة بينما زاد الضرر في النسيج والخلايا بشكل واضح في مجاميع التروية ووصوله الى قمته في مجموعة 1 و 3 ساعة من التروية ووضوح الموت المرضي والموت المبرمج عليها.

في مجموعة التروية بعد 6 ساعات لوحظ عودة النسيج للشفاء مع وجود شظايا النواة (الموت المبرمج) في خلايا النبيبات الكلوية.

من خلال النتائج المبينة نستنتج ظهور وزيادة الموت المرضي والموت المبرمج في مرحلة التروية واكثر من مرحلة الفاقه ،كذلك اثبات كفاءة صبغة الاكريدين المضيئة في اظهارالموت المبرمج والتغيرات الخلوية.

ABSTRACT

Ischemia – reperfusion injury is a complex phenomenon that results in cell damage through a biphasic process. Ischemia initiates the injury by a decrease or complete loss of energy supply needed to maintain homeostasis.

Reperfusion increases cellular damage by a variety of proposed mechanisms such as inflammatory reaction and release of oxygen free radicals. I-R resulting in both pathological cell death (necrosis) and programmed cell death (apoptosis) in human and experiments

This study performed to demonstrate the apoptosis and necrosis the renal tubules under the effect of I/R in by florescent acridine orange.

Twenty five male rats were divided into six groups: control group, ischemia group (40 minute clamping of renal artery) and ischemia – reperfusion group (the removal of renal clamping after 1, 3, and 6 hours).

The samples were prepared for histological technique and the sections stained with acridine orange and examined by florescent microscope.

The results observed mild changes in the ischemic group but the damage of the tissue and cells occurred in the reperfusion groups reaches the maximum effect after 1 and 3 hour, the necrosis and apoptosis were observed clearly.

After 6 hours reperfusion, emit orange fluorescence as evidence of nuclear fragmentation (apoptosis) in cells.

We concluded that the apoptosis and necrosis occurred in the reperfusion after 40 minutes ischemia; also the acridine orange stain is effective stain to investigate the apoptosis and the cellular changes.

INTRODUCTION

There is increasing evidence from animal studies that the kidneys in addition to other central organ systems are particularly sensitive to ischemia followed by reperfusion (1/R). Ischemia is the condition suffered by tissue and organs when deprived from nutrients and oxygen by interfering with blood flow which lead to cell death (1; 2).

Reperfusion injury refers to the tissue damage occurred when blood flow is restarted after an ischemic period. Kidney injury by ischemia and reperfusion manifest a variety of functional defects, prominent among which is impairment of tubular reabsorption of sodium and water. (3; 4)

I/R injury is a complex phenomenon that induces cell damage through a biphasic process. Ischemia starts the injury by deprivation of the energy needed to maintain ionic gradients and homeostasis which may lead to cellular dysfunction and death (5; 6).

Tissue injury resulting from hypoxic – ischemic insult can result in both pathological cell death (necrosis) and (apoptosis) programmed cell death. In humans and experimental models of renal ischemia; tubular cell in various nephron segments undergo (necrosis) and / or apoptotic cell death. Unlike apoptosis that occurs in normal and diseased states, necrosis is induced only when cells or tissues are exposed to severe and acute injury. (7).

Apoptosis and necrosis often occur at the same time in a wide variety of pathological conditions, in cultured cell exposed to physological activators, physical trauma and chemicals (8), and in settings of acute injury such as ischemia-reperfusion injury to the kidney (9).

The study performed for demonstration the apoptosis that occur due to I/R of kidney using the acridine orange and then viewed by florescent microscope.

MATERIALS and METHODS

Twenty five male rats *(Rattus norvegicus albinus*) with a body weight of 250-350 g and age over two months.

Renal ischemia – reperfusion injury model

At the start of the experiments rats were anesthetized with sodium pentobarbital (50 mg / kg) intraperitonially.

Ischemia was induced by clamping the right renal pedicle for a period of 40 minutes using a non traumatic vascular clamp through a midline abdominal incision. After clamp removal the right kidney was inspected for restoration of blood flow, the abdomen was closed and xylocain ointment was applied topically for postoperative pain management.

The animals were divided into 5 groups 5 animals in each group according to the following: control group, ischemic group, after 1 hour, after 3 hours, and the last after 6 hours reperfusion.

The tissue specimens were fixed in 10% formalin for 24 hours the dehydrated in the ascending ethanol alcohol concentration then clearing with xylene for 20 minutes then embedding with paraffin wax (10).

