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Trabajo Original

Otros

Tissular growth factors profile after teduglutide administration on an animal model of intestinal anastomosis

Perfil tisular de factores de crecimiento postadministración de teduglutida en un modelo animal de anastomosis intestinal

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Abstract

Background: Teduglutide is an enterotrophic analogue of glucagon-like peptide-2, with an indirect and poorly understood mechanism of action, approved for the rehabilitation of short-bowel syndrome. This study aims to analyze the response of tissue growth factors to surgical injury and teduglutide administration on an animal model of intestinal anastomosis.

Methods: Wistar rats (n = 59) were distributed into four groups: "ileal resection" or "laparotomy", each one subdivided into "postoperative teduglutide administration" or "no treatment"; and sacrificed at the third or the seventh day, with ileal sample harvesting. Gene expression of *insulin-like growth factor 1 (Igf1), vascular endothelial growth factor a (Vegfa), transforming growth factor β1 (Tgfβ1), connective tissue growth factor (Ctgf), fibroblast growth factor 2 (Fgf2), fibroblast growth factor 7 (Fgf7), epidermal growth factor (Egf), heparin-binding epidermal-like growth factor (Hbegf), platelet-derived growth factor b (Pdgfb) and glucagon-like peptide 2 receptor (Glp2r)* was studied by real-time polymerase chain reaction.

Results: Upregulation of *Fgf7, Fgf2, Egf, Vegfa* and *Glp2r* at the third day and of *Pdgf* at the seventh day was verified in the perianastomotic segment. Teduglutide administration was associated with higher fold-change of relative gene expression of *Vegfa* (3.6 ± 1.3 *vs.* 1.9 ± 2.0, p = 0.0001), *Hbegf* (2.2 ± 2.3 *vs.* 1.1 ± 0.9, p = 0.001), *Igf1* (1.6 ± 7.6 *vs.* 0.9 ± 0.7, p = 0.002) and *Ctgf* (1.1 ± 2.1 *vs.* 0.6 ± 2.0, p = 0.013); and lower fold-change of *Tgfβ1, Fgf7* and *Glp2r*.

Conclusions: Those results underscore the recognized role of *Igf1* and *Hbegf* as molecular mediators of the effects of teduglutide and suggest that other humoral factors, like *Vegf* and *Ctgf*, may also be relevant in the perioperative context. Induction of *Vegfa, Igf1* and *Ctgf* gene expressions might indicate a favorable influence of teduglutide on the intestinal anastomotic healing.

Key words:

Teduglutide. Growth factors. Intestinal anastomosis. Vascular endothelial growth factor. Connective tissue growth factor.

Resumen

Introducción: teduglutida es un análogo intestinotrófico do péptido-2 similar al glucagón, con un mecanismo de acción indirecto y poco conocido, aprobado para la rehabilitación del síndrome de intestino corto. Este estudio propone analizar la respuesta de los factores de crecimiento tisulares a la agresión quirúrgica y a la administración de teduglutida en un modelo animal de anastomosis intestinal.

Métodos: ratones Wistar (n = 59) fueron distribuidos en cuatro grupos: "resección ileal" o "laparotomía", cada uno subdividido en "administración post-operatoria de teduglutida" o "sin tratamiento"; y sacrificados en el tercero o el séptimo día, con recogida de muestras ileales. La expresión génica de *Igf1, Vegfa, Tgfβ1, Ctgf, Fgf2, Fgf7, Egf, Hbegf, Pdgfb* y *Glp2r* fue analizada por qRT-PCR.

Resultados: en el segmento perianastomótico se verificó una sobrerregulación de *Fgf7, Fgf2, Egf, Vegfa* y *Glp2r* al tercer día y de Pdg al séptimo día. La administración de teduglutida se asoció con mayor cambio de la expresión génica relativa de *Vegfa* (3.6 ± 1.3 *vs.* 1.9 ± 2.0, p = 0.0001), *Hbegf* (2.2 ± 2.3 *vs.* 1.1 ± 0.9, p = 0.001), *Igf1* (1.6 ± 7.6 *vs.* 0.9 ± 0.7, p = 0.002) y *Ctgf* (1.1 ± 2.1 *vs.* 0.6 ± 2.0, p = 0.013); y menor cambio de *Tgfβ1, Fgf7* y *Glp2r*.

Conclusiones: estos resultados refuerzan el reconocido papel de *Igf1* y *Hbegf* como mediadores moleculares de los efectos de la teduglutida y sugieren que otros factores humorales, como *Vegf* y *Ctgf*, también pueden ser relevantes en el contexto perioperatorio. La inducción de las expresiones de los genes *Vegfa*, *Igf1* y *Ctgf* podría indicar una influencia favorable de teduglutida en la cicatrización anastomótica intestinal.

Palabras clave:

Teduglutida. Factores de crecimiento. Anastomosis intestinal. Factor de crecimiento vascular endotelial. Factor de crecimiento del tejido conectivo.

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INTRODUCTION

Failure of intestinal anastomotic repair still remains a major source of morbidity and mortality in digestive surgery and one of the most feared postoperative complications (1).

Intestinal anastomotic healing is a complex multi-cellular multi-molecular dynamic process involving a coordinated interplay between multiple signaling networks and a rigorous spacial and temporal control (1-3). Traditionally, wound healing model considers three overlapping phases: inflammatory, proliferative and reparative (2,3). After injury, clotting cascades origin thrombin, an inducer of platelet degranulation, and release of bioactive mediators that stimulate the recruitment of inflammatory cells. Those cells participate in the wound debridement, antigen presentation and phagocytosis and release of reactive oxygen species, inflammatory cytokines, chemokines and growth factors that amplify the repair process (3). Usually, inflammatory phase lasts one to four days (2). Proliferative phase is characterized by re-epithelialization ("restitution" and crypt stem cells proliferation and differentiation), fibroplasia and angiogenesis and, generally, occurs between the fourth and the fourteenth postoperative days (2,3). In the reparative phase, which may elapse up to six months (2), extracellular matrix undergoes continuous synthesis and remodeling by proteolytic enzymes leading to wound maturation and contraction (3).

