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Reversible small bowel obstruction in the chicken foetus

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ABSTRACT

Background: Ligation of the embryonic gut is an established technique to induce intestinal obstruction and subsequently intestinal atresia in chicken embryos. In this study, we modified this established chicken model of prenatal intestinal obstruction to describe (1) the kinetics of morphological changes, (2) to test if removal of the ligature in ovo is possible in later embryonic development and (3) to describe morphological adaptations following removal of the ligature. **Materials and Methods:** On embryonic day (ED) 11, small intestines of chick embryos were ligated micro surgically in ovo. In Group 1 (n = 80) gut was harvested proximal and distal to the ligation on ED 12-19. In Group 2 (n = 20) the induced obstruction was released on day 15 and gut was harvested on ED 16-19. Acetyl choline esterase staining was used as to assess resulting morphological changes. **Results:** A marked intestinal dilatation of the proximal segment can be seen 4 days after the operation (ED 15). The dilatation increased in severity until ED 19 and intestinal atresia could be observed after ED 16. In the dilated proximal segments, signs of disturbed enteric nervous system morphology were obvious. In contrast to this, release of the obstruction on ED 15 in Group 2 resulted in almost normal gut morphology at ED 19. **Conclusion:** Our model not only allows the description of morphological changes caused by an induced obstruction on ED 11 but also-more important - of morphological signs of adaptation following the release of the obstruction on ED 15.

Key words: Chicken model, enteric nervous system, foetal intervention, intestinal atresia, small bowel obstruction

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INTRODUCTION

The insight into the pathogenesis of intestinal atresia and its pathophysiology is still limited. Thus, a number of experimental models have been developed in order to expand knowledge in this field.[1] Among those is the chicken embryo.[2] While most investigaters aim to induce intestinal atresia by coagulation of the mesenteric blood vessels [Table 1][1-15] some investigators studied the effect of pure dilatation on gut development by gut ligation. [Table 2][16-20] In the present study, we studied the effects caused by the release of this ligature after a second foetal intervention on ED 15.

MATERIALS AND METHODS

The chicken embryo is surrounded by extra embryonic membranes and embedded in amniotic fluid. There are four embryonic membranes: The amnion, chorion, yolk sac and allantois. From ED 10 to ED 18, mid-gut loops protrude into the amniotic cavity [Figure 1]. The presence of a physiologic umbilical hernia at this stage allows a simplified surgical intervention in the embryonic/foetal period. According to the findings of Tibboel *et al*. the digestive tract conditions of a white leghorn chicken is comparable to humans in the second trimester of gestation.[2]

Fertilised eggs were received (White Leghorn, *Gallus domesticus*, Co Lohmann, Cuxhaven, Germany) and stored at 4°C until usage. Incubation started at a temperature of 37.5°C with a relative air humidity of 80% in a Bios-Midi incubator (Heraeus, Hanau, Germany) using a standard hatching technology.

Operating procedures and study groups

Prior to surgery, the fertilisation status of the eggs was verified using diaphanoscopy. The air chamber was punctured. This lowered the position of the embryo and reduced the risks of injury to the embryo and its vital structures in the further process. A2 cm × 2 cm

Table 1: Experimentally induced intestinal atresia using the coagulation technique in different animal models since 1955

