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Beneficial effect of preconditioning on ischemia-reperfusion injury in the rat bladder in vivo

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Abstract

We investigated the effect of preconditioning on ischemia-reperfusion injury in the rat bladder. Rat abdominal aorta was clamped with a small clip to induce ischemia-reperfusion injury in the bladder. Twelve-week-old male SD rats were divided into three groups; sham-operated control (Cont), 30 minutes ischemia-60 minutes reperfusion (IR) and three times of 5 minutes ischemia and then 30 minutes ischemia-60 minutes reperfusion (PC) groups. The bladder functions were estimated by cystometric and functional studies. Contractile response curves to increasing concentrations of carbachol were constructed in the absence and presence of various concentrations of subtype selective muscarinic antagonists, i.e. atropine (non-selective) pirenzepine (M1 selective), methoctramine (M2 selective), and 4-DAMP (M1/M3 selective). We also measured tissue levels of malonaldehyde (MDA) and examined possible histological changes in these rat's bladders. Preconditioning partially prevented the reduction of bladder dysfunction induced by ischemia-reperfusion. Estimation of the pA2 values for atropine, pirenzepine, methoctramine, and 4-DAMP indicate that the carbachol-induced contractile response in

bladder dome is mediated through the M3 receptor subtype in all groups. The MDA concentration in the IR group was significantly larger than that of the control group, and preconditioning significantly reduced MDA production in the bladder. In histological studies, the ischemia-reperfusion with or without preconditioning caused infiltration of leukocytes and rupture of microcirculation in the regions of submucosa and smooth muscle without a corresponding sloughing of mucosal cells. Our data indicate that preconditioning has a beneficial effect on ischemia-reperfusion injury in the rat bladder.

Key words: urinary bladder, preconditioning, ischemia-reperfusion, muscarinic receptors

Introduction

The functions of the urinary bladder include urine storage and subsequent micturition. The urinary bladder requires an adequate supply of oxygen and nutrients via the circulation system in order to maintain homeostasis and proper function (Parekh et al., 2001). Both clinical and experimental evidences of ischemia and subsequent reperfusion injury have been reported in many tissues, including such as kidney, liver, stomach, and heart (Rauen et al., 1999). Ischemia and the following reperfusion of the bladder are observed in age-related disorders, urinary retention, atherosclerosis, vasospasm, embolization, and thrombosis (Parekh et al., 2001). Bladder overdistention occurs in patients with acute urinary retention secondary to bladder outlet obstruction (Carpenter et al., 1983). Overdistension, as a physiological or pathological stress, has been shown to result in contractile and metabolic dysfunction of bladder (Lee et al., 2000). Prolonged overdistension can result in injury to the neural pathways responsible for micturition (Tammela et al., 1990), reduce bladder elasticity, alter the biochemical and neuronal responsiveness of the bladder (Carpenter et al., 1983), and subsequently lead to micturition problems. We have reported that, after overdistension,

catheterization/decompression induce reperfusion injury in the bladder and that reactive oxygen species are one of the main contributing factors in this injury (Saito et al., 2001). Ischemia-reperfusion injury may cause dysfunction of the urinary bladder, which results in instability and impairment of detrusor contractility during urination (Greenland et al., 2001). Experimentally we have reported that ischemia and subsequent reperfusion significantly damage the bladder function measured by organ bath studies and histological studies (Saito et al., 1998; Saito et al., 2002; Saito and Miyagawa, 1999).

Ischemic preconditioning (PC) is defined as brief, non-injurious ischemia-reperfusion (IR) periods that render a tissue more resistant to the harmful effects of a subsequent prolonged period of ischemia through endogenous cellular protective mechanisms. Its protective effect from IR injury was first shown by Murry and coworkers on the canine heart (Murry et al., 1986). Subsequently, benefits of PC in many other tissues, such as central nervous system, skeletal muscle, kidney, liver, lung, and mesenteric endothelium have been shown from many groups. PC may play an important role in the development of bladder dysfunction caused by acute/chronic urinary retention and by ischemia-reperfusion. To our knowledge, however, limited information is available

about the effect of PC in the bladder dysfunction. Lorenzi et al. reported the effect of preconditioning in guinea-pig in vitro (Lorenzi et al., 2003). They suggest that in vitro short periods of transient ischemia may be able to protect the guinea-pig bladder from the impairment associate with longer periods of ischemia-reperfusion. However, identified mechanisms of ischemia-reperfusion include altered Ca^{2+} homeostasis, free radical formation, mitochondrial dysfunction, protease activation, altered gene expression, and inflammation (Neumar, 2000). From these points, it is important to perform in vivo study to understand effect of PC on the bladder. In order to clarify the effect of PC on ischemia-reperfusion in the bladder, we investigated the role of PC on ischemia-reperfusion injury in the rat bladder in vivo.

