Production and the characterization of avian crypt-villus enteroids and the effect of chemicals

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Abstract

Background: Three-dimensional models of cell culture such as organoids and mini organs accord better advantage over regular cell culture because of their ability to simulate organ functions hence, used for disease modeling, metabolic research, and the development of therapeutics strategies. However, most advances in this area are limited to mammalian species with little progress in others such as poultry where it can be deployed to study problems of agricultural importance. In the course of enterocyte culture in chicken, we observed that intestinal mucosal villus-crypts self-repair and lead to the formation of enteroid-like structures which appeared to be useful as ex vivo models to study enteric physiology and diseases.

Results: The villus-crypts harvested from chicken intestinal mucosa were cultured to generate enteroids, purified by filtration then re cultured with different chemicals and growth factors to assess their response based on their morphological dispositions. Histochemical analyses using marker antibodies and probes showed the enteroids consisting different cells such as epithelial, goblet, and enteroendocrine cells typical to villi and retain functional characteristics of intestinal mucosa.

Conclusions: The villus enteroids can simulate villus functions with their absorptive cells functionally positioned and exposed to culture medium thus, can help understand the mechanisms of nutrient uptake and their regulation, interactions with alien organisms, and amenable to assays of the factors that may affect gut health.

Background

Organoids are three-dimensional assembly of cells capable of mimicking organ functions in culture hence, have been used for studies relating to regenerative medicine, metabolic research, disease modeling, and therapeutic developments [1–4]. Typically, the organoids are generated from primary tissues, progenitor stem cells, or induced pluripotent cells (IPSC), and maintained using a variety of growth and differentiation factors, and extracellular matrix support [5]. The intestine is a vital organ for nutrient absorption and its epithelial linings protect the organism against harmful antigens, toxins, and pathogens that passage through it [6, 7]. Intestinal organoids, pioneered by studies of Sato et al. [8], have been used to understand their developmental physiology, culture of enteropathogens, and study gastrointestinal problems such as relating to epithelial integrity, enteritis, and autoimmunity [9–11]. However, most advances in this area are limited to mammalian models largely, mouse, human and to some extent, some porcine and bovine models of agricultural importance, where the organoids are often generated using either progenitor stem cells in the crypts or induced pluripotent cells [12–17]. In avian species enteric organoid research is very limited although the poultry constitute a major human food source that are also vulnerable to different enteric problems that can affect their growth and susceptibility to diseases. The lack of pluripotent stem cells to generate intestinal organoids, in avian species further limits the progress although there have been some reports of success using intestinal crypt cells [18–20]. The poultry intestinal organoids can be useful as a screening tool to study the effect

of nutrients to promote growth, understand the factors that can affect intestinal health, pathogen interactions, and screen for antibiotics alternatives to prevent zoonotic diseases. However, many of the current methods of generating intestinal organoids involve complex manipulations such as the use of different factors which often are mammalian specific, inhibitors, and extracellular matrix support, that can be time consuming [21]. Besides, in the stem cell generated organoids, the villus growth being inward, the absorptive cells often, do not align to the culture medium as it would be naturally in the intestinal lumen [22]. Large scale screening assays may be possible if the organoids can be generated rapidly and cost-effectively. In the course of developing chicken enterocyte culture [23, 24], we observed that the villus crypts of intestinal mucosa tend to self-repair to form spheroid-like structures resembling mini organoids that may have potential to study villus physiology. Hence, the objective of this study was to streamline the procedure, purify avian villus enteroids, characterize them, and study their potential as a screening tool using some selective chemicals and growth factors.

Results

Generation of enteric organoids and immunofluorescence characterization

Figure 1 shows the intestinal villi following harvest and the purified enteroids generated after 24 h of culture, and following additional 24 h of their culture. Filtration process yielded substantially cleaner preparation of the enteroids, free of debris, and adherent cells. The purified enteroids on further culture, showed progressive accumulation of cells around them (Fig. 1C). The villus enteroids showed virtual peripheral lining of epithelial cells although in these are spheroids and some also contained pinches of vascular tissues, trapped in the core, visible as orange tinge (not shown).

Immunofluorescence staining of organoids

Immunofluorescence localization of selective antigen markers associated with different cells are shown in the Figs. 2a-h. The enteroids were positive for both keratins type I and II, Na-K-ATPase, pan cadherin, actin, mucin, and alkaline phosphatase. Keratins localized as peripheral bands in the enteroids (Figs. 2a, b). Cadherin, an adhesion molecule which occurs in the epithelium as e-cadherin, a tight junction associated protein [25, 26], was also present in the enteroids (Fig. 2d). Actin which occurs as a crescent band in the apical region of the epithelial enterocytes [24] showed strong presence (Fig. 2e). Goblet cells producing mucin were identified by their binding to Sambucus nigra lectin [24] and anti-mucin antibodies (Figs. 2f, g), and the enteroids were positive for alkaline phosphatase identified by Fast red substrate (Fig. 2h). Some cells in the enteroids, that stained positive for serotonin, chromogranin A, and tryptophan hydroxylase, were presumed to be enterochromaffin cells (Figs. 3a-c) whereas, those positive for lysozyme (Fig. 3d) were inferred to be cells producing antimicrobial factor such as Paneth cells. Because of their positioning on the spheroids, it was not possible to ascertain whether these cells were crypt associated. All those cells other than epithelial cells localized as clusters or isolated populations of cells

