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Allopurinol inhibited free radical generation in the sheep large intestine

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Abstract

The contribution of oxygen-free radicals in intestinal ischaemia-reperfusion injury and the ability of allopurinol in preventing such injury was examined. Ischaemia of the sheep intestine was induced by occlusion of the apex of the caecum for 60 minutes. Allopurinol 50 mg/kg was administered I/V, 15 minutes prior to the induction of ischaemia. Full thickness tissue samples from caecal wall were removed from a normal caecum, 60 mintues after induction of ischaemia and 60 seconds, 60 mintues, and 8 hours following reperfusion.The bowel segments were immediately transferred to glutaraldehyde fixative, processed in a routine manner, and examined using transmission electron microscope. The ultrastructure of the samples treated with allopurinol were well preserved in comparison with those which had not been treated. It is speculated that allopurinol by allowing the conservation of the high pool of purine base allow the recycling of the subterminal hypoxanthine metabolites.

Key words : Allopurinol, Intestine, Ischaemia, Sheep.

Descriptions of morphologic and ultrastructural changes resulting from ischaemia and reperfusion injury in rats, dogs, cats and human are numerous (Chiu *et al.*, 1970; Haglund *et al.*, 1970; Redfors *et al.*, 1984; Megison *et al.*, 1990) but are limited in ruminants. The cellular damage seen after reperfusion of ischaemic tissue is much more severe than the damage seen after ischaemia alone (Granger *et al.*, 1986; Prezyklenk and Kloner 1989; Coetzee *et al.*, 1990). In reperfusion there is reintroduction of blood to the ischaemic tissue. Reestablishment of blood is necessary to save the tissue which is not irreversibly damaged during ischaemia. If the reintroduction of blood to the ischaemic tissue does not happen and the ischaemic period is prolonged, irreversible tissue damage will result. However, reperfusion is a "double edged sword" and while it salvages previously ischaemic tissue, it can lethally injure potentially other viable tissue (Braunwald and Kloner, 1985).

Reperfusion injury is caused by the generation of oxygen-free radicals. Oxygen-free radicals are characterised by having an unpaired electron in thier outer orbit which does not contribute to the bonding within the molecules and has a very short half life; these characteristics result in thier high reactivity (Donald, 1993).

Ischaemia and reperfusion injury may occur in the intestine in cases of strangulation, intussusception, volvulus and stenosis. The free apex of the caecum makes it more susceptible to some of these conditions.

Allopurinol, a xanthine oxidase inhibitor is said to be a very effective free radical scavenger

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provided it is given prior to reperfusion (Jaeschke and Mitchell, 1989). The study reported here was undertaken to extend our knowledge of ultrastructural changes that occur in the sheep caecum subjected to ischaemia and reperfusion and to observe the efficacy of allopurinol in ameliorating the ischaemic-reperfusion injury.

Materials and Methods

Fifty merino rams weighing 28-48kg (average 36kg) with an age range of 3-4 years were randomly divided into 5 groups (10 sheep each). The experimental animals were in healthy condition and acclimatised for at least one week prior to the commencement of the experiment. They were dosed with anthelmintic Ivermectin 200 µg/kg and were fed *ad lib* with chopped lucerne hay during the experiment.Group 1 was the control where no ischaemia or reperfusion was induced.

Animals of group 2,3,4 and 5 were fasted for 24 hours prior to surgery. Anaesthesia was induced with thiopentone sodium 25 mg/kg administered intravenously and maintained with halothane and oxygen in a semi- closed circle system. A right side laparotomy was performed with the animal in left lateral recumbency. After isolating the caecum, the blood supply to its apex was occluded using a pair of De Bakey clamps for 60 minutes.To determine any possible effect of potassium hydroxide (KOH) as the allopurinol solvent and to examine the efficacy of allopurinol in attenuating the reperfusion injury, groups 3,5 were subdivided into two groups (n=5) each. In one subgroup, potassium hydroxide was injected and in the other allopurinol was injected. In group 2, no drug was injected.

The allopurinol and potassium hydroxide (KOH) were injected intravenously. The right saphenous vein was catheterised and connected to an infusion pump to control the rate of injection.

In group 2, the first sample was taken after 60 minutes of ischaemia and the second sample after 60 seconds of reperfusion. In group 3,4 and 5 samples were taken after 60 minutes of ischaemia followed by 60 seconds, 60 minutes and 8 hours, of reperfusion, respectively.

Full thickness tissue samples from the caecal wall were immediately cut into small blocks of about 1mm using a single edged blade in a petri dish containing 3% glutaraldehyde in 0.066M cacodylate buffered with 2.5 mM calcium chloride at pH 7.2. Post fixation was done with 1% osmium tetroxide followed by dehydration in graded acetone series. After polymerisation, the tissues were cut on a Reichert Ultracut E microtome, using glass knives and then mounted on coated and uncoated copper grids stained with uranyl acetate followed by Reynold's lead citrate and viewed with Hitachi 800 transmission electron microscope (at 75-100 kv).

