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LIPID COMPOSITION AND AMINO ACID UPTAKE DURING ROTAVIRUS INFECTION AND PROTECTION WITH TRYPSIN INHIBITOR IN MALNOURISHED INFANT MICE.

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ABSTRACT

The aim of the study was to evaluate the influence of rotavirus (RV) and soyabean trypsin inhibitor (TI) on lipid composition and uptake of glucose and glycine in rotavirus (RV) infected malnourished (PEM) infant mice. Malnutrition was achieved in animals by doubling the litter size. Cholesterol (Ch) and phospholipd (PL) contents, uptake levels of glucose and glycine were determined in jejunum and ileum portion of small intestine. Increase in Ch/PL ratios was observed in PEM + RV group. The levels became comparable in PEM +RV+TI as compared to PEM. Uptake levels of glucose and glycine increased in PEM animals. With rotavirus (PEM+RV+T I group), the levels decreased which became comparable to PEM. Changes in uptake and lipid composition with rotavirus and trypsin inhibitor may be responsible for altering membrane fluidity and organization during rotavirus diarrhea. The results establish the importance of trypsin inhibitor during oral infection with rotavirus.

KEY WORDS

Malnutrition, Rotavirus, Trypsin Inhibitor, Lipids, Amino Acid Uptake, Mice.

INTRODUCTION

Protein energy malnutrition (PEM), one of the biggest public health problems, affects people of all ages, but the results are most dramatic in childhood due to high energy requirements during this period (1). PEM in combination with diarrheal disease is the primary cause of child morbidity and mortality (2). Among the diarrheal diseases, rotavirus (RV) diarrhea is the most common causing 6,00,000 to 8,70,000 deaths each year, accounting for an estimated 20 to 25% of all deaths due to diarrhea and 6% of all deaths among children < 5 years of age (3). Rotaviruses (RV) infect the mature epithelial cells at the tips of the villi of small intestine with subsequent cell lysis and villus blunting, depressed levels of intestinal enzymes (4), resulting in watery diarrhea and dehydration (5). PEM dramatically affects development of the intestinal mucosa specially during the postnatal period. Biochemical and histological alterations in the gut observed in humans and animals with malnutrition result in lower intestinal surface, reduced amino acid uptake and changes in the membrane bound enzymes (6,7) Studies have shown similar metabolic and structural alterations in small intestine of experimental animals affected by malnutrition (8). Several attempts to prevent the disease and supplementation with the nutrients have been encouraging but not highly successful (9). Small intestine brush border is highly specialized to perform variety of digestive and absorptive functions (10) and rotaviruses mainly multiply in cytoplasm (11). So it was of interest to examine these biochemical properties of small intestine and the protective effect of soyabean trypsin inhibitor (TI) during rotavirus diarrhea in infant mice. Rotavirus infection in suckling mouse model, in particular with malnutrition provides an excellent system in which characterization of lipid composition was studied along with the role of soyabean trypsin inhibitor.

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MATERIALS & METHODS

All reagents, buffer components and standards were of the highest grade. Inbred BALB/C mice of 7 day age were selected for the present studies. They were made sure to be born to mothers seronegative for RV antibodies by ELISA (11). All animals were kept in autoclavable polypropylene cages (Tarson, Calcutta, India) and were maintained on pellet diet and water *ad-libitum*. Murine EB rotavirus (Serotype 3) strain used in the present studies was kindly gifted by Dr. H. B. Greenberg California, U.S.A.(11). The virus was

