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Decreased cell proliferation and higher oxidative stress in fibroblasts from Down Syndrome fetuses. Preliminary study

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ABSTRACT

Down Syndrome is the most common chromosomal disease and is also known for its decreased incidence of solid tumors and its progeroid phenotype. Cellular and systemic oxidative stress has been considered as one of the Down Syndrome phenotype causes. We correlated, in a preliminary study, the fibroblast proliferation rate and different cell proliferation key regulators, like Rcan1 and the telomere length from Down Syndrome fetuses, with their oxidative stress profile and the Ribonucleic acid and protein expression of the main antioxidant enzymes together with their activity. Increased oxidized glutathione/glutathione ratio and high peroxide production were found in our cell model. These results correlated with a distorted antioxidant shield. The messenger RNA (*SOD1*) and protein levels of copper/zinc superoxide dismutase were increased together with a decreased mRNA expression and protein levels of glutathione peroxidase (GPx). As a consequence the [Cu/ZnSOD / (catalase + GPx)] activity ratio increases which explains the oxidative stress generated in the cell model. In addition, the expression of thioredoxin 1 and glutaredoxin 1 is decreased. The results obtained show a decreased antioxidant phenotype that correlates with increased levels of Regulator of calcineurin 1 and attrition of telomeres, both related to oxidative stress and cell cycle impairment. Our preliminary results may explain the proneness to a progeroid phenotype.

1. Introduction

Down Syndrome (DS) is the most common chromosomal disease and one of the main causes of intellectual disability. The genes localized in chromosome 21 are linked to mitochondrial energy production, reactive oxygen species metabolism, brain development, neuronal loss, and Alzheimer's type neurodegeneration [1]. Another interesting feature is that individuals with DS have a reduced risk of solid tumors in all the age groups studied [2] and a progeria-like phenotype [3].

The presence of an oxidative stress status in DS patients has been well established [4–8]. The source of the systemic oxidative stress found in DS individuals is not clear, although it is known that Cu/Zn superoxide dismutase (Cu/ZnSOD) activity is increased in DS patients [9] and the ratio Cu/ZnSOD to catalase plus glutathione peroxidase [Cu/ZnSOD / (catalase + glutathione peroxidase)] is increased [10]. The activity of the selenoenzyme GPx could be hampered by the reported lack of selenium levels observed in DS [11]. Thus more hydrogen peroxide is generated by Cu/ZnSOD than catalase and glutathione peroxidase can catabolize, giving rise to an oxidative stress positive feed-back [12]. This process generates a positive feed-back inducing mitochondrial dysfunction [13] and impairing the respiratory complex enzymes [14], which in turn would keep increasing ROS production.

It was reported that cells from DS, or animal model of DS are prone to oxidative stress and apoptosis and have lower proliferation capability [15–19]. This finding could explain, at least in part, some of the progeria-like characteristics of the DS phenotype. Previous reports from our group have emphasized the importance of glutathione (GSH) in cell proliferation [20–22].

The purpose of our study was to clarify the cause of the progeroid-like phenotype and the reported impairment in cell proliferation. Here we provide new information showing that the high oxidative stress previously reported in DS is a very early event in development that is

Abbreviations: DS, Down Syndrome; Cu/ZnSOD, Cu/Zn superoxide dismutase; MnSOD, Mn superoxide dismutase; GPx, glutathione peroxidase; TL, telomere length; ROS, reactive oxygen species; DHR, dihydrorhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Trx1, thioredoxin 1; Grx1, glutaredoxin 1; BrdU, bromodeoxyuridine

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already present in fibroblasts from human fetuses, as reported previously in amniotic fluid form women carrying DS fetuses [23] and in brain fetuses [24,25]. In addition, our findings show a decreased antioxidant shield and an increased production of free radicals. These results partially explain the attrition of the telomere and the increased expression of Rcan1.

2. Material and methods

2.1. Human dermal fibroblast cultures from Down Syndrome (DS) and control (C) fetuses

Abdominal skin biopsies from six human fetuses, products of legal terminations, were collected with informed parental consent and approved by the Ethics Committee of the Hospital, within 2 h postmortem from the Fetal Tissue Bank at the Hospital Universitari Vall d'Hebron (fetaltissuepath@vhebron.net). Three fetuses were diagnosed as having Down Syndrome (DS): one 47, XY + 21, gestational age (GA) 20 weeks (w); one 47, XY + 21, GA 16 weeks; and one 47, XY + 21, t(21;21), GA 22 weeks. Three fetuses were considered as controls (C): one 46, XX, GA 22 weeks, diagnosed with complex cardiac malformation; one 46, XY, GA 22 weeks, diagnosed with anhydramnios with previous membrane rupture and the absence of congenital anomalies; and one 46, XX, GA 14 weeks, diagnosed with occipital encephalocele.

Primary fibroblasts were obtained from skin explants in a 25-cm^2 plastic culture flask with Eagle's Minimal Essential Medium (MEM) supplemented with nonessential amino acids, 10% fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). The flasks were maintained at 37 °C in a 95% humidified air–5% CO_2 atmosphere. The fibroblasts were released by enzymatic digestion with trypsin and sub-cultured in a 75-cm^2 plastic culture flask with 12 ml of medium. The cultures were fed by changing the medium every three days. After reaching confluence, the cells were washed and the pellet resuspended in 1 ml of MEM with 10% FCS and DMSO and frozen (24 h at −80 °C and then in liquid nitrogen). The cells were thawed and reincorporated into the cultures for ensuing experiments.

The cells were subsequently cultured in DMEM (Gibco, Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Sigma-Aldrich, MO, USA) in 5% CO_2 in air at 37 °C at a density of 20.000 cells/cm^2. The sub-cultured method used was digestion with trypsin. All experiments were performed with the same number of passages: 8–10. Studies were performed at different time points in cell culture.

2.2. Analysis of apoptotic cells by flow cytometry

Quantitative analysis of apoptotic cell death was performed by flow cytometry using Annexin V-Alexa Fluor 488 conjugate (Molecular Probes, OR, USA). Cells from culture supernatant and adherent cells collected after trypsinization, were stained with the reactive following the manufacturer's instructions, and 1 μg/ml of propidium iodide (PI) staining at room temperature for 15 min in the dark, followed by cytometric analysis in an Epics Elite cell sorter (Coulter Electronics, LA, USA). Fluorescence emissions were recorded at 515 ± 10 nm.

