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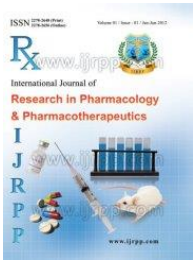

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# A study of role of allopurinol in prevention of ischemic myocardial injury in rabbits

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## ABSTRACT

The pathogenic process following ischemia of heart and brain is responsible a toll of deaths world over. The radicals like superoxide, hydroxyl radical, alkoxy or lipoxy radical can induce a huge damage over cardiovascular system. Xanthine oxidase mediated production of superoxide radicals has been accepted mostly as the key step in production of oxy-radicals mediated myocardial damage. Allopurinol, a xanthine oxidase inhibitor and inhibitor of uric acid formation has been selected as one of the antioxidants in this study. Allopurinol showed decreased mortality in the test animals. It has also reduced the ST elevation in post- reperfusion stage. It has also attenuated the infarct size significantly ($p<0.05$) owing to its capability to prevent excess formation of oxyradicals. It has also been substantiated that not acutely but chronically administered xanthine oxidase inhibitors may have its more profound anti-oxidant effect.

**Keywords-** Reperfusion, Allopurinol, Antioxidant, Xanthine oxidase

## INTRODUCTION

Ischemia induced pathogenic processes of heart and brain are responsible for a great number of deaths world over. A relatively subtle indicator of ischemic injury to a tissue is enhanced capillary permeability resulting in edema. Recent evidence suggests that oxygen derived free-radicals may be abundantly produced in ischaemic tissues, playing its part in the damage.

### Free radical

A free radical is simply a molecule containing an odd number of electrons and thus may be considered to contain an open bond or a half bond, rendering it

chemically reactive. If two radicals react both are eliminated but if a radical reacts with a non radical then a new radical may be formed. This characteristic feature allows free radicals to participate in chain reactions.

## Role of radicals in ischemia

The free radical scavengers led some researchers to believe a role of free radical metabolism in ischaemic injury to the heart. Studies however have shown that the capillary I/V administration of superoxide dismutases provided nearly complete protection against increased permeability in ischaemic ileum in cats.

Major oxygen radicals are-

## Superoxide Anion: 02·

If a single electron is accepted by ground state 02 molecule the product is superoxide radical 02•- Cells known to produce 02 $^{-}$ , include neutrophils, monocytes, macrophages and eosinophils. The major source of superoxide in post- ischemic tissues appears to be the enzyme xanthine oxidase (XD) and is widely distributed in most tissues like intestine, lung and liver in most species. The enzyme is synthesized as xanthine dehydrogenase and this dehydrogenase reduce $NAD^{+}$ .

## Hydroxyl radical: OH

Haemolytic fission of the O-O bond in $H_2O_2$ produces two hydroxyl radicals This can be achieved by heat or ionising radiation. A simple mixture of H2O2 and an iron salt also form the (OH •) radical.

The overall sum of these, in an iron catalysed decomposition of $H_2O_2$ is:-

$$2\,H_2O_2 \xrightarrow{\text{Fe salt}} 2H_2O + O^{2-}$$

## Alkoxy or Lipoxy Radicals (Lipid 0•)

It has been observed that O2 generating systems stimulate the peroxidation of fatty acids or of membranes.

Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from membrane lipids.

$Fe^{++}$ combine with lipid hydroperoxides to give alkoxy radicals. Indicator of oxidative stress and lipid perioxidation of unsaturated fatty acids is malonyldialdehyde (MDA).

# OXYGEN RADICAL SCAVENGERS

There are, however, several systems that contribute to termination or inactivation of free radical reactions These include

## Endogenous or Exogenous oxygen radicals scavenger

These are Vitamin E which may also be called as anti lipid peroxidants .Other scavengers are sulphydril containing compound such as cysteine, glutathione and D-penicillamine; serum proteins such as ceruloplasmin and transferrin.

