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Pentadecapeptide BPC 157 and the esophagocutaneous fistula healing therapy

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ABSTRACT

Esophagocutaneous fistulas are a failure of the NO-system, due to NO-synthase blockage by the NOS-blocker L-NAME consequently counteracted by L-arginine and gastric pentadecapeptide BPC 157 (L-arginine < BPC 157), precipitating a therapeutic benefit. Previously, there was an established BPC 157—NO-system interaction. BPC 157 GEPPPGKPADDAGLV, MW 1419 (LD1 not achieved), is a safe and stable anti-ulcer peptide, successful in inflammatory bowel disease trials, counteracting esophagitis, sphincter failure, gastrointestinal and skin ulcers, gastrocutaneous or colocutaneous fistulas. We treated rats with established cervical esophagocutaneous fistulas throughout four days (both open skin and esophageal defects, with significant leakage) with BPC 157 (parenterally and perorally) and L-NAME (blocking NO genesis) and L-arginine (NO-substrate) alone or in combination. RT-PCR investigated eNOS, iNOS, COX-2 mRNA levels in the fistulas. We evidenced a closely inter-related process of unhealed skin, esophageal defects, unhealed fistulas (up regulated eNOS, iNOS and COX2 mRNA levels), usually lethal, particularly NO-system related and therapy dependent. Generally, the course of fistula healing was accelerated either to a greater extent (with BPC 157 (in particular, less eNOS gene expression) completely counteracting L-NAME effects, in L-NAME+BPC 157 and L-NAME+L-arginine+BPC 157 groups), or to a lesser extent (with L-arginine). Conversely, the process was aggravated, rapidly and prominently (with L-NAME). In particular, BPC 157 was effective either given per-orally/intraperitoneally, in μg- and ng-regimens. Shortly, defects started to heal, with less fistula leakage and no mortality at day 4. Failure of pyloric and lower esophageal sphincter pressure was restored, with practically no esophagitis.

1. Introduction

We focused on stable gastric pentadecapeptide BPC 157 (for review see, i.e., Sikiric et al., 2010, 2011, 2012), NO-system related agents and esophagocutaneous fistula healing in rats.

The healing of esophagocutaneous fistulas is a particular problem (Foster and Lefor, 1996) and various surgical procedures have been suggested (Morita et al., 2010). Meanwhile, a relevant approach in terms of pharmacotherapy is still lacking (Yurtçu et al., 2009) and studies in rats have not been performed (Tobin et al., 1985; Rábago et al., 2002; Yurtçu et al., 2009). Likewise, a relation between esophagocutaneous fistula – skin and esophagitis wounds – and the NO-system has not been determined. While eNOS, iNOS, and COX-2 were up-regulated in esophagitis and skin wounds (Inamori et al., 2006; Patruno et al., 2010; Singh et al., 2011), eNOS, iNOS, COX-2 mRNA levels were not determined in esophagocutaneous fistulas. Recently, the stable gastric pentadecapeptide BPC 157 (known to interact with the NOS-blocker, L-NAME, and the NO-precursor, L-arginine (Balenovic et al., 2009; Boban-Blagaic et al., 2006; Klicek et al., 2008; Lovric-Bencic et al., 2004; Sikiric et al., 1997)) has been shown to heal gastrocutaneous and colocutaneous fistulas (Klicek et al., 2008; Skorjanec et al., 2009) (thereby, simultaneously inducing the healing of various tissues), via the NO-system (Klicek et al., 2008). This would appear to be a rather logical approach in light of the widely suggested importance of NO as an essential signaling pathway, in the gastrointestinal and cardiovascular systems (for review, see Moncada et al., 1991; Whittle et al., 1992), and an important system in wound healing (Frank et al., 1998).

Thus, to resolve the problem of the healing of esophagocutaneous fistulas, we considered that esophagocutaneous fistulas, which are regularly lethal in rats, to be a failure in the healing process that is likely to be NO-system related. Bearing in mind the recognized dual role of NO, where either an inhibition or an uncontrolled excess, could possibly lead to significant damage

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(Lopez-Belmonte et al., 1993; Whittle et al., 1992), a beneficial effect was suggested for L-arginine, the precursor of NO (for review see Klicek et al., 2008; Sikiric et al., 1997; Whittle et al., 1992). Contrary, L-arginine analogues and NO generation inhibitors are consistently claimed to be ulcerogenic (for review see Klicek et al., 2008; Sikiric et al., 1997; Whittle et al., 1992) (but, L-NAME, dose-dependently, prevented the aggravation of indomethacin–gastric lesions in arthritic rats (Kato et al., 2009)). Thereby, to resolve the possible (dual) effect of the NO-system in esophagocutaneous fistulas and the healing effect of BPC 157, both NOS blockade (L-NAME) and L-arginine application will be investigated at the same time, as was the case in our previous BPC 157/NO-system interaction studies (Balenovic et al., 2009; Boban-Blagaic et al., 2006; Grabarevic et al., 1997; Klicek et al., 2008; Lovric-Bencic et al., 2004; Sikiric et al., 1997). In order to ascertain the NO-system relations, we suggest an aggravation that would be caused by L-NAME, a NO-synthase blocker, whose deleterious effects would be corrected by L-arginine. An even greater therapeutic effect with the stable gastric pentadecapeptide BPC 157 should consequently be expected (Balenovic et al., 2009; Klicek et al., 2008; Lovric-Bencic et al., 2004; Sikiric et al., 1997). Besides, by using the reverse transcription–polymerase chain reaction (RT-PCR), this study will investigate whether BPC 157 can improve the esophagocutaneous fistula healing course by specifically influencing eNOS, iNOS, COX-2 mRNA levels in fistulas. Additionally, considering the importance of the sphincter for the integrated function of the whole upper gastrointestinal tract (Sidhu and Triadafilopoulos, 2008) and assuming that that integrity may be largely disturbed by esophagocutaneous fistulas, aside from esophagitis, the effect on lower esophageal and pyloric sphincters was also investigated.

Considering the particular severity of esophagocutaneous fistulas and thereby particular ability to heal such fistulas,—it should be mentioned that the stable gastric pentadecapeptide BPC 157 is likely a novel mediator of Robert's cytoprotection and adaptive cytoprotection (Sikiric et al., 2010, 2011, 2012). Since BPC 157 is a stable compound, not degraded in human gastric juice (more than 24 h) and thereby suitably applied per-orally (i.e., in drinking water), BPC 157 represents a novel wel L-matched anti-ulcer peptide (producing no reported toxicity, and effective alone without carrier (Sikiric et al., 2010, 2011, 2012)), particularly effective in the whole gastrointestinal tract (GEPPPGKPADDAGLV, M.W. 1419, PL 14736, in trials for inflammatory bowel disease)) which could provide a valuable modality for wound treatment (Bilic et al., 2005; Mikus et al., 2001; Seveljevic-Jaran et al., 2006; Sikiric et al., 2003, 2010, 2011, 2012). With stimulation of expression of the early growth response 1 (egr-1) gene (Tkalcevic et al., 2007) and its co-repressor nerve growth factor 1-A binding protein-2 (NAB2), it exhibited a particular effect on collagen, endothelium protection (Hrelec et al., 2009; Sikiric et al., 1994, 2010, 2011, 2012) and prominent angiogenesis (Brcic et al., 2009; Sikiric et al., 1999b, 2006). Accordingly, it may particularly heal both internal and external wounds (Sikiric et al., 2010, 2011, 2012), including esophagocutaneous fistulas. Likewise, BPC 157 also specifically rescued esophagitis and various sphincter failures (Dobric et al., 2007; Petrovic et al., 2006; Sikiric et al., 1999a, 2010, 2011, 2012).