in the enteroids. The enteroids showed cell proliferation indicated by Andy fluor EdU-positive, cells that appeared bright green fluorescent and scattered randomly over the organoids whereas the non-dividing cells appeared orange to red fluorescent (Fig. 3e).

Alkaline phosphatase activity

Measurement of alkaline phosphatase activity with 4-nitrophenyl phosphate substrate using 2 test chemicals showed no substantial difference between the control and treatment groups (Control: 3.21 ± 0.60, cGH: 2.40 ± 0.25; DSS, 2.65 ± 0.61 OD/μg protein, n = 3/group).

The effect of different chemicals on organoid morphology

Figure 4 and Table S2 show some representative images of the enteroids affected by different chemicals after 24 h of treatment. Of three tested growth factors, both EGF and IGF-1 produced enterotrophic effects indicated by budding whereas the bone morphogenetic protein-2 (BMP-2) had no significant effect at 24 h of incubation. In the hormone category, none of the tested hormones except for dexamethasone showed any discernible effect. Dexamethasone appeared to shrink the epithelial cells. Of the two vitamins tested, the trans-retinoic acid showed no effect but 1, 25 dihydroxy vitamin D3 (calcitriol) appeared to shrink the core aspects of the organoids and showed some budding activities. The mycotoxins adversely affected the enteroids causing their fragmentation and breakage by 24 h with deoxynivanelol (DON) showing some delayed effect compared with either aflatoxin B1 or cytochalasin B. The degenerative effect of DON was more pronounced at 48 h of incubation. Similar degenerative effects on enteroids were evident with both Staphylococcus aureus and Clostridium perfringens epsilon enterotoxins which caused vacuolation and eventual disintegration of the enteroids (not shown). Salmonella typhimurium lipopolysaccharide (LPS) appeared produce some shrinkage of core of the enteroids but the peptidoglycans produced no discernible changes up to 48 h of treatment. Indomethacin caused some shrinkage of outer epithelial cells whereas the monensin, an anticoccidial antibiotic, caused degeneration of the enteroids. Phorbol myristate acetate (PMA) did not produce any discernible changes in the enteroids compared with controls. A colitis mimetic, dextran sodium sulfate (DSS), also did not show any morphological changes whereas thiram, a fungicide, caused significant damage and degeneration of the enteroids (Fig. 4).

Discussion

The crypt villi are the major absorptive and protective units of intestine. Hence, understanding their physiology can provide insight into their role in regulating nutrient uptake, growth, and animal health problems. Taking advantage of the self-repairing ability of the villus crypts, we developed a simple method to generate avian villus enteroids that does not involve many complex/ supportive manipulations such as the use of different growth factors, conditioned media, and support with extracellular matrix gels. Each of the enteroids represent a prototype unit of mucosal villus that contained different cell types and show cell turnover and shedding, typical of villi [27, 28]. The cell renewal, shedding and extrusions have been associated to active migration of cells along the villus which is associated with intestinal

homeostasis [28]. Cell shedding by the enteroids was evident by the accumulation of a large number of free cells on subsequent days of culture. The enteroids also showed cell proliferation evident from cell labelling studies which may be responsible for their growth, expansion and budding activities. The presence of different cell types such as the epithelial, goblet, entero-endocrine cells, and the cells that produce antimicrobial peptides, typical of villi [29, 30], were evident using their respective markers. The antibodies against both keratins I and II which are the markers of epithelial cells [31, 32], were strongly reactive with the enteroids as was the antibody against Na-K-ATPase, an ion channel protein, responsible for the maintenance of intestinal health such as the integrity of epithelial tight junction, cell motility and polarization [33, 34]. Decreased levels of Na-K-ATPase activities in the basolateral membrane of intestinal epithelial cells has been linked to chronic intestinal inflammation and malabsorption problems [35]. The enteroids also showed the presence of cadherin, a tight junction associated adhesion protein that maintains epithelial barrier function [26, 36]. The mucin producing goblet cells were identified by their reactivity with SNII lectin [24, 37] and an anti mucin antibody. Of other specialized cells, the identity of enterochromaffin cells were indicated by their reactivity with antibodies specific to serotonin, chicken tryptophan hydroxylase, and chicken chromagranin A which are the markers of these cells [38]. Lysozyme positive cells in the enteroids were indicated by their reactivity to an anti-lysozyme antibody. Paneth cells produce lysozyme but their presence in chickens has been controversial [39, 40] however, in a recent report, Wang et al. [41] showed the presence of these cells in the intestinal crypts. The location of lysozyme producing cells in the enteroids appeared to be surface associated. Many of the specialized cells such as goblet cells and enteroendocrine cells which occur in low numbers, appeared to be scattered or occur as clusters over the organoids. The enteric organoids were positive for alkaline phosphatase, an enzyme that is associated with enterocytes and implicated in the regulation of fatty acid absorption and protection of intestine against bacterial invasion [42]. Alkaline phosphatase attenuates gut inflammation such as in colitis and is amenable to modulation by nutritional factors [43–45]. However, our preliminary tests with 3 different factors, cGH, DSS, and serotonin, showed no modulation of alkaline phosphatase activity of the enteroids; nonetheless, the measurement of alkaline phosphatase can be a useful marker for enteroid function. Based on these characteristics, the villus enteroid can serve as suitable ex vivo model to study villus functions and its regulation.

