Effect of surgical stress on nuclear and mitochondrial DNA from healthy sections of colon and rectum of patients with colorectal cancer

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Surgical resection at any location in the body leads to stress response with cellular and subcellular change, leading to tissue damage. The intestine is extremely sensitive to surgical stress with consequent postoperative complications. It has been suggested that the increase of reactive oxygen species as subcellular changes plays an important role in this process. This article focuses on the effect of surgical stress on nuclear and mitochondrial DNA from healthy sections of colon and rectum of patients with colorectal cancer. Mitochondrial DNA copy number, mitochondrial common deletion and nuclear and mitochondrial 8-oxo-2′-deoxyguanosine content were measured. Both the colon and rectal tissue were significantly damaged either at the nuclear or mitochondrial level. In particular, mitochondrial DNA was more damaged in rectum than in colon. The present investigation found an association between surgical stress and nuclear and mitochondrial DNA damage, suggesting that surgery may generate an increase in free radicals, which trigger a cascade of molecular changes, including alterations in DNA.

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1. Introduction

It has been known for several years that surgical resection causes oxidative stress and results in a wide spectrum of alterations in normal body homeostasis (Anup and Balasubramanian 2000; Thomas and Balasubramanian 2004). The gastrointestinal tract is extremely sensitive to surgical stress, which plays an important role in the development of postoperative complications such as the systemic immune response syndrome (SIRS) and multiple organ failure syndrome (MOFS) (Anup and Balasubramanian 2000; Thomas and Balasubramanian 2004). Many cells, such as endothelial cells and leucocytes, and pro- and anti-inflammatory mediators seem to be involved in the stress response (Thomas and Balasubramanian 2004). However, the foremost effect of stress on the gastrointestinal tract seems to be a decrease in mucosal blood flow, which compromises the integrity of the mucosa barrier. This leads to erosion, which in turn suppresses production of mucus and limits the ability to remove back-diffusing protons.

Hypoperfusion is associated with the impairment of mucosal barrier function, which permits the translocation of bacterial pathogens into systemic circulation. The lack of oxygen supply to the cells during hypoperfusion can result in hypoxia, which in turn leads to oxidative stress. It is well known that reperfusion of an ischaemic tissue increases cellular damage, initiating a cascade of damaging effects including an increase in the reactive oxygen and nitric species (ROS and RNS), which induces membrane peroxidation, DNA oxidation and enzyme denaturation (Potoka *et al.* 2002; Szabo 2003; Cerqueira *et al.* 2005). Several experiments have shown ROS involvement in mucosa

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damage produced by ischaemic reperfusion of intestine (Thomas and Balasubramanian 2004).

Histological analysis of rat intestinal tissue with ischaemia and reperfusion injury showed damage to the mucosa with shortened villi, epithelial lifting and separation of the lamina propria. The damage varied according to the degree of observed protein oxidation and lipid peroxidation (Guven *et al.* 2008). On the other hand, pre-treatment with antioxidants such as epigallocatechin-3-gallate, α-lipoic acid, ebselen and acetylcysteine reduces intestinal ischaemia/reperfusion injury (Cuzzocrea *et al.* 2000; Giakoustidis *et al.* 2008; Ghyczy *et al.* 2008; Guven *et al.* 2008).

The aim of this study was to assess DNA oxidative damage resulting from surgical stress of healthy portions of the colon and rectum of patients with colorectal cancer. In order to make this assessment mitochondrial (mt) DNA copy number, mitochondrial common deletion and nuclear (n) and mt 8-oxo-2′-deoxyguanosine (8-OHdG) content were measured.

2. Materials and methods

2.1 *Patients*

Samples were collected from 15 patients affected by colorectal cancer who had undergone anterior rectal resection at the Hospital of University 'Campus Bio-Medico' of Rome (Italy), from March to December 2008. The study group consisted of 6 males and 9 females, whose mean age was 71.3 years (range 48–89 years) (table 1). All patients underwent a standardized preoperative protocol concerning antibiotic prophylaxis, nutrition and preoperative therapy.

