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Intestinal Edema: Effect of enteral feeding on motility and gene expression

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Abstract

Objective—Edema formation, inflammation and ileus in the intestine are commonly seen in conditions like gastroschisis, inflammatory bowel disease and cirrhosis. We hypothesized that early enteral feeding would improve intestinal transit. We also wanted to study the impact of early enteral feeding on global gene expression in the intestine.

Design—Rats were divided into Sham or Edema ± immediate enteral nutrition (IEN). At 12 hrs, small intestinal transit via FITC-Dextran and tissue water were measured. Ileum was harvested for total RNA to analyze gene expression using cDNA microarray with validation using real-time PCR. Data are expressed as mean ± SEM, n=4-6 and *, ** = $p < 0.05$ vs. all groups using ANOVA.

Results—IEN markedly improved intestinal transit with minimal genetic alterations in Edema animals. Major alterations in gene expression were detected in primary, cellular and macromolecular metabolic activities. Edema also altered more genes involved with the regulation of the actin cytoskeleton.

Conclusions—Intestinal edema results in impaired small intestinal transit and globally increased gene expression. Early enteral nutrition improves edema-induced impaired transit and minimizes gene transcriptional activity.

Keywords

intestinal edema; ileus; enteral feeding; micorarray

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Introduction

The gut is particularly susceptible to injury, which can result in gut edema, ileus, and failure of intestinal defense mechanisms. These factors can delay recovery, increase morbidity and hospital length of stay (1-4). Intestinal edema formation, inflammation and ileus are commonly seen in patients affected with gastroschisis, inflammatory bowel disease and cirrhosis. Excessive intraoperative crystalloid fluid administration during gastrointestinal surgery results in intestinal edema and ileus (5). Fluid overload has been postulated to contribute to feeding intolerance and ileus and bacterial translocation associated with gut edema (6-7). Another major risk factor associated with intestinal ileus is inflammation. Pro-inflammatory mediators like inducible nitric oxide synthase, cyclo-oxygenase-2, interleukin-6 and recruitment of leukocytes to the intestine have been shown to impair gut motility in animal models (8-11). Our group has focused on defining the role gut edema plays in intestinal dysfunction by using a reproducible edema model of acute mesenteric venous pressure elevation and intravenous fluid resuscitation. We have previously shown that gut edema promotes intestinal ileus, depresses intestinal smooth muscle contractility and increases mucosal permeability without ischemia/reperfusion injury (12-13).

Malnutrition is recognized as a cause of increased morbidity and mortality in surgical patients and intestinal dysmotility creates a major dilemma in delivering enteral nutrition in critically ill patients. The timing and route of administration of feeding patients with ileus has not been optimized to date. Moore et al. (14), evaluated tolerance of enteral nutrition after major injury. This study found that 50% of patients undergoing surgery had *fair to poor* tolerance to early enteral nutrition. However, in this study early enteral nutrition was not given until 24 hours after the last surgery. The purpose of this study was to investigate if administration of immediate enteral nutrition (IEN) would improve intestinal transit and modulate gene expression in intestinal edema.

Materials and Methods

All procedures were approved by the University of Texas Animal Welfare Committee and were consistent with the National Institutes of Health *Guide for the Care and Use of Laboratory Animal*.

Operative procedures and experimental groups

Male Sprague-Dawley rats, weighing between 300-350 grams (Harlan Labs, Indianapolis, Indiana, USA), were fasted 16-18 hours prior to surgery and given free access to water. Under general anesthesia with isoflurane using aseptic technique, an intravenous catheter was placed by surgical exposure and direct placement of catheter into the jugular vein. Next, a midline laparotomy incision was made and a silastic catheter introduced into the mid-duodenum via needle puncture and secured with a 6-0 silk purse-string suture. The catheter was brought through the musculature of the left abdominal wall and subcutaneous tissue toward the back of the neck where it was exteriorized through an interscapular incision and fixed to the skin with 4-0 silk suture. Prior to closure of mid-line incision, a 4-0 silk ligature was placed around the superior mesenteric vein (SMV) and tied over a PE-10 silastic tube, which was removed after ligature was secured and 80cc/kg body weight of 0.9% saline administered via the jugular venous catheter. This acute elevation in SMV pressure allowed for the creation of acute intestinal edema (Edema). The experiment included four groups: Sham, Sham given IEN, Edema and Edema given IEN. A micro-pump was attached to a backpack and connected to the duodenal catheter and the enteral formula was started at 0.4 cc/kg/hour and run continuously for 12 hours. Table 1 depicts the enteral formula given to the animals in this study. This formula was adapted and modified to keep the formula isotonic (15).

Intestinal Transit

The effect of IEN on intestinal motility in Sham and Edema animals was measured in vivo using 0.1 ml of a 5 mM solution of non-absorbable FITC-Dextran injected into the duodenal catheter. Intestinal transit for each study was measured at the specified time points above; 0.1 mL of a 5-mmol/L solution of nonabsorbable fluorescein isothiocyanate (FITC)-dextran (molecular weight, 9,400; FITC content, 0.008 mol/mol glucose; Sigma-Aldrich, St. Louis, MO) was injected into the duodenal catheter and flushed with 0.1 mL of normal saline. Twenty-five minutes after FITC-dextran injection, rats were anesthetized with an intraperitoneal injection of ketamine and the entire small intestine was removed and divided into 10 equal segments. The intraluminal contents of each segment were flushed with 3 mL of 5-mmol/L Tris-buffer (pH 10.3) to recover the FITC-dextran. The FITC-dextran concentration was measured using an optical scanner to read the optical density of the FITC-dextran (STORM model 860, Amersham Biosciences, Piscataway, NJ) and expressed as a fraction of total tracer recovered and presented as the geometric center of distribution as described previously in the literature (9,16-17). The distal ileum was harvested and snap frozen and stored at -80°C for RNA extraction. Intestinal transit for each study was measured at the specified time points above

Intestinal Water Determination

The small intestine from the duodenum to the ileum at the ileocecal valve was harvested at 12 hours to determine the tissue water content. Intestinal samples were opened along the antimesenteric border and blotted dry. Wet weight was determined prior to placing tissue in an oven set to 60°C. Tissues were dried until a constant dry weight was obtained over 2-3 days and dry weights were measured and used to determine tissue water content using the following formula: ((wet weight) – (dry weight)/dry weight).

Gene Expression Studies

Rat 30K-Microarray Analysis—The effect of IEN on global gene expression in Sham and Edema animals was measured using a dual-labeled rat 30-K Agilent (Agilent Technologies, Palo Alto, CA) microarray slide. Total RNA was isolated from the distal ileum using RNAzol Bee (Tel-Test, Friendswood, TX), precipitated and washed with chloroform and isopropanol then stored in DEPC-treated water. The RNA samples were checked for degradation and DNA contamination on an Agilent 2100 Bioanalyser using an Agilent RNA 6000 Nano Kit as recommended by the manufacturer. The RNA was labeled with an Agilent Fluorescent Direct Labeling Kit and Perkin Elmer Cyanine-3 or Cyanine-5 dye (Perkin Elmer Life Sciences, Boston, MA). The experiment included three groups (n=4-5 animals pooled RNA): 1) Sham versus 2) Sham given IEN (Sham/IEN), 3) Edema and 4) Edema given IEN (Edema/IEN). The dual-fluorescently labeled cDNA were combined and purified using the Agilent recommended procedure for each microarray chip. Hybridization and post-hybridization washes were performed on an Agilent cDNA microarray using the manufacturers recommended procedure except the hybridization temperature was lowered to 60°C. Following hybridization and washing, microarrays were scanned using an Axon 4200-Two Channel Scanner (Axon, Sunnyvale, CA). The scanned microarrays were analyzed and significant fold changes were determined using GenePix 5.1 software from Axon. Significant fold changes for genes were determined by taking the $\log_2$ ratio of the expression between Edema with or without IEN and Sham/IEN vs. Sham animals ($\log_2$ ratio = Experimental Group/Sham Group). Genes with a $\log_2$ ratio ≥ 1 (> 2-fold up) were considered up-regulated and ≤ 1 (< 2 fold down) were considered down-regulated. Following filtration and normalization of each qualified gene, hierarchical clustering and visualization were carried out using Cluster 3.0 and TreeView software (Eisen Lab, Stanford, CA). Genes were further categorized using the gene ontology DAVID-