For its applications in poultry research such as to understand the effect of nutrients, screening of antibiotic alternatives, and the pathogenesis of enteric diseases, the villus organoids can serve as test models thus, may save both cost and time since they can be generated rapidly within days. Therefore, we investigated the effect of some selective growth factors and chemicals that are likely to affect intestine assessing the gross changes in the morphologies of the enteroids. Our results showed both epidermal growth factor (EGF), a major signaling protein implicated intestine development and repair [46, 47] and insulin like growth factor-1 (IGF-1) that causes intestinal proliferation [48], both, promoted undulation and budding of the organoids within 24–48 h of treatment although the former was much more effective compared with the later. However, the bone morphogenetic protein-2 (BMP-2), another signaling protein and morphogen [49] known to play role in intestinal epithelial differentiation [50] did not produce any significant enterotrophic effect. BMP's effect on intestine has been suggested to be on the level of

mesenchymal cells producing secretory cell differentiation [51]. Thyroxin, similarly, produced no significant morphological effect on the enteroids but dexamethasone produced some shrinkage of epithelial cells without much effect on central cell mass. There are conflicting reports of the effect of thyroid hormones on intestinal epithelial cells with respect to growth although they promote maturation of intestinal mucosa and help in calcium transport process [52]. Previously, using enterocyte culture, we observed the cells treated with thyroxin showed some morphological changes leaning towards more cuboidal shapes compared with the controls [24]; however, it was not possible to discern any such change from the observation of the whole enteroids. The effect of dexamethasone, a synthetic glucocorticoid, was to some extent consistent with the observations of Urayama et al. [53] who have reported its shrinking effects on rat small intestinal cells. Serotonin, which is a product of enterochromaffin cells and other neural cells innervating gastrointestinal tract, affects its motility, and modulates Na+/K + exchange system [54–56]. However, serotonin produced little to no morphological changes in the organoids nor did it have any effect on their alkaline phosphatase activities that may be associated to Na-K-ATPase system. The chicken growth hormone (cGH) produced neither any morphological change nor affected alkaline phosphatase activity of the enteroids although growth hormone is known to stimulate growth of intestinal mucosa and the proliferation of intestinal stem cells [57]. Trans-retinoic acid exhibited no morphological effect on the enteroids but the vitamin D3 (calcitriol) appeared to shrink their central mass. Inhibitory and anti-proliferative effect of trans-retinoic acid has been reported in literature [58], but it was not evident from our results. The effect of vitamin D3 on proliferation or differentiation of enterocytes is little known although it plays a significant role in the absorption of calcium and phosphorous in the intestine [59]. Mycotoxins are fungal metabolites that are toxic to intestine [60]. Compared at the same concentration levels, both aflatoxin B1 and cytochalasin B caused lethal changes resulting in the fragmentation of enteroids within 24 hours of treatment whereas deoxynivalenol (DON) appeared to be relatively less lethal and took longer incubation time, up to 48 h, to produce similar effects. The effects of mycotoxins are presumed to be due to their interfering effects on protein synthesis and cell division, and they disrupt intestinal cell barrier producing cellular apoptosis [61–63] which lead to their degeneration. Similarly, both Staphylococcus aureus and Clostridium perfringens epsilon enterotoxins were lethal to the enteroids and produced severe damage. Neither Salmonella typhimurium lipopolysaccharide nor bacterial peptidoglycan produced any lethal effect in the organoids although both of these products are proinflammatory in many systems [64]. In a previous study with chicken enterocytes, we did not observe any significant effect of LPS on the enterocytes [24] but Salmonella infection has been reported to affect intestinal organoids causing their morphological changes and disrupting epithelial tight junctions [65]. However, it is not known whether the effect of Salmonella can be due to LPS or to the growth and invasion of the bacteria per se. Similarly, the peptidoglycan was ineffective in damaging the enteroids. The Staphylococcus aureus enterotoxin is well known for its wide range of damaging effects on intestine [66] and the enterotoxin of Clostridium perfringens has been identified as major virulent factor in the cause of necrotic enteritis in livestock including poultry [67–70] and the Clostridium difficile toxin disrupts the epithelial barrier function of human intestinal organoids [71]. Both of the enterotoxins caused lethal effect on the enteroids.

