



Chronic Stress Modulates the Mucin Components of the Intestinal Barrier and the Intestinal Morphology

B. M. Habiyambere¹ and E. M. Onyango^{1*}

¹Department of Health Sciences, East Tennessee State University, Box 70673, Johnson City, TN 37614, USA.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors designed the study, wrote the protocol, and managed the experimental process. Author BMH managed the literature searches, and wrote the first draft of the manuscript. Author EMO did statistical analysis and revised manuscript drafts. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2016/23764

Editor(s):

(1) Salomone Di Saverio, Emergency Surgery Unit, Department of General and Transplant Surgery, S. Orsola Malpighi University Hospital, Bologna, Italy.

Reviewers:

(1) Doaa M. Mokhtar, Assiut University, Egypt.

(2) K. Sujathan, Regional Cancer Centre, India.

Complete Peer review History: <http://sciencedomain.org/review-history/13084>

Original Research Article

Received 21st December 2015
Accepted 16th January 2016
Published 27th January 2016

ABSTRACT

Objectives: The aim for this study was to investigate the effects of chronic restraint stress on the expression of mucins that comprises the integral component of the gastrointestinal barrier.

Design and Methods: A completely randomized design was used for the study. Sixteen ICR male mice were randomly divided into treated and control animals. Restraint stress was applied to the treated mice for 21 days. Body weight and feed consumed were regularly recorded. After 21 days mice were euthanized and blood and gastrointestinal tissues were collected. Serum corticosterone levels were determined. Intestinal sections were fixed, paraffin-embedded, sectioned, stained with alcian-blue-periodic acid Schiff stain to visualize goblet cells. Expression of mucosa mucins was determined using qPCR and western blotting.

Results: As expected stressed mice showed higher corticosterone levels than the control mice (5082±1975 vs 1566±1236 pg/ml, $P = 0.002$). At the end of the study the body weight was lower in the stressed group (32±2 vs 36.0±3 g) even though the cumulative food consumed in both groups was not different. Stressed mice had lower goblet cell count in the duodenum, jejunum, ileum and colon (8±2, 7±1, 9±1, and 25±8 vs 14±1, 14±1, 11±2 and 40±9, respectively), and shorter villi in the

*Corresponding author: E-mail: ONYANGO@mail.etsu.edu;

duodenum and jejunum (316 ± 82 and 256 ± 73 vs 440 ± 73 and 357 ± 35 μm , respectively). Expressions of mRNA for Muc1 and Muc4 in the colon of stressed animals were upregulated (1.5 and 2.2 fold increase, and P values of 0.05 and 0.01, respectively). This mRNA upregulation was accompanied by increased expression of Muc1 and 4 proteins in the colon.

Conclusion: These findings suggest that chronic stress affects the intestinal barrier by reducing the number of the mucin-producing goblet cells, shortening the villi and upregulating Muc1 and Muc4 in the colon.

Keywords: Intestinal barrier; mucin; restraint; stress.

1. INTRODUCTION

Stress may be defined as the body's nonspecific response to real or perceived threats/demands [1,2]. The modern fast and demanding pace of life increases the chances of people experiencing stress in their lives. Both the hypothalamic-pituitary-adrenal axis and the sympatho-adrenal axis are involved in the initiation of the generalized stress response [3-5]. Repeated stress may be related to many physical and mental illnesses because of the repeated elevations of the levels of glucocorticoids [6].

The susceptibility of the gastrointestinal tract to stress has been shown in a number of studies [4,6,7-10]. Selye, in studying the stress response, identified the gastrointestinal tract as one of the tissues adversely affected by the stress response in addition to the enlargement of the adrenal glands, and abnormal development of the thymolymphatic system [1,11]. Indeed, in humans, nausea and diarrhea are responses observed in psychological difficulties and fear [4]. Also after severe trauma or prolonged stress gastrointestinal stress ulcers are a commonly observed complication [4] because the integrity of the gastrointestinal wall is usually compromised in these conditions [12]. The bidirectional interaction between the brain and the gut, through the parasympathetic and sympathetic pathways, form the brain-gut axis, and is a very important regulator of many processes such as gastrointestinal motility, absorption of nutrients, ion transport, and blood flow [2]. A stressful event will trigger a cascade of signals along the brain-gut axis that will comprise the stress response of the gastrointestinal tract [5]. They observed that restraining rats for 6 hours for a period ranging from 1 to 21 consecutive days induced changes in the brain and the gut. They noted that restraint induced gastrointestinal ulcers in mice and rats. It has been reported that in the large intestine chronic stress stimulates intestinal permeability to large antigenic molecules thus inducing among other things mast cell activation and decrease of mucin

production in the colon [2,8]. On the other hand, in the small intestine chronic stress was observed to induce water and electrolyte absorption [2]. It has been noted that during psycho-social stress in mice there is a localized loss of goblet cells and crypts in the colon of chronically stressed mice [13].

These studies show that stress affects the functioning of the protective lining of the gastrointestinal tract. This barrier comprises of interactions among the mucosal barrier, the immune system, and the gut microbiota. The gut epithelium, which is part of the gut mucosa, forms a physical barrier between the internal and external environments of the gut. Epithelial cells express both membrane-bound and secreted mucin glycoproteins [14,15]. It is the secreted mucins that form the major component of mucus. Mucin glycoproteins lubricate and protect the mucosa and play a role in various epithelial cell processes such as adhesion, renewal, differentiation and signaling [16]. They also modulate absorption of water and electrolytes and provide attachment sites for commensal and pathogenic microbes [17,18]. Therefore interference with the quality or quantity of mucins may expose epithelial cells lining the gut to various insults and disease conditions. It has, indeed, been shown that in many types of cancers and inflammatory diseases there is deregulation of mucin expression [7,19-22].

