Administration of Nitric Oxide Through a Novel Copper-Chitosan Delivery System in Human Corneal and Limbal Epithelial Cell Injury

Vasiliki Tellios,1 Hong Liu,2 Nikoleta Tellios,2,3 Xiangji Li,2,4 and Cindy M. L. Hutnik2,3,5

1Department of Neuroscience, University of Western Ontario, London, Ontario, Canada
2Lawson Health Research Institute, London, Ontario, Canada
3Department of Pathology and Laboratory Medicine, University of Western Ontario, London, Ontario, Canada
4Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada
5Department of Ophthalmology, Ivey Eye Institute, St. Joseph’s Health Care, London, Ontario, Canada

Correspondence: Cindy M.L. Hutnik, Ivey Eye Institute, St. Joseph’s Health Care, 268 Grosvenor Street, London, Ontario, N6A 4V2, Canada; Cindy.Hutnik@sjhc.london.on.ca.

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PURPOSE. Nitric oxide (NO) has gained attention for its role in facilitating wound healing by promoting cell migration, while being cytoprotective in a variety of cell types. We determined the efficacy of NO, administered using a novel application of copper-chitosan treatments (Cu-Ch), in facilitating corneal epithelial wound healing using an in vitro model of corneal epithelial and limbal epithelial cell injury.

METHODS. Human corneal epithelial (HCE) and human limbal epithelial (HLE) cells were monitored under no-scratch (CON), untreated scratch (CS), scratch + plain chitosan composite (0%), scratch + 1% copper solution Cu-Ch (1%), and scratch + 2% copper solution Cu-Ch (2%) conditions. Cell migration, cytotoxicity, apoptosis, and total nitrate/nitrite concentrations were measured at 24, 48, and 72 hours after injury and treatment. iNOS expression in HLE cells also was determined using Western blot.

RESULTS. Wound closure significantly increased in HCE cells treated with Cu-Ch (1% and 2%) after 72 hours, while HLE cells showed a significant decrease in closure with Cu-Ch (1% and 2%) treatment compared to CS. Cytotoxic fragments decreased significantly with 1% and 2% Cu-Ch treatments in HCE cells. Nitrate/nitrite levels in HLE cells showed a significant increase with 2% Cu-Ch treatment compared to CS. This increase is complemented with an upregulation of iNOS.

CONCLUSIONS. Overall, HCE wound healing was accelerated with administration of Cu-Ch treatment. Differences between HCE and HLE responses may be due to intrinsic differences in NO metabolism, as evidenced by differences in NO production, potentially caused by differences in iNOS expression with treatment.

Keywords: epithelial cell injury, copper-chitosan, nanoparticle, nitric oxide, iNOS

In the presence of an acute mechanical injury to the corneal epithelium, the balance between epithelial cell loss and renewal is disrupted. To resolve this deficit in the corneal epithelium, the physiological process of reepithelialization is modified to induce a greater rate of cell migration and proliferation.1 During this process, only minor cell proliferation is detected in the basal cells situated in the corneal epithelium, while limbal epithelial cells supply the greater portion of proliferative potential.2

Nitric oxide (NO) has gained significant attention over the past decade as a prospective therapeutic signaling biomolecule in facilitating various wound healing processes. NO is produced ubiquitously across various tissues, and is known to modulate wound inflammation and apoptosis, differentiation, migration, and collagen synthesis and deposition, while inhibiting proliferation during an inflammatory response.3–5 Under normal conditions, NO is produced enzymatically at a constant rate via constitutively active isoforms of nitric oxide synthase (NOS) in most cells. This basal rate of NO production is a homeostatic feature that promotes regular cell turnover and viability at specific concentrations.6 In human corneal epithelial (HCE) cells, endothelial nitric oxide synthase (eNOS) is the most abundant constitutively expressed NOS isoform.6 Human limbal epithelial (HLE) cells are known to have a role in corneal wound healing by repopulating the wound site through proliferation.2 Unlike HCE cells, HLE cells characteristically express only iNOS as their main source of endogenous NO, specifically during an inflammatory response.6 In the presence of an injury, the release of cytokines associated with cellular damage activates inducible nitric oxide synthase (iNOS), a nonconstitutive NOS, and, thus, increases the total cellular production of NO, which marks the start of wound healing.4 The upregulation of NO via iNOS in an injury has been reported in various cell types, and is indicative of its potential as a modulator of cellular processes in epithelial injury, while inhibiting proliferation.5,7–8