Materials and Methods

Animal model

All animal experiments were performed in accordance with the guidelines set by the Tottori University Committee for Animal Experimentation. Male Sprague Dawley rats, 12 weeks old and weighing 380-420 g (SLC, Shizuoka, Japan), were divided into 3 groups; sham-operated control (Cont), 30 minutes ischemia-60 minutes reperfusion (IR) and three times of five minutes ischemia and then 30 minutes ischemia-60 minutes reperfusion (PC) group (In each group, n= 6-8). Our protocol is shown in figure 1. The ischemic condition was conducted according to previous reports with minor modifications (Saito et al., 1999). Briefly, under anesthesia with ethyl carbamate (1mg/kg, hypodermoclysis), the abdominal aorta just above the bifurcation of the aorta was clamped with a small clip (Sugita standard aneurysm clip, holding force 145 g, Mizuho Ikakogyo, Tokyo, Japan). The PC condition was initiated that before 30 minutes ischemia, aorta was clamped three times at the same site of aortic occlusion for 5 minutes followed by 5 minutes of reperfusion after each ischemic episode. In our previous study, the clamping the abdominal artery decreased blood flow of urinary

bladder to 5-10 % of the preclamping levels (Saito et al., 1999).

Cystometric studies.

The cystometric studies were performed according to methods used in our previous report (Saito et al., 2007). Cystometry was performed under anesthesia with ethyl carbamate (1.0 mg/kg, subcutaneously). In short, after the experimental periods, each rat's abdomen was opened using a lower midline incision and the bladder was exposed, and cystometry was carried out with a 24 G catheter inserted into the apex of the bladder dome for the purpose of recording pressure and in order to fill the bladder with physiological saline (0.9 % NaCl). External bladder filling was carried out using an infusion pump (5200, TOP, Tokyo) at a constant rate of 0.4 ml/min until micturition was detected. A cystometry catheter was connected to an external pressure transducer (P2310, Gould, Eastlake, OH) for the measurement of intravesical pressure. Intravesical pressure was recorded on the personal computer (Macintosh G3, Apple Computer, Cupertino, CA) via a bridge amplifier (ML112, AD Instruments, Castle Hill, Australia) and multiports controller (Power Lab/16sp, AD Instruments). The

following parameters were evaluated: probability of urination, bladder capacity, maximum detrusor pressure during voiding (Pdet), and residual urine volume. The Pdet was defined as instantaneous pressure minus the post-contraction resting pressure according to our previous reports. Probability of urination was defined as (total number of animals – the number of animals with overdistention) / (total number of animals). In each animal, approximately 5-6 voiding cycles were recorded and then the means of the voiding cycles were calculated.

Tissue preparation and measurement of contractile force in the bladder.

Functional studies were conducted according to methods used in our previous reports (Saito et al., 2007). The rat bladder dome was immediately removed and separated from the bladder base at the level of the ureteral orifices. Razor blades were used to obtain uniform longitudinal strips of the posterior wall of the bladder dome (1.5 x 5 mm). One end was fixed to a hook in the bottom of the muscle chamber, and the other end was fastened to a force displacement transducer. Muscle strips were mounted in organ baths (25 ml) containing Krebs-Henseleit solution, and bubbled with 5% CO_2 and

