



# Resveratrol Supplementation Attenuates Excessive Inflammation and Helps Restore Impaired Restitution in an Intestinal Epithelial Cell Culture Model

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## ABSTRACT

**Aim:** Sustained release of inflammatory mediators, excessive inflammatory response and impaired intestinal epithelial restitution are well-known mechanisms in the pathogenesis of necrotizing enterocolitis. This study focused on the effect of resveratrol on these pathways.

**Materials and Methods:** In this study, the rat crypt intestinal cell line (IEC-6) culture, an application of lipopolysaccharide or a cytokine mixture and a scratch migration assay model were used. Nitric oxide synthase-2 (iNOS) and cyclooxygenase-2 (COX-2), focal adhesion kinase (FAK) and its phosphorylated form (pFAK) levels were assessed.

**Results:** IEC-6 cells covered 88% of the denuded area in the control, 54% in LPS, and 35% in cytomix groups at the 24<sup>th</sup> hour. The treatment with resveratrol at doses of 0.5, 1 and 5  $\mu$ M/L before LPS resulted in the repair of 84%, 87% and 76% of the denuded areas, respectively. Likewise, with cytomix, it was 86%, 82%, and 78%. Resveratrol at a dose of 5  $\mu$ M/L prevented an increase in iNOS levels. All three doses of resveratrol were effective in preventing increases in COX levels. FAK or pFAK expressions remained unchanged in all groups.

**Conclusion:** Resveratrol, being known for its antioxidant features, suppresses excessive inflammatory response and helps preservation of mucosal integrity by conservation of epithelial restitution.

**Keywords:** Necrotizing enterocolitis, resveratrol, restitution, inflammation, nitric oxide synthase-2

## Introduction

Necrotizing enterocolitis (NEC) is a life-threatening condition mainly effecting premature infants in neonatal intensive care units. An inflammatory response within the bowel wall associated with a sustained release of inflammatory mediators results in impairment of microcirculation leading to a spectrum of ischemic changes in the bowel, ranging from focal mucosal injury to total

ischemia of the whole bowel. Despite an overwhelming number of experimental and clinical studies in the literature, its etiopathogenesis still remains elusive (1).

NEC is a disease characterized by a systemic inflammatory response initiated by the intestinal mucosal immune system and a resultant disruption in the integrity of gut mucosal barrier (1). The exact mechanisms triggering this cascade are still unknown despite numerous studies focusing on this

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excessive inflammation and impairment in mucosal healing (2,3). For a long time, encouraging breastmilk feeding was the only prevention strategy with proven effectiveness (1,4,5). Recently, probiotics have been shown to be effective by various studies including several meta-analyses. However, there is still not enough satisfactory data to recommend the use of any specific probiotic strain (6).

Resveratrol is a well-known phytoalexin, mostly cited due to its anti-inflammatory and vasorelaxant features. It has been widely investigated in a wide spectrum of diseases including various cancers, cardiovascular diseases, Alzheimer's disease, and different cascades of inflammation (7). Therefore, we hypothesized that resveratrol may also be beneficial in preventing NEC regarding its effects on various inflammatory pathways. In a previous study by our group, the protective effect of resveratrol was remarkable in an animal model (8). Its dietetic supplementation to newborn rats prevented 'nitric oxide synthase-2 (iNOS)' expression and morphologic changes in an experimental NEC model. In this current study, we aimed to further dissect the mechanisms with which resveratrol prevents excessive inflammation and impaired restitution in an IEC-6 cell culture model.

## Materials and Methods

### Cell Culture and Treatments

The rat crypt intestinal cell line, IEC-6 cells, were maintained as per recommendations of the manufacturer at 37 °C and 5% CO<sub>2</sub>. The tissue culture medium consisted of a combination of Roswell Park Memorial Institute medium (45%), Dulbecco's Modified Eagle's Medium (4.5 g/L glucose: 45%), and heat inactivated fetal bovine serum (10%). The medium also contained 100 µg/mL streptomycin, 100 U/mL penicillin, 4 mM L-glutamine, and 0.1 U/mL insulin. Cells at the passages 15-20 were used for these experiments. Ethanol was used as the vehicle for resveratrol.

All experiments were held in two groups to reproduce the inflammatory environment. Lipopolysaccharide (LPS) at a dose of 50 µg/ml was applied in the first, and a mixture of cytokines consisting of TNF α (10 ng/mL), IFN γ (100 ng/mL) and IL-1β (1 ng/mL) was applied in the second sets of experiments (9-11).

