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THE EFFECT OF CONTINUOUS RELEASE OF METHYLENE BLUE FROM A DRUG DELIVERY SYSTEM ON THE INTESTINES: AN EXPERIMENTAL STUDY IN CHICK EMBRYO GASTROSCHISIS MODEL✣

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SUMMARY

Prolonged exposure to amniotic fluid causes the intestinal changes such as serosal edema, thickening,fibrous coating,and adhesions in gastroschisis. The effect of continuous delivery of methylene blue (CDMB) from a drug delivery system and daily injection of methylen blue (DIMB) on the intestines was evaluated using a chick embryo gastroschisis model.Fifty fertile eggs were divided into5 groups: preliminary study (PSn=10), amnio-allontoic fluid (AAF) control (AAC, n=10), just gastroschisis (JG, n=10), gastroschisis pretreated with daily injection of methylene blue (GP-DIMB, n=10) and gastroschisis pretreated with continuous delivery of methylene blue (GP-CDMB, n=10). The PS group was also divided into 2 subgroups to determine the biochemical differences between the amniotic and allontoic fluid. Gastroschisis was created surgically at the 14th day of incubation. İn GPMB group daily methylene blue injection (5 mg/g) was administered into AAF for 5 days. In GP-CDMB, 25 µg/g methylene blue loaded polymer (Hydroxy-Propyl Methyl Cellulosa-Ethyl Cellulosa "HPMC-EC") was placed into AAF.

A significant decrease in mortality and intestinal damage and higher ganglion number were observed both macroscopically and microscopically in the group GP-CDMB compared to JG (p<0.001). Because of multiple intervention of embryos, higher mortality rate were observed in the group GP-DIMB (75.61%). Pretreatment with continuous delivery of methylene blue from a drug delivery system, prevented intestinal damage, and the complication of multiple interventions of fetuses because of gastroschisis.

Key Words: *Fetal Experiment, Gastroschisis, Methylene Blue, Polymers*

ÖZET

İlaç Taşıyıcı Sistemden Devamlı Salınan Metilen Mavisinin Barsaklara Etkisi: Civciv Embriyosu Gastroşizis Modelinde Deneysel Araştırma

Gastroşizis'de, uzun süreli amniyotik sıvıyla temas, barsaklarda serozal ödem, kalınlaşma, fibröz doku ve yapışıklık gibi değişikliklere sebep olur. İlaç taşıyıcı sistemlerden devamlı salınan metilen mavisi ve günlük uygulanan Metilen Mavisi (MM)'nin etkileri, civciv embryosu gastroşizis modelinde değerlendirildi.Elli adet döllenmiş yumurta 5 gruba ayrıldı: Ön çalışma (PS, n=10), Amniyo-allantoik sıvı kontrol (AAC, n=10), yalın gastroşizis (JG, n=10), günlük metilen mavisi injekte edilerek tedavi edilmiş gastroşizis,(GP-DIMB, n=10) ve ilaç taşıyıcı sistemlerden devamlı salınan metilen mavisiyle tedavi edilen gastroşizis (GP-CDMB).Ön çalışma grubunda amniyotik ve allantoik sıvı biyoşimik farklılıkları iki alt grupta araştırıldı. İnkübasyonun 14. gününde cerrahi gastroşizis yapıldı. GP-DIMB grubunda, AAF içine günlük MM enjeksiyonu (5 µg/g) 5 gün boyunca uygulandı. GP-CDMB grubunda, 25mg/g MM yüklenmiş polimer (Hydroxy-propyl methyl cellulose-Ethyl cellulose) AAF içine yerleştirildi.Mortalite ve barsak hasarlanmasının, polimer ile tedavi edilen GP-CDMB grubunda yalın gastroşizis grubuna kıyasla önemli derecede azaldığı (p<001), ayrıca Auerebach gangliyon hücre sayısının da daha fazla olduğu saptandı.Embryona çoğul girişim nedeniyle GP-DIMB grubunda mortalite daha yüksek oranlarda idi.Polimerlere yüklenen ilaçların, gastroşiziste çoğul grişim komplikasyonlarını ve fetüsda mortalite oranlarını düşürerek postoperatif erken cerrahi girişimleri kolaylaştıran yaralı bir tedavi yöntemi olduğu düşünülmektedir.