Table 1. Patients involved in the study

Patient	Gender	Age	TNM
1	F	85	T3 N2
2	F	72	T4 N2
3	M	88	T2 N1
4	F	89	T1 N0
5	M	48	T1 N2
6	M	61	T3 N0
7	M	68	T3 N0
8	F	56	T3 N1
9	F	56	T4 N2 M1
10	F	70	T3 N0
11	M	77	T3 N1
12	M	83	T4 N1
13	F	58	T2 N1
14	F	48	T1 N0
15	F	81	T1 N1

Patients with connective tissue pathologies, microangiopathies, ulcerative colitis or Crohn's disease or vasculitis were excluded.

All patients provided informed written consent and the study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and approved by the Ethical Committee of University Campus Bio-Medico (reference number 03/08 prot. INT ComEt CBM).

2.2 *Cell culture*

Human umbilical vein endothelial cells (HUVECs) were cultured as described by Guidi *et al.* (2008). Then cells were washed with phosphate-buffered saline (PBS, 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl), harvested by trypsinization and processed for DNA extraction, according to the instructions of the QIAmp DNA mini kit (Qiagen).

2.3 *Isolation of total DNA*

Two 4×5 mm segments of tissues from patients were excised from the edges of the resected specimens, obtaining a fragment section of colon and rectum, respectively. They were immediately frozen at −80°C, and after a few days, total DNA was isolated from tissue using the QIAmp DNA mini kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Total DNA concentration was spectrophotometrically determined at 260 nm (DU-640; Beckman Instruments, Milan, Italy), and quality was analysed on 0.8% agarose gel. DNA samples were immediately analysed or stored at −20°C.

2.4 *Quantitative real-time PCR*

Quantitative real-time PCR was carried out in a Bio-Rad iCycler iQ Multi-Color Real-Time PCR Detection System using 2× Quantitect SYBR Green PCR kit (Qiagen). The sequences of primers used for amplification are listed in table 2. Real-time PCR was performed to determine mtDNA/nDNA ratio and to quantify ND4 lesions. The mtDNA/nDNA ratio was calculated by amplifying both ND1 and ND4 (NADH dehydrogenase complex 1 and 4, respectively) as mtDNA targets and β-actin as nDNA; the ND4 lesion frequency was determined correlating ND1 and ND4, which are both on mt DNA. The PCR conditions were set up as follows: hot start at 95°C for 10 min, then 40 cycles of the two-step at 95°C for 30 s and at 60°C for 30 s. Reaction mix (25 μL final volume) consisted of 12.5 μL Mix Hot-Start (Qiagen), 50 ng/μL DNA template, 2 μL SYBR Green and 0.3 μM of each primer (table 2). Threshold cycle (Ct) was determined on the linear phase of PCRs using the iCycler

Table 2. Employed primers

100 bp, mitochondrial fragment	
ND1 f	5′ACGCCATAAAACTCTTCACCAAAG-3′
ND1 r	5′-TAGTAGAAGAGCGATGGTGAGAGCTA-3′
100 bp, mitochondrial fragment	
ND4 f	5′CCATTCTCCTCCTATCCCTCAAC-3′
ND4r	5′CACAATCTGATGTTTTGGTTAAACTATATT-3′
100 bp, nuclear fragment	
β-actin f	5′-TGACTGGCCCGCTACCTCTT-3′
β-actin r	5′CGGCAGAAGAGAGAACCAGTGA-3′
100 bp, nuclear fragment	
Human ribF1	5′-ATACCTCAGCCGACACATCC-3′
Human ribR1	5′-CTCCTCAAACGCAAGAAAGG-3′
370 bp, mitochondrial fragment	
F2	5′-CCCCTCTAGAGCCCACTGTA-3′
R2	5′-GAGTGCTATAGGCGCTTGTC-3′

iQ Optical System Software version 3 (BioRad, Milan, Italy). The specificity of the amplification products obtained was confirmed by examining thermal denaturation plots and by sample separation in a 3% DNA agarose gel. ND1 and β-actin gene copy number were determined interpolating the threshold cycle (Ct) from standard curves (figure 1) that were obtained using total DNA of the human keratinocytes NCTC 2544 cell line. The ratios were obtained relating these mitochondrial and nuclear DNA quantities.