### Wound Healing Assay

Two perpendicular lines with an intersection at the center of the well were drawn at the outside bottom of the six-well plates before the passaging of the cells. Experimental wounds were made with yellow-tip pipette

parallel to the vertical line to ensure the same area was photographed each time (11).

Each six-well plate was configured as follows; one well as the "control", one well for "treated only with ethanol", one for either "LPS" or "cytomix", and three wells with different doses of "resveratrol" followed by LPS or cytomix application. At least three sets of experiments were performed for each group. The cells in the control group were treated with serum-free medium alone.

The experiments started with 12 hours of serum starvation. Linear wounds were created with yellow-tip pipettes. Three wells were treated with three different doses of resveratrol for one hour. Dose response studies with resveratrol at dosages of 0.1, 0.5, 1, 5 and 10 µM/L were performed. Following this, LPS or cytomix (9-11) were applied to these three wells and one non-treated well for six hours. The medium for cells was used as the vehicle both for LPS and cytomix.

Assessment of migration started with the application of LPS or cytomix. It was monitored with serial photographs of the denuded area taken at 0, 2, 4, 6, 8, 10, 12 and 24 hours under an inverted microscope (Olympus Optical, Tokyo, Japan).

### Western-Blot Analysis

The multiple scrape model to reproduce the conditions of the migration assay was performed (12). For this, cells were grown in 75 cm<sup>2</sup> flasks to reach confluence. The same experimental groups as in the migration model were constituted. At least three sets of experiments for each group were performed. Wound healing assay revealed a statistically significant difference between groups starting at the 12<sup>th</sup> hour. Therefore, cells were exposed to various treatments for 12 hours before sample collection.

iNOS and COX-2 (cyclooxygenase-2) expressions were calculated to assess the inflammatory response. The primary antibodies were rabbit polyclonal iNOS at 1:2,000 dilution, rabbit polyclonal COX-2 at 1:1,000 dilution, and mouse monoclonal β-actin at 1:20,000 dilution. Focal adhesion kinase (FAK) and its phosphorylated form (pFAK) were assessed in order to reveal a possible pathway for their effect on migration. FAK phosphorylated from the tyrosine residue 397 (pFAK<sup>397</sup>) was used. Both FAK and pFAK<sup>397</sup> were rabbit polyclonal and at 1:1,000 dilution. A horseradish peroxidase conjugated mouse/rabbit (according to the primary) antibody was used as the secondary antibody at a dilution of 1:5,000 for iNOS and COX-2, 1:3,000 for FAK and pFAK, and 1:10,000 for β-actin. The medium was

removed after incubation, and SDS-PAGE was performed as previously described (11). Protein concentrations were measured via the Lowry method (13). Bands representing the proteins were visualized using a commercially available chemiluminescence detection kit (ECL Plus; Amersham, GE Healthcare) and images were obtained using a Fusion Solo S imaging system (Vilber, France).

### Materials

IEC-6 cells were obtained from DSMZ<sup>®</sup>, ACC 111 (Leibniz Institute, Braunschweig, Germany). Rabbit polyclonal iNOS antibody, monoclonal anti- $\beta$ -actin, and secondary antibodies (antimouse IgG for  $\beta$ -actin and antirabbit IgG in goat for the others) were from SIGMA (St. Louis, Missouri, USA). Rabbit polyclonal cyclooxygenase-2 (COX-2) was from ABCAM (Biotech Lifesciences, Cambridge UK), FAK and phosphorylated FAK (pFAK) were from Invitrogen (Waltham, Ma, USA). The nitrocellulose membrane used was Hybond-ECL (Amersham, GE Healthcare, Piscataway, NJ, USA). All ingredients of the culture medium were from SIGMA.

### Data Analysis

The closure of the wound, seen in repeated photographs, was measured using 'ImageJ' software (14). The photographs were transferred to ImageJ, a fixed rectangle (with the same width and length) was drawn with one edge on the horizontal marking. The denuded area in this constant rectangle was calculated using the freehand tool in each image (11).

The quantitative analysis of the Western-blot bands was performed using the Fusion Solo S software (Vilber, France). Data are given as the ratio of each protein versus  $\beta$ -actin band density.

