Research Report

The Role of Breastfeeding and the Intake of Bovine Colostrum in Autistic Neonatal Rats with Coeliac Disease

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Abstract

Autoimmune deficiencies are linked to Autism Spectrum Disorder. Coeliac Disease (CD), also known as an auto-immune disease since IgG and IgA antibodies produced by the immune system against specific gluten components, namely gliadin, also target and damage the intestinal tissue, resulting in the characteristic deformed villi which impairs absorption of nutrients. Bovine colostrums are the first milk produced postpartum, and are typically defined as the first six postpartum milkings collected during the period of transition from colostrums to milk. This commentary discusses the role of breastfeeding and the intake of colostrum to overcome coeliac disease in autistic neonatal rats.

Keywords

Autism; Breastfeeding; Coeliac disease; Bovine colostrums

Introduction

Coeliac disease (CD), which develops in genetically susceptible individuals after ingestion of gluten-containing food, is characterized by damaged intestinal mucosa with malabsorption of most nutrients (Marsh, 1992). The disorder, which affects the proximal small intestine, is characterized by crypt cell hyperplasia and villus atrophy. The active component of wheat gluten is a complex mixture of glutamine- and proline-rich proteins called gliadin (Kasarda, 1997). A significant association between maternal history of Coeliac disease and ASDs was observed for the first time (Norgard et al., 1999). The observed associations between familial autoimmunity and ASDs/infantile autism are probably attributable to a combination of a common genetic background and a possible prenatal antibody exposure or alteration in fetal environment during pregnancy (Ludvigsson et al., 1999). In a more recent publication (Wakefield et al., 2008), it was observed that ileal LNH (lymphoid nodular hyperplasia) presented in 93% of affected children with autism and 14.3% of control children. Colonic LNH was present in 30% of affected children and 5.4% of control children. Hyperplasia of the intestinal lymph nodes was found in 88.5% of biopsies of affected children. Active inflammation of the ileum (ileitis) and duodenum was observed in 88% and chronic inflammation of the colon (colitis) was seen in 8% of affected children. The authors characterized the pathology as “a subtle new variant of inflammatory bowel disease that lacks the specific diagnostic features of either Crohn’s disease or ulcerative colitis.” In yet another study (Horvath et al., 1999) used endoscopy with biopsy to examine the upper GI tract of 36 children diagnosed with autism and experiencing abdominal pain, chronic diarrhea, bloating, nighttime awakening, or unexplained irritability. Abnormal findings included reflux esophagitis in 25 of the children, chronic gastritis in 15, and chronic duodenitis in 24. Low activity of intestinal carbohydrate digestive enzymes was observed in 21 children, whereas 27 exhibited increased exocrine secretion of pancreatic-biliary fluid after intravenous administration of the GI hormone secretin. Secretin, a peptide hormone released by endocrine cells within the duodenal mucosa, promotes
sodium bicarbonate and water secretion by the pancreas. It is important to note that this study describes altered function in the upper GI tract of autistic children, whereas the lymphoid nodular hyperplasia described by (Wakefield et al., 2008) was observed in the lowest portion of the small intestine, namely the ileum. The results of these different studies taken together suggest that significant and widespread GI pathophysiology may accompany autism, at least within a subpopulation of patients. As discussed below, the pathology may be central to the etiology of autism. Alternatively, it may simply be a secondary consequence of the disorder. In either case, it is possible that such widespread pathology plays a major role in the symptomatology of the disorder in the affected children. Pathological inflammation of the intestinal mucosa has long been recognized as a primary symptom in Coeliac disease and inflammatory bowel disease. Intestinal inflammation can be regarded as the consequence of the disruption of the complex interaction between all of the cells of the mucosa (immune and nonimmune), as well as the extracellular matrix, the normal interactions being mediated by cell surface and paracrine molecules (Fiocchi, 1997). Colostrums provide all the necessary nutrients, growth factors and immunological components for a healthy term infant needs (Davis et al., 2007). Bovine colostrums are the first milk produced postpartum, and are typically defined as the first six postpartum milkings collected during the period of transition from colostrums to milk (Yu et al., 2007). Several researchers have compared the compositions of colostrums with those of mature milk and concluded that colostrums have higher levels of immunoglobulins and other important immune factors and mediators (Davis et al., 2007). The major differences between bovine colostrums and mature milk are that colostrums have higher protein, lower fat, and a lactose solution rich in immunoglobulins and other important immune factors and mediators (Davis et al., 2007). The major differences between bovine colostrums and mature milk are that colostrums have higher levels of immunoglobulins, vitamins A, B and D, iron, calcium, as well as other vitamins and minerals (Kelly, 2003; Meisel, 1998) reported that the peptides derived from colostrum had immunological enhancing activity. Some peptides, such as Thr-Thr-Met-Pro-Leu-Trp, Pro-Gly-Pro-Ile-Pro-Asn, and Leu-Leu-Try, can help reduce the infection rate of Klebsiella pneumoniae in mice. Lindmark-mansson et al (2000) reported that the lactoferrin derived from colostrums had a high antioxidant activity, could scavenge free radicals generated in the human body, and also had a direct and positive effect on inhibiting atherosclerosis and ageing. Gill et al (2008) reported on the relationship between the assimilated quantity of colostrums and anti-tumor results. Ma et al (2009) found through an indirect model, that bovine colostrums were capable of enhancing the immunity for inducing human leukemic U937 cell death.