EASE software from the National Institute of Health (http://david.abcc.ncifcrf.gov/). This software enables the visualization of molecular interactions from our gene expression data set by overall functional classification and interactive gene maps developed by various gene consortiums in the field of genomics.

SYBR Green Real-Time Quantitative Polymerase Chain Reaction—Microarray validation was performed by using real time- polymerase chain reaction (RT-PCR) for selected gene pathways identified from the DAVID-EASE analysis from the same animals used in the microarray experiment. The primer sequences used for the validation of the microarray experiment are shown in Table 2. cDNA was generated from the total RNA (10 ng) from the distal ileum in the microarray experiment using SuperScript™ First-Strand Synthesis kit (Invitrogen, Carslbad, CA). Primer sequences were designed using AmplifX 1.1 program directed at both the 3′ and 5′ end of the gene sequence. Gene-specific primers used in this study are listed in Table 2.

Real-time quantitative polymerase chain reaction (RT-PCR) was performed using a Sybr Green protocol to confirm microarray results. Due to the inherent inaccuracies in quantifying cDNA by absorbance, the amount of cDNA added to an RT-PCR from each sample was more accurately determined by measuring a housekeeping transcript level in each sample (β-Actin). Selected candidate genes were identified (n=3 per group, randomly selected from the study samples). Relative mRNA expression (C_T) was quantified in triplicate and normalized to β-actin and calculated relative to Sham using the $2^{(-\Delta\Delta CT)}$ method (18-20).

Data analysis

All data for transit, tissue water and histology are expressed as mean ± SEM using a commercial statistical software program (NCSS, Kaysville, UT). Statistical significance of differences among groups was determined by one way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. A P-value <0.05 was considered significant. n=4-6. Significant fold changes were determined by using the GenePix 5.1 software (Axon). Significant fold changes for genes were determined by taking the $\log_2$ ratio of the expression between Edema with or without IEN and Sham/IEN vs. Sham animals ($\log_2$ ratio = Experimental Group/Sham Group). Genes with a $\log_2$ ratio ≥ 1 (> 2-fold up) were considered up-regulated and ≤ 1 (< 2 fold down) were considered down-regulated.

Results

Intestinal Transit

The effect of IEN on intestinal transit in Sham and Edema animals was measured in vivo using FITC-Dextran. As depicted in Figure 1, the average mean geometric center (MGC) in Edema (3.4 ± 0.1*) was significantly decreased compared to Sham (4.7 ± 0.2**) animals. IEN significantly improved intestinal transit in Edema (Edema/IEN: 4.6 ± 0.3**) animals and was comparable to Sham animals with or without administration of IEN (Sham/IEN: 5.8 ± 0.4*) (ANOVA: F statistic = 13.9, p value = 0.00004).

Intestinal Water Determination

We next sought to determine the tissue water content in our acute intestinal edema model and the effects of IEN on intestinal water balance. The tissue wet to dry weight ratio in Edema (3.8 ±0.0) animals was greater than Sham (3.2 ± 0.1*) animals (Figure 2). However, immediate enteral nutrition (3.6 ± 0.1) only slightly decreased the fluid accumulation in the intestine associated with our acute intestinal edema model. IEN also significantly increased

the tissue water weight in Sham/IEN (3.6 ± 0.1) animals compared to Sham animals (ANOVA: F statistic = 5.9, p value = 0.005).

Gene Expression Studies

Feeding effects on global gene expression analysis—The effect of IEN on global gene expression in Sham and Edema animals was measured using a dual-labeled rat 30-K microarray slide. As shown in Figure 3, the global gene changes in Edema animals was greater with a 18% up regulated and 7% down regulated gene effect compared with Edema animals receiving IEN with a 0.2% up regulated and 4% down regulated gene effect. IEN did not alter global gene changes among the Sham animals with a 2% up regulated and 1% down regulated gene effect.

Differentially expressed genes: Cluster and functional classification analysis —Genes identified to be significantly up or down regulated using the Gene 5.1 software from Axon were examined using the Eisen Lab Cluster software. Three major gene clusters were identified and underwent hierarchical clustering analysis using the TreeView software (Figure 4). Cluster 1 contained 608 genes with interactions in the following pathways: oxidative phosphorylation, apoptosis and tight junctions. Figure 5 reflects the differentially regulated genes in the apoptotic gene pathway. The TNF-associated via death domain (TRADD, $\log_2$ ratio: 1.0), Interleukin-3 receptor (IL-3R, $\log_2$ ratio: 1.1), Nuclear factor kappa-B p105 subunit (NF-κB, $\log_2$ ratio: 2.0) and Bcl-2 associated death agonist (BAD, $\log_2$ ratio: 1.1) genes in Edema were up-regulated, but IEN did not alter these genes in Edema or Sham animals. Edema also down-regulated Baculovirus inhibitor of apoptosis (IAP, $\log_2$ ratio: -1.2), Calpain ($\log_2$ ratio: -1.4), Phosphatidylinositol 3-kinase (PI3K, $\log_2$ ratio: -1.6), Caspase 7 (Casp 7, $\log_2$ ratio: -1.2) and Apoptotic death agonist BID ($\log_2$ ratio: -1.2) genes, which were also not changed in the animals receiving IEN. Cluster 2 contained 7 genes with genes from the following functional categories: receptor activity and ion binding. Cluster 3 contained 622 genes with interactions in following pathways: Mitogen activated protein kinase (MAPK), Transforming growth factor-beta (TGF-β) and Janus kinase-signal transducer activating transcription factor (JAK-STAT) signaling. Edema down-regulated several genes: Actin-related protein 3 homolog (ARP3, $\log_2$ ratio: 2.0), Rho A - binding serine/threonine kinase (ROCK, $\log_2$ ratio: -1.7), v-crk avian sarcoma virus CT10 oncogene homolog (CrkII, $\log_2$ ratio: -1.3) and Myosin light chain kinase (MLCK, $\log_2$ ratio: -1.7) involved in the regulators of actin cytoskeleton pathway as depicted in Figure 6. IEN did not change the expression levels above genes except for MLCK, which was up-regulated in Edema/IEN animals. p21 (CDKN1A)-activated kinase 2 (PAK) was down regulated in Edema (log ratio: -2.5) and Edema/IEN ($\log_2$ ratio: -1.2) animals and up regulated in Sham/IEN ($\log_2$ ratio: 1.2) animals. Thymosin beta-4 (Tmsb4) was up regulated in the Sham/IEN and Edema animals, but not changed in the Edema/IEN animals. Figure 7 depicts the DAVID-EASE analysis obtained from the differentially expressed genes among the groups in other functional categories from all three clusters. Primary, cellular and macromolecular metabolic activities accounted for > 50% of the genes among all the groups in the gene ontology analysis. There was a very minimal response in the immune response genes among groups (Figure 7).