The variables were investigated using histograms and Kolmogorov-Smirnov test in order to determine whether they were normally distributed. Repeated measurements ANOVA was used for statistical analysis and  $p < 0.05$  was accepted as significant. Variances were accepted homogeneous and a pairwise post-hoc test (LSD) was used when an overall difference was observed.

## Results

### Dose Titration Studies

Repeated cell subcultures and cell counting with trypan blue showed that the doubling-time for IEC-6 cells at 15-20 passages was around 50 hours. Resveratrol or its solvent ethanol alone had no effect on cell migration or on the expression of the proteins (data not shown). Dose

titration studies with resveratrol at dosages of 0.1, 0.5, 1, 5, and 10  $\mu$ M/L were first performed and the effective doses needed in order to prevent the effects of LPS or cytomix on migration were found to be between 0.5-5  $\mu$ M/L and the remaining experiments were performed with doses of 0.5, 1, and 5  $\mu$ M/L (data not shown).

### Wound Healing Assay

#### "Intestinal restitution is impaired, and resveratrol prevented this impairment"

Representative photographs of the wounds documenting the difference in migration capacity between the groups are shown in Figure 1.

Wound closure was slower from the start in the LPS group compared to all other groups. A statistically significant difference started at the 6<sup>th</sup> hour for the dose of 0.5  $\mu$ M/L and at the 12<sup>th</sup> hour for the remaining groups. Finally, IEC-6 cells covered 88% of the denuded area in the control group, 54% in the LPS group, and 84%, 87%, 76% for the resveratrol+LPS groups at doses of 0.5, 1, and 5  $\mu$ M/L, respectively (Figure 2a).

Similarly, wound closure was slower with the cytomix group. The difference between the cytomix and all other groups was statistically significant starting from the 6<sup>th</sup> hour. Eventually, 35% of the denuded area was repaired in the cytomix groups and 86%, 82%, and 78% for the resveratrol + cytomix groups at doses of 0.5, 1, and 5  $\mu$ M/L, respectively (Figure 2b).

Both the LPS and cytokine mixture were found to significantly impair migration and resveratrol was found to prevent this impairment.

