Role of intracellular second messengers and reactive oxygen species in the pathophysiology of *V. cholera* O139 treated rabbit ileum

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Abstract

*Vibrio cholerae* O139 has pandemic potential and it produces copious amounts of fluid secretion. The levels of various second messengers (intracellular Ca$^{2+}$, cAMP, IP$_3$, PKC) were measured to determine the cause of fluid secretion produced by this strain of *V. cholerae*. There was a significant increase in the levels of these second messengers in *V. cholerae* O139 treated ileum as compared to control ileum (enterocytes). Levels of these second messengers were also assessed in *V. cholerae 569B* induced fluid secretion in rabbit ileum and it was found that the levels were raised more in *V. cholerae* O139 treated ileum than in *V. cholerae 569B* treated rabbit ileum. The intestinal damage was assessed by measuring changes in the extent of lipid peroxidation of the enterocytes. Intracellular second messengers are known to raise the extent of lipid peroxidation. In *V. cholerae O139* treated loops calcium ionophore A23187 enhanced the extent of lipid peroxidation whereas l-verapamil could only marginally decrease the lipid peroxidation. Dantrolene and H$_7$ significantly decreased the extent of lipid peroxidation of enterocytes in *V. cholerae O139* treated rabbit ileum. However, PMA could not enhance further the extent of lipid peroxidation in *V. cholerae O139* treated rabbit ileum. So intracellular calcium and protein kinase C appear to be involved in intestinal damage caused by *V. cholerae O139*. Reactive oxygen species are responsible for causing tissue damage and the extent of oxidative damage depends on the balance between the pro-oxidants and the anti-oxidants. So the changes in the enterocytes' antioxidant level during *V. cholerae O139* mediated intestinal infection was estimated. There was a significant decrease in the enterocyte level of the antioxidant enzymes SOD, catalase, glutathione peroxidase, glutathione reductase, glutathione transferase and glucose-6-phosphate dehydrogenase in *V. cholerae O139* mediated intestinal infection. So a significant decrease in the levels of antioxidant defenses and a significant increase in the levels of second messengers appear to be important in mediating *V. cholerae O139* induced lipid peroxidation which contributes to the changes in membrane permeability and thus to fluid secretion. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cholera causes significant morbidity and mortality in underdeveloped countries. In recent years changing patterns of *Vibrio cholerae* have evolved and classical *V. cholerae* infection has been replaced by Eltor vibrios [1]. Also a large outbreak of cholera-like infections caused by a non-01 serogroup of *V. cholerae* named O139 ‘Bengal’ has been reported from different parts of India. This new strain causes profuse watery diarrhea and induces fluid accumulation in

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the ileal loop assay [2]. Since this strain caused the eighth pandemic of cholera it needs to be studied in depth for its characteristics and potentials.

During diarrheal diseases the mucosal permeability of the gastrointestinal tract is altered. Several studies have shown that oxygen derived free radicals could be the possible cause. Oxygen dependent free radical damage has been implicated in the pathophysiology of certain gastrointestinal diseases [3,4]. A range of reactive oxygen species (ROS) including superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl) and monochloramines are generated both in the mucosa and in the lumen [5,6]. In the lumen materials like ascorbic acid, peroxidized lipids and bacterial metabolites form the source of free radicals. The vascular endothelium has also been seen to produce O$_2^-$ during the inflammatory condition [7]. The ROS are highly chemically reactive species and can damage all kinds of biochemical substances present in the cell, i.e. DNA, lipids, proteins and carbohydrates [8]. The gastrointestinal tract is a rich source of various antioxidants, i.e. superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and $\alpha$-tocopherol, all of which catalyze the inactivation of ROS.

Several studies have shown that Ca$^{2+}$ ions can regulate free radical mediated lipid peroxidation. Protein kinase C (PKC) is a Ca$^{2+}$ and phospholipid dependent enzyme that appears to play a central role in the control of many cellular processes. PKC is activated at low Ca$^{2+}$ ion concentration. Studies by Von Reuker et al. have shown that PKC can also cause lipid peroxidation and cell membrane damage [9].

