Role of xanthine oxidase in small bowel mucosal dysfunction after surgical stress

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Background: The small intestine is highly susceptible to surgical stress even at remote locations. An earlier study using a rat model indicated that oxidative stress plays an important role in this process. The enzyme xanthine oxidase is an important source of free radicals in the small intestine. The role of this enzyme in intestinal damage after surgical stress was examined.

Methods: Rats pretreated with xanthine oxidase inhibitors were subjected to surgical stress by opening the abdomen and handling the intestine, as done during laparotomy. Enterocytes at various stages of differentiation were isolated and the protection offered by xanthine oxidase inhibitors against damage due to surgical stress was determined and compared with normal controls. Protection against structural changes to the mucosa, as well as mitochondrial function was examined.

Results: Surgical stress affected both the villus as well as crypt cells, causing increased superoxide generation, accompanied by increased activity of xanthine oxidase. Xanthine oxidase inhibitors ameliorated the increased superoxide generation, and protected against mitochondrial damage and ultrastructural changes in the intestine.

Conclusion: Surgical stress affects both the villus and crypt cell populations in the small intestine. The enzyme xanthine oxidase may be an important mediator of surgical stress in the intestine.

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Introduction

Surgical stress affects a number of different organ systems throughout the body\(^1\). A major damaging effect of surgical stress is on the intestine, which is highly susceptible even after surgery at remote locations\(^2\). Surgical stress increases intestinal permeability and induces bacterial translocation into the systemic circulation\(^3\), which has been implicated in the multiple organ failure syndrome\(^4\). The intestinal mucosa consists of differentiated absorptive cells that originate in the crypt. The crypt stem cells proliferate to produce the epithelial cells that migrate upwards and undergo differentiation as they reach the villus\(^5\). Thus the intestinal epithelium contains cells at various stages of differentiation. The time taken for this process varies between species, but is about 3 days. Any damage to the proliferating crypt cells or the differentiated villus cells may affect either process, leading to altered cell populations and functional derangement in the intestine. An earlier study from this laboratory indicated that laparotomy with mild intestinal handling, as could occur during any abdominal surgery, can result in oxidative stress in the enterocyte\(^6\). This damage is maximal 60 min after stress and is probably mediated by increased activity of the superoxide generating enzyme, xanthine oxidase, accompanied by mitochondrial dysfunction. The damage is reversible and, by 24 h after stress, most of the parameters returned to normal levels.

To understand more about the mechanism of damage to the enterocyte and its reversibility, the present study was undertaken to investigate the response of the different cell populations in rat small intestine to surgical stress after mild handling. The aim was also to determine the contribution of the superoxide generating enzyme, xanthine oxidase, to cell damage in this model of surgical stress.

Materials and methods

3-(4,5 Dimethylthiazol-2-yl),2,5-diphenyl tetrazolium bromide (MTT), MTT formazan, dimethyl sulphoxide, xanthine, adenosine 5′-diphosphate (ADP), succinate, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA), dithiothreitol, Hepes and bovine serum albumin (BSA) were obtained from Sigma...
Chemical, St. Louis, MO, USA. All other chemicals and solvents used were of analytical grade.

Animals
Adult Wistar rats of both sexes (200–250 g), exposed to a daily 12-h light–dark cycle and fed water and rat chow *ad libitum*, were used for the study. The rats were randomly divided into four groups (*n* = 8): control (no operation), surgical stress (intestinal handling), allopurinol (surgical stress after allopurinol pretreatment) and sodium tungstate (surgical stress after sodium tungstate pretreatment) groups. This study was cleared by the Institutional Animal Experimentation Ethics Committee.

Induction of surgical stress
Overnight fasted rats were anaesthetized by ketamine injection (50 mg/kg body-weight) and the abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was then gently moved and the ileocaecal junction identified. The intestine was then handled along its entire length from the ileocaecal junction proximally, simulating the ‘inspection’ that occurs in a clinical setting. The intestine was then replaced in the abdominal cavity. The whole process was completed within 1–2 min. The abdominal wall was then repaired and the animals were allowed to recover from anaesthesia. The animals were killed by decapitation either at 60 min or 24 h after the surgical procedure. Control rats were injected with ketamine and killed by decapitation without opening the abdominal wall. Control rats were also included in allopurinol- and tungstate-treated groups.

