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Development of an ex Vivo Ileal Explant Culture Method for Amplified Production and Differential Measurement of Nitrite

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Abstract: Quantifying nitrite, a metabolite of nitric oxide (NO), is a well-established marker for the production of reactive oxygen species and an indirect measurement for inflammation. Under optimal culture conditions various cell based systems, like peripheral blood mononuclear cells, abdominal macrophages along with many macrophage based cell lines, would produce measurable nitrite by 24 to 72 h post stimulation with an agonist. We have developed a rapid ex vivo ileal explant culture method that can measure elevated nitrite within 3 h of lipopolysaccharide (LPS) stimulation *in vitro*. The model was developed to measure elevated NO along with the ability to measure differential NO among control and treated groups, with an aptitude to screen potential anti-inflammatory and anti-oxidant candidates. Ileal cross-sections (0.5 cm^2) were cultured from chickens that were challenged for three consecutive d with *Salmonella* Enteritidis in the drinking water. Quantification of NO in these inflamed ileal explants provided a suitable screening model which potentially mimics *in vivo* intestinal conditions. This model could rapidly detect NO, at a greater magnitude than other cell culture methods. The ileal explants produced elevated nitrite by 3 h with a maximal magnitude of 478.42 μM nitrite 6 h post LPS stimulation. The model was also successful in measuring differential NO between the control and groups treated with potential anti-inflammatory compounds. This unique and simple ileal explant culture method provides a rapid screening system for inflammation modulation and the potential to quantify other inflammatory markers that are indicative of other gut pathogens to evaluate candidates for regulating inflammation.

Key words: Nitric oxide, nitrite, ileal explants, inflammation

INTRODUCTION

The innate immune response is modulated through the recruitment of various cellular components upon pathogen exposure. Heterophils, monocytes and macrophages are at the forefront of pathogen recognition and work in combination with effector leukocytes to initiate an immune response. Studies investigating the role of heterophils, monocytes and macrophages begin with quantification of reactive nitrogen species (RNS), reactive oxygen species (ROS), along with cytokines and chemokines (Crippen *et al*., 2003). Though rapid clearance of pathogens has been attributed to ROS (oxidative stress) rather than RNS (nitrosative stress), nitrosative stress is important in chronic and prolonged exposure. The sequential progression from a predominant oxidative stress to the production of nitrosative clearance could optimize the reduction in microbial burden along with minimizing immunopathological consequences of host inflammatory response (Vazquez-Torres and Fang, 2001; Chakravortty and Hensel, 2003). Hence, quantifying metabolites of nitric oxide (NO), such as nitrite or expression of inducible nitric oxide synthase (iNOS), have been the principle for investigating the role of RNS during host inflammatory responses.

Various cell based systems, like peripheral blood mononuclear cells (He *et al*., 2008), abdominal macrophages (Qureshi, 2003) along with cell lines including HD11 (Setta *et al*., 2012), MQ-NCSU (Hussain and Qureshi, 1997), J774 (Korhonen *et al*., 2001; Vitral *et al*., 2010; Takahashi *et al*., 2011), have been employed to test their ability to produce NO in response to various stimuli, in a time and dose dependent manner. Even though these systems have given a lot of information regarding the production of NO and related immunological data, there has been an intrinsic issue regarding quantity and time at which NO is produced. Most of these cell types, under optimal culture conditions, require 24 to 72 h post stimulation with an agonist (e.g., LPS) to produce measurable nitrite in their culture supernatants (Hussain and Qureshi, 1997; Lowry *et al*., 1998; Crippen *et al*., 2003; Lillehoj and Li, 2004; Babu *et al*., 2006; He *et al*., 2006; Setta *et al*., 2012). In most cases, detectable quantities of NO cannot be observed even after 12 to 48 h post stimulation and subsequent increases are slow to

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detect. The mentioned time line for NO production is attributed to the expression of iNOS in these cells, which peaks between 6 to 12 h post stimulation followed by a noticeable lag period, for the accumulation of iNOS mRNA and translation into a functionally active enzyme (Stuehr and Marletta, 1987; Mason *et al*., 1996; Hussain and Qureshi, 1997). Further, studies have shown that the expression of iNOS in macrophages is variable among chickens of several genotypes (Hussain and Qureshi, 1997). Also, employing a single cell type to understand and explain many immunological actions, essentially carried out in concert, may not be comprehensive and fail to represent *in vivo* conditions. For example, as previously mentioned, NO produced by heterophils is not comparable to that of macrophages, under *in vitro* conditions. However, during an inflammatory response to pathogen exposure, heterophils are recruited to the site of infection at much greater numbers than macrophages, resulting in comparable amounts of total NO, as a result of elevated numbers of cells present at the inflammatory site (Kogut *et al*., 1995).