2.5 *Detection of the mitochondrial 4977 bp common deletion by a standard PCR*

To assess the presence of the common deletion (MITOMAP 2009), which employs nucleotides from position 8469 to 13447, we used two primers, F_2 and R_2 (table 1), located at 8283 nt and 13610 nt of the complete *Homo sapiens* mtDNA sequence (accession number: NC_001807.4), respectively. These primers produce a 5347 bp amplicon from intact

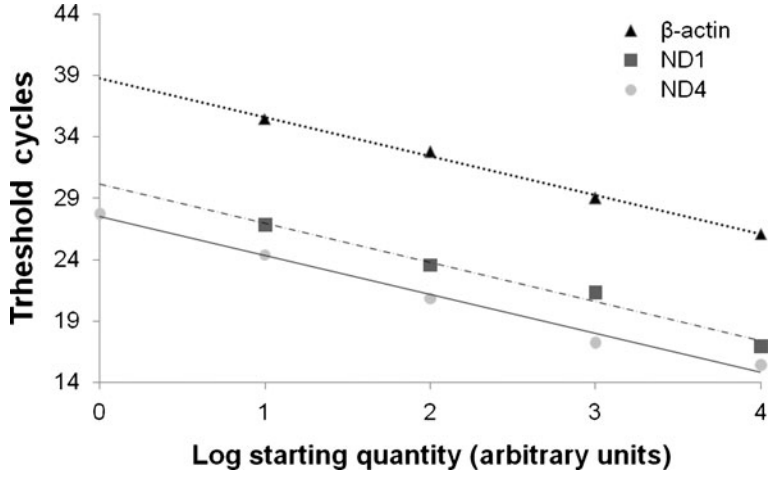

Figure 1. Standard curves of β-actin, ND1 and ND4 amplification in real-time PCR were obtained using human keratinocytes NCTC 2544 cell line DNA. The efficiency of real-time PCR amplification was established from 100 ng to 0.01 ng (10-fold repeated dilutions) cellular DNA, which was allowed to react with primers specific to ND1, ND4 and β-actin genes. The standard curves and correlation coefficient were: $y=-3.219x+38.897$ $R^2=0.997$ (β-actin); $y=-3.198x+26.959$ $R^2=0.984$ (ND1); $y=-3.177x+27.482$ $R^2=0.994$ (ND4).

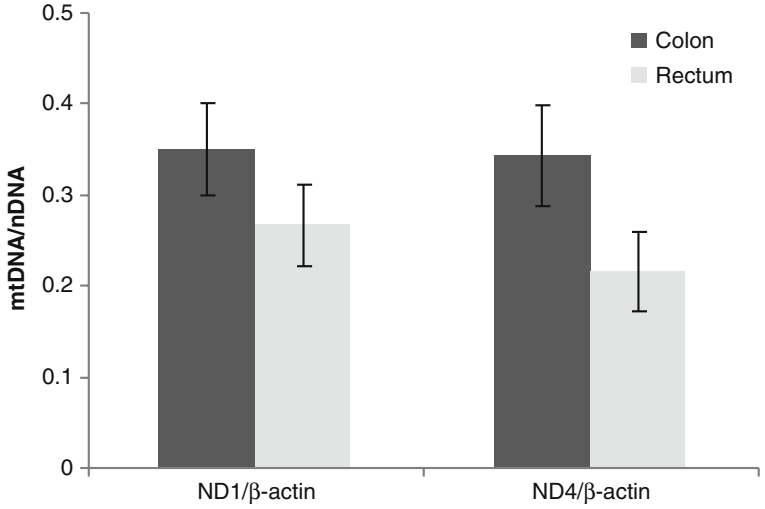

Figure 2. Determination of mtDNA/nDNA ratio as ND4/β-actin and ND1/β-actin by real-time PCR as described in the materials and methods section. Data were expressed as the mean±SEM; all samples were analysed in triplicate. The Mann–Whitney test was performed.

mtDNA and an amplicon of 370 bp in the presence of deletion (5347–4977). PCR reaction was performed in a final volume of 25 µL containing 50 ng genomic DNA, 0.5 µM of each of the above primers, 200 µM dNTPs, 2.5 µL of 10×buffer, 1.5 mM $MgCl_2$ and 1.5 U Taq DNA polymerase (Diatheva s.r.l., Fano, Italy). Reaction conditions were: denaturation at 95°C for 10 min followed by 30 cycles at 94°C for 30 s, 59°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 7 min.