95% O_2 (37℃). One hook was suspended from a transducer (type 45196A, San-ei Instruments, Tokyo, Japan), and the lower hook was fixed to a plastic support leg attached to a micrometer (Mitutoyo, Tokyo, Japan). Each strip was equilibrated unstretched for 30 minutes. A load of 1.0 g was applied to each strip by micrometer adjustment, and the load was readjusted to this level 30 minutes later. Changes in the tone of the strips were measured isometrically by means of force transducers, and the data were recorded on a personal computer (Macintosh G3, Apple Computer, Cupertino, CA) with the use of Chart v 3.6.9 software and a Power Lab/16sp data acquisition system (AD Instruments, Castle Hill, Australia). Cumulative concentration-response curves to carbachol and KCl (100 mM) were constructed. These studies demonstrated that the resting stress at which bladder dome developed maximum contractile forces was in the range of 2.5-3.0 gm./mm^2. Carbachol-induced contractile responses were measured cumulatively in the presence or absence of various concentrations of muscarinic antagonists: pirenzepine (PRZ; M1 selective), methoctramine (MTR; M2 selective), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; M1/M3 selective), and atropine (ATR; nonselective). Antagonists were added 60 minutes prior

to the administration of carbachol. After completion of a concentration-response curve, the tissue was washed until base-line force returned to the resting level, equilibrated for 60 minutes, and then the next consecutive concentration-response curve was constructed.

Measurement of malonaldehyde (MDA) in the bladder

In order to investigate lipid peroxidation of the bladder during ischemia-reperfusion, malonaldehyde (MDA), a marker of lipid peroxidation, concentrations were measured in the experimental rat bladders. The tissues were chopped into small pieces and the pieces were then homogenized in 9 volumes of PBS buffer in 5 mM BHT with five 10 second bursts using the Multi-beads Shocker® (YASUIKIKI, Osaka, Japan) with the speed set at 1800 rpm. Then the MDA concentrations in the bladder were measured by colorimetric assay according to the manufacturer's instructions (BIOXYTECH MDA-586™ kits, OXIS International, Portland, OR). The absorbance was measured at 586 nm. The values were estimated based on the amount of protein in the tissue. Protein was determined using a commercial kit (Protein Assay Rapid Kit_{wako}, Wako

Pure Chemical, Osaka, Japan).

Histological examination of the rat bladder.

After each bladder was transected at the level of the ureteral orifice, the bladder dome was immediately fixed with 10% formalin. After fixation, the tissues were embedded in paraffin. Five micron-thick tissue sections were cut from these paraffin blocks. All of the bladder specimens were stained using Hematoxylin and Eosin (H&E) staining. Each section was viewed under a light microscope at a magnification of x40-400.

Data analysis.

Contractile data were calculated as grams of active force per cross sectional area in square millimeters. The cross-sectional area was calculated using the following equation:

$$\text{cross-sectional area} = \text{weight} / (\text{length} \times 1.05),$$

where 1.05 is the assumed density of the muscle (Saito and Miyagawa, 1999; Saito et al., 2007). The dose ratio was obtained from the ratio of EC_{50} values (the concentration

of agonist that produces half-maximal contractile responses) for carbachol in the presence or absence of an antagonist.

pA_2 values were obtained from Schild plots (Arunlakshana and Schild, 1959). Schild plots were constructed by plotting the log of (dose ratio –1) against the log of the molar concentration ofantagonist. EC_{50} values were calculated as geometric means, whereas E_{max} values were calculated as arithmetic means. A statistical comparison of differences between groups was performed using analysis of variance and Fisher's multiple comparison tests. $P< 0.05$ was regarded as the level of significance.

Drug and Chemicals.

Ethyl carbamate was purchased from Wako Pure Chemical Co. (Osaka, Japan). Carbachol, pirenzepine, methoctramine, 4-DAMP, and atropine were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). All other chemicals used were of reagent grade.

Result

Cystometric studies.

Table1 shows the data of cystometrogram in the experimental rats. In the IR group, the probability of urination was decreased, and in the PC group probability of urination was slightly greater than that in the IR group. The maximum detrusor pressure (Pdet) in the IR group was significantly lower than that of the control group. The Pdet in the PC group did not differ significantly from that in the both IR and Cont groups. In the bladder capacity, although the IR rats treated with PC tended to be improved, the differences in two groups were not significant statistically. The residual urine volume in both the IR and PC rats was markedly greater than that of the controls. In cystometric studies, we note that the PC group was closer to the control group in terms of the probability of urination, Pdet, and the bladder capacity than IR group.

Contractile force in the bladder.

The data obtained by functional studies of the experimental animals are shown in Table 2 and 3. Concentration-response curves to carbachol were shown in Fig. 2.