Results and Discussion

No signs of ischaemic-reperfusion injury were seen in the tissue samples taken from the control group (group 1, n=10). The mitochondria exhibited tightly packed cristae and a moderately electron-dense matrix containing small dense granules (fig.1). The nucleus showed a fine distribution of chromatin and the basement membrane was intact close to the epithelial cell layer (fig.2). The goblet cells showed normal secretory packages with a well preserved border between them (fig.3)

In the sample taken after 60 minutes of ischaemia and before releasing the clumps, except for minor changes (slight fragmentation of mitochondrial cristae) no particular changes were observed (fig.4). The first sign of injury in the sample taken after 60 seconds of reperfusion was disappearance of the dense matrix granules in the mitochondria. Mitochondrial cristae were broken and fragmented, amorphous (flocculent) densities were seen and the outer membrane was ruptured (fig.5). Goblet cells were swollen and fusion of their secretory packages were observed (fig.6).

Separation of basement membrane was clearly seen (fig.7) as compared with the normal control (fig.2). nuclei showed marginal clumping of chromatin granules (fig.7).

In tissue samples taken from the KOH injected subgrcup, tissue damage was clearly seen and was similar to that seen in group 2 where neither KOH nor allopurinol was injected. Mitochondria were badly damaged, flocculent densities were clearly seen and the cristae were fragmented. The basement membrane was separated and there was swelling and fusion of the secretory packages of the goblet cells.

In the allopurinol injected subgroup, the efficacy of allopurinol in preventing reperfusion injury was apparent. Mitochondria, goblet cells, basement membrane and other structures were preserved in thier normal state (fig. 8-9) and therefore were similar to the control group (figs.1-3).

Ultrastructural changes were observed in groups 4 and 5 where tissues had undergone 1 and 8 hours of reperfusion, respectively. The effects of reperfusion injury on the tissues after this period of time were still evident in the subgroups where no treatment was given. Goblet cells were swollen, mitochondria were fragmented and marginal clumping of the nuclear chromatin was seen.

The case was reverse in the subgroups were tissues were protected from reperfusion injury by injecting allopurinol. In these subgroups none of the above-mentioned chages were seen and their structures were in a normal state. (figs. 10-11).

Although considerable, in vitro and in vivo, experimental evidence now supports the fact that free radicals play an important role in inflammatory responses (McCord, 1987; Kehrer, 1993), very few studies have been performed in ruminants.

Mucosal lesions are the characteristic feature of ischaemic- reperfusion injury in the intestine. Such lesions have been observed in various forms of intestinal ischaemia in the dog (Chiu *et al.*,

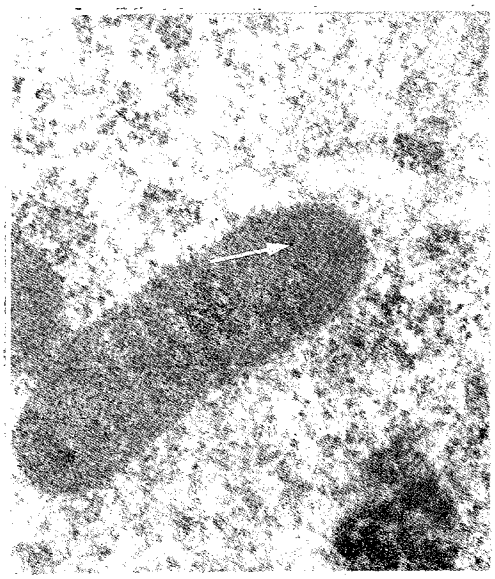

Fig. 1. Electron micrography of a mitochondria from a control (normal) sheep where neither ischaemia nor reperfusion were induced shows dense matrix granules (arrow) and the cristae are in parallel position. Stained with Uranyl acetate and lead citrate. X 34000.

1970), cat (Redfors *et al.*, 1984; Schoenberg *et al.*, 1984), man (Haglund *et al.*, 1970) and rat (Megison *et al.*, 1990). Their presence has been chosen as an important criterion for the evaluation of the intestinal damage (Parks *et al.*, 1990; Clark and Gevertz, 1991).

The events which cause the intestinal damage following ischaemic- reperfusion injury are hypoxia per se and oxygen-free radical generation (Granger *et al.*, 1986; Schoenberg *et al.*, 1984). However, this conclusion is based on indirect observations using pharmacological agents known to influence formation of free radical during reperfusion injury (McCord, 1987).