A systematic review citing the effects of NO in wound healing noted significantly positive effects on the wound healing process corresponding to increases in NO production, specifically an increase in overall wound strength.9 Another
study noted the antimicrobial properties of the molecule when used as a treatment for superficial skin injuries. Determining an optimal concentration of NO in a corneal epithelial environment is critical in positively modulating wound closure of mechanical injuries, including injuries to the cornea. Bonfiglio et al. have assessed the wound healing effects of NO in an in vivo model using rabbit corneas. In their experiments, NO was administered using a widely-used NO donor sodium nitroprusside (SP), and corneal wound healing was compared to controls and conditions treated with iNOS inhibitors. Corneal surfaces treated with SP were completely reepithelialized after 60 hours of treatment, and were coupled with an increase in cell viability at specific concentrations of SP.

NO donor molecules have become increasingly popular in the context of wound healing in light of current studies documenting promising effects of NO in wound healing. Recently, natural biopolymers and nanocomposites have been of interest in terms of designing an effective vessel able to increase NO levels in a cellular environment. For the nanoparticle to be effective in terms of wound modulation, production of NO must be at a controlled rate to produce the cited benefits. Conventional NO donors, such as S-nitroso-N-acetyl-DL-penicillamine (SNAP), SP, and NOC-18 often are found in aqueous solutions, which may result in the hydrolysis of NO into nitrates and nitrates, consequently compromising the experimental effectiveness of the drug. Nanoparticles derived from biocompatible material, such as chitosan, have shown to be a promising tool that can induce synthesis of NO in an injury environment. This biopolymer, when cross-linked with metal ions, has been demonstrated to promote wound healing, while providing antibacterial and cytoprotective activity. The biopolymer proposed in this study (imaged in Fig. 1) is composed of three main ingredients used to facilitate the production of NO. In this composite, copper (I) ions (Cu⁺) are bonded covalently to the organic biopolymer chitosan to form a stable compound. Glucose also is present in the composites, mainly to replenish Cu⁺ ions, while making the composite more malleable. This mechanism of NO production, depicted in Figure 2, is innovative in that it works to recycle wound byproducts, such as nitrates and nitrites, into a proportional concentration of NO.

Given the lack of corneal abrasion treatments that specifically target wound healing, in addition to the heterogeneity associated with NO as a signaling molecule, the purpose of this set of experiments was to assess the efficacy of novel copper-chitosan (Cu-Ch) composites in administering NO for an in vitro model of human corneal and limbal epithelial (HCE and HLE, respectively) injury. A secondary aim of this study sought to address functional differences between HCE and HLE cells under a similar NO stimulus. We hypothesized that the use of NO as a treatment in epithelial injuries will accelerate the wound healing process in addition to positively benefitting cell viability in HCE, while inhibiting the proliferative potential of HLE cells.

MATERIALS AND METHODS

Cell Culture and Cu-Ch Treatments

Primary HCE cells were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA) and ATCC (Manassas, VA, USA), while HLE cells were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Primary HCE and HLE cells within five passages were cultured in corneal epithelial cell basal medium supplemented with the corneal epithelial cell growth kit, both purchased from ATCC. Cells were seeded at 10⁵ cells onto 6-well plates and incubated at 37°C, 5% CO₂ for 48 hours until approximately 80% confluence. Three Cu-Ch treatments were tested, including a vehicle control containing pure chitosan (0%), and 1% and 2% copper solutions. The 0%, 1%, and 2% Cu-Ch composites were kindly supplied by our colleagues at the University of Windsor in the Department of Biochemistry, Bulent Mutus, PhD, and Kathleen Fontana.
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FIGURE 2. Schematic of Cu-Ch mechanism. The active ingredient Cu⁺ interacts with nitrites found naturally in wound exudate. The nitrites are reduced by Cu⁺ to become NO, while the active ingredient is oxidized to its inactive form, Cu²⁺. Cu⁺ concentrations are replenished by the limiting reagent glucose, which reduces Cu²⁺ to its active form.

