Establishment of a chicken enterocyte culture system to screen factors that affect intestinal integrity

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ABSTRACT

Enteric health and integrity is important for overall animal health. Therefore, the understanding of mechanisms how enterocytes interact with different factors such as microbes, toxins, and other chemicals, is essential to evaluate substances that can protect against harmful agents and improve animal health and food safety. Enterocyte culture models afford fast and inexpensive screening method but there is no such model for avian enterocytes. The mammalian systems cannot adequately substitute for species specific screening. To address this need, we developed an enterocyte culture system using intestinal villi, harvested from broiler chickens. These cells are grown in DMEM containing a set of growth factors, polyamines, and serum which support their epithelial cell morphology and favor mucin production. To evaluate the effects of different chemicals, we plated the cells in 48 wells at a concentration of $5X10^3$ cells per well, and on confluency treated them with selective chemicals which included vitamins (1, 25 dihyroxy vitamin D3, Trans retinoic acid, 1 µM), fungicide (thiram 1 µM), metabolic activators (dibuyryl cyclic AMP, a protein kinase A activator and phorbol myristate acetate (PMA), a protein kinase C activator, both at 1 µM, and sodium butyrate 1mM), (Salmonella lipopolysaccharide (LPS) and Staphylococcus peptidoglycan (PGN), both at 1 µg/ml), and screened for changes in cell morphology and viability at 24 h and 48 h of treatment. While none of the treatments affected cellular viability measured by Alamar blue, the retinoic acid and PMA showed significant morphological changes. The PMA treatment showed elongation of cells whereas the retinoic acid favored more flattened epithelial morphology. A preliminary study using label free quantitation proteomic analysis, showed PMA upregulating the pathways of carbohydrate and cytoskeletal metabolism. These results show that the chicken enterocyte culture has potential as a screening tool for chemicals that affect enterocytes and study the mechanisms of their action.

BACKGROUND AND OBJECTIVE

Intestine is a major site for nutrient, microbial, and chemical interactions which affect the health of the organism. The cellular linings of intestine such as the enterocytes and epithelial cells play a significant role in these interactions. Understanding, how these cells behave can help identify factors such as antibiotic alternatives that can protect the gut against harmful factors and pathogens. Experimental models such as enterocyte culture can afford fast, inexpensive, and large scale screening of agents that include chemicals, nutrients, and ATA growth promoters that affect gut and study their mechanisms of action. However, there is no experimental model for avian enterocytes. The mammalian models of intestinal cell culture cannot adequately substitute for species specific screening. To address this need, we developed a chicken enterocyte culture to test its suitability to screen different factors that could affect gut health and its metabolism. The results described here was done using a random choice of metabolic modulators.

METHODS

Cells: Post duodenal intestinal segments from chickens were mildly squeeze-drained into a culture medium (DMEM-F12 medium with 4.5 g glucose/liter, supplemented with glutamine, Hepes, Na-pyruvate, antibiotic/ antimycotic solution) with a pair of tweezers. The mucosal tissue sediment collected after centrifugation at 250 g was treated with Dissociation medium and Accumax (Sigma-Aldrich) by repeated passage through a 22 gauge needle to facilitate dissociation. The cells were centrifuged with Histopaq density gradient medium (1.077) to removes debris and dead cells. The cells at the gradient interface washed 3 times with the same medium then plated in collagen coated flasks reconstituted in same medium containing 10% heat inactivated FBS, 1X polyamine, IX ITS, and 1X epithelial cell growth factor supplements. The enterocytes grow and establish. Few days later the cells are harvested dissociating with Accumax and used for assays. Even though the intestinal villi often, may not be completely dissociated, they may still be plated for the cells to grow out and form colonies.

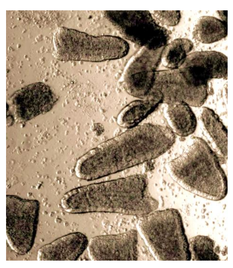
METHODS (continued…)

Evaluation of the chemical and biological agents on the cells: We evaluated the putative biological effectors initially, based on the changes of morphology of the enterocytes then with the viability assay using Alamar Blue. 4-$5X10^3$ cells were seeded in 48 or 96 well plates, grown for 3-5 days to reach a semi confluent (48 well) or confluent state, then added the test agents and incubated for 24-48 h. The morphological changes were evaluated microscopically and photographed. The changes in viability and growth of the enterocytes were evaluated by Alamar blue reduction monitoring the changes in fluorescence 530 Ex/560 Em. The effect on mucin production were evaluated with Dimethylmethylene (DMMB) assay using culture supernatants (it may need additional concentration step due to the sensitivity issues). The concentrated supernatant dissolved in 2 M guanidine HCl for assay.

