Uremic Levels of Oxalic Acid Suppress Replication and Migration of Human Endothelial Cells

Richard I. Levin, Philip W. Kantoff, and Eric A. Jaffe

Patients with chronic renal failure who undergo hemodialysis experience accelerated atherosclerosis and premature death. Since the end-metabolite, oxalic acid, accumulates in plasma in proportion to the severity of renal failure, we studied whether sodium oxalate (0 to 300 μM) is an endothelial toxin and, therefore, might enhance atherogenesis. Exposure to uremic levels of oxalate (>30 μM) for 9 to 28 days depressed endothelial cell replication by 33% to 84% (mean±SD, 54%±15.7%, n=17 experiments, p=0.002). In contrast, replication of fibroblasts exposed to 200 μM oxalate for 45 days was not inhibited. The inhibitory effect of oxalate on endothelial cell replication was both dose- and time-dependent (both p<0.001) and was first detected 3 to 7 days after the initial exposure to oxalate. Further, the inhibitory effect was fully reversible upon removal of oxalate, but only if exposure was limited to 5 days or less. Sodium salts of other carboxylic acids (citric, succinic, glyoxylic, and malonic; 200 μM) as well as HCl (200 μM) did not suppress endothelial cell replication. Oxalate also inhibited endothelial cell migration but had no effect on basal, thrombin-induced, or arachidonate-induced prostacyclin production by endothelial cells. Exposure of endothelial cells to sodium oxalate (200 μM) for as little as 24 hours—a time period sufficient to induce delayed, transient inhibition of replication not detectable until approximately 1 week after exposure— inhibited incorporation of 3H-leucine into protein by 40% (p=0.009). We conclude that sodium oxalate acts as an uremic toxin, inhibiting endothelial cell replication and migration, functions which may be important for constative inhibition of atherosclerosis. (Arteriosclerosis 10:198–207, March/April 1990)

The incidence of both atherosclerosis and its complications is elevated in patients with uremia who are maintained on chronic dialysis.1–17 Further, this morbidity occurs at a much younger age than in the general population,4–6 suggesting that atherosclerosis is both more prevalent and accelerated in patients with chronic renal failure. The mechanism of accelerated atherosclerosis in uremia is probably complex; hypertension,8,9 abnormal carbohydrate metabolism,10,11 a prothrombotic state accompanied by decreased fibrinolysis,12,13 and abnormal factor VIII,14,15 abnormal lipoprotein patterns,16–25 polyamine accumulation,26,27 hyperparathyroidism,28,29 and abnormal trace mineral concentrations30,31 have all been proposed as possible contributing factors.

Physical and metabolic integrity of the endothelium may be critical in preventing or retarding atherosclerosis,32 and therefore, agents that either injure the endothelium or inhibit endothelial cell functions may foster atherogenesis. Oxalic acid is a dicarboxylic acid, which in humans is the end product of the oxidative metabolism of both ascorbic and glyoxylic acids. It is a chelator with no known function in humans but is an inhibitor of intermediary metabolism in cellular and cell-free systems in vitro.33–36 Oxalate is excreted entirely by the kidney and therefore rises in plasma in proportion to the decline in glomerular filtration.37,38,39 Because dialysis does not normalize oxalate concentrations in the plasma of patients with chronic renal failure,37,39 elevated plasma oxalate levels may be one of the atherogenic factors present in chronic uremia. We therefore studied the ability of oxalate to alter endothelial function as an in vitro model of the "response-to-injury hypothesis."32 In this paper, we show that elevated oxalate levels inhibit endothelial cell replication and migration.

Methods

Cell Culture

Human umbilical vein endothelial cells were obtained and cultured as previously described.40 In brief, cells were harvested from umbilical veins with collagenase (0.1%) and maintained in Medium 199 supplemented with 20% pooled human serum to which amphotericin B (2 μg/ml), L-glutamine (1.6 mM), penicillin (40 U/ml), and...
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streptomycin (40 μg/ml) were added. Cells from single primary cultures at confluence were plated in 2 to 8 replicate subcultures containing identical cell numbers in T-25 culture flasks or 6-24-well plates (Corning, Comin, NY). The medium was changed every 3 days, and test materials were added with each change of medium. The conditions applied to subsequent passages are described below. Human lung fibroblasts (Human Genetic Mutant Cell Repository, Rockville, MD), passage 13, were cultured in minimal essential medium (Flow, McLean, VA) with 20% fetal calf serum to which the antibiotics listed above were added.