Observations made in the present experiment indicate that reperfusion injury occurs immediately after reintroduction of blood to the ischaemic tissue, and these results are in line with the findings

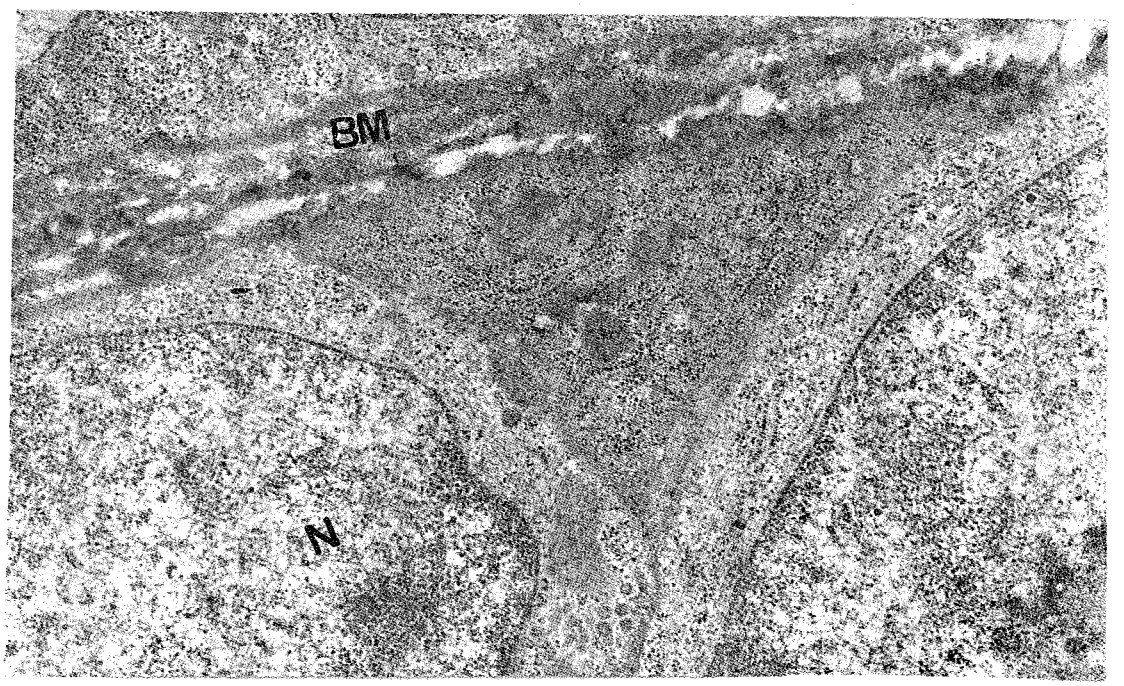

Fig. 2. Electron micrograph of a normal caecum showing intact basement membrane (BM) and the nucleus (N) with finley dispersed chromatin granules. stained with Uranyl acetate and lead citrate. X 19700.

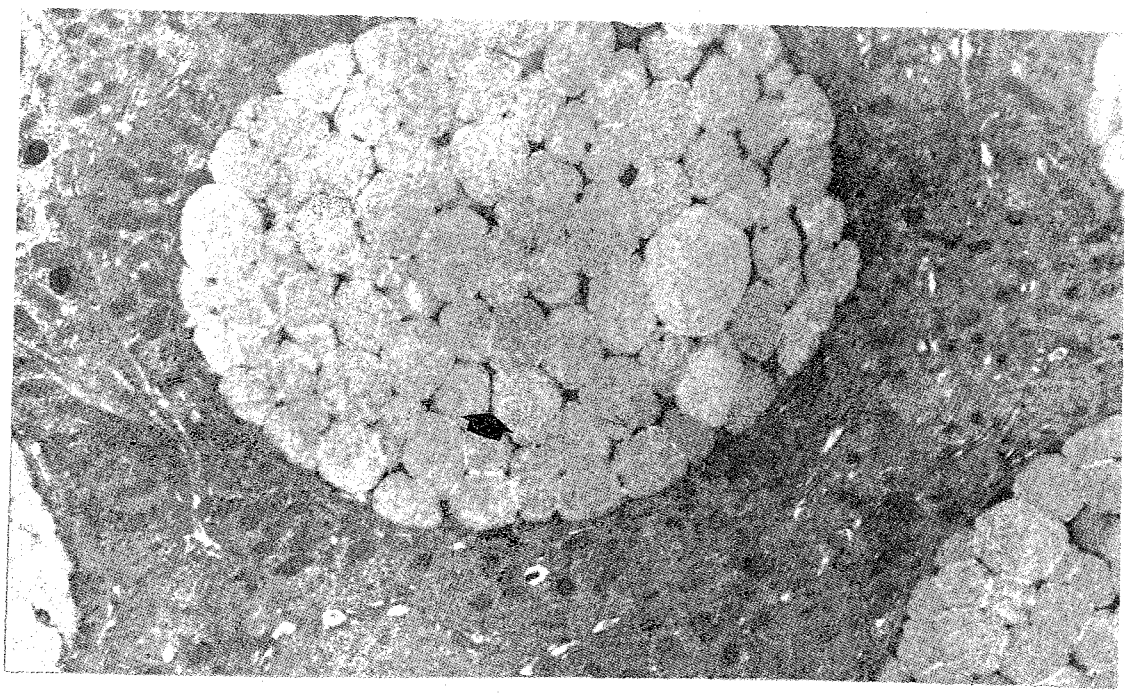

Fig. 3. Goblet cells from a normal tissue sample are shown in this micrograph. Secretory packages are in normal size with well marked border lines between them (arrow). Stained with Uranyl acetate and lead citrate. X 9700.