Because stress affects the functioning of the gut epithelium which is a component of the gut protective barrier, it is important to find out how this in turn affects the quality and quantity of mucins that are expressed by gut epithelium. The studies that have looked at the effect of stress on the gastrointestinal tract [for example 2,13] have not specifically looked at the quantitative and qualitative modulation of individual mucins along the gastrointestinal tract. Because there is changed epithelial functioning during a stressful event, we hypothesize that stress alters the overall quantity and quality of mucin expression in the epithelium of the gastrointestinal tract. The

objective of the present study was to investigate the effects of chronic stress on mucin expression in mice to mimic the chronic exposure to stress in humans [10]. It has been suggested that chronic stress could cause inflammation, changes in the epithelial structure and weaken the defense mechanism in the gut of rodents [10]. Expression of five murine mucins (Muc) in the gastrointestinal tract was examined: 3 membrane-bound mucins (Muc1, Muc3, and Muc4) and 2 secreted gel-forming mucins (Muc2 and Muc5ac) were examined.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Sixteen 4-week-old male weanling ICR Swiss strain mice were used. The mice were housed individually in cages and had a 12-hour light-dark cycle. They were randomly assigned to cages labeled as either test (stress) or control cages. Water and feed were available ad libitum except when the mice were being tested. Mice were weighed twice each week and feed consumption determined also twice weekly for the 3 week-experimental period. Test mice were subjected to restraint/immobilization stress [23] for 6 hours daily for 21 days. Feed and water were withdrawn from the cages for the control mice during the time their test counterparts were undergoing the stress procedure.

At the end of the 21-day period all the mice were euthanized using carbon monoxide, and blood was immediately collected by retro-orbital puncture. The collected blood was left at room temperature to coagulate and was then centrifuged at 1600 x g to obtain serum which was stored at -80°C until needed for the corticosterone assay. The abdomen of each mouse was then opened and the stomach and the intestine harvested. The mucosa from each segment of the gastrointestinal tract (GIT) was collected by scrapping and was then homogenized (Omni TIP Homogenizer, Omni International, Marietta, GA) in Trizol® solution before being stored at -80°C until processed for RNA isolation. A portion, approximately 2 cm, of the different segments of the gut was removed and fixed in methacarn [24] for 24 hours and fixed tissues were transferred to 70% ethanol until further processing for histology.

2.2 Corticosterone Assay

The prepared serum was assayed for the stress hormone corticosterone using the Corticosterone

Enzyme Immunoassay kit (Assay Design Inc, Ann Arbor, MI) following the manufacturer's protocol. Briefly, the corticosterone standards were serially diluted to obtain the following concentrations (pg/ml): 20,000, 4,000, 800, 160 and 32. A 96 well-plate coated with donkey anti-sheep IgG was set up and all the reagents placed in the wells as specified by the manufacturer's instructions. The plate was then sealed and incubated as per instructions. Thereafter wells were decanted, washed before adding the conjugate to the total activity wells. The p-nitrophenyl phosphate substrate was then added to all the wells and the plate was incubated before reading the absorbance using a microplate reader (Benchmark Microplate Reader, Bio-Rad, Hercules, CA) at a wavelength of 405 nm with a correction between 570 and 590 nm.

2.3 RNA Isolation

Total RNA was isolated from mucosa samples following the Trizol® kit manufacturer's protocol briefly described herein. Mucosa was homogenized in Trizol® then frozen until further processing. Samples were thawed, mixed with chloroform, incubated then centrifuged. The mixture separated into 3 phases and the aqueous top phase containing the RNA was transferred to a fresh tube. RNA was precipitated from the aqueous phase using isopropyl alcohol and centrifugation.

Resuspended isolated RNA was subjected to a DNase treatment using the Turbo DNA-free kit (Ambion Inc, Austin, TX) according to the manufacturer's protocol. This was to ensure that no genomic DNA would be carried over to subsequent manipulations. Briefly, all RNA samples were incubated with a DNase buffer followed by buffer inactivation of the buffer and centrifugation to obtain cleaned RNA. A bioanalyzer (Agilent® 2100 Bioanalyzer, Agilent Technology Inc, Santa Clara, CA) was used to determine the RNA concentration and RNA integrity number (RIN) for the isolated RNA.

2.4 Reverse Transcription of RNA

Total RNA was isolated from mucosa samples following the Trizol® kit manufacturer's protocol. The RNA was reverse transcribed using qScript® cDNA Synthesis kit (Quanta Biosciences Inc, Gaithersburg, MD) into complementary DNA (cDNA) using the 2-step protocol as per the manufacturer's instructions. In the reaction tube 33 µl of water, 12 µl of total RNA sample, 12 µl of

5X reaction mix and 3 µl of the reverse transcriptase were combined. The reaction tube was then placed in a thermal cycler (Master cycler gradient, Eppendorf, Hamburg, Germany) programmed to run for one cycle at 22°C for 5 minutes, at 42°C for 30 minutes, and finally at 85°C for 5 minutes. The synthesized cDNA samples were stored at -20°C. Reverse-transcriptase-minus samples were prepared as the other samples with the exception that the enzyme, the reverse transcriptase was not added. This step was taken to rule out any genomic DNA contamination during the actual qPCR (quantitative Polymerase Chain Reaction) run.

2.5 Primers Used in the Study

Specific primers designed for the five mucin genes of interest and one reference gene, *Gapdh*, were used to amplify the cDNA in the qPCR. The primers were:

5'-GATCTCTAGCATCAAGTTCAGGTC-3'
(forward) and
5'- GACTTCACGTCAGAGGCACTAA-3'
(reverse) for *Muc1*;
5'-AACATCACCTGTCCCGACTTCAAC-3'
(forward) and
5'-GGCATGTATGTGATGGAGCCTGA-3'
(reverse) for *Muc2*;
5'-CTTCCAGCCTTCCCTAAACCACA-3'
(forward) and
5'-CTCAGGTCCACAGATCCATGCAA-3'
(reverse) for *Muc3*;
5'-AGGACCAGATGGCTCTGAACCTAA-3'
(forward) and
5'-GTGGTAGCCTTTGTAGCCATCACA-3'
(reverse) for *Muc4*;
5'-ACAGACCTTGATGGCCACTGTTA-3'
(forward) and
5'-CTAACCTCTTGACCACCTGACA-3'
(reverse) for *Muc5ac*;
5'-CCCAATGTGTCCGTCGTGGATC-3'
(forward) and
5'-TGCCTGCTTACCACCTTCTTGA-3'
(reverse) for *Gapdh*.

The resultant amplicon sizes for the designed primers were: 93, 62, 125, 71, 68, 84 base pairs for *Muc1*, *Muc2*, *Muc3*, *Muc4*, *Muc5ac*, and *Gapdh* primer sets, respectively.