2.3. Cellular proliferation assay

Proliferation of DS and control fibroblasts was determined with the "Cell proliferation ELISA BdrU colorimetric" (Roche, NY, USA) as per the manufacturer's recommendations. The cells were cultured in a 96 well plate for 6 h, 24 h, 48 h, and 7 days. The colorimetric final reaction was measured with the spectrophotometer spectra MAXPLUS 384 (Molecular Devices, USA) at 370 nm using as reference wavelength 492 nm, during three consecutive five-minute periods. The variation of absorbance was proportional to the incorporation of BdrU.

2.4. Telomere length (TL)

DNA was extracted from approximately $5 \cdot 10^5$ cells using the DNeasy Blood and Tissue Handbook (Quiagen), and then quantified using the GeneQuant pro UV/Vis (GE Healthcare, Uppsala, Sweden). TL average in fibroblasts was measured with a validated quantitative (Q-PCR) based assay [26,27]. This method measures the average ratio of the telomere repeat copy numbers to a single gene (36B4) copy numbers (T/S ratio) in each sample. All samples were measured in duplicate, and their mean was used. Each of the experiments was performed in triplicate.

Briefly, the Q-PCR technique was performed using a LightCycler thermocycler (LightCycler 480II, Roche Diagnostics, Quebec, Canada) in a 384-well format. Duplicate DNA samples were amplified in parallel: 20 μl PCR reactions included 30 ng of sample DNA, the DNA master SYBR Green I kit (LightCycler® 480 Sybr Green I Master, Roche Diagnostics, USA) and 0.5 pmol/ml of specific primers for the telomere (forward: 5′CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT3′; reverse 5′GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT3′) and for the 36B4 (forward: 5′CAGCAAGTGGGAAGGTGTAATCC3′; reverse: 5′CCCATTCTATCATCAACGGGTACAA3′). The thermal cycling profile for both amplicons began with a cycle at 95 °C for 15 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 1 min and 72 °C for 10 s. The specificity of all reactions was determined by melting point curve analysis using one cycle at 95 °C for 5 s, 70 °C for 1 min, and one cycle at 40 °C for 30 s.

The technique was optimized by developing standard curves using serial dilutions from a reference DNA. This method measures the average ratio of telomere repeat copy number to a single gene (36B4) copy number (T/S ratio) in each sample.

The linear correlation coefficient (r^2) was >0.99 for the telomeric and 36B4 standard curves. The efficiency of the amplification was calculated from the slope of the linear curves. The average efficiency was 2.048 for telomeric amplification and 1.98 for 36B4 amplification. The T/S ratio was calculated using these efficiency values: T/S ratio = $\text{efficiency}^{Cp\ 36B4}/\text{efficiency}^{CpTel}$. The coefficients of variation (CV) within duplicates of the telomere length and 36B4 assays were 2.4% and 0.9%, respectively.

2.5. Peroxide levels: flow cytometry analysis

Acute intracellular production of reactive oxygen species (ROS) was assessed by incubation (30 min) of cell aliquots with dihydrorhodamine (DHR), 0.7 μM. These virtually non-fluorescent probes are oxidized to a fluorescent product. Fluorescence of a single cell was measured by a flow cytometer, Epics Elite cell sorter (Coulter Electronics, LA, USA). Fluorochrome was excited with an argon laser tuned at 488 nm and emission at 515 ± 10 nm. Forward angle and right angle light scattering were measured. Samples were acquired for 15,000 individual cells and ROS production was quantified by mean fluorescence intensities.

2.6. Determination of the GSSG/GSH ratio

The GSSG and GSH concentration was measured using DetectX® Glutathione Fluorescent Detection Kit (Arbors Assay, MI, USA) and the GSSG/GSH ratio was calculated. The cells were grown to confluence in seven days. The washed cell pellet was resuspended in 5% sulfosalicylic acid (w/v), at 10^6 cells/ml, and was lysed and deproteinized by two freeze/thaw cycles in liquid nitrogen. The measurements were performed following the manufacturer's instructions. The fluorescent product was read at 510 nm with excitation at 390 nm in the fluorometer spectra MAX GEMINIS (Molecular Devices, Sunnyvale, USA). Subsequently, we incubated the plate with NADPH and glutathione reductase to convert all oxidized glutathione, GSSG, into GSH, which reacts with the excess ThioStar® to yield the signal related to total GSH content. GSSG was calculated as GSSG = [(GSHtotal − GSHfree) / 2].

2.7. RNA extraction, reverse transcription polymerase chain reaction, and quantitative PCR

Total RNA was isolated from cells using the PARIS™ Protein and RNA Isolation System (Ambion, TX, USA) following the manufacturer's instructions. 1 μg of the purified RNA was reverse transcribed using random hexamers with the High-Capacity cDNA Archive kit (Applied Biosystems, P/N: 4322171, CA, USA) according to the manufacturer's instructions. The mRNA levels were determined by quantitative real-time PCR analysis using an ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystems, CA, USA).

TAqMan gene expression assays for *SOD1*, *SOD2*, *CAT*, *GPX1*, *TXN1* and *GRX1* (Assay-on-demand, Applied Biosystems) were used together with 1× TaqMan® Universal PCR Master Mix (Applied Biosystems, P/N 4304437; CA, USA) and 2 μl of reverse transcribed sample RNA in 20 μl reaction volumes. The PCR conditions were 10 min at 95 °C for enzyme activation, followed by 40 two-step cycles (15 s at 95 °C; 1 min at 60 °C). The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression were measured in all samples to normalize gene expression for sample-to-sample differences in RNA input, RNA quality, and reverse transcription efficiency. Each sample was analyzed in triplicate, and the expression was calculated according to the $2^{-\Delta\Delta Ct}$ method [28].

2.8. Cell lysates and Western blot analysis

Approximately $3 \cdot 10^6$ cells were lysed using lysis buffer (20 mM Hepes, pH 7.4, 1% tritonX-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM activated sodium orthovanadate, 1 mM PMSF, 0.2 % (v/v) protein proteases inhibitor cocktail), in ice for about 15 min and then the suspension was clarified by centrifugation and the supernatants were collected and stored at −80 °C until their use. Total cell extracts were obtained in the absence of reductive agents.