Quantification of Cell Replication

Cell replication was defined as the increase in the number of cells in a given culture over time. The effects on cell replication of sodium oxalate (0 to 300 μM), other carboxylic and organic acids (citric, succinic, glyoxylic, malonic, and hydrochloric; 0 to 200 μM; Sigma, St. Louis, MO), and other metabolites that accumulate in renal failure (creatinine 0 to 1.1 mM; urea 0 to 25 mM) alone and in combination were determined by comparing cell replication in subcultures derived from a single primary culture.

Cells were counted in situ daily by minor modifications of a morphometric technique previously described. At the start of each experiment, four to six permanent counting sites defined by a template were marked on the underside of each flask. The marks allowed absolute positioning of every flask, so that identical fields (same size and same relative position within each flask) could be counted repeatedly. Depending on the experiment, all cells in a 200×, 300×, or 400× field visualized by phase contrast in an inverted microscope (Nikon, Garden City, NJ) were manually counted by using a 90° reticle as a counting aid. The technique yields counts that are tightly and linearly correlated with counts obtained manually with a hemocytometer or automatically with an electronic particle counter (Coulter, Hialeah, FL).

To determine the cumulative cell count represented by the cells in a field on a given day, the actual cell count of the microscopic field was multiplied by the cumulative dilution factor of all previous passages. For example, if cells were diluted at passages 2, 3, and 4 by ratios of 1:2, 1:3, and 1:3, respectively, the cumulative dilution factor in passage 4 would be 18, the product of the individual dilution ratios. Throughout the experiment through multiple passages, all four to six sites in all flasks were counted on the same days, three to seven times per week.

Each experiment began with first-passage cells derived from a single primary culture. Depending on the length of the umbilical cord from which the cells were obtained and the number of experimental conditions to be tested, the primary culture was divided into two to eight first-passage subcultures. On experimental Day 0, before the addition of experimental materials, cells in each flask were counted to verify that identical numbers of cells had successfully plated. If significant differences in cell counts as determined by analysis of variance were present, the culture was discarded. Further, in all experiments, the treatment assigned to a flask was determined randomly by a blinded observer. In some experiments, the blinded observer prepared coded tubes containing the experimental additives, and the investigator(s) performing the daily cell counts were blinded to treatment as well.

Since cell density may itself be a determinant of cell replication, it is necessary to control for potential bias introduced by low plating densities in subsequent passages from cultures containing fewer cells due to retardation of replication. Therefore, cells were passed under three different protocols: 1) the cells of all flasks in an experiment were passed at identical dilution ratios when the first flask became confluent, 2) the cells of each flask were passed when the individual flask became confluent, and 3) the cells of all flasks were passed when the first flask became confluent, but the dilution ratios were adjusted so that all cultures were replated at identical densities. By using all three protocols, it was possible to rule out as major contributors to retardation of replication: 1) the effect of low plating density as produced by protocol 1, 2) the length of time in a given passage as produced by protocol 2, and 3) the stress due to greater replication in the most rapidly dividing culture as produced by protocol 3. Since in all experiments, regardless of the protocol used, oxalate caused significant inhibition of cellular replication, the results from all protocols have been combined in the data presented.

Labeling of Endothelial Cells with ⁵¹Cr

For short-term experiments in which chromium release was studied, endothelial cells were incubated in medium containing ⁵¹Cr (sodium chromate, 5 μCi/ml; New England Nuclear, Boston, MA) for 3 hours and washed twice before use. For long-term experiments, cells were incubated with ⁵¹Cr for 24 hours.

Preparation of Cells for Transmission Electron Microscopy

Cells were washed twice with Medium 199 without human serum and were detached from flasks by incubation at 37°C for 15 minutes with 0.1% ethylenediaminetetraacetic acid and 0.1% collagenase (Type I, Worthington, Freehold, NJ) in a buffer containing 10 mM HEPES (pH 7.4 at 37°C), 137 mM NaCl, 5 mM KCl, and 5.5 mM glucose. Cells were subsequently treated as previously described for viewing in an Elmiskop 1 (Siemens, Iselin, NJ).

