Infarct size limitation by the xanthine oxidase inhibitor, allopurinol, in closed-chest dogs with small infarcts

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SUMMARY The present study was designed to evaluate the ability of allopurinol to limit infarct size following permanent coronary occlusion in the greyhound. Coronary occlusion was produced by injecting 2.5 mm plastic beads into the coronary artery of the closed chest dog. Non-perfused myocardium, the area at risk, was visualised by autoradiography of 14C- cerium labelled microspheres which were infused immediately following coronary embolization. The treated dogs (n = 12) received 400 mg of allopurinol orally one day before surgery. A 25 mg·kg·1 bolus was administered (iv) immediately before occlusion, and repeated every 8 h. 11 dogs served as controls. After 24 h, the dogs were killed and the hearts were sliced into 5.0 mm transverse sections. The infarcted myocardium was visualised by triphenyl tetrazolium chloride staining. The percentage of the risk zone which evolved to infarct was calculated. This percentage was 18.1 ± 3.95% in the allopurinol group vs. 58.4 ± 2.81% in the control group (p < 0.001). We conclude that allopurinol is a potent drug for the limitation of infarct size in the dog with permanent coronary occlusion.

Recent studies implicate oxygen derived free radicals as contributing to the injury incurred by the ischaemic heart. Although several sources of free radical have been proposed for ischaemic myocardium, recent evidence from this laboratory implicates the enzyme xanthine oxidase as a major source. In that study we measured infarct size in dogs experiencing a period of ischaemia followed by reperfusion. The rationale for a reperfusion protocol was that upon reperfusion, oxygen would become available to the enzyme to produce a burst of free radicals. That scenario probably accurately describes events in the deeply ischaemic subendocardium where collateral flow is minimal. But what about the less ischaemic subepicardial regions where salvage is most likely to occur? Is free radical production limited to only the reperfusion period there as well? Simple calculations indicate that any regions experiencing 10% or more residual perfusion will have sufficient oxygen delivery to saturate the xanthine oxidase reaction. Thus, oxygen derived free radical production probably occurs all through the ischaemic period in much of the ischaemic zone. In the regions of the subecardium where residual flow is high, free radical generation may, in fact, be killing cells which otherwise might have had enough flow to survive. If that is the case, then free radical suppression would limit infarct size in permanent occlusion as well as reperfusion protocols.

The present study tests that hypothesis by observing the effect of allopurinol, an inhibitor of xanthine oxidase, on the size of infarcts resulting from permanently occluding a coronary branch in dogs.

Methods

EXPERIMENTAL PROCEDURE

Adult greyhounds of either sex, 19 to 32 kg, were used. The preparation was similar, except for minor modification, to that described in detail previously. Briefly, the dogs were anaesthetised with sodium pentobarbital (30 mg·kg·1 body weight, iv) and
received $1 \times 10^6$ units of penicillin (im) to combat sepsis. All experimental procedures were performed under sterile conditions. The animals were intubated and respired spontaneously with room air. An E-Z catheter (Parke-Davis) was placed percutaneously in the jugular vein for drug administration. The left artery was exposed via a midline cervical incision, and a coronary cannula with a pressure transducer attached to a side arm was inserted. The cannula was advanced into the ascending aorta and inserted into the coronary ostium. Insertion was confirmed when pressure at the tip of the cannula went from aortic to a distal coronary pressure of about 25 mmHg. Coronary occlusion was produced by injecting two plastic beads, 2.5 mm in diameter, through the cannula to embolise a coronary artery. Immediately after coronary embolisation, the cannula was manipulated into the left ventricle. Approximately $2.0 \times 10^7$ radioactivity microspheres, $15.6 \pm 3$ μm in diameter and labelled with $^{141}$ Cesium (3M Co, St Paul, MN), were injected into the ventricle. The cannula was then removed, the carotid artery cut-down repaired and the dogs returned to the kennel. All dogs received an intravenous bolus infusion of heparin (7000 units) every 8 h to prevent clot formation proximal to the emboli.

To evaluate the ability of allopurinol to salvage the myocardium at risk, the dogs were randomly divided into two groups: 1) Control group (13 dogs): These subjects were subjected to ischaemia and received a 20 ml bolus of saline every 8 h; 2) Allopurinol group (19 dogs): These experienced ischaemia and received 400 mg of allopurinol in their food 24 h before surgery. Immediately after anaesthesia, a 25 mg*kg$^{-1}$ bolus of allopurinol was administered intravenously over 10 min and repeated every 8 h. Allopurinol (courtesy of Burroughs Wellcome) for intravenous infusion was prepared in saline as described by Shantney et al.$^6$ Sterility was maintained by injecting the drug through a Millipore filter.