Acridine orange

Staining solution:

1g Acridine orange was dissolved in 1000ml D.W (stock solution). 10ml from this solution was taken & 40 ml of (0.1 M citric acid) with 2.5 ml of (0.3 M Na2 HPO4. 7H2O) was added to form (staining solution). And PH of stain solution was preserved at 2.5 then stored in dark bottle in refrigerator (11).

procedure:

Paraffin blocks were sectioned at 4 µ thickness by Shandon microtome and stained as follow:

1-Sections were dewaxed in xylene (10-15) minutes.

2-Sections were rehydrated in ethanol alcohol (99%, 90%, and 70%) then were passed to distilled water.
3- Slides were stained with Acridine orange for 5 minutes.

4- Slides were rinsed in deionized water & dry for few minutes.
5- Mounting with DPX mounting media.

Tissue sections stained with acridine orange were examined using the Olympus BX41 fluorescence microscope in the medical college, al-nahrain university.

RESULTS

Detection of tubular structural changes by acridine orange stain

Acridine orange is a metachromatic dye which differentially stains, double stranded (ds) and single stranded (ss) nucleic acids. When AO intercalates in the dsDNA it emits green fluorescence upon excitation. It emit red to orange fluorescence when intercalates with ssDNA.

Section of the kidney stained with AO and examined under fluorescence microscopy of the control rats showed normal tubules Fig. (1).

In addition, the nuclei emit green fluorescence; the emission of orange fluorescence of nuclear chromatin fragmentation was seen following 40 minutes of ischemia shown in Fig. (2).

Nuclear chromatin fragmentation with the appearance of apoptotic bodies was more evident after one hour which peaked after three hour following reperfusion shown in Fig. (3&4) respectively.

After six hours, renal tubules like the control group some nuclei still emit orange fluorescence as evidence of nuclear fragmentation (apoptosis) in cells shown in Fig. (5).

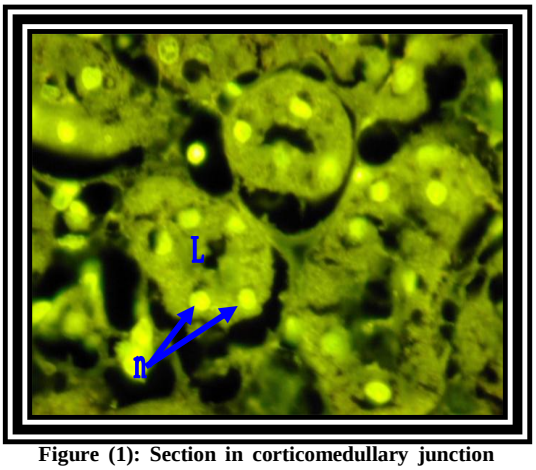

Figure (1): Section in corticomedullary junction of rat kidney in control group showing normal tubule (green fluorescence), normal nuclei (n) occluded lumen (L). AO (X1000)2.8

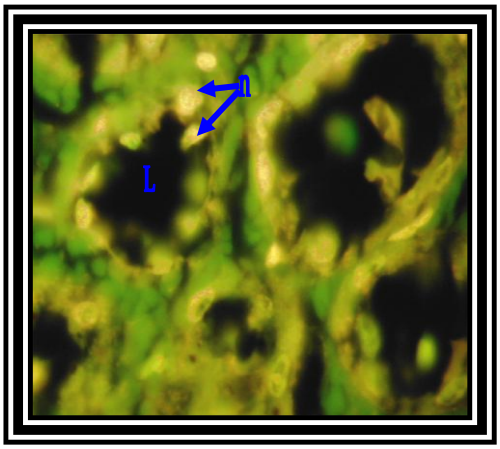

Figure (2): Section in corticomedullary junction of rat kidney in ischemia group showing (orange fluorescence), large lumen (L), and irregular crescent shape nuclei (n). AO (1000)2.8

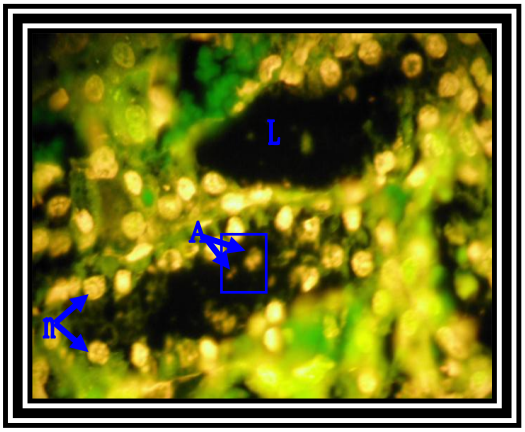

Figure (3): Section in corticomedullary junction of rat kidney after one hour reperfusion showing (orange flu.), large lumen (L), condensed nuclei (n), and Apoptotic body (A). AO(X1000)2.8