2.6 *Determination of the degree of oxidative nDNA and mtDNA damage*

The content of 8-OHdG in nDNA and mtDNA was determined by the quantitative real-time PCR method. If a DNA sample contains 8-OHdG, the amplification efficiency should decrease after treatment of the DNA with hOGG1 enzyme (human 8-oxoguanine DNA glycosylase 1), which removes the 8-OHdG residue to form an abasic site (Sikorsky *et al.* 2004).

The hOGG1 treatment of DNA samples was performed according to Lin *et al. (*2008), as follows: 100 ng of each unknown DNA was digested with/or without 1 unit of hOGG1 at 37°C for 1 h in a final volume of 20 µL, and 2 µL of the treated DNA was amplified by quantitative real-time PCR using nuclear and mitochondrial couples of primers (Human ribF1 and Human ribR1, ND1 f and ND1 r; table 1). The presence of abasic sites in the DNA sample after the treatment with hOGG1 enzyme led to a delay in the amplification cycle. Each analysis was done in duplicate. The significance of oxidative DNA damage was defined by

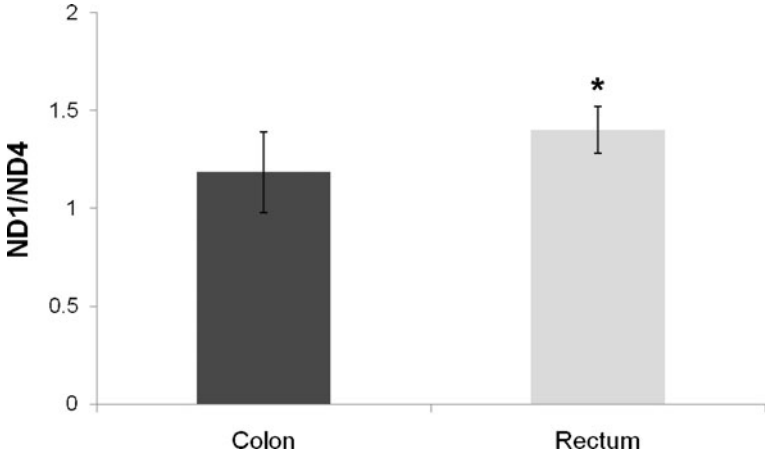

Figure 3. Determination of ND4 lesions as ND1/ND4 ratio by real-time PCR as described in the materials and methods section. Data were expressed as the mean±SEM of all patients analyzed in triplicate; the Mann–Whitney test was performed. *$P<0.0251$.

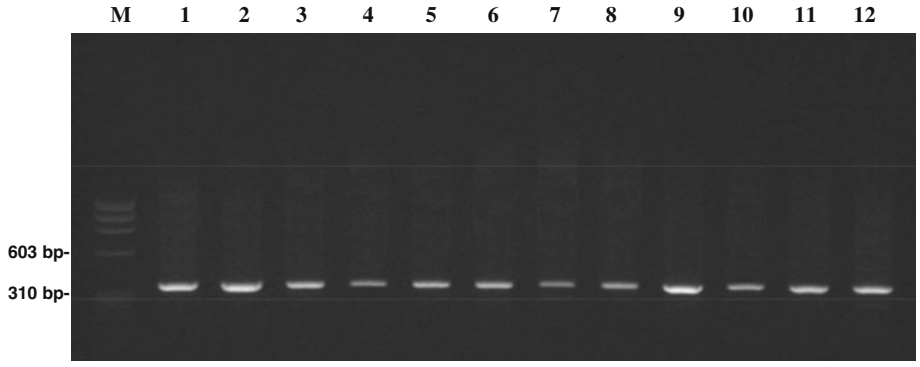

Figure 4. Electrophoretic pattern of 370 bp in length amplicons from healthy rectal sections of patients with colorectal cancer. M=ϕX174 /Hae III molecular weight marker. The amplification of 370 bp in size indicates the presence of 4977 bp mt deletion (see Materials and methods).

comparing hOGG1 untreated and treated sample with the paired *t*-test.