2.6 Generation of Positive Controls/Standards

Standards were prepared by preparing specific pDNA using specific primers then inserting the genes of interest into plasmid vectors.

Fresh PCR products for the genes of interest were prepared using the AccuStart PCR Supermix kit (Quanta Biosciences Inc, Gaithersburg, MD) according to the manufacturer's instructions. The PCR product was prepared by combining in a PCR tube 21 µl of water, 25 µl of AccuStart PCR Supermix, 2 µl of cDNA, 1 µl of forward primer, and 1 µl of reverse primer and amplified, in a thermal cycler (Master cycler gradient, Eppendorf, Hamburg, Germany) for 40 cycles following a specific program created for each specific gene of interest. A small portion of the resulting PCR product was run on agarose gel to confirm that only a single discrete band of the size characteristic of the expected amplicon was present. The rest of the PCR products were cloned into a plasmid vector, pCR®2.1-TOPO® (Invitrogen, Carlsbad, CA). The recombinant plasmids obtained were used to transform competent *E. coli* cells following the kit manufacturer's instructions (TOPO TA Cloning® kit, Invitrogen, Carlsbad, CA).

Colonies of *E. coli* cells with plasmids that had taken up the PCR product insert were inoculated in LB broth, incubated, and the resultant cultures were purified using the PureLink Quick Plasmid Miniprep kit (Invitrogen, Löhne, Germany) to obtain plasmid DNA following the manufacturer's instructions. The concentration of the purified plasmid DNA (pDNA) of each specific clone was determined using a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg, Germany). An aliquot of the pDNA was sequenced at the Molecular Biology core facility (East Tennessee State University, College of Medicine, Johnson City, TN) to confirm that the plasmid (vector) had taken up the intended insert. The pCR® 2.1-TOPO® vector specific primers (M13 primers, Invitrogen, Carlsbad, CA) were used for sequencing the pDNA.

For each gene of interest, the pDNA was serially diluted and a gradient amplification was carried out using a thermal cycler (IQ5 Multicolor Real-Time PCR Detection System, Bio-Rad, Hercules, CA) to determine the optimum annealing temperature at which the gene could best be amplified and also for the construction of a standard curve. The optimum annealing temperature was taken to be one that resulted in the highest efficiency and the best slope of the standard curve. The optimal annealing temperatures (°C) for the 5 target genes (*Muc1*, *Muc2*, *Muc3*, *Muc4* and *Muc5ac*) and one reference gene in the study (*Gapdh*) were 60.5, 60.5, 61.2, 59.0, 59.2, and 61.5 respectively.

2.7 Quantitative PCR Setup and Run

The cDNA prepared from the different sections of the GIT were amplified and quantified by real-time or quantitative PCR (qPCR) to determine the expression of the 5 mucins (Muc1, Muc2, Muc3, Muc4, and Muc5ac). Gapdh, a reference gene, was also amplified and served as the internal control. The plasmid DNA (pDNA) concentration and the size of the plasmid including the insert were used to calculate the copy number, a parameter required to set up the qPCR. Ten-fold serial dilutions of the pDNA standards were made.

Each reaction well on a plate received 2.2 µl of nuclease-free water, 1.5 µl primer mix, 1.2 µl of Sybr Green (Promega, Madison, WI), 0.6 µl of MgCl₂, 7.5 µl of 2x qPCR Supermix (Invitrogen, Carlsbad, CA), and either 2 µl of standard pDNA or sample cDNA. The plate was covered and centrifuged (5804 Eppendorf centrifuge, Eppendorf, Hamburg, Germany) to bring all liquid to the bottom of the wells to remove any air bubbles before loading the plate on a thermal cycler to run for 40 cycles of PCR following a specific program created for each specific gene of interest.

2.8 Preparation of Histological Sections and Staining

Methacarn-fixed tissues were gradually dehydrated, embedded in paraffin wax, sectioned and the resulting thin tissue slices mounted on slides. Mounted tissues were dewaxed, rehydrated before being stained with alcian-blue-periodic acid Schiff (AB-PAS) stain for visualization of mucins. Briefly, tissue slides are immersed in the Alcian blue solution for 30 minutes, rinsed in water, immersed in periodic acid solution for 10 minutes, rinsed in water, immersed in Schiff reagent for 10 minutes, rinsed in water, then gradually dehydrated in ethanol before applying a coverslip on the tissues. The stained slides were examined under a light microscope and the number of goblet cells per villus in the small intestine (or per tubular gland in the colon) counted and also the villi lengths measured using AxioVision software (Carl Zeiss Microimaging GmbH, Gottingen, Germany). Three complete villi in any one slide were chosen for the goblet cell counting and villus length measurement. Two slides per mouse were examined in the stressed and control groups (8 mice per group).

2.9 Western Blotting for Protein Expression

Mucosa tissues were homogenized and lysed using RIPA buffer and protein content measured using bicinchoninic acid assay (BCA). To ensure uniform concentrations, all samples were diluted down to a protein concentration of 2.0 mg/ml as measured by BCA. Protein was separated by running through 10% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked using 5% nonfat milk in Tris-Buffered Saline with Tween-20. They were then incubated with various antibodies against the specific mucins. Chemiluminescence was developed using the SuperSignal West Femto chemiluminescence kit (Thermo).

2.10 Statistical Analysis

The data from the corticosterone assay were analyzed by one-way ANOVA using SAS (version 9.2, SAS Institute Inc. 2002, Cary, NC) to assess any differences between the treated and control mice. The body weight change and feed consumption data were analyzed as repeated measures using SAS mixed model. This analysis was done to investigate whether there were significant differences within groups at the different times when the data were collected and also between treated and control groups. Quantitative histological data of the goblet cell count and villus length were analyzed by one-way ANOVA using SAS to determine whether there was a significant difference between the treated and control mice. The iQ5 software (Bio-Rad, Hercules, CA) was used to calculate the relative expression of each gene after the qPCR was run. The resulting data were subsequently analyzed using SAS. In the analysis the individual mouse was the experimental unit. Differences between treatments were considered significant when the P-value was smaller than 0.05 ($P < 0.05$).