2. Material and methods

2.1. Animals

Wistar Albino male rats (200 g b.w.) were randomly assigned to the experiments (10 animals at least, per each experimental group), all of which were approved by the Local Ethics Committee. Furthermore, all experiments were carried out under a blind protocol, and the effect was assessed by examiners who were completely unaware of the given protocol.

2.2. Drugs

Pentadecapeptide BPC 157 (GEPPPGKPADDAGLV, M.W. 1419), (Diagen, Ljubljana, Slovenia) dissolved in saline, was used in all experiments. The peptide, BPC 157, is part of the sequence of the human gastric juice protein BPC, and is freely soluble in water at pH 7.0 and saline. It was prepared, as described previously (Hrelec et al., 2009; Sikiric et al., 2010, 2011, 2012; Tkalcevic et al., 2007), with 99% high pressure liquid chromatography (HPLC) purity, expressing 1-des-Gly peptide as an impurity. L-NAME (Sigma, USA), L-arginine (Sigma, USA), were accordingly used (Sikiric et al., 2010, 2011, 2012).

2.3. Surgical procedure

In deeply anaesthetized rats, a cervical esophagocutaneous fistula was created (4 mm diameter esophagus, and 4 mm diameter skin defect, with a precise caliper used to verify the initial size of the defect).

2.4. Experimental protocol

BPC 157 was given per-orally, in drinking water (10 μg/kg, 10 ng/kg, i.e., 0.16 μg/ml, 0.16 ng/ml, 12 ml/rat/day) until sacrifice; or, intraperitoneally (10 μg/kg, 10 ng/kg) with first application at 30 min after surgery, last at 24 h before sacrifice. L-NAME (5 mg/kg i.p.) and/or L-arginine (100 mg/kg i.p.) were given alone or together, first application at 30 min after surgery, last at 24 h before sacrifice. BPC 157 (10 μg/kg, i.p. or p.o.), was given with L-NAME (5 mg/kg i.p.) and/or L-arginine (100 mg/kg i.p.). Controls simultaneously received an equivolume of saline (5.0 ml/kg i.p.) or water only. The assessment was at day 1, 2, 3, and 4, as follows (see, 2.5., 2.6., 2.7., 2.8.). Specifically, at day 4, in the fistulas of rats that received BPC 157 10 μg/kg daily in drinking water and in corresponding controls, RNA extraction and RT-PCR were carried out (see, 2.9.).

2.5. Assessment of esophageal and skin defects and fistulas.

A precise caliper was used to verify the final size of the defect (the largest diameter of the skin or esophageal defect (mm)), which was also photographed and further verified using the program ISSA (VAMSTEC Software Company, Zagreb, Croatia). The tissue was processed for further microscopic analysis (Klicek et al., 2008; Skorjanec et al., 2009). To assess fistula leakage, a separate group of animals received a volume of water per-orally, either to leakage induction, or to a maximal volume of 1 ml (Klicek et al., 2008; Skorjanec et al., 2009). In this case, if leakage did not occur after 1 min, the fistula was considered functionally closed.

2.6. Esophagitis assessment

In all rats, macroscopical assessment for esophagitis was completed accordingly, with scores of 0–4, using direct esophagus scanning (ScanMaker i900; Microtek, Willich, Germany) as described previously (Dobric et al., 2007; Petrovic et al., 2006, 2011; Sikiric et al., 2010; Yurtçu et al., 2009): normal, glistening mucosa (score 0); edematous mucosa with focal hemorrhagic spots (score 1); multiple erosions with hematins attached (score 2); tiny esophagus with hemorrhagic and linear yellowish lesions (score 3); tiny esophagus with coalesced hemorrhagic and

Table 1
Primer sequence.

Gene	Nucleotide sequence	Product size (bp)	GenBank Accession No.
GAPDH Glyceraldehyde-3-phosphate dehydrogenase	Sense: TGGCAAGTTCAACGGCACAGT Antisense: TTTGGCCTCACCCTTCAGGT	193	XM_221353
iNOS (NOS-2) Inducible nitric oxide synthase	Sense: TTGGAGCGAGTTGTGGATTGTTGTTC Antisense: GGTGAGGGCTTGCCTGAGTGAGC	126	NM_012611
eNOS (NOS-3) Endothelial nitric oxide synthase	Sense: CTGGCAAGACCGATTACACGA Antisense: TCAGGAGGTCTTGCACATAGG	206	NM_021838
COX-2 Cyclooxygenase-2	Sense: CTGTATCCCGCCCTGCTGGTG Antisense: CCACTTCTCCTCCGAAGGTGC	157	AF233596

yellowish lesions (score 4). Subsequently, the oesophagus samples were placed in 10% formalin and used for histopathological examination (Dobric et al., 2007; Petrovic et al., 2006, 2011).

2.7. Lower esophageal sphincter pressure assessment and pyloric sphincter pressure assessment

As described before (Dobric et al., 2007; Petrovic et al., 2006, 2011; Sikiric et al., 2010, 2011, 2012), manometrical evaluation (cm H_2O) was performed, in all rats, with a water manometer connected to the drainage port of the Foley catheter as described (the values of 68–76 cm H_2O for lower esophageal sphincter, and 68–74 cm H_2O for pyloric sphincter, were considered to be normal as determined before). The proximal side of the esophageal incision, or distal side of the duodenal incision, was ligated to prevent regurgitation (Dobric et al., 2007; Petrovic et al., 2006).

2.8. Weight assessment, food, water consumption, mortality assessment

Animals were weighed before surgery, and thereafter once daily, and before sacrifice. Weight loss (g) was presented as Δ between the initial and final weight (Sever et al., 2009). Mortality was assessed daily.

In separate experiments, rats received a training program in order to acclimate to metabolic cages, and were subjected for 48 h without any treatment, in order to get the basal levels of their eating and drinking behavior (i.e., before treatment). After four days resting in normal cages, they were subjected to the procedure as described above (Section 2.3), then they were subjected to metabolic cages, and food and water consumption was recorded daily. Comparison was made between the groups, before vs. after treatment, and presented as Δ between the initial and final value.

2.9. RNA extraction and RT-PCR

RNA was extracted from five 10 μm thick sections of paraffin embedded fistula tissue using the High Pure RNA Paraffin Kit (Roche, Basel, Switzerland). RNA density was measured using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, United States). Total RNA of 150 ng from each sample was used in RT-PCRs performed in a total volume of 25 μl using the QIAGEN OneStep RT-PCR Kit (Qiagen, Venlo, Netherlands). RT-PCR mixture contained 5 μl 5xQIAGEN OneStep RT-PCR Buffer, 1 μl dNTP Mix, 1.5 μl of 10 μM sense primer, 1.5 μl of 10 μM antisense primer, 1 μl OneStep RT-PCR Enzyme Mix,RNA as described and RNase-free water. RT-PCR was performed in a thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, United States). The samples were incubated at 55 °C for 30 min to enable reverse transcription followed by heating at 94 °C for 15 min to activate the Hot StartTaq DNA Polymerase. After that the samples were incubated at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, 40 cycles with GAPDH, iNOS, eNOS and COX-2 primers (Table 1). At the end, samples were incubated for 10 min at 72 °C. Results were expressed as the ratio of the optical density of the NOS PCR or COX-2 PCR products to the density of the corresponding GAPDH PCR products (Wolfrum et al., 2003).