Keeping in view all these points it was thought that ROS might contribute to the intestinal damage observed during V. cholerae O139 infection. As the extent of oxidative damage is determined by the balance between the pro-oxidants and antioxidants, the levels of antioxidants in the enterocytes might also be affected. Since the underlying mechanism of action of V. cholerae O139 is not known it would be better to study the levels of intracellular second messengers in this infection. Hence, the aim of this study was to evaluate the role of intracellular second messengers and ROS in mediating enterocyte damage during V. cholerae O139 induced fluid secretion and status of antioxidant enzymes.

2. Materials and methods

2.1. Chemicals

Ca$^{2+}$ ionophore, t-Verapamil, dantrolene sodium, phorbol-12-myristate-12-acetate, 1-(-5-isoquinolinylsulfonyl)-2-methylpiperazine, nitroblue tetrazolium (NBT) and NADPH were obtained from Sigma. Thiocticuric acid was obtained from Merck, Germany. All other chemicals were of reagent grade.

2.2. Bacterial strain

V. cholerae 569B and V. cholerae O139 strains were brought from Calcutta. The strains were characterized both morphologically and biochemically on the basis of standard tests. The strains were grown on nutrient agar plates for 24 h at 39°C.

2.3. Animals and treatment protocol

Male New Zealand White rabbits (weighing 1.5–2.0 kg) were used for the study.

Male New Zealand White rabbits were maintained on standard rabbit chow diet with free access to water but not to food 16–18 h before experiment. The rabbit ileal loop assay was done as described [10]. In experimental loops, 1 ml of bacterial growth containing $3 \times 10^7$ CFU was injected, while in control loops 1 ml of phosphate buffered saline (PBS) was injected. Animals were killed with sodium pentobarbital (100 mg/kg i.v.) after 18 h and loop segments were excised quickly for further experimental studies.

Rabbit ileal loops (each 5 cm in length) with spacer loops of 1 cm each were made in fasted and anesthetized rabbits as described above. In the negative control loop 1 ml of PBS pH 7.2 was injected. In the positive control loop PBS containing $3 \times 10^7$ CFU of V. cholerae O139 was injected, whereas in the third loop modulator alone was injected. The fourth loop was the experimental loop in which PBS containing $3 \times 10^7$ CFU of V. cholerae O139 along with modulator was injected.

2.3.1. Modulators/drugs used

To evaluate the role of intracellular mediators in oxidative damage in response to V. cholerae O139...
live culture, the studies were carried out in the presence or absence of various intracellular mediators. These modulators are Ca\(^{2+}\) ionophore A23187 (15 \(\mu\)M), L-verapamil, a Ca\(^{2+}\) channel blocker (200 \(\mu\)M), dantrolene sodium, a drug known to trap calcium inside intracellular stores (25 \(\mu\)M), phorbol myristate acetate (PMA), a PKC activator (200 ng), 1-(5-isooquinolinylsulfonyl)-2-methylpiperazine (H\(T\), 15 mg), a potent specific inhibitor of PKC.

2.3.2. Isolation of enterocytes

Enterocytes were isolated from loop segments as described [11] and purified with slight modifications [12]. The loop segments from control and *V. cholerae* O139 treated rabbit ileum were flushed with warm \(O_2\) saturated PBS (pH 7.5). The loop segments were cut along the mesenteric border and enterocytes were isolated by chelation-elution. The stability of cells was checked by the trypan blue exclusion test [13]. Our cell preparation was found to be >90–95% pure when viewed under phase contrast microscope as well as when stained preparation was examined microscopically.

2.3.3. Preparation of membrane bound PKC

Isolated epithelial cells from both control and *V. cholerae O139* treated ileum were washed twice, suspended in 20 mM Tris-HCl (pH 7.5) and homogenized in a glass homogenizer. The homogenates were centrifuged at 100 000 \(\times\) g for 60 min, the cytosolic fraction was collected. The pellet was resuspended in 20 mM Tris-HCl (pH 7.5), homogenized briefly in the presence of 1% Triton X-100 and recentrifuged as above. The supernatant collected from this centrifugation served as the source of membrane bound PKC.