The E_{max} values of carbachol in the IR rats were markedly smaller than those of the control rats. The E_{max} values were improved by treatment with PC. Contractile responses to 100 mM KCl showed in the same manner as E_{max} values of carbachol in each group. The pA2 values for a series of muscarinic antagonists were similar in all groups and the rank order of the values is as follows;

ATR≧4-DAMP＞MTR＞PRZ

Biochemical analysis.

The tissue concentrations of MDA are also shown in Table 4. The MDA concentrations in the bladder of the IR group were significantly higher than those of the control group. MDA concentrations were significantly decreased in PC rats compared to IR and control groups.

Histological examination.

Fig. 3 shows H&E staining of the rat bladder. In control rats, high levels of normal viability cells were observed. In the IR group, histological damage was observed, with

infiltration of leukocytes and ruptures of microcirculation in the regions of submucosa and smooth muscle without a corresponding sloughing of mucosal cells. In the PC group, ruptures of microcirculation and leukocyte infiltration in the bladder were also observed. Significant protective effects of the PC were not observed in these histological examinations.

Discussion

In the present study, we investigated the effect of PC on ischemia-reperfusion injury in the rat bladder. Our previous and present data indicated that ischemia-reperfusion produced significant damages of bladder function estimated by cystometric and functional studies. Treatment with three times of 5 minutes PC improved this injury. We also demonstrated that one of these preventive mechanisms was to reduce the production of free radicals produced by ischemia-reperfusion in the bladder.

In the past, much attention has focused on the effects of preconditioning on vital organs, such as the heart and brain (Pong, 2004), but little has been reported about the effects of PC in the bladder. Lorenzi and coworkers reported that in vitro short periods of transient ischemia may be able to protect the guinea-pig bladder from the impairment associated with longer periods of ischemia and reperfusion, which might happen in obstructed micturition, and that the phenomenon affects mainly the intrinsic nerves, which are more susceptible to ischemic damage than the smooth muscle (Lorenzi et al., 2003). Furthermore, Yu et al reported that hypoxic PC minimizes oxidative injury induced by overdistension/emptying in the rat bladder (Yu et

al., 2003). In their report, they concluded that hypoxia-reoxygeneration and ischemia-reperfusion lead to the generation of reactive oxygen species (ROS), the induction of Bcl-2 protein expression by hypoxic PC appears to reflect the bladder's up-regulation of the endogeneous antioxidant-induced defense system. The effects enable to survive a subsequent ischemia-reperfusion stress by reducing an oxidative insult and preserving bladder nerve activity and contractile function.

As we suspected that ROS played an important role to prevent ischemia-reperfusion injury in the bladder, we measured the concentrations of MDA, a marker of lipid peroxidation, in the experimental bladder. In the present study, the MDA concentrations in the bladder were significantly increased in the IR group. Treatment with PC significantly decreased MDA production by ischemia-reperfusion, and interestingly, the MDA concentration in the PC group was significantly lower than that of the Cont group. Gurucum and associates recently reported a preventive effect of remote preconditioning in spinal cord ischemia-reperfusion injury (Gurcun et al., 2006). In their study, both direct PC and remote PC caused by occlusion of left renal artery have a preventive effect on spinal cord ischemia-reperfusion injury, and the plasma

MDA concentrations after ischemia-reperfusion with PC were significantly smaller than pre-ischemia levels. These data suggest that at least PC has an effect to reduce ROS production in the ischemia-reperfusion organs. As increases in lipid peroxidation can produce nerve and smooth muscle membrane damage, PC may associate with defensive mechanism that reduce lipid peroxidation.

It is known that PC activates a cellular survival program that requires the integration of several processes including opening of surface K_{ATP} channels, regulation of fatty acid metabolism, ROS production, regulation of the mitochondrial permeability transition and opening of K^+ channels in the mitochondrial inner membrane (Hanley and Daut, 2005). In the functional studies, contractile responses to carbachol and KCl were significantly decreased by ischemia-reperfusion, which was partially prevented by induction of PC. These observed decrease in contractile responses might indicate that ischemia-reperfusion injures or alters the muscarinic receptors on the bladder smooth muscle membrane and their second messenger system. Since we thought a possibility of alterations of these systems, we calculated the pA2 values and their slopes for a series of muscarinic antagonists in order to investigate affinity of receptor. In this study,

there were no significant differences of the pA2 values and slopes between any groups in all muscarinic antagonists. These data indicated that alterations of contractile responses of bladder smooth muscles were due to quantitative rather than qualitative changes of muscarinic receptors and their second messenger system.