Anahtar Kelimeler: *Fetal Cerrahi, Gastroşizis, Metilen Mavisi, Polimer*

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Prolonged exposure to amniotic fluid causes the intestinal damage in gastroschisis, especially in the fetal life. Fetal evisceration of the intestines frequently results in severe postnatal intestinal dysfunction that affects both absorption and motility . Postoperative complications during the management of the patients with gastroschisis are most probably caused by intrauterine intestinal damage in addition to associated anomalies such as intestinal atresia and narcotizing enterocolitis. Intrauterine urinary and gastrointestinal waste products were blamed for intestinal damage in gastroschisis (1-3). Nitric oxide is a powerful, multifunctional neurotransmitter that is vital to normal bowel function and is produced by the enzyme Nitric oxide syntase (NOS). Abnormalities of gastrointestinal NOS have been increasingly implicated as a cause of malabsorption and dysmotility in gastroschisis and several other gastrointestinal diseases(4) . Methylene blue (methylthionine chloride; MB) is a thiazine dye used in the treatment of methemoglobinemia (5,6). MB is known to be an inhibitor of soluble guanylate cyclase and have shown that it is a direct inhibitor of NOS (4-7). The study was performed to determine whether using methylene blue from a drug delivery system could prevent the ID in gastroschisis.

Materials and Methods

One hundert two fertile chick eggs (Gallus domesticus), weighing 54-70 g, were incubated at 37.5 °C in 80% humidity. The eggs were divided into five groups of 10 eggs each. The eggshell windows were opened by electric hand separator which is used from Dentist.

1. Preliminary study group (PS, n=10): Intestines, amniotic and allantoic fluid obtained from chicken embryos on the 19^{th} day of incubation, without any intervention.
2. AAF Control group (AAC, n=10): Through an eggshell windows the allantoic and amniotic membranes were opened without creating gastroschisis on the 14^{th} day of incubation that resembles the amniotic cavity in humans. Intestines and AAF were harvested on the 19^{th} day of incubation.
3. Just gastroschisis group (JG, n=10): Gastroschisis was created by surgical intervention on the 14^{th} day of incubation. AAF and intestines were harvested on the 19^{th} day of incubation without any treatment.
4. Gastroschisis pretreated with daily injection of methylene blue group (GP-DIMB, n=10) Gastroschisis was created on the 14^{th} day of incubation and methylene blue (5 mg / g) which dissolved in 0.75% NaCl solution (Physiologic serum saline for birds) was instilled (0.1 mL / 50 g) into the AAF on the 15^{th}, 16^{th}, 17^{th} and 18^{th} days of incubation. AAF and intestines were harvested on the 19^{th} day of incubation.
5. Gastroschisis pretreted with Continuous delivery of methylene blue group

(GP-CDMB,n=10): Gastroschisis was created on the 14^{th} day of incubation and methylene blue (25 mg / g) loaded polymer (HPMC -EC) was placed into the AAF. AAF and intestines were harvested on the 19^{th} day of incubation.

By wide distruption of amnio-allantoic membrane, an amnio-allantoic cavity was created surgically to resemble the human amniotic cavity in all groups except PS group. Surgical intervention for creation of gastroschisis was performed by previously described method on the 14^{th} day of incubation(6,7). In a short describtion ,after disruption of amnio - allantoic membranes, a defect was created at the base of the umblical stalk, and intestines were gently pushed out of the abdominal cavity.

The solution of methylene blue used for pretreatment were daily instilled into AAF using a sterile 26 –gauge needle and an injector.

After surgical intervention, the eggshells were sealed with sterile plastic dressing and incubated. By daily inspection the survival of the embryos was checked and 52 dead embryos were discharged. Ten chickens from each group were evaluated during the study. The eggshells were opened on the19^{th} day of incubation and embrionic fluid samples were harvested to

investigate the glucose, pH, urea, creatinine, uric acid, electrolytes (Na, K, Cl), total bilirubine, indirect bilirubine, direct bilirubine and amylase. After macroscopic evaluation, the intestines were harvested and kept in 10% formalin solution.

A microscopic ruler (ocular micrometer) was attached to the objective, the same pathologist measured intestinal wall thicknessand ganglion cells in a blind manner (M.Trichrom and H&E).

The intestines were graded between 0 to 4 microscopically according the degree of the damage such as normal intestine (=0, no changes), minimal changes (=1, serosal edema), mild (=2, fibrous coating), moderate (=3, fibrous coating and intestinal thickening), severe (=4, fibrous coating and intestinal thickening and adhesions).

The results were presented as the means $\pm$ SEM. Statistical analysis was performed by analysis of variance followed by Kruskal–Wallis 1-way Anova (8). Differences were considered significant when p values were less than 0.05.