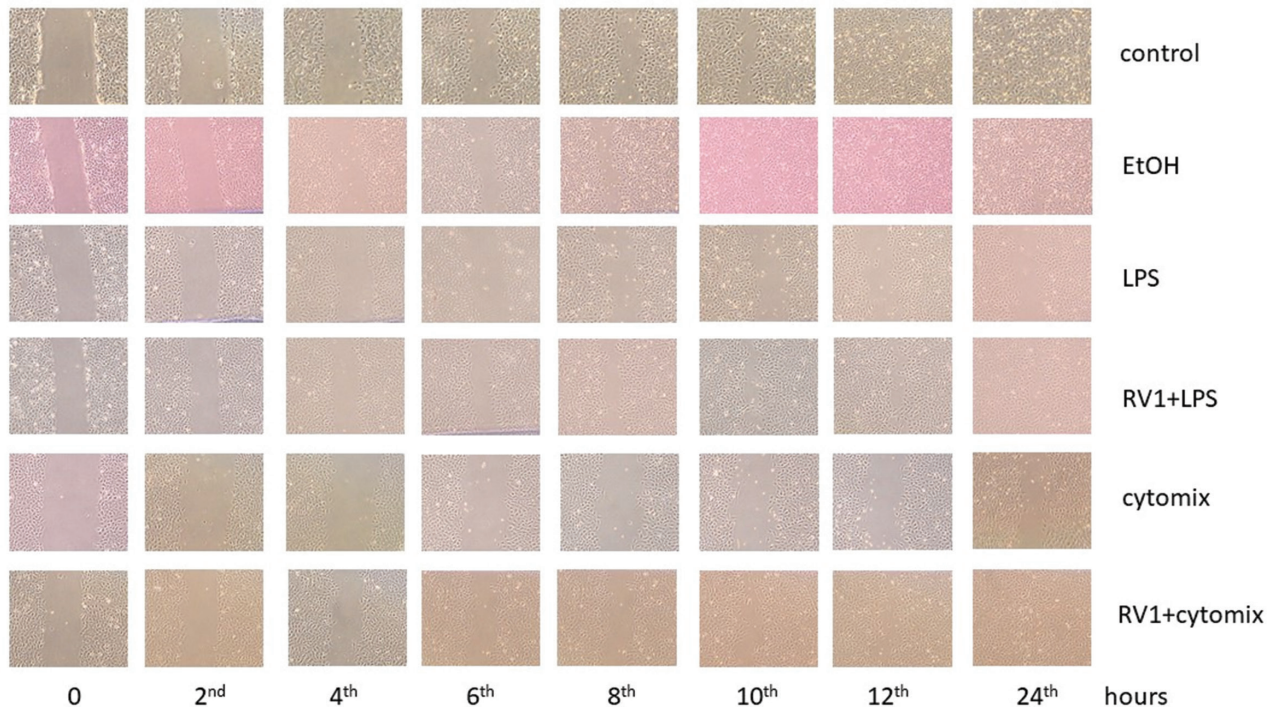
### Western Blot Analysis

#### "iNOS and COX-2 are elevated, and resveratrol prevented this alteration"

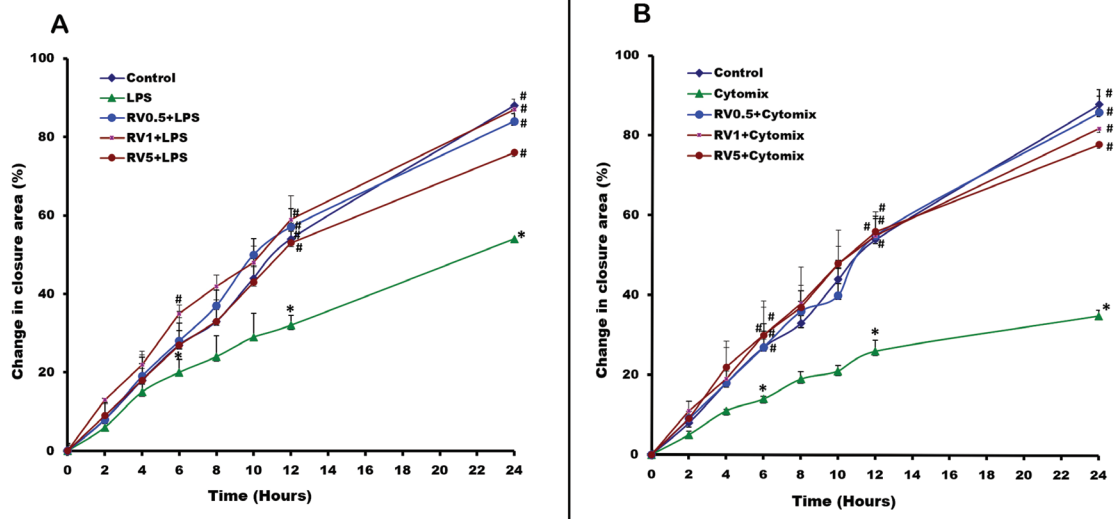
Both LPS and cytomix were found to increase iNOS and COX-2 expression. Resveratrol at doses of 0.5 and 1  $\mu$ M/L did not prevent the increase in iNOS levels after LPS or cytomix administration but it was found to be effective at a dose of 5  $\mu$ M/L (Figure 3). On the other hand, all three doses of resveratrol were found to be effective in preventing the increase in COX levels after LPS or cytomix (Figure 4).

"Total FAK expression or FAK phosphorylation (pFAK) remained unchanged in our experimental model"

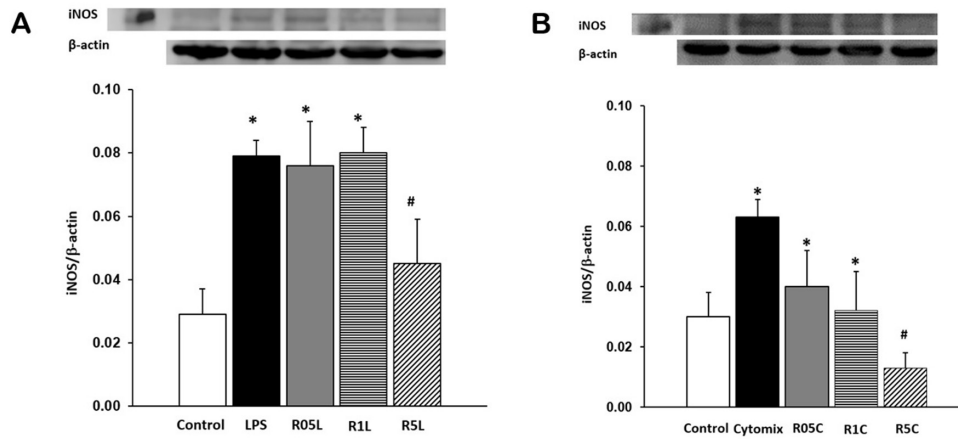
Neither LPS nor cytomix were found to alter total FAK or pFAK levels, and there was no significant change with resveratrol (Figures 5 and 6). The pFAK/FAK ratio also did not change (data not shown).



