Oxidative stress gene expression profile in inbred mouse after ischemia/reperfusion small bowel injury[1]

Perfil da expressão gênica do estresse oxidativo em camundongos isogênicos após lesão de isquemia e reperfusão intestinal

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ABSTRACT

PURPOSE: To determine the profile of gene expressions associated with oxidative stress and thereby contribute to establish parameters about the role of enzyme clusters related to the ischemia/reperfusion intestinal injury.

METHODS: Twelve male inbred mice (C57BL/6) were randomly assigned: Control Group (CG) submitted to anesthesia, laparotomy and observed by 120min; Ischemia/reperfusion Group (IRG) submitted to anesthesia, laparotomy, 60min of small bowel ischemia and 60min of reperfusion. A pool of six samples was submitted to the qPCR-RT protocol (six clusters) for mouse oxidative stress and antioxidant defense pathways.

RESULTS: On the 84 genes investigated, 64 (76.2%) had statistic significant expression and 20 (23.8%) showed no statistical difference to the control group. From these 64 significantly expressed genes, 60 (93.7%) were up-regulated and 04 (6.3%) were down-regulated. From the group with no statistical significantly expression, 12 genes were up-regulated and 8 genes were down–regulated. Surprisingly, 37 (44.04%) showed a higher than threefold up-regulation and then arbitrarily the values was considered as a very significant. Thus, 37 genes (44.04%) were expressed very significantly up-regulated. The remained 47 (55.9%) genes were up-regulated less than three folds (35 genes – 41.6%) or down-regulated less than three folds (12 genes – 14.3%).

CONCLUSION: The intestinal ischemia and reperfusion promote a global hyper-expression profile of six different clusters genes related to antioxidant defense and oxidative stress.

Key words: Oxidative Stress. Antioxidants. Gene expression. Ischemia. Reperfusion. Real-Time Polymerase Chain Reaction. Mice.

RESUMO

OBJETIVO: Determinar o perfil de expressão dos genes associados com estresse oxidativo e contribuir para estabelecer parâmetros sobre o papel das familias de enzimas relacionadas com a lesão de isquemia / reperfusão intestinal.

MÉTODOS: Doze camundongos machos isogênicos (C57BL/6) foram distribuídos aleatoriamente: Grupo Controle (CG) submetido à laparotomia anestesia, e observado por 120min; Grupo isquemia/reperfusão (IRG) submetido à anestesia, laparotomia, 60min de isquemia do intestino delgado e 60min de reperfusão. Um *pool* dos seis camundongos de cada grupo foi submetido ao protocolo de

qPCR-RT (seis famílias) para o estresse oxidativo e defesa antioxidante

RESULTADOS: Dos 84 genes investigados, 64 (76,2%) tiveram expressão estatística significante e 20 (23,8%) não apresentaram diferença estatística com o grupo controle. Dos 64 genes expressos de forma significante, 60 (93,7%) foram hiper-expressos e 04 (6,3%) foram hipo-expressos. Do grupo sem expressão estatisticamente significante, 12 genes foram hiper e 8 genes foram hipo-expressos. Surpreendentemente, 37 (44,04%) apresentaram expressão três maior que o limiar de normalidade e arbitrariamente os valores foram considerados como altamente significantes. Assim, 37 genes (44,04%) foram hiper-expressos de modo muito significante. Nos demais, 47 (55,9%) dos genes foram hiper-expressos menos de três vezes (35 genes - 41,6%) ou hipo-expressos menos de três vezes(12 genes - 14,3%).

CONCLUSÃO: A isquemia e reperfusão intestinal promoveu um perfil de hiper-expressão global das seis familias de genes relacionados com estresse oxidativo antioxidante e defesa antioxidante.

Descritores: Estresse Oxidativo. Antioxidantes. Expressão Gênica. Isquemia. Reperfusão. Reação em Cadeia da Polimerase em Tempo Real. Camundongos.

Introduction

The oxygen is a critical substrate in the alleviation of hypoxia, anoxia, and ischemia, but paradoxically, it also functions as a deleterious metabolite during the reperfusion of previously ischemic tissues[1-3]. Ischemia reperfusion injury (IRI) is often seen in organ transplants, major organ resections and in shock[4].

Reactive oxygen species (ROS) generated during the reperfusion phase overwhelms the scavenging capacities of antioxidant enzymes, and result in oxidative damage[4,5]. The oxidative stress occurs early (i.e., minutes) after reoxygenation and will inflict further damage to numerous cellular elements such as mitochondria, proteins, nucleic acids and cellular membranes[4,5]. The stress may lead either to immediate cell death (necrosis), controlled cell death (apoptosis) or trigger changes of the cell phenotype in response to the injury[6,7].