Radioimmunoassay for 6-keto-PGF₁α

Endothelial cells were grown in medium alone or in medium containing sodium oxalate (0 to 200 μM) for various times. The cells were fed with fresh medium containing appropriate concentrations of oxalate for 24 hours before stimulation. The cells were washed twice with HEPES-buffered saline (10 mM HEPES [pH 7.4 at 37°C], 137 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl, and 5.5 mM glucose) and were stimulated with 2 μM sodium arachidonate for 2 minutes or with human thrombin (0.1 U/ml) for 5 minutes, since prostacyclin (PGI₂) production was maximal at these times for these agonists. Supernates were removed, snap-frozen, and subsequently analyzed by specific radioimmunoassay for
the stable metabolite of PGI₂, 6-keto-PGF₁α, as previously described.45

Effect of Oxalate on Endothelial Cell Migration

Confluent monolayers of endothelial cells were wounded with a razor blade according to the method of Schleef and Birdwell46 at 60 minutes after being treated with mitomycin C (10 μg/ml). The distance of cellular migration into the wound approximately 70 hours after wounding was determined by using phase-contrast microscopy and aligning the original edge of the wound with the "0" line on a 10 × 10 grid-reticle and counting the number of boxes from the line containing cells in each of the 10 rows defined by the reticle.

Effect of Oxalate on Macromolecular Incorporation of ³H-Leucine and ³H-Thymidine

Endothelial cells were exposed to medium alone or to medium containing sodium oxalate (200 μM) for 24 hours. Either ³H-leucine (10 μCi/ml) or ³H-thymidine (7 μCi/ml; both New England Nuclear) was then added to the medium, and the cells were labeled overnight. In some experiments, cells were pretreated with cycloheximide (3 μg/ml). After labeling, the cells were washed three times in cold, HEPES-buffered saline. The cells were disrupted with three cycles of freeze-thawing, the macromolecules were precipitated with 10% trichloroacetic acid, and the pellets and supernates were counted in a liquid scintillation counter (Tri-carb 4500, Packard, Sterling, VA). Since both uptake of label into cells (for ³H-leucine: 7.8%±2.5% in control vs. 9.1%±3.8% in oxalate-treated cultures [n=9, p>0.5]; for ³H-thymidine: 10.1%±5.2% in control vs. 13.8%±6.9% in oxalate-treated cultures [n=9, p>0.5]) and cell counts between the precipitate and supernatant fractions obtained from the cell lysates.

Statistical Analysis

The significance of the effect of various treatments and their interactions on cell replication was determined by analysis of variance (ANOVA) or repeated-measures ANOVA as appropriate, and post-hoc pairwise comparisons were made with the least-significant-difference test. The significance of the effect of sodium oxalate on endothelial cell PGI₂ production was also determined by ANOVA. The significance of the effect of sodium oxalate on endothelial cell migration, a noncontinuous variable, was determined by the Mann-Whitney U test. Analyses were performed by computer programs contained either in the Hewlett-Packard statistics library (09815-15001) or in PC Statistician/PC ANOVA (Human Systems Dynamics, Northridge, CA). Data are presented as mean±SD unless stated otherwise.