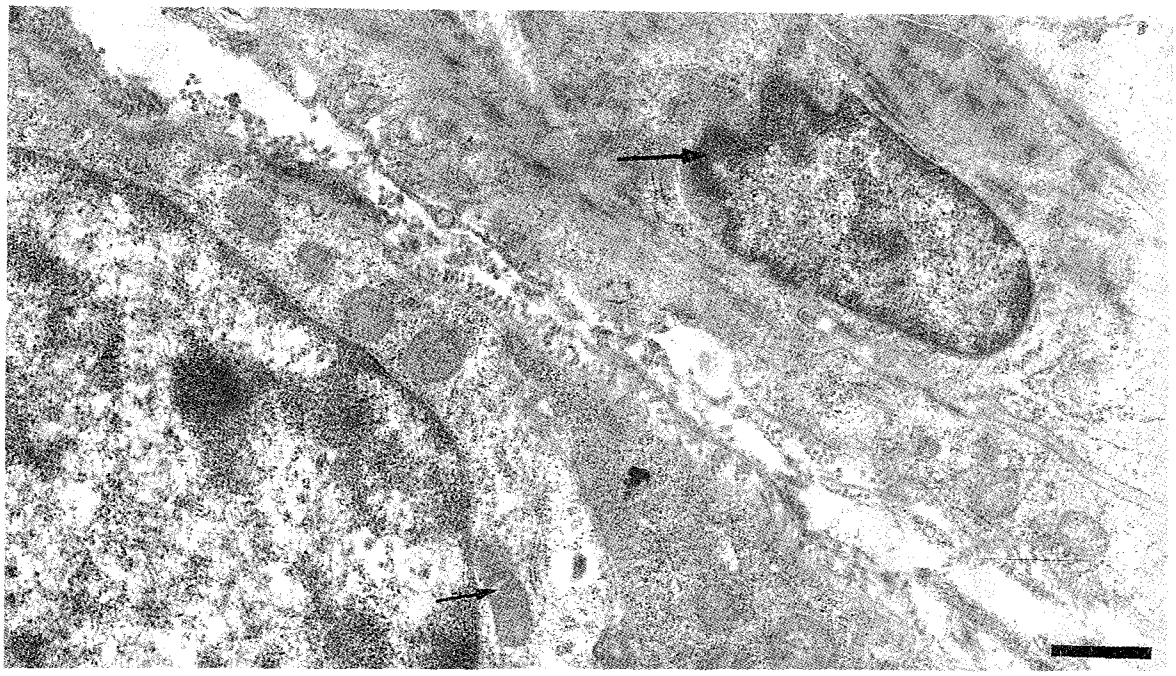

Fig. 4. Electron micrograph from a tissue sample undergone 60 minutes of ischaemia. Slight fragmentation of cristae are seen (small arrow), one nucleus is almost in normal shape where as the other one has clumping of the chromatin granules on its edge (large arrow). Stained with uranyl acetate and lead citrate. X 13800.