Quantitative Validating Real-Time PCR—Microarray validation was performed by using real time- polymerase chain reaction (RT-PCR) for selected gene from cluster 1 and 3 identified from the DAVID-EASE analysis. The quantitative PCR results compared to the microarray experiment for this study shown are shown in Figure 8.

Discussion

Our study shows that early enteral nutrition improved intestinal transit associated with gut edema and returned a majority of genes back to baseline expression. Although the majority of studies have focused on surgical manipulation and ischemia/reperfusion role in ileus, our group has focused on defining the role gut edema plays in intestinal dysfunction by using a reproducible acute intestinal edema model of acute mesenteric venous pressure elevation and intravenous fluid resuscitation. Intestinal transit has been used by our group and others (9, 11-12) as a surrogate marker for evaluating ileus. In this present study intestinal edema delayed intestinal transit. Delivering an intraluminal volume of a nutrient dense solution improved delayed intestinal transit associated with intestinal edema and only slightly improved intestinal tissue water. Acceleration in intestinal transit is also demonstrated in Sham animals receiving IEN. Owens et al. demonstrated in their neonatal enteral nutritional pup model that intestinal water weight increased as the % of enteral nutrition was increased and the migrating motor complex was greater in pups receiving more enteral nutrition compared to pups that did not receive enteral nutrition (21). This may account for the similar results since in our Sham/IEN group receiving enteral nutrition. Grossie et al. showed similar improvements in intestinal transit with delivery of enteral nutrition and intraluminal delivery of saline in a model of ischemia/reperfusion injury (15). We have also show in prior studies of resuscitation-induced gut edema and ischemic preconditioning (12, 22), that there was a threshold level of edema that needed to be generated before there was a delay in intestinal transit. In our resuscitation-induced gut edema model, gut edema was produced by acute mesenteric venous hypertension and crystalloid resuscitation, intestinal transit was shown to be depressed (12). In this same study, fluid resuscitation alone was enough to create intestinal edema, but there was a threshold level of edema that needed to be generated before there was a delay in intestinal transit. Late IPC produced comparable levels of fluid accumulation (edema) seen in the group of rats receiving fluid resuscitation only; however, in the setting of IPC, gut edema did not reach the threshold level necessary to delay intestinal transit seen in that study. In our current study of IEN, the Sham/IEN group produced tissue water levels comparable to the animals from our prior gut edema study that received crystalloid resuscitation without partial venous occlusion. Similar to the crystalloid group, the intestinal edema seen in our Sham/IEN did not reach the threshold level necessary to delay intestinal transit. Most of the prospective studies on early enteral feeding showed that early enteral feeding into the small intestine is well tolerated and reduces the duration of postoperative ileus (23-24); however all these studies consider ‘early’ > 24 hours after insult. Limited data exists on immediate enteral feeding in critically ill patients.

Smooth muscle contractility plays an important role in intestinal motility. The majority of studies evaluating ileus in the setting of intestinal injury have focused on inflammation in models of hemorrhagic shock, ischemia/reperfusion and surgical manipulation; yet few reports have investigated the role of cytoskeletal stress fiber alterations relationship to ileus after intestinal injury. Newer research on smooth muscle cell signaling and remodeling has provided evidence for mechanical stress induced alterations in smooth muscle contractility. We have previously shown that our edema model results in a 4-fold interstitial pressure within the submucosa (mechanical stress), which results in approximately a 4-fold increase in engineering stress applied to the smooth muscle (25). Radhakrishnan et al. performed detailed studies on the effects of intestinal edema on the tissue elastic modulus of intestinal tissue and its relationship to cytoskeletal proteins. As intestinal edema formation increased, elasticity increased with a concomitant delay in intestinal transit. Intermediate filaments like calponin and vimentin and cytoskeletal proteins like globular actin are the main tissue determinants of intestinal stiffness and they were decreased in the muscular layers of edematous intestine (25-26). Smooth muscle contractility is also affected by both extracellular matrix components and myosin light chain phosphorylation. Uray et al.

demonstrated that intestinal edema decreased myosin-light chain contractility by decreasing phosphorylation of the myosin light chain (13). In this study we used cDNA microarray to help explore the differentially regulated genes related to contractile force proteins that may contribute to the delay in intestinal transit seen in our edema model. Our microarray data showed that edema had the greatest effect on cytoskeletal gene expression (Figure 7) with decreased expression of myosin light chain kinase mRNA (Figure 5) and increased calponin expression. IEN had the opposite effects on both MLCK and calponin mRNA expression, which may play a role in maintenance of the contractile apparatus in Edema/IEN animals.

Mechanical stress not only effects stress fiber formation, but has also been found to inhibit smooth muscle cell proliferation, promoting apoptosis and up-regulation of TGF-β transcription stimulating collagen deposition (28-30). In our study, intestinal edema altered more genes in the apoptotic pathway. Edema up-regulated the death domain adaptor molecule (Figure 6), TRADD which interacts with the tumor necrosis factor receptor mediating programmed cell death signaling and nuclear factor-kappa b (NFκ-B) activation (31-32). TRADD also reduces recruitment of proteins known as inhibitor-of apoptosis, these genes were also down-regulated by edema. The finding that NFκ-B is up-regulated is consistent with recent data from our group demonstrating that NFκ-B is activated in intestinal edema and associated with decreased smooth muscle contraction; however, pharmacological inhibition restores intestinal smooth muscle contractility in the setting of intestinal edema.

One limitation of our study is the use of full-thickness intestinal tissue for the gene expression experiments. There may be a difference in gene expression in the intestinal mucosa, submucosa or muscularis layers and/or local immune cells. These shortcomings could be ultimately overcome by immunohistochemistry and separating mucosa from the muscularis of the intestine. Nevertheless, these data begin to explore the global effects of acute intestinal edema and early enteral nutrition on gene expression in the intestine. Further studies are needed in our edema model to explore alterations in the pathways identified in our study on intestinal dysfunction.

In conclusion, we have shown that early delivery of nutrients to the intestine helps to resolve ileus associated with gut edema. Administration of immediate, early enteral nutrition helped to restore the delayed intestinal motility pattern seen in intestinal edema. These data are important for not only establishing a link between cellular homeostasis and intestinal dysmotility, but may lead to further insight into how intestinal edema delays intestinal transit. These data provide further evidence that edema can alter gene expression patterns in the intestine promoting dysfunction.