Protein content was determined by a modified Lowry method [29]. Aliquots of cell lysates (30–40 μg) were added to sample buffer with 10% β-mercaptoethanol and fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electroblotted (Bio-Rad) onto nitrocellulose membrane. Membranes were incubated with primary antibodies that recognize MnSOD (1:1000, Stressgen, Ann Arbor, MI, USA), Cu/ZnSOD (1:1000, Stressgen, Ann Arbor, MI, USA), catalase (1:5000, Sigma-Aldrich), GPx1 (1.5 μg/ml, Abcam, Cambridge, MA, USA), Trx1 (1:1000, Cell Signaling Technology, Inc. MA, USA), Grx1 (1:1000, Abcam, Cambridge, MA, USA), Rcan1 (anti DSCR1), a synthetic peptide corresponding to amino acids 235–252 of human DSCR1 (Gene ID 1827) (1:500 Sigma-Aldrich), and α-tubulin (1:1000, Santa Cruz BioTech.) as a loading control, overnight at 4 °C. The blots were incubated for 1 h with a secondary antibody (mouse, rabbit, or goat, according to the antibody) conjugated with horseradish peroxidase-linked. Finally, detection was carried out using the ECL™ Western Blotting Detection Reagents as specified by the manufacturer (Amersham GE Healthcare). Chemiluminescent signals were assessed using a Fujifilm scanning densitometer (Fujifilm LAS-1000 plus).

Western blotting of total extract in non-reducing buffer and electrophoresis conditions were performed to determine glutathionylated proteins, using 20 μg of total protein. The primary antibody used was anti-glutathione antibody (1:1000, Virogen, Grater Boston, MA, USA).

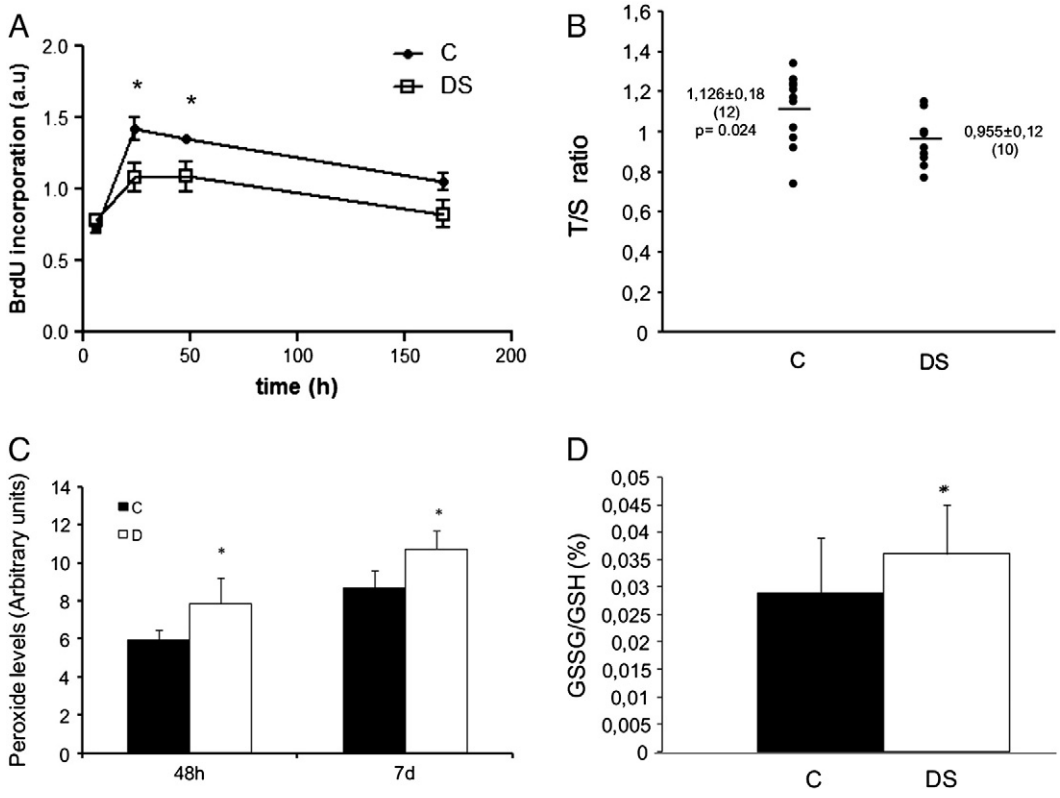

Fig. 1. Cell proliferation, telomere length (TL) and oxidative stress profile for Down Syndrome (DS) and control fibroblasts (C). (A) Cell proliferation: Cells were cultured at different time-points and then incubated with BrdU for 3 h. BrdU incorporation was determined using a BrdU antibody as described in the Material and methods section. The bar graph shows mean ± SD. Each experiment was repeated twice, n = 6. Statistically significant differences ($p < 0.05$) were compared with the control average. (B) TL of DS and C fibroblasts: Results are shown as individual values and mean ± SD; the number of determinations is stated in brackets. Experiments were performed in triplicate (two DS values were outliers). The T/S ratio was calculated using the efficiency of the amplification values: T/S ratio = $\text{efficiency}^{Cp\ 36B4}/\text{efficiency}^{CpTel}$. Statistically significant differences ($p < 0.05$) compared with the control average. (C) Cellular levels of peroxide were measured by flow cytometry detection 48 h and 7 days after cell culture, as described in the Material and methods section. The results are mean ± SD of three different experiments with three different DS patients and three different controls in each experiment. (D) GSSG/GSH cellular ratio evaluated by fluorimetric detection kit, graph bars show mean ± SD of a total of three experiments. Statistically significant differences ($p < 0.05$) compared with the control average.

The level of protein oxidation was determined by the Oxy Blot protein Oxidation Detection Kit (Merck Millipore, MA, USA) which detects carbonylated proteins. To determine oxidized protein carbonyl groups, 20 μg of total extracts was derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The derivatized samples were separated by SDS-PAGE electrophoresis followed by Western blotting and immunodetection protocols as previously described.