The published literature to evaluate the IRI is focused on morphological and biochemical changes involved in the cascade of oxidative stress which, either directly by ROS or through lipoperoxidation products, may initiate the activation of specific transcription factors and expression of appropriate target genes as adaptation in response to the damage[3-6]. Knowledge of the interrelationships of these genes must be useful as potential targets for therapeutic intervention against IRI[8,9].

This research attempts to bring into the spotlight some pertinent developments regarding to the gene expression associated to the oxidative stress on the ischemia injury of small bowel. This hypothesis was tested in the present study using array technology for quantitative real-time polymerase chain reaction[10] (qRT-PCR) which provides a powerful tool to simultaneously analyze expression of these specific genes in intestinal tissue.

The objective was to determine the profile of gene expressions associated with oxidative stress and thereby contributes to establish parameters about the role of enzyme clusters related to the ischemia/reperfusion intestinal injury.

Methods

The experimental protocol (#1379/08) was approved by the Ethics Committee of the Federal University of Sao Paulo (UNIFESP), Brazil. The study was designed as a randomized controlled trial with a blinded assessment of the outcome using inbred mice (C57BL/6).

Twelve male inbred mice - C57BL/6 (Center for the Development of Experimental Models for Medicine and Biology – CEDEME/UNIFESP) that weighed 35 to 40g were housed under temperature and light controlled environmental conditions with a 12 hours light-dark cycle. The animals had free access to water and standard pellet chow until six hours prior to the surgical procedures. All procedures were conducted in the laboratories of Experimental Surgery, Department of Surgery at Federal University of Grande Dourados (UFGD), Brazil. The animals were randomly assigned to one of two groups: Control Group (CG) submitted to anesthesia, laparotomy and observed by 120min; Ischemia/reperfusion Group (IRG) submitted to anesthesia, laparotomy, 60min of small bowel ischemia and 60min of reperfusion.

The animals received an intramuscular combination of 44mg/kg of ketamine, 2.5 mg/kg of xylazine and 0.75mg/kg of acepromazine. Body temperature was maintained at 37.8°C using a homeothermic soft blanket. After the samples were collected, the animals were dead by beheading.

Under aseptic conditions and using a magnification device (4x), all 12 animals underwent a midline laparotomy. In the IRG (n=6) the superior mesenteric artery was carefully dissected and then occluded by a microvascular clamp during 60min. Ischemia was confirmed by observing the pale appearance of the clamped

small bowel and absent of beats in the mesenteric branches artery. The surgical wounds remained covered with wet gauze wrappings to minimize evaporative loss. After 60min the clamp was removed, reperfusion was evaluated based on immediate color recovery and artery beats. Following 60min of reperfusion there was a collected sample (3cm each) of small bowel 20cm far from the duodenojejunal flexure. In the CG (n=6) was performed the anesthesia, laparotomy, superior mesenteric artery dissection without occlusion, and following the 120min was a collected sample (3cm) of small bowel. All the samples were longitudinally open, gently washed in saline solution and less than one and half minute was involved in aluminum sheet and harvested in liquid nitrogen.

RT² Profiler™ PCR array from SA Biosciences (Frederick, Maryland; cat # PAMM-065) was performed for mouse oxidative stress and antioxidant defense pathways according to manufacturer's protocol. Briefly, total RNA was extracted from CG and IRG small bowel tissues using Trizol reagent (Life Technologies, Grand Island, NY, USA) and step crossed purified (Rneasy MiniKit Qiagen, Co - USA). The concentration of total RNA samples was determined by spectrophotometry and quality assessed using the same analysis on agarose gel 2%. One microgram of total RNA was used to make first strand complementary DNA (cDNA) using RT2 First Strand Kit (SA Biosciences). Equal amount of cDNA was mixed with Master Mix - SYBR Green Company SA Biosciences (Qiagen, Co) and aliquoted to each well of the PCR array plate containing the pre-dispensed gene-specific primer sets, and performed PCR according to manufacturer's instruction. The PCR was done in 96 well plates with 84 genes related to oxidative stress, five housekeeping genes (Actin B, Gapdh, Hsp90ab1, Hprt1, Gusb) used for normalizing the PCR array data, and one negative control for genomic DNA contamination, a primer set that specifically detects non-transcribed, repetitive genomic DNA with a high level of sensitivity, and three wells of reverse transcription controls (RTC) to verify the efficiency of the RT reaction with a qPCR assay that specifically detects template synthesized from the first strand synthesis kit's built-in external RNA control. The replicate positive PCR controls (PPC) were also used to check the efficiency of the polymerase chain reaction itself. These elements use a pre-dispensed artificial DNA sequence and the primer set that detects it. The two sets of replicate control wells (RTC and PPC) also test for inter-well and intra-plate consistency. The instrument's software (MxPro Equipment Real Time Systems – Stratagene - GE, Co) calculates the threshold cycle (Ct) values for all the genes in the array. Finally, it calculates fold changes in gene expression for pair wise comparison using the ΔΔCt method from the raw threshold cycle data. This method used in our study to determine the relative expression levels of genes of interest for each sample are contained in the spreadsheet itself for analysis of plates of sheet PCR Array Data Analysis v3.3 (SA Biosciences - Qiagen, Co).