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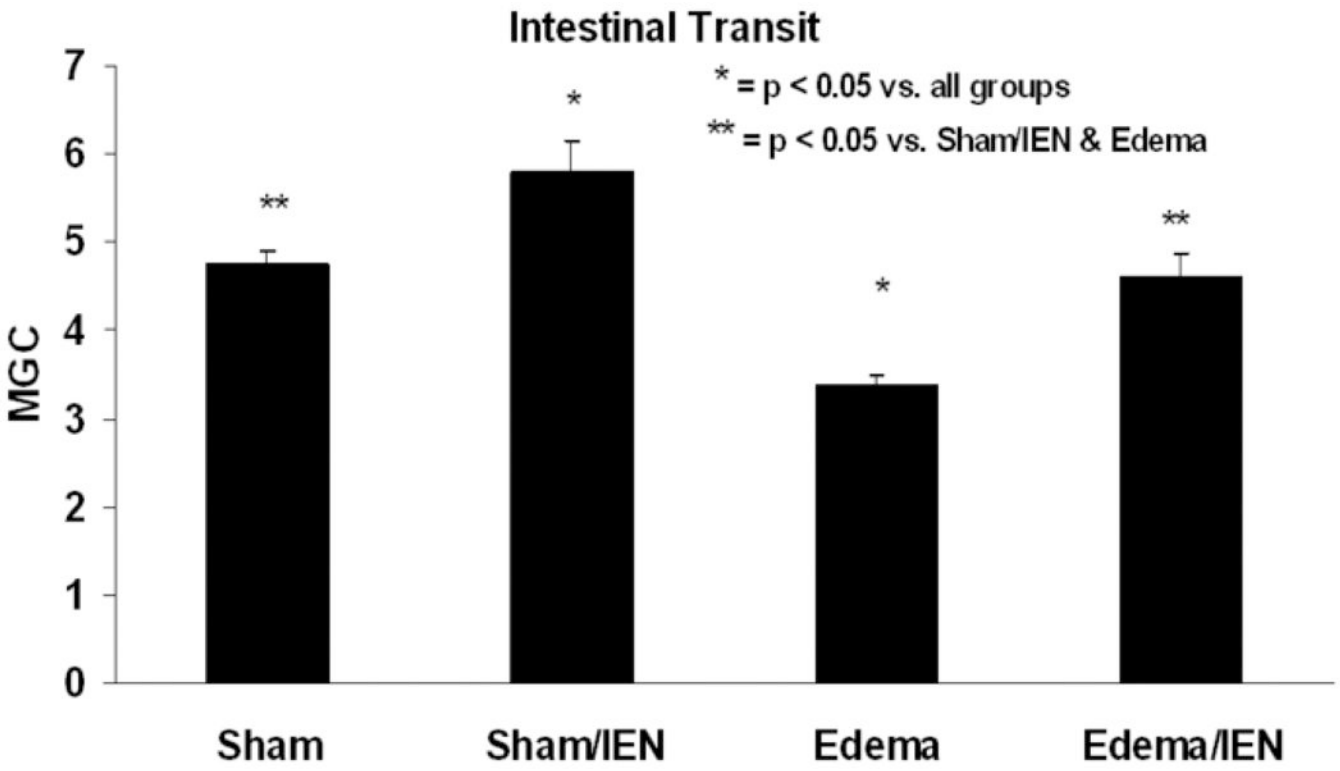


Figure 1. Intestinal transit

Rats were subjected to Sham surgery with and without IEN (Sham and Sham/IEN), acute elevation of SMV pressure and crystalloid resuscitation with and without IEN (Edema and Edema/IEN). Intestinal transit of tracer along intestinal segments was then measured as detailed in "Methods." The mean geometric center (MGC) of tracer is presented. *P < 0.05 for Edema versus all other groups, using one-way ANOVA. n = 5-6.

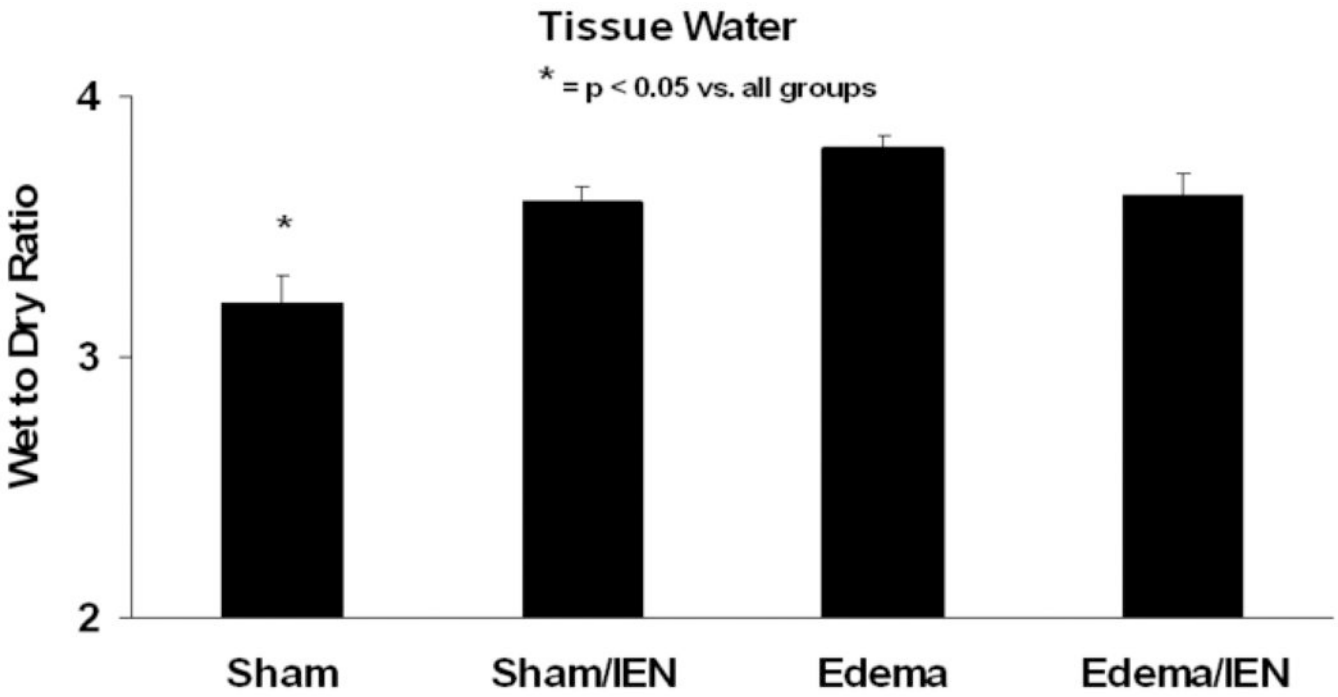


Figure 2. Tissue water weight

Rats were subjected to Sham surgery with and without IEN (Sham and Sham/IEN), acute elevation of SMV pressure and crystalloid resuscitation with and without IEN (Edema and Edema/IEN). Tissue water weight is calculated as a wet weight to dry weight ratio and expressed. *$P < 0.05$ for Sham versus Sham/IEN, Edema and Edema/IEN, using one-way ANOVA. n = 5-6.