2.9. Measurement of antioxidant enzymatic activities

2.9.1. Measurement of Cu/ZnSOD and MnSOD activity

The cells were treated as described by the Cayman Chemical Company "Superoxide Dismutase Assay kit" following the manufacturer's instructions (Ann Arbor, MI, USA) to determine total SOD, MnSOD, and Cu/ZnSOD activities. The final absorbance was measured at 450 nm using a SPECTRA MAXPLUS 384 spectrophotometer (Molecular Devices, CA, USA).

2.9.2. Measurement of catalase activity

Catalase enzymatic activity was determined by measuring the product of the reaction of catalase in the cells with peroxide. Cells were lysed using a freeze (liquid N_2, 10 s) and thaw (ice, 15 min) procedure repeated three times. After centrifugation of the cell lysate at 13,000 g, for 10 min at 4 °C, 98% methanol and 84 mM H_2O_2 were added. The formaldehyde produced reacted with 35 mM purpald reagent (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) dissolved in 0.5 M HCl for 10 min, at room temperature. Finally, the absorbance at wavelength 540 nm was measured with a SPECTRA MAXPLUS 384 spectrophotometer (Molecular Devices, CA, USA).

2.9.3. Measurement of glutathione peroxidase activity

To determinate GPx activity the cells were treated as described by the manufacturer for "GPx Assay kit" (Cayman Chemical Company, MI, USA). The rate of decrease in absorbance at 340 nm (1 mU/ml GPx) was measured by the SPECTRA MAXPLUS 384 spectrophotometer (Molecular Devices, CA, USA).

2.10. Statistical analyses

Results are expressed as mean ± standard deviation. A non-parametric Mann–Whitney *U* test for independent samples was applied to compare control and DS results. The null hypothesis considered the lack of differences between the group means and was rejected for p values <0.05. The statistical software used was IBM SPSS Statistics 20.0.0.

3. Results

3.1. Impairment of cell proliferation

Fetal fibroblast proliferation from control samples determined by bromodeoxyuridine (BrdU) showed a high rate of proliferation up to

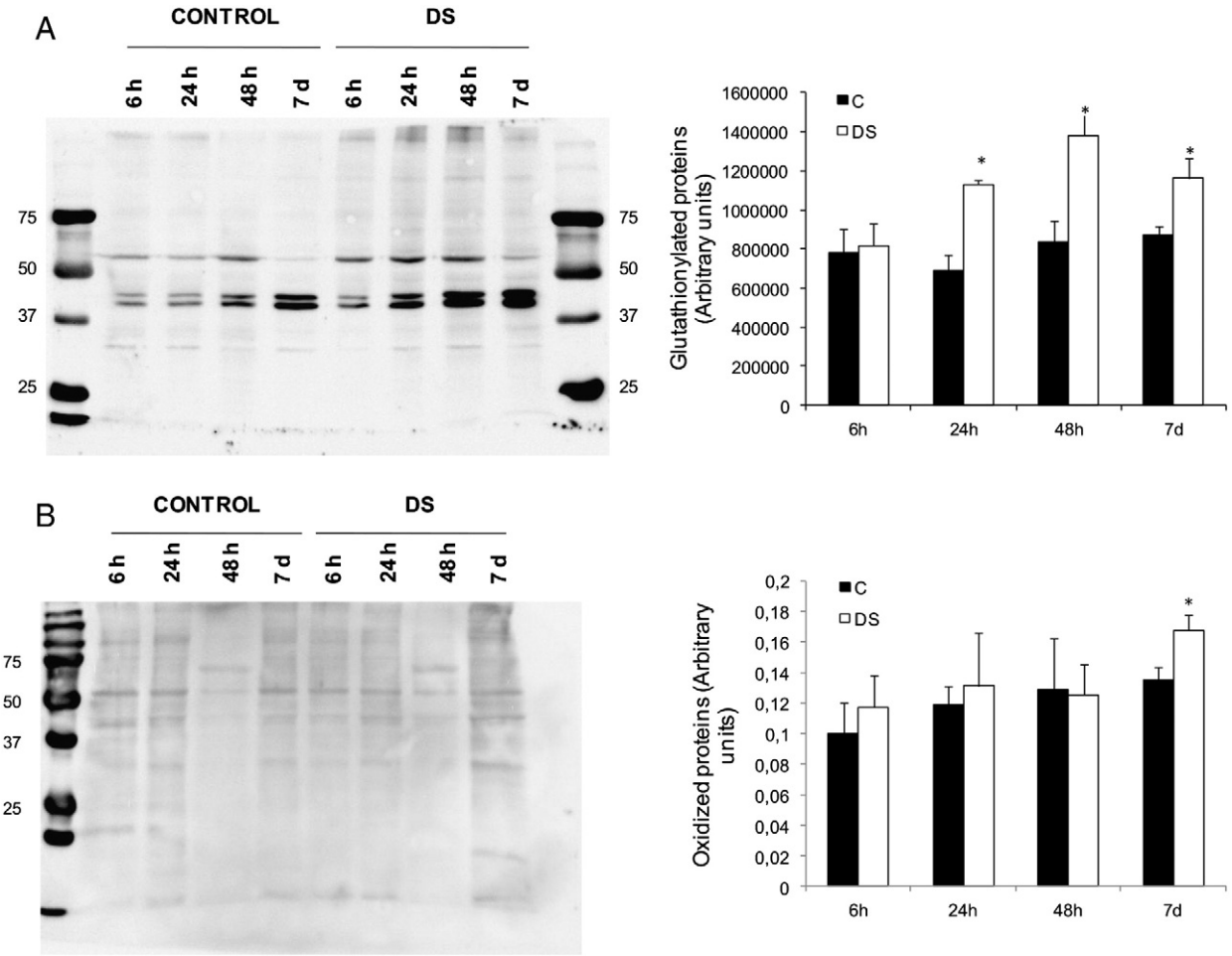

Fig. 2. Glutathionylated and oxidized total proteins for Down Syndrome (DS) and control cells. Each experiment was performed in triplicate. Statistically significant differences ($p < 0.05$) compared with the control average. (A) Glutathionylated total proteins in DS and control fibroblasts at different time points in culture (6 h, 24 h, 48 h, and 7 days). Western blot of a representative experiment is shown. (B) Oxidized total proteins in DS and control fibroblasts at different time points in culture (6 h, 24 h, 48 h, and 7 days). Western blot of a representative experiment is shown.

the first 24 h of culture. Afterwards cell proliferation reached a plateau for the rest of the culture. However, proliferation of fibroblasts from DS fetuses was significantly lower, mainly during the first 24 h of culture and remained lower until cells had been in culture for seven days (see Fig. 1A). No changes in basal apoptosis were detected between DS and control fetal fibroblasts (results not shown).