According to the discoveries appear to verify what parents and physicians have long suspected, namely, that many autistic children have coeliac disease with abnormal GI function. So we try in this investigation to examine neonatal autistic rats, coupled with an effective treatment (bovine colostrum) to normalize GI tract (especially in the duodenum), may prove effective in lessening the severe impact this disorder has on the autistic child. Additionally, our work aimed at studying morphologic changes in the duodenum with changes in biochemical parameters, of suckled pups that received gliadin in the neonatal period and comparing it with pups that received gliadin + bovine colostrum and with breast-fed controls.

1 Results
1.1 Changes in body weight and postnatal growth on day 0, 7, 14 and 21

As shown in Table 1, Figure 1, pup weight was significantly decreased (P<0.001) in autistic neonatal pups PND 0 as compared to normal control pups (1.6 ±0.1, 1.5±0.4 vs 3.53±0.3). The animals from group 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>PND 0</th>
<th>PND 7</th>
<th>PND 14</th>
<th>PND 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.53±0.3</td>
<td>10.1±2.0</td>
<td>17.5±1.6</td>
<td>33.40±2.70</td>
</tr>
<tr>
<td>Group 1</td>
<td>1.60±0.1**</td>
<td>5.5±0.1**</td>
<td>14.2±2.0**</td>
<td>21.40±2.36**</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.50±0.4**</td>
<td>9.3±1.0**</td>
<td>16.8±0.1</td>
<td>32.41±1.75**</td>
</tr>
</tbody>
</table>

Note: Changes of pup weight; The data of body weight were presented as mean ± S.D. Statistical difference was shown as: *P<0.05, **P<0.001, with respect to control; # P<0.05, ##P<0.001, with respect to group 1.
Figure 1 Mean values ± SD of the mean body weights (g) in the studied groups

(Suckling pups + gliadin) showed a statistically significantly (P<0.001) reduced increase in body weight till weaning in comparison with normal control (21.40±2.36) g vs (33.4±2.7) g. While significant increase (P<0.001) in body weight was detected in bovine colostrums treated pups on PND 7 and 21 as compared to suckling pups in group 1. But no significant difference was noticed between the two groups on PND 0 and 14. The animals showed long bones malformation on PND 0 and the stools were sticky & loose in group 1 on PND 14 (results not showed).

1.2 Changes of biochemical parameters in control group and study 1 on PND 0

Table 2 shows the changes of biochemical parameters of control groups and autistic pups. The mean change in study 1 group was significantly increased in epinephrine, norepinephrine and serotonin compared with that in control group (P<0.001). Mother administered a single intraperitoneal injection of 600 mg/kg sodium valproate on day 12.5 after conception results in significantly reduced calcium and Vit D levels in pups (study 1) as compared with normal control groups (P<0.001).

1.3 Changes of biochemical parameters in control and study 2 on PND 21 (mean±S.D.)

Considering the key roles of gliadin in the development of coeliac disease in autistic pups, it was necessary and important to investigate the changes of the chemical parameters for Coeliac disease concomitant with autism, which were displayed in Table 3. Almost all the above adverse effects induced by autism in Table 2 were suppressed by bovine colostrums treatment in group 2 as follows:

1.3.1 Mean vitamin D (Vit D) (ng/mL)

As shown in Table 3, Figure 2, significant difference of vit D level was observed between group 2 and normal control groups (p<0.05) (41.16±0.622) ng/mL vs (44.36±0.302) ng/mL, respectively. Furthermore, 21-day-old rat pups, group 1 (suckling pups + gliadin) significantly lowered the mean vit D level as compared to normal control group (p<0.001) (9.4±0.225) vs (44.36±0.302). While treatment with bovine colostrums significantly increased vit D levels (p<0.001) as compared to suckling pups in group 1. Treatment with bovine colostrum inhibited the decreased level of vit D in autistic pups with Coeliac disease.