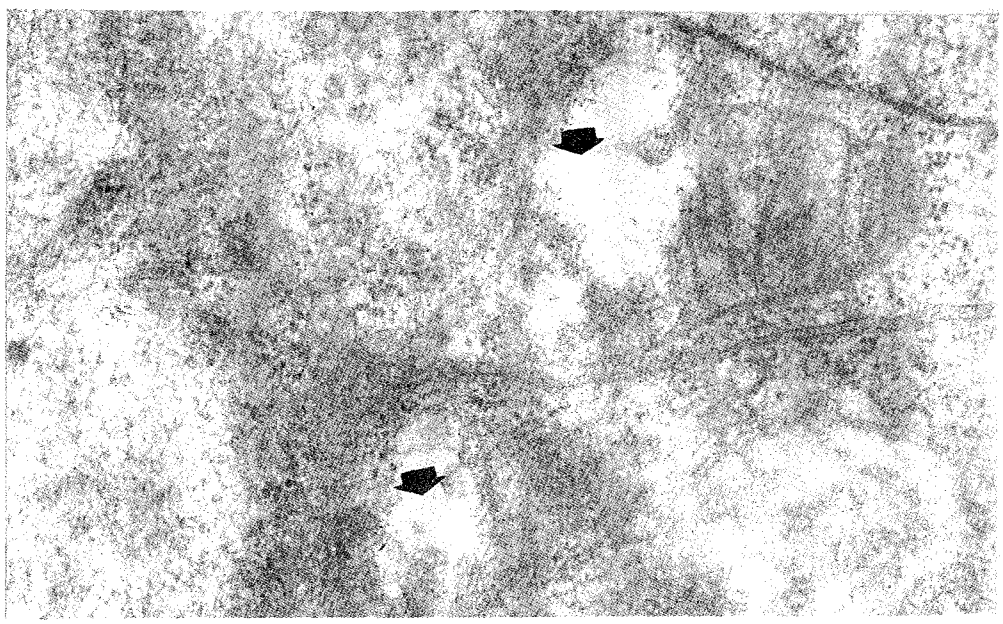

Fig. 5. Electron micrograph from a tissue sample taken after 60 minutes of ischaemia followed by 60 seconds of reperfusion where neither potassium hydroxide nor allopurinol were injected. Fragmentation of the cristae and rupture of the outer membrane of the mitochondria (arrows) are clearly seen in this micrograph. Stained with Uranyl acetate and lead citrate. X 43300.

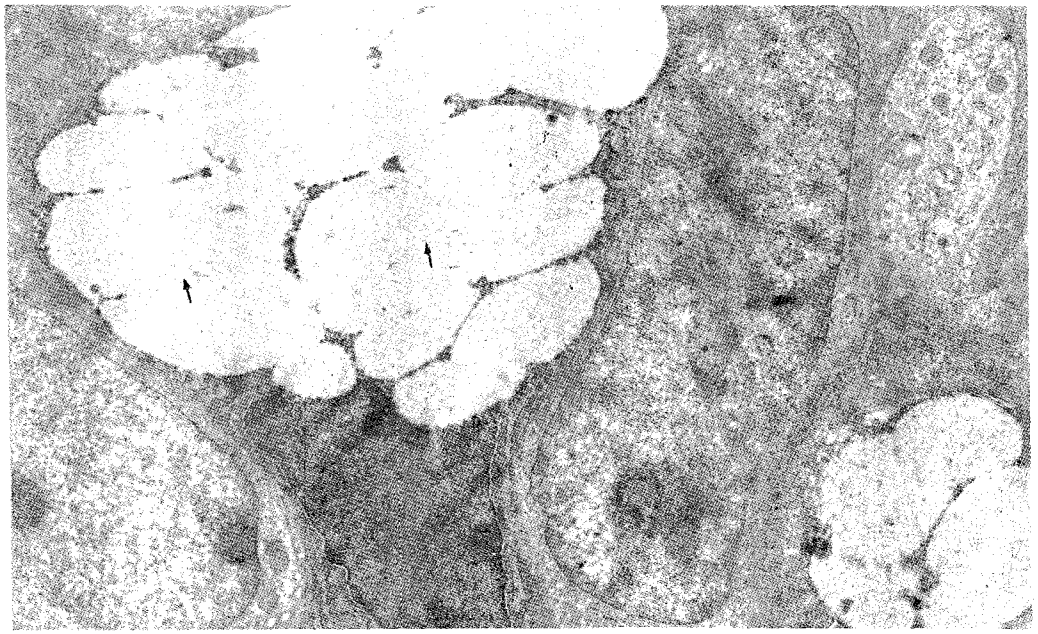

Fig. 6. Electron micrograph taken at the same time frame as in the figure 5 shows swelling of the goblet cells with the fusion of the secretory packages and disappearances of the border line between the secretory packages (arrows). Stained with Uranyl acetate and lead citrate. X 8900.