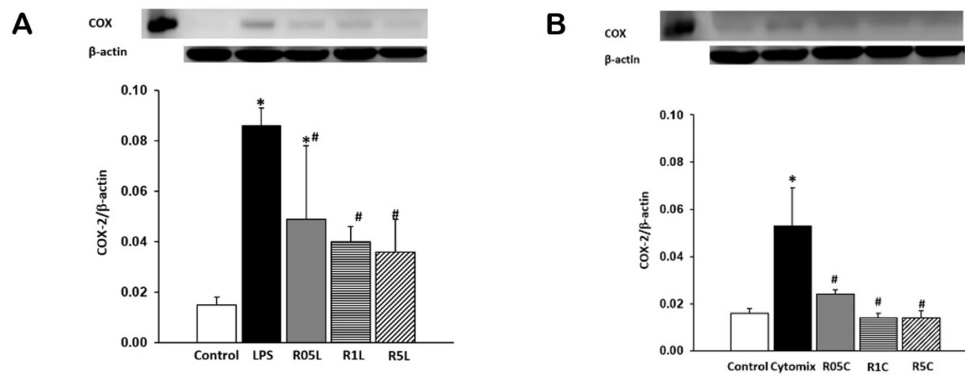
**Figure 1.** Representative images of the denuded area photographed for 24 hours. (EtOH: ethanol, LPS: lipopolysaccharide, RV1+LPS: treated with 1  $\mu$ M/L resveratrol followed by LPS, RV1+cytomix: treated with 1  $\mu$ M/L resveratrol followed by cytomix)



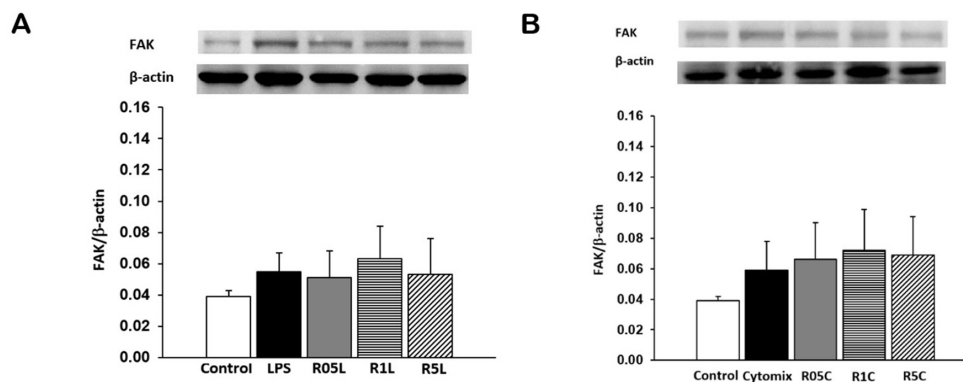
**Figure 2.** Time-response curves of wound closure (%) # represents statistically significant difference from the control and \* from the lipopolysaccharide (LPS) or cytomix groups. A= Groups treated with LPS; B= Groups treated with cytomix



**Figure 3.** Western blot analysis of iNOS expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; R05L= treated with 0.5  $\mu$ M/L resveratrol followed by LPS; R1L= treated with 1  $\mu$ M/L resveratrol followed by LPS; R5L= treated with 5  $\mu$ M/L resveratrol followed by LPS; R05C= treated with 0.5  $\mu$ M/L resveratrol followed by cytomix; R1C= treated with 1  $\mu$ M/L resveratrol followed by cytomix; R5C= treated with 5  $\mu$ M/L resveratrol followed by cytomix)