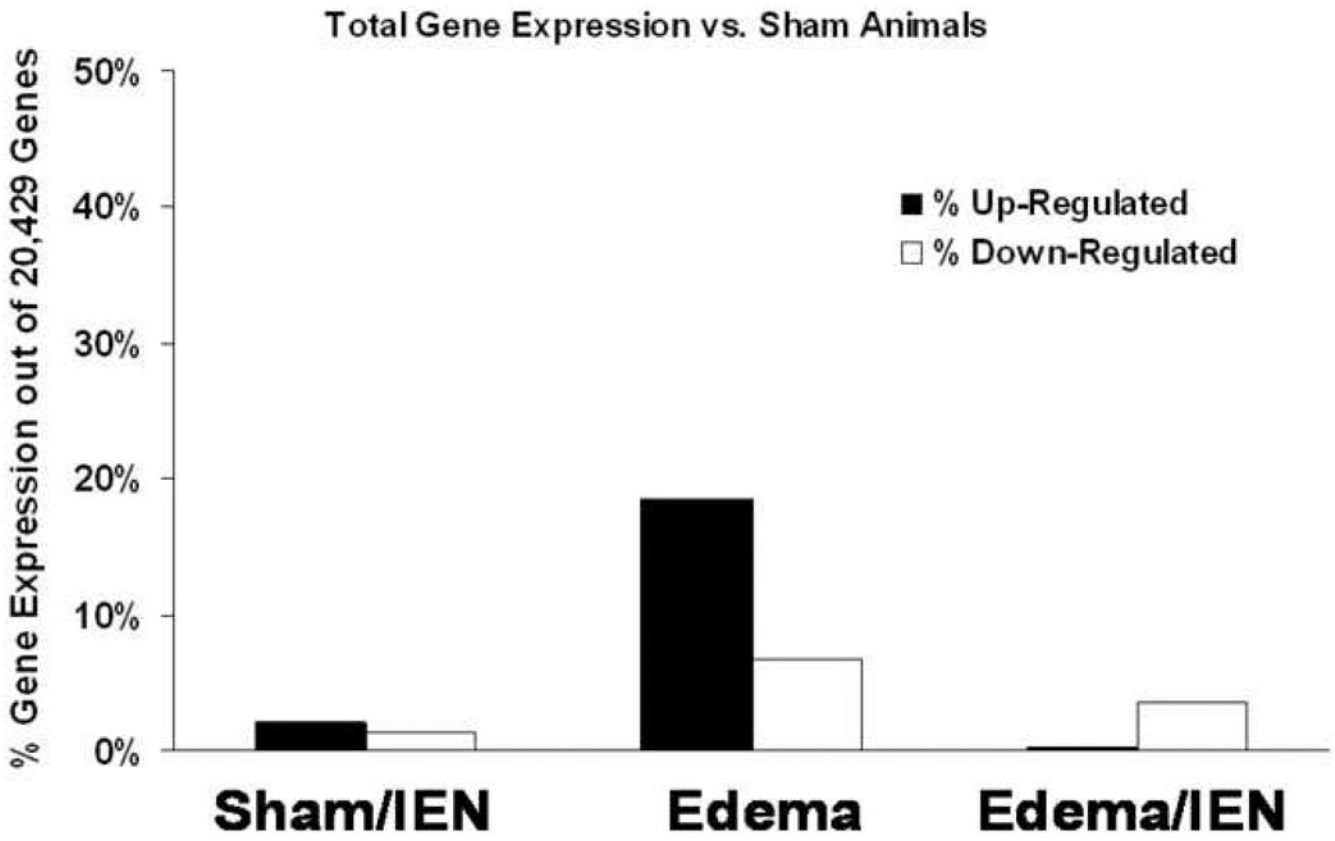


Figure 3. Global gene expression versus Sham animals

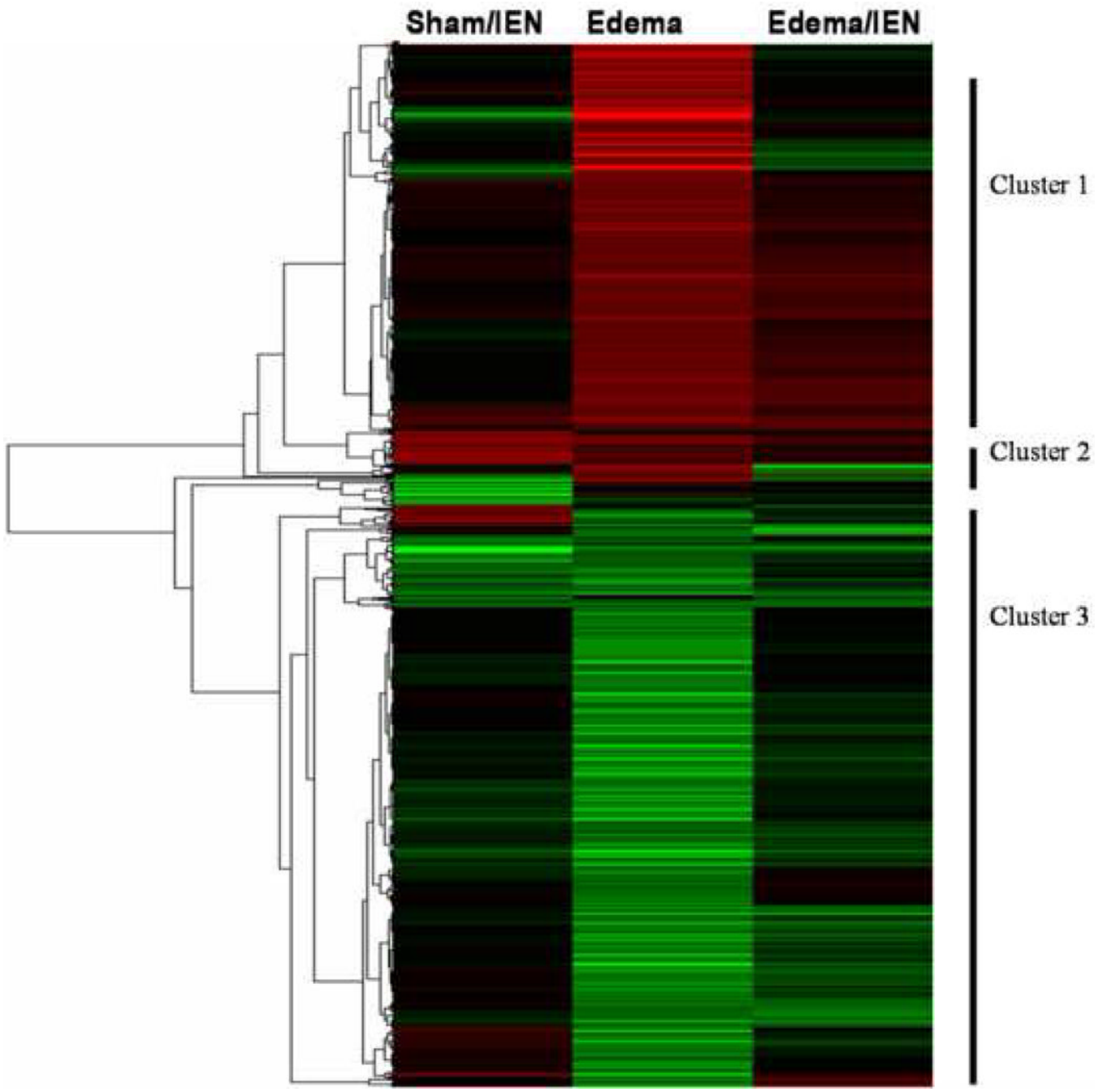


Figure 4. Hierarchical clustering of differentially regulated genes

Cluster was performed by Treeview software. Intensity in the red and green color spectrum denotes up-regulated and down-regulated genes respectively. The black intensity denotes no change in gene expression levels compared to Sham animals. n = 4-5.