These highly reactive RNS and ROS free radicals have been shown to have clinical effects during pathogenesis. As seen with clinical salmonellosis and coccidiosis, the targets of inflammation are not necessarily limited to the invading pathogen, but also extended to host tissue (Vazquez-Torres and Fang, 2001). The inflammatory responses that are aimed at controlling these luminal pathogens, interestingly, have been shown to promote their outgrowth in the gut lumen, enhancing their infection. For example, the epithelial transmigration of neutrophils in mammals (heterophils in chickens) into the intestinal lumen followed by the release of ROS provides a respiratory electron acceptor, tetrathionate to *Salmonella* and facilitates its outgrowth in the intestine (Winter *et al*., 2010; Thiennimitr *et al*., 2012).

We have developed a rapid ex vivo ileal explant culture method that detects increased NO within 3 h of LPS stimulation *in vitro*, in ileal explants from *Salmonella*-challenged chickens. We have also evaluated this model for its capability to measure differential NO in explants treated with potential anti-inflammatory and anti-oxidant plant extracts with active poly-phenolic compounds (Sandoval-Chacon *et al*., 1998; Puupponen-Pimia *et al*., 2005; Jang *et al*., 2007; Hassan *et al*., 2008; Wang *et al*., 2008; Allen-Hall *et al*., 2010). Ileal cross-sections were cultured from chickens that were previously challenged with *S.* Enteritidis, in drinking water, for three consecutive days. Chronically pre-exposing *Salmonella* to chickens was assumed to result in nitrosative stress and infiltration of various immune cells to the site of infection causing inflammation. The inflamed gut environment would theoretically recruit all cell types including heterophils, monocytes, macrophages, which until now have been cultured and tested individually. Culturing such an inflamed intestinal explant would provide us with a model that decreases the time required to trigger innate immune effector functions, like NO production, with a significant increase in NO magnitude, hence mimicking *in vivo* mucosal conditions. Since the immune cells are pre-infiltrated into the explant, a simple stimulation with an agonist like LPS should produce NO quickly at measurable amounts from the culture supernatants. Further, the developed method was evaluated for the ability to measure differential NO levels between control and treated groups, four plant extracts, Cat's claw (CC), *Senna reticulate* (Senna), Black Walnut (BW) and Clove (CL), all of which are reported to have anti-inflammatory and anti-oxidant properties (Sandoval-Chacon *et al*., 1998; Puupponen-Pimia *et al*., 2005; Wang *et al*., 2008).

MATERIALS AND METHODS

Animal source: Day-of-hatch, off-sex broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR, USA) and were placed in isolators, in a controlled environment. Chickens were provided *ad libitum* access to water and a balanced non-medicated corn-soybean diet meeting the nutrition requirements of poultry recommended by NRC (1994). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas.

Salmonella Enteritidis

A primary poultry isolate of *Salmonella enterica* subspecies *enterica* serovar Enteritidis, bacteriophage type 13A (*Salmonella* Enteritidis), was obtained from the USDA National Veterinary Services Laboratory. This isolate was resistant to novobiocin (25 µg/mL, catalog no. N-1628, Sigma) and was selected for resistance to nalidixic acid (20 µg/mL, catalog no.N-4382, Sigma) in our laboratory. For the present studies, 100 µL of *Salmonella* Enteritidis from a frozen aliquot was added to 10 mL of tryptic soy broth (Catalog no. 22092, Sigma) and incubated at 37°C for 8 h and passaged every 8 h to ensure that all bacteria were in log phase. Post incubation, bacterial cells were washed 3 times in sterile 0.9% saline by centrifugation at 1,864 x g, quantified with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific) and diluted in sterile 0.9% saline to a concentration of approximately 10^8 cfu/mL. Concentrations of *Salmonella* Enteritidis were determined retrospectively by serial dilution and further plating on brilliant green agar (Catalog no. 70134, Sigma) with novobiocin and nalidixic acid for enumeration of actual cfu used to challenge the chickens.