The intestine from control and surgically treated rats was removed for the study of various cell populations. Enterocytes at various stages of maturation were isolated into separate fractions as described previously using the metal chelation method. The cell fractions at various levels were numbered 1–9, fraction 1 being villus tip cells, fraction 9 being crypt stem cells and the middle fractions (8–2) containing cells with progressively increasing differentiation. For studies using mitochondria, total enterocytes were obtained from the intestine and mitochondria were isolated from the enterocytes as described previously.

Inhibition of xanthine oxidase activity
Xanthine oxidase was inhibited by treatment of the animals with either allopurinol or sodium tungstate. Rats were given an intraperitoneal injection of allopurinol 100 mg/kg body-weight 1 h before laparotomy or were fed sodium tungstate (0.7 g per kg body-weight) in drinking water for 5 days before surgery.

Assays
Superoxide generation was assessed by dye reduction, using the tetrizolium dye MTT that is reduced by superoxide radicals. The MTT assay was performed on the cell suspension or mitochondria using a microplate reader as described before. Xanthine oxidase was measured using a spectrophotometric assay based on the production of uric acid, which is measured at 295 nm. Protein was measured using bovine serum albumin as standard.

Mitochondrial function
Oxygen uptake was determined polarographically using a Clark type electrode in 2 ml respiratory medium (sucrose 225 mmol/l, magnesium chloride 5 mmol/l, monopotassium phosphate 10 mmol/l, potassium chloride 20 mmol/l, Tris 10 mmol/l and Hepes 5 mmol/l, pH 7.4) containing succinate 5 mmol/l as respiratory substrate. A mitochondrial protein of concentration of 2 mg/ml was used. Oxygen uptake was stimulated with ADP 0.3 mmol/l. Swelling of control and surgically stressed mitochondria was determined by the decrease in absorbance at 540 nm up to 10 min.

Histological studies
Mucosal tissues from control rats and those obtained at 60 min and 24 h after surgical stress, with and without pretreatment with xanthine oxidase inhibitors, were fixed in 2.5 per cent glutaraldehyde, post fixed in osmium tetroxide and embedded in epoxy resin. Sections 1 µm thick were cut and stained with toluidine blue. Suitable areas for ultrastructural study were chosen after examining the 1-µm sections under the light microscope.

For ultrastructural study, ultrathin sections were cut on an LKB UM4 ultramicrotome with a diamond knife (Diatome, Biel, Switzerland). The sections were mounted on copper grids and stained with uranyl acetate and lead citrate. The grids were examined under a Philips EM201C electron microscope (Philips, Eindhoven, The Netherlands)

Statistical analysis
Data are expressed as mean(s.d.). Statistical analysis was performed with Student *t* test.

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Results

Superoxide generation

The intestinal epithelium undergoes a continuous turnover and the cells along the villus–crypt axis are thus at various stages of differentiation. To determine whether cells from any particular population are more susceptible to surgical stress-mediated injury, cells were isolated from villus, middle and crypt fractions, and assayed for superoxide production using MTT reduction at different time intervals after surgical stress in comparison to control cells (Fig. 1). There was progressive MTT reduction 60 min after stress seen throughout the intestinal villus–crypt axis. By 24 h after stress, levels had returned close to control values in most of the fractions.

![Graph](image1)

**Fig. 1** Superoxide generation measured by 3-(4,5 dimethylthiazol-2-yl),2,5-diphenyl tetrazolium bromide (MTT) reduction in various cell fractions from the villus–crypt axis in rat intestine after surgical stress. Values are mean(s.d.) from three separate experiments. *P*<0.05 versus control, †P<0.05 versus 60 min (Student t test)

![Graph](image2)

**Fig. 2** Xanthine oxidase activity in various cell fractions from the villus–crypt axis in rat intestine after surgical stress. Values are mean(s.d.) from three separate experiments. *P*<0.05 versus control, †P<0.05 versus 60 min (Student t test)
Xanthine oxidase activity

An earlier study using isolated total enterocytes demonstrated an increased activity of xanthine oxidase after surgical stress. In the present study xanthine oxidase was assayed in various enterocyte populations separately, to determine whether the increased activity was confined to any particular cell population of the mucosa. The increase in xanthine oxidase activity after stress was most marked in the middle and crypt fractions. This activity returned to the control level by 24 h after surgical stress (Fig. 2). The intestinal crypt cells had no detectable xanthine oxidase activity in control rats or 24 h after surgical stress. Activity was detectable 60 min after stress in treated rats, although the activity was lower than that in villus cells.