2.3.4. PKC assay

The PKC levels were estimated in both cytosolic and membrane fractions of enterocytes isolated from control and *V. cholerae O139* treated rabbit ileum as described by Thomas et al. [14]. The enzyme was assayed with histone III S (10 \(\mu\)g), phosphatidylserine (5 \(\mu\)g), diolein (2.5 \(\mu\)g), CaCl\(_2\) (0.5 mM), MgCl\(_2\) (20 mM), Tris-HCl (20 mM, pH 7.5), leupeptin (2 mg), ATP (10 \(\mu\)M) and 1.0 \(\mu\)Ci [\(\gamma\)-32P]ATP (specific activity 3500 \(\mu\)Ci/mM) in a final volume of 100 \(\mu\)l. The reaction was started by addition of enzyme and stopped after incubation at 30°C for 10 min by transferring 60 \(\mu\)l of reaction mixture to 3 mm Whatman filter paper (2\(\times\)2 cm) as described [14] and washed thoroughly with 10% trichloroacetic acid (TCA), then with Tris-mannitol and finally it was dried. Radioactivity was determined using a liquid scintillation counter (12 15 Rackbeta). The experiments were done in duplicate and repeated six times, protein content was measured as described by Lowry et al. [15].

2.3.5. Measurement of cAMP levels

The cAMP levels were measured in enterocytes isolated from control and *V. cholerae O139* treated rabbit ileum. The cAMP levels were measured by RIA. The isolated enterocytes were collected as a pellet. The pellet was resuspended in acidic ethanol (1 ml 1 N HCl/ml ethanol) and allowed to stand for 5 min. After centrifugation at 12 000 \(\times\) g the supernatant was collected. The precipitate was washed with 1 ml of ethanol/water (2:1, v/v) and centrifuged. The supernatants were combined and evaporated to dryness at 60°C under a stream of nitrogen. The results are expressed as pmol of cAMP/mg protein.

2.4. Measurement of inositol triphosphate (IP\(_3\)) levels

2.4.1. Labelling of cells with \(H_3\)PO\(_4\)

Isolated enterocytes were labelled with \(32\)P according to the method of Sugimoto et al. [16]. The cells were resuspended in Tris-mannitol buffer (pH 7.4) and incubated with carrier free \(32\)P (50 \(\mu\)Ci/ml cell suspension) for 60 min at 37°C. Subsequently, the excess of extracellular \(32\)P was removed by thoroughly washing with ice cold buffer and resuspending in the same buffer. Thereafter the cells were treated with *V. cholerae O139* for 15 min. Incubation was terminated by addition of ice cold chloroform/methanol/HCl (2:1:1.6 ml, v/v). Phase separation was carried out by centrifugation at 2000 rpm.

2.4.2. Identification of inositol compounds

Water soluble inositol compounds present in the polar phase of cell extracts were separated on a small column containing 5 ml of Dowex AG 1 \(\times\) 8 in formate form by the method of Emilson and Sundler [17]. The column was washed with distilled water (10 ml) to remove myo-inositol. Phosphate esters were
then eluted by stepwise addition of (i) 0.02 M ammonium formate (IP), (ii) 0.5 M ammonium formate (IP2) and (iii) 1 M ammonium formate (IP3).

2.4.3. Measurement of intracellular free calcium (Ca^{2+})

Intracellular free calcium (Ca^{2+}) levels were measured in enterocytes isolated from control and V. cholerae O139 treated rabbit ileum. Isolated enterocytes were loaded with 6 μM Fura-2AM. The free intracellular calcium Ca^{2+} was estimated according to Tsien et al. [18] using a kDa for Fura-2 of 224.