MEASUREMENT OF PLASMA LEVELS OF ALLOPURINOL AND OXYPURINOL
Blood samples were drawn from 4 of the dogs in the treatment group in order to determine the plasma levels of allopurinol and its metabolite oxypurinol. Samples were drawn prior to injection of drug and at 5 min, 2, 4, and 6, h post-injection. The blood samples were centrifuged for 15 min at 1000 $\times$ g to remove the formed elements and the blood proteins were removed with Trichloro-acetic acid. Drug levels were determined by high pressure liquid chromatography.

MEASUREMENT OF INFARCT SIZE AND REGION AT RISK
Twenty-four hours following embolisation, the dogs were killed with $60$ mg$\cdot$kg$^{-1}$ sodium pentobarbital, and the hearts were excised, weighed, and put into a freezer. At that time the location of the beads embolising the coronary arteries was noted. Once the hearts were frozen, they were sliced into 5.0 mm thick transverse sections.

The tissue slices were rinsed in cold water to remove blood and residue. These slices were then placed in a solution of 2, 3, 5-triphenyl tetrazolium chloride (TPT) (100 mg, Sigma Chem. Co., St Louis, MO), and 300 ml of phosphate buffer (32 mol$\cdot$litre$^{-1}$, pH 7.4) for a 20 min incubation at 37°C. At the end of the incubation period, the slices were dipped in 10% formalin, which not only fixed the tissue, but also enhanced the colour contrast.

TPT, a formazan stain for myocardial dehydrogenase enzyme activity, delineates infarcted myocardium.$^6, 9$ The tissue slices were then placed on an acrylic sheet, basal surface up, and covered by a layer of Saran Wrap. This was followed by a sheet of Kodak AR X-ray film. Finally, a second acrylic sheet was placed on top of the film and the two acrylic sheets were kept tightly in place by a wooden press. This package was put in a light-proof box and kept in a freezer for the exposure period, from 24 to 72 h, depending on the radioactivity of microspheres. The film was then removed and developed. The $^{141}$ Cesium-labelled microspheres produced sharp spots in the film. Therefore, the autoradiogram revealed the area of preserved blood flow around the field of the embolised artery and defined the "cold area" as the risk zone.

Areas of the basal surface of the infarct and risk zone were measured by computerised planimetry of each slice by an investigator not informed as to the origin of the material. The volume of the infarct and the risk zone were calculated by multiplying by the thickness (5.0 mm) of the slice. The values of the sequential slices were summed to provide the total volumes for each heart. The percentage of the region at risk which was infarcted was then determined. To further normalise the sizes of the infarct and risk zones, infarct volumes were also multiplied by 1.05 (specific gravity of the tissue) and were expressed as a percentage of total ventricular weight.

Results
A total of 35 greyhound dogs were used for this study. There were three deaths as a consequence of aortic rupture prior to coronary embolisation. Two dogs in the control group and four dogs in the allopurinol group died post operative, probably of a lethal arrhythmia. These dogs were excluded from this study because of unsatisfactory autoradiograms. At the conclusion of the investigation, there were 11 dogs in the control group and 12 in the drug group.
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- **MEAN**: Mean values for each group.
- **SE**: Standard error of the mean.
- **05-04**: Date range from May 4th to May 9th.
- **04-29**: Date range from April 29th to May 4th.
- **05-09**: Date range from May 9th to May 17th.
- **03-10**: Date range from March 10th to May 9th.
- **05-17**: Date range from May 17th to May 30th.
- **05-05**: Date range from May 5th to May 9th.

### Notes:
- The data represents the volume of the infarct in cm³. INFARCT VOLUME = INFARCT RISK (FIRST ORDER). Volumes were compared between the control and experimental groups using an ANOVA test. The results indicate a significant difference between the groups at the p<0.005 level. The drug significantly different from the control group at the p<0.005 level.

### References:
Allopurinol and infarct size

HAEMODYNAMIC DATA
Values for heart rate, aortic pressure and left ventricular end-diastolic pressure are summarised in table 1. These haemodynamic parameters were not different between the groups before coronary embolisation. Intravenous administration of allopurinol caused no significant haemodynamic changes. Following embolisation, however, aortic pressure fell by a similar degree in the two groups.

TISSUE DATA
Table 1 summarises the results. The upper half of the table is the drug group and the lower half is the control group. Notice that the drug group is segregated into two sections. The last two rows of the drug group present data from two dogs which had abnormally small risk zones (note the column headed risk). Since it has been proposed that the size of the risk zone is an important determinant of the percentage of the risk zone which infarcts, we analysed the drug group both with these two dogs included and without them. The first group of means and standard errors are for the entire data set while those below them represent only the first ten drug dogs. The lower half of the table summarises the control dogs.