The effect of some metabolic modulators, indomethacin, a prostaglandin inhibitor, PMA, a protein kinase C activator, and the monensin, an ionophore, anticoccidial antibiotic, on the enteroid morphologies were tested. Indomethacin produces ulcers in human and rat small intestines [72] however, it appeared only to shrink epithelial aspects of the enteroids whereas PMA showed no significant effect on the enteroids although our previous study with the effect of PMA on enterocytes showed significant cachectic changes in the enterocytes [23, 24]. Monensin, on the other hand, damaged the organoids, however, it has been a preferred anticoccidial drug in the poultry and some early studies suggested its toxic and necrotic effect in the intestine [73]. Tetramethyl thiuram disulfide (thiram) is a fungicide and an endocrine disruptor, produces many toxic effects in chickens including gastrointestinal problems and growth retardation [74]. Thiram exhibited severe toxic effect on the enteroids leading to their disintegration but sodium dextran sulfate, a widely used inducer of experimental colitis, showed no lethal effect on the enteroids although its effect in vivo is attributed to the changes in the mucosal permeability activating inflammatory cells [75–77]. Overall, these results highlight how different modulators may affect the intestinal villi and the villus enteroids may contribute to understand their mechanisms of action.

Conclusion

The major highlight of this study is the method to generate chicken crypto-villus enteroids using ileal mucosal tissues in a relatively simple and cost-effective manner that does not require much complex media formulation and extracellular matrix support, therefore, can be potentially useful for routine in vitro assays to assess their interactions with chemicals and pathogens, nutrient uptake, and enteric disease problems. An advantage offered by this model, stems from the positioning of the enterocytes which directly expose to the culture medium as it would in the lumen of intestine. Thus, the enteroids can be amenable to a variety of metabolic, developmental, genetic, and pathological studies. However, maintaining the enteroids for long term in culture appears to be the problem because the cell loss by shedding appear to exceed growth that may require additional maneuver such as the use of different growth maintenance factors, and inhibitors as have been developed for different mammalian tissues [21] which will then facilitate their preservation, storage, and retrieval. Nonetheless, this avian villus enteroid model appears promising for its use in poultry research to study metabolic and developmental aspects of avian intestine.

Methods

Harvest of intestinal villi and culture to generate enteroids

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Arkansas. Day-old male broiler chicks obtained from a local hatchery were killed by decapitation and the intestine segments between the pancreatic loop and Meckel's diverticulum removed, and placed in Dulbecco's modified minimum essential medium (DMEM)-F12 (1:1) containing glutamine, HEPES, sodium bicarbonate (HiMedia Laboratories, LLC) supplemented with sodium pyruvate, and antibiotic-antimycotic solutions (Sigma Aldrich) (Complete medium). The mucosal tissues were extruded

from the intestine by longitudinal draining using tweezers then triturated to disperse the tissues. The tissues were centrifuged at 200 g for 10 minutes, and the supernatant discarded. This process was repeated once after which the tissue pellets were re suspended in the same medium additionally supplemented with 10% Hyclone fetal bovine serum (FBS) (GE health Sciences, Logan UT), 1X insulin transferrin selenite (ITS), 1X polyamine (www.sigmaaldrich.com), and bovine pituitary extract (Cell Applications Inc., San Diego, CA) (Culture medium) then incubated in six well hydrophobic suspension cell culture plates (Sarstedt, Germany) overnight. This allows the sheared ends of the villi to self-repair giving rise to spheroid shaped enteroids. The cultures containing cell debris and enteric spheroids were gently triturated to remove the attached cells then strained through 40 μm Falcon cell strainers (www.vwr.com) by three successive transfers and washes using complete medium. The enteroids were concentrated by centrifugation at 200 g for 10 min and reconstituted in fresh culture medium containing FBS and growth factors as described above for different assays. The density of the spheroids were adjusted to an approximately 100–150 enteroids per ml. and cultured in hydrophobic suspension culture wells. The villus crypts and the enteroids were photographed using a BX Olympus inverted microscope equipped with a CoolSnapPro camera (http://www.mediacy.com).