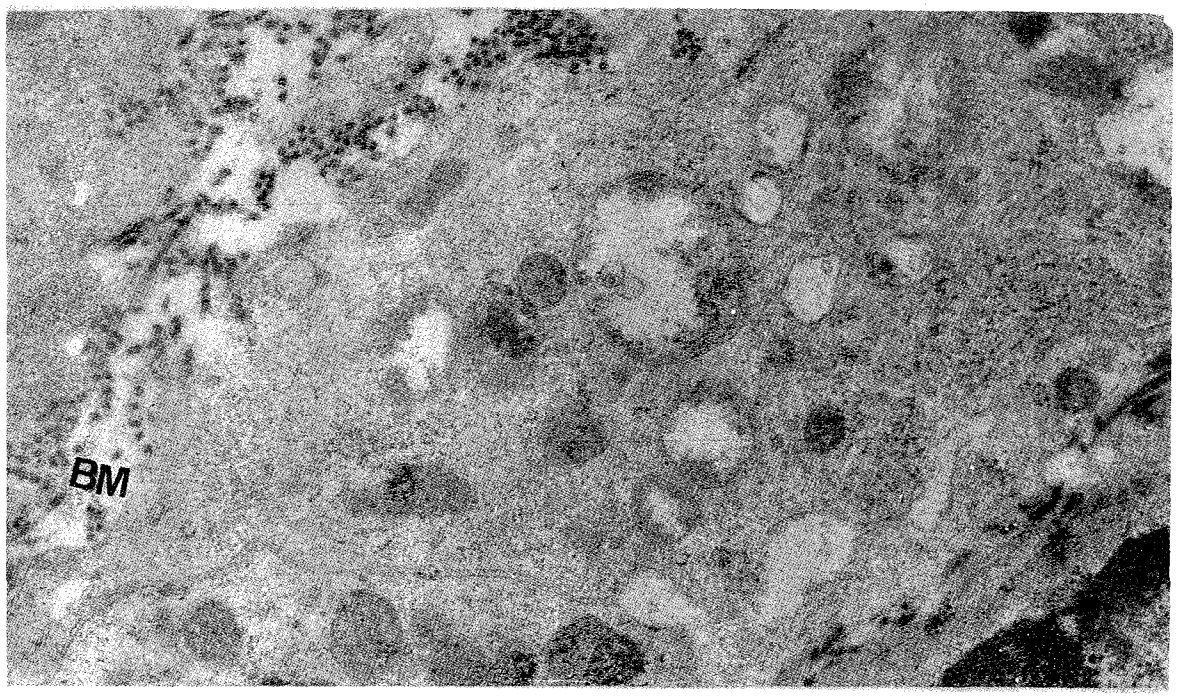

Fig. 7. Electron micrograph taken after at the same time frame as in the figure 5. Lifting of the basement membrane (BM) is seen as compared with the normal micrograph (fig. 2), nucleus (N) is seen with marginal clumping of chromatin granules. Stained with uranyl acetate and lead citrate. X 21460.

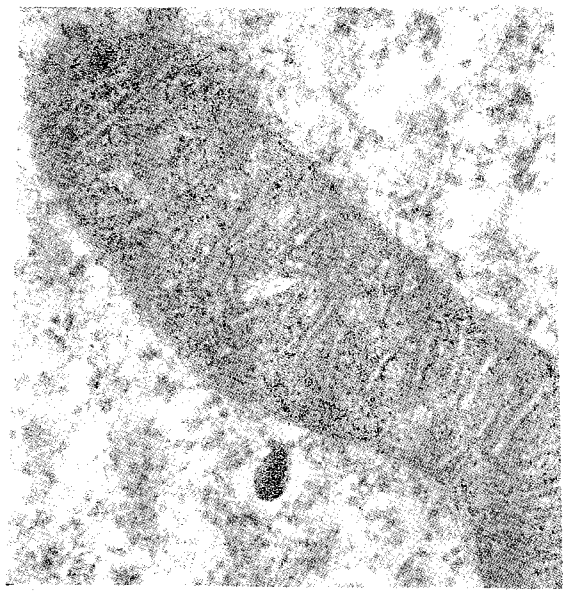

Fig. 8. Mitochondria with well preserved structure is shown in this micrograph taken after 60 minutes of ischaemia followed by 60 seconds of reperfusion treated with allopurinol. Stained with Uranyl acetate *and lead citrate. X 5000.*

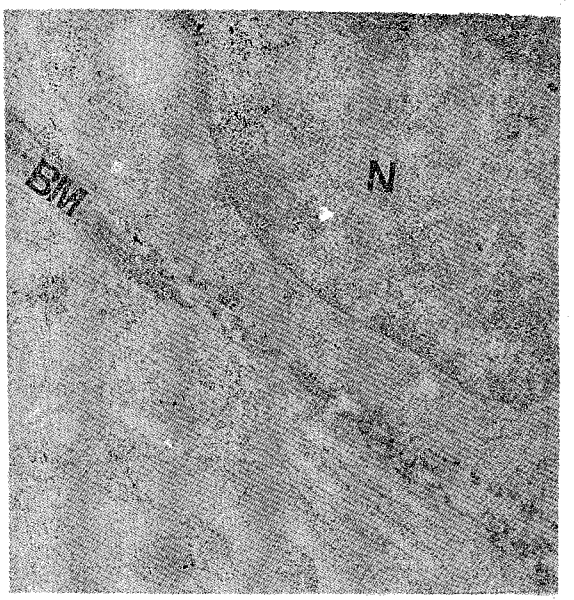

Fig. 9. Electron micrograph taken at the same time frame as in figure 8 shows intact basement membrane (BM) and the nucleus (N) with finley dispersed chromatin granules. Stained with Uranyl acetate *and lead citrate. X 15000.*