Plant extracts: Black Walnut (*Juglansnigra*), Clove (*Syzygiumaromaticum*) and Senna (*Senna reticulata*) liquid extracts were manufactured and obtained from

Ozark Natural Foods, Fayetteville, AR, USA, containing 1000 mg/ml herb weight equivalence and 48-58% alcohol by volume. Powdered form of Cat's Claw (*Uncariatomentosa*) inner bark was also obtained from the same source and manufactured by Now Foods, Bloomingdale, IL. All plant extracts, to be tested as treatments, were adjusted for a final concentration of 100 µg/ml of RPMI 1640 medium with 5% bovine serum, 1.5 mM/L glutamine and 1 mL of antibiotic-antimycotic solution (containing 10,000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin-Sigma-aldrich, St. Louis, MO).

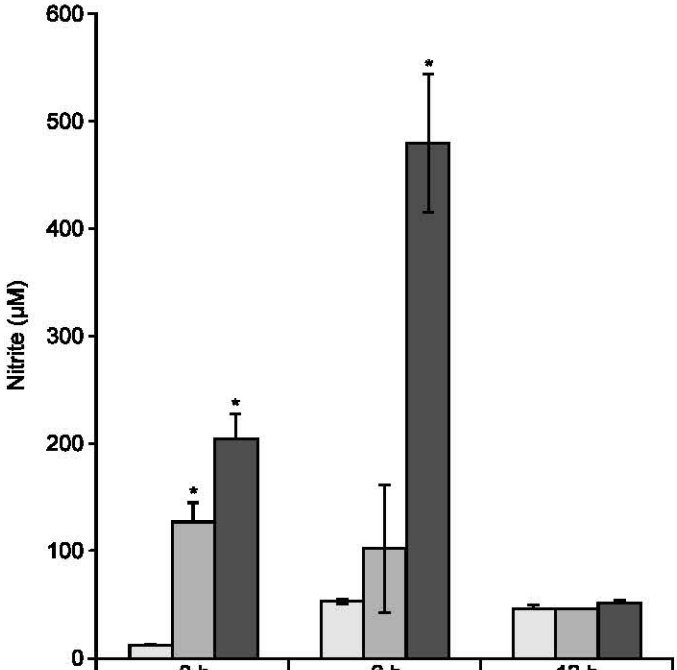
Explant culture: Day-of-hatch off sex broiler chicks were randomly assigned into 2 groups (n = 12/group). At 2 wk of age, one group was challenged with *Salmonella* Enteritidis (10^8 cfu/chick) in the drinking water for 3 consecutive d, with an objective to chronically infect the birds, causing inflamed gastrointestinal conditions. The other group received sterile saline and served as a control group. Three d post challenge, the birds were humanely killed by CO_2 asphyxiation and ileal explants were aseptically removed and cultured. Briefly, the entire ileum from the inflamed gut was aseptically removed, cleaned by infusing sterile 0.9% saline through the ileal section to remove all of the ingesta. The cleansed ileum section was then incised longitudinally exposing the mucosal surface. Ileal sections (0.5 cm^2) were made using a sterile surgical blade and placed in a 24 well culture plate, with one explant per well. Care was taken in placing the tissue explants, with the serosa facing down and in contact with the well bottom and the mucosa facing up, exposed to the media components. Circular metal meshes measuring approximately 7.5 mm in radius with 3 mm height were used to keep the floating explants in place (Menconi *et al*., 2013). These meshes were made of steel 316 L, the same material used in construction of fermenters and bioreactors. The material is non-reactive, non-additive, non-absorptive and non-corrosive and hence a safe choice to be used with a culture medium. Further care was taken to autoclave these meshes before use, to avoid any contamination.

Explants (n = 12) for group 1 were obtained from non-challenged chickens, with one ileal explant per chicken. Explants (n = 12 for each group) for group 2 through 7 were obtained from chickens that were previously challenged with *Salmonella*. The ileal explants cultured in 24 well culture plates were then transferred to a laminar airflow hood and 1 mL of RPMI 1640 medium with 5% bovine serum, 1.5 mM/L glutamine and 1 mL of antibiotic-antimycotic solution (containing 10,000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin -Sigma-aldrich, St. Louis, MO) was added to each well. The explants were incubated for 1 h at 40°C, 5% CO_2 for acclimatizing the explants to culture conditions.

