Research Paper

Standardization of *in vitro* Cell-based Model for Renal Ischemia and Reperfusion Injury

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Renal ischemia reperfusion injury contributes patho-physiological imbalance of acute renal failure that comprises of generation of reactive oxygen species, nitric oxide and peroxynitrite and inflammation involving cytokine/adhesion molecule cascade, finally leads to cell death. Oxygen deprival associated with ischemia that in turn lead to decline ATP production is the characteristic feature usually addressed in the development of *in vitro* cell based ischemic model. In order to create oxygen deficit in the cell lines different approaches like chemical induction, enzymatic induction and anaerobic chamber models are widely used. However efficiencies of these models were varied and the present study was aimed to compare the suitability of these models in creating *in vitro* ischemia reperfusion in cell culture. In the chemical induced method we used different concentrations of rotenone, antimycin and sodium azide to inhibit electron transport chain and thereby reduced the ATP production, measured indirectly by cell viability assay. Among the chemical induced model, antimycin mediated cell injury was more reliable for ischemia reperfusion study. In the enzymatic model, comprises of glucose oxidase (3mM/s) and catalase (998 s^{-1} at 10:1 ratio) was used and found to be best among the three approaches as it can create injury in short experimental time and are reproducible. However anaerobic chamber method was not suitable for ischemia reperfusion study as it need more time to induce significant cell injury.

Key words: Renal ischemia reperfusion, LLC PK1, Rotenone, antimycin, enzymatic hypoxia, hydrogen sulfide

Renal ischemia reperfusion injury (I/R) is one of the major causative factors for acute renal injury that is becoming growing reason/s for mortality rates, results from a generalized or localized impairment of oxygen and nutrient delivery to, and waste product removal from, cells of the kidney[1]. Renal I/R injury is characterized to have oxidative, inflammatory and apoptotic injury along with activation of inflammatory processes within tissues[2] but also the modulation of mitochondrial function, changes in intracellular calcium levels.

A hallmark of ischemia reperfusion injury is loss of the apical brush border of proximal tubular cells that in turn will affect the stability of cytoskeleton[3]. In fact patho physiology of I/R is reflected in all segments of nephron, but most commonly injured is epithelial cells of proximal tubular cell[4]. This is because these cell types has a high metabolic rate for ion transport and have limited capacity to undergo anaerobic glycolysis. Moreover owing to the marked microvascular hypoperfusion and congestion in this region after I/R injury, that may persists and mediates continued ischemia even when cortical blood flow might have returned to near normal levels[4].

In order to get a great insight in to the mechanism triggered and regulate tubular cell I/R injury, most of the researchers rely on *in vitro* cell culture models. Even though *in vitro* cell based experimental models mimic some of the features of tissue ischemia and I/R based patho physiology, it cannot reproduce the *in vivo* tissue environment precisely[5]. However *in vitro* cell based model provide sufficient reliable and authentic data for further *in vivo* analysis. Hence it is very relevant and important for any researcher to standardize their *in vitro* experimental model for dependable results.

Renal I/R is a multifaceted disease with multi factorial reasons for its patho physiology. Mimicking the exact *in vivo* pathological environment in cell based model is a challenge. Since deprival of ATP

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concentration resulted from low blood supply leads to I/R injury, most of the cell based cultural model focussed to inhibit the production/synthesis of ATP. Chemically induced I/R[6], enzymatic induction of I/R[7] and anaerobic chamber[8] mediated I/R are the main experimental approach to induced I/R in cell cultural model. In the present study, we compare the different above mentioned approaches to induce I/R in epithelial cell line from Pig Kidney, namely LLC PK1, to study the efficacy of H_2S mediated renal protection.

MATERIALS AND METHODS

Cell culture:

LLC-PK1, derived from the renal epithelial cells of Hampshire pigs PK1 (American Type Culture Collection, Manassas, VA) was grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal calf serum, 50µg/ml streptomycin, and 50 U/ml penicillin at 37° in a CO_2 incubator (95% air, 5% CO_2), and then, 0.5% (w/v) trypsin was used to detach the cells from the flasks. The cells were subsequently centrifuged, re-suspended in DMEM, and sub-cultured in 75 cm^2 culture flasks or 96-well.