Statistical analysis

The analysis of gene expression by real-time PCR used in this work represents a relative quantification of genes of interest. To support the analysis 06 endogenous controls were part of the PCR reactions array for each of the samples tested, genes whose expression was used do not present a statistically significant variation between samples. The control samples for each experimental group were used as reference baseline. The results were transformed into log2 scale for the calculation of averages and standard errors and for statistical analysis. Fold-Change [2^ (- Delta Delta Ct)] is the normalized gene expression [2^ (- Delta Ct)] in the Test Sample divided the normalized gene expression [2^ (- Delta Ct)] in the Control Sample. Fold-Regulation represents fold-change results in a biologically meaningful way. It was considered that fold-change values greater than one indicates a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. The p values are calculated based on a Student's t-test of the replicate 2^ (- Delta Ct) values for each gene in the control group and treatment groups. P values less than 0.05 was considered significant in all experiments and indicated by symbols.

Results

Given the 84 genes related to oxidative stress and antioxidant defense in the inbred mice (C57BL/6), 64 (76.2%) genes had statistic significant expression and 20 (23.8%) showed no statistical difference to the control group. From these 64 significantly expressed genes, 60 (93.7%) were up-regulated and 04 (6.3%) were down-regulated. From the group with no statistical significantly expression, 12 genes were up-regulated and 8 genes were down–regulated. On the 84 genes investigated, 37 (44.04%) showed a higher than threefold up-regulation. The gene bank, symbol abbreviation (alphabetical order), description of genes, the fold up or down-regulation, and the *p* value were depicted on the sequence of Tables 1, 2 and 3. In the Figure 1 the scatter plot allows the visualization of up and down fold-regulation between the test sample and control in the 84 genes expression investigated.

Six major functional gene clusters allowed by qRT-PCR kit were subjected to hierarchical clustering analysis using

the ΔΔCt method from the raw threshold cycle data: glutathione peroxidase (ten genes), antioxidants peroxiredoxin (eight genes), antioxidants peroxidases (16 genes), reactive oxygen species (ROS), superoxide metabolism (16 genes), oxidative stress (22 genes) and genes involved in oxygen transporters metabolism (12 genes).

With regard to genes related to glutathione peroxidases (GPx) the expression of 70% showed fold up-regulation; genes involved with the antioxidants peroxiredoxin (TPx), 75% showed significant fold up-regulation; genes involved with the antioxidants peroxidases, 87.5% showed significant fold up-regulation; genes involved in reactive oxygen species (ROS) and superoxide metabolism, 93.7% showed significant fold up-regulation; genes involved in oxidative stress responsive metabolism, 84.6% showed significant fold up-regulation; genes involved in oxygen transporters metabolism, 91.7% showed significant fold up-regulation.

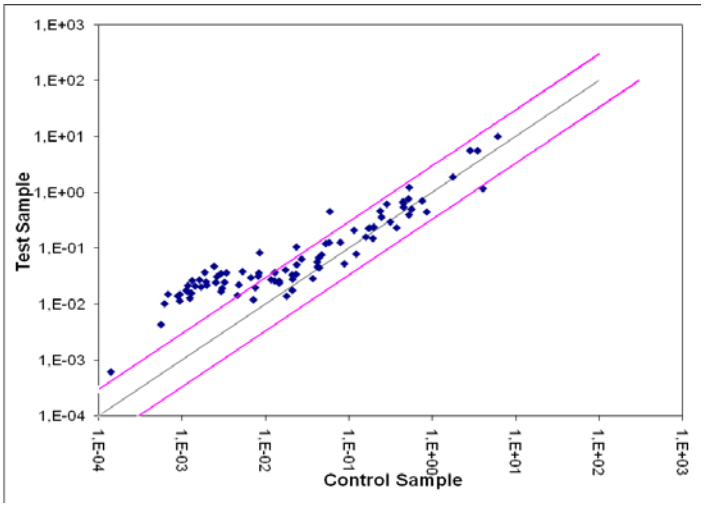

FIGURE 1 - Scatter plot allows the visualization of up and down fold-regulation of all gene expression investigated. The black line indicates fold changes [(2 ^ (-DC$_t$)] of 1. The pink lines indicate the threefold-change in gene expression threshold.