Results

Effect of Sodium Oxalate on Cellular Replication

Sodium oxalate significantly inhibited endothelial cell replication in a time-dependent manner. In each experiment, the cells were counted repeatedly in one to four flasks for each condition, and within each flask at four to six sites when T-25 flasks were used and six sites when T-75 flasks were used. As shown in Figure 1A, the replication-retarding effect of oxalate was usually detectable after 1 week of incubation, while the earliest significant difference in any experiment was seen at 3 days. As shown in Figure 1B, the effect of sodium oxalate was both dose- and time-dependent (both p<0.0001); in some experiments, prolonged exposure to oxalate led to the death of the culture. When the mean cell counts from 17 experiments were analyzed, exposure of human endothelial cells to sodium oxalate (mean±SD, 182±38 μM; range, 30 to
Figure 2. Reversibility of oxalate-induced retardation of replication. A primary culture of human umbilical vein endothelial cells was divided into eight replicate subcultures and was grown in medium alone or in medium containing 200 \( \mu M \) of sodium oxalate for 0 to 17 days. The cultures were exposed to medium containing oxalate for 0, 1, 3, 5, 8, 10, 12, or 15 days as shown and were then grown in medium alone for the remainder of the experiment. By Day 12, the subculture never exposed to oxalate had a total, cumulative cell count of 312±61 cells/300× field. In contrast, by Day 12, subcultures exposed to oxalate for any and all times shown were inhibited by an average of 62% (to 118.5±13.8 cells/300× field, \( p<0.0001 \)). Cultures exposed to oxalate for 1, 3, or 5 days showed delayed inhibition of replication, which began on Day 8, persisted through Day 12, and then abated so that by Day 15, these subcultures contained cell counts that were virtually identical to the subcultures never exposed to oxalate (274.1±10.6 vs. 276.8±67.1, respectively). However, by Day 15, the cell counts in subcultures exposed to oxalate for 8, 10, 12, or 15 days remained significantly different from the controls (mean»127.7±20.5, \( p<0.0001 \)). This pattern persisted through the end of the experiment, i.e., to Day 17.

Further, when each of the 17 experiments was analyzed individually, oxalate significantly inhibited cellular replication in every experiment.

Figure 2 demonstrates that the replication-retarding effect of oxalate was reversible and that reversibility depended on the length of exposure. Exposure to 200 \( \mu M \) of oxalate for as little as 24 hours and for up to 5 days resulted in a significant, but delayed and transient, inhibition of cellular replication \( (p<0.0001) \). This inhibition persisted until the 15th day, at which time cell counts in these subcultures, which had been exposed to oxalate for up to 5 days, were virtually identical with the cell count in the control subculture, which was never exposed to oxalate. In contrast, subcultures exposed to oxalate for longer than 5 days had not recovered by the termination of the experiment at Day 17 and demonstrated persistent inhibition of cellular replication. Virtually identical results were obtained in two additional experiments.

To determine the specificity of this phenomenon, several experiments were performed. As shown in Figure 3, 300 \( \mu M \) for a mean time of 19.2±4.8 days (range, 9 to 28 days), inhibited replication by 54.3%±15.7% \( (p=0.002, n=17, \text{range of inhibition, 28.7% to 84.0%}) \). Further, when each of the 17 experiments was analyzed individually, oxalate significantly inhibited cellular replication in every experiment.

Calcium Chelation and Inhibition of Cellular Replication by Sodium Oxalate

Sodium oxalate binds ionized calcium and, while it is unlikely that chelation of 200 \( \mu M \) of calcium in a medium containing 1.8 mM calcium would be of significance in contributing to the inhibition of cellular replication, we studied this directly. Four replicate subcultures from a
single primary culture were grown, respectively, in medium alone, medium containing 200 μM sodium oxalate, medium to which 200 μM calcium chloride had been added, and medium containing 200 μM sodium oxalate to which 200 μM calcium chloride had been added. Cells were counted repeatedly over a 33-day period at six sites in each flask. Control and calcium-treated cultures exhibited virtually identical growth curves with mean, cumulative cell counts on Day 33 of 35 568±4420 and 36 288±3267, respectively (p>0.5). In contrast, the oxalate-treated and oxalate/calcium-treated cultures began to show inhibition of replication on Day 8 and were inhibited by virtually identical amounts by Day 33 (20.4% and 20.2%, respectively) when mean cumulative cell counts were 28 296±2215 and 28 368±3517, respectively (effect of oxalate, p<0.0001; effect of calcium, p>0.5). Further, there was no interaction between calcium and oxalate; i.e., the addition of an equimolar amount of calcium chloride did not alter the inhibition of cell replication induced by sodium oxalate (interaction of calcium and oxalate, p>0.5). Thus, it is unlikely that chelation of calcium by oxalate is responsible for the oxalate-induced inhibition of cellular replication.

**Effect of Sodium Oxalate on \( ^{51} \text{Cr} \) Release from Endothelial Cells**

While the replication curves do not suggest that oxalate has an acutely toxic, lethal effect, we studied this possibility directly by determining whether sodium oxalate enhanced the release of \(^{51}\text{Cr} \) from endothelial cells. Sodium oxalate had no effect on \(^{51}\text{Cr} \) release from endothelial cells after exposure for 0 to 4 hours or 1 to 10 days (data not shown), suggesting that the inhibition of replication is, in fact, due to slowing of replication rather than to increased cell death.