In this study, we demonstrated the effect of PC during ischemia-reperfusion in the bladder, and we also demonstrated that one of these mechanisms is to reduce production of ROS in the bladder. However, roles of opening of surface K_{ATP} channels, regulation of fatty acid metabolism, nitric oxide production, regulation of the mitochondrial permeability transition and opening of K^+ channels in the mitochondrial inner membrane are not clear. In order to understand the precise mechanisms of PC, it is important to investigate these effects on the bladder. However, the detailed mechanism of this preventive effect remains unclear, and warrants further study.

Conclusion

Ischemia-reperfusion injury significantly reduces the contractile force of the bladder smooth muscle, which is partially prevented by PC. However, the changes of the receptor characteristics concerned with the contractile responses to stimulus from muscarinic receptors were not observed. Our data indicate that PC has a beneficial effect on ischemia-reperfusion injury in the rat bladder, and one of the mechanisms is to reduce ROS production in the bladder.

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up-regulation of Bcl-2 in the rat. Journal of Physiology 554(3), 815-828.

Figure legends

Fig 1. Protocol in this study.

Fig. 2. Contractile responses of rat bladder smooth muscle to carbachol.

Contractile data were calculated as grams of active force per cross sectional area in square millimeters.

Fig 3. Typical H&E staining in the rat bladder.

Ruptures of microcirculation (short arrows) and leukocyte infiltration (long arrows) in the regions of submucosa and smooth muscle of the bladder were observed in IR and PC Groups (X 200).

Table 1. cystometrogram data in the experimental rats

Group	Probability of urination	Pdet ($cm\ H_2O$)	Bladder capacity (ml)	Residual urine (ml)
Cont	100 %	42.8 ± 2.3	0.70 ± 0.18	0.034 ± 0.010
IR	53.8 %	34.0 ± 2.1*	0.53 ± 0.10	0.233 ± 0.057*
PC	61.5 %	35.4 ± 2.9	0.59 ± 0.14	0.430 ± 0.105*

Data are shown as mean ± S.E.M. of five to nine separated determinations in each group. Pdet means maximum contraction pressure of the detrusor. *:significantly different from the Cont group.

Table 2. Functional studies in the experimental rats

Group	E_{max}/cross-sectional area (g/mm_2)	EC_{50} ($\times 10^{-6}$ M)	KCl/ cross-sectional area (g/mm_2)
Cont	2.25 ± 0.16	4.3 ± 0.5	1.50 ± 0.15
IR	1.61 ± 0.10*	3.1 ± 0.3*	1.04 ± 0.67*
PC	1.87 ± 0.17	2.0 ± 0.2**	1.09 ± 0.90*

Data are shown as mean ±S.E.M. of six to eight separated determinations in each group. E_{max} and ED_{50} values are for carbachol. KCl means contractile force to 100 m mol/l KCL. *: significantly different from the Cont group. **: significantly different from the other groups.

Table 3. pA2 values and Slopes of Schlid Plots for muscarinic antagonists in the experimental rat bladder

Group	ATR		PRZ		MTR		4-DAMP	
	pA2 (± S.E.M.)	Slope	pA2 (± S.E.M.)	Slope	pA2 (± S.E.M.)	Slope	pA2 (± S.E.M.)	Slope
Cont	9.57	0.950 ± 0.055	7.29	0.924 ± 0.096	7.94	0.533 ± 0.087	9.39	0.95 ± 0.055
	(9.44 - 9.77)		(7.23 – 7.37)		(7.81 – 8.12)		(9.28 – 9.54)	
IR	9.35	0.977 ± 0.095	7.46	0.917 ± 0.062	7.98	0.568 ± 0.1	9.70	0.977 ± 0.095
	(9.26 – 9.47)		(7.37 – 7.57)		(7.77 – 8.40)		(9.62 – 9.81)	
PC	10.0*	1.101 ± 0.099	7.22	1.028 ± 0.026	7.82	0.541 ± 0.108	9.44	1.101 ± 0.099
	(9.88 – 10.18)*		(7.16 – 7.29)		(7.64 – 8.14)		(9.36 – 9.54)	