Ischaemia-coagulation or ligation of the mesenteric vessels

Author	Year	Animal model	Day of surgery (average gestation)	Day of evaluation	Animals included	Assay type
Louw and Barnard[1]	1955	Dog	NA (63)	3 and 12 postdelivery	2/NA	Macroscopic evaluation of intestinal atresia model
Louw[3]	1966	Dog	45-55 (63)	1-14 postdelivery	38/51	Macroscopic evaluation of intestinal atresia model
Koga *et al*.[4]	1975	Dog	45-55 (63)	1-5, 7, 11 postoperation	13/13	Body weight, length of intestine. LM: H and E, elastic van Gieson, Masson's trichrome, Pap's silver. Gut wall morphology
Abrams[5]	1968	Lamb	80-100 (150)	78-107 prebirth	5/16	Macroscopic evaluation of intestinal atresia model
Touloukian[6]	1978	Lamb	90-100 (150)	5 prebirth	5/5	LM, SEM, TEM: Gut wall and epithelial morphology
Tepas *et al*.[7]	1979	Lamb	85 (150)	At delivery	8/15	LM: AChE, ATPase; neurotransmitter activity and contractile muscular functions
Blanc and Silver[8]	1962	Rabbit	20-23 (32)	4-7 postoperation	17/69	Macroscopic evaluation of intestinal atresia model
Tsujimoto *et al*.[9]	1972	Rabbit	18-27 (32)	1-9 postoperation	69/136	Macroscopic evaluation of intestinal atresia model; LM: H and E
Serrano *et al*.[10]	1991	Rat	16-20 (22)	1 prebirth	18/55	LM, TEM: Epithelial morphology; enzyme assay of brush border enzymes: Maltase, lactase
Earlam[11]	1972	Chicken	9-17 (21)	1-7 postoperation	3/38	Macroscopic evaluation of intestinal atresia model
Tibboel *et al*.[2]*	1979	Chicken	16 (21)	At hatching	200/497	Macroscopic evaluation of intestinal atresia model
Tovar *et al*.[12]	1991	Chicken	12 (21)	4 postoperation	61/427	Macroscopic evaluation of intestinal atresia model. LM, SEM: wall and epithelial morphology
Masumoto *et al*.[13]*	1999	Chicken	11 (21)	2 posthatching	54/196	LM: NADPH-d, vip, substance-P, alpha smooth muscle actin
Baglaj *et al*.[14]	2001	Chicken	12 (21)	3, 5, 7, 9 postoperation	108/409	LM, SEM, TEM: epithelial morphology
Parisi *et al*.[15]	2004	Chicken	12 (21)	3, 5, 7, 8 postoperation	65/189	LM: vip, substance-P: Evaluation of enteric nervous system

*Masumoto and Tibboel *et al*. eviscerated loop of midgut and cut antimesenterically. AchE: Acetyl choline esterase staining; LM: Light microscopy; NA: Not applicable; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy

Table 2: Review of literature of experimental induced intestinal atresia using the ligation technique in different established animal models since 1955

Chronic mechanical obstruction - ligation of midgut

Author	Year	Animal model	Day of surgery (average gestation)	Day of evaluation	Animals included	Assay type
Pickard *et al*.[16]	1981	Lamb	90-115 (150)	At delivery	8/8	Macroscopic evaluation of intestinal atresia model; LM: AChE, ATPase; neurotransmitter activity and contractile muscular functions
Trahair *et al*.[17]	1993	Lamb	90-95 (150)	At delivery	5/5	SEM, TEM: Epithelial morphology
Schoenberg[18]	2002	Chicken	11 (21)	8 postoperation	95/87	LM: AChE, silver staining, TEM: enteric nervous system
Fiegel *et al*.[19]	2006	Chicken	11 (21)	1-7 postoperation	NA/NA	LM: AChE, silver staining
Fourcade *et al*.[20]	2010	Rat	16-20 (22)	1 prebirth	65/190	Macroscopic evaluation of chronic obsruction model; LM: H and E, gut wall morphology

AchE: Acetyl choline esterase staining; LM: Light microscopy; NA: Not applicable; SEM: Scanning electron microscopy; TEM: Transmission electron microscopy

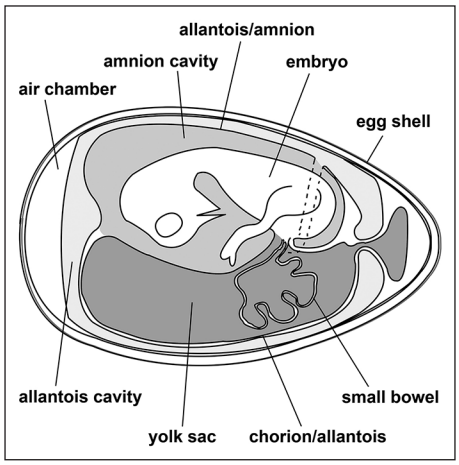

Figure 1: Physiological conditions of an 11 day old chicken embryo. Citation deleted and is included in table 1 The presence of the physiologic umbilical hernia (arrow) allows a simplified surgical intervention during the foetal period

window was established in the eggshell by removing it with the lining shell membrane attached.

