Digital morphometric and stereologic analysis of small intestinal mucosa in well-nourished and malnourished children with persistent diarrhea

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Abstract

Objective: to test the hypothesis that the proximal small intestines of children with persistent diarrhea present morphometric and stereologic changes proportional to their nutritional status, using microscope images stored in a computer.

Methods: cross-sectional study with 65 pediatric patients, whose ages ranged from 4 months to 5 years, with persistent diarrhea for over 14 days. The nutritional assessment was performed according to the z-scores for weight/age (W/A), weight/height (W/H) and height/age (H/A) ratios, divided into: well-nourished = z > 2SD and malnourished = z < -2SD; well-nourished = z > 2SD, nutritional risk = z < -1SD and malnourished = z < -2SD; and continuously, in descending order, using the NCHS charts. After obtaining the computer images using the software Scion Image, villous height, crypt depth, mucosal thickness, total mucosal thickness, and villous/crypt ratio were measured in the fragments of the small intestinal mucosa, enlarged 100 times. When images were enlarged 500 times, enterocyte height, nuclear height and brush-border height were measured. Stereologic analysis was performed using cycloid arcs.

Results: for W/A, W/H and H/A z-scores, divided into two nutritional status categories, no statistically significant difference was observed in regard to villous height, crypt depth, mucosal thickness, total mucosal thickness and villous/crypt ratio. Enterocyte height presented the most significant difference between well-nourished and malnourished groups, for W/A and W/H ratios, with a 500x enlargement, although this difference was not statistically significant. When z-scores were subdivided into three nutritional status categories, a digital morphometric analysis showed a statistically significant difference for villous/crypt ratio between the well-nourished and slightly malnourished group and the well-nourished and mild to severe malnourished group (p = 0.048). The villous/crypt ratio was higher among well-nourished children. Using the Spearman coefficient, the variables enterocyte height, height of enterocyte nucleus and brush-border height presented a clear association with the W/A ratio (r = 0.25; p = 0.038), W/H ratio (r = 0.029; p = 0.019). The height of the enterocyte and the brush-border height were associated with W/H ratio.

Conclusions: the observed associations between nutritional status and the analyzed small intestinal mucosa variables showed a positive correlation with patients’ weight. Although these associations were of a slight to moderate magnitude, we observed a tendency of enterocyte size reduction, as well as a reduction in the size of its nucleus and brush-border, as the level of malnutrition increases.

Introduction

According to the World Health Organization, severe malnutrition is a social and medical problem and is associated with 29% of child deaths in the 0 to 4 years age group.\(^1\) Pelletier et al.\(^2\) however, believe that this percentage is not a true reflection of reality and consider that 56% of child deaths are due to damage resulting from milder forms of malnutrition. The pathogenesis of persistent diarrhea associated with malnutrition is multifactorial, with early weaning, poverty, infectious and nutritional factors and allergic phenomena contribute to its perpetuation. The final result of these events is damage to the small intestine mucosa. An understanding of the complex interactions which take place in the luminal-mucosal interface of the small intestine is fundamental to understanding the nature of this type of diarrhea.\(^3\)

Clinical and experimental studies of humans have shown that nutritional disturbances can produce morphological and structural alterations to the intestinal mucosa.\(^4,5\) These alterations can be reflected in morphometry and in the surface/volume ratio of the intestinal mucosa.\(^6\)

In order to study these alterations, sensitive, refined techniques which can be easily reproduced must be used. Computer programs which allow image capture, the calculation of the dimensions or number of objects designated by the user and the digital storage of this data can enable a precise and minute study of tissues, including the small intestine mucosa.\(^6,7\) Image capture enables analysis to be done by more than one observer, reduces the subjectivity of the examination and facilitates the comparison of studies in this area.

Morphometric studies of small intestine mucosa are beginning to be used to evaluate the extent of alterations found in malnourished patients. Some of these studies reveal a clear correlation between nutritional status and small intestine mucosa alterations,\(^8\) while others have not corroborated these findings.\(^3,12-15\) However, the morphometric techniques employed for this research are not uniform making comparison of the results difficult. In a previous study,\(^16\) the morphometric analysis of the small intestine mucosa of 85 children, between 4 months and 10 years old, was performed using a micrometer, coupled to an optical microscope which was the only method available at the time. The data analysis, using the Spearman correlation coefficient, showed a positive correlation between: the height of the villi and the z score W/A; the total mucosa thickness and W/A and W/S z scores and between the villus/ crypt ratio and W/A and W/S z scores.