maintained throughout in an infective form by serial passaging in *vivo* in 7 day old mice as described earlier (11). The 100 ID50 of stock RV (P9) was determined according to the method of Reed and Muench (12). Mild to moderate PEM was induced by doubling the litter size. Litters of infant mice (n = 144) were divided into four groups: healthy controls, malnourished (PEM), PEM+RV and PEM+RV+TI (n=36 each). Control and PEM animals were orally inoculated with 50 µl normal saline each. Each animal of PEM+RV group was orally inoculated with 100 ID 50 RV (P_9) and in PEM+RV +TI group each animal was orally inoculated 0.6 mg TI/g body body weight / 100 ID50 RV (P9). Sufficient time was allowed for the animals to swallow the liquid. Animals of control and PEM groups were kept separately from the infected animals. A dose of 0.6 mg TI/g body weight /50ul of 100 ID 50 of RV stock was selected for each animal to be given orally after a pilot study was conducted as used by Ebina and Tsukada (13). Animals were sacrificed under light chloroform anesthesia on 0, 1, 3, 5, 7 and 10 days post inoculation (dpi). All animals were sacrificed mid-afternoon to eliminate diurnal variations in the estimating parameters. Whole small intestines were separately homogenized in cold 0.9% normal saline. Virus antigen load in intestine of mice was estimated according to the method described earlier (11). Small segments of both jejunum and ileum were removed and weighed. ($U-^{14}C$) – Gylcine and glucose uptake in vitro was measured by the method of Miller and Crane (14). Amino acid uptake was initiated with the addition of weighed amount of sample to 500 µl, uptake buffer (maleic acid 0.2 mol/L, Tris buffer 0.5 mol/L, NaCl 1.54 mol/L, CaCl2 0.252 mol/L, and MgSO4 0.242 mol/L). The reaction mixture was incubated at 37 °C in waterbath shaker for 5 min. The reaction was stopped by adding ice cold Tris – maleate stopping buffer (pH 7.4). The contents were filtered through Whatman paper No. 1 after sufficient washing with a known amount of buffer. The tissues were dried and dissolved in 200 µL of 10% potassium hydroxide.Counts were checked in beta liquid scintillation counter (Packard instrument company, Downers Grove, USA) and expressed as counts per minute (CPM) /g tissue/min. Lipids were extracted and washed using the method of Bligh and Dyer (15) and were finally redissolved in small volumes of chloroform. Cholesterol contents were measured by applying the method of Zlatkis *et al.* (16). Lipid phosphorus was determined by the modified method of Marinetti (17). The protein contents of the fractions were estimated by Lowry *et al.*, (18).

STATISTICAL ANALYSIS

Student's t-test was employed for the statistical analyses of data to compare each group. The data was expressed as mean ± standard error (SE) of six replicates. P-value less than 0.05 was taken as the significant value. Ethical approval from the Post Graduate Institute of Medical Education& Research Ethical Committee was obtained .

RESULTS

When suckling mice were distributed and maintained in litters' 12-14 pups with a single mother, they were mild to moderately malnourished by 7 day of age. Decrease in 28.3% body weight was found compared to the controls. The mean intestinal length of small intestine was also significantly decreased by doubling the litter size compared to the controls (16.6 ± 0.97 Vs 15.4 ± 0.41 cms). All rotavirus infected animals exhibited yellowish watery diarrhea on palpation of stomach which was evidenced by pronounced fecal staining in comparison to controls with no clinical signs of rotavirus infection. This fecal staining of infected animals correlated well with the significant decrease in body weights. Diarrhea was not observed in groups to be treated (PEM + RV and PEM + RV + TI) prior to inoculation with rotavirus and / or TI. Rotavirus antigen levels increased significantly in PEM+RV group, which reduced significantly ($p< 0.001$) and became comparable to the controls in PEM+RV+TI animals. Cholesterol content in jejunum was significantly increased on all scheduled days in PEM animals compared to the controls. In PEM+RV, the levels rose further on the peak day of infection (5 dpi). However, in PEM+RV+TI the levels became comparable to the PEM animals. In ileum the levels increased significantly on 3 dpi in PEM + RV group compared to PEM which became comparable to PEM when TI was inoculated along with the RV. Phospholipids were elevated in PEM animals in both anatomical portions of intestine compared to the controls. RV and TI had no effect in PEM animals in both the portions compared to the PEM alone animals. Cholesterol / Phospholipid (Ch/PL) ratio was not changed in PEM animals compared

SEVERITY OF DIRRHOEA

GROUPS	0 dpi	1 dpi	3 dpi	5 dpi	7 dpi	10 dpi
Control	-	-	-	-	-	-
PEM		-	-	-	-	+
PEM + RV	-	+	++	+++	++	+
PEM + RV + TI	-	-	+	++	+	-