Induction of ischemia reperfusion (I/R) in LLC-PK1:

Ischemia was induced in LLC PK1 by three methods namely enzymatic, chemical and anerobic chamber methods and the results were compared. I/R was induced *in vitro* according to the well-studied and extensively characterized model[9]. Briefly, when the cells cultured in 96-well plates became confluent, ischemia was created by changing the culture medium to DMEM without glucose and serum but contain either enzyme or chemicals that induce ischemia. However for induction of ischemia in anerobic chamber model, only the normal growth culture media was replace by glucose and serum deprived DMEM media and were placed in anerobic chamber.

For the induction of ischemia by enzymatic method, GOX/CAT system consisting of glucose oxidase (GOX) and catalase (CAT) and 2-deoxyglucose (a non-metabolizable isomer of L-glucose) were used. GOX/CAT system was prepared by diluting glucose oxidase and catalase at a constant 10:1 ratio in cell culture medium (both Sigma cat. No. C3155 and G0543). Enzyme activities of stock solutions were 3 mM/s for GOX and 998 s^{-1} for CAT. To obtain a defined, stable oxygen concentration of 2% on cell surface stock solutions were diluted by 1:10,000 for GOX and 1:1,000 for CAT. The *in vitro* reperfusion was achieved by incubating cells in glucose-replete complete growth medium.

Chemical like rotenone, antimycin and sodium azide were used to induce ischemia. LLC PK1 cells were incubated for one hour with either rotenone, antimycin or sodium azide of different concentrations to create ischemia and followed by replacement of ischemic media with glucose rich growth medium to induce reperfusion injury.

For the induction of ischemia by anaerobic chamber method, LLC PK1 cells were incubated for one hour in anerobic chamber to create ischemia and then incubate in a CO_2 incubator (95% air, 5% CO_2), at 37° for 3 h to create reperfusion injury.

Experimental protocol:

In general, 80% confluent LLC PK1 cells were selected for the study. DMEM media was replaced by fresh media in control group alone where as in other groups ischemic media comprises of DMEM without glucose + 2-deoxyglucose + [GOX/CAT]/ rotenone/ antimycin/ azide was added and incubated for different time intervals such as 1, 3, 6 and 12 h at 37º for the induction of ischemia. At the end of this stage fresh DMEM was used to replace ischemic medium for reperfusion effect for 3 h.

Another set of experiments were designed to study the efficacy of the model that comprises of following groups namely: Control, ischemic control, reperfusion control, NaSH (positive control) and NaCl (negative control) treated: In the NaSH and NaCl groups, cells were preincubated with NaSH (1 mM) and NaCl (10 mM) respectively for 1 h before the induction of 1 h ischemia and 3 h reperfusion.

Cell viability tests:

Cell viability tests were done by two methods namely crystal violet and sulforhodamine B assays. Briefly for crystal violet assay, cells (grown on 96 well plates with 80% confluence) were washed and stained with 0.5% crystal violet in methanol for 8-10 min at 22°, then washed three times with 1X phosphate-buffered saline solution. The absorption measured at 550 nm was used as an index for cell viability.

For sulforhodamine B (SRB) colorimetric assay[10] cells (grown on 96 well plate with 80% confluence) were washed and fixed with by means of protein precipitation with 50% trichloroacetic acid at 4º (final concentration 10%) for 1 h. After five washing with tap water, cells were stained for at least 15 min with 0.4% SRB dissolved in 1% acetic acid and subsequently washed four times with 1% acetic acid to remove unbound stain. The protein-bound dye is dissolved in 10 mM Tris base solution for optical density determination at excitation wavelength of 488 and emission wavelength of 585 nm.

Assay of lactate dehydrogenase leakage:

The activity of cytoplasmic lactate dehydrogenase (LDH) leakage into culture media was determined as described previously[11]. After the experimental protocol, 100 µl of media were collected, and the LDH activity was assayed in 2.4 ml of phosphate buffer (0.1 mol/l, pH 7.4) with 100 µl of NADH (2.5 mg/ml phosphate buffer). The rate of NADH oxidation was determined by following the decrease in absorbance at 340 nm at 25° with the use of a spectrophotometer.

Statistical analysis:

Data are reported as means±SD. The comparison between values of the same group, at various time points along the experiment was conducted using Student's t-test or ANOVA. Differences in variables between groups for a specific time point were analyzed using one-way ANOVA. Values of $P<0.05$ were considered significant.