In the present study, the use of a computer enabled capture and storage of images of the small intestine mucosa, and also computerized morphometry of a proportion of the material that had been used previously. Furthermore, capturing the images at 500x magnification allowed measurements to be made of the height of the enterocyte, the height of the nucleus and its brush border. In order to study possible alterations to the mucosa surface the technique of counting cycloid curves superimposed upon the images was used.

The current study used an easily accessed program which is available on the Internet (Scion Image Rasband W. Scion Image Beta2 on line version),\(^17\) in order to evaluate, by means of computerized morphometry, alterations caused by malnutrition to the small intestine mucosa of well-nourished children and of children with differing degrees of malnutrition.

Methods

The histological material was obtained from stored blocks of wax from 85 hospitalized children, the majority at the Pediatric Gastroenterology Unit at the Santa Casa de Misericórdia (Blessed House of Mercy) in Porto Alegre, in the period between May 1989 and November 1991. For the current study children were selected aged between 6 months and 5 years, with diarrhea of more than 14 days’ duration who had had a small intestine biopsy as part of the work up for to investigate the diarrhea. Nutritional status was assessed by means of z scores for weight/age (W/A), weight/stature (W/S) and stature/age (S/A), using the NCHS tables.\(^18\) The z scores were divided into 2 nutritional status categories (well-nourished: z \(\geq\) 2DP, malnourished: z < -2DP), into three categories (well-nourished: z \(\geq\) -1DP, nutritional risk: z \(<\) -1DP to z \(-\)2DP and malnourished: z \(<\) -2DP) and were also sorted in descending order.

This was a retrospective cross-sectional study. The small intestine mucosa fragments were sliced and stained with hematoxylin and eosin for histological study. Patients were included if their mucosa fragments, cut perpendicular to the longitudinal axis of the tissue, had, at least six villi and six crypts for morphometric and stereological evaluation, giving a total of 65 fragments. The new fragments were measured with the micrometer, as in the earlier study. After images of the intestinal mucosa had been captured, performed by means of a microscope coupled to a computer, computerized morphometry was used to compare the two methods. The small intestine mucosa variables studied, by micrometer and digital morphometry, at 100 times magnification, were: the height of the villi, the depth of the crypts, mucosa thickness, total mucosa thickness and the villus/ crypt ratio (Figure 1). Additionally, computerized morphometry was performed of the average height of at least 30 enterocytes, of the nuclei of the enterocytes and of the brush border (Figure 2), at 500 times magnification. The cells selected had a basal nucleus and were located in the central third of the villus. The average height of each variable was calculated, dividing the sum of the measurements by the number of units observed. Additionally, in order to estimate the surface area of the small intestine mucosa fragments, stereological analysis was performed by means of a count of cycloid curves.
from images captured at 40 times magnification (Figure 3). In order to measure surface area the number of times that the cycloid curve intercepted the epithelial surface was counted and the subjacent tissue was evaluated by counting the number of points which fell between the epithelium and the lamina propria. The resulting number was an absolute number. The estimate of surface area was made using the formula:

\[ S_v = 2 \cdot \left( \frac{I}{L} \right) \]

\( I \) = intersection: the sum of the number of times that the cycloid curve and the points touched the epithelial surface; \( L \) = total length of the test lines; i.e. it’s entire grid and it’s a constant: 4.9297mm.

Morphometry was performed by the first author of the study, blind to the nutritional status of the patient. In order to capture images of the small intestine mucosa, a conventional optical microscope was used coupled to a video camera linked to a microcomputer with a video capture board and image analysis software. The images were captured as *tag image format*. The software used for digital morphometric analysis was Scion Image (Rasband W. Scion Image Beta2 on line version at: http://www.scioncorp.com ed: National Institutes of Health, modified by Scion Corporation - Frederick, MD, USA; 1997). In considering three groups analysis of variance (ANOVA) of classification criteria was used with significant differences identified by the Student-Newman-Keuls post-hoc test. Evaluation of linear correlation was made using the Pearson (r) correlation coefficient. The significance level adopted was \( \alpha = 0.05 \). Data was processed and analyzed using SPSS, version 10.0 and Sigma Plot v. 2.1.