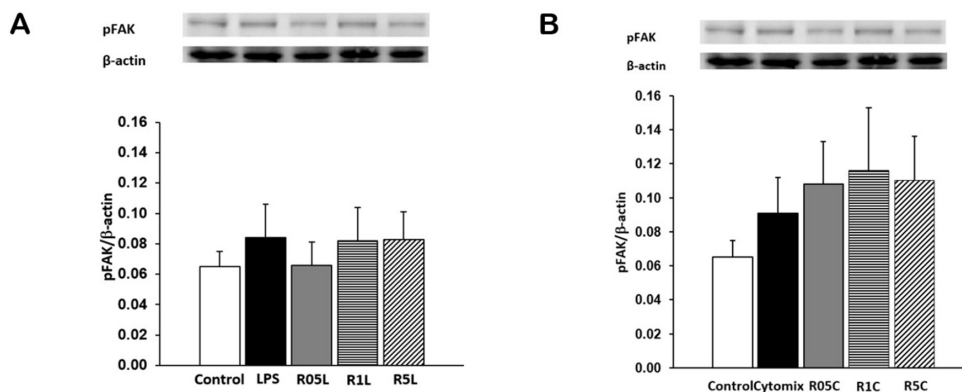


**Figure 4.** Western blot analysis of COX expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; R05L= treated with 0.5  $\mu$ M/L resveratrol followed by LPS; R1L= treated with 1  $\mu$ M/L resveratrol followed by LPS; R5L= treated with 5  $\mu$ M/L resveratrol followed by LPS; R05C= treated with 0.5  $\mu$ M/L resveratrol followed by cytomix; R1C= treated with 1  $\mu$ M/L resveratrol followed by cytomix; R5C= treated with 5  $\mu$ M/L resveratrol followed by cytomix)



**Figure 5.** Western blot analysis of FAK expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; R05L= treated with 0.5  $\mu$ M/L resveratrol followed by LPS; R1L= treated with 1  $\mu$ M/L resveratrol followed by LPS; R5L= treated with 5  $\mu$ M/L resveratrol followed by LPS; R05C= treated with 0.5  $\mu$ M/L resveratrol followed by cytomix; R1C= treated with 1  $\mu$ M/L resveratrol followed by cytomix; R5C= treated with 5  $\mu$ M/L resveratrol followed by cytomix)





**Figure 6.** Western blot analysis of pFAK expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; R05L= treated with 0.5 μM/L resveratrol followed by LPS; R1L= treated with 1 μM/L resveratrol followed by LPS; R5L= treated with 5 μM/L resveratrol followed by LPS; R05C= treated with 0.5 μM/L resveratrol followed by cytomix; R1C= treated with 1 μM/L resveratrol followed by cytomix; R5C= treated with 5 μM/L resveratrol followed by cytomix)

## Discussion

NEC is a disease characterized by a systemic inflammatory response triggered by the intestinal mucosal immune system activated by microbial antigens and enteral feeding (15). After stimulation of the mucosal immune system, an increase in the production of pro-inflammatory cytokines and some inflammatory enzymes such as COX-2 and iNOS synthase occurs. LPS and this excessive inflammation induce intestinal epithelial cell injury and enterocyte apoptosis, resulting in defects in gut mucosal integrity, followed by bacterial translocation and sepsis (1,15).

Healing of the mucosal injury starts with the migration of enterocytes, which is called restitution. In NEC, LPS and inflammatory cytokines are also shown to impair restitution and therefore the healing of mucosal defects as well as the initial injury to the immature intestine (3). Therefore, suppression of inflammatory cytokines and the preservation of the ability of enterocyte migration has a critical role in the prevention and treatment of this highly fatal disease.

Phytoalexins are antimicrobial substances produced *de novo* by plants. Some of them also have antioxidant features. Resveratrol (3,4,5 trihydroxystilbene) is a phytoalexin produced by some spermatophytes in response to injury (7). Resveratrol is a free-radical scavenger and a modulator for some fundamental enzymes in the cell cycle. Many studies have revealed its antioxidant, anti-inflammatory, anti-mutagenic, vasorelaxant, anti-aggregant and hepatoprotective features (7). The previous animal model study carried out in our department revealed a remarkable protective role of resveratrol against NEC. With this current

study, we aimed to investigate the possible pathways which may explain the mechanism of action and provide insights for future studies.

Previous studies have shown that sustained overexpression of intestinal iNOS plays a critical role in the pathogenesis of NEC by inducing enterocyte apoptosis with resultant intestinal barrier failure (1,15). Pro-inflammatory cytokines such as COX-2 are also known to be over-expressed in the bowel with NEC (1). We therefore hypothesized that resveratrol may prevent the exaggerated inflammatory state by suppressing the overexpression of iNOS (16) and cytokines such as COX-2 (17) due to its antioxidant and anti-inflammatory features. We used COX-2 in our experiments as it is one of the most widely investigated cytokines in NEC pathogenesis (1) and also the inhibitory effect of resveratrol on COX-2 has been shown (7,17).