2.7 *Statistical analysis*

The statistical analysis was performed using Statistica 6.1, Vigonza (PD). The differences between groups were considered significant when $P<0.05$.

3. Results

Because increased levels of ROS can modify the content of mtDNA, the mtDNA/nDNA ratio was evaluated performing real-time PCR of mitochondrial ND1 and ND4 genes normalized with nuclear β-actin gene (figure 2). The mt/nDNA analysis showed a greater content of colon mtDNA than rectal mtDNA, although the difference was not statistically significant. The ND1/ND4 ratio showed a reduction of ND4 copy number in rectum ($*P<0.0251$; figure 3).

As ND4 is involved in the 4977 deletion, the mitochondrial common deletion, we can assume that this ratio represents the deletion quantisation. These results showed that in the patients under study, rectum was more damaged than colon.

Common deletion presence was confirmed by a standard PCR (as reported in materials and methods), which amplifies a fragment of 370 bp in length in the presence of deletion. Both tissues of all patients under analysis showed common deletion (figure 4).

The content of 8-OHdG was also investigated as a marker of oxidative damage. Both levels of nuclear and mitochondrial 8-OHdG were assayed by a quantitative real-time PCR method. To ensure that the 8-OHdG formation is not dependent on the extraction method, but represents a possible consequence of surgical stress, two pairs of primers, nuclear and mitochondrial, were used to perform real-time on DNA

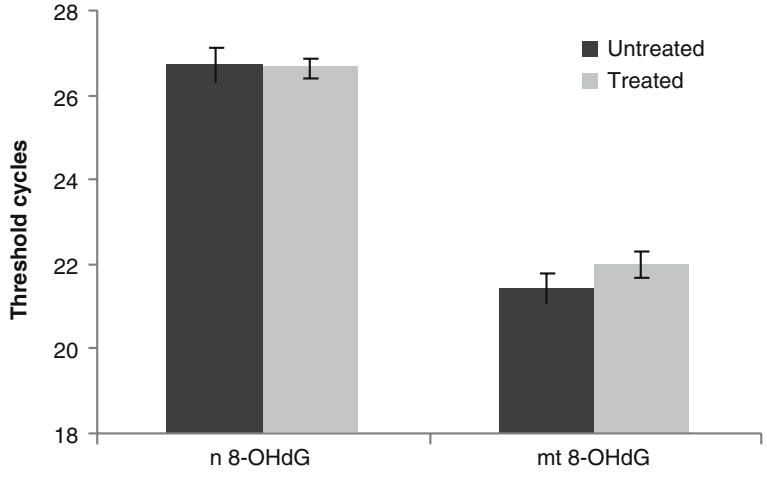

Figure 5. Determination of the degree of oxidative nDNA and mtDNA damage in DNA from untreated HUVECs and those treated with hOGG1.