Scanning Electron Microscopy

Topographical morphology of the Cu-Ch treatments was imaged using the Hitachi s3400 Scanning Electron Microscope supplied by the Biotron facility at the University of Western Ontario. Cu-Ch particles were mounted and coated with a mixture of gold and palladium, and viewed at 25 kV.

MTT Assay

To optimize the concentration of Cu-chitosan for the scratch assay trials, the primary HCE cells were treated with 2, 4, and 6 µg Cu-chitosan treatments over the course of 24, 48, and 72 hours in a 96-well plate. The MTT reduction assay (Sigma-Aldrich Corp.; Oakville, ON, Canada) was used as an index of cellular metabolic activity and viability, and was performed according to the manufacturer’s instructions. Absorbances were normalized to the untreated control cultures, which represented 100% viability.

Wound Healing Assay

HCE and HLE cells were seeded in 6-well culture plates and grown to 80% confluence in serum-free medium. A linear mechanical abrasion was made using a sterile scalpel in the cell monolayer to simulate a wound. Mechanically-injured cells and cell fragments were not removed, and 8 µg of Cu-Ch treatment (0%, 1%, 2%) were administered immediately after the abrasion (final treatment volume adjusted for culture dish area and volume of medium). Migration of cells into the scratched area was monitored across 24, 48, and 72 hours in comparison with untreated scratch (CS) conditions. Griess reagents were used to detect nitrate/ite levels in the medium and used as an inference of NO production, measured in µM, performed according to the manufacturer’s instructions (Thermo Fisher Scientific). Absorbances were normalized to the treatments’ respective protein levels using the Micro BCA assay (Thermo Fisher Scientific) and again to the CON condition, which represented 100% of total cytotoxicity.

Cell Death ELISA

A Cell Apoptosis ELISA kit was purchased from Sigma-Aldrich Corp. HCE and HLE cells were mechanically injured and treated as described previously. Cells were lysed after 24, 48, and 72 hours using 400 µL of lysis buffer (using PBS, 1 mM EDTA, and 0.2% Tween-20) per sample. Samples were centrifuged at 2655g for 30 minutes and the supernatant was collected. The cell death ELISA was used as an index for cellular death in the form of apoptosis, performed according to the manufacturer’s instructions. Absorbances were normalized to the unscratched control (CON) condition, which represented 100% of total apoptosis. Absorbances were normalized further according to the amount of protein present in each well, measured using a micro bicinchoninic acid assay (Micro BCA assay; Thermo Fisher Scientific).

LDH Assay

Medium from the wound healing assay was collected, and total nitrate/ite production was measured after 24, 48, and 72 hours of treatment with 8 µg of 0%, 1%, and 2% Cu-Ch treatments and compared to CON and untreated scratch (CS) conditions. Griess reagents were used to detect nitrate/ite levels in the medium and used as an inference of NO production, measured in µM, performed according to the manufacturer’s instructions (Thermo Fisher Scientific). Absorbances were normalized according to the basal level of nitrates/ites found in the serum-free medium.

Western Blotting

Proteins from the HLE treatment conditions mentioned were lysed after 24 hours using IP lysis buffer (Pierce, Thermo Fisher Scientific), and measured using a Micro BCA assay (Thermo Fisher Scientific). Proteins were run through SDS-PAGE using an 8% polyacrylamide gel at 100 V and transferred to a nitrocellulose membrane. Membranes were blocked in 5% BSA solution, and later incubated with primary anti-iNOS rabbit monoclonal antibody (1:1000; Sigma-Aldrich Corp.) and secondary anti-rabbit IgG HRP conjugate (1:3000, Bio-Rad Laboratories, Hercules, CA, USA). iNOS protein expression levels were normalized to housekeeping protein GAPDH (1:3000; Santa Cruz Biotechnology, Dallas, TX, USA).