Our study provided evidence that resveratrol is effective in suppressing excessive iNOS and COX-2 production caused by both LPS and/or inflammatory cytokines. Although a variety of pathways are most likely active in NEC pathogenesis, resveratrol was shown to be effective in regulating anti-inflammatory cascades.

Our second hypothesis was that resveratrol could also improve mucosal healing by restoring the migration capability of enterocytes. The second part of our study therefore focused on restitution. Intestinal epithelial cells have an impressive capacity to repair mucosal defects. This relies on the migration, proliferation, and differentiation capability of intestinal crypt cells. Before the much slower proliferation and differentiation phases, viable cells bordering the damaged area migrate to cover any defects.

This process is called restitution and it is known to be accomplished within 15 to 60 minutes (18). Migration and therefore restitution have been shown to be impaired in NEC in both *in vivo* and *in vitro* experimental studies (15).

In our study, we initially performed a time-course analysis in order to determine and confirm the doubling time of IEC-6 cells, and this doubling time was found to be around 50 hours at 15-20 passages, which was consistent with the manufacturers statement. Therefore, closure of the defects was a result of cell migration (restitution) rather than the doubling of the IEC-6 cells.

Having seen its effects on epithelial restitution, we then investigated a possible pathway which could alter this directional motility. FAK is an important regulatory protein which can modify the migration process, leading either to the formation or turnover of focal contacts. FAK protein levels or their regulation by phosphorylation (pFAK) have been asserted to be associated with the modulation of intestinal epithelial restitution (12,19). Cetin et al. (19) have shown the inhibition of intestinal restitution by endotoxin through increased focal adhesions and the increase in pFAK expression in enterocytes caused by nitric oxide with a consequent increase in the formation of focal adhesions in an experimental NEC model (20). We therefore investigated any relationships between resveratrol and total FAK expression or its phosphorylation. The tyrosine residue 397 (pFAK<sup>397</sup>) is known to be the major site of auto-phosphorylation and has been linked with some important pathways including migration (21). However, we were unable to show any alteration in FAK or pFAK levels in our experimental cell culture model. The former study by Cetin et al. (20) was an animal model. One plausible explanation could be the association of other *in vivo* factors which might be responsible for FAK induction which were not represented in our cell culture model.

There is a wide range for the doses of resveratrol in the literature, and treatment with lower doses are mostly attributed to exhibit its anti-inflammatory and antioxidant effects (22); thus, dose titration studies were performed using 0.1, 0.5, 1, 5, and 10  $\mu\text{M/L}$  of resveratrol. The doses 0.1 and 10  $\mu\text{M/L}$  were then discarded due to poor response. Another interesting finding of our study were the better responses in migration with lower doses, and the better responses in iNOS reduction with the higher doses we used. The reduction in COX-2 was similar across all three doses. These discordant results support the presence of other possible *in vivo* pathways involved. Both our previous *in vivo* data (8) and our current *in vitro* data favors the possible protective role of resveratrol in NEC.

### Study Limitations

This study was performed in order to evaluate the possible protective effects of resveratrol on NEC. *In vitro* studies cannot represent the actual disease process as there are several other pathways involved. Also, our findings do not claim any causative effect. Further studies looking into other possible pathways, studies involving chemical inhibitors or genetic modifications to solidify any causative effect, and clinical studies to support these results are required.

### Conclusion

Resveratrol, being known for its antioxidant and vasorelaxant features, was found to regulate the overwhelming inflammatory response and help maintain mucosal integrity by the conservation of epithelial restitution. We found an association with iNOS and COX, but none with FAK or its phosphorylation. Other pathways should also be explored in order to fully explain its possible protective role, which was demonstrated in our previous NEC animal model.

### Ethics

**Ethics Committee Approval:** Ethics committee approval is not required as it is a cell culture study.

**Informed Consent:** Informed consent is not required as it is a cell culture study.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Concept: S.T., A.E., M.O.E., Design: S.T., A.E., M.O.E., Data Collection or Processing: S.T., A.E., Analysis and Interpretation: S.T., A.E., M.O.E., Literature Search: S.T., Writing: S.T.

**Conflict of Interest:** The authors declared that there were no conflicts of interest.

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