3. RESULTS

3.1 Corticosterone Levels in the Sera

The results presented in Fig. 1 indicated high levels of the stress hormone, corticosterone, in the treated group. Two mice from the stressed group had extremely high levels of corticosterone, the rest ranged from 3274 to 7293 pg/ml of serum for the stressed mice, while in the control mice corticosterone levels ranged

from 44 to 2971 pg/ml. When the 2 outlier mice were excluded, the corticosterone levels (means \pm SD; pg/ml) were 5082 ± 1975 for the stressed and 1566 ± 1236 for the control mice (Fig. 1). There were significant differences between the stressed and control mice ($P = 0.002$) with stressed mice showing higher corticosterone levels compared with control mice.

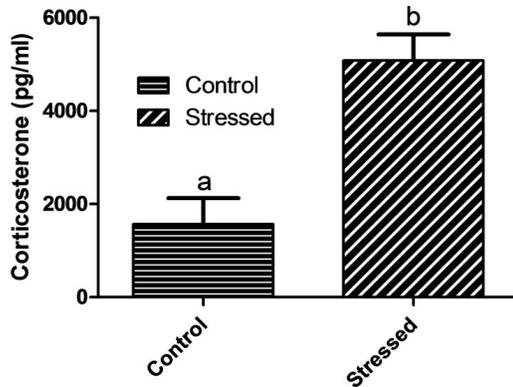


Fig. 1. Corticosterone levels in the sera (pg/ml) of stressed and control (non-stressed) mice at the end of the 21-day study period

Each bar represents a mean \pm SD of 8 mice. Different letters (a, b) on the bars indicate a significant difference between the stressed and the control mice ($P = 0.002$)

3.2 Body Weight and Feed Consumption

At the beginning of the study, the weight (mean \pm SD) of the stressed mice was 32.6 ± 1.2 g, while the control mice weighed 31.8 ± 2.5 g. After the study period of 3 weeks, the final weight for the stressed mice was 32.4 ± 1.7 g and 36.0 ± 3.0 g for the control mice. The control mice increased their weight consistently throughout the 3-week study period and the group weighed significantly more than the stressed mice ($P = 0.001$, Fig. 2).

The total feed (mean \pm SD) consumed by each of the stressed mice during 21-day period ranged from 105 to 126 g with a mean of 115 ± 7 g per mouse, while the control mice consumed feed ranging from 93 to 121 g per mouse and a mean of 110 ± 10 g per mouse. Statistical analysis of the feed consumed indicated no significant difference ($P = 0.26$) between the stressed and control mice (Fig. 3).

3.3 Goblet Cell Count

Intestinal villi morphology and goblet cells in the two groups of mice is shown, for example, in the jejunum in Figs. 4 and 5. The number of goblet cells

was determined in the duodenum, jejunum, ileum and colon portions of the intestine. The count (mean \pm SD) for the control group was higher than in the stressed group in all the three intestinal sections. The count for stressed mice ranged from 6 to 11 cells in the duodenum with a mean of 8 ± 2 . For the control mice the count ranged from 13 to 16 and the mean was 14 ± 1 . In the jejunum the count ranged from 5 to 8 with a mean 7 ± 1 in stressed mice and the goblet cell count ranged from 13 to 16 with a mean 14 ± 1 for control mice. In the ileum the count ranged from 7 to 10 in stressed and a mean 9 ± 1 . The control mice ranged from 9 to 14 and had a mean of 11 ± 2 (Fig. 6). The goblet cell count per tubular gland in the colon ranged from 13 to 34 in stressed and a mean 25 ± 8 . The control mice ranged from 29 to 52 and had a mean of 40 ± 9 . The goblet cell counts were lower in the stressed mice than in the control mice for all the intestinal sections ($P < 0.05$, Fig. 6). The respective P values for the duodenum, jejunum, ileum and colon were 0.0001, 0.0001, 0.01 and 0.003.

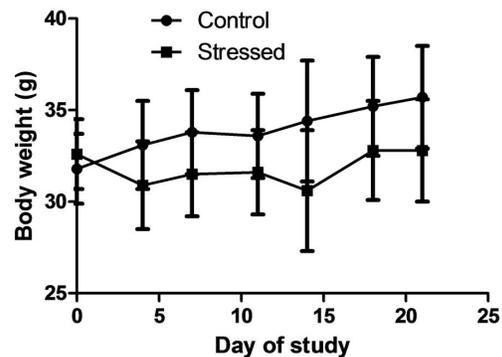


Fig. 2. Body weight (g) of stressed and control (non-stressed) mice during the 21-day study period

Each point in the line graph represents a mean \pm SD of 8 mice. There was a significant difference between the stressed and the control mice ($P = 0.001$)

3.4 Intestinal Villi Length

The mean villus length for the stressed mice was $316 \mu\text{m}$ in the duodenum, $256 \mu\text{m}$ in the jejunum, and $230 \mu\text{m}$ in the ileum (with the standard deviation of 82, 73 and 73 respectively). The mean villus length for the control group was $440 \mu\text{m}$ in the duodenum, $357 \mu\text{m}$ in the jejunum and $253 \mu\text{m}$ in the ileum (with the standard deviation of 73, 35, and 10 respectively). Villi were shorter in the stressed mice than in control mice in the duodenum and jejunum (P values of 0.01 and 0.003, respectively, Fig. 7).

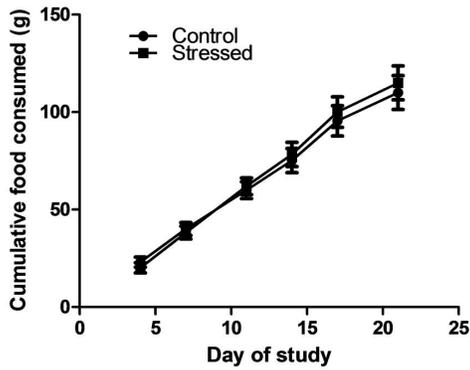


Fig. 3. Cumulative feed consumed (g) by stressed and control (non-stressed) mice for the 21-day study period

Each point in the line graph represents a mean \pm SD of 8 mice. There was no significant difference between the stressed and the control mice ($P = 0.26$)