3.2. Telomeric length in fibroblasts from Down Syndrome fetuses

Telomere length is a well-known marker of cell proliferation and an index of cellular senescence. Fig. 1B shows a consistent and statistically significant 15.16% attrition of telomeres in the fetal DS fibroblast primary cell lines when compared with their control counterparts.

Thus, cells from DS have lower potential for proliferation than fibroblasts from control fetuses in the early stages of human development.

3.3. Oxidative stress profile in cells from Down Syndrome fetuses

Since previous reports have emphasized the role of Cu/ZnSOD in the production of high hydrogen peroxide levels in cells from DS patients and as a systemic oxidative stress has been previously reported by different authors, a number of different parameters of oxidative stress and antioxidants were determined in our cell model.

As expected, peroxide levels were significantly higher in fibroblasts from DS fetuses (Fig. 1C), and were 38% higher 48 h after culture and 28.75% 7 days after plating. Thus a consistent and continuous oxidative stress took place throughout the cell culture. Similarly, we determined the GSSG/GSH ratio as a measure of oxidative stress. In keeping with peroxide levels, the GSSG/GSH ratio was significantly higher in fibroblasts from DS fetuses (Fig. 1D). However, the independent concentrations of GSH and GSSG did not show significant differences between groups (results not shown). These results suggest a clear oxidative stress during cell proliferation in fibroblasts from DS when compared with controls. Cellular proteins may become prone to S-glutathionylation and protein carbonylation as a possible consequence of this oxidative stress status. Indeed, Fig. 2A shows increased protein glutathionylation 24 h, 48 h, and 7 days after seeding. Protein carbonylation was also higher at day seven of culture (Fig. 2B).

3.4. Antioxidant defense in Down Syndrome fibroblasts

In order to explain the reported oxidative status of DS fetus fibroblasts, we determined a wide array of antioxidant cellular components. Fig. 3A shows a consistent decrease in the expression of *TXN1* gene, the gene that codifies in humans for thioredoxin 1 (Trx1) one of the main members of the thioredoxin family. The thioredoxin family is one of the major regulators of the thiol redox state. Thioredoxin is a general protein disulfide reductase being an important component of the antioxidant cellular shield that has not been studied previously in DS fibroblasts. Expression was almost absent when compared with control cells, after 48 h and 7 days in culture.

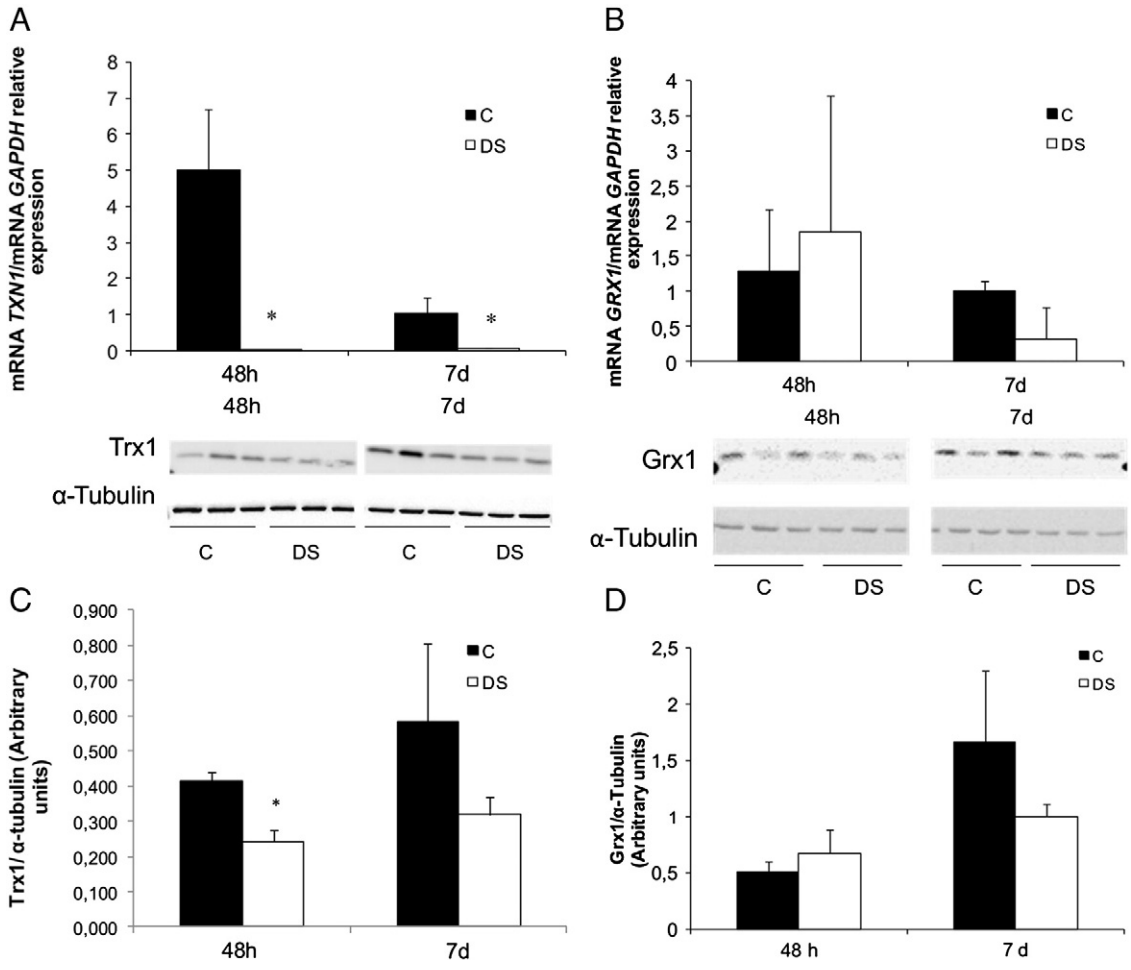

Fig. 3. Analysis of thioredoxin 1 and glutaredoxin 1 in Down Syndrome (DS) and control fibroblasts (C). (A) mRNA levels for *TXN1* determined by qRT-PCR. (B) mRNA levels for *GRX1* determined by qRT-PCR (C) Trx1 protein levels analyzed by Western blot, a representative experiment, and quantification of Western blot of Trx1of three different experiments. Graph bars show mean ± SD of a total of three experiments. (D) Grx1 protein levels analyzed by Western blot, a representative experiment, and quantification of Western blot of Grx1 of three different experiments. Graph bars show mean ± SD of a total of three experiments. Statistically significant differences ($p < 0.05$) compared with the control average.