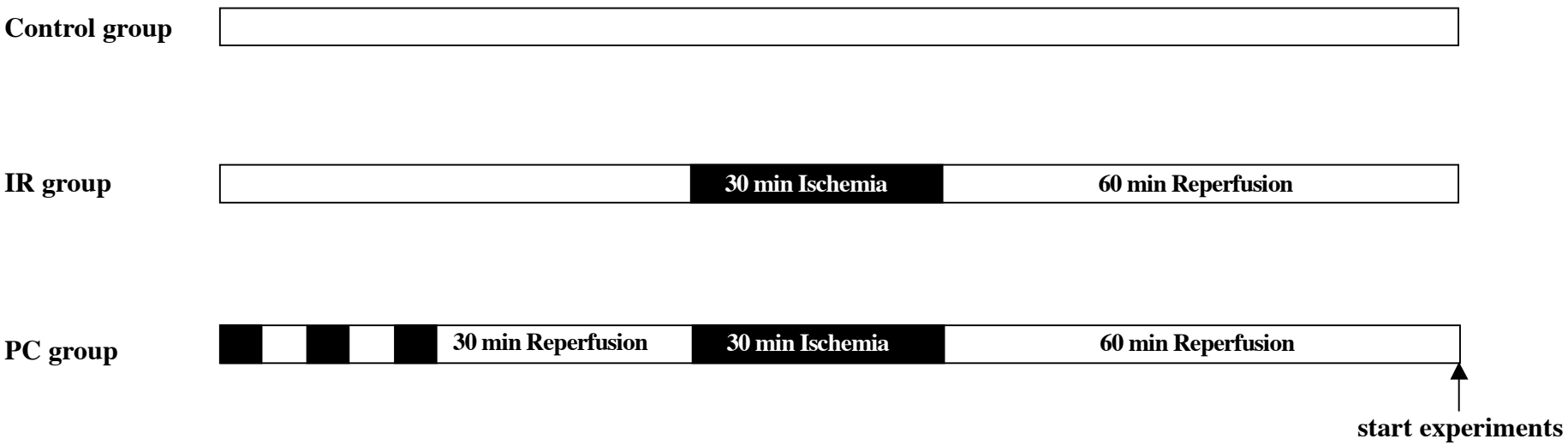
Data are shown as mean ±S.E.M. of five to nine separated determinations in each group. *:significantly different from the other group. ATR: atropine; PRZ: pirenzepine; MTR: methoctoramine