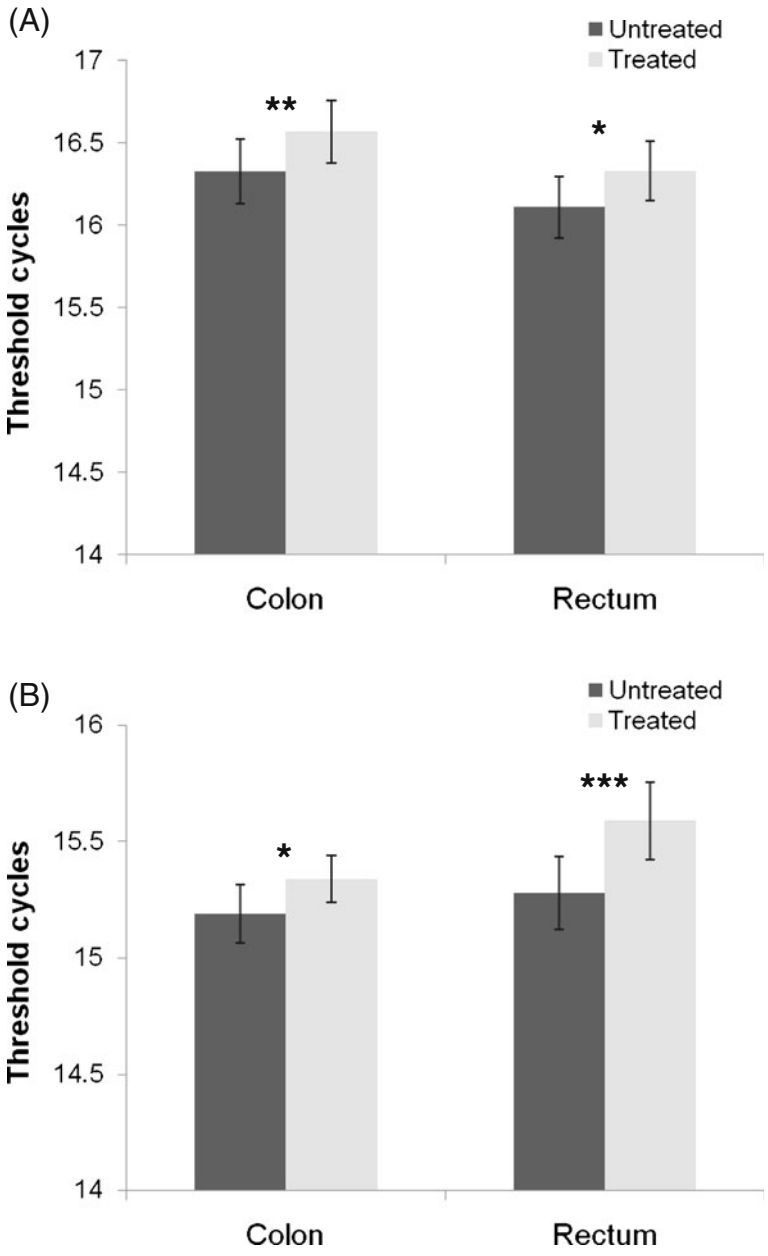

Figure 6. (**A**) Determination of the degree of oxidative damage from colon and rectum nDNA (untreated and treated with hOGG1). (**B**) Determination of the degree of oxidative damage from untreated colon and rectum mtDNA and colon and rectum mtDNA treated with hOGG1. The presence of abasic sites after treatment with hOGG1 reduces the PCR efficiency and thus increases the Ct value. Paired *t*-test was performed to compare untreated and treated samples.

extracted from HUVECs with the same extraction kit used for colon and rectum, and treated with hOGG1 or untreated. As reported in figure 5, no significant difference was found between DNA treated with hOGG1 and the untreated DNA, suggesting that 8-OHdG formation cannot be attributed to extraction procedure (figure 5). The colon and rectum were then analysed and found to be significantly damaged (figure 6) at both levels. A greater quantity of 8-OHdG was found in mt DNA of rectum, confirming previous results obtained with mt/n DNA content analysis.

4. Discussion

Assuming that surgical stress generates oxidative stress (Anup and Balasubramanian 2000; Thomas and Balasubramanian

2004), molecular characterizations were performed in order to identify potential DNA modifications linked to ROS production. Both n- and mtDNA were analysed, because they coexist in eukaryotic cells and have different sensitivity to ROS species, probably due to their different locations and conformations (Guidi *et al.* 2008). Nuclear genome consists of linear molecules located in the nucleus, while the human mitochondrial genome, completely sequenced in 1981 (MITOMAP 2009), is a 16.569 bp closed circular double-strand molecule, present in high copy number per cell and widely varying among cell types (Anderson *et al.* 1981). mtDNA has been observed to be more susceptible to damage than nuclear DNA because of several factors. These factors include mtDNA's exposure to high levels of ROS produced during oxidative phosphorilation (Yakes and Van 1997), the lack of protective histones and limited DNA repair pathways (Bohr 2002), having a robust base excision repair (BER) system but not a nucleotide excision repair (NER) one (Sawyer and Van 1999). Oxidative damage to mtDNA may lead to loss of membrane potential, reduced ATP synthesis and cell death (Van *et al.* 2006).