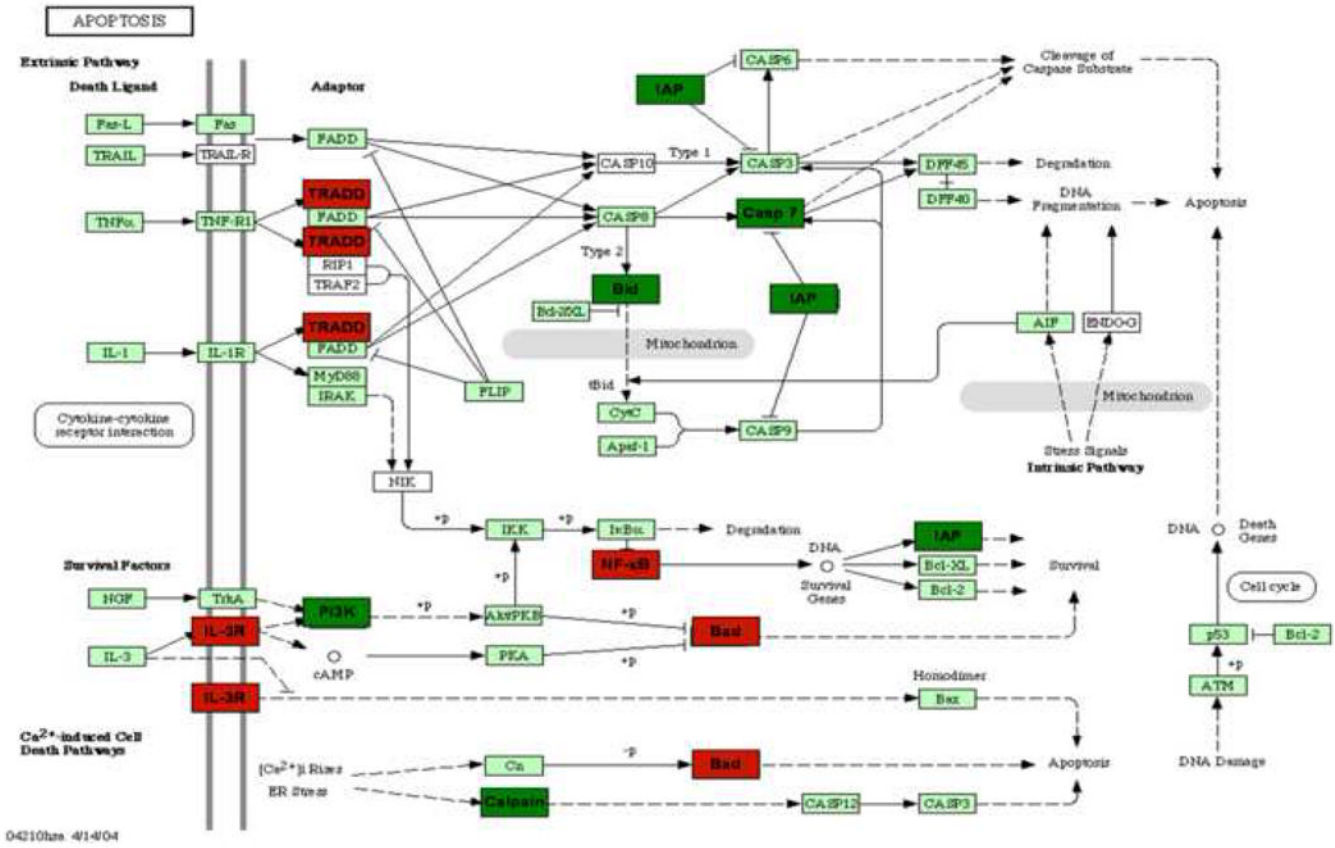


Figure 5. Apoptosis pathway differentially regulated genes

Intensity in the red and green color spectrum denotes up-regulated and down-regulated genes respectively. n = 4-5.

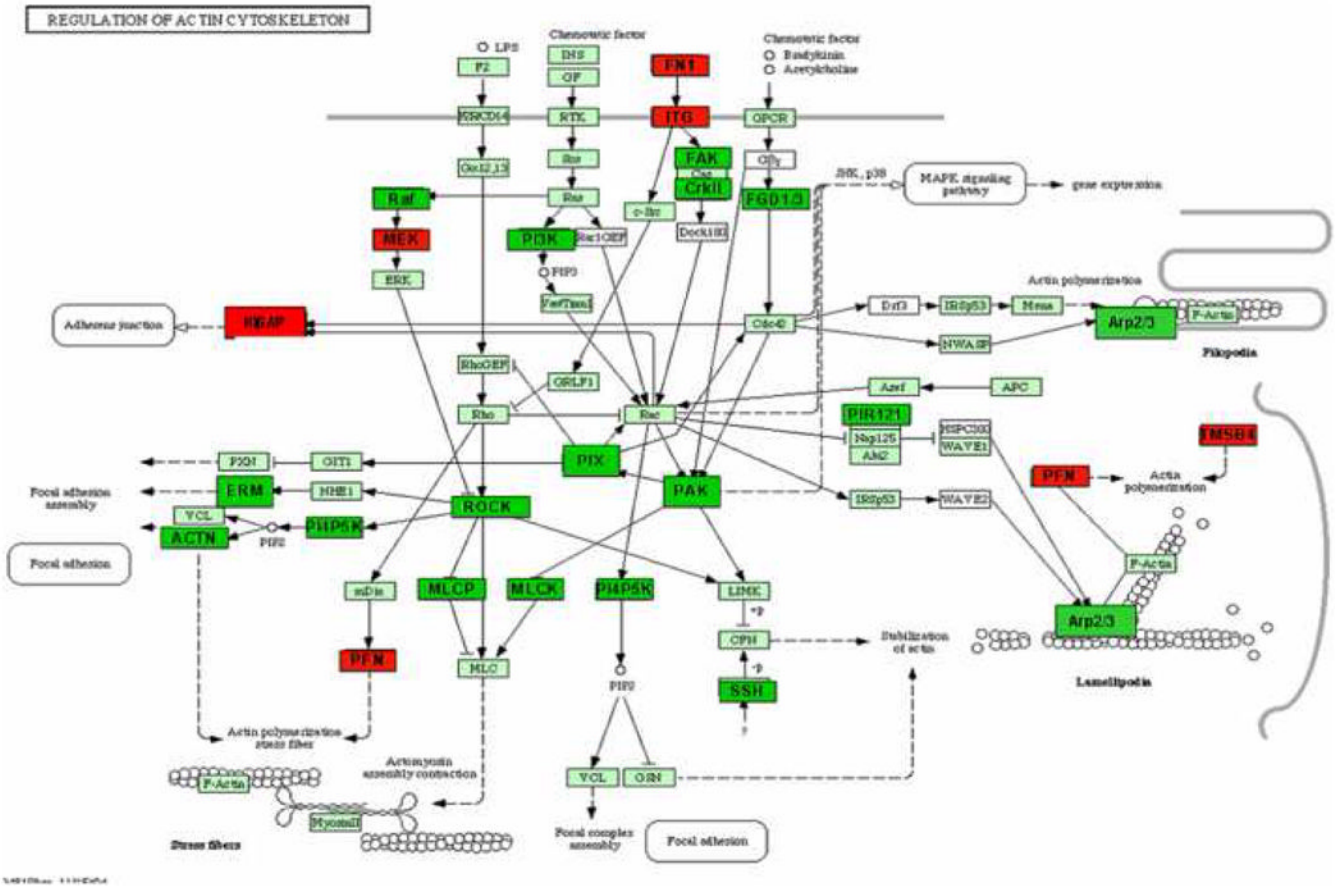


Figure 6. Regulators of Actin Cytoskeleton differentially regulated genes

Intensity in the red and green color spectrum denotes up-regulated and down-regulated genes respectively. n = 4-5.