2.4.4. Measurement of lipid peroxidation

Malondialdehyde (MDA) estimation was done as described [19]. The absorbance was recorded at 535 nm spectrophotometrically. In parallel experiments, MDA standard 1,1,3,3-tetraethoxypropane at concentrations ranging from 5 to 50 nM was also run. The lipid peroxidation was estimated as nmol of MDA formed/mg protein and it is expressed as units/mg protein. The MDA levels were also measured in the presence or absence of modulators as described above.

2.4.5. Estimation of enzymatic antioxidant defense system

The enzymes were assayed in subcellular fractions (SF) of enterocytes. For this, enterocytes were sonicated for 3 min in burst of 30 s with cooling at 0°C. The sonicate was centrifuged at 105,000 × g for 1 h [20]. Supernatant was used for estimation of enzymes.

2.4.6. Superoxide dismutase (SOD) assay

SOD was estimated as described [21]. The percentage inhibition in the rate of NBT reduction was noted and one unit of enzyme was expressed as the inverse of the amount of SF protein required to inhibit the reduction of NBT by 50%.

2.4.7. Catalase assay

The activity of catalase was assayed as described [22] in which decomposition of H_{2}O_{2} was followed spectrophotometrically at 240 nm. The change in absorbance was read at 240 nm for 60 s at 10 s intervals and specific activity was calculated using a molar absorbance index for H_{2}O_{2} of 43.6. The specific activity was expressed as nmol H_{2}O_{2} decomposed/min/mg protein.

2.4.8. Glutathione peroxidase (GSH-Px) assay

The activity of the enzyme was assayed as described [23]. The change in absorbance was recorded at 1 min intervals at 340 nm and the specific activity was calculated using an extinction coefficient of 6.22 cm/μmol for NADPH. The activity was expressed as nmol NADPH oxidized/min/mg protein.

2.4.9. Glutathione S-transferase (GST) assay

The enzyme GST was assayed as described [24]. The change in absorbance was recorded at 1 min intervals at 340 nm. The specific activity was calculated using an extinction coefficient of 9.6 mM^{-1} cm^{-1}. The specific activity was expressed as nmol product formed/min/mg protein.

2.4.10. Reduced glutathione (GSH) levels

The reduced glutathione levels were estimated as described [20]. The glutathione content was measured at 412 nm. For each set of assays a standard curve using reduced glutathione (100 μM) was run. Glutathione content was expressed as μM of GSH/mg protein.

2.4.11. Glutathione reductase (GR) assay

GR was assayed according to the method described by Carlberg and Mannervik [25]. The enzyme activity was measured at 340 nm. One unit of enzyme was defined as the amount of enzyme that catalyzed the oxidation of 1 nmol of NADPH per min at 25°C. The specific activity was expressed as enzyme units/mg protein.

2.4.12. Glucose-6-phosphate dehydrogenase (G-6-PDH) assay

The activity of G-6-PDH was estimated according to the method of Babior and Cohen [26]. The specific activity was calculated using an extinction coefficient of 6.22 × 10^{3} M^{-1} cm^{-1} for NADP. The activity was expressed as nmol of NADP reduced/min/mg protein.

2.4.13. Vitamin E and vitamin A assay

Vitamin E and A levels were measured by high
performance liquid chromatography as described [27]. Briefly, 200 μl of sample preparation was treated with ethanol and extracted with heptane. The sample was vortexed and centrifuged at 3000 rpm for 10 min. The upper layer was transferred to another tube and dried under a stream of nitrogen. The dried sample was reconstituted in the mobile phase (acetonitrile/methanol/chloroform, 47:47:6 v/v). 20 μl of sample preparation was injected into the system. The flow rate was 1.8 μl/min and total run time was 10 min. The absorption was measured at 292 and 325 for vit E and vit A respectively.

2.4.14. Protein estimation

Protein estimation was carried out as described [15]. Bovine serum albumin was used as standard.