Results

The survival rates were decreased in the chick embryos with creation of gastroschisis compared with PS and AAC groups. The survival rates were greater in the chick embryos with GP-CDMB (76.92%) group compared with the GP-DIMB(24.39%) and JG (41%) group ($p<0.001$). Because of multiple intervention of embryos in the GP-DIMB groups mortality was highest (75.61%), (Table.1).

Table 1: The Experimental Groups Mortality and Survival Rate of the Chick Embryos

Group	Mortality	Alive (%)	Total
PS	0	10 (100)	10
CG	4	10 (71.42)	14
JG	14	10 (41.66)	24
GP-DIMB	31	10 (24.39)	41
GP-CDMB	3	10 (76.92)	13
Total	52	50 (49.01)	102

Abbreviations:PS; Preliminary study group, CG; Control group, JG; Just gastroschisis group, GP-DIMB: Gastroschisis pretreated with methylene blue group,GP-CDMB: Continuous delivery of methylene blue group

The biochemical parameters, histopathologic changes of the intestines, intestinal wall thickness and Auerbach ganglions of the chick embryos are summarized in Table. 2

Table 2: The Biochemical Parameters, Macroscopic and Histopathological Changes of the intestines, Intestinal Wall Thickness and Number of Auerbach Ganglion Cells of the Chick Embryos

Group	Amn - PS	All - PS	CG	JG	GP-DIMB	GP-CDMB
Glucose (mg/dL)	8.6 ± 0.2	44.8 ± 1.0	6.6 ± 0.3	12.1 ± 0.6	8.6 ± 0.3	6.2 ± 0.1
Ürea (mg/dL)	7.6 ± 0.1	93.5 ± 2.1	81.4 ± 1.7	34.8 ± 0.9	47.60 ± 0.9	46.90 ± 0.8
Creatinine (mg/dL)	0.4 ± 0.1	3.6 ± 0.4	2.43 ±0.4	0.8 ± 0.1	0.77 ± 0.1	0.91 ± 0.1
Ü.acid (mg/dL)	31 ± 0.5	298.2 ± 9.1	204 ± 8.7	80.9± 4.3	83.60 ± 3.3	76.78 ± 5.7
T.bilirubine(mg/dL)	0.6 ± 0.1	2.4 ± 0.4	1.71 ± 0.6	0.4 ± 0.1	0.53 ± 0.1	0.53 ± 0.3
D.bilirubine(mg/dL)	0.4 ± 0.1	0.3 ± 0.1	0.17 ± 0.1	0.1 ± 0.1	0.13 ± 0.1	0.03 ± 0.0
İnd. Bil (mg/dL)	0.2 ± 0.1	2.08 ± 0.4	1.53 ± 0.2	0.3 ± 0.1	0.40 ± 0.1	0.5 ± 0.1
Amylase (mg/dL)	61.4 ± 3.4	31 ± 0.5	108.8 ± 7.5	248.1 ± 10.3	177.4 ± 8.3	282.6 ± 9.3
Na (mEq/L)	122 ± 6.5	45.16 ± 2.1	83.1 ± 3.1	87.04 ± 3.3	90.6 ± 3.3	88.6 ± 3.9
K (mEq/L)	21.1 ± 0.9	20.6 ± 0.7	20.9 ± 0.8	29.39 ± 0.9	33.11 ± 0.8	33.85 ± 0.3
Cl (mEq/L)	18.08 ± 0.6	33.9 ± 1.2	57.8 ± 1.1	72.05 ± 1.1	73.90 ± 0.7	69.9 ± 0.7
PH	7.6 ± 0.4	6.3 ± 0.5	6.67 ± 0.4	7.26 ± 0.4	7.35 ± 0.1	7.22 ± 0.3
Int. Macroscopic-Grade	0 ± 0.0	0 ± 0.0	0 ± 0.0	2.50 ± 0.1	1.00 ± 0.1	1.00 ± 0.1
Int. Microscopic-Grade	0 ± 0.0	0 ± 0.0	0 ± 0.0	3.0 ± 0.1	2.0 ± 0.1	1.0 ± 0.1
Intestinal Thickness (hm)	288.61 ± 17.5	280.24 ± 18.6	305.34 ± 24.4	818.59 ± 121.4	401.30 ± 66.5	402.03 ± 18.7
Auerbach Ganglion (number)	19.0 ± 3.7	18.4 ± 3.6	17.3 ± 4.4	11.5 ± 2.8	12.2 ± 5.9	14.6 ± 3.3

Abbreviations: PS; Preliminary study group (Amn – amnion control, All – allantois control), CG; Control group, JG; Just gastroschisis group, GP-DIMB: Gastroschisis pretreated with methylene blue group,GP-CDMB: Continuous delivery of methylene blue group

Urea, creatinine, uric acid, total bilirubine, indirect bilirubine and glucose concentrations were higher in the allantoic fluid of PS group whereas lower in the GP-DIMB and GP-CDMB groups compared with the AAC group ($p<0.05$).