1.3.2 Mean serotonin (μg/L)

As shown in Table 3, Figure 3, a significant increase in mean serotonin levels was detected in group 1 treated control, as compared to normal control pups (30.5±0.565) vs (9.12±0.126) (p<0.001). While a significant decrease in serotonin level was detected in group 2 pups treated with bovine colostrums as compared to group 1, (12.55±0.2976) ug/L vs (30.5±0.565) ug/L respectively (p<0.001).
Table 2 Changes of biochemical parameters in control and study 1 on PND 0 (mean ± S.D.)

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>vit D</th>
<th>Serotonin</th>
<th>Epinephrine</th>
<th>Nor epinephrine</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit</td>
<td>ng/mL</td>
<td>ug/L</td>
<td>ng/L</td>
<td>ng/L</td>
<td>mg/dL</td>
</tr>
<tr>
<td>Method</td>
<td>CMIA</td>
<td>HPLC</td>
<td>HPLC</td>
<td>HPLC</td>
<td>Calorimetric</td>
</tr>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>34.26±0.514</td>
<td>7.26±0.604</td>
<td>16.54±0.185</td>
<td>101.32±1.683</td>
<td>10.01±0.211</td>
</tr>
<tr>
<td>Study 1</td>
<td>6.21±0.13</td>
<td>33.3±0.244</td>
<td>84.25±595</td>
<td>184.29±8.44</td>
<td>6.52±0.169</td>
</tr>
</tbody>
</table>

Note: * P<0.05, ** P<0.01, compared with control group

Table 3 Changes of biochemical parameters in control and study 2 on PND 21 (mean ± S.D.)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vit D</th>
<th>Serotonin</th>
<th>Epinephrine</th>
<th>Norepinephrine</th>
<th>Transglutaminase</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit</td>
<td>ng/mL</td>
<td>ug/L</td>
<td>ng/L</td>
<td>ng/L</td>
<td>g/L</td>
<td>mg/dL</td>
</tr>
<tr>
<td>Method</td>
<td>CMIA</td>
<td>HPLC</td>
<td>HPLC</td>
<td>HPLC</td>
<td>ELISA</td>
<td>Calorimetric</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>44.36±0.302</td>
<td>9.12±0.126</td>
<td>21.73±0.290</td>
<td>110.46±0.990</td>
<td>0.9±0.066</td>
<td>10.91±0.241</td>
</tr>
<tr>
<td>Group 1</td>
<td>9.4±0.225</td>
<td>30.5±0.565</td>
<td>76.22±1.15**</td>
<td>204.87±1.83**</td>
<td>3.1±0.149**</td>
<td>7.31±0.411</td>
</tr>
<tr>
<td>Group 2</td>
<td>41.16±0.622</td>
<td>12.55±0.297</td>
<td>35.29±0.338</td>
<td>183.72±1.42**</td>
<td>1.1±0.101**</td>
<td>9.71±0.325</td>
</tr>
</tbody>
</table>

Note: * P<0.05, ** P<0.01, compared with control group; # P<0.05, ## P<0.01, compared with group 1.

1.3.3 Mean epinephrine (ng/L)

As shown also in Table 3, Figure 4 mean epinephrine was significantly increased (P<0.001) in all treated groups whether in group 1 (Suckling pups + gliadin), group 2 (bovine colostrums + gliadin) (on PND 21) as compared to controls (76.22±1.15) ng/L, (35.29±338) ng/L vs (21.73±290) ng/L, respectively. But epinephrine level was significantly (P<0.001) decreased in group 2 treated pups as compared to group 1 treated pups (35.29±0.338) vs (76.22±1.15).