2.10. Statistical analyses

Statistical analysis was performed by a non-parametric Kruskal–Wallis ANOVA test and, later, a Mann–Whitney U-test, to compare groups. Fisher exact probability test was used for mortality assessment. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Control

Weight loss was in accordance with the advance of esophagitis, sphincter failure and the persistence of both defects, with continuous leakage of fistulas, that was not restored by day 4 (Table 2). Also, the mortality rate was 70% by the 4th day (Table 3). The rats could not survive thereafter. RNA extraction and RT-PCR showed up-regulated eNOS, iNOS and COX2 expression in fistulas (Table 4).

3.2. BPC 157

Both defects started to heal within a short period of time, with less fistula leakage (Table 2). There was no mortality at day 4 (Table 2); and practically no esophagitis in any animal during the whole experiment. Also, higher PS pressure was observed. Likewise, failure of LES pressure was restored in BPC 157 rats (Table 1). Interestingly, the same efficacy was noted when BPC 157 was given per-orally or intraperitoneally, in μg- and ng-regimens (Tables 2 and 3). In fistulas, BPC 157 specifically affects gene expression (i.e., decreased eNOS-expression while COX2 and iNOS-expression remained up-regulated) (Table 4).

3.3. L-NAME

An already deleterious course of the cervical esophagocutaneous fistulas had further deteriorated. Increased weight loss was in accordance with an advanced and severe deterioration of esophagitis. An increase of both defects persisted. Increased continuous leakage of the fistula, unrestored by day 4, along with sphincter failure had been equally presented (Table 2). Also, the mortality rate was 70% by the 4th day (Table 3). The rats could not survive thereafter.

Table 2
Treatment with BPC 157, L-arginine, L-NAME and their combinations.