## Enzymes

a) Superoxide dismutase: It converts superoxide to $H_20_2$

b) Catalase- It is present in peroxisomes and decomposes $H_2O_2$

c) Glutathione peroxidase : It catalyses the reduced glutathione (GSH) to release hydrogen from -SH to a hydroxyl radical thus forming oxidised glutathione (GSSG)

## Exogenous Substances

1. Xanthine Oxidase inhibitor: Allopurinol blocks the formation of superoxide radicals from xanthine and oxygen. This is also used here in this study.
2. Protease Inhibitors: Saquinavir blocks the conversion of dehydrogenase to xanthine oxidase.
3. Iron Chelators: Desferoxaminee chelate the ferrous ions from the ischaemic area so that it cannot react with $H_20_2$ to form OH • radicals.
4. Captopril : ACE Inhibitors
5. N-acetyl Cysteine - GSH precursor
6. Inhibitors of Lipid Peroxidation.

a) Beta Carotenes 20 Mg/Kg. Orally

b) Phenol antioxidants (Konocalova G. G. et al,19S9)

- Dibunol, ionol
- Ceruloplasmin, 50 mg/Kg.

## Clinical Relevance

In the context of ischemic heart disease the importance of oxygen radical lies in the possibility of limiting infarct size by the action of the radical scavengers, such as superoxide dismutase, catalase, and glutathione reductase. The role of oxygen free radicals in causing injury during myocardial

reperfusion and myocardial stunning has been extensively studied in vitro. This study has been performed to observe the protective role of oxygen radical scavengers on cardiac physiology as well as body physiology during myocardial ischemia.

## MATERIAL AND METHODS

### Selection and Care of animals

Male albino rabbits weighing 900 gms to l.3 kg were selected for the present study, which has been conducted in strict accordance with the GUIDE FOR THE CARE AND USE LAB ANIMALS by National Institute of Health. All rabbits received standard diet ad libitum before all experimentation.

### Anaesthesia

All animals were anaesthetized by injection of sodium pentobarbitone 30mg/kg dissolved as 1% solution in warm saline, given intraperitoneally or intravenously.

### Experiment Protocol

A total of 10 albino rabbits were taken and then divided into two groups of 5 each. Control group was given glucose 10mg OD, while the test group was fed with allopurinol 10mg/kg/day.

### Recording of Blood Pressure & ECG

The blood pressure tracings and the electrocardiogram (EKG) were recorded on a multichannel recorder (biophysioscillograph - Polyrite, India). The B. P. was recorded from the femoral artery of the animal by using pre-calibrated pressure transducer - filled with heparinised saline. 1 mm of rise on paper corresponds to pressure 20 mm of mercury. The EKG was recorded by placing suitable disc electrodes on the naked skin of all the 4 limbs and throughout the experiment records were taken on standard limb lead II. Recordings were done on polygraph with paper speed of 50mm/min.

### Tracheal Intubation

A mid line incision extending from superior end of the larynx to the suprasternal notch was given on the ventral aspect of the neck. The muscles and the fascia were retracted to sides and trachea was explored. A polyvinyl Tracheal cannula was pushed into the trachea after making an incision between the rings and secured with the silk threads. The cannula was connected to positive pressure animal respirator set at 20 ml/kg tidal volume at the rate of 50/min. The animal was kept on ventilation for about 10 minutes before the thoracotomy was performed and to ensure the normal respiratory hemodynamics. The pH of blood was intermittently monitored to keep it in the optimal range.

### Femoral Cannulation

Inguinal region of the animal was exposed and femoral sheath was identified and femoral artery and femoral vein were separated out. Both were cannulated with polyvinyl tubings and were secured by silk thread. Venous cannula was further used for drug administration and arterial cannula was connected to the pressure transducer for blood pressure recording.

### Coronary Ligation

A left intercostal incision extending from margin of sternum to the vertebral column was given in the 4th intercostal space and the ribs were retracted with the rib retractor.

The lungs were also retracted to expose the posterolateral aspect of the left ventricle and the aortic root. The anterior descending branch of left coronary artery was identified and ligated using 5(0) round bodied curved needle with silk thread by a slip ligature so that whenever desired the reperfusion can be done. The resulting coronary ischaemia, was maintained for 30 minutes, which was indicated by pallor and cyanosis of the myocardium. **Hoshida et al, (1995)** [5]

Throughout the experiment ECG was recorded continuously to catch the period of maximum arrhythmias and to record the ischaemic changes indicated by the ST elevation of more than 0.5 millivolts.

### Reperfusion

The reperfusion was achieved by releasing the slip ligature 30 minutes after CAL(coronary artery ligation) indicated by hyperemia of the ischemic zone. Subsequent changes were recorded on electrocardiogram and observation done at 30 and after 60 mins after reperfusion.