**Morphological Characterization of Endothelial Cells Treated with Sodium Oxalate**

Elevated concentrations of oxalate in both hereditary oxalosis and chronic uremia are associated with the formation of oxalate crystals, which may be associated with vasculitis.47-49 We explored the possibility that crystal formation or destruction of intracellular organelles might be responsible for retardation of replication by microscopic observations of cellular morphology. Within a week to 10 days of growth in medium containing sodium oxalate (200 μM), there were no differences noted in morphology of cells viewed under phase-contrast microscopy compared to cells grown in medium alone, and no crystals were observed under polarizing microscopy. As cultures aged, they developed a typical "senescent" appearance with many giant cells, large nuclei, and extensive vacuolization; these changes appeared much earlier in oxalate-treated than in control cultures. No other obvious morphologic differences at the level of light microscopy between cells exposed to medium alone or to medium containing sodium oxalate were noted. The absence of crystal formation and intracellular disruption was confirmed by transmission electron microscopy (data not shown).

**Interaction of Sodium Oxalate with Other Metabolites that Accumulate in Renal Failure**

Since urea is known to enhance the solubility of oxalate,50 we determined whether uremic concentrations of these two metabolites interacted in altering endothelial cell replication. We also studied the interaction of oxalate and creatinine. As shown in Figure 5A, by Day 26 in culture while both urea (25 mM or 150 mg/dl [equivalent to a blood urea nitrogen level of approximately 70]) and oxalate (150 μM) alone suppressed endothelial cell replication (by 22% and 66%, respectively, both p<0.0001), their combination was additive (90% suppression). This result was obtained in two of three experiments, while in the third, urea alone did not inhibit cell replication, and there was no additive effect of oxalate and urea in inhibiting cell replication. As shown in Figure 5B, creatinine (1.1 mM or 12.4 mg/dl) and oxalate also independently inhibited endothelial cell replication (by 11% and 84% respectively, both p<0.001), and their combination was additive (99% suppression). Similar to the variability described above for the effect of urea, the inhibitory effect...
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15,000
10,000
5,000
0

0 5 10 15 20
Day

Cumulative Cell Count

Control
Oxalate
Citrate
Succinate
Glyoxylate
Malonate

Figure 4. Specificity of sodium oxalate as an inhibitor of cell replication. A primary culture of endothelial cells was divided into six replicate subcultures and was grown in medium alone or in medium containing 200 μM of the sodium salts of the carboxylic acids listed. At Day 16, the total, cumulative cell counts/300x field in each of these subcultures were: control, 9024±897; oxalate, 5664±1105; citrate, 9824±1049; succinate, 10 404±1603; glyoxylate, 8976±1627; and malonate, 9528±891. The inhibition of cellular replication induced by oxalate was significant (p<0.0001), and cell counts in the oxalate subculture differed significantly from each of the other subcultures (all p<0.01), while none of the cell counts in the subcultures exposed to the other acids differed significantly from control or each other.

of creatinine was seen in only one of two experiments. Thus, both urea and creatinine, independently, are weak inhibitors of endothelial cell replication compared to oxalate, and their interactions with oxalate are variable.

Effect of Sodium Oxalate on Endothelial Cell Prostacyclin Production

To determine whether oxalate also altered endothelial function, as represented by eicosanoid synthesis, the effect of sodium oxalate on PGI2 production by endothelial cells was determined both in the basal state and after stimulation by thrombin and sodium arachidonate. Table 1 demonstrates that exposure of near-confluent endothelial cells to various concentrations of sodium oxalate (0 to 200 μM) for 4 days had no effect on PGI2 production. Similarly, when the endothelial cell conditioned media from four different cultures grown with or without oxalate were examined serially (three to six changes of medium spanning 15 to 24 days) and the production of PGI2 was normalized for cell count, sodium oxalate had no effect on PGI2 production (data not shown). Thus, the complex cascade of events that generates PGI2 from arachidonic acid was not altered by prolonged exposure to oxalate.