Figure 4 Mean values ± SD of epinephrine (ng/L) in the studied groups

1.3.4 Mean norepinephrine (ng/L)

As shown in Table 3, Figure 5 mean norepinephrine was significantly increased (P<0.001) in all treated groups whether in group 1 (Suckling pups + gliadin) and 2 (bovine colostrums + gliadin) (on PND 21) as compared to controls (204.87±1.83) ng/L, (183.72±1.42) ng/L, vs 110.46±0.990) ng/L, respectively. Moreover, no significant change was detected in group 2 treated pups as compared to normal control pups. Treatment

Figure 5 Mean values ± SD of norepinephrine (ng/L) in the studied groups

1.3.5 Mean transglutaminase (g/L)

As shown also in Table 3, Figure 6 mean transglutaminase was significantly increased (P<0.001) in group 1 (Suckling pups + gliadin) (on PND 21) as compared to controls (3.1±0.149 g/L vs 0.9±0.066 g/L, respectively). Furthermore, mean transglutaminase level was significantly (P<0.001) decreased in group 2 treated pups as compared to group 1 treated control. Moreover, no significant change was detected in group 2 treated pups as compared to normal control pups. Treatment
with bovine colostrum inhibited the increased level of transglutaminase in autistic pups with Coeliac disease.

1.3.6 Mean calcium (mg/dl)
The calcium level was significantly decreased in group 1 as compared to normal control (7.31±0.411 mg/dL vs 10.91±0.241 mg/dL) (P<0.05), respectively. Furthermore, significantly increased in group 2 as compared to group 1 (9.71±0.325 vs 7.31±0.41). No significant difference of mean calcium level was observed between group 2 and normal control groups (P>0.05). This means that bovine colostrums treatment was more effective than mother's milk treatment in increasing calc ium level as evidenced by the detected significant changes between the two groups (Figure 7).

1.4 Gene expression in different studied groups
1.4.1 Met gene expression in study 1 on PND0
The expression of Met gene shows approximately 6.5 fold up regulated. After normalization Met gene was 5.5 up regulated compared to control as shown in Figure 8.

1.4.2 Met and DQ2 genes expression in study 2 (group 1 and group 2) on PND21
As shown in Figure 9, the expression of Met and DQ2 genes shows in Group I, 5.0 and 4.2 fold expression respectively. After normalization 4.0 and 3.2 fold expression of Met and DQ2 gene compared to control respectively. In Group II, gene expression of DQ2 and Met gene were 1.5 and 1.3 respectively. Whereas, after normalization of DQ2 and Met gene were 0.5 and 0.3 respectively.

1.5 Scanning electron microscopy of the duodenal villi on PND 21 from the following groups:
1.5.1 Control group
The scanning electron microscope demonstrated the duodenal villi with intact tips and borders. The openings of goblet cells were observed on the surface of the villi (Figure 10).
1.5.2 Group I
Studying the surface of the duodenal villi using scanning electron microscope, some villi appeared sloughing with their tips and other villi were seen to be deformed (Figure 11). The villi of the duodenum of this group showed distorted appearance with necrotic tips reflecting ulceration (Figure 12). There was accumulation of mucous on the surface of the villi (Figure 12).

1.5.3 Group II
The scanning electron microscope of this group demonstrated the duodenal villi similar to that of control group with intact tips and borders (Figure 13).

2 Discussion
As with many complex diseases, genetic and environmental factors including diet, infections and xenobiotics play a critical role in the development of autism (Schultz et al., 2006). Additionally, it has been shown that stress exacerbates autistic behaviours and that these effects may be due to abnormal regulation of the hypothalamic-pituitary axis (HPA) and stress hormones (Schultz et al., 2006). The increased level of serotonin (33.3±0.244), epinephrine (84.25±0.595) and norepinephrine (184.29±8.44) in autistic pups study 1 due to mother prenatal exposure to VPA. In a recent survey of 500 parents of autistic children, almost one-half reported that their children had loose stools or frequent diarrhea (Goodwin et al., 1971). Food intolerance was noted particularly for wheat and casein. In an early study, autistic children were coincidentally afflicted with Coeliac disease, a disorder characterized by marked atrophy of the intestinal villi caused by a response of the intestinal immune system to gliadin, a peptide in gluten (Lightdale et al., 2001). In accordance with the previous studies, our results with scanning electron microscopy of 21-day-old rat pups, group 1 (suckling pups + gliadin) showing deformed villi and the other villi show sloughing of their tips (↓), notice short villus (▲) (x 330).