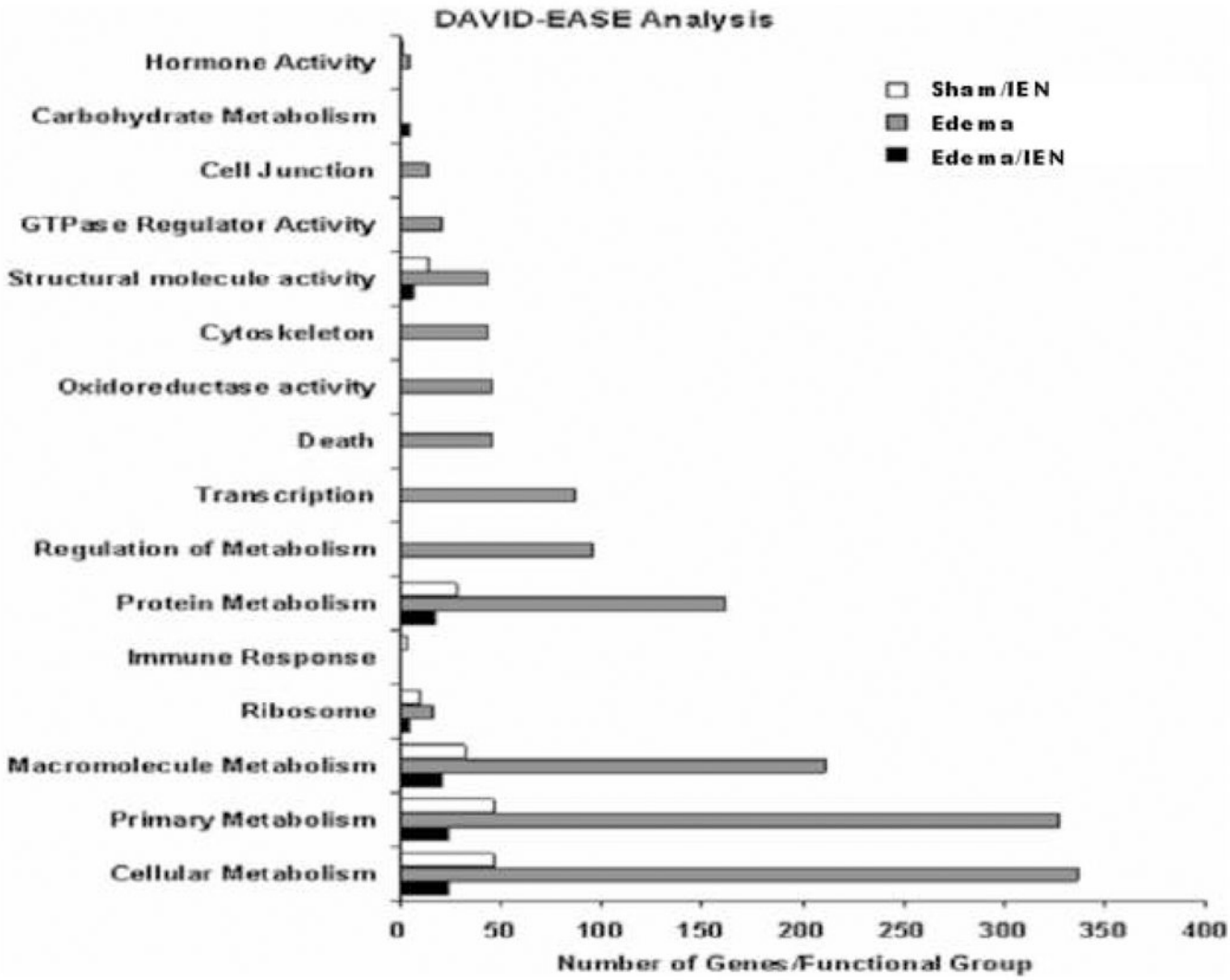
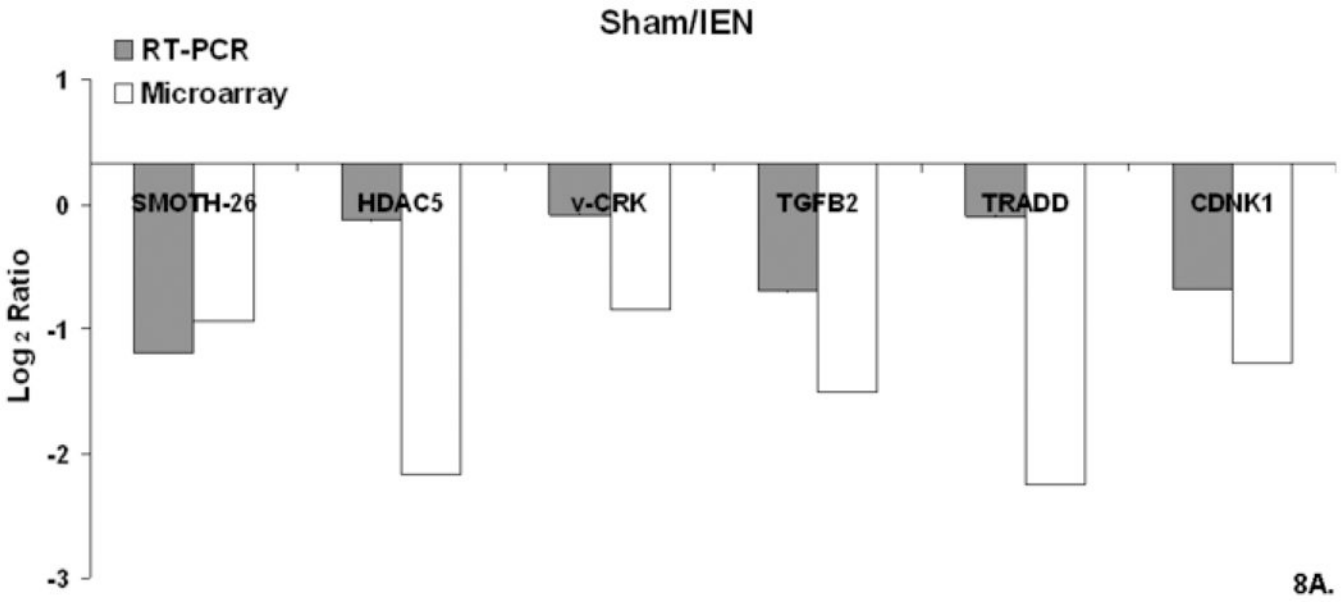
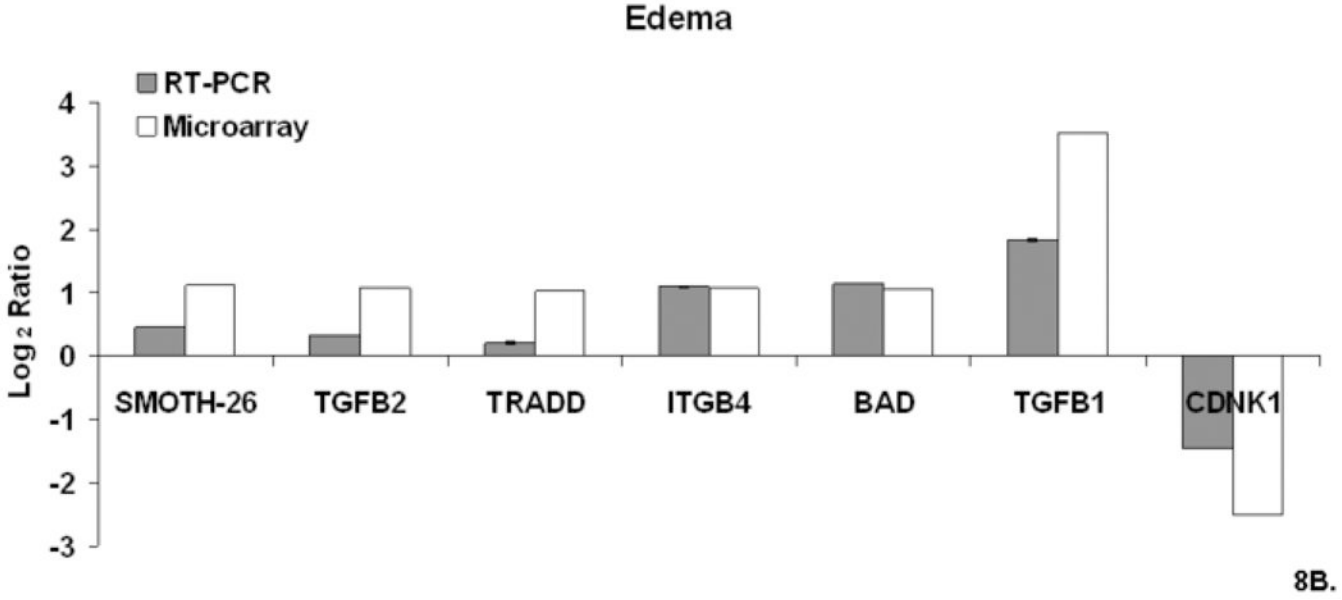