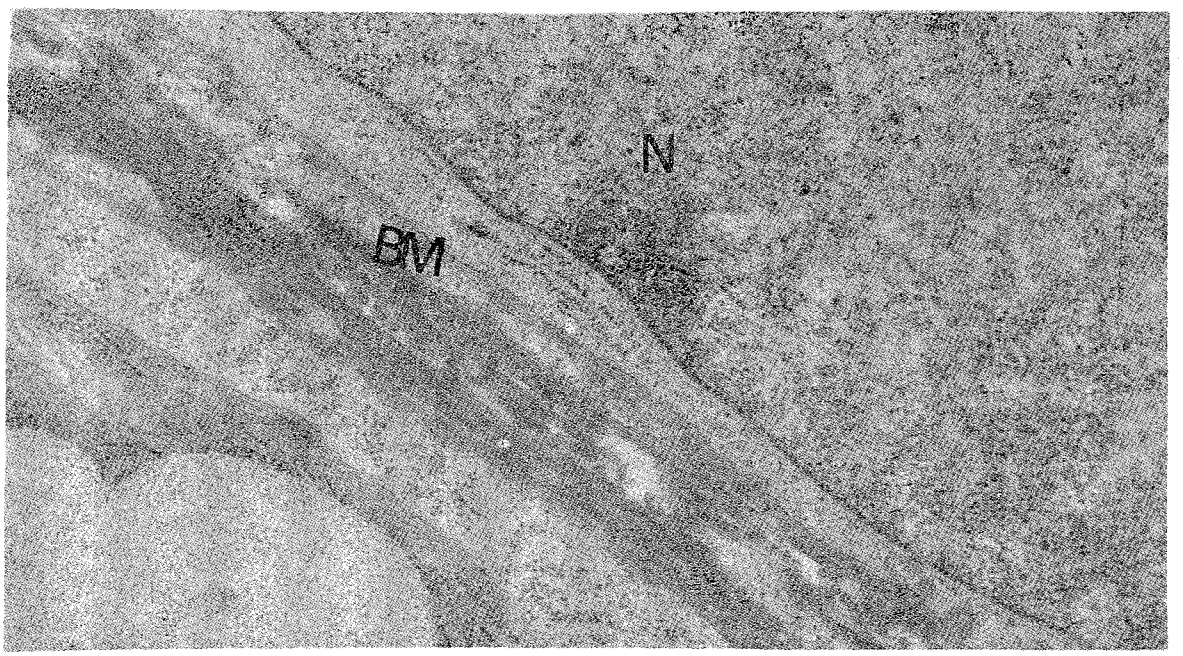

Fig. 10. Electron micrograph from a tissue taken after 60 minutes of ischaemia followed by 60 minutes of reperfusion after administration of allopurinol shows intact basement membrane (BM) and a nucleus (N) with finely dispersed chromatin granules. Stained with Uranyl acetate and lead citrate. X 20530.

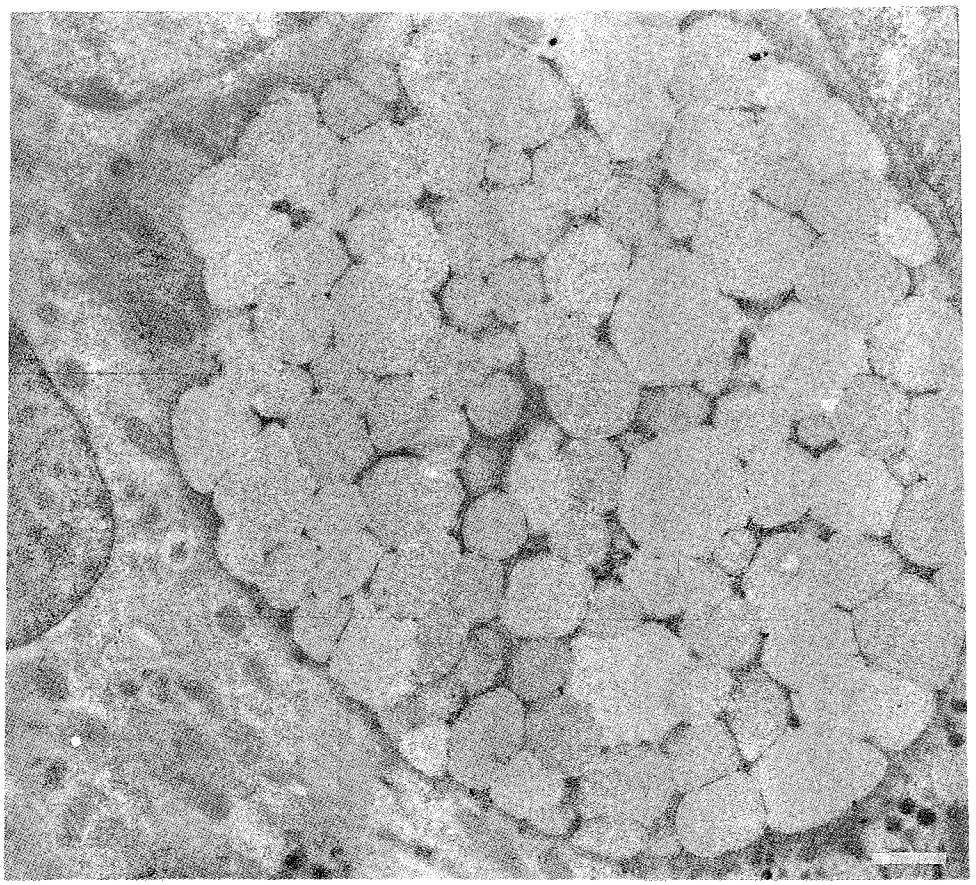

Fig. 11. Normal goblet cell with well preserved secretory packages is seen in this micrograph taken after 60 minutes of ischaemia followed by 8 hours of reperfusion treated with allopurinol. Stained with Uranyl acetate and lead citrate. X 8400.

of other investigators (Zweier *et al.,* 1987; Das and Gerald, 1991).