In addition, distorted appearance of the villi with accumulation of mucous (Figure 12). Gluten-sensitive enteropathy (CD) is characterized by intolerance to the cereal protein gluten. The disease is found in individuals genetically predisposed to having the HLA-DQ 2 molecule (Mölberg et al., 1997). Our results revealed the expression of DQ2 in group 1 with 4.2 fold up regulated as compared to control, whereas, 1.3 fold expression in group 2 was estimated. This result revealed that bovine colostrum could minimize the expression of DQ2 in autistic rats with coeliac disease.
CD is a human disease. Among animals, a similar enteropathy was described only in Irish Setter dogs (Hall and Batt, 1992), which show partial villous atrophy after gluten intake. Many researchers tried to develop an experimental model for CD in rats and mice by oral and parenteral administration of gluten, but the resulting structural changes in intestinal mucosa were only marginal (Troncone and Ferguson, 1991). However, later studies showed that substantially increased gluten concentration in the diet caused major changes in the mucosal architecture in mice (Troncone et al., 1994). Intestinal permeability has an important role in the development of intestinal diseases. In this context, the model of young rats is highly suitable, especially because, until weaning, the rat intestine features vacuolized enterocytes and the intestinal barrier is open for the transport of immunoglobulins. During this period, positively charged peptides and lectins can be absorbed in significant amounts (Simonski et al., 1986). In humans, vacuolized enterocytes are found in the fetal period up to the 20th gestation week; they are then replaced by enterocytes functionally analogous to adult enterocytes, and the intestinal barrier is closed (Moxey and Trier, 1997). These facts notwithstanding, it has been stated that the maturation of the intestinal barrier in humans is not terminated before 2 years of age (van Elburg et al., 1992). A number of studies imply that breast-feeding in humans has a protective effect against the appearance of CD (Auricchio et al., 1983). In our study, we used this unique model to induce gluten enteropathy and found that concurrent administration of interferon-γ intraperitoneally and gliadin intragastrically in group 1, followed by a second intragastric dose of gliadin, caused Coeliac crisis on day 14—loose stool and mucosal lesions with deformation and distortion of duodenal villi by scanning electron microscope (on day 21). These disturbances were not manifested in group 2 with bovine colostrums uptake till weaning as confirmed with scanning microscopy in (Figure 13). Coeliac disease is assumed to develop only when oral tolerance to gluten has been abrogated. Increased production of interferon-γ by immunoreactive T-cell clones appears to be involved. In baby rats, interferon-γ applied after birth increases macro-molecular transport across Peyer patches before gut maturation (Sütas et al., 1997), and we assumed that this would include gluten in our experiments. Repeated application of gliadin together with interferon-γ administration in this investigaton after birth results in Coeliac crisis in suckling baby autistic rats, probably reflecting a disturbance of oral tolerance due to autism also. The authors found that deamination would be central to the disruption of gluten tolerance in coeliac-disease patients that increases the binding affinity of gliadin peptides for DQ2 (Arentz-Hansen et al., 2000). Another authors concluded the increased level of expression of TG2 in the mucosa of coeliac-disease patients seems to be related to inflammation (Haroon et al., 1999). Inflammation, as induced by an infection, might, in addition, breach the epithelial barrier and lead to further influx of gluten peptides into the lamina propria. So, it is probable that the generation of deamidated gluten peptides is linked closely with inflammation, a risk factor for coeliac disease (Haroon et al., 1999). In agreement with the aforementioned studies, our results revealed the upregulation of DQ2 may increase the binding affinity of gliadine peptide in group 1 as shown in (Figure 9). Furthermore, the increased tissue transglutaminase antibody tTG level was noticed also in group 1 suckling autistic pups due to repeated application of gliadin together with interferon-γ administration after birth that results in Coeliac crisis.