Statistical Analyses

Data are given as the mean of three technical replicates of three individual biological donors per cell type. All data were analyzed using GraphPad Prism 6.0 software. Comparisons of multiple treatments over various time points were performed using a 2-way ANOVA with a Bonferroni post hoc test. For multivariate comparisons over one time point, a 1-way ANOVA was used with a Tukey post hoc test. P ≤ 0.05 was considered statistically significant. All data are reported as the mean ± SEM.
RESULTS

Optimization Profile of HCE and Limbal Cells

To optimize the dosage of Cu-Ch for use in subsequent experiments, cellular metabolic activity was assessed in a time- and dose-dependent manner. HCE cells treated with 2, 4, and 6 μg of each 0%, 1%, and 2% treatments over 24, 48, and 72 hours showed a significant time- and dose-dependent decrease in cellular metabolic activity (Fig. 3). At 72 hours, cellular metabolic activity decreased significantly to approximately 16% and 13% of that of the CON with 6 μg of 1% and 2% treatment, respectively (P < 0.0001). The 2% treatment significantly decreased cellular metabolic activity at all concentrations at 72 hours (P < 0.0001), while the 2 and 4 μg treatments of 1% Cu-Ch at 72 hours did not significantly decrease cell metabolic activity compared to CON.

The same treatment in limbal cells showed a similar time- and dose-dependent result in terms of cellular metabolic activity (Fig. 4). At 72 hours, cellular metabolic activity significantly dropped to 7% of that of the CON with 6 μg of the 1% and 2% treatments (P < 0.0001). Only the 2 μg treatment of the 1% particle after 72 hours did not present a significant decrease in cellular metabolic activity compared to CON. The lowest dose of Cu-Ch in both cell types showed the least deviation in metabolic activity compared to the control, and this concentration was carried on to the wound healing assay.
Cu-Ch Treatment Accelerates HCE Wound Healing After 72 hours

To determine the functional effects of NO on HCE and HLE cells, cellular migration was assayed in the presence of Cu-Ch treatments. After in vitro mechanical injury, HCE and HLE cells were treated with 8 μg of each treatment, or left untreated. In HCE cells, 1% and 2% particles at 8 μg concentrations presented visible increases in wound closure percentage compared to CS at 72 hours (Fig. 5). Semiquantitative analyses of the wound area showed a significant increase in wound closure percentage with 1% Cu-Ch treatment at 72 hours, while 2% Cu-Ch treatment elicited a significantly greater wound healing response across all time points compared to CS (Fig. 5B).

HLE Wound Closure Impeded With Cu-Ch Treatments

In contrast, treatment with 1% and 2% Cu-Ch on HLE cells resulted in visibly decreased wound closure compared to CS at 72 hours (Fig. 6A). Semiquantitative analyses of the wound area showed significant decreases in wound closure percentage compared to CS at the 48- and 72-hour time points (P < 0.001; Fig. 6B).

Cell Cytotoxicity Associated With Necrotic Death Decreases in HCE Cells Treated With Cu-Ch

Treatment with NO may cause necrotic or apoptotic death in an in vitro setting. To determine whether Cu-Ch treatment induced either necrotic or apoptotic death in HCE and HLE cells, we next wanted to characterize the cell death profile within each cell type after Cu-Ch treatment. Apoptosis levels in HCE cells after 72 hours of 8 μg treatment of Cu-Ch, as measured by levels of histone-associated DNA fragments read through an ELISA cell death detection kit, were not significantly increased or decreased compared to CON and CS (Fig. 7A). In HLE cells, apoptosis levels after 72 hours of 8 μg treatment of 1% and 2% beads showed trending elevations compared to CS, although this change of approximately 1.5-fold was not significant (Fig. 7B).

Cytotoxicity levels, as measured by levels of LDH in HCE cells after 72 hours of 1% and 2% Cu-Ch treatments, were significantly lower compared to CS (P < 0.0001; Fig. 8A). In HLE cells, a 2-fold increase in cytotoxicity levels was noted in 1% and 2% treatments compared to CS, although this increase was not significant (Fig. 8B).

NO Production Increases in HLE Cells With Cu-Ch Treatment

After noting functional differences in HCE and HLE cell types with Cu-Ch treatment, we next wanted to discover whether comparable differences in NO production were present using the Griess reduction method. With the addition of 1% and 2% treatments, no significant changes were seen in [NO₃⁻] in treated HCE cells compared to CS (Fig. 9A). With the addition of the 2% treatment in particular, [NO₃⁻] significantly increased to 5.39 μM ± 0.63 compared to CS (P < 0.05) in HLE cells (Fig. 9B).