Numerous experimental studies have been published on the effect of adjuvants of intestinal anastomotic repair. Perioperative strategies to improve anastomotic healing described in those studies included tissue adhesives (4), growth factors, stem cells-based therapies (5), artificial matrixes and patches (4,6,7), topical or systemic pharmacological interventions (8,9), perfusion reinforcement techniques (10), among others. Nevertheless, despite extensive research, no substantial evidence was documented to justify the implementation of any of those strategies for daily clinical use (2,4).

Several experimental studies, analyzing the effects of growth factors on the gastrointestinal anastomotic healing, suggest participation in all phases of tissue healing and potential benefit from insulin-like growth factor 1, vascular endothelial growth factor, epidermal growth factor, heparin-binding epidermal growth factor, transforming growth factor-β, fibroblast growth factor 2 and keratinocyte growth factor (2,9).

Glucagon-like peptide 2 (Glp2) is a potent gastrointestinal growth factor, produced in enteroendocrine L cells, with intestinotrophic, antisecretory, transit-modulating and anti-inflammatory effects (11). Glp2 demonstrates a complex, indirect and poorly understood mechanism of action with intricate signaling pathways and multiple mediators' participation (including insulin-like growth factor 1, ErbB superfamily of ligands, fibroblast growth factor 7, vasoactive intestinal polypeptide and endothelial nitric oxide synthase) (11). Teduglutide is a long-acting dipeptidylpeptidase IV-resistant analogue of GLP2 recently approved for the pharmacological rehabilitation of adult patients with short-bowel syndrome (12). Response of tissue growth factors, key mediators of the anastomotic repair, to teduglutide administration in the perioperative context of intestinal anastomosis is yet to be defined.

This study aims to analyze the response of tissue growth factors to surgical injury and teduglutide short-term administration on an animal model of intestinal anastomosis.

METHODS

STUDY PROTOCOL AND SURGICAL PROCEDURES

Study was approved by the Ethics Committee of the Faculty of Coimbra, Coimbra, Portugal (Official Letter nº32-06-2009) and performed according to institutional and national animals' protection guidelines.

Adult male Wistar *albinus* rats were randomly allocated into four groups: "*ileal resection and anastomosis*" ("*Res*") or "*laparotomy*" ("*Lap*"), each one subdivided into "*postoperative teduglutide administration*" ("*Ted +*") or "*no treatment*" ("*Ted -*"). Evaluation was performed at the operation and sacrifice moments, at the third or the seventh postoperative day (eight subgroups), with ileal segment harvesting and blood collection (Fig. 1). Blinded assessment was guaranteed in the laboratorial analysis.

Animals weighting 250 to 300 g were harboured in ventilated cages with a controlled environment of temperature (22 ± 1 °C), relative humidity (50 ± 10%) and light-dark cycles of 12 hours; and with free access to water and standard rodent diet.

All the surgical interventions were performed by the same surgeon after a period of two hours solid fasting, with clean surgical technique and under anaesthesia with intraperitoneal ketamine hydrochloride (75 mg/kg; Pfizer Inc., NY, USA) and chlorpromazine (3 mg/kg; Laboratórios Vitória, Amadora, Portugal). In "*Res*" groups, a 10 cm length ileal resection was undertaken, 5 cm upstream of ileocecal valve, after a 3 cm abdominal wall midline incision. A standard end-to-end anastomosis was constructed with eight equidistant full-thickness polydioxanone USP 6/0 stitches (PDS II, Ethicon, Johnson-Johnson Intl). Abdominal wall was closed with muscle-aponeurotic and cutaneous running sutures of braided coated polyglactin 910 USP 4/0 (Surgilactin, Sutures Limited, United Kingdom) and natural silk USP 4/0 (Surgisilk, Sutures Limited, United Kingdom), respectively. In "*Lap*" groups, animals were subjected to a 3 cm midline laparotomy (without resection) with gentle manipulation of the small bowel.

In "*Ted +*" groups, teduglutide (American Peptide Company) was administered in the postoperative period (from the operation day), 200 µg/kg/day, subcutaneously, dissolved in 0.25 ml phosphate buffered saline pH 7.4 (PBS, pH 7.4, Gibco, Life Technologies), after preparation according to the manufacturer's recommendations.

In the first postoperative day, ingestion of water with 5% glucose at a 1:1 ratio was allowed and then unrestricted oral hydration and chow were reassumed. Daily surveillance was performed and operative mortality and morbidity were registered. At the third or seventh postoperative day, animals were sacrificed by cervical displacement and a re-laparotomy with ileal resection was performed (10 cm length, preserving distal 3 cm).