Persson et al (2002) revealed that colostrum is the first milk produced after the delivery and is particularly rich in growth factors, immunoglobulins, antimicrobial peptides, and other bioactive molecules. They added that the major peptide growth factor constituents of bovine colostrum includes EGF, IGF-I and II, TGF (transforming growth factor)-alpha, TGF-beta family, lactoferrin and others. Among these factors, IGF-I and TGF-beta family are present in high concentrations in bovine colostrum compared with bovine milk and human colostrum. Another authors found that IGF-I was used to improve impaired intestinal barrier function induced by sepsis (Playford et al., 2000) and burn injuries, (van Hooijdonk et al., 2000) and to increase the uptake and
utilization of glutamine by the bowel. In our study, gliadin has been found to induce marked atrophy of the intestinal villi with destructive changes as manifested by scanning electron microscope in group 1 while, Bovine colostrums supplements have been found to overcome with reduction of the damage score as compared to treated suckling rats. Furthermore, we found a significant decrease in serum vitamin D and calcium levels in autistic neonatal rats study 1 and group 1. Also both epinephrine and norepinephrine were significantly increased in autistic pups on PND0 and treated control on PND 21 as compared to normal control. More recently, researchers (Kalueff et al., 2006) have suggested that vitamin D, acting as a neurosteroid, offers “neuroprotection, antiepileptic effects, immunomodulation, impact on several brain neurotransmitter systems and hormones, as well as regulation of behaviors,” stressing the importance of prenatal, neonatal, and postnatal vitamin D supplementation for normal brain functioning. Another researcher added that vitamin D-binding protein (DBP), also called Group Specific Component (Gc) is a glycoprotein present in the plasma of most vertebrates and more than 120 genetic variants have been recorded. It is the main carrier for vitamin D and its hydroxylated metabolites in the plasma of vertebrates, showing the highest affinity for 25-hydroxy-cholecalciferol (Braun et al., 1990). DBP binds vitamin D metabolites in serum act as a carrier for these metabolites preventing cells from a massive uptake and its toxic effects (Ena et al., 1992). In addition, DBP has also been detected in the mature milk of several species, but in levels much lower than in serum; the level in human milk is only about 2% that of serum. However, DBP concentrations in cow’s colostrum and early milk are much higher than in definitive milk. Interestingly, the highest concentration of DBP was found in the first milking (250 mug/mL and 111 mug/mL for bovine and human colostrum, respectively) (Brandtzæg, 2003). In this study, diminished growth of longitudinal bones was found in neonatal offspring born to valporeic acid and also when treated with gliadin in group 1 suckling pups with reduced level of 25-OH vit D and calcium after weaning. In contrast, the effects of bovine colostrum till weaning on Vit-D and calcium levels appear to be more efficient than mother’s milk, since measurements of sera from these two groups indicated significant differences (P<0.001) between treated groups as compared to each other. Breastfeeding provides the immunological integration between mother and neonate. Breastfeeding has immunological advantages as it protects against infections. Moreover, there is evidence that it protects against cardiovascular disorders, obesity, Crohn’s disease, colitis ulcerosa, allergies, Diabetes mellitus type I and other (autoimmune) disorders as C (Yoshizawa et al., 1997). The risk of these disorders could increase if the duration of breastfeeding is less than 3-6 months (Kovacs et al., 1996). Several studies describe the detection of wheat gliadins and other gluten peptides in breast milk, as well as specific IgA-antibodies against gliadin (Auricchio et al., 1983). The low levels of gluten in breast milk could potentially be involved in the induction of oral tolerance for gluten in breastfed infants. The concentration of IgA antigliadin is the highest in colostrum and reduces after a month (van Hooijdonk et al., 2000). An important systematic review and meta-analysis of observational studies on breastfeeding and CD by Namgung et al (1993), concludes that breastfeeding offers protection against the development of CD. However, it is unclear if breastfeeding protects permanently against the development of CD or whether it only delays the onset of symptoms (Auricchio et al., 1983). The mechanism of protection against CD by breast milk is not well understood (Akobeng et al., 2006) suggest that breastfeeding modulates the early exposure of the neonate’s intestinal mucosa to microbes and limits bacterial translocation through the gut mucosa. In addition, by preventing inflammation in the gut, breastfeeding should also diminish the passage of gluten peptides into the lamina propria and thereby prevent the trigger to CD development (Namgung et al., 1993; Hanson et al., 2002). Another possible preventive mechanism is that human milk may decrease tissue transglutaminase tTG expression in the gut and diminish the generation of deamidated gluten peptides (Namgung et al., 1993). This finding may explain previous data (Aeschlimann et al., 1991) suggesting extracellular tTG accumulation by a hitherto unknown mechanism other than leakage from the cells in Coeliac disease. Tissue transglutaminase has been regarded primarily as an intracellular enzyme. However, extracellular matrix-bound tTG has been demonstrated after cell injury or membrane perturbation (Aeschlimann et al., 1991). This is in
accordance with the findings of (Lock et al., 1999) who demonstrated that tTG was distributed in both rodent and primate tissues identically with the extracellular reticulin-endothelial-staining pattern of sera from Coeliac patients. Ivarsson (2005) suggested that CD may be prevented by improving early feeding. Since that time more infants were still breastfed when gluten was introduced in smaller amounts and this was followed by a sharp decline of the previous incidence level of CD in children. These findings open the way to possible prevention strategies, by introducing the adequate quantity of gluten at the optimal time, during the period of breastfeeding. In our study we compare and clarify the role of early bovine colostrums or (ongoing) breastfeeding with gluten introduction in the prevention of the development of CD. Breast feeding in our study never prevented CD crisis when gluten was introduced till weaning in group 1 suggesting the passage of gluten peptide to the lamina propria that cause inflammation in the gut and intestinal mucosa due to increased level of tTG in this group as confirmed by scanning electron microscope. Another possible explanation that the introduction of gliadin with different doses concomitant with interferon-γ abrogated the incidence level of Coeliac disease that may interfere with milk composition with respect to the duration of breastfeeding in our experiment. In addition, in rodents, two growth factors play an important role in the process of regeneration and proliferation of enterocytes: epidermal growth factor (EGF) and transforming growth factor (TGF)-α. However, TGF-α has not been found in rat maternal milk (Dvorak et al., 1994). In our investigation, the scanning electron micrographs in group 1 showed deformed villi in some areas while others exhibited sloughing in their tips due to the increased level of tTG. Thus, it is likely that the increased introduction to gluten in small intestine could affect the synthesis of EGF and TGF-α that may enhance the deficit of maternal milk to protect against coeliac crisis.