### Histopathological analysis

After 1 hour of the reperfusion, the rabbit was sacrificed by giving overdose of pentobarbitone sodium. Heart was resected out with the aorta, with

ligature in position and was perfused with saline through the aorta to wash out the residual blood. Reocclusion of the coronary artery was done by same silk thread which was left in the same place. Evan's blue 2% was introduced through coronary sinus to estimate the area perfused by the occluded artery and was termed as **ischemic region. (Hoshida et at, 1995)** [5]

The left ventricle was then cut into six pieces, vertically in the apex base axis, which were then incubated with 1% triphenyl tetrazolium chloride at 37ºC for 10 minutes, to stain the non ischaemic region. The unstained zone of the tissue was termed as "infarct region".

The ischaemic, infarct and the non ischaemic regions were separated with scissors and weighed and the "area at risk" and the "infarct size" were calculated as follows **(Kuzuya et al, 1990)**

$$\text{Area at risk} - \frac{\text{Weight of Ischaemic Region in gms}}{\text{Weight of Left Ventricle (gms)}} \times 100$$

$$\text{Infarct size} - \frac{\text{Weight of Infarct region in gms}}{\text{Weight of Ischemic Region (gms)}} \times 100$$

## Source of Drugs

Allopurinol as Tab. Zyloric available as 100 mg. Allopurinol Tablet (Burrough'sWellcome).

## Statistical Analysis

The data of area at risk and infarct size were expressed as percentage. The differences in ST elevation were compared in millivolts and expressed as mean ± standard error of mean. Difference between means of various sets of observations of ST elevation were examined by student't' test at 95% level of confidence ($p < 0.05$). Difference between the standard error of proportions of various sets of results in estimation of mortality, area at risk and infarct size were tested for significance by 'z' test.

## RESULTS

Following parameters were observed in order to observe the efficacy of allopurinol as an antioxidant:-

1. ST Elevation in millivolts (mv.).
2. Histopathological analysis
   - -Area at risk as percentage.
   - -Infarct size as percentage

ST elevation is an electrophysiological indicator of myocardial damage due to infarction.

Two sets of ST elevation at specific periods were included in the observations-

(i) Before reperfusion i.e. during the phase of coronary ischemia of myocardial membranes. (Table 1)

(ii) 60 min. after reperfusion when major inflow of free radicals has taken place and maximum amount of damage due to lipid peroxidation has been done by them during 60 minutes. (Table 2)

**Table1- Significance of difference in ST segment elevation before reperfusion**

| Group 'n' | Mean ST Elevation (mv) | Standard Error of Mean | 't' value | 'p' value |
|---|---|---|---|---|
| Control (3) | 1.50 | + 0.08 | 2.5 | >0.05 |
| Test (4) | 1.25 | +0.06 | | |

**Table 2- Significance of difference in ST segment elevation after 60 minutes of reperfusion**

| Group 'n' | Mean ST Elevation (mv) | Standard Error of Mean | 't' value | 'p' value |
|---|---|---|---|---|
| Control (1) | 1.50 | + 0.20 | 17.57 | <0.01 |
| Test (3) | 0.50 | +0.13 | | |

In allopurinol group although there was lesser grade of ST elevation in test group but it could not attain statistically significant levels (C 1.5 ± 0.08; T 1.25 ± 0.06; $p > 0.05$).

## Area at risk

Area at risk was measured after histopathological staining indicated the area that suffered ischemia but not myocardial damage. It was observed that there was no significant difference in area at risk regarding test animals. (Table 3)

**Table 3- Significance of difference of proportion in area at risk in different groups**

| Group 'n' | Area at risk (%) | Standard Error of Mean | 'z' value | 'p' value |
|---|---|---|---|---|
| Control (4) | 50% | + 2.8% | 0.05 | >0.05 |
| Test (4) | 47% | + 2.4% | | |

## Infarct Size

Infarct size denotes the amount of myocardium which is actually damaged. In Allopurinol group there was significant reduction in infarct size in test animals ($p < 0.05$). **(Table 4)**

**Table 4- Significance of Difference of Proportion in Infarct Size in Various Groups**

| Group (n) | | Infarct Size (%) | Standard error of proportion | "z" value | "p" value |
|---|---|---|---|---|---|
| Control | (4) | 95.5 | +2.8% | 2.16 | <0.05 |
| Test | (4) | 15.0 | +2.4% | | |

# DISCUSSION

There has always been an endeavor to decrease mortality and morbidity in acute myocardial infarction by limiting the pathogenic processes that result after myocardial ischemia. Various interventions have been directed towards controlling the ischemia and enhance the recirculation of myocardium. But very few steps have been taken towards controlling the cellular damage at molecular level, the seeds of which are already sown by the hypoxic insult at the time of coronary ischemia. Free radical, a highly reactive moiety with unpaired electron in outer shell are abundantly produced at the time of ischemia, and become the major source of cell damage the recent fact which has also been indicated by our experimental study.