Investigation of iNOS Expression in Response to Exogenous NO Production

Considering [NO₃⁻] levels were increased significantly upon treatment of Cu-Ch in HLE cells, we next wanted to test whether this increase was related to changes in iNOS protein expression, the fundamental NO-synthesizing enzyme in this cell type. iNOS expression was analyzed after 24 hours of treatment postmechanical injury (Fig. 10). Although the 2% treatment condition exhibited an approximate 4-fold increase compared to CON, this difference was not statistically significant. Each donor, however, experienced a marked increase in iNOS expression, particularly with 2% Cu-Ch treatment, compared to their respective control conditions. (Fig. 10A).

DISCUSSION

The effects of NO in terms of wound healing have been shown to be beneficial at optimal concentrations and in specific contexts. In this study, the addition of a Cu-Ch treatment (1% and 2%) in a simulated HCE cell injury has been shown to increase wound closure significantly, while reducing the amount of cytotoxic cell fragments related to necrotic pathways resulting from mechanical injury at 72 hours. When incorporated into a HLE cell injury, however, wound closure significantly decreased in 1% and 2% Cu-Ch treatments. This corresponded with a significant increase in [NO₃⁻] production, specifically in the 2% treatment, at 72 hours after injury.

The results from the MTT assay for optimization purposes also revealed the effects of NO in a noninjury environment. The MTT data in both cell types showed an increased sensitivity to the Cu-Ch treatment in increasing doses and over longer time periods. These results corresponded to previous data showing a decrease in cell proliferation within an NO-supplemented environment. The Cu-Ch treatment in this condition may be reacting to basal levels of nitrates and nitrates released into the extracellular environment, the by-products of constitutively active NOS enzymes.

Overall, the HCE cells has been shown to act more favorably to Cu-Ch treatment, and consequently the effects of increased NO synthesis, in a trauma environment compared to HLE cells. The increase in wound closure documented in an in vitro environment in this study is similar to the wound closure results found in in vitro studies involving the cornea. NO has been shown to upregulate cell migration in the presence of an injury via the cGMP-PKG-Rho GTPase signaling pathway, ultimately altering cytoskeleton arrangement leading to locomotion. This pathway may be implicated in the cell migration process in HCE cells, and should be considered for future studies. The HCE cells also experience the added benefit of a significantly lower cytotoxic environment with the addition of 1% and 2% treatments compared to CS, as it has been shown previously that NO exhibits antimicrobial and cytoprotective effects, particularly in conjunction with chitosan biomaterials.

The HLE cells, in contrast, did not respond as favorably to exogenous NO. In the CS and 0% vehicle trials, HLE cells showed a very high potential for cell proliferation and migration (Fig. 6A), and this is consistent with much of the literature documenting the physiologic limburs. However, under both Cu-Ch treatments in HLE cells, cell apoptosis and necrosis markers were elevated, but the change was not statistically significant. This finding may be an indicator of a potential mechanism that is characteristic of NO-mediated cell death, associated with the hyperproduction of NO in an in vitro environment. The differences in results between the HCE and HLE cells may suggest fundamental mechanistic variances associated with the NO signaling pathway, and prompt further study into the dissimilarities between these closely connected cell types.
FIGURE 5. Cu-Ch treatment promotes wound closure in mechanically injured HCE cells. (A) HCE cells were mechanically injured and treated immediately after injury. Wound areas across treatments were compared across all time points. Black lines denote the visually determined wound borders, while dotted lines represent baseline wound area. (B) When quantified, significant increases in wound closure were noted after 72 hours of 1% and 2% treatments compared to CS. Data were analyzed using a 2-way ANOVA with Bonferroni post hoc (*P < 0.05; **P < 0.01; n = 3).
FIGURE 6. Cu-Ch treatment deters wound closure in mechanically injured HLE cells. (A) HLE cells were mechanically wounded and treated with 8 μg of each previously mentioned treatment, immediately after injury. Black lines denote the visually determined wound borders, while dotted lines represent baseline wound area. (B) When quantified, significant decreases after 48 and 72 hours were noted in the 1% and 2% treatments compared to CS. Data were analyzed using 2-way ANOVA with Bonferroni post hoc (****P < 0.0001; n = 3).
Significant and accelerated wound healing in epithelial cells under NO treatment has been documented previously multiple times, and this finding remains consistent with the results shown in this study regarding the corneal epithelium. The basal value of NO measured in HCE cells, averaged in this study to 3.25 ± 0.85 μM, is consistent with NO levels of the corneal epithelium referenced in the literature (4.12 ± 0.95 μM). The results concerning the NO data (Fig. 9) clearly illustrated the delicate concentration balance needed when applying NO as a signaling molecule during injury. The hyperproduction of NO,
as seen in its significant increase after 72 hours in HLE cells, has shown to heavily impede fundamental processes associated with wound healing through the overproduction of reactive oxygen and nitrogen species (ROS/RNS), such as peroxynitrates.\(^{28}\) ROS/RNS production can lead to oxidative stress, which can impede wound healing processes.\(^{29}\) However, the Cu-Ch treatment in HCE cells did not induce an over production of NO, but rather slight increases that facilitated key wound healing components, such as cell migration. This may be due to the basal activity of eNOS within HCE cells—