RESULTS AND DISCUSSION

Reperfusion of ischemic tissues is often associated with microvascular dysfunction and is exhibited in different organs like heart[12,13], kidney and liver, that are predominantly undergoing transplantation. But the contribution of reperfusion to the extent of injury in ischemic renal tissue is unclear. Some studies indicate that reperfusion further aggravate the ischemic injury, whereas other suggests that it unmask the irreversible ischemic injury. Yet another group have an opinion that reperfusion injury is an independent entity. Resolving this issue is not easy as the contribution of ischemia and reperfusion to the injury is difficult to quantify in intact renal tissue as reperfusion injury may initiates within seconds of reoxygenation. Cultured cardiac myocytes may be useful in this problem by allowing the serial measurement of cell viability during the ischemia and reoxygenation phases. Ischemic tolerance has been described in a variety of primary cell culture system, but similar *in vitro* model for renal ischemia reperfusion injury have not been well established. The present study demonstrates anti ischemic reperfusion effect of NaSH (H_2S donor) after standardizing ischemia reperfusion model in kidney epithelial cell lines namely LLC PK1.

Using a model of ATP depletion in LLC PK1 cells by chemical inducers like rotenone, antimycin and azide (electron transport chain respiration inhibitors), we created ischemia for specific time and compared the resulted injury. Mitochondrial respiratory chain complex I inhibitor, rotenone could induce cell death in a variety of cells mainly by reducing ATP synthesis and by releasing reactive oxygen species[14]. Since ischemia and reperfusion is also characterized to have limited ATP and increased ROS, we used rotenone for the induction of ischemia in LLC PK1 cells.

Tables 1 and 2 shows results of cell death induced by different concentration of rotenone in LLC PK1 cells. Early reports suggest that extent of I/R injury depends of duration of ischemic time and thus we create and standardize cell injury by perfusing the cell for 3 h (by replacing the cells with fresh normal growth medium) after having varied ischemic time intervals like 1, 3, 6 and 12 h. Cells incubated with higher concentration of rotenone did not show significant damage with 1 h incubation

TABLE 1: SULFORHODAMINE B CYTOTOXICITY ASSAY: ROTENONE- AND ANTIMYCIN-INDUCED ISCHEMIA REPERFUSION

Groups	Control	1 µM	5 µM	10 µM	20 µM
		Rotenone-induced ischemia			
A	4772±212	4644±491	4597±555	4445±511	4379±401
B	4847±98	4365±401	4137±487	*3822±421	*3503±321
C	4897±122	*3492±367	*3310±591	*3058±401	*2802±291
D	5097±156	*2771±291	*2638±441	*2442±391	*2229±251
		Antimycin-induced ischemia			
A	4838±132	4702±173	*4147±287	*3315±210	*3125±298
B	4870±84	*3762±121	*3317±139	*2652±242	*2500±232
C	4945±91	*3009±96	*2654±111	*2121±132	*2000±205
D	4945±91	*1595±47	*1406±58	*1124±109	*1060±102

Group A: 1 h ischemia and 3 h reperfusion; Group B: 3 h ischemia and 3 h reperfusion; Group C: 6 h ischemia and 3 h reperfusion; Group D: 12 h ischemia and 3 h reperfusion. Different concentration of rotenone and antimycin were used to induce ischemia reperfusion injury to the cells. Reperfusion time was fixed for 3 h where as ischemic time was varied to standardized ischemia reperfusion injury. Results are expressed as mean±SD of n=4-6 independent assays. (*) $P<0.05$, statistically different from the control group

TABLE 2: CRYSTAL VIOLET CYTOTOXICITY ASSAY: ROTENONE- AND ANTIMYCIN-INDUCED ISCHEMIA REPERFUSION

Groups	Control	1 µM	5 µM	10 µM	20 µM
			Rotenone-induced ischemia		
A	0.803±0.017	0.796±0.014	0.775±0.037	0.793±0.021	0.793±0.017
B	0.803±0.015	0.778±0.021	0.702±0.044	0.703±0.062	*0.643±0.041
C	0.821±0.024	*0.623±0.043	*0.561±0.0203	*0.562±0.045	*0.514±0.034
D	0.821±0.037	*0.436±0.033	*0.393±0.011	*0.393±0.017	*0.360±0.028
			Antimycin-induced ischemia		
A	0.894±0.02	0.789±0.06	0.720±0.02	0.731±0.034	*0.641±0.04
B	1.014±0.02	*0.783±0.02	*0.691±0.05	*0.552±0.02	*0.490±0.03
C	1.030±0.03	*0.627±0.04	*0.552±0.05	*0.442±0.07	*0.392±0.02
D	1.030±0.05	*0.332±0.03	*0.293±0.02	*0.234±0.02	*0.207±0.03