Medication given daily after creation of fistulas per-orally (p.o.) in drinking water, or intraperitoneally (i.p.) once time daily	Post-operative day	Daily sickness course in rats with esophagocutaneous fistulas					
		Skin defect, mm, means ± SD	Esophageal defect, mm, means ± SD	Fistula leaking, ml H_2O, means ± SD	Esophagitis, score (0–4), min/med/max	Pressure within lower esophageal sphincter, cm H_2O, means ± SD	Pressure within pyloric sphincter, cm H_2O, means ± SD
Drinking water (12 ml/rat/day) (control p.o.)	Day 1	3.4 ± 1.1	3.3 ± 1.1	0.15 ± 0.03	0/1/2	61 ± 2.9	49 ± 2.0
	Day 2	4.3 ± 1.2	3 ± 1.1	0.15 ± 0.02	1/2/2	54 ± 2.4	45 ± 1.0
	Day 3	5.1 ± 1.4	3.4 ± 1.2	0.15 ± 0.02	1/2/2	52 ± 2.1	40 ± 2.1
	Day 4	3.8 ± 1.2	3.1 ± 1.0	0.15 ± 0.02	1/2/3	50 ± 2.2	40 ± 2.1
BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	1.75 ± 0.3[a]	1.5 ± 0.3[a]	0.35 ± 0.11[a]	0/0/1[a]	69 ± 2.2[a]	65 ± 2.0[a]
	Day 2	2 ± 0.7[a]	1.75 ± 0.4[a]	0.35 ± 0.14[a]	0/0/1[a]	72 ± 2.1[a]	67 ± 2.0[a]
	Day 3	1.3 ± 0.3[a]	1 ± 0.2[a]	0.45 ± 0.17[a]	0/0/1[a]	67 ± 2.5[a]	68 ± 2.0[a]
	Day 4	1.3 ± 0.4[a]	1.5 ± 0.3[a]	0.55 ± 0.21[a]	0/0/1[a]	70 ± 1.1[a]	66 ± 1.0[a]
BPC 157 10 ng/kg p.o. (0.16 ng/ml)	Day 1	2 ± 0.6[a]	1.8 ± 0.3[a]	0.30 ± 0.09[a]	0/0/1[a]	68 ± 2.3[a]	59 ± 2.1[a]
	Day 2	2.25 ± 0.6[a]	1.7 ± 0.4[a]	0.35 ± 0.13[a]	0/0/1[a]	70 ± 3.1[a]	57 ± 2.0[a]
	Day 3	1.75 ± 0.4[a]	1.6 ± 0.5[a]	0.40 ± 0.10[a]	0/0.5/1[a]	66 ± 2.1[a]	60 ± 2.3[a]
	Day 4	1.2 ± 0.5[a]	1.4 ± 0.4[a]	0.56 ± 0.19[a]	0/0.5/1[a]	71 ± 3.1[a]	62 ± 2.3[a]
L-NAME 5 mg/kg i.p.	Day 1	5 ± 1.5[a]	6 ± 2.4[a]	0.08 ± 0.02[a]	1/1/2	57 ± 2.7	45 ± 2.0
	Day 2	6.4 ± 1.4[a]	6 ± 2.6[a]	0.08 ± 0.03[a]	1/2/2	55 ± 2.0	43 ± 1.0
	Day 3	6.4 ± 1.7[a]	5 ± 1.8[a]	0.06 ± 0.03[a]	2/2.5/3	48 ± 2.5	41 ± 2.1
	Day 4	6.2 ± 1.8[a]	5 ± 2.2[a]	0.05 ± 0.01[a]	2/4/4[a]	51 ± 2.2	41 ± 2.1
L-arginine 100 mg/kg i.p.	Day 1	2.9 ± 1.0	3.4 ± 1.2	0.16 ± 0.04	0/1/1	67 ± 2.2[a]	55 ± 2.1[a]
	Day 2	3.3 ± 1.1[a]	2 ± 0.7[a]	0.19 ± 0.04	1/1/2	65 ± 2.0[a]	61 ± 2.0[a]
	Day 3	3 ± 1.0[a]	2.5 ± 1.1[a]	0.17 ± 0.03	1/2/2	68 ± 2.8[a]	62 ± 2.0[a]
	Day 4	2.9 ± 1.4[a]	2.5 ± 0.9[a]	0.3 ± 0.11[a]	1/2/2	66 ± 2.4[a]	60 ± 2.0[a]
L-NAME 5 mg/kg i.p.+L-arginine 100 mg/kg i.p.	Day 1	4.1 ± 1.4	3.25 ± 1.8	0.13 ± 0.01	1/1.5/2	60 ± 2.6	46 ± 1.8
	Day 2	3.2 ± 1.3	3.5 ± 1.3	0.2 ± 0.08	1/2/2	61 ± 2.1[a]	50 ± 2.2[a]
	Day 3	3.5 ± 1.2	3.5 ± 1.3	0.22 ± 0.07	1/2/2	63 ± 2.1[a]	55 ± 2.4[a]
	Day 4	3 ± 0.9	3.5 ± 1.5	0.21 ± 0.07	1/2/2	65 ± 2.2[a]	55 ± 2.4[a]
L-NAME 5 mg/kg i.p.+BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	3.8 ± 1.7	3.6 ± 1.5	0.20 ± 0.06	0/1/1	69 ± 2.4[a]	65 ± 2.6[a]
	Day 2	3 ± 1.2[a]	2.5 ± 1.1[a]	0.27 ± 0.06[a]	0/0/1[a]	70 ± 2.1[a]	63 ± 2.4[a]
	Day 3	3.4 ± 1.1[a]	2.5 ± 1.2[a]	0.51 ± 0.17[a]	0/0/1[a]	71 ± 3.2[a]	67 ± 2.0[a]
	Day 4	2.4 ± 0.9[a]	2 ± 0.8[a]	0.55 ± 0.16	0/0/1[a]	71 ± 2.9[a]	66 ± 1.1[a]
L-arginine 100 mg/kg i.p.+BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	3.2 ± 0.8	2.5 ± 0.7[a]	0.17 ± 0.04	0/1/1	69 ± 2.2[a]	63 ± 2.4[a]
	Day 2	3 ± 1.1[a]	2.4 ± 0.7[a]	0.19 ± 0.03	0/0/1[a]	65 ± 2.4[a]	57 ± 2.5[a]
	Day 3	3 ± 0.9[a]	1.5 ± 0.4[a]	0.56 ± 0.27[a]	0/0/1[a]	66 ± 1.8[a]	57 ± 2.4[a]
	Day 4	2.25 ± 0.5[a]	1.2 ± 0.2[a]	0.45 ± 0.15[a]	0/0/1[a]	70 ± 2.7[a]	63 ± 2.6[a]
L-NAME 5 mg/kg i.p.+L-arginine 100 mg/kg i.p.+BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	3.6 ± 0.7	2.3 ± 0.9[a]	0.11 ± 0.02	0/1/1	68 ± 2.5[a]	59 ± 2.0[a]
	Day 2	2.4 ± 1.0[a]	2.25 ± 1.2[a]	0.42 ± 0.15[a]	0/0.5/1[a]	65 ± 2.8[a]	57 ± 2.1[a]
	Day 3	2.75 ± 0.8[a]	1.5 ± 0.4[a]	0.45 ± 0.11[a]	0/0/1[a]	66 ± 2.5[a]	60 ± 2.8[a]
	Day 4	2.5 ± 1.2[a]	1.3 ± 0.6[a]	0.48 ± 0.12[a]	0/0/1[a]	71 ± 3.1[a]	62 ± 2.7[a]
0.9% NaCl 5 ml/kg i.p. (control i.p.)	Day 1	3.4 ± 1.2	3.3 ± 1.6	0.17 ± 0.04	0/1/2	60 ± 2.6	49 ± 2.0
	Day 2	4.3 ± 1.6	3 ± 1.5	0.15 ± 0.03	1/2/2	55 ± 2.4	45 ± 1.0
	Day 3	5.1 ± 1.9	3.4 ± 1.4	0.14 ± 0.02	1/2/3	51 ± 2.4	41 ± 2.1
	Day 4	3.8 ± 1.4	3.1 ± 1.3	15 ± 0.03	1/2/3	50 ± 2.1	40 ± 2.1
BPC 157 10 μg/kg i.p.	Day 1	1.5 ± 0.3[a]	1.6 ± 0.5[a]	0.35 ± 0.12[a]	0/1/1[a]	69 ± 2.1[a]	65 ± 2.0[a]
	Day 2	1.5 ± 0.4[a]	1.5 ± 0.5[a]	0.35 ± 0.13[a]	0/0/1[a]	72 ± 2.7[a]	67 ± 2.0[a]
	Day 3	1.3 ± 0.3[a]	1 ± 0.4[a]	0.45 ± 0.17[a]	0/0/1[a]	67 ± 2.1[a]	68 ± 2.0[a]
	Day 4	1.3 ± 0.2[a]	1.3 ± 0.4[a]	0.55 ± 0.26[a]	0/0/1[a]	70 ± 2.8[a]	66 ± 1.0[a]
BPC 157 10 ng/kg i.p.	Day 1	2 ± 1.0[a]	1.8 ± 0.5[a]	0.30 ± 0.12[a]	0/1/1	68 ± 2.3[a]	59 ± 2.4[a]
	Day 2	2.25 ± 0.6[a]	1.7 ± 0.4[a]	0.35 ± 0.13[a]	0/0/1[a]	70 ± 2.1[a]	57 ± 2.3[a]
	Day 3	1.75 ± 0.5[a]	1.6 ± 0.3[a]	0.43 ± 0.13[a]	0/0.5/1[a]	66 ± 2.0[a]	60 ± 2.6[a]
	Day 4	1.2 ± 0.3[a]	1.4 ± 0.3[a]	0.57 ± 0.27[a]	0/0.5/1[a]	71 ± 3.0[a]	62 ± 2.7[a]
L-NAME 5 mg/kg i.p.+BPC 157 10 μg/kg i.p.	Day 1	3.8 ± 1.1	3.6 ± 1.5	0.22 ± 0.04	0/1/1	69 ± 2.2[a]	65 ± 2.4[a]
	Day 2	3 ± 0.9[a]	2.3 ± 0.7[a]	0.25 ± 0.06[a]	0/0/1[a]	70 ± 2.4[a]	63 ± 2.2[a]
	Day 3	3.4 ± 1.2[a]	2.5 ± 1.0[a]	0.54 ± 0.22[a]	0/0/1[a]	71 ± 2.2[a]	67 ± 3.0[a]
	Day 4	2.4 ± 0.9[a]	2 ± 0.9[a]	0.58 ± 0.18[a]	0/0/1[a]	71 ± 2.7[a]	66 ± 2.6[a]
L-arginine 100 mg/kg i.p.+BPC 157 10 μg/kg i.p.	Day 1	3.2 ± 1.4	2.5 ± 0.8[a]	0.17 ± 0.04	0/1/1	69 ± 2.2[a]	63 ± 2.6[a]
	Day 2	3 ± 1.3[a]	2.4 ± 1.2[a]	0.19 ± 0.05	0/0/1[a]	65 ± 2.4[a]	57 ± 2.4[a]
	Day 3	3 ± 1.2[a]	1.5 ± 0.4	0.56 ± 0.19[a]	0/0/1[a]	66 ± 1.9[a]	57 ± 2.2[a]
	Day 4	2.25 ± 1.0[a]	1.2 ± 0.4[a]	0.45 ± 0.17[a]	0/0/1[a]	70 ± 2.1[a]	63 ± 2.1[a]
L-NAME 5 mg/kg i.p.+L-arginine 100 mg/kg i.p.+ BPC 157 10 μg/kg i.p.	Day 1	3.6 ± 1.5	2.5 ± 1.3[a]	0.26 ± 0.06	0/1/1	66 ± 2.5[a]	59 ± 2.1[a]
	Day 2	2.4 ± 1.1[a]	2.45 ± 0.7[a]	0.44 ± 0.16[a]	0/0.5/1[a]	65 ± 2.0[a]	57 ± 2.2[a]
	Day 3	2.75 ± 0.8[a]	1.5 ± 0.5[a]	0.45 ± 0.15[a]	0/0/1[a]	66 ± 2.4[a]	60 ± 2.4[a]
	Day 4	2.5 ± 0.8[a]	1.3 ± 04[a]	0.43 ± 0.13[a]	0/0/1[a]	71 ± 2.5[a]	62 ± 2.6[a]

Daily sickness course in rats with esophagocutaneous fistulas, skin defect, mm, esophageal defect, mm, fistula leaking, ml H_2O, pressure within sphincter, lower esophageal and pyloric, cm H_2O, means ± SD, esophagitis, score (0–4), min/med/max. Medication was given daily after creation of fistulas per-orally (p.o.) in drinking water till the end of experiments, or intraperitoneally (i.p.) once time daily, first application at 30 min, last at 24 h before sacrifice.

[a] $P < 0.05$, at least, vs. control.

3.4. L-arginine

The healing of both defects could not be seen before the second post-operative day; meanwhile, the fistula leakage was decreased at day 4 (Table 2). There was no mortality at day 4 (Table 3); the esophagitis presentation was not affected. Also, we noted a higher PS and LES pressure (Tables 2 and 3).

Table 3
Treatment with BPC 157, L-arginine, L-NAME and their combinations.