For the statistical analysis, the quantitative data was initially described as mean average and standard deviation. Comparisons between two groups were made with the Student \( t \) test, for independent samples, with estimates of average difference and the 95% confidence interval (CI). In considering three groups analysis of variance (ANOVA) of classification criteria was used with significant differences identified by the Student-Newman-Keuls post-hoc test. Evaluation of linear correlation was made using the Pearson (r) correlation coefficient. The significance level adopted was \( \alpha = 0.05 \). Data was processed and analyzed using SPSS, version 10.0 and Sigma Plot v. 2.1.
This study was approved by the Commission for Ethics in Research of the Research and Post Graduate Unit at the Hospital de Clínicas de Porto Alegre.

Results

The average age of patients in this study was 1 year and 5 months (+ 9 months), 50 of them (77%) were less than 24 months old and Caucasians predominated. The average duration of diarrhea was 5.3 (+ 5.4) months. There was no relationship between the duration of diarrhea and nutritional status. Nutritional assessment into two categories was as follows: z W/A = 29 WellN and 36 MalN; z W/S = 41 WellN and 24 MalN; z S/A = 45 WellN and 20 MalN. Nutritional assessment into three categories showed: z W/A = 17 WellN, 12 nutritional risk (NRisk) and 36 MalN; z W/S = 22 WellN, 19 NRisk and 24 MalN; z S/A: 28 WellN, 17 NRisk and 20 MalN. Five patients presented nutritional edema. The average values for the variables studied at 100 times magnification, including all patients from the earlier study and those from the current study are given in Table 1. The average values for the variables studied at 500 times magnification, including 65 patients, were: height of the enterocyte: 28.7 ± 4 µm; height of the nucleus: 8.1 ± 1 µm; height of the brush border: 1.9 ± 1 µm.

Table 1 - Comparison of the characteristics of the small intestine mucosa through morphometry measured with micrometer

<table>
<thead>
<tr>
<th>Variable</th>
<th>First study* (85 patients)</th>
<th>Current study (65 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mucosa thickness</td>
<td>485.1 ± 111.8 µm</td>
<td>491.7 ± 80.8 µm</td>
</tr>
<tr>
<td>Height of the villi</td>
<td>268.3 ± 87.5 µm</td>
<td>286.6 ± 62.9 µm</td>
</tr>
<tr>
<td>Depth of the crypts</td>
<td>112.9 ± 33.8 µm</td>
<td>103.9 ± 16.8 µm</td>
</tr>
<tr>
<td>Mucosa thickness</td>
<td>210.6 ± 73.2 µm</td>
<td>205.5 ± 49.1 µm</td>
</tr>
<tr>
<td>Villus/crypt ratio</td>
<td>2.7 ± 1</td>
<td>2.8 ± 0.7</td>
</tr>
</tbody>
</table>

* Reference 18
Employing the Student \( t \) test, the morphometric analysis revealed that when the patients were divided into two nutritional status categories, according to z score for W/A, W/S and S/A, there were no statistically significant differences between the variables height of the villi, depth of the crypts, mucosa thickness and total mucosa thickness and the villus/crypt ratio, performed on 62 fragments. When the patients were divided into three nutritional status categories, the digital morphometric analysis revealed statistically significant differences for the villus/crypt ratio between well-nourished and those at nutritional risk and between well-nourished and malnourished. However, there was no difference between mildly malnourished and moderately and severely malnourished patients. The villus/crypt ratio was greatest among well-nourished patients.

In our previous study, the analysis performed using the Spearman correlation coefficient showed a positive correlation between the height of the villi and the W/A ratio, total mucosa thickness and the W/A and W/S scores and between the villus/crypt ratio and the W/A and W/S ratios, with lower values being returned for the more severely malnourished children. In terms of the variables height of the enterocyte, height of the nucleus and of the brush border, assessed across 65 fragments, taking into account the malnutrition classification by W/A, when the \( t \) test was used the characteristic which presented the greatest difference between the well-nourished and malnourished groups was the height of the enterocyte (well-nourished: 29.8 \( \mu \)m, malnourished: 27.9 \( \mu \)m). The same was observed of the classification according to W/S (well-nourished: 29.4 \( \mu \)m, malnourished: 27.6 \( \mu \)m). Despite this, none of the differences achieved statistical significance.

Nevertheless, when the Pearson correlation coefficient was used to evaluate correlations between the different digital morphometry parameters and the z scores, a clear association was observed between the height of the enterocyte and the W/A and W/S z scores; between the height of the nucleus and the W/A z score and between the height of the brush border and the W/A z score, although the strength of this association was of low to, at most, moderate magnitude. Table 2 contains the correlation matrix, showing Pearson linear correlation coefficients and statistical significance for digital morphometry parameters against nutritional status assessment.

