The role of gut-liver axis in the restriction of intrauterine growth in a model of experimental gastroschisis[1]

O papel do eixo intestino-fígado na restrição de crescimento intra-uterino em um modelo de gastrosquise experimental

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ABSTRACT

PURPOSE: To evaluate the intrauterine growth restriction (IUGR) by the expression of IR-β, IRS-1, IRS-2, IGF-IRβ and Ikappaβ in experimental model of gastroschisis.

METHODS: Pregnant rats at 18.5 days of gestation were submitted to surgery to create experimental fetal gastroschisis (term = 22 days) were divided in three groups: gastroschisis (G), control (C) and sham (S). Fetuses were evaluated for body weight (BW), intestinal (IW), liver (LW) and their relations IW/BW and LW/BW. IR-β and IGF-IRβ receptors, IRS-1 and IRS-2 substrates and Ikappaβ protein were analyzed by western blotting.

RESULTS: BW was lower in G, the IW and IW / BW were greater than C and S ($p<0.05$) groups. The liver showed no differences between groups. In fetuses with gastroschisis, compared with control fetuses, the expression of IGF-IRβ ($p<0.001$) and Ikappaβ ($p<0.001$) increased in the liver and intestine, as well as IR-β ($p<0.001$) which decreased in both. In contrast to the intestine, IRS-1 ($p<0.001$) increased in the liver and IRS-2 decreased ($p<0.01$).

CONCLUSION: The axis of the intestine liver has an important role in inflammation, with consequent changes in the metabolic pathway of glucose can contribute to the IUGR in fetuses with gastroschisis.

Key words: Gastroschisis. Fetal Growth Retardation. Receptor, Insulin. Fetal Development. Rats.

RESUMO

OBJETIVO: Avaliar a restrição de crescimento intra-uterino (RCIU) pela expressão de IR-β, IRS-1, IRS-2, IGF-IRβ e a via inflamatória do Ikappaβ no modelo de gastrosquise experimental.

MÉTODOS: Ratas grávidas com 18,5 dias de gestação foram submetidas a cirurgia experimental para criar gastrosquise fetal (termo = 22 dias) e os fetos foram divididos em três grupos: gastrosquise (G), controle (C) e sham (S). Os fetos foram avaliados quanto ao peso corporal (BW), intestinal (IW), fígado (LW) e suas relações IW/BW e LW/BW. Os receptores IR-β e IGF-IRβ, os substratos IRS-1 e IRS-2 e a proteína Ikappaβ foram analisados por western blotting.

RESULTADOS: O BW de G foi menor, o IW e IW/BW foram superiores a C e S ($p < 0.05$). O fígado não apresentou diferenças

entre os grupos. Nos fetos com gastrosquise, quando comparados com fetos controles, a expressão de IGF-IRβ ($p<0.001$) e Ikappaβ ($p<0.001$) aumentou no fígado e intestino, assim como IR-β ($p<0.001$) que diminuiu em ambos. Inversamente ao intestino, IRS-1 ($p<0.001$) aumentou no fígado e IRS-2 diminuiu ($p<0.01$).

CONCLUSÃO: O eixo do intestino fígado tem um papel importante na inflamação, com consequentes alterações na via metabólica de glicose que pode contribuir para a RCIU em fetos com gastrosquise.

Descritores: Gastrosquise. Retardo de Crescimento Intrauterino. Receptor de Insulina. Desenvolvimento fetal; Ratos.

Introduction

Gastroschisis is a congenital defect characterized by an opening on the abdominal wall, usually right to the umbilical cord, which affects five in 10,000 live births[1]. The permanent exposure of the bowel loops to the amniotic fluid (AF) changes the intestinal morphology, affecting motility and nutrients absorption[2].

A proper intestinal length is required for fetal somatic growth. Intestinal anomalies such as gastroschisis may cause intrauterine growth restriction (IUGR) with birth weight less than expected for gestational age[3,4]. IUGR is frequently seen in fetuses with gastroschisis. Although the causes of IUGR in gastroschisis are still not known, it might be due to the defect itself or to secondary causes like the metabolic response of the bowel in contact with the amniotic fluid[5]. In the rat model of gastroschisis the body weight is decreased and there is an inverse relationship between the length of exposure of the bowel to the amniotic fluid and body weight, so that longer exposure leads to a more decreased body weight in fetuses with gastroschisis[6].