Medication given daily after creation of fistulas per-orally (p.o.) in drinking water, or intraperitoneally (i.p.) once time daily	Post-operative day	Daily sickness course in rats with esophagocutaneous fistulas			
		Water intake, (ml), Δ, before–after, means ± SD	Food intake, (g), Δ, before–after, means ± SD	Weight loss, (g), Δ, before–after, means ± SD	Number of rats, dead/survived
Drinking water (12 ml/rat/day) (control p.o.)	Day 1	−3.1 ± 0.5	13.7 ± 0.5	20 ± 3	3/7
	Day 2	−3.0 ± 0.3	14.0 ± 0.4	48 ± 4	3/7
	Day 3	−3.2 ± 0.5	12.4 ± 0.6	47 ± 5	5/5
	Day 4	−2.9 ± 0.4	13.1 ± 0.5	58 ± 4	7/3
BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	2.1 ± 0.3[a]	11.1 ± 0.5[a]	6 ± 2[a]	0/10[a]
	Day 2	2.2 ± 0.5[a]	10.2 ± 0.5[a]	11 ± 3[a]	0/10[a]
	Day 3	2.2 ± 0.6[a]	10.4 ± 0.5[a]	27 ± 4[a]	0/10[a]
	Day 4	2.0 ± 0.2[a]	10.7 ± 0.5[a]	30 ± 4[a]	0/10[a]
BPC 157 10 ng/kg p.o. (0.16 ng/ml)	Day 1	1.4 ± 0.4[a]	10.8 ± 0.5[a]	10 ± 2[a]	0/10[a]
	Day 2	1.3 ± 0.1[a]	10.5 ± 0.3[a]	22 ± 4[a]	0/10[a]
	Day 3	2.1 ± 0.3[a]	10.4 ± 0.3[a]	31 ± 5[a]	0/10[a]
	Day 4	1.9 ± 0.4[a]	10.0 ± 0.2[a]	40 ± 4[a]	0/10[a]
L-NAME 5 mg/kg i.p.	Day 1	−4.8 ± 0.3[a]	15.4 ± 0.1[a]	44 ± 5[a]	3/7
	Day 2	−5.0 ± 0.6[a]	15.7 ± 0.2[a]	59 ± 7[a]	3/7
	Day 3	−5.2 ± 0.7[a]	15.2 ± 0.5[a]	73 ± 8[a]	5/5
	Day 4	−5.3 ± 0.8[a]	16.0 ± 0.5[a]	75 ± 6[a]	7/3
L-arginine 100 mg/kg i.p.	Day 1	0.4 ± 0.3[a]	13.2 ± 0.3	22 ± 3	0/10[a]
	Day 2	0.5 ± 0.3[a]	13.7 ± 0.4	47 ± 4	0/10[a]
	Day 3	0.5 ± 0.5[a]	13.0 ± 0.2	47 ± 4	0/10[a]
	Day 4	0.6 ± 0.4[a]	14.0 ± 0.7	63 ± 5	0/10[a]
L-NAME 5 mg/kg i.p.+L-arginine 100 mg/kg i.p.	Day 1	0.3 ± 0.1[a]	13.2 ± 0.9	30 ± 2	0/10[a]
	Day 2	0.9 ± 0.5[a]	13.7 ± 0.7	43 ± 3	0/10[a]
	Day 3	0.7 ± 0.6[a]	13.1 ± 0.8	40 ± 4	0/10[a]
	Day 4	0.6 ± 0.6	14.0 ± 0.9	55 ± 6	0/10[a]
L-NAME 5 mg/kg i.p.+BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	2.1 ± 0.5[a]	9.9 ± 0.3[a]	9 ± 2[a]	0/10[a]
	Day 2	2.1 ± 0.8[a]	10.4 ± 0.4[a]	15 ± 3[a]	0/10[a]
	Day 3	2.4 ± 0.7[a]	10.0 ± 0.8[a]	30 ± 4[a]	0/10[a]
	Day 4	2.2 ± 0.3[a]	10.2 ± 0.1[a]	35 ± 4[a]	0/10[a]
L-arginine 100 mg/kg i.p.+BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	1.8 ± 0.5[a]	10.7 ± 0.5[a]	12 ± 2[a]	0/10[a]
	Day 2	1.7 ± 0.4[a]	10.2 ± 0.8[a]	14 ± 3[a]	0/10[a]
	Day 3	2.0 ± 0.5[a]	10.0 ± 0.9[a]	24 ± 3[a]	0/10[a]
	Day 4	2.1 ± 0.7[a]	10.4 ± 0.3[a]	27 ± 4[a]	0/10[a]
L-NAME 5 mg/kg i.p.+L-arginine 100 mg/kg i.p.+BPC 157 10 μg/kg p.o. (0.16 μg/ml)	Day 1	1.9 ± 0.3[a]	10.5 ± 0.4[a]	10 ± 2[a]	0/10[a]
	Day 2	2.0 ± 0.4[a]	10.1 ± 0.8[a]	18 ± 3[a]	0/10[a]
	Day 3	2.2 ± 0.7[a]	10.0 ± 0.6[a]	27 ± 2[a]	0/10[a]
	Day 4	2.1 ± 0.3[a]	10.6 ± 0.7[a]	30 ± 4[a]	0/10[a]
0.9% NaCl 5 ml/kg i.p. (control i.p.)	Day 1	−3.3 ± 0.5	13.2 ± 0.5	20 ± 4	3/7
	Day 2	−2.9 ± 0.4	13.8 ± 0.5	48 ± 7	3/7
	Day 3	−3.2 ± 0.5	13.3 ± 0.5	47 ± 6	5/5
	Day 4	−3.3 ± 0.5	15.0 ± 0.5	58 ± 8	7/3
BPC 157 10 μg/kg i.p.	Day 1	1.8 ± 0.4[a]	10.0 ± 0.3[a]	10 ± 2[a]	0/10[a]
	Day 2	1.7 ± 0.7[a]	9.6 ± 0.8[a]	15 ± 3[a]	0/10[a]
	Day 3	1.6 ± 0.5[a]	9.4 ± 0.9[a]	25 ± 3[a]	0/10[a]
	Day 4	2.0 ± 0.5[a]	9.7 ± 0.7[a]	38 ± 4[a]	0/10[a]
BPC 157 10 ng/kg i.p.	Day 1	1.5 ± 0.5[a]	10.2 ± 0.8[a]	11 ± 2[a]	0/10[a]
	Day 2	1.3 ± 0.3[a]	9.3 ± 0.6[a]	20 ± 3[a]	0/10[a]
	Day 3	2.0 ± 0.6[a]	9.4 ± 0.8[a]	37 ± 4[a]	0/10[a]
	Day 4	2.1 ± 0.3[a]	10.3 ± 0.8[a]	40 ± 4[a]	0/10[a]
L-NAME 5 mg/kg i.p.+BPC 157 10 μg/kg i.p.	Day 1	2.2 ± 0.5[a]	10.2 ± 0.8[a]	9 ± 2[a]	0/10[a]
	Day 2	2.5 ± 1.5[a]	9.1 ± 0.6[a]	15 ± 3[a]	0/10[a]
	Day 3	2.3 ± 1.1[a]	9.1 ± 0.7[a]	30 ± 5[a]	0/10[a]
	Day 4	2.0 ± 0.4[a]	9.3 ± 0.9[a]	35 ± 5[a]	0/10[a]
L-arginine 100 mg/kg i.p.+BPC 157 10 μg/kg i.p.	Day 1	2.1 ± 0.6[a]	10.4 ± 0.4[a]	12 ± 2[a]	0/10[a]
	Day 2	2.2 ± 0.8[a]	9.2 ± 0.6[a]	14 ± 2[a]	0/10[a]
	Day 3	1.9 ± 0.7[a]	10.6 ± 0.7[a]	24 ± 4[a]	0/10[a]
	Day 4	2.0 ± 0.3[a]	9.7 ± 0.8[a]	27 ± 4[a]	0/10[a]
L-NAME 5 mg/kg i.p.+L-arginine 100 mg/kg i.p.+BPC 157 10 μg/kg i.p.	Day 1	1.8 ± 0.5[a]	9.7 ± 0.7[a]	10 ± 2[a]	0/10[a]
	Day 2	1.7 ± 0.4[a]	9.6 ± 0.6[a]	18 ± 2[a]	0/10[a]
	Day 3	2.0 ± 0.9[a]	9.8 ± 0.7[a]	27 ± 3[a]	0/10[a]
	Day 4	2.0 ± 0.7[a]	10.1 ± 0.8[a]	30 ± 4[a]	0/10[a]

Daily sickness course in rats with esophagocutaneous fistulas, water intake (ml), food intake (g), weight loss (g), (Δ, before–after), means ± SD, number of rats dead/survived. Medication was given daily after creation of fistulas per-orally (p.o.) in drinking water till the end of experiments, or intraperitoneally (i.p.) once time daily, first application at 30 min, last at 24h before sacrifice.