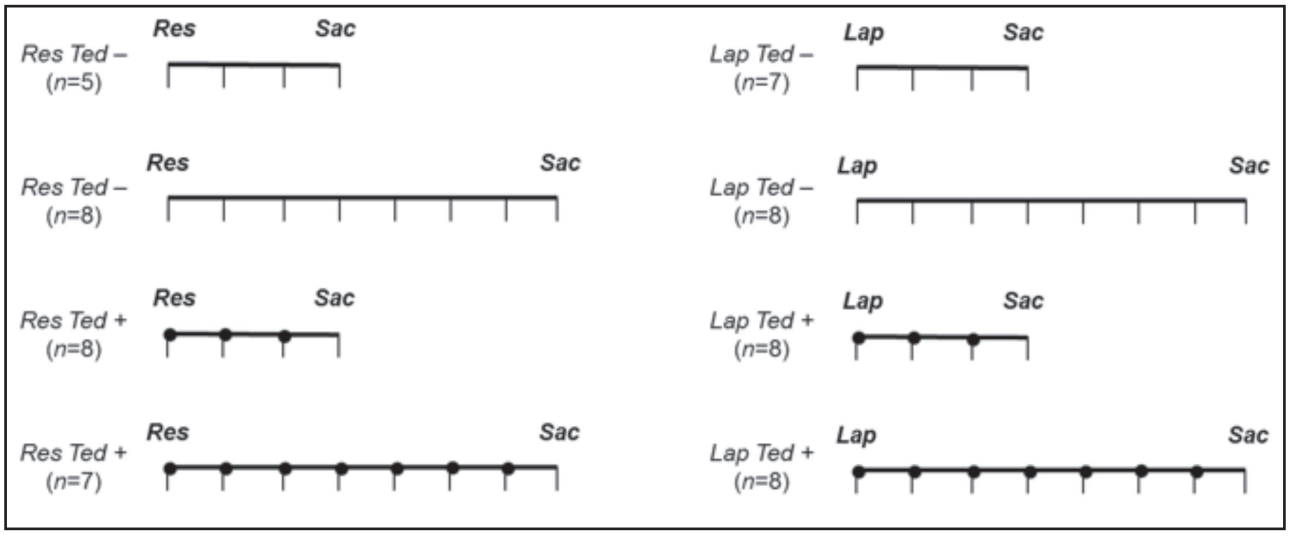

Figure 1.

Study design. Adult male Wistar *albinus* rats were randomly distributed into four groups: *"ileal resection" ("Res") versus "laparotomy" ("Lap")*, each one subdivided into *"postoperative teduglutide administration" ("Ted +") versus "no treatment" ("Ted -").* Evaluation was performed at the moments of the operation and sacrifice (Sac), at the third or the seventh postoperative day (eight subgroups), with ileal segment harvesting and blood collection. Baseline values of *"ileal resection"* groups were considered for comparison with postoperative results of the "*laparotomy*" groups; tissue samples recovered at the sacrifice in those animals corresponded to the perianastomotic segments. • Teduglutide administration.

TISSUE AND BLOOD HARVESTING

Three similar longitudinal strips of the most distal 4 cm length of each ileal operative specimen, each one corresponding to one third of the circumference, were carefully recovered, after gentle washing with normal saline solution, for homogenization and additional procedures, respectively. Tissue baseline values of "*ileal resection*" groups were considered for comparison with postoperative results of the "*laparotomy*" groups; tissue samples recovered at the sacrifice in those animals corresponded to the perianastomotic segments.

Blood samples of 1 ml were drawn in the morning, before the operations, from the tail vein, into polyethylene therephthalate K3 ethylenediaminetetraacetic acid (K3EDTA) vacutainers. Samples were stabilized immediately with 0.1 mg/ml of aprotinin from bovine lung (Sigma-Aldrich) and 0.037 mg/ml of dipeptidylpeptidase IV competitive inhibitor nicotinonitrile dihydrochloride hydrate (Sigma-Aldrich) and centrifuged for 20 min at 1500x *g* and 4 °C. Plasma aliquots were stored at -80 °C.

INTESTINAL TISSUE HOMOGENIZATION

Briefly, fragments from one ileal longitudinal strip recovered according to previous description, with approximately 1 ml, were rapidly introduced in a mixture of protease inhibitors in a proportion of 1 ml/100 mg and submitted to mechanical homogenization. Proteases cocktail was previously prepared by adding aprotinin from bovine lung (Sigma-Aldrich), leucopeptin hemisulfate salt (Sigma-Aldrich) and pepstatin A (Sigma-Aldrich) (1 µl of each, all diluted in a 10 mg/ml stock concentration) to 10 ml of phosphate buffered saline (PBS, pH 7.4, Gibco, Life Technologies) and stored on ice. Preparation was sonicated twice with one short pulse of ten seconds, cooled during ten seconds and distributed into two tubes of 1.5 ml. Sonication (one pulse of ten seconds) was repeated and centrifugation was undertaken, 14000x *g*, for 10 min, at 4 °C. Supernatant was removed to a new tube and pellet was preserved on ice for posterior ribonucleic acid (RNA) extraction.

ANALYSIS OF GROWTH FACTORS AND Glp2 RECEPTOR GENE EXPRESSIONS

Gene expression of growth factors in the rats ileum was determined by quantitative estimation of messenger ribonucleic acid (mRNA) using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Studied growth factors and receptor included: *insulin-like growth factor 1, transcript variant (Igf1), vascular endothelial growth factor A, transcript variant 2 (Vegfa), transforming growth factor, beta 1 (Tgfß1), connective tissue growth factor (Ctgf), fibroblast growth factor 2 (Fgf2), fibroblast growth factor 7 (Fgf7), epidermal growth factor (Egf), heparin-binding Egf-like growth factor (Hbegf); platelet-derived growth factor beta polypeptide (Pdgfb)* and *glucagon-like peptide 2 receptor (Glp2r)*.