Xanthine oxidase activity causes generation of free radicals and oxidative stress, which was maximal 60 min after surgical stress using the present model. Therefore superoxide generation measured using MTT reduction was assessed in xanthine oxidase-deficient animals and controls in the various cell fractions (Fig. 3). Pretreatment of animals with the xanthine oxidase inhibitors allopurinol or sodium tungstate protected against the increased MTT reduction by isolated enterocytes after surgical stress. This protection was more evident in villus cells than middle or crypt cells. No xanthine oxidase activity could be detected in any cell fractions from xanthine oxidase inhibited (allopurinol or sodium tungstate) animals (data not shown).

Mitochondrial function

It has previously been observed by the authors that surgical stress results in enterocyte mitochondrial dysfunction due to uncoupling of mitochondria (R. Anup, P. Susama and K. A. Balasubramanian, unpublished observation). To determine whether pretreatment with xanthine oxidase inhibitors could protect against this, MTT reduction was assayed using mitochondria isolated 60 min after stress in rats treated with allopurinol or sodium tungstate (Fig. 4a). Inhibition of xanthine oxidase activity prevented the increase in MTT reduction caused by surgical stress. In addition, uncoupling of respiration, determined by the fall in mitochondrial respiratory control ratio, seen 60 min after surgical stress, was prevented by xanthine oxidase inhibition (Fig. 4b). Another characteristic feature of mitochondrial damage, mitochondrial swelling, was also prevented by xanthine oxidase inhibition (Fig. 4c).

Histological changes

An earlier study from this laboratory demonstrated ultrastructural changes in the intestinal mucosa following surgical stress, resulting in widening of the intercellular spaces, most prominent at 60 min after intestinal handling. To complement the biochemical studies, histological studies were carried out in the present study to determine whether inhibition of xanthine oxidase could prevent the ultrastructural changes due to surgical stress. On light
microscopic examination, rats subjected to surgical stress showed mild patchy widening of intercellular spaces of the villus epithelium and focal oedema with vasodilatation of the lamina propria (Fig. 5). The changes were reduced or absent in samples obtained from rats pretreated with xanthine oxidase inhibitors. On ultrastructural examination (Fig. 6), mucosa subjected to surgical stress showed widening of the intercellular spaces of adjacent epithelial cells over the villi. The widening was more prominent at the basal aspect with altered gap junctions. Control groups did not display any such changes. At 24 h after surgical stress, these changes were absent or extremely focal. The changes were less marked among animals pretreated with allopurinol or sodium tungstate, in which the intercellular widening was reduced by 50 and 68 per cent respectively.

**Discussion**

The intestine is highly susceptible to surgery, even at remote locations, and an earlier study using a rat model indicated that handling of the intestine following laparotomy caused oxidative stress in the enterocyte, accompanied by functional alterations in the intestine. Since the intestinal epithelium consists of cells at various stages of differentiation, it was of interest to determine whether the damage caused by surgical stress was confined to a particular cell population of the epithelium or was widespread. In the present study there was increased MTT reduction, 60 min after surgical stress, throughout the cells of the villus–crypt axis. The tetrazolium dye MTT can be reduced by superoxide radicals and dye reduction is a measure of superoxide generation.

Free radicals have been implicated in surgical stress-induced damage and lipid peroxidation, a marker of oxidative stress, has been shown to occur after surgery. A definite time course of oxidative stress markers in arterial blood exists during open heart surgery. Antioxidants can reverse liver metastasis induced by surgical stress and damage after coronary bypass surgery. There are a number of putative free radical generating sites in the cell that include the mitochondrial respiratory chain and the enzyme xanthine oxidase.