Figure 7. DAVID-EASE Gene Ontology of differentially regulated genes

Gene ontology was performed using the DAVID-EASE software. The y-axis denotes the functional category assigned to individual gene ID numbers. The x-axis denotes the total number of genes both up and down-regulated in the groups. n = 4-5.



8A.



8B.

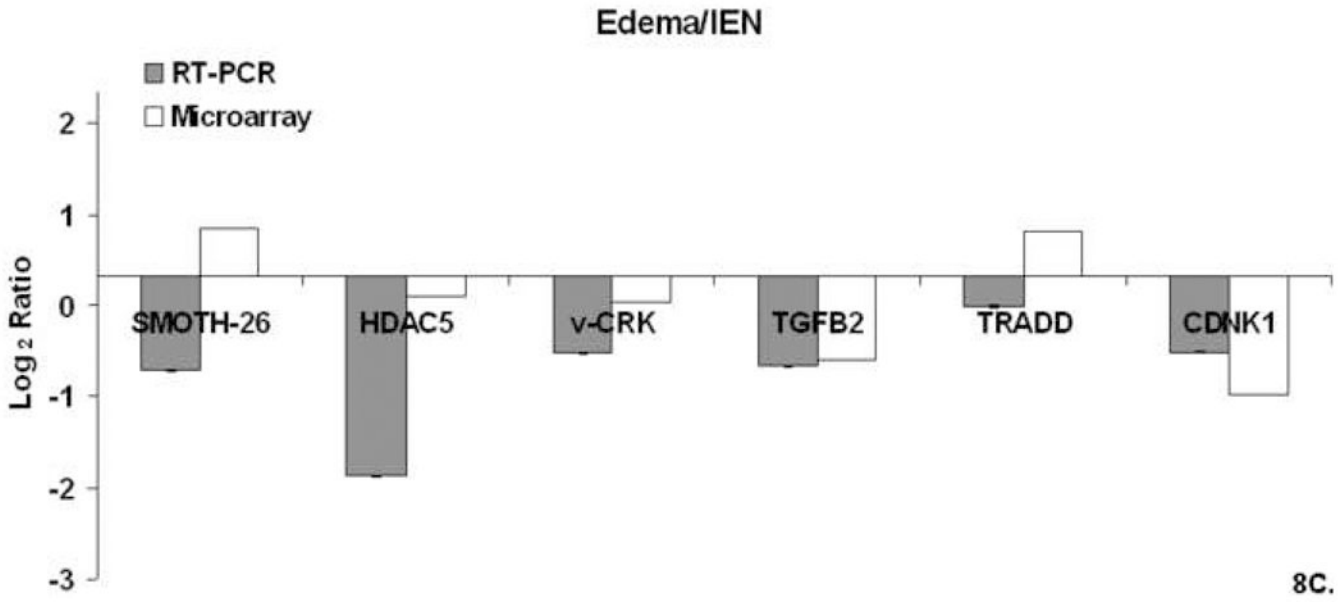


Figure 8. A-C. Real Time QT-PCR

Relative mRNA expression (C_T) was quantified in triplicate and normalized to β-actin and calculated relative to Sham using the $2^{(-\Delta\Delta CT)}$ method. Figure 8A. Sham/IEN QT-PCR. Figure 8B Edema QT-PCR. Figure 8C Edema/IEN QT-PCR.

Table 1

Composition of nutrient solution

Nutrient solution	mL/L*
Travasol 10%†	500
D50W	500
Electrolytes‡	20
Potassium phosphates‡	6
MVI-12°	5

*Amount of the respective solutions added

†Contains the following: essential amino acids leucine (730 mg), isoleucine (600 mg), lysine (580 mg), valine (580 mg), phenylalanine (560 mg), histidine (480 mg), threonine (420 mg), methionine (400 mg), and tryptophan (180 mg); non-essential amino acids alanine (2.07 g), arginine (1.15 g), glycine (1.03 g), proline (680 mg), serine (500 mg), and tyrosine (40 mg).

‡Abbott Laboratories, North Chicago, IL, USA.

°Astra Pharmaceuticals, Westborough, MA, USA.

D50W, 50% dextrose injection, USP; TPN, total parenteral nutrition.

Table 2

Primer Sequences

Gene Name	Forward Primer	Reverse Primer
Actin	5′-TCT GGA GAA GAG CTA TGA GCT GCC TG -3′	5′-TCG TGC CAC CAG ACA GCA CTG TGT TG -3′
SMOTH-26	5′-TAA GTG CGT CTT CAC CTA CGT GCA-3′	5′-TGC ACG TAG GTG AAG ACG CAC TTA-3′
HDAC5	5′-CCT GTT CGC TGA GTT CCA GAA ACA-3′	5′-TGT TTC TGG AAC TCA GCG AAC AGG-3′
v-CRK(Crkll)	5′-ATT CCT GTC CCT TAC GTG GAG AAG-3′	5′-CTT CTC CAC GTA AGG GAC AGG AAT-3′
TGF-B2	5′-TGA AAC GGA AGC GCA TCG AA-3′	5′-TT CGA TGC GCT TCC GTT TCA-3′
TRADD	5′-TGA GCT CTG CAA ACT GAC GTG T-3′	5′-CAC GTC AGT TTG CAG AGC TCA-3′
ITGB4	5′-ATT CAT CCA ACA TCG TGG AGC TGC-3′	5′-GCA GCT CCA CGA TGT TGG ATG AAT-3′
Cdnk1	5′-TTG GAG AAG CAC TGC CGA GAT ATG-3′	5′-CAT ATC TCG GCA GTG CTT CTC CAA-3′
BAD	5′-AAC ACA GAT GCG ACA AAG CGC-3′	5′-GCG CTT TGT CGC ATC TGT GTT-3′
TGF-B1	5′-TGT TCG TGA CGT GAG GGA GTT TTG-3′	5′-CAA AAC TCC CTC ACG TCA CGA ACA-3′