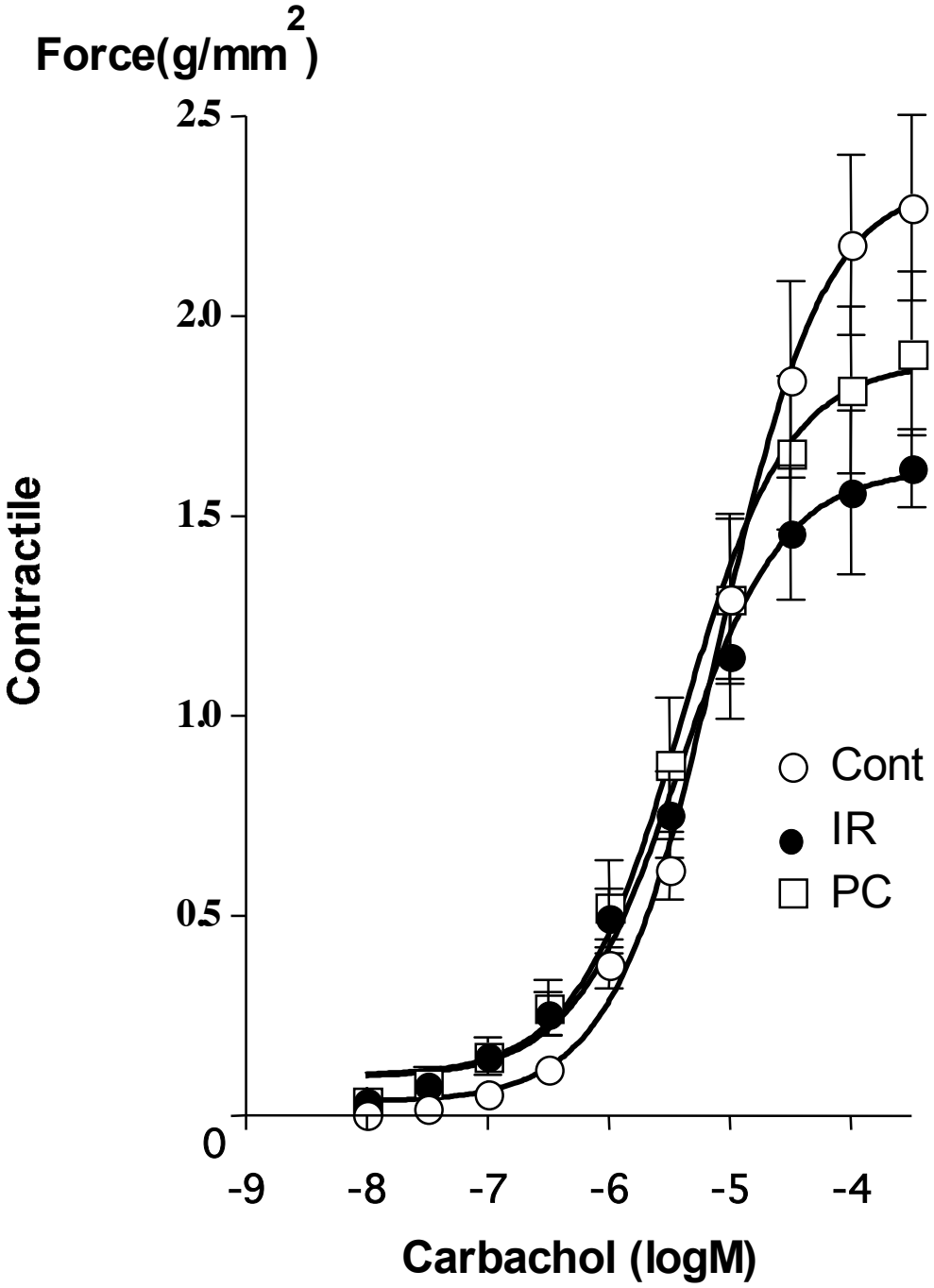
Table 4. MDA concentrations in experimental rat bladders

Group	MDA concentrations (n mol/mg protein)
Cont	3.87 ± 0.16
IR	4.72 ± 0.29*
PC	3.05 ± 0.24**

Data are shown as mean ±S.E.M. of six to eight separated determinations in each group.

*: significantly different from the Cont group. **:significantly different from the other groups.


Control group
IR group
30 min Ischemia
60 min Reperfusion
PC group
30 min Reperfusion
30 min Ischemia
60 min Reperfusion
start experiments
5-min Ischemia
5-min Reperfusion
Ischemia
Reperfusion


Force(g/mm 2)
Contractile
2.5
2.0
1.5
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Carbachol (logM)
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IR
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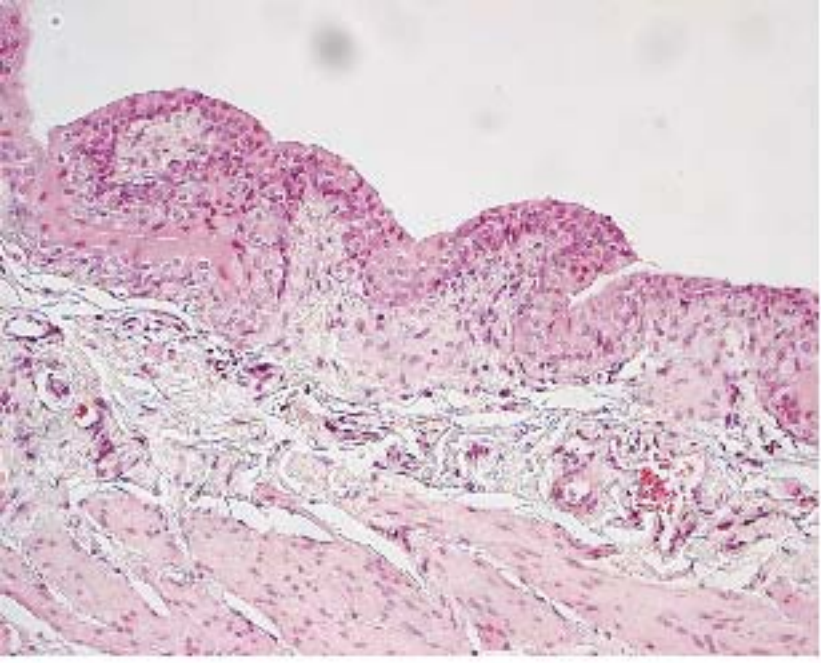