There were significant differences between samples from subgroups where the free radical scavenger (allopurinol) was injected as compared with those samples treated with only KOH. Organelles in the allopurinol-treated subgroups were well preserved in their ultrastructure and they were similar to the control (normal tissue) group.

Mechanism(s) of action of allopurinol is not well known but speculations have been made based on its function (Moorhouse *et al.*, 1987). Uric acid, an end product of hypoxanthine activity in xanthine-xanthine oxidase reaction in formed by deamination of adenine and guanine in nucleic acid. Allopurinol being a well known xanthine oxidase inhibitor (Das and Gerald, 1991; Godin, 1991) maintains high pool of purine base with in the body which are mainly directed towards the reutilization of depleted stores, rather than being converted into the end product, uric acid.

References

Braunwald, E. and Klomer, A.1985. Myocardial reperfusion: a double-edgedsword. J. Clin. Invest. **76**:1713

Chiu, C.J., McCardle, A.H., Brown, R., Scott, H.J.and Gurd, F.N. 1970. Intestinal mucosal lesions in low flow states. Arch. Surg. **101**:478

Clark, E.T. and Gaevertz, B.L.1991. Intermittent ischaemia potentiates intestinal reperfusion injury. J.Vasc.Surg. **13**:601

Coetzee, W.A., Owen, P., Dennis, S.C., Samman. S. and Opie, L.H.1990. Reperfusion damaged-free radicals mediate delayed membrane changes rather than early ventricular arrhythmia. Cardiovasc. Res. **24**:156

Das, D.K. and Gerald, A.C. 1991. Hi ı performance liquid chromatography detection of hydroxylated benzoic acid as an indirect measure of hydroxyl radical in heart: its' possible link with mycardial reperfusion injury.J.Chromatography. **536**:273

Donald, C.B. 1993. Oxygen free radical and tissue injury. *In*: Oxygen free radicals in tissue damage. Merrill, T.and samason, F., (eds). Birkhauser:Boston: 13

Godin, D.1991. Allopurinol and ischaemia-reperfusion injury: new use for an old drug. Can. J. Cardiol. **7**:163

Granger, D.N.; Hollwrath, M.E. and Parks, D.A.1986. Ischaemia- reperfusion injury: role of oxygen derived free radicals. Acta.Physiol. Scand. **548**:47

Haglund, U., Huten, L., Ahren, C. and Lundgren, O. 1970. Mucosal lesions in human small intestine in shock. Gut. **16**:979

Jaeschke, H. and Mitchell, J.R. 1989. Mitochondria and xanthine oxidase both generate reactive oxygen species in isolated perfused rat liver after hypoxic injury. Biochem. Biophysic. Communication **160**:140

Kehrer, J.P. 1993. Free radicals as mediators of tissue injury and diseases. Critical Rev. Toxicol. **23**:21

McCord, J.M. 1987. Oxygen derived free radicals: a link between reperfusion injury and inflammation. Fed. Proc. **46**:2402

Megison, S.M., Horton, J.W., Chao, H. and Walker, B. 1990. Prolonged survival and decreased mucosal injury after low-dose enteral allopurinol prophylaxis in mesenteric ischaemia. J. Ped. Surg. **25**:917

Moorhouse, P.C., Grootveld, M., Halliwell, B., Quinlan, J.G. and Gutteridge, J.M.C. 1987. FEBS. IET. **213**:23

Parks, P.O., Haglund, U., Bulkley, G.B.and Falt, K. 1990. The sequence of development of intestinal injury after strangulation ischaemia and reperfusion. Surgery **107**:574

Przyklenk, K.and Kloner, R.A. 1989. Reperfusion injury by oxygen derived free radicals. Circ. Res. **64**:86

Redfors, S., Hallback, D.A., Haglund, U., Jodal, M. and Lundgren, O. 1984. Blood flow distribution, villus tissue osmolality and fluid and electrolyte transport in cat small intestine during regional hypotension. Acta. Physiol. Scand. **121**:193

Schoenberg, M.H., Muhl, E., Sellin, O., Youness, M., Scheildberg, S.W. and Haglund, U. 1984. Post hypotension generation of superoxide free radical: possible role in the pathogenesis of intestinal mucosal damage. Acta. Chir. Scand. **150**:301

Zweier, J.L., Rayburn, B.R. and Flaherty, J.T.1987. Recombinant superoxide reduces oxygen derived free radicals concentration in reperfused myocardium. J.Clin. Invest. **80**:1728

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