Group A: 1 h ischemia and 3 h reperfusion; Group B: 3 h ischemia and 3 h reperfusion; Group C: 6 h ischemia and 3 h reperfusion; Group D: 12 h ischemia and 3 h reperfusion. Different concentration of rotenone and antimycin were used to induce ischemia reperfusion injury to the cells. Reperfusion time was fixed for 3 h where as ischemic time was varied to standardized ischemia reperfusion injury. Cell injury was assessed by crystal violet assay. Results are expressed as mean±SD of n=4-6 independent assays. (*) P<0.05, statistically different from the control group

while increasing the incubation time to 3, 6 and 12 h impart significant injury to the cells. However, dose dependent cellular injury was not observed. Evidence from early report suggests that energy deprivation and ROS may be the main mechanism of rotenone-induced cell death[14] and it may destroy primary dopaminergic neurons in a dose- and time-dependent manner[15]. In agreement to these findings, insignificant cell damage in 1h rotenone incubation suggests that LLC PK1 cells may be resistant/adapted to low rotenone incubation irrespective of their different concentrations.

In vitro chemical anoxia model characterized to have depleted ATP concentration through antimycin incubation to LLC PK1 cells are well established[16]. True anoxia is practically impossible and thus in order to mimic ischemic condition along with antimycin we replaced normal growth media with ischemic growth media where instead of glucose, deoxyglucose has been added, thereby resulted in graded depletion of ATP that can cause apoptosis, the characteristic feature of ischemia reperfusion injury[16]. Tables 1 and 2 shows viability of cells subjected to different time duration of ischemia reperfusion. Similar to rotenone, cell death was elevated when the ischemic time was increased but contrary to rotenone results even in 1h incubation with antimycin, resulted significant cell desath. Epithelial cells such as LLC PK1 are glycolytic and can utilize amino acids through gluconeogenesis to produce glucose[17] and therefore not susceptible to rapid hypoxic cell death, thus need more cellular incubation with chemical ischemic agent for a desired cell death for the experimental set up. However, antimycin, know to be mitochondrial electron transport chain complex blocker at cytochrome bc_1 region[18] was also reported to inhibit gluconeogenesis indirectly by inhibiting oxygen uptake, makes antimycin mediated ischemic injury more suitable for I/R model.

In another experiment we use sodium azide as the chemical ischemic agent to induce ischemia reperfusion in LLC PK1 cells. Previous studies showed that sodium azide[19] inhibited oxidative phosphorylation *via* inhibition of cytochrome oxidase, the final enzyme in the mitochondrial electron transport chain, thereby resulting in a rapid depletion of intracellular ATP that in turn can increase mitochondrial K_{ATP} channel activity. According to our results shown in Tables 3 and 4, dose-dependent cell damage was observed in LLC PK_1 cells incubated with different doses of sodium azide when there was short ischemic time interval. Dose-dependent cell damage was negated once the ischemic duration was increased. This may be due to the indirect effect of K_{ATP} channel activity.

In order to create oxygen deficiency that leads to impaired oxidative phosphorylation, ultimately resulted in ATP depletion was created in LLC PK1 cells by using two enzymes system consisting of glucose oxidase (GOX) and catalase (CAT), (GOX/CAT). Glucose oxidase rapidly removes oxygen from the cell culture medium or buffer by reducing it with glucose, generating peroxide in the process. It has been reported that, in contrast to hypoxia chambers, the GOX/CAT system more rapidly induces hypoxia within minutes at a defined rate. Further H_2O_2 levels are solely controlled by the ratio of GOX and CAT activities[7]. They can be adjusted at non-toxic or toxic dosages over 24 h. Thus, the GOX/CAT system

mimics a non-phosphorylating respiratory chain and can be employed to address many questions ranging from redox signalling to ischemia/reperfusion studies in transplantation medicine. As expected, GOX/CAT system imparts injury to cells significantly in 1 h of ischemic condition itself (Tables 3 and 4). More than 50% cellular damage was shown by this system in 3 h of ischemia with high concentration of the enzymes makes suitable for ischemia reperfusion study in cell lines. On the other hand ETC inhibitors need more incubation time to insult 50% cellular injury, there by experiments with longer duration of ischemia and reperfusion is not recommended. Among the ETC inhibitors antimycin is widely used for hypoxia and ischemia reperfusion study. Anaerobic chamber method is not suitable for short term ischemia reperfusion model as it required more time for the model to get 50 % of cellular damage (Table 5).