Inflammatory reactions, as seen in the intestines of fetuses with gastroschisis, can affect fetal hormone production and glucose and amino acid transport by altering fetal differentiation and development. For example, changes in the insulin receptor (IR) and their substrates (IRS-1, IRS-2) interfere with the glucose metabolism and fetal growth[7]. Changes in type I insulin-like growth factor receptor (IGF-IR), responsible for the fetal growth and development[8], may explain the relationship between IUGR and cardiovascular diseases and type II diabetes in adults. Furthermore, the study of nuclear transcription factor kappaB (NF-kappaβ) and its inhibitor, Ikappaβ, can contribute to a better understanding of the relationship between IUGR and inflammation in gastroschisis[9].

Thus, in order to better understand the cause of IUGR in the gastroschisis experimental model we assessed the expression of IR-β and IGF-IRB receptors, IRS-1 and IRS-2 substrates and Ikappaβ protein.

Methods

This research was submitted to and approved by the State University of Campinas (UNICAMP) Animal Research Committee and followed guidelines for the care and use of laboratory animals, internal number #1452-1.

Female Sprague-Dawley rats, weighing 200 to 250g, were mated and the presence of sperm in the vaginal smear the following day, was defined as gestational day 0 (term=22 days). On day 18.5 of gestation, anesthesia was induced by intramuscular injection of Ketamin 50mg/ml (Ketamina®-Pfizer do Brasil Ltda.) associated with Xylazin 10mg/ml (Rompum®-Bayer do Brasil Ltda.). This anesthetic association (Ketamin 175mg/kg and Xylazin 2,5mg/kg) keeps animals in deep anesthesia for three hours and promotes six to 12 hours of painless recovery. The rats were operated under sterile conditions, on an electrically warmed table regulated at 37°C (Harvard Apparatus®-USA). The abdomen was shaven with an electric razor (Sunbeam®-USA) and cleaned with chlorhexidin solution (Chlorohex®). The abdominal wall was opened through a median longitudinal incision and the uterus was exposed and kept warm by intermittent irrigation of warm saline solution (38°C). Fetuses were operated according to the technique described by Correia-Pinto *et al*[10] by partially eviscerating the bowel through a right paramedian opening of the abdominal wall. We exposed both legs of the fetus and made an incision with a direct view of the umbilical cord. This position permitted a safe and controlled incision without damage to the umbilical vasculature. The fetuses (n=60) were divided into three groups (n=20 each): gastroschisis (G), control (C) and sham (S). The control fetuses were left undisturbed and sham fetuses were exposed by histerotomy and manipulated but no abdominal wall incision was performed. The first and last fetuses of each uterine horn were not used. Each pregnant rat had at least two fetuses submitted to gastroschisis. Fetuses were harvested by cesarean section on 21.5 day of gestation. Fetuses were weighed on a precision balance OHAUS 360 (Denver Instruments, Denver, CO-USA) and then sacrificed by occipital puncture and a lethal dose of anesthetic. The intestinal tract was removed from pylorus to rectum and weighed (intestinal weight, IW) as well as the liver (liver weight, LW), after that, samples were immediately frozen for western blotting. The intestinal weight to body weight (IW/BW) ratio and liver weight to body weight (LW/BW) ratio were

calculated.

Western blotting

After weighing, liver and intestine from six fetuses per group were individually homogenized immediately in extraction buffer, 4°C, using a Polytron PTA 20S generator (Brinkmann Instruments, Westbury, N.Y., USA, model PT 10/35) operated at maximum speed for 30s. The extraction buffer was composed of 100 mmol/l Tris (pH 7.4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium sodium fluoride, 10 mmol/l EDTA, 10mmol/l sodium vanadate, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 0,1 mg/ml aprotinin and 1% Triton-X100. The extracts were centrifuged at 15000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA, USA) for 45 min to remove insoluble material, and the supernatants were used for protein quantification. Proteins were denatured, run on SDS-PAGE and transferred to nitrocellulose membranes. Antibodies used for immunoblotting were anti-IRβ, anti-IRS-1, anti-IRS-2, anti-IGF-IRB and anti-Ikappaβ non-phosphorylated (Santa Cruz Biotechnology Inc., CA, USA). Blots were exposed to preflashed Kodak X-ray film with Cronex Lightning Plus intensifying screens at −80°C for 12–48h. Band densities were quantified by optical densitometry (Scion Image software, ScionCorp, Frederick, MD, USA) of the developed radiographs.

Statistical analysis was performed using ANOVA test with Tukey's post-test. Significant statistical difference was considered when $p<0.05$.