No other differences were found between the two groups, even when the two dogs having abnormally small risk zones were included in the analysis. Using the infarct as a percentage of the risk zone, probably the most accurate expression of infarct size, it becomes clear that allopurinol limited infarct size to only about 1/5 of that in the nontreated group (58.4% vs 18.1%).

The data appear in graphic form in the fig. All 12 of the drug treated dogs are represented in the fig. The two outilers, however, are represented as open circles. The means and confidence limits in all three panels are based on all 12 dogs. Statistics for the selected group are presented in table 1 only. Again a striking limitation of infarct size is apparent in the drug group. Analysis of the tissue and the autoradiogram revealed that virtually all of the salvage had occurred at the subepicardium.

PLASMA LEVELS OF ALLOPURINOL AND OXYPURINOL
Table 2 shows the plasma concentrations of allopurinol and its metabolite oxypurinol in 4 dogs over the first drug cycle. Allopurinol levels started out high and progressively fell through the cycle to about 1/5 of the 25 \( \mu \text{g} \cdot \text{ml}^{-1} \) starting value. 25 \( \mu \text{g} \cdot \text{ml}^{-1} \) is based on the starting dose of 25 mg \( \text{kg}^{-1} \). It was assumed that allopurinol became uniformly distributed in the plasma and tissues. Unfortunately, residual drug in the jugular catheter caused gross contamination of the 5 min allopurinol determinations and they were thus, unavailable to confirm that assumption. Oxypurinol levels rose steadily through the cycle from a low value of 0.6 \( \mu \text{g} \cdot \text{ml}^{-1} \) at 5 min to 2.6 \( \mu \text{g} \cdot \text{ml}^{-1} \) at 6 h. In no dog did the sum of allopurinol plus oxypurinol fall below 1.0 \( \mu \text{g} \cdot \text{ml}^{-1} \). That level is considered sufficient to effectively inhibit the xanthine oxidase enzyme.

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<th>Time</th>
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<th>Oxypurinol</th>
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<td>Before IV injection</td>
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<td>0.6–5.0 ( \mu \text{g} \cdot \text{ml}^{-1} )</td>
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<tr>
<td>After IV injection</td>
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<td>5 min</td>
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<tr>
<td>2 h</td>
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<td>4 h</td>
<td>9.4±2.0</td>
<td>(0.8–16.8)</td>
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<tr>
<td>6 h</td>
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Values are presented as mean ± standard error. The range appears in parentheses.
Discussion

The results indicate the allopurinol pretreatment caused a remarkable limitation of infarct size in these dogs. The infarcts in the treatment group were only 1/3 the size of the controls. Although previous studies indicated that allopurinol could limit infarct size in a reperfusion setting, the present findings indicate that it is not a type of collateral flow. Before we discuss the possible mechanisms responsible for this salvage we must consider possible artifacts.

In the present study, we measured infarct size with tetrazolium staining. Tetrazolium stain has been criticized recently on the grounds that it underestimates infarct size. Loss of dehydrogenase enzymes and cofactor by dead myocytes reportedly cannot be complete when tetrazolium evaluation is made only 6 h following coronary occlusion. At that time, much of the ischemic tissue stained as living is actually dead by histological criteria. Furthermore, we have found that one drug, flubiprofen, can indeed delay the rate of early enzyme loss and yet not affect the long term loss. To avoid this problem, we performed our evaluation 24 h after coronary occlusion. Due to the kinetics of enzyme loss by necrotic myocardium, it is very unlikely that any transplanted dead and still active enzymes and cofactor after 24 h. It seems equally unlikely that the process could be delayed by allopurinol to the point of producing a serious artefact, although we acknowledge that such a possibility cannot be completely excluded at this time. The only conclusive proof that the "salvaged" tissue was truly preserved will be a demonstration that its mechanical function was retained. This must obviously be the object of future study.

Another possible artifact with this preparation might be that the drug could, somehow, cause the bead to migrate downstream between the time of injection of microspheres and the time of removal of the heart for evaluation. We checked for this possibility in a previous study with the powerful dilator, nifedipine and could not detect any bead movement. Such movement seems even more unlikely in the present study: a) this dose of the drug is not a coronary dilator in our experience, and b) drug was already present when the microspheres were injected.

The ability of allopurinol to limit infarct size could have been mediated by three possible mechanisms: Protecting the intracellular purine pool, increasing collateral blood flow by vasodilation, or prohibiting the formation of oxygen derived free radicals.