TABLE 1 - Alphabetical sequence of genes investigated and the fold up (+) or down (-) regulation (Bold=up-regulation/ threefold). (*Student's* t-test). (*=significant value p<0.05).

#	Gene Bank	Symbol	Gene description	Fold Up/down	*p* value
1	NM_013930	Aass	Aminoadipate-semialdehyde synthase	**+19.64**	**0.000001***
2	NM_028717	Als2	Amyotrophic lateral sclerosis 2 homolog	+1.47	**0.001659***
3	NM_007462	Apc	Adenomatosis polyposis coli	-1.07	0.550495
4	NM_009696	Apoe	Apolipoprotein E	+1.94	**0.002533***
5	NM_009702	Aqr	Aquarius	+2.35	**0.000257***
6	NM_019864	Atr	Ataxia telangiectasia and rad3 related	+2.84	**0.000036***
7	NM_009804	Cat	Catalase	+2.31	**0.000234***
8	NM_016892	Ccs	Copper chaperone/ superoxide dismutase	+2.37	0.124457
9	NM_001081339	Xirp1	Xin actin-binding repeat containing 1	**+15.85**	**0.000232***
10	NM_007798	Ctsb	Cathepsin B	+1.46	0.084244
11	NM_007806	Cyba	Cytochrome b-245. alpha polypeptide	-1.55	0.077515
12	NM_030206	Cygb	Cytoglobin	**+6.37**	**0.001317***
13	NM_001039520	Dnm2	Dynamin 2	-1.34	0.095871
14	XM_130483	Duox1	Dual oxidase 1	**+12.08**	**0.000466***
15	NM_153068	Ehd2	EH-domain containing 2	**+12.05**	**0.000047***
16	NM_007946	Epx	Eosinophil peroxidase	**+16.85**	**0.002598***
17	NM_007949	Ercc2	Excision repair cross-complementing 2	**+4.34**	**0.001642***
18	NM_001081221	Ercc6	Excision repair cross-complementing 6	+2.17	**0.003671***
19	NM_007985	Fancc	Fanconi anemia. complementation C	**+7.25**	**0.000015***
20	NM_018881	Fmo2	Flavin containing monooxygenase 2	+1.60	**0.019633***
21	NM_021356	Gab1	Growth factor receptor bound protein 2	+1.10	0.197265
22	NM_008160	Gpx1	Glutathione peroxidase 1	+1.61	**0.001431***
23	NM_030677	Gpx2	Glutathione peroxidase 2	-1.30	0.208667
24	NM_008161	Gpx3	Glutathione peroxidase 3	-1.18	0.226815
25	NM_008162	Gpx4	Glutathione peroxidase 4	-1.93	0.000234*
26	NM_010343	Gpx5	Glutathione peroxidase 5	**+4.42**	**0.001774***
27	NM_145451	Gpx6	Glutathione peroxidase 6	**+12.28**	**0.000015***
28	NM_024198	Gpx7	Glutathione peroxidase 7	**+16.01**	**0.000362***
29	NM_027127	Gpx8	Glutathione peroxidase 8 (putative)	+1.78	0.072334
30	NM_010344	Gsr	Glutathione reductase	+1.36	0.149896
31	NM_029555	Gstk1	Glutathione S-transferase kappa 1	+1.32	**0.002691***

TABLE 2 - Alphabetical sequence of genes investigated and the fold up (+) or down (-) regulation (Bold=up-regulation/ threefold). (*Student's* t-test). (*=significant value p<0.05).