The content of immunoglobulins in colostrum and milk is highly dependent on the animal species (Butler et al., 2005). Thus, for many species the proportion of IgA increases between colostrum and milk (Mix et al., 2006). For animals like rats, mice, dogs and ungulates, uptake of colostrum of adequate quality and sufficient quantity is important for the offspring to boost the systemic immune function in the short term. Immune factors from bovine colostrum supplementation are not digested and absorbed, but remain intact and active in the intestinal tract. As such, colostrum participates in gut-associating lymphoid tissue (GALT) activity, and therefore plays a role in both immune health and gastrointestinal function (Mix et al., 2006). Notably, recent studies have suggested the occurrence of an innate immune response to gluten in Coeliac patients (Meresse et al., 2004; Maiuri et al., 2005). Furthermore, the requirement for calcium activation of the tTG enzyme has been disputed. Our finding that calcium is not essential is in agreement with recent reports (Haroon et al., 1999). In our investigation, pups fed bovine colostrums were significantly improved than suckling pups when assessed by their body weight. The mean body weight measured on group 2 samples were the highest by the end of weaning (32.41±1.75) and significantly differ from those observed on the other days) among the other groups of pups. In summary, our data for the first time suggest that the uptake of bovine colostrum could minimize the severity of GI lesions in coeliac disease concomitant with autism.

3. Materials and Methods
3.1 The valproic acid rat model of autism and Coeliac disease
Female Wistar rats with controlled fertility cycle were mated overnight and the morning when spermatozoa were found was designated as the first day of gestation. Females were on a standard diet and received a single intraperitoneal injection of 600 mg/kg sodium valproate on day 12.5 after conception. Control females were kept on normal standard diet and injected with physiological saline at the same time. Sodium valproate (Sigma) was dissolved in saline at a concentration of 250 mg/mL. Administration of this dose to rats during embryogenesis has been shown to result in a maximum level of total VPA (900 μg/mL) in maternal plasma in less than 1 h, with a mean plasma elimination half-life of 2.3 h (Binkerd et al., 1988). Dams were housed individually and were allowed to raise their own litters. 1st and 2nd experimental groups were treated with interferon-γ (1 000 U per animal, administered intraperitoneally) after birth. Gliadin (0.5 and 3 mg) was intragastrically administered to the pups of the 1st and 2nd groups on days 0 and 3, and a 30 mg challenge dose was given on day 20 (24 hours before the termination of the experiment) (Štěpánková et al., 2003).
### 3.2 Animals

Male Wistar neonatal rats originating from Valproate-treated females were randomly selected from the two experimental groups (ten pups from each group) before any of the above treatment and were subjected to blood tests to investigate autism (study 1). Thirty newborn rats (of sixty five) were used as follows: the 1st group served as treated control and were assigned randomly to be mother-fed after injection of interferon-\(\gamma\) and administration of gliadin (10 pups). Ten pups for the second group were collected from their mothers immediately after birth to prevent sucking of maternal milk. No foster nursing took place because the major objective was to study pup viability. The animals were weighed and then placed in an infant incubator to control body temperature and assigned to hand-fed of bovine colostrum every 3–4 h using a silicone rubber tube. This method is time and labor demanding yet essential because gastrostomy of newborn rats is associated with a very high surgery related death rate. In addition, ten normal dams fed normal littermates served as normal controls in this study.