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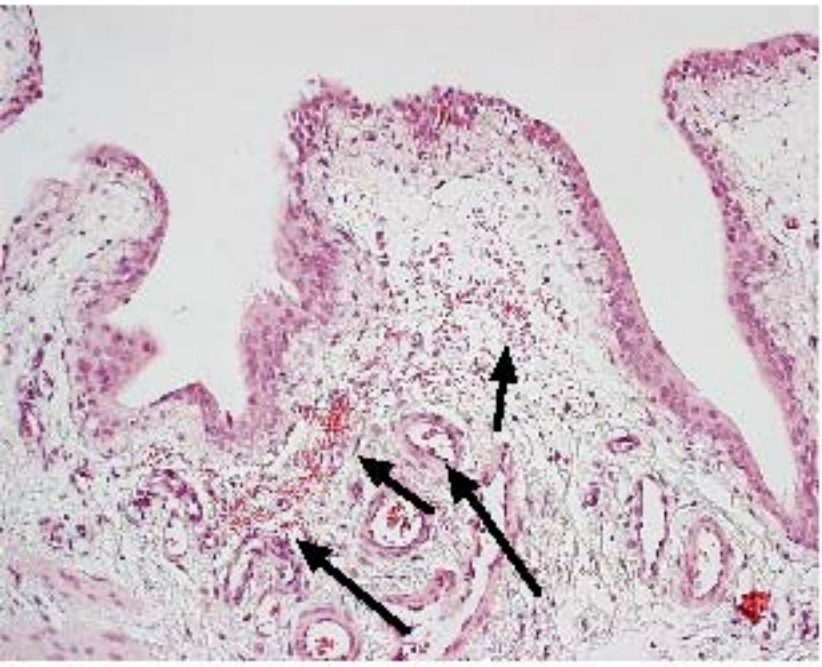
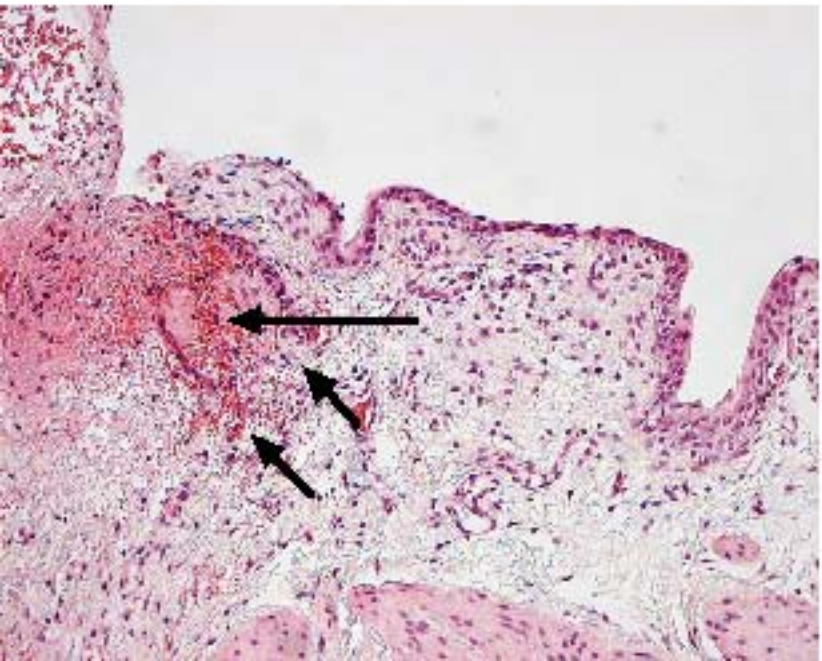


IR



PC