Total RNA was extracted from homogenates of rats longitudinal strips of ileum using the Isolate II RNA Mini Kit (Bioline). One microgram of isolated total RNA was used for reverse-transcription, which was performed with the Tetro cDNA Synthesis Kit (Bioline) and using random hexamer. Real-time PCR primers were designed with Bea-

con Designer software (Premier Biosoft, PA, USA) and were obtained from Sigma-Aldrich (Sintra, Portugal). All the genes included in this study were described in the National Center for Biotechnology Information (NCBI) Gene database (http://www.ncbi.nlm.nih.gov/) as indicated in table I. *Hypoxanthine phosphoribosyltransferase* (*Hprt*) was used as housekeeping gene. qRT-PCR was performed on a Bio-Rad iQ5 real-time PCR instrument (BioRad, Hercules, CA, USA) using the SensiFAST™ SYBR & Fluorescein Kit (Bioline). For each sample, PCR was performed in duplicate.

Data were analyzed by relative quantification (13). All values were normalized to the values of the reference gene of those samples.

DETERMINATION OF PLASMA Glp2 CONCENTRATION

Plasma Glp2 levels were determined by competitive enzyme immunoassay (EIA) using the glucagon-like peptide 2 (Glp2) EIA Kit 96-Well Plate (Phoenix Europe GmbH, Karlsuhe, Germany), in accordance to the manufacturer's recommended protocol. All determinations were done in duplicate. Glp2 plasma concentrations were calculated using the corresponding standard curve and the microplate reader with Gen5 software (Synergy HT, Biotek, Winooski, VT, USA), and expressed in ng/ml.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 18 software (SPSS, Chicago, IL, USA). Type of distribution of variables was determined using Shapiro-Wilk and Kolmogorov-Smirnov-Lillifors tests. Data were indicated as median and interquartile range (median±IQR). Non-parametric continuous variables were compared by Mann-Whitney *U* test and analysis of variance by ranks (Kruskall-Wallis test) with pairwise comparisons; correlations were determined by the Spearman's rank correlation coefficient (σ). Differences were considered statistically significant at a level of 95% ($p < 0.05$).

Table I. Primers used in the analysis of gene expression levels of growth factors and Glp2 receptor by qRT-PCR

Gene[a]	GenBank Accession nº	Orientation	Sequence	Size (bp)
Rat Igf1	NM_001082477	Forward	GCCATTAGCCCTGCCCTTTCTT	78
Rat Igf1	NM_001082477	Reverse	GCCACCCAGTTGCTATTGCTTTCG	
Rat Vegfa	NM_001110333	Forward	CAGCGACAAGGCAGACTATTC	193
Rat Vegfa	NM_001110333	Reverse	CCGAAGTAATTTGAGGGAGTGAAG	
Rat Tgfb1	NM_021578	Forward	GTGGACCGCAACAACGCAATCT	100
Rat Tgfb1	NM_021578	Reverse	GTTCTGGCACTGCTTCCCGAATG	
Rat Ctgf	NM_022266	Forward	TGCCGGTAACAAGCCAGATT	394
Rat Ctgf	NM_022266	Reverse	GCAGCAAACACTTCCTCGTG	
Rat Fgf2	NM_019305	Forward	TGCTCTAGGGGACTGGAGATT	86
Rat Fgf2	NM_019305	Reverse	GACCAGCCTTCCACCCAAAG	
Rat Fgf7	NM_022182	Forward	TGGCAATCAAAGGGGTGGAA	249
Rat Fgf7	NM_022182	Reverse	TAGGAAGAAAGTGGGCCGTT	
Rat Egf	NM_012842	Forward	TGACCTTAGAACCACCGAGACCAT	92
Rat Egf	NM_012842	Reverse	TCTGCTGTGCTGTGACACTGAG	
Rat Hbegf	NM_012945	Forward	AGAGAGGACGGATGAGTGGT	99
Rat Hbegf	NM_012945	Reverse	GGAGGGTCCAAACAGCAGAT	
Rat Pdgfb	NM_031524	Forward	GCACAGAGACTCCGTAGAC	77
Rat Pdgfb	NM_031524	Reverse	CCGACTCGACTCCAGAATG	
Rat Glp2r	NM_021848	Forward	ACCTGTTCGCTTCGTTCA	106
Rat Glp2r	NM_021848	Reverse	GACATCCATCCACTCTCATCAT	
Rat Hprt1	NM_012583.2	Forward	CTCCTCAGACCGCTTTTC	86
Rat Hprt1	NM_012583.2	Reverse	CTGGTTCATCATCACTAATCAC	

[a]*Igf1: insulin-like growth factor 1, transcript variant 1, mRNA; Vegfa: vascular endothelial growth factor A, transcript variant 2, mRNA; Tgfb1: transforming growth factor, beta 1, mRNA; Ctgf: connective tissue growth factor, mRNA; Fgf2: fibroblast growth factor 2, mRNA; Fgf7: fibroblast growth factor 7, mRNA; Egf: epidermal growth factor, mRNA; hbegf: Heparin-binding EGF-like growth factor, mRNA; Pdgfb: platelet-derived growth factor beta polypeptide, mRNA; Glp2r: glucagon-like peptide 2 receptor, mRNA; Hprt1: hypoxanthine phosphoribosyltransferase 1, mRNA (housekeeping gene) (Rattus norvegicus).*

RESULTS

Fifty-nine animals were studied and included into the different groups (Fig. 1).