After establishing I/R model in LLC PK1 cells by approaches, we studied the efficacy of H_2S as protective agent against I/R. H_2S is believed to have protection against I/R injury in different organ system predominantly by opening K_{ATP} channels[20]. Endogenous H_2S is normally metabolized in mitochondria of different organ system. But protective effect of exogenous H_2S mechanism to I/R is not clearly understood. Indeed, cell line models are gaining interest in the scientific community especially to study the bioavailability, absorption and transport apart from the biological effect to specific cell types. LLC PK1 cells are well characterize for differentiation, formation of tight junction and transport study[21]. We used LLC PK1 cells to understand the significance of endogenous hydrogen sulfide release in renal ischemia reperfusion during exogenous thiosulfate incubation.

Table 6 shows the effect of H_2S as anti ischemic reperfusion agent in different ischemia reperfusion models in LLC PK1 cells when the cells were pre incubated with H_2S for 1h before the induction of ischemia. Pretreated LLC PK1 cells with H_2S for 1h showed significant cell viability as compared to NaCl (negative control) and ischemia reperfusion control (Table 6). Even though both chemical and enzymatic model showed the protective effect of H_2S, the result was prominent in the model that used GOX/

TABLE 3: SULFORHODAMINE B CYTOTOXICITY ASSAY: SODIUM AZIDE AND ENZYME-INDUCED ISCHEMIA REPERFUSION

Groups	Control	1 mM	5 mM	10 mM	20 mM
		Sodium azide-induced ischemia			
A	4662±612	4336±115	3907±171	3517±198	*3090±109
B	4451±406	4076±102	3673±162	3293±190	*2881±102
C	4529±356	*2998±189	*2706±264	*2433±205	*2137±156
D	4528±399	*2452±212	*2209±112	*1989±116	*1747±201
Groups	**Control**	**Glucose oxidase- and catalase system-induced ischemia**			
		H_2O_2 (1 µl/L)	H_2O_2 (5 µl/L)	H_2O_2 (10 µl/L)	H_2O_2 (20 µl/L)
A	4836±203	4232±125	*3732±155	*2983±289	*2812±273
B	4962±159	*3432±102	*3027±212	*2420±156	*2281±187
C	4965±100	*3056±123	*2695±121	*2154±116	*2031±142
D	4798±160	*2821±85	*2488±112	*1989±162	*1875±182

Group A: 1 h ischemia and 3 h reperfusion; Group B: 3 h ischemia and 3 h reperfusion; Group C: 6 h ischemia and 3 h reperfusion; Group D: 12 h ischemia and 3 h reperfusion. Different concentration of sodium azide and glucose oxidase and catalyse system (10:1 ratio of GOX (3 mM/s) and catalase (998 s^{-1}) were used to induce ischemia reperfusion injury to the cells. Reperfusion time was fixed for 3 h where as ischemic time was varied to standardized ischemia reperfusion injury. Results are expressed as mean±SD of *n*=4-6 independent assays. (*) *P*<0.05, statistically different from the control group

TABLE 4: CRYSTAL VIOLET CYTOTOXICITY ASSAY: SODIUM AZIDE- AND ENZYME-INDUCED ISCHEMIA REPERFUSION

Groups	Control	1 mM	5 mM	10 mM	20 mM
		Sodium azide-induced ischemia			
A	0.888±0.06	0.788±0.04	0.810±0.02	*0.690±0.03	*0.653±0.05
B	0.927±0.05	0.849±0.03	0.765±0.05	*0.686±0.04	*0.565±0.04
C	0.943±0.04	*0.624±0.04	*0.563±0.04	*0.506±0.03	*0.419±0.03
D	0.943±0.03	*0.510±0.07	*0.460±0.03	*0.414±0.05	*0.342±0.02
Groups	**Control**	**Glucose oxidase- and catalase system-induced ischemia**			
		H_2O_2 (1µl/L)	H_2O_2 (5µl/L)	H_2O_2 (10µl/L)	H_2O_2 (20µl/L)
A	1.007±0.06	*0.881±0.03	*0.777±0.04	*0.621±0.03	*0.551±0.03
B	1.033±0.05	*0.715±0.03	*0.630±0.03	*0.504±0.02	*0.447±0.05
C	1.033±0.04	*0.636±0.06	*0.561±0.06	*0.448±0.03	*0.398±0.03
D	0.999±0.03	*0.587±0.05	*0.518±0.07	*0.414±0.07	*0.367±0.03