Animals were divided into four groups.

1 Intestinal ligature at ED 11. After the procedure, incubation was continued and starting at day 12, vital embryos were harvested on each day between ED 12 and 19 for morphological analysis.
2 Intestinal ligature at ED 11. Incubation was continued until ED 15. In vital embryos, the ligature of the gut was cut when clear distension of the gut proximal to the ligation was present, and signs of atresia formation were missing. Incubation was continued and starting at ED 16, vital embryos were harvested at ED 16, 17, 18 and 19 for morphological analysis.

3 Embryos were sham operated on ED 11 and analysed on EDs 12 up to 19.
4 Incubation of embryos without any surgical intervention from ED 12 until ED 19.

After the insertion of a window in the egg shell, the embryos were staged. At ED 11 they have reached stage 37 according to Hamburger and Hamilton 1951.[21]

In these embryos, the chorioallantoic membrane was opened sharply in an avascular area. Then the omphalomesenteric artery was used as a landmark to identify the insertion of the umbilical cord vessels into the embryo. Under visual control (Stemi 2000C, Zeiss, Germany) the physiologically protruding midgut loop in the vicinity of the umbilical cord vessels was legated with a 9-0 propylene suture (Ethicon) [Figure 2]. Throughout the surgery, care was taken to protect the mesenteric blood supply and to ensure that the thread was tight but not penetrating the intestinal wall. Subsequently the bowel was repositioned, and the eggshell closed using a parafilm (Pechiney Laboratory Safety Products and Apparel Inc., Chicago, IL, USA). This is important to prevent embryo death due to fluid loss. Incubation was continued and vitality of the embryos checked on a daily basis until ED 19 and/or embryos were removed for morphological analysis.

In the second group, ligation was performed on ED 11 and released on ED 15 with incubation continuing to ED 19. Release of the ligature was done using micro scissors, taking care not to damage the intestinal wall and the revascularised chorioallantoic blood vessels [Figure 3]. If damage occurred during surgery, the embryo was excluded from further study. Again incubation was continued until ED 19 and morphological and histological conditions were analysed daily at ED 16-19.

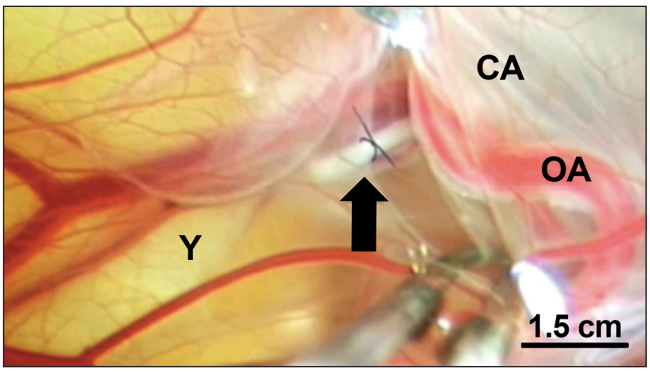

Figure 2: Binocular view of the midgut after ligature placement at embryonic day 11. Black arrow indicates ligature localisation on midgut loop. The omphelomesenteric artery (OA) served as a landmark for preparation. Chorioallantoic membrane (CA) and yolk sack (Y) are marked

The third group was sham operated on, on ED 11. The fourth group was without surgical intervention and served as a control group. Both groups were incubated till ED 19 and analysed as for Groups 1 and 2.