RESPONSE OF TISSUE GROWTH FACTORS GENE EXPRESSION TO ILEAL RESECTION AND ANASTOMOSIS

In the perianastomotic segment, upregulation of gene expression of *Fgf7* (fold-change: 12.6 ± 2.8), *Fgf2* (fold-change: 6.1 ± 2.2), *Egf* (fold-change: 2.7 ± 2.2), *Vegfa* (fold-change: 2.7 ± 1.0) and *Glp2r* (fold-change: 2.3 ± 2.1) at the third postoperative day (Fig. 2A); as well as of *Pdgfb* (fold-change: 2.0 ± 1.5), *Igf1*, *Egf*, *Hbegf*, *Vegfa* and *Glp2r* at the seventh day was verified (Fig. 2B). Moreover, downregulation of *Igf1* (fold-change: 0.2 ± 0.3), *Hbegf* (fold-change: 0.4 ± 0.5), *Tgfβ1* (fold-change: 0.4 ± 0.3) and *Ctgf* at the third day (Fig. 2A); as well as of *Tgfβ1* (fold-change: 0.5 ± 0.3), *Fgf7* (fold-change: 0.5±0.4), *Ctgf* (fold-change: 0.5 ± 0.3) and *Fgf2* at the seventh day was also observed (Fig. 2B).

G*lp2r* expression was upregulated after ileal resection and anastomosis, particularly at the third postoperative day, while it was downregulated after isolated laparotomy.

In the perianastomotic segment, lower fold-change of relative gene expression of *Vegfa* and *Ctgf* at the third day and of *Fgf7* at the seventh day was observed in comparison with the ileal sample recovered after isolated laparotomy (Figs. 2A and B).

RESPONSE OF TISSUE GROWTH FACTORS GENE EXPRESSION TO ISOLATED LAPAROTOMY

In the ileal samples recovered after isolated laparotomy, upregulation of gene expression of *Vegfα* (fold-change: 4.1 ± 1.1), *Ctgf* (fold-change: 3.4 ± 1.6) and *Egf* at the third postoperative day and of *Egf* (fold-change: 3.4 ± 1.3), *Fgf7* (fold-change: 3.1 ± 1.0), *Vegfa* and *Hbegf* at the seventh day was documented (Figs. 2A and B). Furthermore, downregulation of *Tgfβ1* (fold-change: 0.1 ± 0.1), *Pdgfb* (fold-change: 0.3 ± 0.1), *Fgf2* (fold-change: 0.5 ± 0.3), *Igf1*, *Fgf7*, *Hbegf* and *Glp2r* at the third day after the operation; as well as of *Ctgf* (fold-change: 0.2 ± 0.1), *Glp2r* (fold-change: 0.5 ± 0.4), *Tgfβ1* (fold-change: 0.5 ± 0.1), *Fgf2* and *Pdgfb* at the seventh day was also evident.

RESPONSE OF TISSUE GROWTH FACTORS GENE EXPRESSION TO TEDUGLUTIDE POSTOPERATIVE ADMINISTRATION

At the third postoperative day (in the anastomotic segment and in the ileal sample recovered after isolated laparotomy), teduglutide was significantly associated with a higher fold-change of relative gene expression of *Igf1* (5.6 ± 8.0 *vs.* 0.7 ± 0.7, p = 0.0001) and *Hbegf* (1.5 ± 2.8 *vs.* 0.6 ± 0.4, p = 0.001) and lower fold-change of *Fgf2* (0.5 ± 0.2 *vs.* 0.8 ± 5.5, p = 0.002) and *Fgf7* (0.2 ± 0.7 *vs.* 1.3 ± 11.4, p = 0.0001) (Fig. 3). At the seventh postoperative day, teduglutide was significantly associated with higher fold-change of relative gene expression of *Vegfa* (3.6 ± 2.0 *vs.* 1.5 ± 0.6, p = 0.0001), *Ctgf* (1.1 ± 2.4 *vs.* 0.3 ± 0.4, p = 0.0001) and *Hbegf* (3.0 ± 1.6 *vs.* 1.3 ± 0.7, p = 0.004) and lower fold-change of *Tgfβ1* (0.02 ± 0.04 *vs.* 0.5 ± 0.5, p = 0.0001), *Fgf7* (0.4 ± 0.4 *vs.* 0.9 ± 2.6, p = 0.007) and *Glp2r* (0.4 ± 0.2 *vs.* 1.0 ± 0.7, p = 0.0001) (Fig. 3).

In all the animals (n = 59), at the sacrifice, teduglutide was significantly associated with higher fold-change of relative gene expression of *Vegfa* (3.6 ± 1.3 *vs.* 1.9 ± 2.0, p = 0.0001), *Hbegf* (2.2 ± 2.3 *vs.* 1.1 ± 0.9, p = 0.001), *Igf1* (1.6 ± 7.6 *vs.* 0.9 ± 0.7, p = 0.002) and *Ctgf* (1.1 ± 2.1 *vs.* 0.6 ± 2, p = 0.013) and lower fold-change of *Tgfβ1* (0.1 ± 0.4 *vs.* 0.4 ± 0.4, p = 0.002), *Fgf7* (0.4 ± 0.6 *vs.* 1.2 ± 3.0, p = 0.0001) and *Glp2r* (0.5 ± 6.1 *vs.* 1.0 ± 0.9, p = 0.042) (Fig. 4).

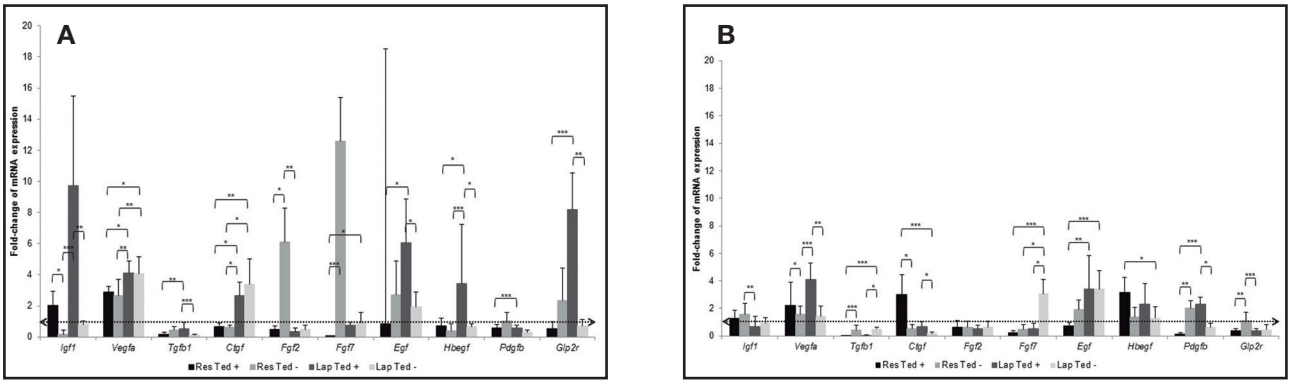

Figure 2.