Glutaredoxins (Grxs) are part of the Trx protein family. Grxs use glutathione as electron donor. However, *GRX1* mRNA expression (Fig. 3B) was not significantly different in either cell type. The protein expression results of both antioxidant enzymes (Fig. 3C and D) confirmed our RT-PCR results. As occurred for the respective gene expression, thioredoxin 1 protein was lower in DS (mainly 48 h after culture). There was an absence of a statistically significant difference in glutaredoxin 1 protein expression.

It was previously reported that Cu/ZnSOD activity was higher in DS patients. As expected, we found higher *SOD1* (the gene that codifies for Cu/ZnSOD) mRNA levels, in fibroblasts from DS fetuses (Fig. 4A, left). Expression of Cu/ZnSOD proteins was also higher after 48 h in culture (Fig. 4B and C), although the differences were not statistically significant. *SOD2* (the gene that codifies for MnSOD) mRNA levels were significantly lower in DS fibroblasts seven days after culture (Fig. 4A, right). However these results were not correlated with statistically significant changes in the levels of the MnSOD protein (Fig. 4B and C).

Since SOD action must be coordinated with catalase and glutathione peroxidase (GPx) activity in order to metabolize the H_2O_2 produced by SOD enzymes, we focused our attention on these H_2O_2 antioxidant enzymes. The results presented in Fig. 5A and B showed no changes in *CAT* mRNA and catalase protein levels. However a significant decrement, more than a 50% decrease, in mRNA and protein expression was found for GPx in cell extracts from DS fetal fibroblasts. In order to obtain a complete picture of the antioxidant imbalance in the antioxidant enzymes, we determined the enzymatic activity of Cu/ZnSOD, MnSOD, catalase, and GPx. These results showed a significant increase in Cu/ZnSOD (Fig. 6A) and a decrease in GPx activity (Fig. 6D), with non-significant changes in MnSOD (Fig. 6B) and catalase activity (Fig. 6C).

Following the example of different authors, [30,31] we calculated the [Cu/ZnSOD / (catalase + GPx)] activity ratio as the best index to explain the chronic oxidative stress found in DS cells (Fig. 7). As expected, a significant increase in this ratio was found in DS fetus fibroblasts,