Immunofluorescence staining of enteroids and cell proliferation assay

The presence of different marker antigens in the enteroids were assessed by immunofluorescence localization using chicken antigen specific antibodies and other probes. The enteroids on second day of culture were transferred to glass slides coated with 2% Biobond™ (Electronmicroscopy Science, www.emsdiasum.com) using glass capillaries, fixed with 4% para-formaldehyde in phosphate buffered saline (PBS) for 15 minutes in vapor phase, and stored for subsequent histochemical procedures. For antigen localization, the slides were successively washed twice 5 min each in excess PBS then the enteroids were permeabilized with 0.5% Triton X-100 in PBS, and blocked with 10% goat serum for 10 minutes. Primary antibodies diluted per manufacturer suggested concentrations where available, or mouse monoclonal antibodies (diluted to an approximate concentration of 5 μg/ml 0.1% bovine serum albumin (BSA), were placed on the enteroids, and incubated overnight (Table 1) at 4 °C in a humidified chamber. The enteroids were washed 3 times with PBS for 5 minutes each and layered over with secondary antibodies such as goat anti-mouse IgG or goat anti-rabbit IgG conjugated to Alexa fluor 488 (Abcam, Inc) using the manufacturer suggested concentrations as probes against their respective primary antibodies, incubated for 1 hour at room temperature, and rinsed 3 times with PBS. Appropriate negative control tests were done using only the secondary antibodies omitting the primary antibodies. In preliminary screenings some of the primary antibodies that were not chicken specific showed no binding. Following incubation with the antibodies, the enteroids were counterstained with 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI; 0.2 μg/ml PBS), and applied Cytovista™ tissue cleaning agent (www.thermofisher.com) for 10 minutes, washed twice with excess PBS, and mounted with ProLong gold anti-fade reagent (www.thermofisher.com). Actin was stained using Alexa fluor 535 labelled Actistain Phalloidin (www.cytoskeleton.com) following the manufacturer's protocol. Tetramethyl rhodamine (TRITC) conjugated Sambucus nigra lectin (SNAII, EY laboratories Inc., San Mateo, CA) was used to

identify mucin producing cells [24, 37]. The fluorescent and bright field images were photographed with an Olympus BX microscope equipped with Infinity 3-3UR camera (www.lumenera.com). The images were processed and superposed using proPremier software (http://www.mediacy.com/imagepro). In some instances, the labelled target cells were pseudo colorized to enhance contrast and visibility of the antigen positive cells. The antibody and the probe reagents are listed in Table 1.

The proliferation of the cells in the enteroids was monitored using iClick™ EdU Andy Fluor 488 imaging kit (ABP Biosciences, www.abpbio.com). The enteric spheroids were plated in 48 well plates and incubated overnight with 10 μM 5-ethynyl-2'-deoxyuridine (EdU). Twenty-four hours later, the enteroids were washed 2 times in DMEM/ F-12 media and transferred to slides prepared as described above, fixed in 4% para-formaldehyde. The enteroids were permeabilized with 0.5% Triton X-100, washed 3 times in PBS, layered over with ~ 0.5 ml of iClick reaction cocktail as suggested by the manufacturer, and incubated for 30 minutes at room temperature in dark. The enteroids were then counter stained with propidium iodide (1 μg/ml PBS) as nuclear stain, and the fluorescent images visualized under microscope. The proliferating cells were bright green fluorescent whereas the non-proliferating cells appeared orange to red.

Alkaline phosphatase immunohistochemistry and assay

Intestine is a major source of alkaline phosphatase which helps in the absorption of fatty acids and is an important protective enzyme against inflammation [78, 79]. We used a Fast-Red chromogen kit (Abcam) for immunochemical identification of alkaline phosphatase in the enteroids. Quantitative measurement of the alkaline phosphatase activity was done using 4-nitrophenyl phosphate (4-NPP) substrate in diethanolamine buffer pH 9.7. The effect of cGH, DSS, and serotonin on enteroid alkaline phosphatase activities were measured incubating ~ 12–20 enteroids in 100 μl of culture media in 96 well suspension culture plates (Sarstedt, Germany), the agents added at the concentrations of 1 μg/ml for 24 h then transferred to microtubes, and centrifuged at 1000 g to remove supernatant. The enteroid pellets were additionally washed in 0.5 ml of Hanks Balanced salt solution (HBSS), centrifuged at 10,000 g, and the resulting pellet dissolved in 30 μl of M-Per lysis buffer (Thermo Fisher Scientific). The lysates were centrifuged at 20,000 g and the alkaline phosphatase activity of the supernatants measured incubating an aliquot of the supernatant with 100 μl of substrate solution prepared dissolving 4-NPP (1 mg/ml) and incubating at 37^{o}C for 30 min when the reaction was terminated adding an equal volume of 1N NaOH [80]. The optical density (OD) of 4-nitrophenol was determined at 405 nm. The protein concentrations of the extracts were determined using BCA reagent (Thermo Fisher Scientific) using bovine serum albumin (BSA) as standard. Alkaline phosphatase activity was calculated against the protein content of the extract. The assays were done using triplicate cultures, results evaluated statistically using GLM procedure [81] and expressed as OD / μg protein at 30 minutes of incubation at 37^{o}C.