### 3.3 Postnatal growth and maturation development

On PND 0, pup weights were determined and examined for malformations. Measurement of pup weights was repeated on PND 7, 14, and 21 when the offspring were weaned from their mothers for all experimental groups.

### 3.4 Blood test

#### 3.4.1 Study 1 (to investigate autism)

Blood samples ten pups/each experimental group that will serve as group 1 and group 2 in study 2 were pooled for the analyses before administration of interferon - \(\gamma\) and gliadin. In addition, ten pups from control females that were kept on normal standard diet and injected with physiological saline were served as control. The samples were taken from the eye ball using capillary tubes and collected in EDTA tubes containing aprotinine at 0°C, centrifuged at 1 600 g for 15 minutes. Plasma was analyzed for the following: 1-Epinephrine and norepinephrine Plasma catecholamine concentrations were determined by High Performance Liquid Chromatography HPLC as previously described (Lang et al., 1989).

The blood samples were taken from the eye ball using capillary tubes and collected in eppendorf tubes, Serum was analysed after centrifugation for the following: 2-Serotonin in plasma was measured by High Performance Liquid Chromatography HPLC (Kluge et al., 1999). 3-Calcium & 25-OH vitamin D were measured by using Chemiluminescent Microparticles Immuno- assay CMIA (Peterlik, 2009).

#### 3.4.2 Interferon \(\gamma\)

Recombinant rat interferon-\(\gamma\) (PRP24; Serotec, Oxford, United Kingdom) was used. Interferon-\(\gamma\) was lyophilized (0.1 mg), reconstituted in 0.3 mL distilled water, and stored until use in portions of 50 \(\mu\)L (10 000 U) at \(\text{°C} -70\). A dose of 1 000 U was used for application per one newborn rat. After application of interferon-\(\gamma\) the pups were fed with mother's milk or bovine colostrum.

### 3.4.3 Gliadin administration

Gliadin (from wheat gluten, G-3375; Sigma, St. Louis, MO, USA) was diluted in 0.02 mol/l acetic acid (pH 2.9) and administered intragastrically by means of a silicon tube. Young rats were repeatedly given gliadin in the following doses: day 0, 0.5 mg in one intragastric dose; day 3, 3 mg in one intragastric dose. The pups in each group were killed at 21 days of age. They received a provocative dose of 30 mg gliadin per animal 24 hours before the killing (on PND 20).

#### 3.4.4 Blood test

Study 2 (to investigate Coeliac disease in autistic pups). After application of interferon-\(\gamma\) in group 1 (Suckling pups + gliadin) and group 2 (Bovine colostrum + gliadin). Serum tissue transglutaminase tTG antibody titres were measured quantitatively by an enzyme-linked immunosorbent assay (QuantaLite tTG, Inova Diagnostics, Ca, USA).

All the previous chemical analysis for study 1 and control group on PND 0 were repeated also on PND 21 in addition to serum tTG assay.

### 3.5 Gene expression from blood

About 250 \(\mu\)L of blood was collected from different groups (study 1 on PND0 for \(\text{Met}\) gene expression, study 2 on PND21 for \(\text{Dq2}\) and \(\text{Met}\) gene expression as compared to control for each study) in PAXGene Blood RNA tubes (Qiagen Inc., Valencia, CA, USA) for downstream RNA isolation, Further RNA was quantified using NanoDrop2000 and Real Time-PCR analysis (Enitan et al., 2007) was carried out for \(\text{DQ2}\) and \(\text{Met}\) gene expression using GapDH as a housekeeping gene.
3.6 Scanning electron microscopy
Duodenal I samples from each experimental group on PND21 were taken for scanning electron microscopy and were fixed in 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.2. Dried specimens mounted on aluminium specimen holders with double-sided adhesive tape were coated with gold in a Polaron sputter-coater.

3.7 Statistical analysis
SPSS13.0 was used for the statistical analysis. The data were expressed as mean ± SD, and were analyzed by one-way ANOVA, followed with post hoc test for the multiple comparisons. Differences were considered significantly at \( P < 0.05 \) level.

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