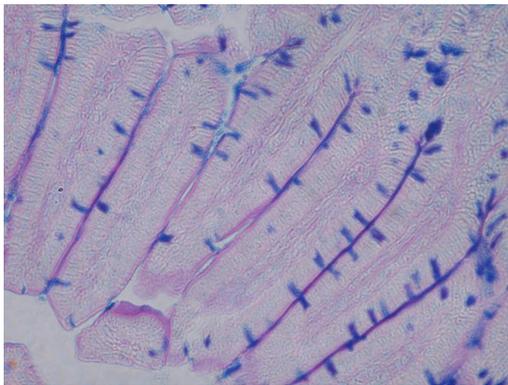


Fig. 4. Villi showing goblet cells in the jejunum of control (non-stressed) mice at the end of 21-day study period (magnification of 400x, alcian-blue-periodic acid Schiff (AB-PAS) stain)

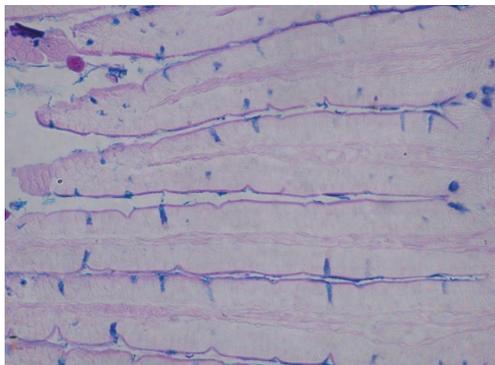


Fig. 5. Villi showing goblet cells in the jejunum of stressed mice at the end of 21-day study period (magnification of 400x, alcian-blue-periodic acid Schiff (AB-PAS) stain)



Fig. 6. Goblet cell count in the duodenum, jejunum, ileum and colon of stressed and control (non-stressed) mice at the end of 21-day study period

Each bar represents a mean \pm SD of 8 mice. Different letters (a, b) on the bars indicate a significant difference between the stressed and the control mice ($P < 0.05$)

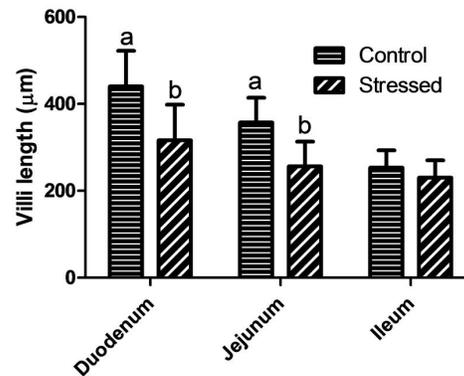


Fig. 7. Villus length (mm) measured in the duodenum, jejunum, and ileum of stressed and control (non-stressed) mice at the end of 21-day study period

Each bar represents a mean of $8 \pm$ SD mice. Different letters (a, b) on the bars indicate a significant difference between the stressed and the control mice ($P < 0.05$)

3.5 Expression of Mucins in the Gastrointestinal Tract

The integrity or quality of RNA isolated and used for the determination of expression of mucins was measured in RIN values. The values ranged from 3 to 9.8 on a scale of 1-10 with 10 being non-degraded RNA. A majority of the RNA samples were of good quality as reflected in the high RIN values.

Muc1 expression was lowest in the jejunum while it was highest in the stomach (Table 1). The

expression of Muc1 in the colon was significantly higher (1.5-fold) in stressed than in control animals ($P = 0.05$, Table 1). The other intestinal sections did not exhibit any significant differences between the treatments.

Muc2 was expressed consistently in the intestinal tract with the highest expression found in the colon followed by the jejunum, ileum, and duodenum (Table 2). The stomach had the lowest expression of Muc2. There were no significant differences between the stressed and the control mice in all the gastrointestinal sections assessed.

Muc3 was expressed throughout the length of the whole the gastrointestinal tract (Table 3). The jejunum showed a relatively higher expression of Muc3 compared to other gastrointestinal sections. Muc3 expression between the 2 treatments in the duodenum was tending to

significance ($P = 0.06$). The stressed mice showed a 4.7-fold increase in expression when compared to the control mice (Table 3).

Muc4 expression in the colon showed a significant difference between the 2 groups ($P = 0.01$, Table 4). Muc4 showed a more than 2.2-fold increase in expression in the stressed mice compared to the control group.

Muc5ac had very low expression in the colon, duodenum and jejunum (Table 5). The stomach had the highest expression of Muc5ac followed by the ileum. There were no significant differences in expression of Muc5ac between stressed and control mice.

Expression of mucin proteins were shown to be significantly different in mRNA expression were also shown to be different in Western blots (Figs. 8 and 9).

Table 1. Muc1 expression in the gastrointestinal tract (GIT) of control (non-stressed) and stressed mice at the end of 21-day study period

GIT Section	Control Mice*	Stressed Mice*	Pooled SD	P-value
Stomach	0.2517	0.3949	0.2323	0.24
Duodenum	0.0026	0.0018	0.0049	0.74
Jejunum	0.0009	0.0010	0.0007	0.67
Ileum	0.1453	0.1370	0.2641	0.95
Colon†	0.0302	0.0465	0.0151	0.05

*Values are means of 8 mice. †Significant difference between the stressed and the control mice ($P < 0.05$).

Table 2. Muc2 expression in the gastrointestinal tract (GIT) of control (non-stressed) and stressed mice at the end of 21-day study period

GIT Section	Control Mice*	Stressed Mice*	Pooled SD	P-value
Stomach	0.0146	0.0081	0.0302	0.67
Duodenum	0.0875	0.0989	0.0824	0.79
Jejunum	0.6959	0.5892	0.4501	0.64
Ileum	0.6002	0.5228	0.3053	0.62
Colon	1.4337	1.9396	1.0928	0.37

*Values are means of 8 mice

Table 3. Muc3 expression in the gastrointestinal tract (GIT) of control (non-stressed) and stressed mice at the end of 21-day study period

GIT Section	Control Mice*	Stressed Mice*	Pooled SD	P-value
Stomach	0.3974	0.2501	0.6984	0.68
Duodenum	0.2198	1.0305	0.7901	0.06
Jejunum	3.0356	3.5001	2.2744	0.70
Ileum	1.0767	1.1081	1.0781	0.95
Colon	1.5649	1.2239	0.6488	0.33

*Values are means of 8 mice.