to controls in both anatomical regions of small intestine. In PEM+RV animals, the Ch/PL ratio increased significantly on 3 and 5 dpi in jejunum and on 3,5, and 7 dpi in ileum compared to PEM animals. This ratio in PEM+RV+TI decreased on the peak days infection i.e. 3 and 5 dpi compared to the PEM + RV animals in both jejunum and ileum and became comparable to the PEM animals Glucose uptake levels were significantly increased in PEM animals compared to controls in jejunum and ileum. In PEM+RV animals, uptake levels decreased significantly in jejunum and ileum on 3 and 5 dpi compared to PEM group of animals. Uptake levels in PEM+RV+TI animals increased significantly on these peak days of infection and became comparable to the PEM animals. Glycine uptake levels were similarly elevated as glucose levels with PEM compared to controls in both jejunum and ileum on all days of experimental schedule. In PEM+RV, the uptake levels decreased significantly on 3 and 5 dpi in jejunum and ileum both compared to PEM animals. When TI was inoculated to PEM+RV animals, uptake levels were increased significantly on 3 and 5 dpi in jejunum and became comparable to the controls. However, in ileum the levels in PEM+RV+TI were less as compared to the PEM animals on 3 and 5 dpi and thereafter became comparable to the PEM animals showing delayed recovery with inhibitor.

DISCUSSION

Reducing the protein energy intake in infant mice by doubling the litter size compared to controls led to mild to moderate malnutrition as evident from significant decrease (28.3%) in body weight of animals. Previously also, malnutrition was induced through expansion of litter size (19). Decrease in body weight upto 30% compared to controls indicate presence of mild -moderate PEM according to Gomez et al.(20), whose classification is made on the weight for age criteria. In humans, PEM is often associated with infection, a factor that limits the study of the effects of protein energy restriction itself on the structure and function of small intestine. Also, the availability of small intestinal specimens is very limited in humans with gastroenteritis. The present model was chosen as the pathogenesis and clinical manifestations of rotavirus infection are similar in suckling mice and human infants (21).

Rotavirus induced severe watery diarrhea in infected malnourished mice within 1 dpi and was cleared by 10 dpi. In rotavirus alone animals diarrhea appeared on 3 dpi and was cleared by 7 dpi indicating that infection was for longer duration in malnutrition. In the present study, in PEM+RV+TI group diarrhea appeared on 3 dpi and was cleared by 7 dpi demonstrating that trypsin inhibitor intake during rotavirus enteritis decreases the duration of diarrhea. These data are consistent with precious evidence that early re-feeding of children with enteritis decreases the duration of diarrhea (22). Similar results were observed in our previous studies (23).

An important observation in the present study was the reduction in the length of small intestine with malnutrition. This must have resulted due to reduction of the total absorptive surface of intestine. Similar results have been reported earlier (19). In the present study, uptake of amino acid and glucose was increased during malnutrition in infant mice. It is quite clear that during malnutrition in neonates, there is a need for large amounts of energy for vital cellular processes. So, during this process, elevated levels of gluconeogenic enzyme which in turn break down stored glycogen (24), increase lipolysis and ketogenesis to provide more glucose to the system via the gluconeogenic pathway. It has been demonstrated that protein deficient rats exhibit increase in pinocytic activity and a deterioration of apical junctions with movement of protein molecules directly between the cells (25). In support of these observations, we found augmentation of amino acid and glucose uptake levels in malnourished animals.

The results of present studies are in consistent to the earlier reports which might be due to reduced absorptive capacity of damaged epithelial lining. An interesting observation which we made for the first time was that, addition of TI restored the uptake levels in the animals showing its protective role during rotavirus infection. Similarly, in the membrane fluidity study, it was found that the membranes become more fluid during malnutrition. Membrane fluidity is known to be affected during several physiological conditions including PEM and infections (26). In the present studies no effect of PEM on membrane fluidity was observed which might have been due to mild to moderate PEM induced in the animals. When rotavirus infection was given to malnourished animals, drastic alterations took place, increasing the Ch/ PL ratio which indicated the decrease in membrane fluidity. This could be probably the cause of decreased amino acid and glucose uptake during rotavirus infection in malnourished infant mice. We are reporting for the first time the lipid characterization during rotavirus infection in malnourished infant mice. Interestingly, TI was found to be effective in restoring the lipid levels and hence the membrane fluidity of PEM+RV+TI animals was comparable to PEM animals showing its protective efficacy.

In conclusion, the present studies provide clear evidence that feeding/supplementing trypsin inhibitor during diarrhea episode is important for rapid recovery

of small intestine following rotavirus infection in animals.

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