Fig. 1. Values are expressed as mean ± S.E.M. of six animals. Six rabbit ileal loops (5 cm in length) with spacer loops of 1 cm were made in fasted and anesthetized rabbits as described in Section 2. In negative control loop 1 ml of PBS was injected. In positive control loop PBS containing $3 \times 10^7$ cfu of V. cholerae O139 was injected. In the third loop Ca$^{2+}$ ionophore A23187 was injected. In the experimental loop (fourth) $3 \times 10^5$ cfu of V. cholerae O139 along with A23187 was injected. In the fifth loop verapamil alone was injected whereas in the sixth loop (experimental loop) $3 \times 10^5$ cfu of V. cholerae O139 along with verapamil was injected. The animals were killed 18 h post inoculation. The enterocytes were isolated as described in Section 2. The extent of lipid peroxidation as judged by MDA levels in terms of units/mg protein was determined. *P < 0.001 as compared to control. **P < 0.01 as compared to the absence of dantrolene within the same group.

2.5. Statistical analysis

The data were analyzed by Student’s t-test (unpaired). A P value < 0.05 was taken as significant. All values were expressed as mean ± S.E.M. of six observations unless otherwise indicated.

3. Results

3.1. In vivo effect of Ca$^{2+}$ ionophore A23187 and l-verapamil on V. cholerae O139 mediated lipid peroxidation

V. cholerae O139 when injected alone in positive control loops induced a significant increase (P < 0.001) in MDA levels (2.8 ± 0.11 units/mg protein). In negative control loops in which saline was injected there was negligible lipid peroxidation. Fig. 1 shows that Ca$^{2+}$ ionophore induced a significant (P < 0.001) increase in MDA levels when injected alone in loops (2.1 ± 0.16 units/mg protein). Ca$^{2+}$
ionophore further increased the MDA levels (P < 0.001) significantly when added with *V. cholerae* O139 (3.5 ± 0.21 units/mg protein). L-Verapamil decreased MDA levels marginally in experimental loops as compared to positive control loops (0.8 ± 0.01 units/mg protein). It did not affect MDA levels of control loops.

### 3.2. In vivo effect of dantrolene on *V. cholerae* O139 mediated lipid peroxidation

Dantrolene when added to control loops did not alter the basal extent of lipid peroxidation of the enterocytes (Fig. 2). Dantrolene significantly (P < 0.01) decreased the extent of lipid peroxidation of enterocytes isolated from experimental loops (1.23 ± 0.09 units/mg protein) as compared to enterocytes isolated from positive control loops (2.51 ± 0.08 units/mg protein).

### 3.3. In vivo effect of PMA and H7 on *V. cholerae* O139 mediated lipid peroxidation

Fig. 3 shows that the PKC activator PMA when injected alone in control loops significantly (P < 0.01) increased the extent of lipid peroxidation. MDA levels in negative control loops were 0.49 ± 0.07 units/mg protein and in positive control loops 2.6 ± 0.13 units/mg protein. The effect of PMA was significant (P < 0.001) as compared to negative control loops. The addition of PMA with *V. cholerae* O139 in experimental loop had no significant effect on extent of lipid peroxidation of enterocytes as compared to positive control loops (2.23 ± 0.25 units/mg protein).

Furthermore, H7 was found to significantly (P < 0.001) decrease the extent of lipid peroxidation of enterocytes isolated from experimental loops (1.1 ± 0.02 units/mg protein) as compared to positive control loops. However, the presence of H7 had no significant effect on the extent of lipid peroxidation of enterocytes isolated from control loops.

### 3.4. Measurement of second messengers

Levels of cAMP, intracellular calcium, inositol triphosphate and protein kinase C were measured in

![Fig. 3. Values are expressed as mean ± S.E.M. of six animals.](image)

The rabbit ileal loops were made in fasted and anesthetized rabbits. cAMP, Ca²⁺, IP₃ and PKC levels were measured as described in Section 2. The results are expressed as nmol adenosine hydrolyzed/mg protein for cAMP, nmol/l for Ca²⁺. Values are mean ± S.E.M. of six determinations. *P < 0.01 as compared to control; **P < 0.001 as compared to control.
enterocytes isolated from control and *V. cholerae* O139 treated rabbit ileum. The levels of these second messengers obtained from *V. cholerae* O139 treated loops were compared with levels obtained from *V. cholerae* 569B treated loops. There was a significant (*P* < 0.001) increase in the levels of these second messengers in *V. cholerae* O139 treated loops as compared to control loops. However, *V. cholerae* O139 could raise the levels of these second messengers to a much greater extent than *V. cholerae* 569B (Table 1).