Table 4. Muc4 expression in the gastrointestinal tract (GIT) of control (non-stressed) and stressed mice at the end of 21-day study period

GIT Section	Control Mice*	Stressed Mice*	Pooled SD	P-value
Stomach	0.0325	0.0245	0.0132	0.24
Duodenum	0.0090	0.0072	0.0079	0.65
Jejunum	0.1854	0.1460	0.1478	0.60
Ileum	0.0421	0.0544	0.0316	0.45
Colon†	0.4837	1.0570	0.4020	0.01

*Values are means of 8 mice. †Significant difference between the stressed and the control mice ($P < 0.05$).

Table 5. Muc5ac expression in the gastrointestinal tract (GIT) of control (non-stressed) and stressed mice at the end of 21-day study period

GIT section	Control Mice*	Stressed Mice*	Pooled SD	P-value
Stomach	0.0106	0.0107	0.0065	0.97
Duodenum	0.0002	0.0000	0.0000	0.56
Jejunum	0.0000	0.0000	0.0000	0.43
Ileum	0.0020	0.0012	0.0037	0.69
Colon	0.0000	0.0092	0.0170	0.30

*Values are means of 8 mice.

4. DISCUSSION

Restraint is a stressful process for animals [25]. To assess the effect of this stressor on the experimental mice required to determine first and foremost the corticosterone level in the serum of the mice. The restraint-stress applied on the test mice induced high corticosterone levels in the serum. Two mice in the stressed group were more agitated and always resisted being restrained more than the other mice. Consistent with the observed behavior, the corticosterone assay results indicated that their stress hormone levels were very high and were considered outliers. The control mice had elevated levels of corticosterone probably due to the method used to euthanize the animals at the end of the study. A quick decapitation might have had less stressful impact than the use of carbon dioxide.

Because all the mice were sacrificed the same way, the method used would not make any notable difference; it only caused a shift of the baseline.

The detection of high corticosterone levels in the serum or plasma could be an indication of stress. Findings in the present study were consistent with those of Gu and colleagues [26] when they analyzed the serum corticosterone levels of stressed and control mice. It has been reported that the level of corticosterone in chronic stress is less than in acute stress [27]. This could be attributed to some degree of adaptation [25]. The goal of the corticosterone assay was to validate that the protocol used was effective in inducing stress in the mice as demonstrated by the levels of corticosterone in the serum.



Fig. 8. Western blot image of Muc1 protein expression in the gastrointestinal tract (GIT) of control (non-stressed) and stressed mice at the end of 21-day study period

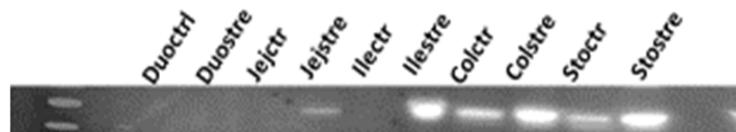


Fig. 9. Western blot image of Muc4 protein expression in the gastrointestinal tract (GIT) of control (non-stressed) and stressed mice at the end of 21-day study period

Mice were weighed regularly to assess whether stress would impact their body weight during the study period depending on the treatment they were subjected to. It was noted that from measurement to measurement there was a difference in body weight. The control mice as expected gained weight consistently throughout the experimental period. In the beginning the stressed mice lost weight, and then they started to regain some weight and stayed somewhat on that trend. This could be due to some adaptation mechanism to cope with the stressor. There were significant differences in the overall body weight between the 2 groups of mice. The findings in the present study are consistent with those of Gu and colleagues [26] who conducted a study in which they compared the body weight of control and stressed mice. The protocol they used to induce chronic mild stress involved the mice being subjected to a combination of a period of continuous illumination, a 45° tilt for the cages, and dirty cages. They noted that the body weight increased in both groups even though the stressed mice weighed less than the control mice over time. Stress had an effect on body weight.

The protocol followed in the present study allowed mice to have free access to feed except during the 6-hour restraint period and it was noted that there was no difference between the stressed and control mice in terms of the amount of feed they consumed. Although there was no significant difference between the amounts of feed consumed, the control mice, nevertheless, gained more weight than the stressed mice. Perhaps the struggle and the stress hormones produced during restraint stress protocol was a drain on the body energy in the stressed mice leading to a smaller increase in bodyweight during the study period.

Mucin-producing cells play a very crucial role in maintaining the mucus protective layer that covers the epithelium of lumen and ducts in the body. Stress has been shown to affect the functioning of the gastrointestinal wall including mucin production [2,8]. In the present study the effect of chronic stress on mucin-producing cells (goblet cells) was assessed. When comparing the goblet cells in the duodenal, jejunal, ileal and colon sections of the intestinal tract of stressed and control mice, it was noted that there were significant differences in the goblet cell count in these intestinal sections. There was a decrease in goblet cell counts in the intestinal sections of stressed animals when compared to control mice. These findings were consistent with those

of Castagliuolo and colleagues [28,29] who found that rats subjected to immobilization-stress had increased mucin secretion in the colon while the goblet cells were decreased in number. Repeated exposure to restraint-stress has been shown to induce a reduction of mucin secretion as well as the number of goblet cells in the colon [30]. On the other hand, Rubio and colleagues [31] investigated the effect of stress on the mucus of the colon in rats. They observed an initial drop in goblet cells followed by an increase of mucin-producing cells.

Duodenal, jejunal, and ileal sections stained with AB-PAS were also examined to assess any difference in the morphology of the epithelium of stressed and control mice. The results revealed that there were significant differences in the heights of villi in the duodenum and jejunum of stressed and control mice. There was a significant decrease in height of villi of stressed mice in the duodenal and jejunal sections of the intestinal tract. Nevertheless, there was no significant difference in villi length between the 2 groups in the ileum. These findings are contrary to those of a study conducted in birds by Burkholder and others [32] who noted that stress did not significantly affect the villi length of intestinal tissues from birds. The different findings could possibly have been as a result of the type of stress and the duration of exposure of the birds to the stressor. In the study by Burkholder et al. [32] the birds were exposed to the stress of feed withdrawal accompanied with high temperatures (30°C) for a period of 24 hours.

In the present study Muc1 was one of the predominant mucins expressed in the stomach and colon under normal physiological conditions. Muc1 was highly expressed in the stomach with no significant differences noted between the stressed and control mice. The colon showed significant differences between the stressed and control mice in the present study. Upregulation of Muc1 and Muc4 has been reported in the colon of mice during acute colitis but its expression was diminished in chronic colitis indicating a possible role of these mucins in the inflammatory process [33]. Similarly, the findings of the present study showed that the 2 mucins (Muc1 and Muc4) were expressed more in the colon of chronically stressed mice. Upregulation of mucins has been associated with disease conditions such as cancer in humans. For example, in gastric carcinoma MUC1 expression has been noted to be associated with smaller

tumors while the expression of MUC3 was linked to larger tumors and metastasis [34].