Embryos were sacrificed by decapitation. The extra embryonic membranes were opened, and bowel loops were carefully inspected using a stereo microscope. Full thickness circumferential biopsies of the intestinal wall were obtained 5 mm proximal and distal to the intestinal obstruction [Figure 6b]. Equivalent midgut specimens were excised from control and sham-operated embryos.

Samples were snap frozen in liquid nitrogen and stored at minus 80°C. The frozen samples were sectioned at 8 µm using a Leica CM3000 cryostat (Leica Instruments GmbH, Nussloch, Germany). The slides were air-dried at room temperature for 12-24 h and then either processed directly or stored at −30°C.

Haematoxylin and eosin and acetyl choline esterase (AChE) staining was used according to a standard protocol.[22] AChE staining was used as a simple indicator to estimate the morphological changes seen after the onset of distension and its release.

RESULTS

A total of 658 embryos underwent microsurgical manipulation assigned to one of the three intervention groups. The survival rate of the 40 control and the 40 sham-operated embryos was 100% and 65%, respectively. In Group 1, 364 embryos underwent ligation of the midgut as described above, 80 showed signs of a successful operation and survived up to ED 19. In Group 2, a total of 254 embryos had a release of

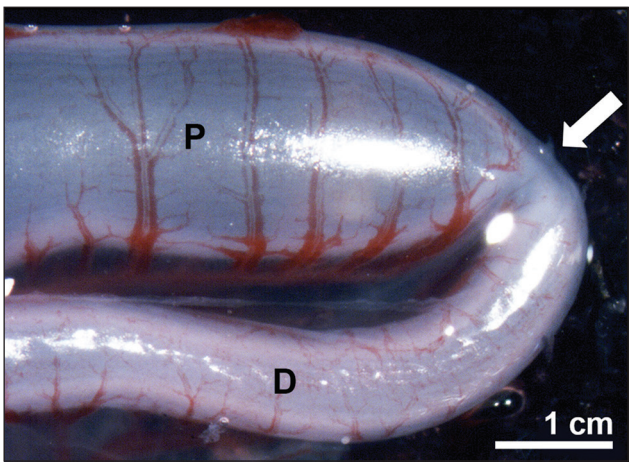

Figure 3: Macroscopic situation directly after release of ligature at embryonic day 15. Former position of ligature is indicated by white arrow between proximal (P) and distal (D) part of a small intestine

the ligature at ED 15 after initial gut ligation on ED 11. Of these, 25 survived up to ED 19 (10%). Criteria for a successful operation were:

i. Vitality of the chicken embryo at the time point of the harvest,
ii. macroscopic evidence of intestinal obstruction or intestinal atresia and
iii. no additional malformations.

At ED 12, 1 day after ligation, there are no changes in gut dimensions visible [Figure 4a]. In the first 2 days, only minor effects were seen [Figure 4b]. The proximal gut increased in diameter compared to the distal part [Figure 4c]. At ED, 15 clear signs of bowel distension caused by artificial obstruction were identified [Figure 4d]. The grade and intensity of the distension progressed rapidly until ED 17. The findings on ED 18 and 19 did not differ significantly from that on ED 17, even though the proximal bowel was more dilated, and the distal bowel remained thin and narrow. Between ED 13 and ED 19, the diameter of the proximal bowel got approximately 8 times wider than the untreated controls. In contrast to this, the distal bowel showed signs of disuse with its maximal diameter attaining only half of the diameter of untreated controls on ED 19.

After ligation, complete obstruction (intestinal atresia) was noticed for the first time on ED 17 in 50% of all vital embryos. This was followed by 90% on ED 18 and 100% on ED 19. In the majority of the cases (18/25), Type I atresia with a massively dilated proximal and small distal intestine was identified. In our experimental model, only a couple of cases (2/25) showed features of Type II intestinal atresia with a short fibrotic cord joining the proximal and distal portion of the intestine. In five cases (5/25) we observed a complete interruption of the gastrointestinal tract (Type IIIa intestinal atresia [Figure 5]). Classification was done according to Davies *et al*.[23] No intra-abdominal pathology was observed in the control nor in the sham-operated group.