**FIGURE 9.** [NO\(_x\)] production was increased significantly in HLE cells treated with 2% Cu-Ch treatment, but not HCE cells. (A) After 72 hours of injury, [NO\(_x\)] production was measured using the Griess assay. No significant changes in [NO\(_x\)] were detected. Data were analyzed using 1-way ANOVA with Tukey’s post hoc (\(n = 3\)). (B) After 72 hours, [NO\(_x\)] production is significantly higher compared to CS in the 2% treatment condition in HLE cells. Data were analyzed using 1-way ANOVA with Tukey’s post hoc (*\(P < 0.05\); \(n = 3\)).

**FIGURE 10.** iNOS expression is not increased significantly with Cu-Ch following 24 hours of treatment in HLE cells. (A) Relative changes in iNOS expression are detectable, specifically with 2% Cu-Ch treatment, compared to CON and CS (\(n = 3\)). (B) Quantification using densitometry depicts an approximate 4-fold upregulation in iNOS expression compared to CON, (not statistically significant). Changes presented are fold changes normalized to the CON condition. Data were analyzed using 1-way ANOVA with Tukey’s post hoc (\(n = 3\)).
these cells may adjust well to higher concentrations of NO, while cells not primed with a constitutive NOS isoform, such as HLE cells, may have difficulty compensating for comparable NO concentrations. The Griess reagent used in this study is successful in detecting changes at the μM level, which is sensitive enough to detect pathologic increases in NOx species; this may explain nonsignificant increases in NOx species in treated HCE cells.6

The increase in [NOx] species in HLE cells may be explained by an increase in iNOS protein expression, the most dominant NO contributor in this cell type.6 Overall iNOS expression was assayed at the 24-hour time point, where increases in protein expression are crucial in affecting fundamental changes in the wound healing process. In particular, the latent phase of corneal wound healing, which is devoted to protein synthesis, occurs within the first 24 hours of injury to the cornea.1,2,7

Within this study, iNOS expression in individual donors increased qualitatively with 2% Cu-Ch treatment after 24 hours relative to CON and CS conditions (Fig. 10A). However, these increases were not statistically significant when the data from the three donors were averaged and an analysis of densitometry was performed. Observed changes may be due to individual donor and biological variance, which may involve certain demographic factors including age, race and sex.30 These factors may affect the activity of the iNOS enzyme as well as its expression in the presence of an injury. These results suggested the presence of a potential positive feedback mechanism induced by the presence of NO, which has been shown previously to differentially affect iNOS expression and activity depending on cell type and concentration.31 In the context of our study, further investigation is required to determine the role of iNOS and its importance in the wound healing process.

In conclusion, HCE cells respond favorably to Cu-Ch treatments in the presence of a mechanical injury, while the hyperproduction of NO in HLE cell injury impedes the fundamental processes of wound healing in an in vitro setting. This finding ultimately highlights underlying mechanistic differences between the two cell types at the level of NO signaling processes and metabolism. Cu-Ch may become a viable and effective treatment in facilitating and consequently accelerating the wound healing process in mechanical injuries, such as corneal abrasions, and may be extended to target other epithelial cell injuries. Future studies should include translation of this research to animal models to determine systemic effects of this treatment.

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**References**