### 3.5. Antioxidant defense parameters

Figs. 4 and 5 show the changes in the enterocyte antioxidant levels in control and *V. cholerae* O139 treated rabbit ileum loops. In the present study, there was a significant decrease in the activity of all the antioxidant enzymes studied. Furthermore, a significant decrease in the vit E levels was also observed (Table 2).

#### 4. Discussion

Cholera in its most severe form is characterized by severe watery diarrhea, vomiting and muscle cramps. Recent outbreaks of severe cholera in India and Bangladesh have been associated with a *V. cholerae* strain of the novel serogroup O139.

The intestinal microvillus membrane is highly specialized to perform transport and enzymatic functions essential for normal digestion and absorption. Since it is believed that lipid and lipid-protein interactions in the membrane play a major role in their function, it is important to study the effect of free radicals on these dynamic features in membrane components [3].

The cytotoxic effects of ROS result mainly from the peroxidation of the lipid contents of cellular and mitochondrial membranes [28]. A measure of the lipid peroxidation products such as MDA is an indication of the extent of peroxidation. In the present study, a significant increase in lipid peroxidation of
enterocytes isolated from treated rabbit ileum as compared to control was observed as judged by the increased production of MDA. Thus *V. cholerae O139* besides causing fluid accumulation also causes damage to mucosal membrane by peroxidation of lipids in epithelial cells.

Intracellular messengers are proposed to directly regulate the intestinal electrolyte transport [29]. In intestinal epithelium cyclic nucleotides (cAMP, cGMP), Ca$^{2+}$, PKC and phospholipid metabolites have been implicated in the action of microbes and their toxins on transepithelial transport of electrolytes and water [30]. Their concentration increases by receptor mediated stimulus provided by microbe or microbial toxins. The role of intracellular calcium stores in the pathogenesis of diarrhea has attracted much attention in the past few years [31]. In the present study we have shown that there were rises in the levels of cAMP, intracellular Ca$^{2+}$, IP$_3$ and PKC in enterocytes isolated from *V. cholerae O139* treated rabbit ileum as compared to control ileum. It was also observed that *O139* could raise the levels of these intracellular messengers to a much greater extent than *V. cholerae 569B*. This proves that *V. cholerae O139* is a much more potent strain than *V. cholerae 569B*. This is the reason that it could cause the eighth pandemic.

Intracellular messengers have been reported to cause intestinal damage. In this study an attempt was made to determine the role of intracellular messengers in *V. cholerae O139* mediated intestinal damage, the extent of lipid peroxidation was taken as a measure of intestinal damage. The Ca$^{2+}$ ionophore A23187 is a lipophilic antibiotic which produces a net shift of Ca$^{2+}$ from the extracellular milieu to the cell. Verapamil is a Ca$^{2+}$ antagonist and is known to inhibit Ca$^{2+}$ influx [32]. It was observed in this study that extracellular calcium does not contribute significantly in inducing lipid peroxidation. But we saw that there was a rise in the levels of intracellular Ca$^{2+}$ in enterocytes isolated from *V. cholerae O139* treated rabbit ileum.

The increased intracellular Ca$^{2+}$ may have its source either from increased Ca$^{2+}$ entry from the extracellular milieu to the cell or from the release of Ca$^{2+}$ from intracellular calcium stores [33]. Since extracellular calcium does not play a significant role, the increased calcium may thus result from the release of calcium from intracellular calcium stores. In the present study, the role of intracellular calcium stores in intestinal damage was studied by the use of dantrolene. Dantrolene is a drug which is used clinically as a skeletal muscle relaxant and is known to trap calcium in intracellular calcium stores in skeletal muscles [34]. We found that dantrolene significantly decreased the extent of lipid peroxidation of enterocytes as compared to its absence. The role of PKC in mediating *V. cholerae O139* induced lipid peroxidation was also studied. The role of PKC was studied by using PKC activator PMA and its inhibitor, H$_2$. It was observed that PMA could not alter the extent of lipid peroxidation of enterocytes as compared to its absence within the same group. However, PMA induced a significant increase in the extent of lipid peroxidation in control studies, and H$_2$ significantly inhibited the *V. cholerae O139* induced lipid peroxidation. The stimulatory and inhibitory effects of PMA and H-7 on lipid have been reported earlier [9].