#	Gene Bank	Symbol	Gene description	Fold Up/down	*p* value
32	NM_175000	Hbq1	Hemoglobin. theta 1	+**18.39**	**0.000126***
33	NM_010497	Idh1	Isocitrate dehydrogenase 1 (NADP+).	-1.13	0.266264
34	NM_026298	Ift172	Intraflagellar transport 172 homolog	+**7.68**	**0.000029***
35	NM_001009940	Il19	Interleukin 19	+**12.93**	**0.000010***
36	NM_016971	Il22	Interleukin 22	+**19.90**	**0.000083***
37	NM_010628	Kif9	Kinesin family member 9	+**12.24**	**0.000681***
38	NM_080420	Lpo	Lactoperoxidase	+**22.32**	**0.000020***
39	NM_013593	Mb	Myoglobin	+**10.31**	**0.000024***
40	NM_010824	Mpo	Myeloperoxidase	+**11.93**	**0.000008***
41	NM_145143	Mpp4	Membrane protein. palmitoylated 4	+**7.77**	**0.000001***
42	NM_010877	Ncf2	Neutrophil cytosolic factor 2	+**4.48**	**0.000018***
43	NM_022414	Ngb	Neuroglobin	+**11.08**	**0.000386***
44	NM_010927	Nos2	Nitric oxide synthase 2. inducible	+**4.64**	**0.000117***
45	NM_172203	Nox1	NADPH oxidase 1	+**14.76**	**0.000077***
46	NM_015760	Nox4	NADPH oxidase 4	+**12.26**	**0.000005***
47	NM_172204	Noxa1	NADPH oxidase activator 1	+**3.13**	**0.000083***
48	NM_027988	Noxo1	NADPH oxidase organizer 1	+1.32	**0.000177***
49	NM_008706	Nqo1	NAD(P)H dehydrogenase. quinone 1	+1.19	0.194275
50	NM_172527	Nudt15	Nudix type motif 15	+**9.77**	**0.000018***
51	NM_008750	Nxn	Nucleoredoxin	+2.03	**0.002268***
52	NM_020569	Park7	Parkinson disease 7	+1.82	**0.001012***
53	NM_133819	Ppp1r15b	Protein phosphatase 1. Subunit 15b	-1.01	0.962143
54	NM_011034	Prdx1	Peroxiredoxin 1	+1.30	**0.031985***
55	NM_011563	Prdx2	Peroxiredoxin 2	+1.47	**0.015081***
56	NM_007452	Prdx3	Peroxiredoxin 3	+1.52	**0.030003***
57	NM_016764	Prdx4	Peroxiredoxin 4	+1.21	0.270081
58	NM_012021	Prdx5	Peroxiredoxin 5	-1.61	0.008534*
59	NM_007453	Prdx6	Peroxiredoxin 6	-1.29	0.010835*
60	NM_177256	Prdx6-rs1	Peroxiredoxin 6. related sequence 1	+1.02	0.848407

TABLE 3 - Alphabetical sequence of genes investigated and the fold up (+) or down (-) regulation (Bold=up-regulation/ threefold). (*Student's* t-test). (*=significant value p<0.05).

#	Gene Bank		Symbol	Gene description	Fold Up/down	*p* value
61	NM_011170	61	Prnp	Prion protein	+**5.77**	**0.001140***
62	NM_011186	62	Psmb5	Proteasome subunit. beta type 5	+1.20	0.078442
63	NM_008969	63	Ptgs1	Prostaglandin-endoperoxide synthase 1	+1.55	**0.012124***
64	NM_011198	64	Ptgs2	Prostaglandin-endoperoxide synthase 2	+**9.65**	**0.000003***
65	NM_009020	65	Rag2	Recombination activating gene 2	+**14.60**	**0.000293***
66	NM_058214	66	Recql4	RecQ protein-like 4	+**10.76**	**0.000121***
67	NM_009127	67	Scd1	Stearoyl-Coenzyme A desaturase 1	+**7.65**	**0.000420***
68	NM_173052	68	Serpinb1b	Serine (or cysteine) peptidase inhibitor.	+1.62	0.065882
69	NM_134086	69	Slc38a1	Solute carrier family 38. member 1	+**4.47**	**0.000106***
70	NM_027868	70	Slc41a3	Solute carrier family 41. member 3	-1.29	0.391744
71	NM_011434	71	Sod1	Superoxide dismutase 1. soluble	+1.41	0.062857
72	NM_013671	72	Sod2	Superoxide dismutase 2. mitochondrial	+1.61	**0.009249***
73	NM_011435	73	Sod3	Superoxide dismutase 3. extracellular	+2.62	**0.002347***
74	NM_029688	74	Srxn1	Sulfiredoxin 1 homolog (S. cerevisiae)	-1.67	0.000594*
75	NM_021883	75	Tmod1	Tropomodulin 1	+**15.80**	**0.000161***
76	NM_009417	76	Tpo	Thyroid peroxidase	+**20.12**	**0.000043***
77	NM_023719	77	Txnip	Thioredoxin interacting protein	+1.95	**0.000260***
78	NM_015762	78	Txnrd1	Thioredoxin reductase 1	+1.63	0.058357
79	NM_013711	79	Txnrd2	Thioredoxin reductase 2	+1.68	**0.000027***
80	NM_153162	80	Txnrd3	Thioredoxin reductase 3	+2.36	**0.001705***
81	NM_009464	81	Ucp3	Uncoupling protein 3	+**16.70**	**0.000102***
82	NM_011701	82	Vim	Vimentin	+2.16	**0.000155***
83	NM_011728	83	Xpa	Xeroderma pigmentosum.	+2.14	**0.002744***
84	XM_127602	84	Zmynd17	Zinc finger. MYND domain containing 17	+**3.87**	**0.000172***