Results

Morphological

A total 14 pregnant rats with 60 fetuses were studied. There was no mortality in C and S groups but the mortality rate was 10% (2/22) in fetuses with gastroschisis. We observed a significantly lower body weight and increased intestinal weight and IW/BW ratio in fetuses with gastroschisis compared with controls and shams ($p<0.05$). The liver weight, as well the LW/BW, were similar among the three groups (Table 1).

TABLE 1 - Results of body weight (BW), intestinal weight (IW), liver weight (LW) and the ratios IW/BW and LW/BW of fetuses from the three studied groups.

Weight (mg)	Gastroschisis (n=20)	Control (n=20)	Sham (n=20)	P
Body (BW)	5147 (±617)	5672 (±444)	5332 (±491)	<0.05
Intestine (IW)	334 (±169)	233 (±52)	223 (±55)	<0.05
Liver (LW)	379 (±103)	386 (±76)	350 (±81)	NS
IW/BW	0.063 (±0.029)	0.041 (±0.010)	0.042 (±0.011)	<0.05
LW/BW	0.073 (±0.015)	0,068 (±0.012)	0,066 (±0.014)	NS

Western blotting

Liver. The expression of IRS-1 and IGF-IRB was higher in G ($p<0.001$) and S ($p<0.001$) groups compared with C group. Hepatic expression of IRβ was lower in G group compared with S ($p<0.01$) and C ($p<0.001$) groups. The expression of IRS-2 was lower in G ($p<0.01$) compared with C group. On the other hand, the expression of non-phosphorylated Ikappa-β was higher in G group compared with C ($p<0.001$) and S ($p<0.01$) groups (Figure 1).

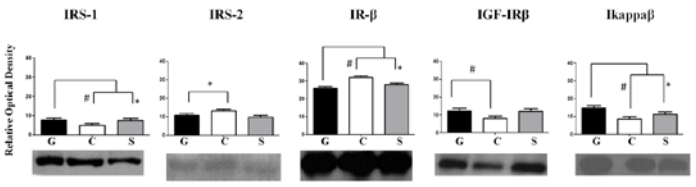

FIGURE 1 - Expression of IR-β, IRS-1, IRS-2, IGF-IRβ and Ikappaβ from liver of G, C e S fetuses on 21.5 gestational day by western blotting. G: Gastrochisis, C: Control, S: Sham. * $p<0.05$ # $p<0.001$

Intestine. The expression of IRβ and IRS-1 was lower in G group compared with S ($p<0.01$) and C ($p<0.001$) groups. Intestinal expression of IRS-2 was higher in G and C groups compared with S group, but it was not statistically different. Expression of non-phosphorylated Ikappa-β was higher in G ($p<0.001$) and S ($p<0.001$) groups compared with C group, while the expression of IGF-IRB was higher in G ($p<0.001$) and C ($p<0.001$) groups compared with S group (Figure 2).

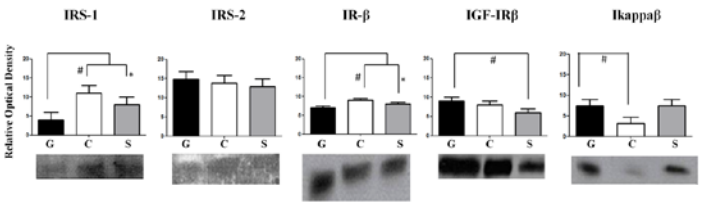

FIGURE 2 - Expression of IR-β, IRS-1, IRS-2, IGF-IRβ and Ikappaβ from intestine of G, C e S fetuses on 21.5 gestational day by western blotting. G: Gastrochisis, C: Control, S: Sham. * $p<0.05$ # $p<0.001$

Discussion

Several metabolic diseases in adulthood that result from permanent endocrine and metabolic changes, such as hypertension and diabetes mellitus, can be associated with the fetal adaptation to decreased nutrition. The fetal genotype can lead to different phenotypes depending on pre and postnatal conditions, while one of the essential mechanisms of IUGR is related to the insulin receptors (IR)[11].

After binding to insulin, the IR undergoes a conformational change and self-phosphorilates, increasing their tyrosine-kinase activity, consequently phosphorilating their substrates. Ten different IR substrates (IRS) have been described. Phosphorilation in tyrosine residues of protein in the IRS family creates sites for recognition of molecules with domains similar to the system Src2, with phosphatidilinositol-3 kinase (PI3K). PI3K is an essential molecule in the regulation of mitosis, cellular differentiation and glucose transportation through the cytoplasmic membrane[7].