Muc2 was expressed in the entire intestinal tract of stressed and control mice. No significant differences were noted between the 2 groups of mice. Expression of this mucin was, however, low in the stomach of the control mice. This low or no expression of Muc1 or MUC1 in stomach tissues is consistent with the findings of [35] who reported absence of MUC1 in the normal human gastric tissue. They noted that MUC2 was expressed in the stomach of human patients with intestinal tumors [35]. In the present study, Muc2 expression was the lowest in the stomach, while the highest expression site was the colon. Muc2 is mainly secreted by goblet cells and is one of the major proteins that comprise the mucus layer that protects and lubricates the epithelia of ducts and lumens [36]. Because Muc2 is the major intestinal mucin [37-39], it was surprising to note that there were no significant differences in its expression between the control and stressed mice. Presumably restraint-stress does not significantly affect the specific signaling pathway through which Muc2 is controlled in the gastrointestinal tract.

It has been reported that MUC3 is expressed throughout the human gastrointestinal tract with the highest expression in the jejunum and ileum [37,40]. In the present study in mice differences bordering significance were noted in Muc3 expression in the duodenum between the 2 treatments. There was an upregulation of Muc3 in the stressed mice by about 5-fold compared to control mice in the present study. Growth factors and cytokines seem to have the ability to control the secretion of Muc3 in the gastrointestinal tract [41]. It is possible that chronic stress could trigger growth factors or cytokines as a way of regulating the expression of Muc3.

Muc4, in the present study, was expressed to some extent in all portions/sections of the gastrointestinal tract of both stressed and control mice. Similar expression sites for Muc4 or MUC4 have been noted [16,40]. Muc4 expression in the colon showed a significant difference between the stressed and control mice. Muc4 expression was upregulated in the stressed group compared to the control group. Upregulation of MUC4 in humans has been observed in many cancers [42,43] and has been suggested to facilitate tumor formation by allowing cancer cells to proliferate [42].

Muc5ac was poorly expressed in the gastrointestinal tract of mice with the exception of some expression in the stomach and the ileum. There were, however, no significant differences in its expression in these two sections of the gastrointestinal tract between stressed and control mice. It has been suggested that MUC5AC in humans is upregulated in the airway epithelium under cytokines regulation but the mechanisms are not fully understood [44].

Upregulation of many mucins is often associated with a number of cancers. By causing elevation of some mucins, notably Muc1, Muc3 and Muc4, chronic stress may possibly be a preliminary stage in the pathogenesis of more serious deregulation of these mucins in the gastrointestinal tract.

5. CONCLUSION

In conclusion, chronic stress in mice caused a decrease in body weight, a decrease in intestinal goblet cell count, a decrease in villi length in the duodenum and jejunum, and an increase in expression of Muc1 and Muc4 in the colon.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved (P0700701 of 2008) by the University Committee on Animal Care (UCAC) at East Tennessee State University, Johnson City, TN, a AALAC (the Association for Assessment and Accreditation of Laboratory Animal Care International) accredited research institution.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Marla Perna, Rhessa Dykes, Michelle Duffourc, Justin Kotala and Beverly Ngeny for technical assistance, and Dr Sandra Gendler and Dr Gunnar Hansson's labs for graciously providing antibodies against Muc 1, Muc2 and Muc3. This study was funded by a grant from the East Tennessee State University Research Development Committee Major Grants Program.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Selye H. Forty years of stress research: Principal remaining problems and misconceptions. *CMAJ*. 1976;115:53-55.
2. Bhatia V, Tandon RK. Stress and the gastrointestinal tract. *J Gastroenterol Hepatol*. 2005;20:332-339.
3. Herd JA. Cardiovascular response to stress. *Physiol Rev*. 1991;71:305-30.
4. Habib KE, Gold PW, Chrousos GP. Neuroendocrinology of stress. *Endocrinol Metab Clin North Am*. 2001;30:695-728.
5. Caso JR, Leza JC, Menchén L. The effects of physical and psychological stress on the gastrointestinal tract: Lessons from animal models. *Curr Mol Med*. 2008;8:299-312.
6. Du J, Wang Y, Hunter R, Wei Y, Blumenthal R, Falke C, et al. Dynamic regulation of mitochondrial function by glucocorticoids. *Proc Natl Acad Sci USA*. 2009;106:3543-48.
7. Devine PL, McKenzie IF. Mucins: Structure, function, and association with malignancy. *Bioessays*. 1992;14:619-25.
8. Gabry KE, Chrousos GP, Rice KC, Mostafa RM, Sternberg E, Negrao AB, et al. Marked suppression of gastric ulcerogenesis and intestinal responses to stress by a novel class of drugs. *Mol Psychiatry*. 2002;7:474-84.
9. Tsukada F, Sawamura K, Kohno H, Ohkubo Y. Mechanism of inhibition of small intestinal motility by restraint stress differs from that with norepinephrine treatment in rats. *Biol Pharm Bull*. 2002;25:122-4.
10. Gareau MG, Silva MA, Perdue MH. Pathophysiological mechanisms of stress-induced intestinal damage. *Curr Mol Med*. 2008;8:274-81.
11. Selye H. A syndrome produced by diverse noxious agents. *Nature*. 1936;138:32.
12. Söderholm JD, Perdue MH. Stress and the Gastrointestinal Tract. II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol*. 2001;280:G7-G13.
13. Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH, et al. Adrenal insufficiency and colonic inflammation after a novel chronic psychosocial stress paradigm in mice: Implications and mechanisms. *Endocrinology*. 2007;148:670-82.
14. Shekels LL, Hunninghake DA, Tisdales AS, Gipson IK, Kielizenski M, Kozak CA, et al. Cloning and characterization of mouse intestinal Muc3 mucin: 3' sequence contains epidermal-growth-like domains. *Biochem J*. 1998;330:1301-8.
15. Williams SJ, McGuckin MA, Gotley DC, Eyre HJ, Sutherland GR, Antalis TM. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. *Cancer Res*. 1999;59:4083-89.
16. Andrianifahanana M, Moniaux N, Batra SK. Regulation of mucin expression: Mechanistic aspects and implications for cancer and inflammatory diseases. *Biochim Biophys Acta*. 2006;1765:189-222.
17. Chang JF, Zhao HL, Phillips J, Greenburg G. The epithelial mucin, MUC1, is expressed on resting T lymphocytes and can function as a negative regulator of T cell activation. *Cell Immunol*. 2000;201:83-88.
18. Robbe C, Capon C, Coddeville B, Michalski JC. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem J*. 2004;384:307-16.
19. Gendler SJ, Spicer AP. Epithelial mucin genes. *Annu Rev Physiol*. 1995;57:607-34.
20. Hollingsworth MA, Swanson BJ. Mucins in cancer: Protection and control of the cell surface. *Nat Rev Cancer*. 2004;4:45-60.
21. Moniaux N, Andrianifahanana M, Brand RE, Batra SK. Multiple roles of mucins in pancreatic cancer, a lethal and challenging malignancy. *Br J Cancer*. 2004;91:1633-38.
22. An G, Wei B, Xia B, McDaniel JM, Ju T, Cummings, RD, et al. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. *J Exp Med*. 2007;204:1417-1429.
23. Bonneau RH, Sheridan JF, Feng N, Glaser R. Stress-induced modulation of the primary cellular immune response to herpes simplex virus infection is mediated by both adrenal-dependent and independent mechanisms. *J Neuroimmunol*. 1993;42:167-76.