Xanthine oxidase has been implicated in surgical stress and increased plasma xanthine oxidase activity has been shown after limb surgery and sepsis. Xanthine oxidase inhibition also reduces haemorrhagic shock-induced bacterial translocation and the present authors’ earlier study demonstrated increased xanthine oxidase activity 60 min after surgical stress. In the present study, it was found that the increase in xanthine oxidase activity seen after surgical stress was more prominent in the middle and crypt regions of the epithelium, compared with the villus. Xanthine
Fig. 5  Histological sections of small intestine from surgically stressed rats. Dilated blood vessels and widened intercellular spaces (arrows) are seen 60 min after surgical stress. a Original magnification × 90; b original magnification × 390 (toluidine blue stain)

Fig. 6  Electron microscopy of small intestinal villus epithelium in rats subjected to surgical stress showing normal appearance in controls (a). Widened intercellular spaces (asterisks) are seen at 60 min after surgical stress (b), which are decreased by pretreatment with sodium tungstate (c). (Original magnification × 35 500)
oxidase is highly active in the enterocyte and is an important source of free radicals in the mucosa during ischaemia–reperfusion. Xanthine oxidase can also produce site-specific oxidant injury to organs remote from its site of release. Xanthine oxidase activity can be inhibited completely by allopurinol, which protects against bacterial translocation and intestinal mucosal lipid peroxidation. In the present study, xanthine oxidase activity could not be detected either in control rats or 60 min after surgical stress after pretreatment with allopurinol (data not shown). The increased MTT reduction after surgical stress was attenuated in allopurinol-treated rat intestine and this protection was more prominent in villus than crypt cells. Allopurinol is also an antioxidant, in addition to its inhibitory effect on xanthine oxidase so experiments were repeated with another xanthine oxidase inhibitor, sodium tungstate, which depletes molybdenum, a cofactor for the enzyme. Xanthine oxidase activity was similarly undetectable in enterocytes after treatment with tungstate (data not shown). In tungstate-treated rats inhibition of MTT reduction was similar, indicating that the increase in MTT reduction was to a certain extent caused by activation of xanthine oxidase, especially in cells of the villus region.

Another source of superoxide in the cell is the mitochondria, since approximately 1–2% of the oxygen consumed by the mitochondria constitutes results in generation of superoxide. Uncoupling of mitochondrial respiration may contribute to generation of free radicals, which are capable of altering mitochondrial function. Mitochondrial function can be affected by surgical stress and earlier observations from this laboratory indicated that there was mitochondrial dysfunction 60 min after surgical stress, with an increased MTT reduction in the presence of respiratory substrate and a decreased respiratory control ratio (R. Anup, P. Susama and K. A. Balasubramanian, unpublished observation). In the present study, pretreatment of animals with allopurinol or sodium tungstate protected mitochondria against the decrease in respiratory control ratio and concomitant increase in MTT reduction. Another characteristic feature of mitochondrial damage observed in the earlier study was initiation of the mitochondrial permeability transition. This damage can be effected by a number of inducers, including oxidants, and is the result of opening of a non-specific megapore in the inner mitochondrial membrane. Inhibition of xanthine oxidase by allopurinol or sodium tungstate offered partial protection against this damaging phenomenon.

On histological examination, the intestine from surgically stressed rats showed vasodilatation and lamina propria oedema under light microscopy. Electron microscopy indicated increased intercellular spaces 60 min after stress, which suggested increased fluid movement between epithelial cells. This may also result in bacterial translocation seen in other studies. Inhibition of intercellular widening, by pretreatment with allopurinol or sodium tungstate, implies that xanthine oxidase activation influences the widening of intercellular spaces and supports the hypothesis that xanthine oxidase mediates bacterial translocation.

In conclusion, these studies have shown that surgical stress produced by intestinal handling causes widespread damage to the intestinal epithelium, with both villus and crypt cells being affected. Increased generation of superoxide radicals in both villus and crypt cells was seen 60 min after stress, which was partly due to activation of xanthine oxidase. This activation was more prominent in the middle and crypt regions than the villus cells, and inhibition of xanthine oxidase activity by two separate inhibitors indicated an important role for this enzyme in superoxide generation. The xanthine oxidase inhibitors also protected against the damaging effects of intestinal handling on enterocyte mitochondria, including a decrease in the intercellular space widening seen in the intestinal epithelium. Xanthine oxidase may be an important mediator of surgical stress in the intestine.

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References