Effect of Sodium Oxalate on Endothelial Cell Migration After Wounding

Figure 6 shows that sodium oxalate inhibits migration of endothelial cells. After exposure to 200 μM of sodium oxalate for 10 days, migration 70 hours after wounding the monolayer was inhibited by 45% (p<0.0001). Similar to the time requirement for inhibition of endothelial cell replication, inhibition by oxalate of endothelial cell migration was also time-dependent, being present after a 10- but not a 3- or 7-day exposure.

Effect of Sodium Oxalate on Incorporation of 3H-Leucine and 3H-Thymidine

Since there were no gross morphological abnormalities in endothelial cells exposed to sodium oxalate that might explain its replicative inhibitory effect, we sought to determine whether oxalate altered protein or DNA synthetic activity as a mechanism of retardation of replication. Because exposure to oxalate for as little as 24 hours inhibits cellular replication (see Figure 2) and because substantial reductions in synthetic rate would be expected late in the course of an experiment when the rate of cell replication is dramatically reduced, we studied the effect of sodium oxalate (200 μM) on synthetic rates after only a 24-hour exposure. As shown in Table 2, sodium oxalate inhibited leucine incorporation by approximately 40% (p<0.0001) but decreased thymidine incorporation by only 32%, a change that was not statistically significant (p=0.071). Thus, it is possible that oxalate inhibits endothelial cell replication by interfering with protein synthesis.

Discussion

In this study, we have shown that elevated levels of oxalate, an end-product of human metabolism that
area may be important in retarding atherogenesis. In this context, it is of interest that hyperglycemia, the metabolic derangement of diabetes, which is a major risk factor for atherosclerosis, has also been shown to inhibit endothelial cell replication and metabolism. Therefore, the inhibition in vitro of endothelial replication and migration by concentrations of sodium oxalate, which are present in uremic patients undergoing chronic dialysis, may be viewed as a potentially atherogenic phenomenon that may contribute to the increased prevalence and acceleration of atherosclerosis in the uremic population.

Normal plasma oxalate concentrations determined by modern techniques, including in vivo isotope dilution studies, range between approximately 1 and 5 μM. In renal failure, oxalate concentration rises linearly in proportion to the creatinine and therefore is dependent on the severity of renal failure. In patients undergoing chronic dialysis, pre-dialysis serum oxalate concentrations of 20 to 200 μM and immediate, post-dialysis levels are approximately 10 to 50 μM. Thus, the concentrations of oxalate utilized in our studies, 30 to 300 μM, encompass the concentrations of oxalate present in uremic patients undergoing chronic dialysis and induce a dose-dependent inhibition of endothelial cell replication (Figure 1). Further, oxalate and urea were additive in inhibiting cell replication in some experiments (Figure 5), and in vivo the oxalate binds calcium and have approximately equal affinity constants for calcium, and oxalate inhibits endothelial cell replication, while citrate does not (Figure 4). Further,
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Table 1. Effect of Sodium Oxalate on Stimulated Prostacyclin Production

<table>
<thead>
<tr>
<th>Oxalate concentration (μM)</th>
<th>Agent</th>
<th>0</th>
<th>10</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na A</td>
<td>5.7±0.9</td>
<td>6.1±3.0</td>
<td>6.1±1.4</td>
<td>4.6±1.8</td>
<td>3.7±1.6</td>
<td>5.4±0.9</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>3.2±0.4</td>
<td>3.6±0.6</td>
<td>4.0±0.4</td>
<td>3.1±0.1</td>
<td>3.0±0.2</td>
<td>3.7±0.5</td>
<td></td>
</tr>
</tbody>
</table>

Values of PG2 are given in ng/0.1 ml.

Table 2. Effect of Sodium Oxalate on Incorporation of 3H-Leucine and 3H-Thymidine by Endothelial Cells

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Oxalate</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H-leucine</td>
<td>Control</td>
<td>Oxalate</td>
<td>%</td>
</tr>
<tr>
<td>4.1±3.5</td>
<td>2.5±2.4</td>
<td>39.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3H-thymidine</td>
<td>10.3±4.5</td>
<td>7.1±4.0</td>
<td>31.8</td>
</tr>
</tbody>
</table>

The results are the means±SD of three experiments of three replicates each.

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