Post initial incubation, medium for explants from group 1 through 3 were replaced with 1 mL of fresh medium, whereas, fresh medium containing BW, CL, CC, or Senna plants extracts were added to groups 4 through 7. The cultures were further incubated at 40°C, 5% CO_2 for 3 h. Following incubation, the explants from group 3 through 7 were replaced with 1 mL of fresh culture medium containing LPS from *Salmonella* Enteritidis (Sigma-aldrich, St. Louis, MO) at a concentration of 100 µg/ml, while groups 1 and 2 received fresh medium without LPS. Overall, a total of 7 groups of explants were cultured with or without plant extract treatments and with or without LPS stimulation. Group 1 (n = 12 explants) were from non-challenged birds and were not stimulated with LPS nor treated and hence served as a double negative control (*Salmonella* $^{(-)}$/LPS $^{(-)}$). Group 2 (n = 12 explants) was from *Salmonella* challenged birds but was not stimulated with LPS *in vitro* (*Salmonella* $^{(+)}$/LPS $^{(-)}$) and was not treated with plant extracts. Group 3 (n = 12 explants) served as double-positive control, since they were both challenged with *Salmonella* and stimulated with LPS *in vitro* (*Salmonella* $^{(+)}$/LPS $^{(+)}$). Groups 4 through 7 were all from the *Salmonella* challenged birds, treated with different plant extracts and then stimulated with LPS *in vitro*. The medium containing plant extract was removed from the explant tissue and then fresh complete RPMI was replaced and further incubated for 12 h during which supernatant fluids were collected at 3, 6 and 12 h post stimulation with LPS *in vitro* for the nitrite assay.

Nitrite assay: The Greiss reaction assay, which colorimetrically quantifies nitrite, was used to measure the nitrite accumulated over time in the culture medium, which served as an indirect measure of NO produced by the explants. The assay was carried out in a 96 well microtitre plate to which 100 µL of culture supernatant from the explants (at 3, 6 and 12 h) was added in triplicates, followed by an equal volume of Greiss reagents. First, 50 µL of 1% sulfanilamide (Sigma-aldrich, St. Louis, MO) in 5% phosphoric acid, was added and incubated at room temperature for 10 min followed by 50 µL of 0.1% N-(1-napthyl) ethylenediamine dihydrochloride (Sigma-aldrich, St. Louis, MO) in water and incubated further for a visible colored reaction to develop. Nitric oxide was measured at 540 nm using a Bio-Tek uQuant microplate reader (Bio-Tek, Winooski, VT). Optical density (OD) for each explant sample was compared to known amounts of sodium nitrite (1.25, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 µM) and extrapolated using a standard curve equation.

Data and statistical analysis: The production of nitrite was measured at 3, 6 and 12 h post stimulation with LPS and results were expressed as mean±SEM of micromolar amounts of nitrite for each group of explants



	3 h	6 h	12 h
Salmonella-/LPS-	9.85	52.29	45.81
Salmonella+/LPS-	126.83	100.10	45.28
Salmonella+/LPS+	203.69	478.42	51.12

Fig. 1: Comparison of nitric oxide production. Nitrite was measured in the supernatant of explants stimulated with 100 µg of lipopolysaccharides (LPS)/ml and measured at 3, 6 and 12 h. Bars represent means (n = 12 explants/group) and standard error of 3 groups. Treatment means were partitioned and asterisks above the bars indicates significant difference ($p \leq 0.05$) as compared to the double negative control non-challenged nor treated with LPS (non-challenged-LPS)

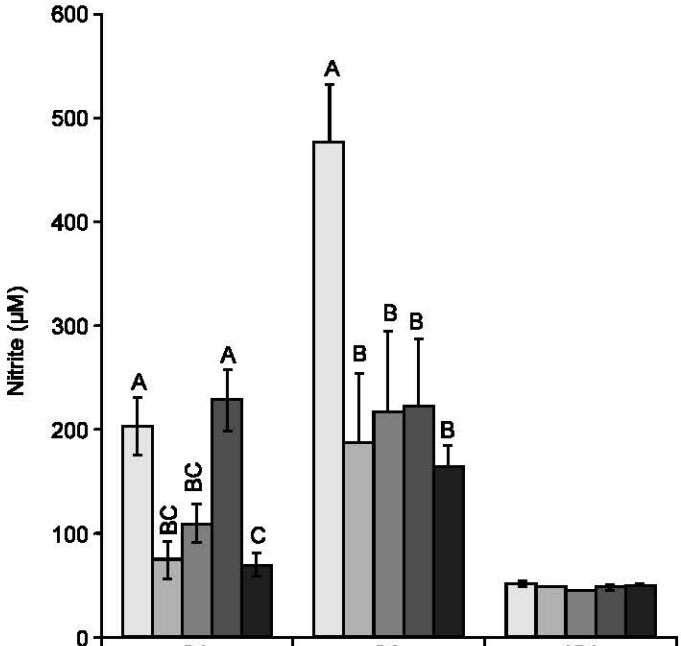
(n = 12 explants group). Significant differences in nitrite production between non-stimulated, LPS stimulated and plant extract treated groups were determined by ANOVA (SAS Institute, 2002) and deemed significant if the $p \leq 0.05$. The data were also subjected to mean separation using Duncan's multiple range test at 5% level of significance.