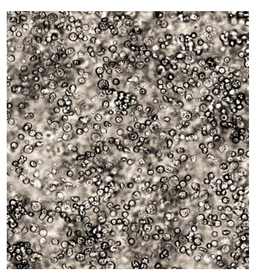
Test chemicals: We used some selective chemicals such as **vitamins** (**1, 25 dihyroxy vitamin D3, trans retinoic acid** both at 1 µM), **fungicide** (**thiram**, 1 µM), **metabolic activators** (**dibuyryl cyclic AMP**, a protein kinase A activator and **phorbol myristate acetate (PMA)**, a protein kinase C activator, both at 1 µM, and **Sodium butyrate,** 1 mM), **inflammation mediators** (Salmonella **lipopolysaccharide (LPS)** and Staphylococcus **peptidoglycan (PGN)** both, at 1 µg/ml). All results reported were based on 48 h assays.

Proteomics: We evaluated the effect of PMA using mucin production and quantitative proteomics. Cells seeded in 6 well plates were grown to full confluency, washed 2 times with Hank's balanced salt solution and replaced with serum and antibiotic free medium and triplicate cultures treated with control or PMA (1 µg/ml) for 48 h. The supernatant removed for mucin assay. The cell layer was treated with β-D glucopyranoside lysing solution, reduced, alkylated, and subjected to trypsin digestion, desalted and subjected to LC-MS-MS. The identified proteins were evaluated using Skyline (http://proteome.gs.washington.edu/software/skyline) software statistics and the differentially elevated proteins subjected to string bioinformatics analyses (http://string-db.org).

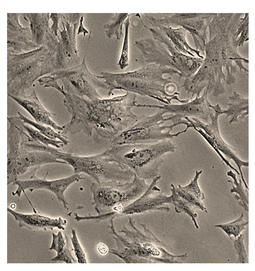
RESULTS



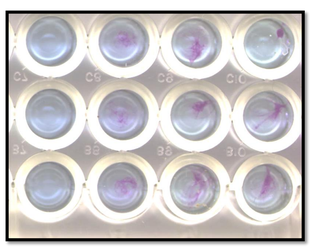
Villi



Isolated enterocytes

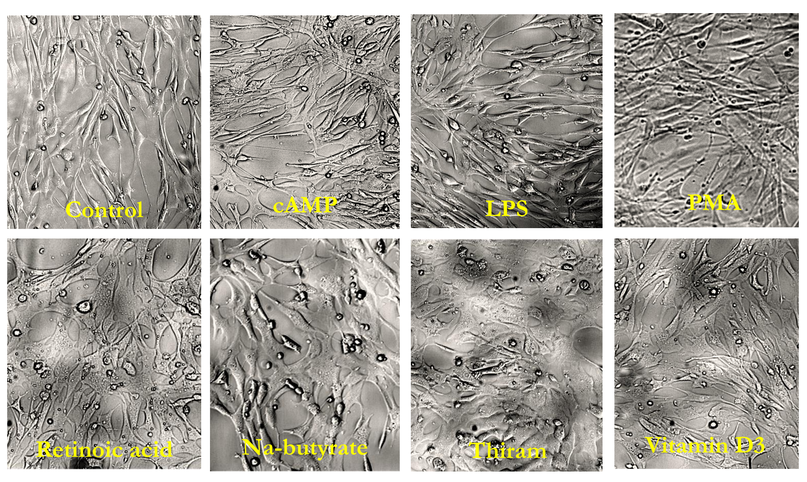


Epithelial enterocytes



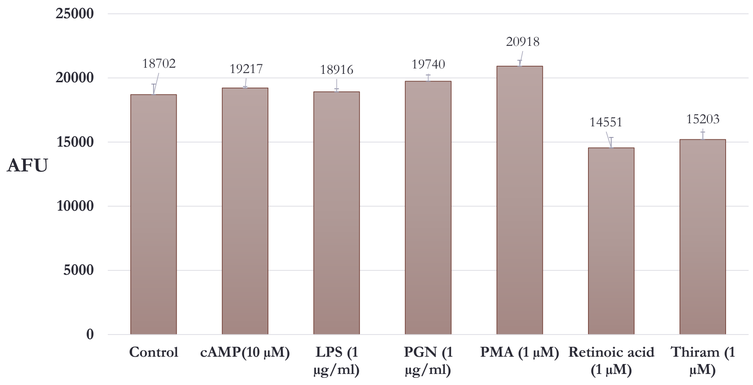
Mucin is seen as red precipitate in DMMB binding assay

