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# Multidirectional and simultaneous evaluation of gastroschisis-related intestinal damage in chick embryos ☆

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ABSTRACT

*Purpose:* In a chick model of gastroschisis, we aimed to investigate the morphological/cellular, molecular, and ultrastructural changes taking place in gastroschisis-related intestinal damage (GRID).
*Methods:* 13-Day fertilized eggs were divided into two groups. Control group: chorio-amnio-allontoic membranes opened and abdominal wall exposed. Gastroschisis group: an anterior abdominal wall defect created after opening membranes. Embryos from both groups were surgically removed on post-fertilization day 19. Intestinal samples were obtained for histopathology, immunohistochemistry, molecular biology, and electron microscopy.
*Results:* The histopathological grade of intestinal damage which primarily involved mucosal structures was significantly higher in the gastroschisis group when compared to the control group ($p < 0.001$). Immunohistochemically, E-cadherin and synaptophysin immunoreactivity in the gastroschisis group was significantly lower than control group ($p < 0.05$ and $p < 0.01$, respectively), whereas there was no significant difference in laminin and type-4 collagen immunoreactivity between the groups ($p > 0.05$). Molecular analyses indicated a significant decrease in NFκB and IκB expression in the gastroschisis group ($p < 0.05$ and $p = 0.001$, respectively). Electron microscopy showed that the gastroschisis group had considerable ultrastructural damage, manifested by apoptosis in all layers.
*Conclusions:* GRID affected all layers but was more prominent in mucosa. The damage may depend on E-cadherin and synaptophysin downregulation. Increased apoptotic activity, associated with decreased NFκB and IκB expression, may be an important component of this multifactorial damaging process.


Gastrointestinal dysfunction is a common problem in newborns with gastroschisis. This condition can cause severe clinical complications and mortality, because it is difficult to ensure optimum enteral nutrition in these infants [1–3]. Although many studies of gastroschisis have been published, the reasons for and the molecular/ultrastructural characteristics of the intestinal damage that leads to gastrointestinal dysfunction are still unclear. The etiopathogenetic factors proposed for gastroschisis-related intestinal damage (GRID) include continuous/long-term contact with amniotic fluid and/or vascular injury. These factors may lead directly or indirectly to inflammation, which is attributed to amniotic waste products or to ischemia owing to chronic mechanical constriction [4–7].

Many experimental studies of the aforementioned etiopathogenetic factors have been conducted to determine the structural and functional changes occurring in the bowel [8–12]. From the histopathologic point of view, light microscopy is a basic means of determining intestinal damage through visual evaluation, morphometric analyses, and damage scoring/grading. In addition, the effects of certain tissue-building proteins, such as E-cadherin, type 4 collagen, laminin, and synaptophysin have been investigated to clarify the mechanisms of the intestinal damage that affects cells and/or extracellular structures, such as intercellular junctions and basal membrane [13–15]. Other immunohistochemical studies, based on the TUNEL assay and the Ki67 marker, have also been conducted to determine the role of apoptotic/proliferative processes [16]. Molecular studies have shown that fibronectin, ICAM-I, nuclear factor kappa B (NFκB), and inhibitor of kappa B (IκB) are transcription factors specific to pleiotropic, reactive oxygen radical activation genes that are involved in the cell cycle, multiplication, and differentiation by independent and complementary means [13,17–19]. However, very few human and animal studies have evaluated the molecular basis of GRID. Similarly, there are few reports of the ultrastructural changes seen in fetal intestines [20,21].

In this study, we designed a single experiment to evaluate several endpoint effects of gastroschisis on the intestines in a chick model. Accordingly, the methods that we used simultaneously included

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histopathological, immunohistochemical, molecular biologic, and electron microscopic analyses.

## 1. Materials and methods

This study was started after being approved by the animal care committee of our institution. Chicken eggs (50–70 g) with known fertilization dates were kept under standard incubation conditions (CIMUKA Egg Incubator; 37.5 °C, 80% humidity, and mechanical tilting at 2 hourly intervals). Surgical procedures were performed by adapting the method defined by Aktug et al. [22], and all surgical procedures were conducted under sterile conditions with a surgical loupe (3.5×). On post-fertilization day 13, the eggs were randomly divided into two groups.

### 1.1. Group 1 (control group)

After opening the egg shell at the blunt end (air-sac side), the chorionic, allantoic, and amniotic membranes were carefully opened to ensure a common allantoic–amniotic cavity and to expose the anterior abdominal wall. Then, the shell defect was closed with a sterile drape, leaving the abdominal wall intact.

### 1.2. Group 2 (gastroschisis group)

The amniotic cavity and chick embryo were accessed as described for the control group. At the next stage, the umbilical stalk was gently retracted and an abdominal wall defect was created by making a small incision at the paraumbilical region. Gentle abdominal pressure was applied if the intestinal segments did not eviscerate spontaneously. Afterwards, the shell defect was closed.