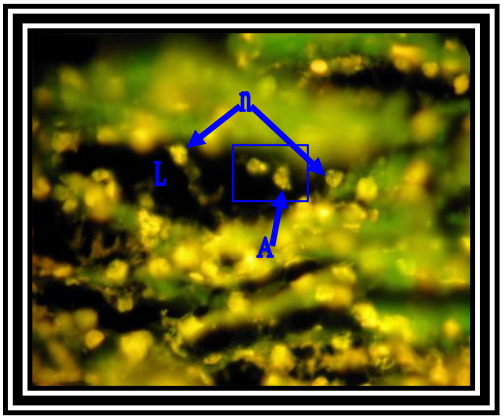

Figure (4): Section in corticomedullary junction of rat kidney after 3 hour reperfusion showing (orange flu.), large lumen (L), condensed nuclei (n), and apoptotic body (A). AO (1000)2.8

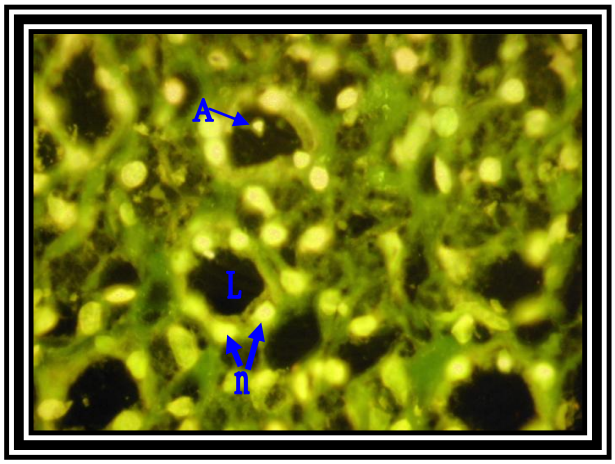

Figure (5): Section in corticomedullary junction of rat kidney after six hors reperfusion showing lumen (L), nuclei (n), and the apoptotic body (A) AO (1000)2.8

DISCUSSION

Healthy kidneys consumed relatively large amount of oxygen which is used to maintain oxidative phosphorylation and synthesis of ATP which is required for tubular reabsorption activity, during ischemia and reperfusion of the kidney, tubular cells are deprived of oxygen and substrates and exposed to accumulating potentially toxic metabolite, Thus, ATP depletion and cytoskeletal derangement are rapidly induced by ischemia which may resolve quickly during reperfusion, providing that the ischemic phase is not too severe. As the reperfusion phase restores the delivery of oxygen and substrates and removes the metabolic products, however reperfusion itself may introduce or amplify mechanisms for example ROS (Reactive oxygen species) and leukocytic dependent mechanisms that leads to cell injury (4).

In the present study, rat kidney was exposed to ischemia in vivo for a period of 40 minutes. This period of kidney ischemia was chosen in accordance with various studies exposing rat kidney to I/R in vivo (12; 13; 14).

Apoptotic and necrotic forms of cell death coexist in I/R of renal tissue. The relative contribution of the two modes of cell death after I/R insult depended on the severity of the injury and the level of ATP depletion (15). Previous studies reported that mild to moderate ATP depeletion induced apoptosis while severe ATP depletion resulted in necrosis. (4)

Furthermore the emission of orange fluorescence upon excitation of nucleus stained with acridine orange in contrast to the emission of green fluoresce in the control group, as an early evidence of chromatin condensation . It was reported that chromatin condensation is an early event of apoptosis and the condensed chromatin is much more sensitive to DNA denaturation than

normal chromatine (16). These findings are in line with various in vivo and in vitro reports showing that renal apoptosis after ischemia is induced by hypoxia (17) and ATP depletion (18).

A process of resolution and recovery in the cytoskeletal derangement in tubular cells was observed after six hours of reperfusion and take the semi appearance and the structures in the normal kidney.

These reasons of previous studies showed above interpreted the changes, necrosis and apoptosis that occurred during the ischemia for 40 minutes and the reperfusion for different times till six hours and the risks from the reperfusion and the damage that occurred also the study showed the efficiency of this acridine orange for demonstration of apoptosis in the histological studies.

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