There were significant signs of intestinal damage in the JG group while intestinal damage was partially prevented in the GP-DIMB and GP-CDMB groups The intestines were thickened and covered by fibrous peel in the JG group. In the GP-CDMB and GP DIMB groups the intestinal thickness was nearly normal and there was no fibrine deposition on serosal surface (Figs1-4). There was no adhesion in the GP- CDMB and GP-DIMB groups compared with the JG group ($p<0.001$), (Fig.3). There were no significant signs of intestinal damage between GPMB and GP-CDMB groups ($p>0.05$). The total number of the ganglion cells was higher in the GP-CDMB group than the JG group ($p<0.05$).

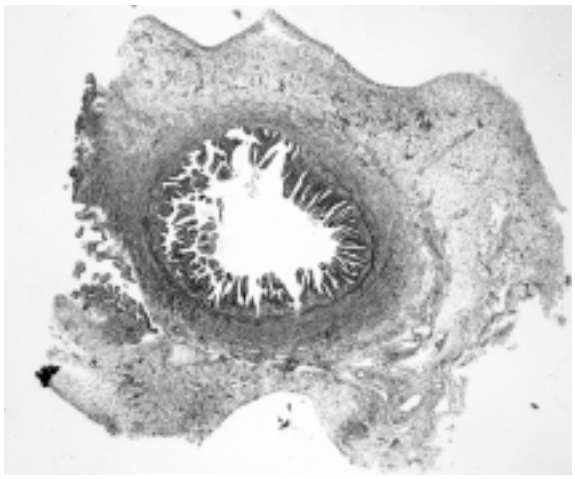

Figure 1. Intestinal wall thickening in the just gastroschisis group(No treatment) (Masson's Trichrome x 28)

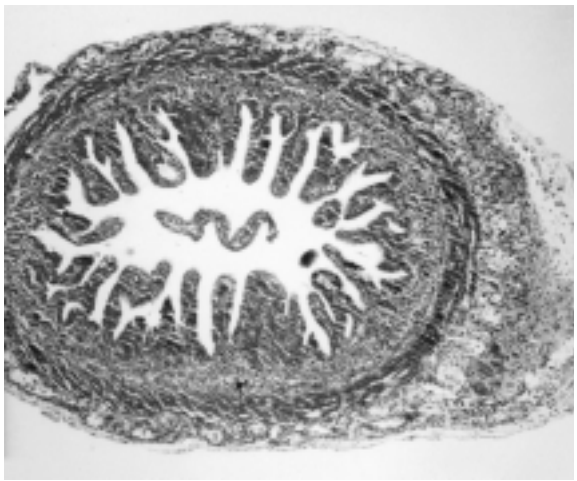

Figure 2. Nearly normal appearance of intestinal wall which was treated with polymer (Continuous Delivery of Methylene Blue Group) (H&E x 115)

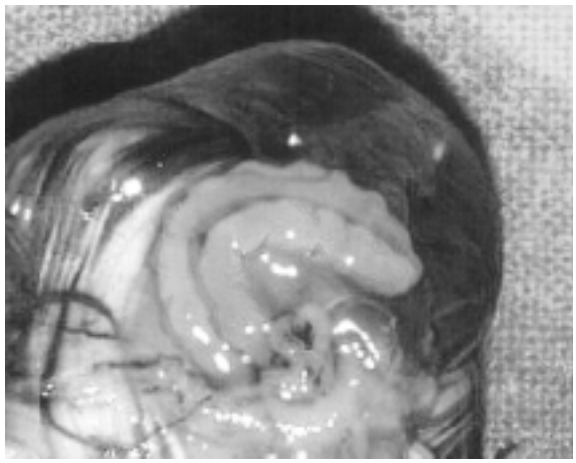

Figure 3. Macroscopic appearance of intestinal wall thickening, edema and adhesion was seen in the just gastroschisis group(No treatment)