Ischaemia rapidly increases the dephosphorylation of high energy phosphates. This process continues with the further breakdown of nucleotides to nucleosides, terminating in the irreversible conversion of hypoxanthine to uric acid by xanthine oxidase. Conversion to uric acid causes the purine bases to become irreversibly lost from the nucleotide pool. It has been postulated that the nucleotide pool would be protected by allopurinol after periods of oxygen depletion. Kmetec et al reported that allopurinol increases the ratio of ATP + ADP to AMP in cultured heart cells. Recent reports also indicate that a beneficial effect is realised when the purine pool is replenished exogenously.

The arguments against this mechanism are twofold. First, the unphosphorylated purines, adenosine, inosine, hypoxanthine and xanthine, are all lipid soluble and can be washed out of the tissue. Since collateral flow persists in the ischemic tissue at a rate of 0.1 to 0.2 ml per minute per gram tissue this would result in a half-life of the nonphosphorylated purines in the tissue of only 3.5 to 7 min. Thus, blockade of xanthine oxidase alone would not be expected to keep the purines from being lost by the ischemic zone. Secondly, there is little evidence that the loss of unphosphorylated purines is the lethal event during ischemia.

Although the collateral circulation of dogs is variable from animal to animal, it has been well documented that the collateral flow is one of the major determinants of infarct size subsequent to coronary occlusion. Stanley et al compared the effects of allopurinol and nitroglycerin on the coronary resistance and coronary blood flow. They reported that allopurinol produced a greater and more prolonged dilatation in the coronary arteries than nitroglycerin. Arnold et al using dogs with 2 h coronary ligation investigated the effect of allopurinol on the collateralisation by examining a postmortem coronary angiogram. Allopurinol-treated dogs showed numerous small collaterals in the ischemic area which were not present in the control dogs. It was our experience, however, that allopurinol is not a vasoactive substance in this dose. For example, it had no effect on aortic pressure in this study and in a previous study with allopurinol where coronary flow was measured we found no effect on either flow to the normal regions or to collateral dependent tissue. Furthermore, the modest reduction in ischemic zone size (about 30%) reported by Arnold et al may have been a chance artifact as only 6 animals were measured in each group. Our opinion, then, is that augmentation in collateral flow represents the least likely explanation for allopurinol's effect.

It is our conclusion that allopurinol protected these hearts through free radical suppression. The ability of allopurinol to prevent free radical formation is based on the ability of xanthine oxidase to generate free radicals. Fridovich proposed that a biological system
which contained xanthine oxidase, hypoxanthine and/or xanthine is capable of producing the superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), both of them being cytotoxic. The competitive inhibition of xanthine oxidase by allopurinol could significantly reduce the levels of these two compounds, and thereby decrease the amount of tissue injury. Recently Parks et al.$^{25}$ demonstrated that pretreatment with either allopurinol or superoxide dismutase abolished the lesions in the mucosa produced by $3$ h of intestinal ischaemia. Their conclusion was that allopurinol was as effective as superoxide dismutase in limiting infarcts in reperfused myocardium.$^3$

Free radical damage might be expected to be more pronounced in a reperfusion model since oxygen is abundant during the reperfusion period. On the other hand, sufficient oxygen may be delivered by the collateral flow to provide enough substrate for the xanthine oxidase reaction to produce damage in the permanent occlusion model as well. In a recent study we assayed for xanthine oxidase activity in dog heart.$^3$

The enzyme was found to be in two forms. Well perfused tissue has the enzyme only as a dehydrogenase which uses NAD$^+$ as its electron acceptor and does not produce free radicals. In the ischaemic heart when we found that allopurinol was as effective as superoxide dismutase in limiting tissue injury. Recently Parks et al.$^{25}$ suggest that the collateral development which presumably occurs in the early stages of coronary artery disease may well augment collateral function such that a sizeable mass of tissue will fall in the salvage window in many MI patients as well.

The second criteria is that the drug be given before the injury becomes irreversible. Unfortunately, it is currently not possible to begin treatment in most coronary patients much earlier than $3$ h after the onset of the occlusion. We don’t know how late after occlusion the drug can be given and still realize a beneficial effect, but $3$ h is probably too late to realize much salvage. Because of the unique properties of allopurinol, however, that problem could be circumvented by using allopurinol as a prophylactic agent in high-risk populations. It is inexpensive and well tolerated in long term therapy.

**Clinical Implications**

Could allopurinol be of benefit to the nonreperfused coronary patient? The answer is probably yes if two key criteria are met. First the patient must have a significant quantity of tissue which falls in the "salvage window". That tissue must have enough flow such that it could survive if free radicals were suppressed. The dog, which has more coronary collaterals than does healthy man, obviously has a large amount of tissue which falls in the salvage window. Recent studies by Lee et al.$^{26}$ suggest that the collateral development which presumably occurs in the early stages of coronary artery disease may well augment collateral function such that a sizeable mass of tissue will fall in the salvage window in many MI patients as well.

**References**