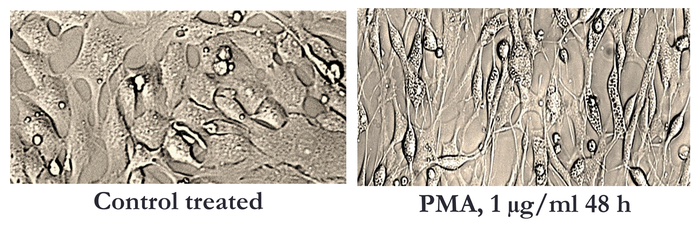
Morphological changes induced by different biological and chemical agents



RESULTS (continued….)

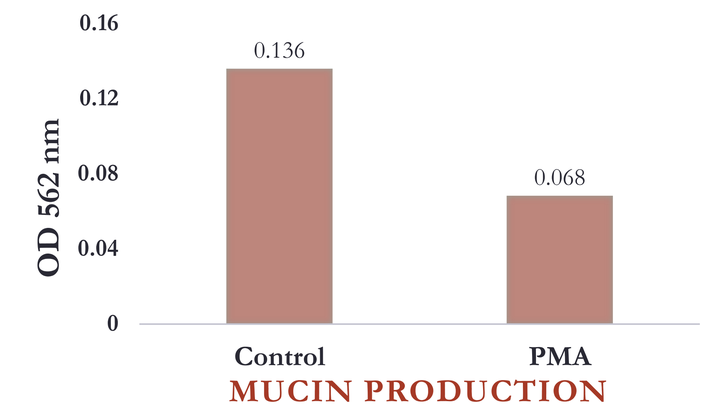
Alamar Blue assay





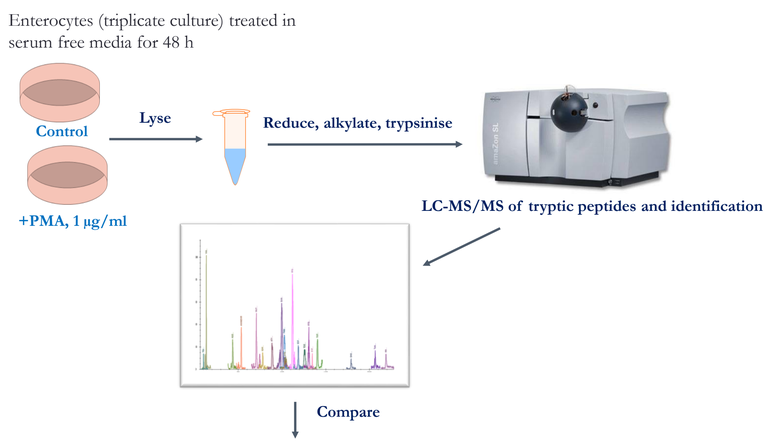
Control treated — PMA, 1 µg/ml 48 h

PMA induces dehydration and enteritis-type effect



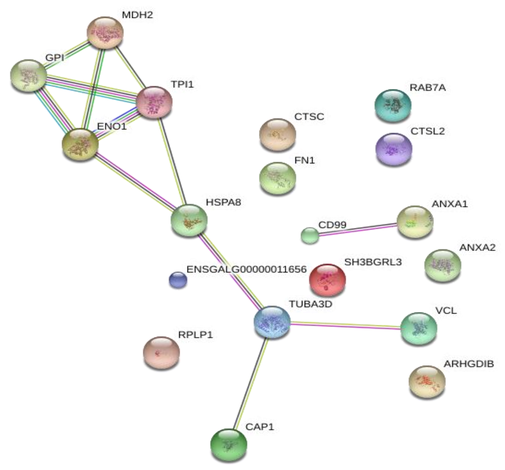
MUCIN PRODUCTION

Proteomic changes analysis



Differentially expressed proteins subjected to bioinformatics analysis

PMA induces increased expression of carbohydrate metabolic enzymes



CONCLUSIONS

The above results show that the chicken enterocyte system can be useful to screen different chemical and biological agents for their effects and mechanisms of action. We believe that such screen may also help identify factors that may protect against harmful agents such as chemicals, food, pathogens, toxins, and to identify antibiotic alternatives.