The IR can also, under special circumstances, phosphorilate in serine residue, which attenuates signaling transmission, causing negative feedback and could result in insulin resistance. This situation can be observed in chronic or acute inflammatory states, when there is release of pro-inflammatory cytokines such as interleukin 1 (IL-1), tumor necrosis factor alfa (TNF-α), and interferon gama (IFN-γ). These phosphorilation in serine residues determines a decreased efficacy in the trophic and metabolic pathways and, consequently, changes in the triggers for growth, replication, and development of cellular organelles[7,12]. Consequently, the timing systems of fetal cellular growth are changed, resulting in IUGR.

We noted a decreased intestinal and hepatic expression of IRβ, probably indicating that the insulin level is increased, leading to a downregulation of the receptor. It is known that newborns small for gestational age present low levels of circulating insulin and type 1 insulin-like growth factor (IGF-1), followed by an increase during the first years of life[13]. Our model is fetal, so the hormone levels control may still not be well defined, what could explain these results. In addition, we also observed an increased hepatic IRS-1 and a decreased intestinal IRS-1, and the lower the expression, the greater the fetal insulin resistance and IUGR. In the study of intestine-liver axis, the liver could try to compensate changes that occurred during the intestinal inflammatory process of gastroschisis, and for this reason, the liver IRS-1 increased.

A way to control the insulin resistance would be to increase the expression of IRS-2, which partially compensates the lower amount of IRS-1, allowing a regulation of glucose levels in fetal organism, so that hyperglycemia does not happen[12]. Our findings show that intestinal IRS-2 increased, although no statistical difference between groups was shown, while it decreased in the liver when compared with C group. For this reason, the fetus could present hyperglycemic levels, since there may be abnormalities in insulin action on peripheral tissues and this may be due to translation of the intermediate glucose metabolism deviation with decreased production of energy for fetal growth.

Analyzing the IGF-IRβ levels, we found that it is increased in the liver and intestine of fetuses with gastroschisis, similar results were found on the IUGR model in rats[14]. In a fetal sheep model, it was also found an increased IGF-IRβ, although on skeletal muscles, suggesting that the increase is a response to low circulating hormone concentrations in fetuses with IUGR[13].

The inflammatory process that the amniotic fluid triggers in the intestinal loops in gastroschisis can be a sufficient stimulus to activate the transcription factor NF-kappa-B and promote increased inflammation of the bowel and liver. The involvement of the gut-liver axis by activation of the NF-kappaβ, or intestinal injury affecting the liver was tested in the gastroschisis model[15].

NF-kappaβ is a transcription factor that is involved in activation of many genes, including those related to liver cell injury, endotoxemia and oxidative stress. NF-kappaβ is a heterodimeric complex of p50 and p65 subunits that interacts with the family of inhibitors Ikappaβ. When the cells are stimulated, Ikappaβ is phosphorylated with subsequent release of NF-kappaβ. Once released, NF-kappaβ translocates into the nucleus, where it is connected to a specific sequence in the promoter region of target genes. Among the genes activated by NF-kappaβ genes are pro-inflammatory cytokine genes and cytotoxic genes, and genes that participate in the prevention of apoptosis during liver regeneration.

In our study, we analyzed the inhibitory protein Ikappaβ that increase its expression in the two studied organs. We believe that this result is due to the intense inflammatory process that occurs in the bowel loops during the gastroschisis, in which the organism tries to soothe inflammation by inhibiting NF-kappaβ. Because NF-kappaβ is increased in the studied organs, the

intestine-liver axis tries to increase the inhibition to reduce the inflammation that occurs in gastroschisis. However, it is important to remember that, even with this increase in inhibitory proteins, is not sufficient to block NF-kappaβ action and prevent inflammation in gastroschisis.

Our results showed that gastroschisis increased the hepatic and intestinal expression of Ikappaβ which could reinforce the concept that these fetuses have fetal inflammatory response syndrome. It may be possible that the inflammatory process in the bowel consumes energy that should be directed for growth. It appears that the signals for glucose uptake involving insulin receptors are compromised and the fetus with gastroschisis may have a transitory insulin resistance[15].

Conclusion

The gut-liver axis has an inflammatory role in the development associated changes of the metabolic pathway of glucose which could contribute to the IUGR in fetuses affected by this disease.

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Conflict of interest: none
Financial source: Sao Paulo Research Foundation (FAPESP)

[1]Research performed at Laboratory of Experimental Fetal Surgery, School of Medicine of Ribeirao Preto, University of Sao Paulo (USP), Ribeirao Preto-SP, Brazil.