The effect of different factors on enteroids

To find the effect of different chemicals on the enteroids, we used a panel of selective growth factors, hormones, micronutrients, toxins, and chemicals (Table 2). Approximately 7–10 enteroids were placed in

100 µl culture medium in 96 well suspension culture plates and treated with the chemicals for 24–48 h and the overall changes in the enteroids were assessed under microscope. The test chemicals were first dissolved in their respective solvents such as complete medium (growth factors), or ethanol (for hydrophobic chemicals) as recommended by the manufacturer then diluted further with complete medium such as the final concentration of ethanol not to exceed over 1% in culture. Respective solvents were used as controls and diluted similarly. For serotonin we used ascorbic acid (20 mg/ml) as diluent and antioxidant. The comparisons were done utilizing only a single concentration of all chemicals and growth factors, 1 µg/ml although some preliminary tests were run using log dilutions of some of the chemicals. Solvents diluted appropriately were used as controls and each assay was done in triplicate and repeated twice at 2 different times. Enteroids were observed for any significant changes in their morphologies and photographed using a BX Olympus microscope as described above. The subjective but independent assessments of the morphological changes were done by 2 individuals. The overall effects on the villus enteroids are shown in Fig. 4 and Table 2.

Abbreviations

BCA: Bicinchoninic acid

BMP-2: Bone morphogenetic protein-2

cGH: Chicken growth hormone

DON: Deoxynivanelol

DSS: Dextran sulfate sodium

EGF: Epidermal growth factor

IGF-1: Insulin like growth factor 1

LPS: Lipopolysachharide

PMA: Phorbol myristate acetate

SNII: *Sambucus nigra* lectin II

Declarations

Ethics approval and consent to participate

The study was approved by University of Arkansas, Animal Care and Use Committee.

Consent for Publication

Not applicable. The manuscript does not contain any human data.

Availability of data and materials

The methods used in this study are available from corresponding author and the first and second authors.

Competing interests

The author declare that they have no competing interests.

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Author's contributions

NCR and MA conceived and designed the studies. MA and KA performed the experiments. NCR, MA, KA, AD and RL wrote the manuscript. All authors read and approved the manuscript.

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Figures

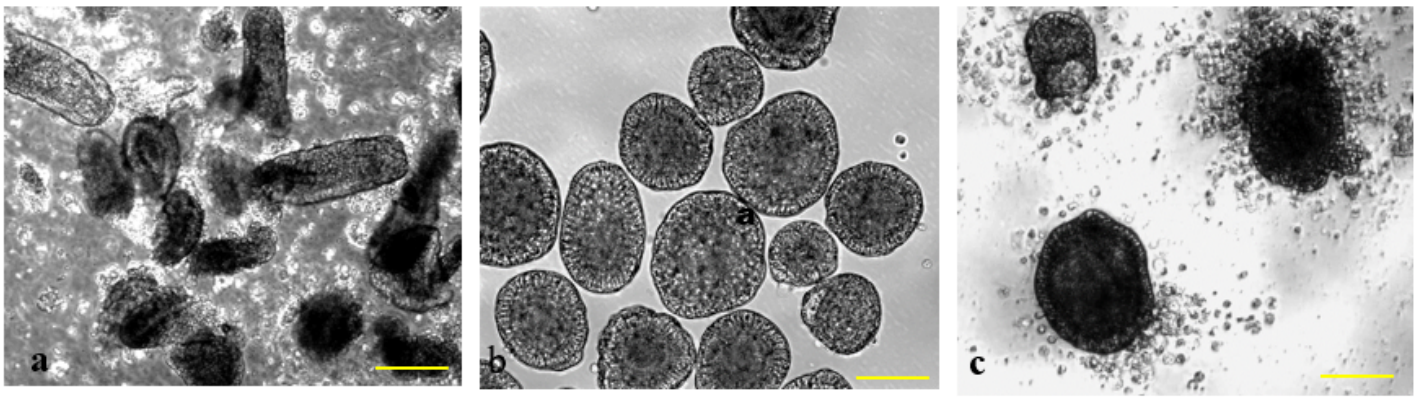

Figure 1

Intestinal villi after harvest (a), 24 h following culture and purification of the enteroids (b), and 24 h after the culture of the enteroids (c). Magnification 200X. (Bar =100 μm).