Our work was directed to neutrilise or scavenges these free radicals by allopurinol as an antioxidant. After an episode of myocardial ischaemia the event of hypoxia converts xanthine dehydrogenase enzyme in cardiomyocyte to xanthine oxidase. The second event of ATP accumulation enhances the production of xanthine by degradation. Xanthine in presence of high concentration of oxygen obtained from reperfusion, forms superoxide free radicals. This is the key step in the production of oxyradical mediated myocardial damage.

Allopurinol inhibits xanthine oxidase and retards formation of superoxide radicals during reperfusion. We have employed male albino rabbits in our experiments because of the extremely minimal native collateral supply in this species (Maxwell et at, 1987) and also histological and anatomical similarity with human coronary artery to a greater extent.

A short acting barbiturate, sodium pentobarbitone, used in the present study, has been widely used as a standard general anaesthetic for rabbits. (Hoshida et al, 1995) As compared to other experirnental anaesthetic agents like chloralose and sodium pentothal, when given in appropriate dose (30mg/Kg./ dose i.v) pentobarbitone does not interfere with the hemodynarnic parameters like blood pressure and heart rate and causes minimal change in respiration.

There are invasive and non-invasive techniques of producing coronary ischeamia. One of the non-invasive techniques accepted is by injection of isoproterenol rng/kg., every 24 hrs. interval which produces ischaemic changes in ECG (Kela et al, 1980). This technique has certain short comings viz. inability to produce ischemic damage by reperfusion, whereas reperfusion has been implicated as the main factor for myocardial damage in an ischaemic episode and difficulty in assessment of area at risk and infarct size as isoprotenol produces global ischaemia. Hence, we have employed the accepted invasive technique for producing myocardial ischaemia by the ligation of LAD branch of coronary artery (Hoshida et al, 1995). In this technique, only an anticipated area is infarcted which can be separated out by the staining of the vasculature.

## ECG Changes in Experimental Myodcardial Ischemia

Three pathophysiologic events occur, either in sequence or simultaneously namely ischemia, injury due to reperfusion and infarction. ECG manifestations of these processes include changes in T wave during ischemia, ST segment changes during injury and changes in QRS complex during infarction.

The earliest T wave change of acute ischemia is tall peaked T wave followed later by symmetrically inverted T wave. Myocardial injury produces ST segment elevation of more than 0.5 millivolts. As the period of acute injury resolves, the ST segment returns to baseline. Pathologic Q waves are the QRS manifestation of a transmural myocardial infarction and necrotic damage. Inverted T waves and pathologic Q waves are more or less permanent and persist till the end of the experiment. We did not use T waves as our parameters as it varies with duration of ischemia. It takes a longer time to produce pathologic Q wave invariably in experimental models. So we employed ST segment as our parameter which is universally accepted as a marker for myocardial injury. (Hoshida et al, 1995)

## Histopathological Parameters

Estimation of "area at risk" and "infarct size" have been accepted as the most sensitive and accurate parameters to measure myocardial damage in experimental models. By this experimental technique, unless a method is devised to produce mycardial ischaemia in same way as atherospasmogenic mechanisms in human ischaemic heart diseases. So, in our study also the drug could not improve the "area at risk" in test animals significantly which is in accordance with similar findings using the same parameter of area at risk (Hoshida et al, 1995).

In our study we ligated the LAD branch of coronary artery more distally to achieve the area at risk between 45% to 75%. As discussed before, infarct size is the assessment of necrosed tissue in the area at risk owing to cytokine and free radical mediated cellular injury, stimulated by anoxia and hyperoxia. This is the ultimate and most accurate parameter to quantitatively assess the grade of myocardial infarction and compare the effects of various drugs which neutralise cytokine and free radical induced this myocardial damage. (Banka et ale, 1981; Fishbein et al., 1995). We have also found significant results in regards with infarct size.