Fold-changes of relative gene expression of growth factors and Glp2 receptor in the rats' ileum, at the third (A) and the seventh (B) days after operation determined by qRT-PCR. Animals *(n = 59)* were submitted to ileal resection and anastomosis *("Res")* or laparotomy *("Lap")* and sacrificed at the third or at the seventh postoperative days; in groups *"Res Ted +"* and *"Lap Ted +"*, teduglutide was administered after the operation. Samples recovered at the sacrifice from rats that underwent ileal resection corresponded to the anastomotic segment. Values were normalized to *Hprt* gene and fold-changes were generated by comparing with baseline values of rats submitted to ileal resection (n = 28). Results were expressed as median±interquartile range. Kruskal-Wallis test with pairwise comparisons was used. *p < 0.05; **p < 0.01; ***p < 0.001.

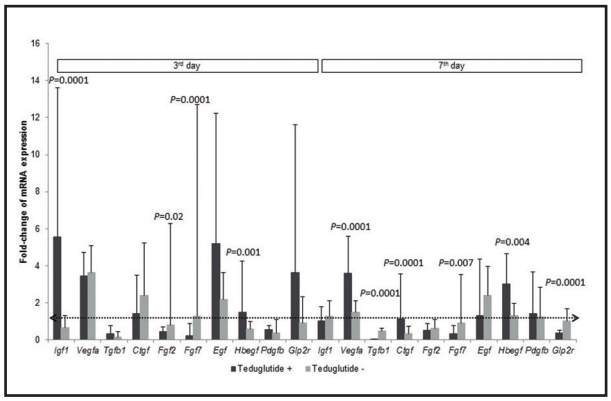

Figure 3.

Fold-change of relative gene expression of growth factors and *Glp2 receptor*, in the ileum of rats from all groups *(n = 59)*, according to teduglutide administration. Relative gene expression was determined at the moment of sacrifice (third or seventh day after ileal resection and anastomosis or after laparotomy) by qRT-PCR. Samples recovered at the sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Values were normalized to *Hprt* gene and fold-changes were generated by comparing with baseline values of rats submitted to ileal resection *(n = 28)*. Results were expressed as median ± interquartile range. Mann-Whitney U test was used.
Teduglutide + Postoperative teduglutide administration; *Teduglutide* - Without teduglutide administration.

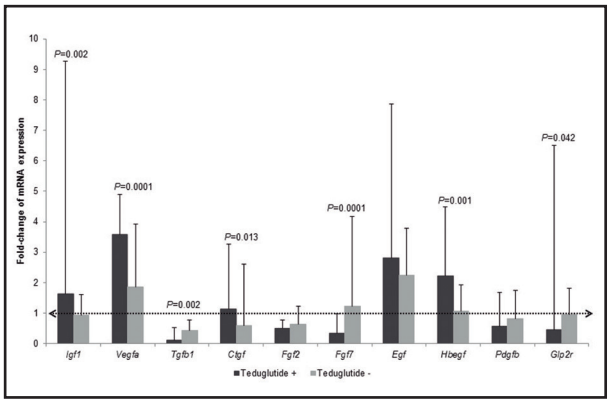

Figure 4.

Fold-change of relative gene expression of growth factors and *Glp2 receptor*, in the ileum of rats from all groups *(n = 59)* at the moment of sacrifice, according to teduglutide administration. Relative gene expression was determined at the moment of sacrifice (third or seventh day after ileal resection and anastomosis and after laparotomy) by qRT-PCR. Samples recovered at the sacrifice from rats submitted to ileal resection corresponded to the anastomotic segment. Values were normalized to *Hprt* gene and fold-changes were generated by comparing with baseline values of rats submitted to ileal resection *(n = 28)*. Results were expressed as median ± interquartile range. Mann-Whitney U test was used.
Teduglutide + Postoperative teduglutide administration; *Teduglutide* - Without teduglutide administration.

CORRELATIONS BETWEEN TISSUE GROWTH FACTORS GENE EXPRESSION AT THE SACRIFICE

At the sacrifice, *Glp2r* relative gene expression correlated directly with *Egf*, *Hbegf*, *Ctgf, Fgf7* and *Igf1* gene expressions and inversely with Glp2 plasma levels (Table II).

DISCUSSION

Results of present study, namely the upregulation of *Fgf7, Fgf2*, *Egf* and *Vegfa* gene expression levels at the third postoperative day and of *Pdgfb*, *Vegfa*, *Egf* and *Igf1* at the seventh day in the perianastomotic segment, re-enforce the recognized participation of those growth factors in the wound healing process (2,3). Upregulation of *Pdgfb* and *Igf1* gene expression occurred in the proliferative phase; whereas that of *Vegfa* was verified in both inflammatory and proliferative stages, as expected (2,3). However, downregulation of *Tgf*β1 and *Ctgf* gene expressions observed in the anastomotic segment at the third and at the seventh postoperative days was unexpected, giving the relevant participation of those growth factors in the anastomotic repair (2,14). In fact, Seigert GJ et al. (14) demonstrated recently a consistent upregulation of tissue *Tgf*β, *Ctgf* and *Igf1* gene expressions after ileo-ileal anastomosis.