Discussion

Our data demonstrated the effective detection of differential expression of 84 gene transcripts which are involved in oxidative stress and antioxidant-related gene expression in ischemia/reperfusion injury of small bowel by using quantitative RT-PCR array. Surprisingly, the high values of up-regulation were unexpected findings and then arbitrarily the threshold of three folds values of up- or down-regulation of six samples pool of inbred mice of IRG in comparison to CG we considered as a very significant. Thus, 37 genes (44.04%) were expressed very significantly up-regulated. The remaining 47 (55.9%) genes were up-regulated less than three folds (35 genes – 41.6%) or down-regulated less than three folds (12 genes – 14.3%), as compared to control small bowel sample.

Determining protein functions from genomic sequences is a challenge. The studies in literature are based in the assumption that proteins that function together in a pathway or structural complex are likely to evolve in a correlated fashion[11].

The findings explored in the present study attempt to signal the possible implications of current knowledge and future lines of inquiry to elucidate this relationship. The following clusters analysis is based on the available literature on gene expression in general, since it specifically in relation to gut the reports are limited.

The glutathione and peroxiredoxin are two families of thiol peroxidases catalyze the reduction of hydroperoxides by thiols, essentially by similar mechanisms. The similarity of Gpx and Prx mechanisms and specificities should, however, not be considered indicative of a common phylogenetic ancestor; it rather is an example of convergent evolution. Just focusing on the situation in mammals, we have to encounter eight Gpx- and six Prx-type peroxidases on top of catalase, other heme peroxidases, and GSH-S-transferases doing similar jobs[12].

The peroxidase activity of glutathione play the biological role to protect the organism from oxidative damage reducing lipid hydroperoxides to their corresponding alcohols and reducing free hydrogen peroxide to water. It is expressed in nearly every mammalian cell. There are several isozymes encoded by eight different genes isoforms, which vary in cellular location and substrate specificity.

In our results we highlight the over expression of Gpx5, Gpx6 and Gpx7. Gpx7 is unrelated to the other isoforms but has high intensity expression profile in human tissue with high-confidence ($p<0.04$) mainly in smooth muscle[13]. The integrity of the muscle layer directly affects bowel motility, although it has greater resistance to necrosis and apoptosis in ischemic conditions than the mucosal layer[13-16]. The up-regulation of Gpx7 may be a promising factor in further research to clarify their involvement in the whole antioxidant process and as a monitor of therapeutic procedures. Moreover, the up-regulation of other isoforms of the cluster of glutathione can be investigated with the same purpose.

The peroxiredoxin (Prdx) represents an important emerging family of sulfhydryl-linked antioxidant proteins, apparently ubiquitously present in all known organisms. The antioxidant function of the peroxiredoxin complements that of other enzymic and non-enzymic systems within the cell. In addition, there is increasing evidence that the peroxiredoxin play a role in cell signaling, by controlling and/or sensing hydrogen peroxide and peroxynitrite levels[17].

On the cluster of 08 genes involved with the Prdx genes 06 (75%) of them were up-expressed. However, Ehd2 were significantly expressed over than threefold. This gene, as the Gpx7, was report as of high intensity expression profile in human tissue with high-confidence ($p<0.04$) mainly in smooth muscle[18]. The over expression of the peroxiredoxin cluster genes and their relationship with the antioxidant pathway is a challenging task.

Peroxidases are a large family of catalyst enzymes. For many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. Peroxidases can contain a heme cofactor in their active sites, or redox- active cysteine or selenocysteine residues. While the exact mechanisms have yet to be elucidated, peroxidases are known to play a part in modulation of oxidative stress[19]. On the 16 peroxidases allowed by the kit of PCR array 04 (25%) were up regulated less than threefold (Cat, Ctsb, Ptgs1, Serpinb1b) and 10 (62.5%) showed up regulation over than threefold (Aass, Duox1, Epx, Kif9, Lpo, Mpo, Ptgs2, Rag2, Tmod1, Tpo). The Aass (Alpha-aminoadipic semialdehyde synthase - mitochondrial) is a bifunctional enzyme that catalyzes the first two steps in the mammalian lysine degradation pathway[19]. It up-regulation may mean an adjustment of the respiratory chain to the conditions of hypoxia in intestinal tissue. The Epx and Tpo in turn are from a family of mammalian peroxidases, that includes Lpo and Mpo, and all then related to electron transporter activity, oxide reductase activity, peroxidase activity and response to oxidative stress. Among the genes over expressed draws our attention the Kif9 (Kinesin family member 9). The kinesin is a protein belonging to a class of motor proteins found in eukaryotic cells. It moves along microtubule cables powered by the hydrolysis of ATP. Their active movement supports several cellular functions including mitosis, meiosis and transport of cargo such as axonal transport.