RESULTS

The ability of this ileal explant culture model to produce increased nitrite and measure differential NO, over a period of 12 h in response to LPS stimulation, is presented in Fig. 1 and 2. The explants from *Salmonella* $^{(+)}$/LPS $^{(+)}$ produced up to 200 µM nitrite in 3 h and a maximum of 478.5 µM of nitrite by 6 h post LPS stimulation *in vitro*. Further, we were able to measure differential nitrite among the plant extract treated groups compared to *Salmonella* $^{(+)}$/LPS $^{(+)}$ using this model.

The ability of the explants to produce elevated nitrite in a short period in response to LPS-stimulation was measured (Fig. 1). *Salmonella* $^{(-)}$/LPS $^{(-)}$ explants produced up to 10 µM nitrite, which was comparable to most other cell culture models. *Salmonella* $^{(+)}$/LPS $^{(-)}$ produced 126.83 µM nitrite by 3 h, which is acceptable given the fact that the explants in this group were from *Salmonella* challenged birds. *Salmonella* $^{(+)}$/LPS $^{(+)}$ produced 203.69 µM nitrite in just 3 h that was significantly higher than *Salmonella* $^{(-)}$/LPS $^{(-)}$ ($p \leq 0.05$). A similar trend was seen at 6 h, *Salmonella* $^{(-)}$/LPS $^{(-)}$, *Salmonella* $^{(+)}$/LPS $^{(-)}$ and *Salmonella* $^{(+)}$/LPS $^{(+)}$ producing up to 52.29 µM, 100.1 µM and 478.42 µM nitrite, respectively ($p \leq 0.05$).

The efficiency of the model to measure differential nitrite over time was evaluated comparing the *Salmonella* $^{(+)}$/LPS $^{(+)}$ with that of the plant extract treated groups (Fig. 2). To recall, all 5 of these groups that were compared originated from *Salmonella* challenged birds, further stimulated with LPS *in vitro* and treated or not treated with BW, CL, CC, or Senna with plant extracts. Three h post stimulation, BW, CL and CC significantly reduced the NO production measuring 74.41 µM, 108.93 µM and 69.55 µM nitrite when compared to *Salmonella* $^{(+)}$/LPS $^{(+)}$ control which measured as high as 203.69 µM ($p \leq 0.05$). In contrast, NO production was unaffected by Senna treatment 228.21 µM nitrite. At 6 h post LPS stimulation *in vitro*, the *Salmonella* $^{(+)}$/LPS $^{(+)}$ control group continued



	3 h	6 h	12 h
Salmonella +/LPS+	203.69	478.42	51.12
Salmonella +/LPS +/BW+	74.41	187.30	48.02
Salmonella +/LPS +/CL+	108.93	217.07	44.75
Salmonella +/LPS +/Senna+	228.21	222.82	47.55
Salmonella +/LPS +/CC+	69.55	164.52	50.31

Fig. 2: Differential nitric oxide production among treated groups. Nitrite was measured in the supernatant of explants treated with one of the plant extracts (Cat's claw, CC; *Senna reticulate*, Senna; Black Walnut, BW and Clove, CL) and then stimulated with 100 µg of lipopolysaccharides (LPS)/ml and measured at 3, 6 and 12 h. Bars represent means (n = 12 explants/group) and standard error of treated groups along with the double positive control challenged with *Salmonella* (challenged+LPS). Treatment means were partitioned and literals above the bars indicate significant difference ($p \leq 0.05$) as compared to the positive control

to increase NO production at 478.42 µM nitrite. BW, CL and CC also significantly reduced the relative NO production 187.3 µM, 217.07 µM and 164.52 µM ($p \leq 0.05$). Senna did reduce the relative NO production at 6 h when compared to the control group and was measured to be 222.82 µM. Nitrite was also measured at 12 h post stimulation, where all groups attained a basal level averaging 47 µM nitrite.