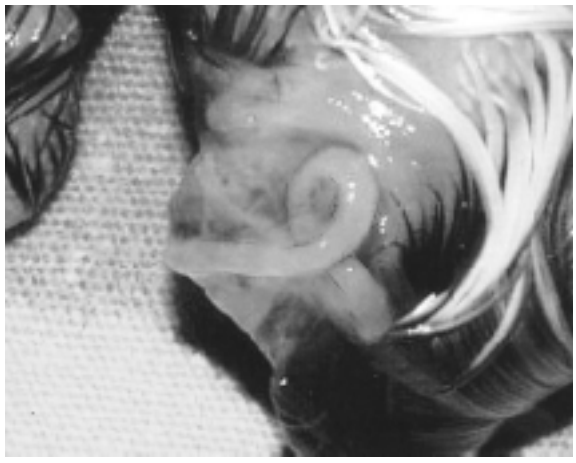

Figure 4. Macroscopic appearance of nearly normal intestinal wall which was treated with polymer (Continuous Delivery of Methylene Blue Group)

Discussion

Evaluation of embryonic fluids from chicken embryo gastroschisis model shows remarkable similarities with human amniotic fluid on composition. Chicken embryo discharged urinary and intestinal waste products to allantoic cavity, however, human fetus discharged to the amniotic cavity. Common amnio-allantoic cavity of chicken embryo resembles human amniotic cavity (9). The etiology of intestinal damage in gastroschisis is still uncertain and attributed to either direct exposure to the amniotic fluid or ischemia and lymphatic congestion caused by abdominal wall compression of the eviscerated mesentery (9 -11).

Kanmaz et al found a correlation between intestinal damage and pH of amnioallantoic fluid (AAF) in a chick gastroschisis model and they concluded that acidity of AAF is responsible for this damage (1).

Dilsiz et al showed that nitric oxide is an important mediator of the ID in gastroschisis and they concluded that the inhibition of NOS with L-NAME could prevent ID in gastroschisis (2). Tibboel et al have suggested that intestinal damage was the result of venous and lymphatic obstruction at the site of narrow abdominal defect. They showed that the normal human fetus defecates routinely during fetal life and the intestinal damage in gastroschisis is a result of prolonged contact with gastrointestinal waste products in amnio-allantoic fluid (10,12). Klück et al found a correlation between ID and exposure to amnioallantoic fluid (AAF) and concluded that urinary products are responsible for this damage (3). There was no intestinal damage when the protruded bowels were not related with allantoic fluid in their study.According to Langer et al intestinal dysfunction in gastroschisis must have been caused by both amniotic fluid exposure and chronic vascular compromise. They have suggested that amniotic fluid was responsible for intestinal adhesions, vascular compromise for intestinal damage, and both together for malabsorption and dysmotility (9,10).

In the present study drug delivery system was investigated first time to treat the ID in the chick gastroschisis model. The aim was to determine whether the ID in gastroschisis could be prevented by controlled release of methylene blue from a drug delivery system (HPMC – EC). Methylene blue (methylthionine chloride; MB) is a thiazine dye used in the treatment of methemoglobinemia (6,7). It may represent a new class of anti-oxidant drugs, which competitively inhibits the reduction of molecular oxygen to superoxide by acting as an alternative electron acceptor for tissue oxidases . It is a widely accepted pharmacological tool in the analysis of the nitric oxide – pathway.

MB is known to be an inhibitor of soluble guanylate cyclase and have shown that it is a direct inhibitor of NOS and other iron containing enzymes (5-7). Increased NOS-2 gene expression has been shown in the intestinal mucosa of patients with inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and necrotizing enterocolitis (4). In the current study, because of NOS inhibition of MB, intestinal mucosa, submucosa, muscular, serosal thickening and ID were significantly decreased in GP-DI MB and GP-CDMB groups. These findings show us that NO is an important mediator of the ID in gastroschisis. However, the mortality rate was higher in the GP-DIMB group because of multiple interventions to the embryo. The survival rates were greater in the GP- CDMB (76.92%) group than the GP-DIMB (24.39%) group. The adverse effects of AF on the intestines may be preventable by altering the compositions of AF by either exchange or injecting appropriate solution into the amniotic cavity. This could be achieved by use of an amniotic cavity catheter or repeated amniocentesis, however, in these groups the mortality rate were high (1,2,11,13,14). Controlled drug release system provides localized delivery, lowers the risk of systemic side effects and prevents multiple intervention.

As a first preliminary study, we believe that drug delivery system could be a new simple technique and alternative for human fetuses with gastroschisis. We hope the use of drug delivery system of an appropriate drug for treatment, will prevent ID of gastroschisis totally in the future by eliminating the effect of amniotic cavity catheter and multiple amniocentesis.

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