[a] $P < 0.05$, at least, vs. control.

3.5. L-NAME+L-arginine

Co-administration of L-arginine counteracted the aggravation that was induced by L-NAME. There was no mortality at day 4 (Table 3); the esophagitis presentation was attenuated. Also, we noted a higher PS and LES pressure than in corresponding controls (Table 2).

3.6. L-NAME+BPC 157

Generally, BPC 157 counteracted the L-NAME-aggravation even below control values. Both defects start to heal at day 2, with less fistula leakage and counteracted esophagitis (Table 3). There was no mortality at day 4 (Table 3). Also, higher PS

Table 4
Densitometric analysis of RT-PCR products showing the effect of BPC 157 on eNOS mRNA levels.

Medication given daily after creation of fistulas per-orally (p.o.) in drinking water	eNOS/GAPDH	iNOS/GAPDH	COX-2/GAPDH
	means ± SD	means ± SD	means ± SD
Drinking water (12 ml/rat/day) (control p.o.)	1.49 ± 0.31	1.57 ± 0.32	1.65 ± 0.24
BPC 157 10 μg/kg p.o. (0.16 μg/ml)	0.48 ± 0.13[a]	1.69 ± 0.27	1.80 ± 0.34

Results were expressed as the ratio of the optical density of the eNOS PCR and COX-2 products to the density of the corresponding GAPDH PCR products and standardized to 1.0 for controls (relative intensity). Medication was given daily after creation of fistulas per-orally (p.o.) in drinking water till the end of experiments, at the day 4. Data represent means ± SD of four experiments.

[a] $P < 0.05$, at least, vs. control.

pressures were observed. Likewise, a failure of LES pressure was abolished, like in BPC 157 rats (Table 2). As before, the same efficacy was noted when BPC 157 was given either per-orally or intraperitoneally (Tables 2 and 3).

3.7. L-arginine+BPC 157

Compared with controls, a beneficial effect is clearly presented, however, no potentiating beneficial effect could be seen with L-arginine+BPC 157. Skin defects start to heal at day 2; the esophageal defect even earlier, with less fistula leakage and a counteracted esophagitis (Table 2). There was no mortality at day 4 (Table 3). Also, a higher PS pressure was observed. Likewise, failure of LES pressure was abolished, as with rats that received BPC 157. As before, the same efficacy was noted as when BPC 157 was given per-orally or intraperitoneally (Tables 2 and 3).

3.8. L-NAME+L-arginine+BPC 157

Like in the case of L-NAME+BPC 157, the values of L-NAME+L-arginine+BPC 157

are generally below the control values. As with L-arginine+BPC 157, skin defects start to heal at day 2; the esophageal defect starts to heal even earlier, with less fistula leakage and counteracted esophagitis (Table 2). There was no mortality at day 4 (Table 3). Also, a higher PS pressure was observed. Likewise, failure of LES pressure was abolished, like in BPC 157 rats (Table 2). Noteworthy, the same efficacy was noted when BPC 157 was given per-orally or intraperitoneally (Tables 2 and 3).

In addition to the described effects of BPC 157, L-arginine and L-NAME, there was an evident presentation of weight loss (increased (with L-NAME); no influence with (L-arginine; L-NAME+/L-arginine); decreased with (BPC 157, BPC 157+L-NAME, BPC 157+L-arginine, BPC 157+L-NAME+L-arginine), drinking increased with (L-NAME); no influence with (L-arginine; L-NAME+/L-arginine); decreased with (BPC 157, BPC 157+L-NAME, BPC 157+L-arginine, BPC 157+L-NAME+L-arginine) and the process of eating worsened with L-NAME; no influence with (L-arginine; L-NAME+/L-arginine); ameliorated with (BPC 157, BPC 157+L-NAME, BPC 157+L-arginine, BPC 157+L-NAME+L-arginine) (Table 3).

Generally, microscopic presentation followed the described macroscopical healing (Table 2, Figs. 1–3). After 4 days, the control animals showed severe necrosis along the fistula wall, including a large necrotic area of the superficial epithelium and a broad band of necrotic subcutaneous tissue and muscle. Abundant, predominantly polymorphonuclear, infiltration was present along the channel. The inflammation extended to the adipose tissue. In contrast, in BPC 157, the luminal surface of the esophagus shows only milder and focal ulcerative lesions with focal inflammation restricted to the epidermis and upper dermis.

4. Discussion

We challenged the previous assumptions and attempts to treat esophagocutaneous fistulas with biological fibrin glue, pedicle flaps or cyanoacrylate (Rábago et al., 2002; Tobin et al., 1985; Yurtçu et al., 2009). Here, the esophageal, skin and fistula defects were more aggravated (L-NAME) and unhealed (along with up regulated eNOS, iNOS and COX2 expression). Thereby, BPC 157 and L-arginine and their beneficial effects, prompt (BPC 157) and delayed (L-arginine), signify this particular and complex healing process.

Since they are reciprocally dependent, fistulas generally indicate an underlying active disease (for review see, i.e., Present, 2003; Vuksic et al., 2007) which impacts the therapeutic effect and vice versa. Here, esophagocutaneous fistulas are interrelated with the progression of esophagitis and the failed pressure in lower esophageal and pyloric sphincters (considering the sphincter as a specialized region, characteristic for coordinated function of the esophagogastric portion of the gastrointestinal tract (Sidhu and Triadafilopoulos, 2008)), being aggravated in severity with the NOS-blocker, L-NAME. In contrast there is almost no esophagitis (BPC 157-rats) with rescued sphincter pressure (L-arginine-, BPC 157-rats). Healing activity appears at day 1 with BPC 157-regimens whereas L-arginine starts after. These might in general explain the role of the NO system (in both NOS-substrate L-arginine application and NOS-blockade by L-NAME). Presenting a more down-hill course (with L-NAME) vs. the consequent beneficial therapeutic interventions (null mortality, BPC 157 > L-arginine). Particularly, in fistulas, BPC 157 specifically affects gene expression (i.e., eNOS-expression decreased while COX2 and iNOS-expression remained up-regulated).

Also importantly, besides the general congruence – based on effective therapeutic interventions – some peculiarities occurred throughout the complex healing interplay with the NO-system, after creation of esophagocutaneous fistulas (i.e., course of initial lesions; course of associated damages, i.e., failure of sphincters and esophagitis). First, regarding the course of esophagocutaneous fistulas, all these parameters share the same pathway through the NO-system. Specifically, skin defects, esophageal defects and fistula leakages follow a course of improvement per viam BPC 157 and L-arginine (in order BPC 157 > L-arginine) vs. the corresponding course of aggravation per viam L-NAME. Namely, L-NAME/L-arginine is a relationship of mutual counteraction; meanwhile BPC 157 completely eliminates all deleterious effects of L-NAME, bringing all (+L-NAME)-lesions markedly below control values.