Most of then walk towards the plus end of a microtubule which, in most cells, entails transporting cargo from the centre of the cell towards the periphery[21]. The up-regulation of Kif9 can be taken as an attempt by cells to maintain their integrity of microtubules and further studies should consider this factor as a monitor of modulation or predictive biomarker on the effects of intestinal ischemia/reperfusion injury. Similarly over expressions of other catalases studied deserve further studies to take advantage of the capability of the findings.

Most of the oxygen consumed by aerobic organisms is reduced to water but a significant proportion of the oxygen molecules are converted to superoxide anion radicals. Chemically-reactive molecules containing oxygen such as oxygen ions and peroxides are highly reactive due to the presence of unpaired valence shell electrons. A cascade of enzymes, some of them inside the mitochondria themselves, scavenges superoxide anions in order to protect cells from oxidative damage induced by reactive oxygen species (ROS). The yield of superoxide generation and subsequently ROS production depend mostly on oxygen concentration[22].

Effects of ROS on cell metabolism have been well documented in different tissues and organs in a large number of species. These include not only roles in apoptosis but also positive effects such as the induction of host defense genes and mobilization of ion transport systems. This reactive oxygen species (ROS) form as a consequence of the normal metabolism of oxygen and play important roles in cell signaling. ROS levels can increase dramatically into a situation of hypoxia and is associated with increases in the expression of genes critical for tissue oxygen consumption[23]. The present study found changes in the quantitative expression level of key genes of ROS. On the cluster of 16 genes involved with the ROS genes, nine (56.3%) were significantly up-regulated. The up-regulation of IL22 and IL19 drew attention. Interleukin-22 is related as extracellular protein involved to acute-phase of inflammatory response by a mechanism of cell-cell signaling, while interleukin-19 is also an extracellular protein related to cytokine activity and signal transduction of immune response[24]. Our data suggest that the IR small bowel injury triggers a response in the production of proteins whose function is to promote an early inflammatory response and this signals a pathway that should be explored more carefully in future research.

The nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) generates superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen to produce the superoxide. Under normal circumstances, the complex is latent in neutrophils and is activated during respiratory chain. It is well known the role of neutrophils adherence and the production of inflammatory substances during oxidative stress situations[25]. In the present research the gene expression of Nox1, Nox4, Noxa and Noxo1 were up-regulated. Does it show the attempt of small bowel tissue in to protect itself from ROS or should be a deleterious effect that worsening the IRI? Drug therapies or procedures of ischemic preconditioning can make contribution to the better understanding of how actually the mitochondrial respiratory chain acts on the phenomenon and how were the best options to reduce or eliminate their effects.

Considering now the gene expression related of 22 genes involved in the cluster of oxidative stress responsive metabolism, 19 (86.3%) of them showed significant fold up-regulation. The highest over expression was due to genes Ucp3, Nudt15, Mpp4, Prnp, Ercc2 and Zmynd17. Ucp3 is member of the larger family of mitochondrial anion carrier proteins and facilitate the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane. It also reduce the mitochondrial membrane potential in mammalian cells. This gene's protein product is postulated to protect mitochondria against lipid-induced oxidative stress[2,27]. Nudt15 mediates the hydrolysis of some nucleoside diphosphate derivatives and in vitro is associated to preventing misincorporation of 8-oxo-dGTP into DNA thus preventing A:T to C:G transversions. Its substrate specificity *in vivo* however remains unclear but may have a role in DNA synthesis and cell cycle progression thought the interaction with proliferating cell nuclear antigen (PCNA)[28]. The Prnp gene provides instructions for making a protein called the prion protein (PrP), which is active in the brain and several other tissues. Although the precise function of PrP is unknown, it is probably involved in the transport of charged copper atoms (copper ions) into cells. Researchers have also proposed roles for PrP in cell signaling, cell protection, and the formation of synapses, which are the junctions between nerve cells (neurons) where cell-to-cell communication occurs[29]. Could it be that the up regulation is associated with the protecting of the myoenteric plexus? It is a path to be explored. Our results with this cluster point to a significant over expression of genes encoding the oxidative stress responsive metabolism, but they need more researches to prove and establish correlations between these findings and the possibility of interfering on them.