**Table 2 -** Correlation matrix, showing Pearson linear correlation coefficients and statistical significance for digital morphometry parameters against nutritional status assessment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Weight/Age (z score)</th>
<th>Classification Weight/Height (z score)</th>
<th>Height/Age (z score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height of the villi - ( \mu )m</td>
<td>0.06</td>
<td>0.13</td>
<td>-0.01</td>
</tr>
<tr>
<td>at 100 times magnification, ( n = 62 )</td>
<td>p = 0.631</td>
<td>p = 0.309</td>
<td>p = 0.924</td>
</tr>
<tr>
<td>Depth of the crypts, ( \mu )m</td>
<td>-0.03</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>at 100 times magnification, ( n = 62 )</td>
<td>p = 0.803</td>
<td>p = 0.783</td>
<td>p = 0.858</td>
</tr>
<tr>
<td>Mucosa thickness, ( \mu )m</td>
<td>-0.15</td>
<td>0.02</td>
<td>-0.20</td>
</tr>
<tr>
<td>at 100 times magnification, ( n = 62 )</td>
<td>p = 0.231</td>
<td>p = 0.855</td>
<td>p = 0.125</td>
</tr>
<tr>
<td>Total mucosa thickness, ( \mu )m</td>
<td>0.08</td>
<td>0.24</td>
<td>-0.06</td>
</tr>
<tr>
<td>at 100 times magnification, ( n = 62 )</td>
<td>p = 0.551</td>
<td>p = 0.060</td>
<td>p = 0.638</td>
</tr>
<tr>
<td>Villus/crypt ratio, ( \mu )m</td>
<td>0.13</td>
<td>0.13</td>
<td>-0.22</td>
</tr>
<tr>
<td>at 100 times magnification, ( n = 62 )</td>
<td>p = 0.314</td>
<td>p = 0.307</td>
<td>p = 0.867</td>
</tr>
<tr>
<td>Surface/volume</td>
<td>-0.02</td>
<td>0.19</td>
<td>-0.14</td>
</tr>
<tr>
<td>at 40 times magnification, ( n = 62 )</td>
<td>p = 0.871</td>
<td>p = 0.128</td>
<td>p = 0.281</td>
</tr>
<tr>
<td>Height of the enterocyte, ( \mu )m</td>
<td>0.25*</td>
<td>0.29*</td>
<td>0.16</td>
</tr>
<tr>
<td>at 500 times magnification, ( n = 65 )</td>
<td>p = 0.038</td>
<td>p = 0.019</td>
<td>p = 0.179</td>
</tr>
<tr>
<td>Height of the nucleus, ( \mu )m</td>
<td>0.24*</td>
<td>0.16</td>
<td>0.23</td>
</tr>
<tr>
<td>at 500 times magnification, ( n = 65 )</td>
<td>p = 0.054</td>
<td>p = 0.188</td>
<td>p = 0.063</td>
</tr>
<tr>
<td>Height of the brush border, ( \mu )m</td>
<td>0.26*</td>
<td>0.27*</td>
<td>0.23</td>
</tr>
<tr>
<td>at 500 times magnification, ( n = 65 )</td>
<td>p = 0.032</td>
<td>p = 0.030</td>
<td>p = 0.062</td>
</tr>
</tbody>
</table>

* Statistically significant results.
Stereological analysis with cycloid curves, performed across 62 fragments, did not reveal any statistically significant results in terms of nutritional status.

Discussion

Persistent diarrhea occurs most often during the first two years of life. Of the patients in this study, 77% were less than two years old, confirming the vulnerability of this age group to increased diarrhea prevalence. For Lentze, it is difficult to distinguish which came first, the diarrhea or the malnutrition when dealing with malnourished children.