The results presented herein showed that n- and mtDNA from healthy sections of colon and rectum of patients affected by colorectal cancer were damaged in both tissues. However, mtDNA from the rectum was more damaged than that from the colon either when common deletion was quantified by ND1/ND4 ratio, or when content in mt- and n-8-OHdG was evaluated (figures 3 and 6).

The common deletion, an indicator of tissue deterioration in aging and in energetic, has been found in several cancers, stressed tissues, aging and even normal-looking tissues (Cortopassi and Arnheim 1990; Simonetti *et al.* 1992; Lezza *et al.* 1994; Lee *et al.* 1994; Hsieh *et al.* 1994; Fukushima *et al.* 1995; Maximo *et al.* 2001; Chen *et al.* 2011). Maximo *et al.*, in studies on thyroid tumours, have found that 100% of Hürtle adenoma and cancer cells carried the common deletion and 0–33% of the adjacent parenchyma carried the same change (Maximo *et al.* 2002). High prevalence of this deletion (95%) has also been found in esophageal tissue adjacent to tumour in patients with esophageal squamous cell cancer from which no evidence was found in peripheral blood (Abnet *et al.* 2004). Chen *et al.* investigated the common deletion in tumour tissue and paired non-tumour areas from colorectal cancer patients in the Wenzhou area of China. They found that the common deletion was more likely to be present in patients of younger age (≤65 years) and its level decreased as the cancer stage advanced (Chen *et al.* 2011). In the present study we found that 100% of non-malignant colon and rectal tissues carried the deletion, although it was found to be more prevalent in the rectum. However, in order to establish if this common deletion could represent a marker of colorectal carcinoma risk, further analysis of this deletion in subjects without cancer is required. Such an analysis would allow us to determine if the deletion can be directly associated with colorectal cancer or if it is simply a consequence of aging.

For several years, 8-OHdG has been the most frequently investigated marker of oxidative DNA damage. It can pair both with cytosine stable *syn* conformation and with adenine during DNA replication. If the A/8-OHdG intermediates are not repaired, G:C to A:T transversion are induced (Wood *et al.* 1990; Shibutani *et al.* 1991; Michaels and Miller 1992; Cheng *et al.* 1992; Moriya 1993). 8-OHdG mismatches of adenine with 8-OHdG are mainly removed by BER, the most important cellular protection mechanism. The key enzymes in the BER pathway are specific glycosylases (OGG1 and OGG2).They remove different types of modified bases and the deoxyribose moieties of the nucleotides, leaving apurinic/apyrimidinic sites (AP) as intermediates, which are substrates for endonucleases (Lu *et al.* 2001; Cooke *et al.* 2003). Since 8-OHdG is not metabolized, it is excreted in the urine (Cooke *et al.* 2003; Crohns *et al.* 2009). Indeed, many studies show the presence of 8-OHdG in urine or blood of patients with various cancers such as head and neck (Roszkowski *et al.* 2008), breast (Haghdoost *et al.* 2001), lung or colorectal cancer (Bialkowski *et al.* 1996; Crohns *et al.* 2009). This type of assay is the average 8-OHdG damage in the body and therefore may also depend on diet, the mechanisms of cell death and DNA repair (Wu *et al*. 2004), and it does not allow us to distinguish the specific damage to the tissues. The most innovative aspect of the present study compared with previous investigations is the quantification of 8-OHdG directly in the DNA of the tissue under study.

The data presented herein show that after surgical resection, nDNA and mtDNA from healthy sections of colon and rectum of patients with colorectal cancer were altered, and that mtDNA from the rectum was more damaged than the colon mtDNA.

In this study, in addition to surgical stress, the subjects' age, lifestyle and epigenetic factors, including diet, may differentially contribute to the pattern of mtDNA and nDNA alterations. Hence, in order to reduce the numbers of these variables, which can act on DNA and to be able to attribute the observed DNA alterations to surgical stress, an animal model is required because it represents a homogenous sample.

Further studies are also needed in order to understand why the mtDNA from rectum shows more damage than colon mtDNA.

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