The transport of oxygen is related to the directed movement of oxygen into, out of or within a cell, by means of some agent such as a transporter or pore. Within the red blood cell the hemoglobin molecule is subjected to modulation mechanisms,

which optimize its functional behavior to the specific physiological requirements. At the cellular level, these modulation mechanisms are utilized to perform a number of other functions that are not minor with respect to the basic function of oxygen transport[30]. A dynamic mathematic model of RBC metabolism, involving the O_2 sensing mechanism of hemoglobin and the hypoxia-induced activation correlated with their release to predict temporal alterations in intracellular metabolites and cellular energetic in response to hypoxia[31].

In present work from 12 genes involved in oxygen transporters metabolism, 11 (91.6%) were up-regulated, but eight (66.7%) of them were up-regulated than threefold, three (25%) were up-regulated less than twofold and only one (8.3%) was down-regulated. Hbq1 a member of the human alpha-globin gene family that involves five functional genes and two pseudo genes showed the highest expression among the oxygen transport genes investigated. Instead the predictive interactions of Hbq1 with the others four alpha globins, their expression in the different tissues and exactly function is until now unknown[32]. Myoglobin (Mb) is an iron- and oxygen-binding protein found in the muscle tissue (mainly in the heart and skeletal muscle) of vertebrates in general and in almost all mammals. It is related to hemoglobin, which is the iron- and oxygen-binding protein in blood, specifically in the red blood cells. The only time myoglobin is found in the bloodstream is when it is released following muscle injury. It is an abnormal finding, and can be diagnostically relevant when found in blood[33]. In the present research the gene was up-regulated. Recent studies suggest that NO can serve as an antioxidant of the high oxidizing ferryl myoglobin which has been proposed at least in part responsible for the oxidative damage caused by the reperfusion of ischemic tissues. The relationship between NO and myoglobin probably plays a role when NO was completely consumed and large amount of nitrite are still present[34]. A further research in our experimental model should try to establish the relationship between gene expression observed and the presence of myoglobin into the bloodstream. It would also be highly relevant to associate with such events of IRI to the intestinal smooth muscle. Ngb is a gene that encodes an oxygen-binding protein that is distantly related to members of the globin gene family. It is highly conserved among other vertebrates. It is expressed in the central and peripheral nervous system where it may be involved in increasing oxygen availability and providing protection under hypoxic/ischemic conditions. Neuroglobin (Ngb) and citoglobin (CYgb) are two globins, whose functions are still a matter of debate. Its physiological role is unknown but, like hemoglobin, myoglobin, and cytoglobin/histoglobin, it may transport oxygen, detoxify reactive oxygen species, or serve as a hypoxia sensor. A potential role of detoxification of ROS is suggested mainly in neuronal tissue[35]. In the present work we observed a significantly up-regulation of Ngb and Cygb suggesting that the simultaneous study of the myenteric plexus should be a promise line of investigation. Fancc is gene related to protect primary blood cells from cytotoxic and genotoxic effects of cross-linking agents in a specific illness (Fanconi anemia) but also exhibits functions in hematopoietic normal cells in addition to its role in the complex corrects the defect in Fanconi anemia complementation group C cells[36]. Our data showed a significantly up-regulation (+7.25) that could be related to attempt to protect the blood cells from ischemia damage.

To sum up, the RT-qPCR tool allowed an evaluation of a wide range of 84 genes correlated with oxidative stress in a model study of ischemia and reperfusion injury in the intestine of inbred mice. The results indicated a clear hyper-expression of genes that are known to be involved in antioxidant defense mechanism. The literature review did not show any other work involving this type of model or somewhat similar. This unprecedented study opens perspectives to a more detailed analysis for each gene family. The results obtained here on the gene expression of the oxidative stress could be compared with the effect of the different antioxidants drugs, scavengers of ROS, the procedures for ischemic preconditioning, hyperbaric oxygen, and other proceedings commonly used for relieve or abolish ischemia and reperfusion intestinal injury. The report also points to an ultimate and practical application study of the phenomena of ischemia and reperfusion with a chance of applicability on monitoring of clinical conditions such as those of intestinal transplantation.

Conclusion

The intestinal ischemia and reperfusion promote a global hyper-expression profile of six different clusters genes related to antioxidant defense and oxidative stress.

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