Furthermore, failure of both sphincters presents an alternative NO-system route (i.e., improvement by L-arginine and BPC 157 (approaching the values in normal non-operated rats), with no effect by L-NAME and thereby confirming no mutual counteraction by–L-arginine/L-NAME, while BPC 157 with L-NAME maintained its original beneficial effect). Therefore, presenting the importance of the NO-system in the function of esophageal

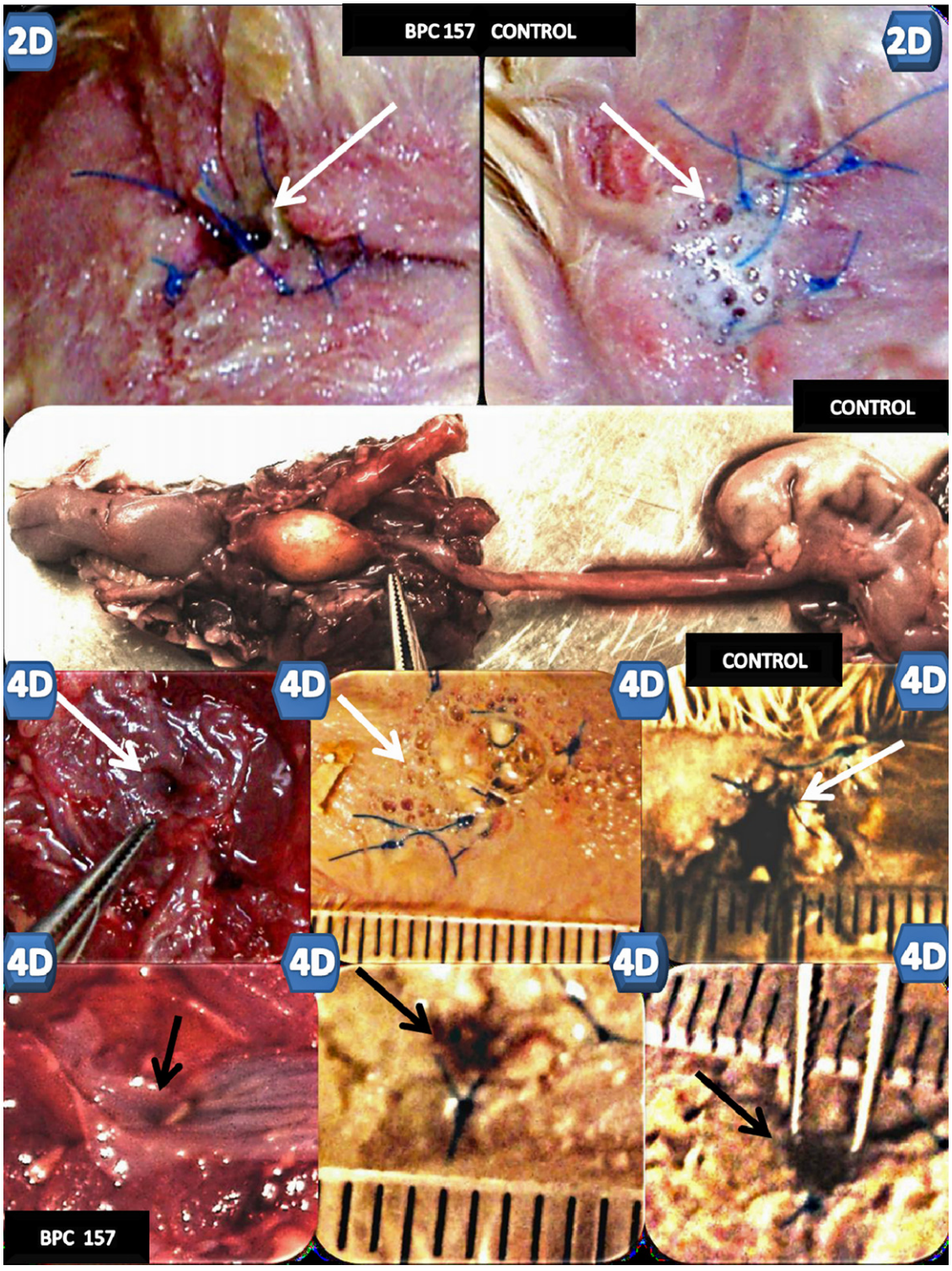

Fig. 1. Characteristic macroscopic presentation of rats with esophagocutaneous fistulas (arrows). At day 2 (2D). Upper row. Continuous saliva leaking through esophagocutaneous fistula in controls (right), no leaking at all in BPC 157 treated rats. At day 4 (4D). Middle, upper row. Sac full of the food content close to the fistula in controls. Middle, lower row. Controls. Still open both esophageal defect (left), continuous fistula leaking (middle), and significant skin defect at the sacrifice (left). Lower row. BPC 157 treated rats. Nearly closed esophageal defect (left), no fistula leaking (middle), nearly closed skin defect (right) (scale in mm).

sphincters (Sidhu and Triadafilopoulos, 2008) and the crucial importance of sphincter function for the whole esophageal integrity, it seems that this distinction in NO-system-processes may have a particular significance, especially since the mortality, with respect to the given agents and their combinations, follows the same presentation.

The special point is the presentation of esophagitis (i.e., improvement by BPC 157 vs. aggravation by L-NAME; no effect of L-arginine; and then interestingly, L-arginine+L-NAME where L-arginine counteracted L-NAME aggravation to control values; meanwhile, BPC 157 with L-NAME maintained its original beneficial effect). This course adopts the presentation noted previously

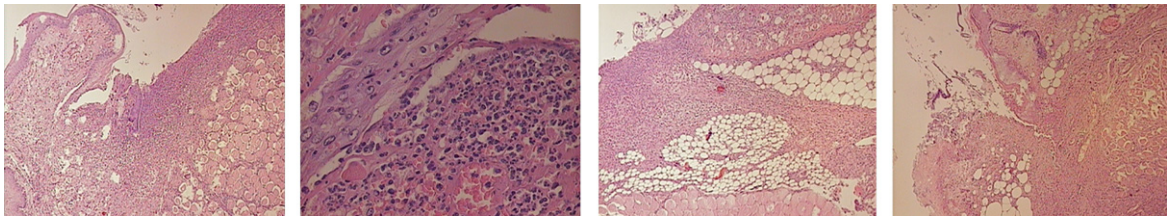

Fig. 2. Characteristic microscopic presentation in control rats with esophagocutaneous fistulas at day 4. Hematoxylin and eosin. The luminal surface of the esophagus covered by necrotic tissue and a great number of neutrophils present (objective ×5 (left), objective ×40 (middle, left)). The inflammation extends to the adipose tissue (objective x5 (middle, right)). Ulcerative epidermis (objective ×5 (right)).

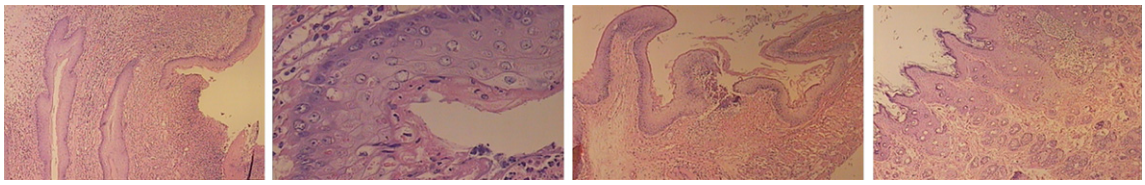

Fig. 3. Characteristic microscopic presentation in BPC 157 rats with esophagocutaneous fistulas at day 4. Hematoxylin and eosin. Luminal surface of the esophagus shows milder and focal ulcerative lesion (BPC 157, ng, objective ×5 (left); objective ×40 (middle, left), BPC 157 μg, objective ×5 (middle, right). Focal inflammation restricted to epidermis and upper dermis (BPC 157 μg, objective ×5 (right)).

in the in rats with colocutaneous fistulas or digitalis intoxication treated with BPC 157, L-arginine and L-NAME applications (Balenovic et al., 2009; Klicek et al., 2008). Interestingly, the presentation of weight loss, drinking (increased/no influenced/decreased), and eating (decreased, more or less) are in accordance with the state of illness severity.