In this experiment, teduglutide administration was significantly associated with higher fold-change of relative gene expression of *Igf1*, *Hbegf*, *Vegfa* and *Ctgf*, as well as with lower fold-change of *Tgf*β1 and *Fgf7* in the postoperative period.

Induction of *Igf1* messenger RNA expression in teduglutide-treated animals documented in this study was according to the literature (11) and was observed at the third day, both in the anastomotic segment and in the ileal sample recovered after isolated laparotomy. Igf1 is considered a critical mediator of the enterotrophic effects of Glp2 (15), although not systematically required (11). Igf1 promotes proliferation of small intestinal epithelium and participates in the fibroplasia, modulating the proliferation of fibroblasts and myofibroblasts and the collagen synthesis (15,16). Several studies demonstrated that this growth factor administration improves healing parameters, on animal models of colonic anastomosis, in high risk contexts (2,9).

An increase of *Hbegf* messenger RNA expression levels in teduglutide-treated animals was verified in present study, at both postoperative time points (although statistically significant only at the seventh day after isolated laparotomy). Involvement of the ErbB ligant-ErbB signaling pathway in the proliferative actions of Glp2 was suggested in previous experiments (11). Studies about the refeeding-induced mucosal proliferation revealed also the importance of ErbB signaling for the actions of endogenous Glp2 (11).

Hbegf has mitogenic and chemotactic effects on epithelial cells, smooth muscle cells and fibroblasts; promotes extracellular matrix synthesis and angiogenesis; modulates vasodilatation and preserves microcirculatory blood flow; improves intestinal motility and demonstrates anti-inflammatory effects (2,17,18). Moreover, this growth factor preserves the intestinal mucosa and restores gut barrier function after intestinal injury (17). Indeed, a potent intestinal cytoprotective effect of Hbegf on intestinal epithelial cells (including stem cells), endothelial cells, pericytes, immunocytes and neuronal cells has been demonstrated in animal models of

Table II. Correlations between relative gene expression of growth factors and Glp2 receptor in rats' small intestine and plasma levels of Glp2 at the moment of sacrifice (n = 59)[a]

σ / p	Igf1	Vegfa	Tgfβ1	Ctgf	Fgf2	Fgf7	Egf	Hbegf	Pdgfb	Glp2r	Plasma [Glp2]
Igf1			34.2% p = 0.009		-27.7% p = 0.036	-31.4% p = 0.016				30.4% p = 0.02	
Vegfa			-28.6% p = 0.029			-41.3% p = 0.001					
Tgfβ1	34.2% p = 0.009	-28.6% p = 0.029		-28.2% p = 0.032		34% p = 0.009	36.7% p = 0.005		29,6% p = 0,024	48.2% p = 0.0001	
Ctgf			-28.2% p = 0.032				27.3% p = 0.038		-52,6% p = 0,0001	37% p = 0.004	-40.7% p = 0.002
Fgf2	-27.7% p = 0.036					30.3% p = 0.021	53.2% p = 0.0001	31.8% p = 0.015			
Fgf7	-31.4% p = 0.016	-41.3% p = 0.001	34% p = 0.009		30.3% p = 0.021		44.4% p = 0.0001			37.6% p = 0.004	-40.5% p = 0.002
Egf			36.7% p = 0.005	27.3% p = 0.038	53.2% p = 0.0001	44.4% p = 0.0001		44.3% p = 0.001		52.7% p = 0.0001	-31.9% p = 0.016
Hbegf					31.8% p = 0.015		44.3 % p = 0.001			39.9% p = 0.002	
Pdgfb			29.6% p = 0.024	-52.6% p = 0.0001							
Glp2r	30.4% p = 0.02		48.2% p = 0.0001	37% p = 0.004		37.6% p = 0.004	52.7% p = 0.0001	39.9% p = 0.002			-30.6% p = 0.022
Plasma [Glp2]				-40.7% p = 0.002		-40.5% p = 0.002	-31.9% p = 0.016			-30.6% p = 0.022	

[a]*Relative gene expression of growth factors and Glp2 receptor and postoperative plasma levels of Glp2 ([Glp2]) were determined by qRT-PCR and competitive enzyme immunoassay, respectively. Spearman's rank correlation coefficient (σ) and level of significance (p) were presented.*
Igf1: insulin-like growth factor 1, transcript variant 1, mRNA; Vegfa: vascular endothelial growth factor A, transcript variant 2, mRNA; Tgfb1: transforming growth factor, beta 1, mRNA; Ctgf: connective tissue growth factor, mRNA; Fgf2: fibroblast growth factor 2, mRNA; Fgf7: fibroblast growth factor 7, mRNA; Egf: epidermal growth factor, mRNA; Hbegf: heparin-binding EGF-like growth factor, mRNA; Pdgfb: platelet-derived growth factor beta polypeptide, mRNA; Glp2r: glucagon-like peptide 2 receptor, mRNA.

necrotizing enterocolitis, ischemia/reperfusion injury, and hemorrhagic shock and resuscitation (17). Furthermore, in 2011, Radulescu A et al. (18) demonstrated, on an animal model, that exogenous Hbegf promoted intestinal anastomotic repair and that *Hbegf* (-/-) knockout mice had worse healing scores and higher morbidity and mortality rates after intestinal anastomosis.

Induction of *Vegfa* and *Ctgf* gene expression levels observed in this study in teduglutide-treated animals at the seventh day suggest that these growth factors may be also relevant as downstream mediators of Glp2 effects in the perioperative context.