Group A: 1 h ischemia and 3 h reperfusion; Group B: 3 h ischemia and 3 h reperfusion; Group C: 6 h ischemia and 3 h reperfusion; Group D: 12 h ischemia and 3 h reperfusion. Different concentration of sodium azide and glucose oxidase and catalyse system (10:1 ratio of GOX (3 mM/s) and catalase (998 s^{-1}) were used to induce ischemia reperfusion injury to the cells. Reperfusion time was fixed for 3 h where as ischemic time was varied to standardized ischemia reperfusion injury. Results are expressed as mean±SD of *n*=4-6 independent assays. (*) *P*<0.05, statistically different from the control group

TABLE 5: SULFORHODAMINE B AND CRYSTAL VIOLET CYTOTOXICITY ASSAY: ANEROBIC CHAMBER MEDIATED ISCHEMIA REPERFUSION

Control	3 h Isc+3 h I/R	6 h Isc+3 h I/R	12 h Isc+3 h I/R	24 h Isc+3 h I/R
	Sulforhodamine assay			
4772.7±122	4644.5±156		*3289.4±156	*2758.9±166
	Crystal violet assay			
0.9943±0.06	0.9676±0.05	0.8428±0.06	*0.6853±0.07	*0.5409±0.05

Ischemia reperfusion was created by placing the cells in anaerobic chamber for different time interval and the injury was assessed by sulforhodamine B and crystal violet assay. Results are expressed as mean±SD of *n*=4-6 independent assays. (*) *P*<0.05, statistically different from the control group

TABLE 6: EFFECT OF HYDROGEN SULFIDE AS ANTIISCHEMIA REPERFUSION AGENT

Ischemic agent	C	I/R	NaCl (10 mM)	NaSH (1 mM)
Sulforhodamine assay				
Rotenone	5029±161	*3771±126	*3922±142	4526±133
Antimycin	4842±150	*2670±143	*2597±157	4360±142
Sodium Azide	4662±185	*3252±132	*3391±145	4195±122
Enzymatic system (glucose oxidase and catalase system)	4459±178	*2771±133	*2864±123	4208±136
Anaerobic Chamber	4772±123	4104±151	4200±171	4295±156
Crystal violet assay				
Rotenone	1.047±0.037	*0.785±0.033	*0.817±0.052	0.942±0.017
Antimycin	1.008±0.017	*0.556±0.052	*0.541±0.041	0.908±0.033
Sodium Azide	0.971±0.023	*0.677±0.032	*0.706±0.036	0.874±0.062
Enzymatic system (glucose oxidase and catalase system)	0.929±0.041	*0.577±0.041	*0.596±0.027	0.876±0.053
Anaerobic Chamber	0.994±0.052	0.855±0.052	0.875±0.017	0.894±0.032

Hydrogen sulfide donor namely NaSH (1 mM) was added to the cells 1 h prior to the induction of ischemia and reperfusion. NaCl (10 mM) was used as negative control for the study. Results are expressed as mean±SD of n=4-6 independent assays. (*) P<0.05, statistically different from the control group

CAT and antimycin to create ischemia. Based on the above observations, we conclude that cell based model for renal ischemia reperfusion can be studied with the help of chemical inducer, enzymatic method and anaerobic chamber incubation. Among these, enzymatic method is most suitable for short term ischemia reperfusion study.

ACKNOWLEDGEMENTS

The authors thank SASTRA University management especially honourable Vice Chancellor, for supporting the study by sanctioning the grants from Prof. T. R. Rajagopalan Research Fund, SASTRA University, India

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Accepted 30 June 2014
Revised 27 June 2014
Received 04 August 2013
Indian J Pharm Sci 2014;76(4):348-353