Therefore, based on how BPC 157, L-arginine, and L-NAME (and their mutual interactions) affect the assessed parameters, we suggest that at least three distinctive NO-system pathways (i.e., esophageal/skin defects and fistula healing; sphincters; esophagitis) are all particularly involved in the NO-system processes. Considering the presentations of weight loss, drinking and eating disturbances and mortality, it may be speculated that the "esophagitis-pathway" or "sphincter-pathway" may prevail, or, it may be a consequence of how the local injury (esophageal and skin defect and fistula) affects systemic disturbances, due to fistula leakage and the lack of adequate drinking and eating. Very likely, these NO-system-pathways, when they were activated separately or together, may explain the fast and more advanced therapeutic interventions which are apparent with BPC 157 and the delayed and slower therapeutic interventions which appear with L-arginine. The fastest and the most general effect of BPC 157 (very rapid and simultaneous onset of healing of esophageal and skin wounds and fistulas, along with lesser weight loss, improved course of esophagitis and the rescue of both lower esophageal and pyloric sphincters) should be considered together with the more specific effects of L-NAME and L-arginine. The most prominent L-NAME aggravation (advanced weight loss; hindered healing of esophageal and skin wounds; increased leaking of fistulas) occurred far before the aggravation of the course of esophagitis, without an effect on both sphincters. The fastest L-arginine benefit was on both sphincters and then, on weight loss, the healing of esophageal, skin wounds and fistulas, respectively, with no influence on the course of esophagitis.

Therefore, since esophageal and skin defect healing was made worse by L-NAME and given that this process is particularly sensitive to BPC 157 application – BPC 157 nullifies the effect of L-NAME – esophageal and skin defects and fistula healing should be seen primarily through the application of BPC 157. Thus, considering the esophagocutaneous fistulas in wound healing terms and the overall effect of BPC 157 in wound healing (for review see, i.e., Sikiric et al., 2010, 2011, 2012), it is likely that applications of BPC 157, L-arginine and L-NAME for esophagocutaneous fistula healing, implicate that NO functions as an endogenous wound healing factor (Frank et al., 1998). In theory, a possible further improvement over control values would be in congruence with a suitable counteraction of disturbed or even blunted NO-generation and a clear amelioration of the healing induced by the given agent (Frank et al., 1998). Thereby, within the same dose-range, BPC 157 accordingly heals fistulas as before (Klicek et al., 2008; Skorjanec et al., 2009), as a result of suited healing of both the skin and esophageal defects; attenuates esophagitis as before (Dobric et al., 2007; Petrovic et al., 2006; Petrovic et al., 2011) (since fistula healing also involves healing of the underlying disease as well (Vuksic et al., 2007)); and rescues pressure failure of sphincters as before (Dobric et al., 2007; Petrovic et al., 2006; Petrovic et al., 2011) (assuming that sphincters function better with the coordinated function of the esophagogastric tract (Sidhu and Triadafilopoulos, 2008)).

Contrary to the uniform beneficial effect of BPC 157 – based on the corresponding L-NAME effects – with different efficacy levels, L-arginine is more restricted. It primarily affects sphincters (i.e., to counteract that, more L-NAME would be required), has a lesser effect on skin/esophageal defects, even less on fistulas (postponed healing skin and esophageal defects completely counteracted with L-NAME; fistulas healing only at latest interval) while it does not reduce esophagitis. Thus, we could imagine that more than one molecular mechanism, could be vice versa responsible. While the essential question remained unresolved, as previously noted (Balenovic et al., 2009; Klicek et al., 2008; Lovric-Bencic et al., 2004; Sikiric et al., 1997), higher effects of BPC 157 could be not potentiated by L-arginine and therefore, they likely represent two separate ways in the NO-system.

In this, BPC 157's activity implies a particular balancing effect on eNOS, assuming the special functions of eNOS, i.e., the principal regulator of integrated arterial function in rats (Gaballa et al., 1999). In esophagocutaneous fistula rats, when presented with more lesions, reflecting an increased severity, the noted up-regulation of eNOS, iNOS and COX2 levels in esophagocutaneous fistulas is probably stretched to the limit, consequential to general deterioration. Thus, it is likely that fewer lesions in BPC 157-fistula rats with a lesser activity of eNOS suggests an additive and/or synergistic effect, with the activity of BPC 157 administration itself, having a particular effect in BPC 157-esophagocutaneous rats. As such, this particular effect of pentadecapeptide BPC 157 (eNOS decreased, iNOS and COX2 levels

remained up-regulated), where all deleterious effects of L-NAME were eliminated, suggests that BPC 157 administration outweighs the previously increased activity of eNOS (iNOS and COX2) in esophagocutaneous fistulas-rats. Certainly, such an effect of BPC 157 implies the npreserved integrity of the endothelium. This may be essential for the advanced healing in esophagocutaneous fistulas rats. Besides the evidenced effect on eNOS-expression, in support of the preserved endothelium integrity are: the effect on increased serum endothelin values along with cardiac failure reversal (Lovric-Bencic et al., 2004); the counteracting effect of BPC 157 on L-NAME parallel with its endothelium protection and mucosal integrity maintenance (Sikiric et al., 1997); in rats with abdominal aorta anastomosis BPC 157 both prevents thrombotic clot formation and destroys already formed thrombosis (Hrelec et al., 2009) and also, after amputation, BPC 157 reduces bleeding time and thrombocytopenia after heparin, warfarin or aspirin (Stupnisek et al., 2012).

Finally, the same relations with L-NAME (NOS-blockade) and/or L-arginine (NOS-substrate)-application were obtained using both intraperitoneal and per-oral BPC 157 regimens. Thereby, these BPC 157/NO-system interactions could be seen as useful extensions of the already known interactions of BPC 157/NO-system (Balenovic et al., 2009; Boban-Blagaic et al., 2006; Klicek et al., 2008; Lovric-Bencic et al. 2004; Sikiric et al., 1997). Thus, along with other types of fistula healing (Klicek et al., 2008; Skorjanec et al., 2009) these findings show that parenteral or peroral applications of BPC 157 (thereby, having both local and systemic effect) would be generally responsible for the survival and the successful counteraction of regular fistula healing failure. Particular for esophagocutaneous fistulas and skin and esophageal wound healing, the BPC 157 peptide, stable in human gastric juice (more for 24 h) (for review see, i.e., Sikiric et al., 2010, 2011, 2012) which is recognized to be a basal protectant in the saliva and gastric juice, can be seen as a mediator of Robert's cytoprotection (for review see, i.e., Sikiric et al., 2010, 2011, 2012). BPC 157, with its prominent effect on wound healing and collagen formation (for review see, i.e., Sikiric et al., 2010, 2011, 2012), including its effect on egr-1 and NAB2 genes (Tkalcevic et al., 2007), is likely responsible for the quick initiation of the fistula healing process that may rescue rats with esophagocutaneous fistulas. However, how and whether this experimental advantage (i.e., particular effect on eNOS gene), may translate into an enhanced clinical performance remains to be determined.

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