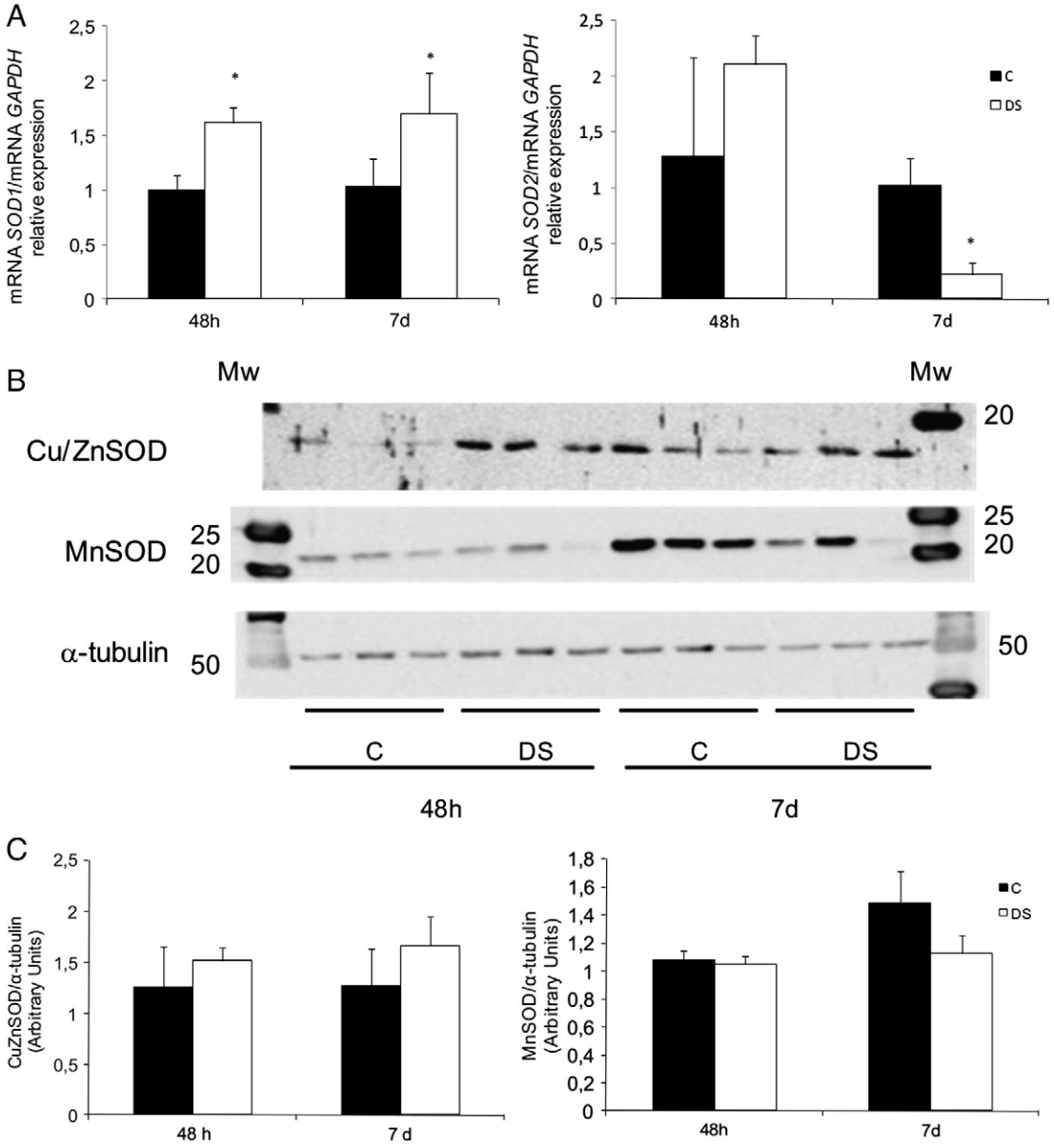

Fig. 4. Analysis of superoxide detoxifying enzymes in Down Syndrome (DS) and control fibroblasts (C). (A) mRNA levels for *SOD1* (the gene that codifies for Cu/ZnSOD), determined by qRT-PCR (left). mRNA levels for *SOD2* (the gene that codifies for MnSOD), determined by qRT-PCR (right). (B) Cu/ZnSOD and MnSOD protein levels analyzed by Western blot, a representative experiment. (C) Quantification of Western blot of Cu/ZnSOD (left) and MnSOD (right). Graph bars show mean ± SD of a total of three experiments. Statistically significant differences ($p < 0.05$) compared with the control average.

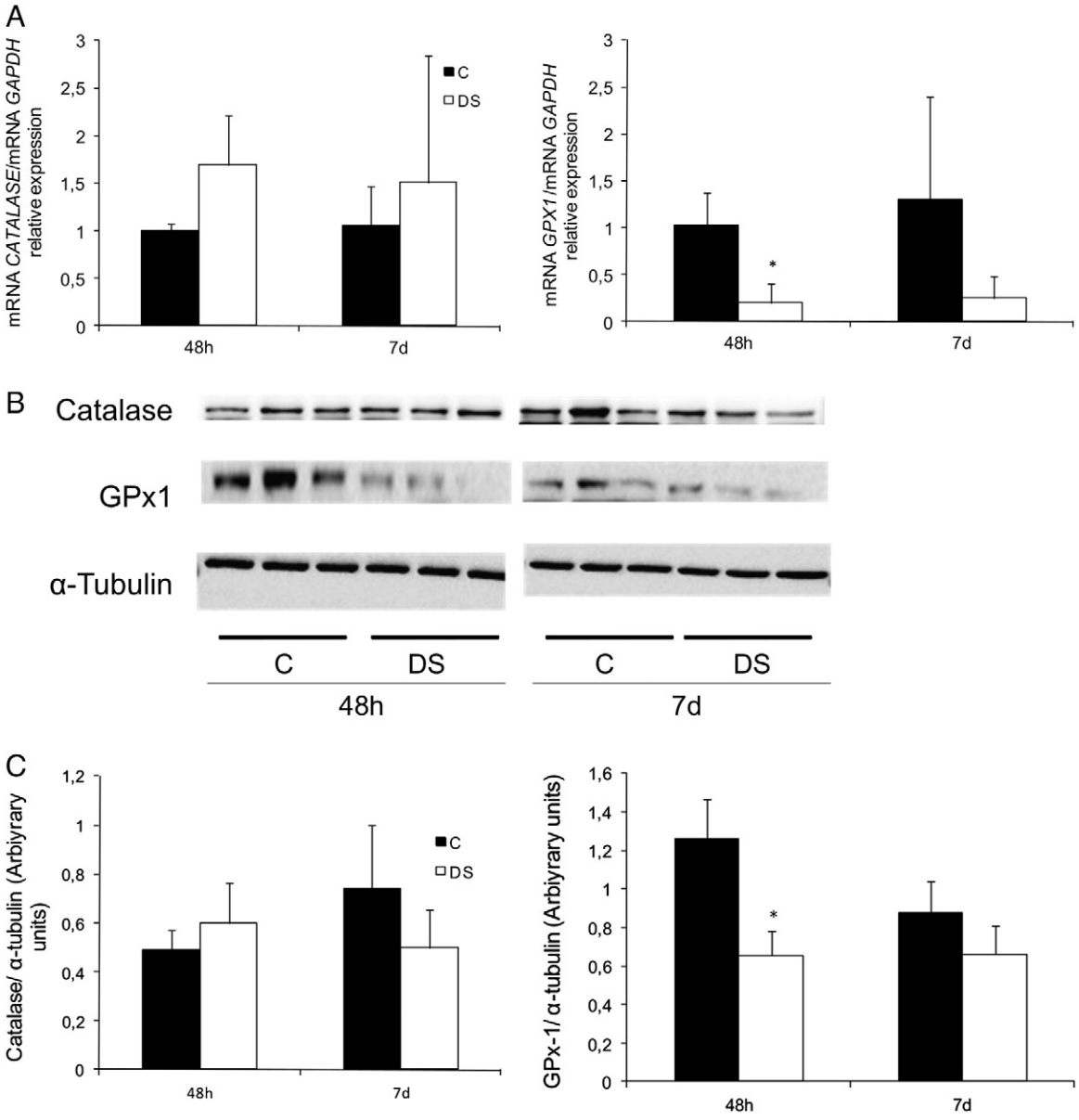

Fig. 5. Analysis of H_2O_2 detoxifying enzymes. (A) mRNA levels of *CAT* gene (left) and mRNA levels of *GPX1* gene (right). (B) Determination of catalase and GPx1 protein levels by Western blot, a representative experiment. (C) Quantification of Western blot of catalase (left) and GPx (right). Graph bars show mean ± SD of a total of three experiments.

indicating that during fetus development a sustained imbalance in antioxidant defenses takes place.

3.5. Rcan1 protein levels in Down Syndrome fibroblasts

Down Syndrome critical region gene 1, also known as DSCR1 or Rcan1 is present in chromosome 21. Its gene product interacts with calcineurin A and inhibits calcineurin-dependent signaling pathways of genetic transcription. Numerous reports consistently underscore the low incidence of solid tumors in DS patients [32–35]. It has been proposed that this is due to the increased expression of RCAN1 [36]. This gene encodes a protein that is able to suppress the vascular endothelial growth factor (VEGF) [37–42], thereby decreasing cell proliferation. The purpose was to show a parallelism between the previously reported high expression of Rcan1, with oxidative stress parameters and low proliferative capacity in a cellular model during early development in human Down Syndrome fibroblasts.