While it is known that luminal nutrients are essential to the integrity of intestinal mucosa, the role played by malnutrition in the pathogenesis of malabsorption and intestinal enteroPATHY remains controversial. Steatorrhea and villi atrophy have been described in children with kwashiorkor and marasmus. The milder forms of malnutrition do not appear to have a greater impact on the structure and function of intestinal mucosa and, probably, do not explain the alterations to the mucosa architecture found in the tropics. The alterations described with patients suffering from protracted diarrhea, malnourishment or tropical enteropathy, are particularly related to alterations to the morphology of the mucosa, the mucosa surface area and enterocytes. Kallas et al., comparing children with diarrhea due to classic enteropathogenic Escherichia coli with those suffering from environmental enteropathy, observed higher values in the crypt compartment amongst the environmental enteropathy sufferers. In Venezuela, Romer et al., studying the small intestine mucosa of 24 children with diarrhea and varying degrees of malnutrition, grouped according to the Gomez classification system, did not observe a correlation between morphological alterations and the degree of malnutrition. Ferreira and Fagundes Neto, in São Paulo, found alterations of varying intensity to the intestinal mucosa of children with protracted diarrhea which were not related to their nutritional status. Sullivan, assessing the jejunal morphometry by micrometer, is probably a result of the exclusion of 20 patients which included those older than 5 and those whose fragments produced less than six villi and six crypts for morphometric analysis.

In relation to the study of enterocytes, when the height of these cells was measured in 19 controls, children with no diarrhea (32.3 ± 3.8 µm) and 23 with persistent diarrhea (31.5 ± 5.5 µm), without mentioning nutritional status, Penna et al. report that no differences were detected between the groups. However, a comparison between 24 patients with normal histology and 18 with partial villi atrophy (34.1 ± 3.4 µm and 28.9 4.7 µm, respectively), statistically significant differences between the groups were found. Bhan et al. evaluated the enterocyte heights of different groups of children: well-nourished, with protracted diarrhea of varying etiologies, with coeliac disease and severe malnourishment. The values for enterocyte height were greater among the well-nourished subjects (31 ± 2.7 µm) and, in descending order, among those with severe malnutrition (25.3 ± 1.7 µm); the enteropathogenic E. Coli carriers (20.9 ± 2.6 µm) and those with untreated coeliac disease (15.5 ± 2.6 µm). Bahn et al., in common with this study, found higher values for enterocyte height among well-nourished subjects when compared with severely malnourished patients.

The values observed in this study are similar to those found by Penna et al. in patients with partial villi atrophy, and also those detected in a study of adult patients by Wood et al. who found statistically significant differences between Caucasian English patients (30.2 µm) Indian immigrants (28.3 ± 2.7 µm) and Afro-Caribbeans (27.2 ± 2.7 µm). The authors proposed that these findings “..reflect the ‘normal’ morphology of immigrant patients, determined by environmental factors “.

When the enterocyte size of the patients in this study were analyzed with the Student t test in terms of W/A and W/S z score classifications, there were greater differences between well-nourished and malnourished patients, although these never achieved statistical significance. However, analysis by means of the Spearman linear correlation coefficient revealed statistically significant differences between enterocyte height, height of the nucleus and height of the brush border and the W/A ratio and between enterocyte height and height of the brush border and the W/S ratio. There is a tendency for the height of these structures to reduce in proportion to the extent that the degree of malnutrition increases. This finding suggests that malnutrition per se may be associated with the reduction in enterocyte height. Reductions in enterocyte nucleus and brush border measurements were also found among the most malnourished patients.

Stereological analysis to measure the absorbent intestinal surface area, performed by means of cycloid curves in this study, did not reveal a positive correlation with nutritional status. Ribeiro et al. however, in an experimental study of the epithelium of the small intestine using rats with protein-
energy malnutrition, found reduced jejunal absorbent surface area while there was no observable alteration to villi height. They concluded that malnutrition can affect intestinal function by reducing adsorbent surface area with no apparent structural alterations. Wood, Gearty and Cooper,26 as in our research, also found no statistically significant difference in terms of surface area/volume, when studying Indian, Afro-Caribbean and English adult patients. These authors suggested that the similarities between the surface area/volume ratios of these groups may be explained by a small intestine mucosa layer which is of lesser thickness among immigrant patients.

We did not find any studies using stereological assessment to evaluate the intestinal mucosa of pediatric patients in the scientific literature.

In conclusion, in common with our previous study, it was found that observed correlations were always related to patient weight. Thus, the most significant associations detected were between nutritional status and enterocyte morphology. Although these associations were moderate to weak, there is a tendency for the enterocyte, its nucleus and its brush border to reduce in size in proportion to the degree to which malnutrition increases. The significance of studies of the small intestine mucosa enterocyte is in the fact that it has a role to play in the immunological protection of this mucosa.28 The interactions between immunological and morphological and structural alterations to the small intestine mucosa with protein-energy malnutrition need to be studied further with the aim of improving the treatment of malnourished children.29

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References