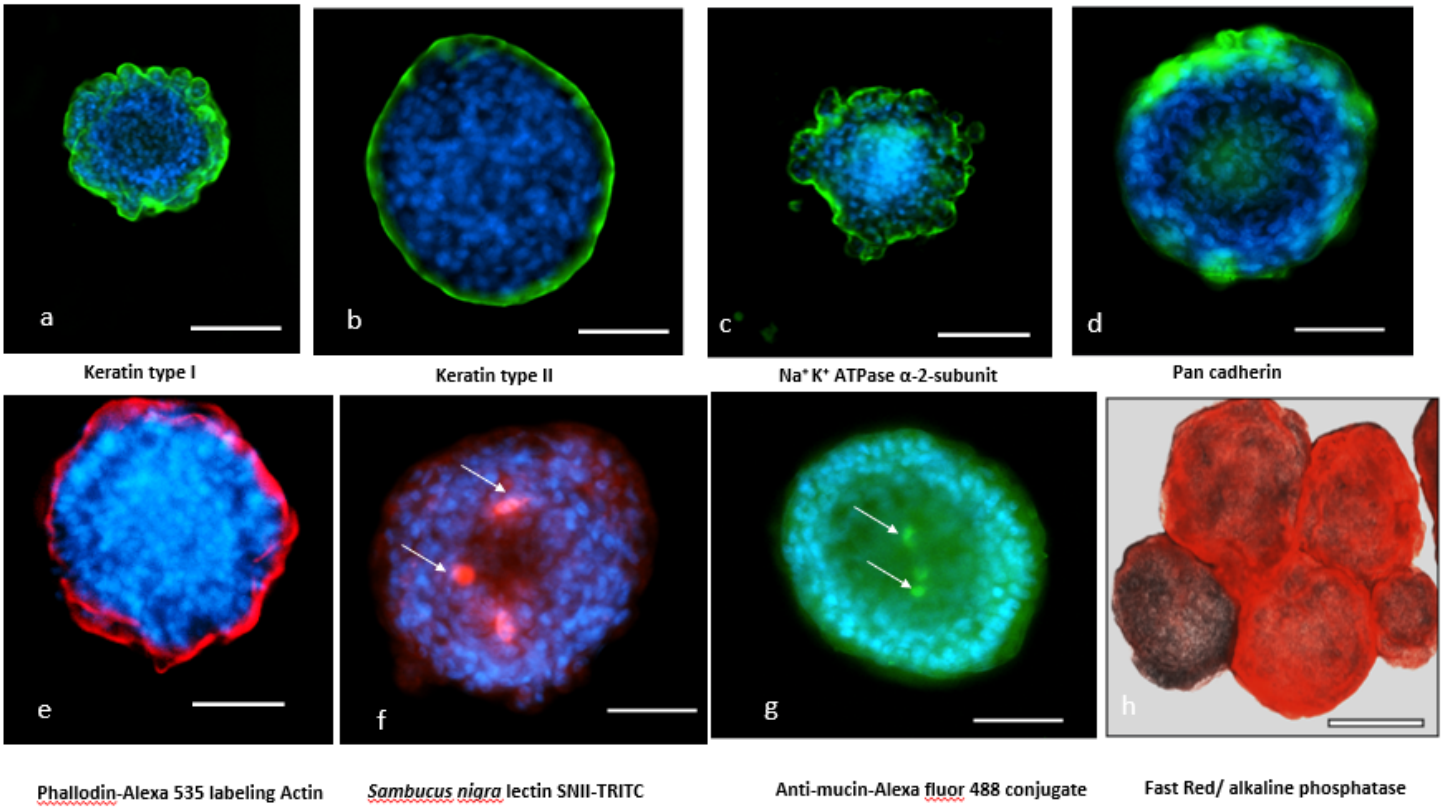

Figure 2

Immunolocalization of antigens in the villus enteroids: a and b. keratins types I and II; c. Na-K-ATPase α-2-subunit; d. Pan cadherin, e. Phalloidin-alexa 535 labelled actin, f. Sambucus nigra lectin SNII-TRITC labeled goblet cells, g. Anti-mucin antibody labelled goblet cells, and h. and Fast red positive alkaline phosphatase. The nuclei are stained blue with DAPI in all pictures. The magnification of the images in a, c, and h are 200 X, bar=80 µm and the rest 400X, bar=40 µm.