Regeneration capacity was estimated by the ability of the gut to recover from structural changes caused by the ligature. The main advantage of our model is the possibility to resolve the obstruction by removing the suture in a second intervention. We selected ED 15 for this second intervention because macroscopically severe pathological changes were frequently observed at this time point after ligation while signs of intestinal atresia were missing. After the release of the ligature, the proximal dilatation diminished until ED 19. Macroscopically embryos on ED 19 showed a pattern almost comparable to the control groups.

Untreated animals show the typical layers of the bowel wall consisting of tunica mucosa, tela submucosa, tunica muscularis and serosa. The AChE staining allows localisation of the enteric nervous system (ENS). The plexus myentericus is located between the circular and the longitudinal muscle layers of the gut and complemented by the plexus submucosus located in the sub mucosal layer. This was analysed on ED 19 [Figure 6a].

Treated animals with persistent ligature were sacrificed, and tissue sampled proximal [Figure 6d] and distal of the ligation [Figure 6e]. At ED 19 we noticed not only a clear distension of the proximal gut but also a somewhat flattened villi with a reduced absorptive surface area. In the ENS, a reduction of ganglia size and disturbance of ganglia distribution is also noted. At ED 19, proximal to the ligature, a loss of density is seen in the plexus submucosus and plexus myentericus [Figure 6d]. The ENS in the distal gut part is comparable to controls with ganglia distribution similar to untreated

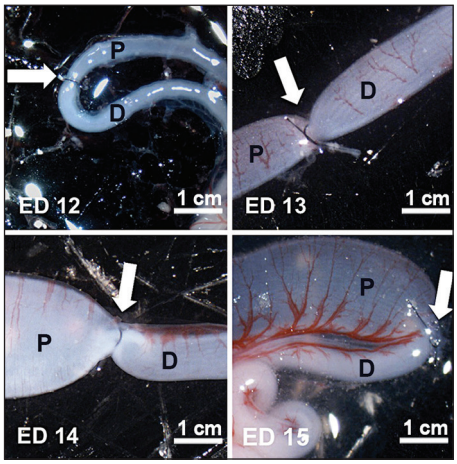

Figure 4: Macroscopic picture of gut dilatation following ligature placement on embryonic day 11. White arrow = ligature position. P = proximal D = distal part of the small intestine (see text for details).

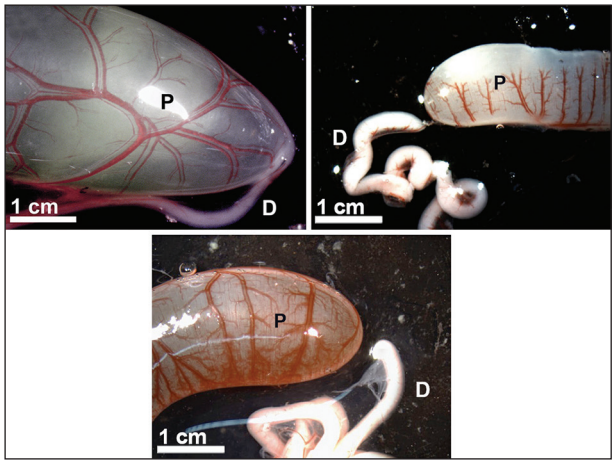

Figure 5: Observed forms of intestinal atresia. Citation deleted and mentioned in text

cohort [Figure 6e]. Nevertheless, the post-stenotic lumen is collapsed probably due to loss of amniotic fluid [Figure 6b and c]. After removal of the ligature, the ENS recovered in the following 4 days. At ED 19 almost no differences to the untreated controls were visible.