DISCUSSION

The ileal explant culture method produced elevated nitrite in a shorter period (3 h), compared to other end point nitric oxide analysis methods that rely on at least 24 h incubation (Crippen *et al*., 2003). The ileal explant method is a suitable model for screening of mucosal immune functions, potentially mimicking *in vivo* conditions. Development of the ileal explant model exhibited various interesting insights regarding immune functions, with NO production as an indicator. It was noteworthy to observe (Fig. 1) that the (*Salmonella* $^{(+)}$/LPS $^{(-)}$) group nitrite production decreased over time (126.83 µM at 3h, 100.1 µM at 6h and 45.28 at 12 h rather than increasing at 6 h) providing additional support that these immune cells require additional stimulation *in vitro* irrespective of pre-exposure to *Salmonella* under *in vivo* conditions. However, comparison of (*Salmonella* $^{(+)}$/LPS $^{(+)}$) exposed to a pathogen showed a significant decrease in the time required to trigger the immune effector functions along with a significant shift in its magnitude. In addition, explants from non-challenged birds without any stimulation with LPS (*Salmonella* $^{(-)}$/LPS $^{(-)}$) produced low amounts of nitrite that was expected considering that these ileal explants remained naïve to any agonist. Typically, cell culture models have a delayed response with a lower signal with the potential that primary monocytes/macrophages collected after exposure to the *Salmonella* were naïve to the pathogen as seen by reduced monocyte/macrophage cell numbers within 1 h of an LPS injection (Hussain and Qureschi, 1997; Lowry

et al., 1998; Crippen *et al*., 2003; Lillehoj and Li, 2004; Babu *et al*., 2006; He *et al*., 2006; Bowen *et al*., 2009; Setta *et al*., 2012). Nitric oxide amounts returned to basal levels by 12 h post LPS stimulation, which can be attributed to depletion of available medium resources and the loss of ileal explant viability that resulted in the decrease of nitrite detected. Although large amounts of antibiotics were used to prevent overgrowth of normal flora that grow on the epithelial cells in the gastrointestinal tract, some bacteria were able to grow and remain in the culture medium depleting available resources in the medium. Even though the explant viability for treatments was not exclusively tested, further assay development would require an acceptable explant viability test for the model. Use of sensor dyes, like Calcein (Calceinacetoxymethyl ester-Sigma-17783) which is a non-fluorescent cell permeable derivative which becomes fluorescent on hydrolysis by a living cell and DAF-FM (4-Amino-5-methylamino-2',7'-difluoro-fluorescein diacetate-Sigma-D2321) which also measures real time NO with high specificities, could be used for this purpose. A modified MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay to measure explant viability originally employed to test the viability of epi-ocular explants in toxicological studies may also be utilized for this model (Salem and Katz, 2003).

This model is a rapid screening tool for the effect of inflammatory NO production inhibitors. The marked decrease in NO production by treatment with plant extracts (BW, CL, Senna and CL) tested in this model (Fig. 2) provides additional support for the use of this model to rapidly and sensitively screen of anti-inflammatory or anti-oxidant compounds. However, further studies *in vivo* are necessary to authenticate the effects of these plant extracts to establish their mode of action.

Conclusion: In summary, we have developed a new ex vivo ileal explant culture model that is capable of inducing elevated NO levels and has the ability to measure nitrite in a very short time period, potentially mimicking *in vivo* inflammatory intestinal conditions. The model was successfully tested to produce elevated amounts of NO in as little as 3 h in comparison to previously reported cell culture models, which is vastly quicker than 12 to 24 h necessary to build up enough nitrite in supernatant fluid of cell culture models. This ileal explant culture method is a useful screening model for testing anti-inflammatory candidates. This ileal explant model better mimics intestinal inflammation than primary cell culture (NO measurement from PBMCs or use of cell lines) considering that both endothelial cells and monocytes and macrophages would be present in culture at the same time allowing NO production from both eNOS and iNOS activation to affect the measurement. Future studies will include NO production measurement from gut scrapings rather than the use of ileal explant sections because the same cells would be present in culture without the rest of the delicate intestinal tract tissue interfering with the culture method.

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