Vegfa has an important participation in wound healing as it promotes the early events of angiogenesis (namely endothelial cell migration, proliferation and differentiation) and lymphangiogenesis (2,19). In 2012, Enestvedt CK et al. (20) demonstrate a favorable impact of recombinant *Vegf* gene therapy on the healing of an ischemic esophagogastrostomy on an animal model, including enhanced neovascularization, blood flow and bursting pressure. After intraoperative local Vegfa administration in a rabbit model of colonic anastomoses, Ishii M et al. (21) found improved bursting pressure, increased hydroxyproline levels and, also, significantly enhanced submucosal capillary vascular counts.

Ctgf is considered a key determinant in the formation and maintenance of connective tissues and in the wound repair process (19,22,23). In fact, this growth factor promotes proliferation, differentiation and chemotaxis of fibroblasts, epithelial-mesenchymal transition, extracellular matrix formation and remodeling, re-epithelialization (by stimulation of cell migration) and angiogenesis (19,22,23). Discrepant responses of *Ctgf* and *Tgf*β1 gene expressions to teduglutide administration, observed in our study, were surprising because Ctgf is controlled by Tgfβ1 in a Smad-dependent way and acts as a downstream mediator of Tgfβ action on connective tissue cells (19,22).

Our data indicated that teduglutide administration was associated with downregulation of *Tgfβ1* gene expression in the anastomotic segment at both moments of evaluation. This fact raises concern about a potential negative impact on the anastomotic healing, since Tgfβ participates in all phases of wound healing (2,19).

Tgfβ is a pleiotropic polypeptide hormone that modulates the mucosal immune response and tissue remodeling in the gut. In fact, Tgfβ downregulates the production of proinflammatory cytokines, promotes the differentiation of regulatory T-cells and induces the production of Immunoglobulin A (22,24). This growth factor also regulates extracellular matrix turnover and exerts an important role in tissue physiologic remodeling and wound repair in the intestine. Indeed, Tgfβ promotes the recruitment, proliferation, differentiation and activation of extracellular matrix-production cells (16,19,22,24) and the epithelial- and endothelial-mesenchymal transition (22); it also stimulates extracellular matrix production and deposition (including types I and III collagens, fibronectin and proteoglycans) and inhibits its degradation (including inhibition of matrix metalloproteinases 1, 3 and 9) (16,19,22). Tgfβ promotes epithelial restitution inducing the migration of epithelial cells across the wound margin (22,24), stimulates the recruitment of inflammatory cells and macrophage-mediated tissue debridement (19), promotes angiogenesis (thought upregulation of Vegf) (19,22) and participates in wound contraction (19). Adenoviral-mediated transfer of *Tgfβ*1 on an animal model of colonic anastomoses, through intraluminal local administration, was associated with a significant increase of the anastomotic bursting pressure (25).

Downregulation of *Fgf7* gene in animals submitted to teduglutide treatment documented in our study (statistically significant only in the anastomotic segment at the third day and in the ileal sample recovered after isolated laparotomy at the seventh day) was also unexpected because this growth factor has been proposed as one of the mediators of Glp2 action, particularly on the colonic mucosa (11). Fgf7 (also known as keratinocyte growth factor 1, Kgf1) is a mitogenic growth factor with an important role in the intestinal epithelial growth, maintenance and repair and in preservation of the barrier function (2,19,26). A favorable effect of this growth factor on intestinal mucosal protection has been demonstrated, on experimental studies, in chemically induced inflammatory bowel disease, chemotherapy and radiation mucositis, ischemia/reperfusion syndrome, short bowel syndrome and total parenteral nutrition contexts (26). Intraperitoneal administration of truncated Kgf on an animal model of colonic anastomosis was associated with enhanced anastomotic bursting pressure, lower inflammatory activity on histological examination and higher crypt cell proliferation rates (27).

As outlined above, most of the Glp2 effects are indirect and secondary to endocrine, paracrine, autocrine and neural signaling activated by the Glp2r (11). In present study, higher correlation coefficients were expected between relative mRNA expressions of *Glp2r* and *Igf1*, *Hbegf* and *Fgf7* gene expression levels, because those growth factors have been recognized as molecular downstream mediators of the Glp2r signaling in gastrointestinal tract (11).

Gene expressions of *Fgf2* and *Vegfa*, two of the most important mediators of neoangiogenesis (3), did not correlated significantly. In relation to the Glp2/Glp2r axis, our study suggests that tissue G*lp2r* expression may be negatively affected by increased Glp2 plasma concentrations.

In conclusion, results of present study underscore the recognized role of *Igf1* and *Hbegf* as molecular mediators of the effects of teduglutide and suggest that other humoral factors, like *Vegfa* and *Ctgf* may be also relevant in the perioperative context of intestinal anastomosis. A negative influence of teduglutide on postoperative *Tgfβ*1 relative gene expression was also indicated.

Albeit the negative impact on postoperative tissue *Tgfβ*1, induction of *Vegfa*, *Igf1* and *Ctgf* gene expressions might indicate a favorable influence of teduglutide on the intestinal anastomotic healing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Study was approved by the Ethics Committee of Faculty of Medicine, University of Coimbra, Coimbra, Portugal (License nº32-06-2009) and undertaken according to institutional and national animals' protection guidelines.

AUTHORS' CONTRIBUTIONS

- Study conception and design: Costa B.
- Acquisition of data: Costa B, Gonçalves AC, Alves R, Abrantes AM, Matafome P.
- Analysis and interpretation of data: Costa B.
- Drafting of manuscript: Costa B.
- Critical review: Seiça R, Sarmento-Ribeiro AB, Botelho MF, Castro Sousa F.

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