The eggs were placed back in the incubator following the surgical procedures. On post-fertilization day 19, the surviving chicks were first recovered and then killed by decapitation. Intestinal tissue samples (approximately 1.5–2 cm long) were obtained. The samples were divided into three equal parts to be used in histopathological, immunohistochemical, molecular, and electron microscopic examinations and then sent to the laboratories. When our team members evaluated the specimens, they were blinded with respect to which group the specimens came from. The experiment continued until ten live chick embryos had been obtained from each group on post-fertilization day 19. A total of 43 embryos underwent surgical intervention, including 21 controls and 22 embryos with gastroschisis.

### 1.3. Histopathological evaluation

Tissue samples were embedded in paraffin blocks after dehydration in a series of alcohol dilutions. Sections 5 μm in thickness were obtained and stained with hematoxylin–eosin. Park's classification was used to score the intestinal damage observed by light microscopy [23]. In Park's classification, tissue integrity is graded on a scale of 0–8, ranging from normal mucosa to transmural necrosis (Table 1). Additional histopathological changes, such as inflammatory cell infiltration, lymphatic abnormalities, and tissue edema, were also investigated.

### 1.4. Immunohistochemical evaluation

Sections 4 μm in thickness obtained from tissue samples were placed on slides with polylysine and kept in a 50 °C incubator for 2 hours to melt the paraffin. For deparaffinization and clarification process, the sections were first placed into an incubator to melt their paraffin content, then washed with tap water followed by distilled water. Citrate buffer was then added, and the sections were boiled in the microwave with antigen-retrieval solution (pH 6.5) for 40 min and blocked with $H_2O_2$. Later, the tissues were incubated with E-cadherin (1:25 dilution, 36B5; Neomarkers), laminin (1:50, RB-082-A; Neomarkers), type-4 collagen (1:50, PHM-12 + CIV 22; Neomarkers), and synaptophysin (1:100, SP11; Neomarkers) antibodies at room temperature (25 °C). After washing with distilled water and phosphate-buffered solution, slides were incubated with a secondary antibody for 10 min and then incubated with streptavidin–avidin for 10 min. Hematoxylin–eosin was applied for reverse staining.

E-cadherin, laminin, and type-4 collagen immunostaining was performed to evaluate intercellular connections and basal membrane. The presence of ganglion cells and their structural appearance were evaluated by synaptophysin immunostaining. Slides of each embryo were examined separately, and the results were reported as either “positive” or “negative,” according to the immunohistochemical staining results. Weak and/or scarce immunoreactivity was reported as “negative.”

**Table 1**
Park's classification for intestinal damage [23].

| Grade | Description |
|---|---|
| 0 | Normal mucosa |
| 1 | Subepithelial space at villus tip |
| 2 | Extended subepithelial space |
| 3 | Epithelial lifting along the villus side |
| 4 | Denuded villi |
| 5 | Loss of villous structure |
| 6 | Crypt layer injury |
| 7 | Transmucosal infarction |
| 8 | Transmural infarction |

### 1.5. Molecular biologic analyses

Sample lysates obtained from surgically dissected intestinal tissues were treated as follows for Western blotting and quantitative real-time PCR analysis. GAPDHs were used as an internal control for both analyses.

### 1.6. Western blotting

All specimens from both groups were separately homogenized in Triton X-100 buffer with 500 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 1.2% Triton X-100. Lysates were clarified by centrifugation (12,000 × *g* for 1 min), and equal amounts of proteins were subjected to SDS-PAGE on 10% polyacrylamide gels. Proteins were then electrophoretically transferred onto a nitrocellulose sheet overnight at 40 V and analyzed by immunoblotting with the corresponding antibody. Antibodies against NFκB (SC-372, Santa Cruz Biotechnology) and IκB (SC-371, Santa Cruz Biotechnology) were utilized to detect protein expression in tissue from both groups. As an internal control, GAPDH expression was detected by GAPDH antibody (SC-25778, Santa Cruz Biotechnology).

### 1.7. Quantitative real-time PCR

Real-time PCR analyses were performed for both groups to detect mRNA expression of NFκB-p65, IκB, and the internal control GAPDH. RNA was isolated with the Magna Pure LC mRNA Isolation Kit (cat# 3172627, Roche Applied Science), and LightCycler-RNA Amplification Kit Hybridization Probes (cat# 2015145, Roche Applied Science) were used. Gene-specific primers and probes were designed with LightCycler Probe Design Software. One-step RT-PCR protocol was performed in accordance with the manufacturer's instructions. For each reaction, standard curve was created with LightCycler Control Kit RNA (Roche Applied Science) and relative quantification was represented as percentage.

### 1.8. Electron microscopic analysis

Intestinal segments from the chick embryos were first prefixed in 2.2% glutaraldehyde solution, post-fixed in 1% osmium tetroxide