The gastrointestinal tract is a rich source of the antioxidant enzymes, GSH and α-tocopherol, all of which detoxify these oxidants [35]. However, when the rate of production of ROS exceeds the capacity of the antioxidant defenses, substantial tissue damage occurs.

In the present study, there was a marked decrease in the activity of all the antioxidant enzymes, i.e. SOD, GSH-Px, GST and catalase, in the enterocytes isolated from both experimental groups as compared to the control group. A significant decrease in the GSH and vit E levels was also observed. However, there was no change in the vit A levels. It seems that the decrease in the level of antioxidant defenses sensitized the enterocytes to an increased flux of ROS which led to membrane damage and cell death.

The significant decrease in the levels of SOD enzyme in the enterocytes isolated from *V. cholerae O139* treated ileum might result from either a reduced local concentration of enzyme or an increased concentration of superoxide radicals. SOD mediated tissue protection against injury related to ROS has been seen in experimental colitis [36].

Catalase is a major antioxidant defense component that converts H$_2$O$_2$ to water [37]. In our study the decrease in catalase activity might be correlated with an increase in the level of lipid peroxidation.
Glutathione plays an important role in the detoxification reactions. Depletion of glutathione content potentiates the induction of tissue injury as it plays an important role in maintaining tissue integrity [38]. In our study the significant decrease in the GSH content of the enterocytes might be due to its oxidation upon exposure to the free radicals. Recently, reduced levels of glutathione and increased lipid peroxidation have been reported in patients with active duodenal ulcer [39].

Furthermore, in our study decreased levels of GSH were accompanied by a significant decrease in the GR and G-6-PDH activities. Siegers et al. [40] have also observed that a marked decrease of GSH level is accompanied by a concomitant decline of GR activity. Reduced glutathione is a specific substrate for GSH-Px [41] and GST [42]. In the present study the decrease in the level of GSH probably led to a significant decrease in GSH-Px and GST activity. Reduced glutathione is a specific substrate for GSH-Px [41] and GST [42]. In the present study the decrease in the level of GSH probably led to a significant decrease in GSH-Px and GST activity. With a significant decrease in activities of GSH-Px and catalase, it is reasonable to expect that H₂O₂ is not as efficiently catabolized. It would then accumulate to a point where its reaction with iron initiates the peroxidation of lipids to a faster or greater extent than can be controlled by the cells. With a significant decrease in the level of GST the lipid peroxides cannot be efficiently detoxified. Thus a significant decrease in the level of GSH probably led to an increase in the level of lipid peroxidation.

Tocopherol can scavenge free radicals and during this process it is depleted in the membrane. A significant decrease in the tocopherol content of the enterocytes as shown in the present study might expose the cells to oxidative stress and further contribute to the chain propagation of lipid peroxidation.

From the findings of our study it is suggested that V. cholerae O139 mediated intestinal infection is accompanied by a significant increase in the levels of intracellular second messengers. Further, the infection is accompanied by a significant increase in the generation of ROS and a significant decrease in the level of endogenous antioxidant defenses. This decrease in the levels of antioxidant defenses sensitized the enterocytes to an increased flux of ROS which led to an increase in the extent of lipid peroxidation of the enterocyte membrane. Intracellular calcium stores and PKC seem to be important in mediating V. cholerae O139 induced lipid peroxidation. Thus increased fluid secretion in this infection might be due to the changes in membrane permeability which take place as a result of increased lipid peroxidation.

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