DISCUSSION

In 2002, Schoenberg and Kluth studied changes of the ENS in an embryonic model of intestinal obstruction.[18] To exclude collateral damage on surrounding tissues by uncontrolled interference with mesenteric blood supply, they developed the thread technique: At a defined position, a thread was placed around the circumference of the gut and knotted in a manner that the thread was tight but not penetrating the intestinal wall [Figure 2]. Normal growth of the gut with the thread in place caused the stenosis and later completes atresia.

The present study is an extension of the original study by Schoenberg. Chicken embryos were also used as they offer several advantages over other animal models.

1 The approach to the embryo through the egg shell is easy.
2 The further development can be easily studied on the same embryo by simple removal of the parafilm.
3 Further experimental interventions on the same embryo with limited stress for the individual.

For these reasons Tibboel *et al*. stated that the chicken embryo might be a useful model to study surgical procedures in the foetal period.[2]

The objective of the current study was twofold:

1 To study morphological changes of the gut and the ENS in a stepwise fashion on consecutive days after the initial ligature.
2 To test, if a reversion of these changes is possible after the second procedure.

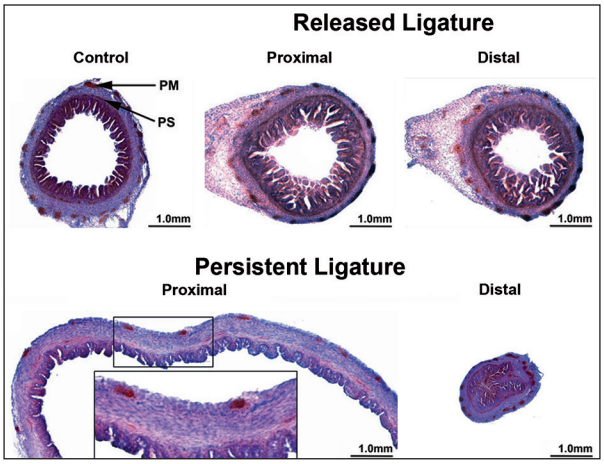

Figure 6: Microscopic evaluation of small bowel cross sections (see text for details)

It must be highlighted that the proposed chicken model is not a model to study intestinal atresia. Intestinal atresia is rather a secondary phenomenon after the initial ligation then the initial aim of the procedure. Our main interest was to describe the natural history of gut development and distension caused by occlusion compared to the processes that take place after the release of the occlusion and the normalisation of the gut diameters. Schoenberg and Kluth have already shown the detrimental effect on the developing ENS and the cells of Cajal that can be observed at ED 19 after placing a ligature on ED 11.[18] Therefore, for the purpose of this study, a rough assessment of the changes caused in the general morphology and the morphology of the ENS by the release of the ligature seems to be sufficient.

Narrowing of the gut at the place of the ligature is obvious once the intestine grows in diameter. The full picture of intestinal atresia can be seen after ED 17 and onwards. The rapid increase of the diameter of the occluded gut (up to eight times until ED 19) causes mechanical forces that result in stretching of the tissue. Microscopic evaluation of the stretched gut showed no alteration in the gut wall layers and is thus conserved proximal and distal to the ligature up to ED 19. After AChE staining we noted that ganglia of the ENS are reduced in number and size up to a complete loss in the later stages ED 17, 18 and 19.

A number of experimental techniques had been described in the literature to induce intestinal atresia in the foetal period. Coagulation of the mesenteric blood vessels is a popular procedure to provoke intestinal atresia formation. However, the disadvantage of this technique is an incalculable number of factors released by this procedure. This makes it a less useful model if the sole effect of distension on the developing ENS is the aim of the study. Furthermore, intestinal obstruction caused by intervention on the blood supply is not reversible as in our model: The removal of the ligature 4 days after initial ligature placement (ED 15) was fully successful in 25 embryos (10% of the embryos included in this experimental group). Removal of the ligature resulted in the recovery of the small bowel within 4 days (ED 19). After that the gut diameter as well as the distribution of the ENS was comparable to untreated animals. These results make this animal model a promising tool to study the impact of intestinal obstruction - and its release on the developing ENS.

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