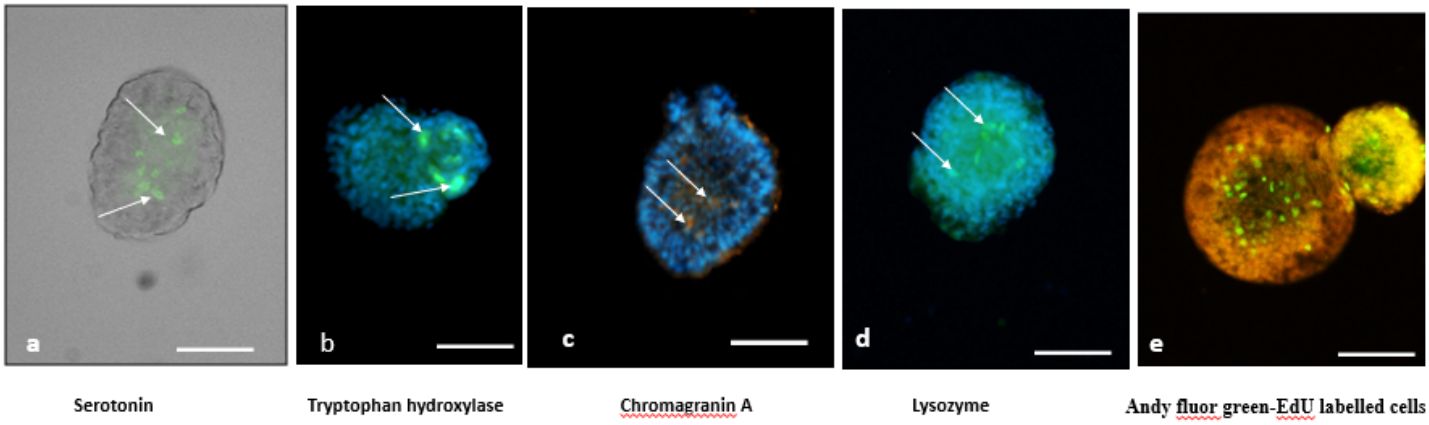

Figure 3

Immunofluorescence localization of antigen specific cells and proliferating cells. (a), immunofluorescence localization of serotonin (a), tryptophan hydroxylase (b), chromogranin A (c), and

lysozyme (d), and Andy fluor green fluorescent EdU labeled proliferating cells (e). The nuclei are stained blue with DAPI except in (e) where they were stained with propidium iodide showing orange-red color. Images are magnified to 200X, bar= 40 µm.

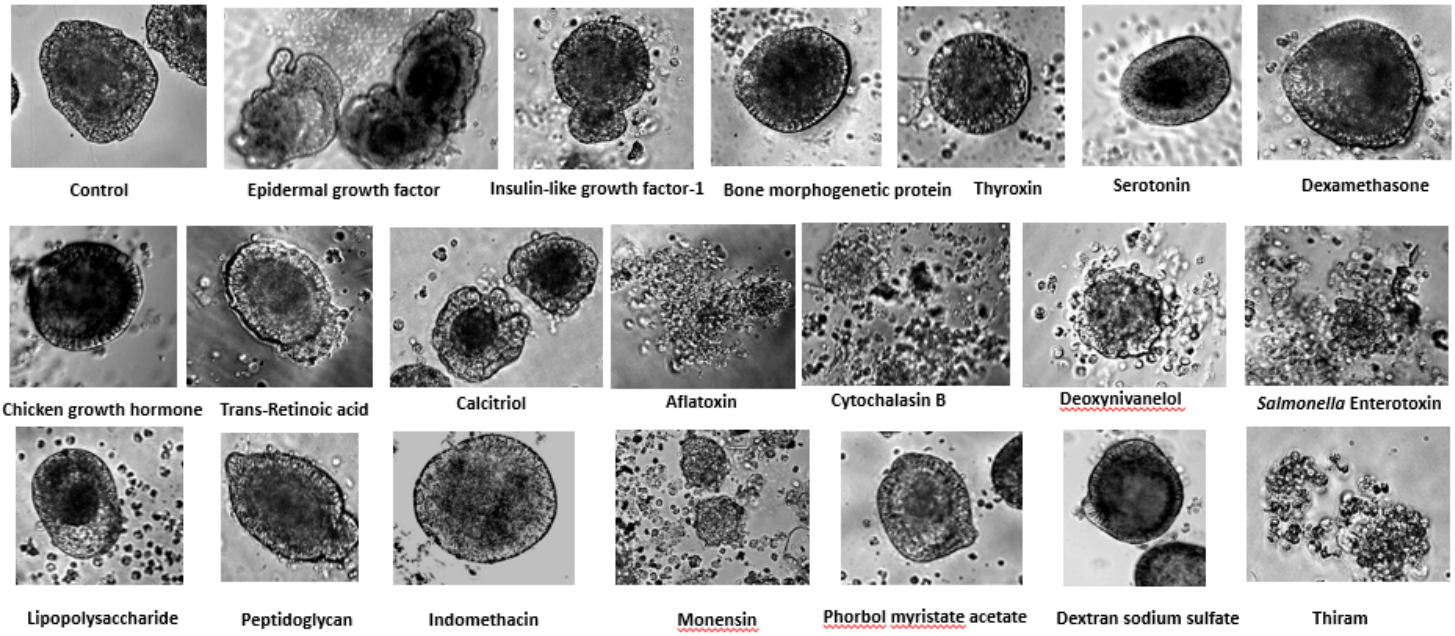

Figure 4

Showing the effect of different chemicals on the enteroids after 24 h incubation with 1 µg/ml of each factor. Magnification 200X.

Supplementary Files

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