## Role of Allopurinol Used In This Study on Mortality, ST Elevation and Infarct Size

Xanthine oxidase mediated production of superoxide radicals has been accepted mostly as the key step in production of oxy-radicals mediated myocardial damage (Mc. Cord J.M., 1988). Therefore, allopurinol, a xanthine oxidase inhibitor and clinically used as inhibitor of uric acid formation has been selected as one of the antioxidants in this study. Recently, Chambers et al., (1985) have also used allopurinol with promising results i.e. it has reduced zone of infarction significantly in experimental ischemia.

Allopurinol reduced the ST elevation in post-reperfusion stage more significantly than other antioxidants in our study ($C=1.5$ $t=0.5$ $p<0.01$), as it prevents excess formation of superoxide radicals by inhibiting xanthine oxidase, when major oxygen inflow is there during reperfusion. This step is the key event in free radical induced cell injury. It is not protective in pre- reperfusion stage as high oxygen concentration is not available to combine with xanthine and produce superoxide radicals significantly. So, if allopurinol is administered acutely just before or during reperfusion, it could have its maximum effects in preventing formation of superoxide radicals. This step was beyond the scope of this study as parenteral preparation of allopurinol is not available.

Allopurinol attenuated the infarct size significantly ($p<0.05$) owing to its capability to prevent excess formation of oxyradicals. Allopurinol could not reduce ST elevation in the pre reperfusion stage as there were insignificant production of oxyfree radicals, because of low availability of oxygen. Therefore, the step of conversion of xanthine to superoxide free radicals could not be carried out effectively. Hence there is no role of xanthine oxidase inhibitor at this stage. Attenuation of ST elevation along with infarct size was in accordance with the study of Chamber et al., (1985). On the contrary only one study of **Downey J. M. et al., (1987**) does not correlate with our work. This study states that xanthine oxidase is not a source of free radicals in ischaemic rabbit heart, because they could not detect xanthine oxidase and dehydrogenase in rabbit's heart before and during ischaemia. This was

further supported by an ischaemic rabbit's heart model in which they found that giving allopurinol 75mg PO 24 hrs. before the experiment and 30 mg./kg 5 minutes before occlusion did not produce any significant change in infarct size as compared to the control (control 67.5 ± 3.8%; Allopurinol 65.8 ± 8.7%), while a combination of enzymes superoxide dismutase and catalase reduced the infarct size to 35.4 ± 3.3%. They have found detectable changes of xanthine oxidase activity in rat's myocardium during ischaemia (6% activity in non- ischaemic stage to 25% of activity in ischaemic stage). Also the co-author of this work Chambers et al. (1985) in a separate study have found that xanthine oxidase levels increased to 300% during ischaemia in dogs and allopurinol reduces the infarct size significantly. So there does not seem to be any reason of xanthine oxidase activity to be absent in rabbits and findings of **Downey et al, (1987)** may be limited to some specific species of rabbits in which xanthine oxidase deficiency is genetically and racially predetermined and is subject to further evaluation by larger sample of different species of rabbits all over the world. The difference in the findings of infarct size reducing potential of allopurinol in the work of Downey et al (1987) and our study may also be due to the use of allopurinol in a lower dose by Downey et al (1987) (75mg. Vs. 100mg.) and for a lesser duration (24 hrs. prior to ischaemia vs. 15 days prior to ischaemia). However due to non availability of parenteral preparation of allopurinol we could not use it 5 minutes before ischaemia. But chronically administered xanthine oxidase inhibitors may have its more profound anti-oxidant effect.

## CONCLUSION

With the results in the present study we conclude that the coronary ligation of LAD branch produces detectable ischaemia in the rabbit myocardium and the use of allopurinol offers myocardial protection by limiting the infarct size as well as bringing about improvement in ST changes in electrocardiogram. However these protective changes are phase related. Allopurinol exerts its cardio protective antioxidant role mainly in the post-reperfusion stage. The possible mechanism of the effect of allopurinol in the present study seems to be that allopurinol acts by inhibiting key step in the cascade of free radical mediated cardiac damage i.e. the production of superoxide radicals of xanthine with oxygen during reperfusion, by action of xanthine oxidase, thus it bring down ST elevation and infarct size effectively but only during post-reperfusion stage, as maximum superoxide radicals are formed during the time of reperfusion and allopurinol decrease its levels only in post-reperfusion stage.

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