24. Puchtler H, Waldrop FS, Meloan SN, Terry MS, Conner HM. Methacarn (methanol-carnoy) fixation. Practical and theoretical considerations. *Histochemie*. 1970;21:97-116.
25. Kvetnansky S, Mikulaj L. Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. *Endocrinology*. 1970;87:738-43.
26. Gu H, Tang C, Peng K, Sun H, Yang Y. Effects of chronic stress on the development of atherosclerosis and expression of toll-like receptor 4 signaling pathway in adolescent apolipoprotein E knockout mice. *J Biomed Biotechnol*; 2009. DOI: 10.1155/2009/613879
27. Rich EL, Romero LM. Exposure to chronic stress downregulates corticosterone responses to acute stressors. *Am J Physiol Regul Integr Comp Physiol*. 2005;288: R1628-36.
28. Castagliuolo I, Leeman SE, Bartolak-Suki E, Nikulasson S, Qiu B, Carraway RE, et al. A neurotensin antagonist, SR 48692, inhibits colonic responses to immobilization stress in rats. *Proc Natl Acad Sci USA*. 1996;93:12611-15.
29. Castagliuolo I, Wershil BK, Karalis K, Pasha A, Nikulasson ST, Pothoulakis C. Colonic mucin release in response to immobilization stress is mast cell dependent. *Am J Physiol Gastrointest Liver Physiol*. 1998;274:1094-100.
30. Pfeiffer CJ, Qiu B, Lam SK. Reduction of colonic mucus by repeated short-term stress enhances experimental colitis in rats. *J Physiol Paris*. 2001;95:81-87.
31. Rubio CA, Huang CB, Porwitt A, Kock Y, Stenling R. Quantification of the colonic mucous cell population during protracted stress in rats. *Stress Med*. 1991;7:145-51.
32. Burkholder KM, Thompson KL, Applegate TJ, Patterson JA. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella enteritidis* colonization in broilers. *Poult Sci*. 2008;87:1734-41.
33. Hoebler C, Gaudier E, De Coppet P, Rival M, Cherbut C. MUC genes are differently expressed during onset and maintenance of inflammation in dextran sodium sulfate-treated mice. *Dig Dis Sci*. 2006;51:381-89.
34. Wang RQ, Fang DC. Alterations of MUC1 and MUC3 expression in gastric carcinoma: Relevance to patient clinicopathological features. *J Clin Pathol*. 2003;56:378-84.
35. Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, et al. Heterogeneity of mucin gene expression in normal and neoplastic tissues. *Cancer Res*. 1993;53:641-51.
36. Rakha EA, Boyce RWG, El-Rehim DA, Kurien T, Green AR, Paish EC, et al. Expression of mucins (MUC1, MUC2, MUC3, MUC4, MUC5AC and MUC6) and their prognostic significance in human breast cancer. *Mod Pathol*. 2005;18:1295-1304.
37. Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, Aubert JP. Expression of human mucin genes in respiratory, digestive tracts ascertained by in situ hybridization. *J Histochem Cytochem*. 1993;41:1479-1485.
38. Weiss AA, Babyatsky MW, Ogata S, Chen A, Itzkowitz SH. Expression of MUC2 and MUC3 mRNA in human normal, malignant, and inflammatory intestinal tissues. *J Histochem Cytochem*. 1996;44:1161-66.
39. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci USA*. 2000;105:15064-69.
40. Corfield AP, Myerscough N, Longman R, Arul S, Pignatelli M. Mucins and mucosal protection in the gastrointestinal tract: New prospects for mucins in the pathology of gastrointestinal disease. *Gut*. 2000;47:589-94.
41. Shekels LL, Ho SB. Characterization of the mouse Muc3 membrane bound intestinal mucin 5' coding and promoter regions: Regulation by inflammatory cytokines. *Biochim Biophys Acta*. 2003;1627:90-100.
42. Moniaux N, Chaturvedi P, Vashney GC, Meza JL, Rodriguez-Sierra JF, Aubert J-P, et al. Human MUC4 mucin induces ultrastructural changes and tumorigenicity in pancreatic cancer cells. *Br J Cancer* 2007;97:345-57.
43. Senapati S, Chaturvedi P, Sharma P, Venkatrman G, Meza JL, El-Rifai W, et al. Deregulation of MUC4 in gastric adenocarcinomas: Potential pathological implication in poorly differentiated non-signet ring cell type gastric cancer. *Br J Cancer*. 2008;99:949-56.

44. Song KS, Lee W-J, Chung KC, Koo JS, Yang EY, Choi JY, et al. Interleukin-1 β and Tumor Necrosis Factor- α induce MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. J Biol Chem. 2003;278:23243-50.

© 2016 Habiyambere and Onyango; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
<http://sciedomain.org/review-history/13084>