Over-expression of *RCAN1* has been reported in fibroblasts and in neurons from adult DS patients [36]. Fig. 8 shows that an over-expression of Rcan1 took place in fibroblasts from fetal DS. It is known that Rcan1 induces a decrease in proliferation of several cell lines by the repression of different transcription factors and a decrease in synaptic development and activity [36,37]. Our results show that in early fetal development, and not only in brain developmental cell lines, the expression of Rcan1 is increased, in keeping with an increase in oxidative stress parameters and attrition of telomeres. This suggests an involvement in the impairment of DS fetus fibroblast proliferation.

4. Discussion

Different regulators of the proliferative cell capability were decreased in the cell model we studied. These changes impaired proliferation of DS fibroblasts when compared with control fibroblasts from fetuses (Fig. 1A). Indeed, the telomere length of fibroblasts, a well-known marker of the proliferative cell potential, was significantly shorter than its control counterpart (Fig. 1B), suggesting that attrition of the telomeres had a possible causative role in the decreased proliferative capability of these cells. Varizi et al. [43] showed increased age-dependent telomere

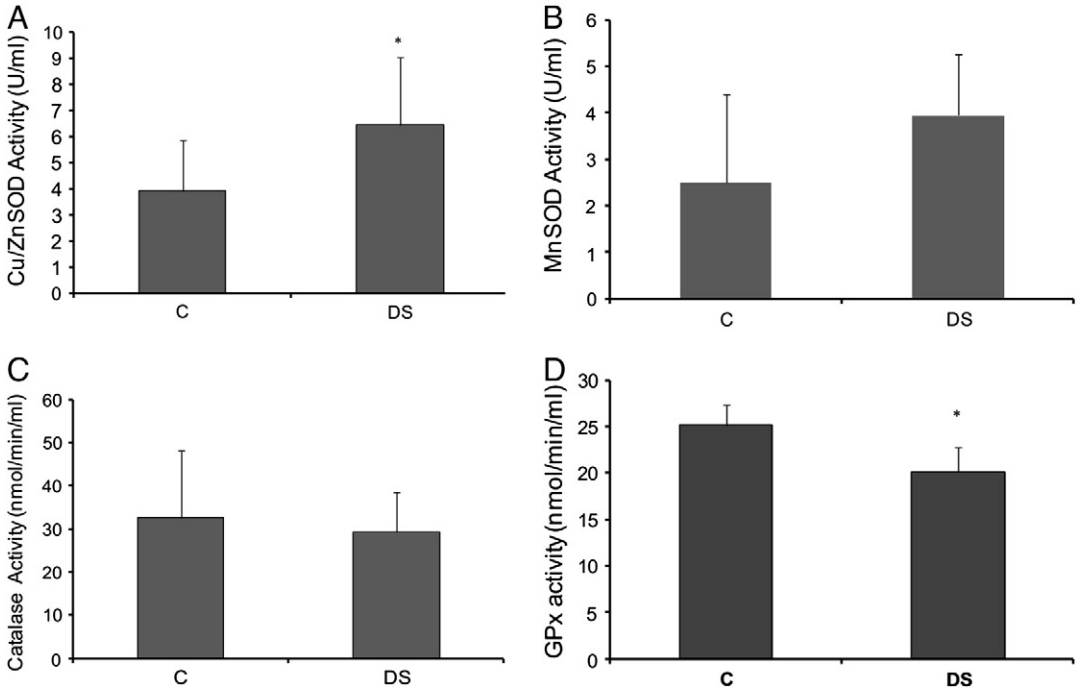

Fig. 6. Analysis of antioxidant enzyme activities in Down Syndrome (DS) and control fibroblasts (C). (A) Cu/ZnSOD activity and (B) MnSOD activity evaluated using the Superoxide Dismutase Assay kit (Cayman, Ann Arbor, MI, USA) as describe in the Material and methods section. (C) Catalase activity was analyzed spectrophotometrically measuring the formation of the purpald-formaldehyde adduct at 540 nm. (D) Glutathione peroxidase activity determined by the glutathione peroxidase assay kit (Cayman, Ann Arbor, MI, USA) as describe in the Material and methods section. All measurements were made in three fibroblast cell lines from Down Syndrome patients and three control cell lines. Results are represented as mean (±SD). Statistically significant differences ($p < 0.05$) compared with the control value.

attrition in neutrophils and lymphocytes from DS when compared with control cells. Our TL results in embryonic DS human fibroblasts correlated with the decrease in the length of telomeres found in hematopoietic progenitor cells from DS fetuses [44].

Von Zglinicki [45] demonstrated that oxidative stress is a major cause of telomere attrition. In different pathological situations, like Fanconi anemia, a similar correlation was found between oxidative stress, TL, and proliferative impairment [46]. Previous results have shown impaired proliferation in fibroblasts from adult DS patients when compared with healthy individuals [10,47]. Kimura et al. [31] used fibroblasts from established cell lines from a repository of both child and adult male DS patients and showed an initial impairment of cell proliferation, but normal population doublings and lack of correlation with the TL. However, here we show that, even in fibroblasts obtained from DS fetuses, the proliferation rate is significantly lower than in control cell lines, suggesting that the impaired proliferative capability is a very early event in DS and not just a consequence of a chronic physiopathological mechanism. A higher number of DS samples and their respective matched controls would probably allow a statistical correlation

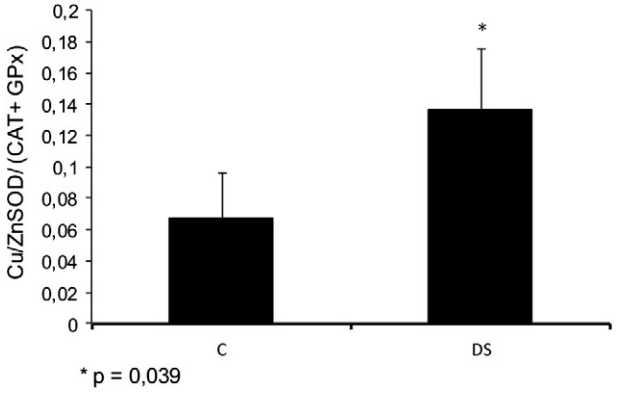

Fig. 7. Ratio between the anti-oxidant enzyme activities. Cu/ZnSOD activity units (superoxide detoxifying enzyme) divided by the decrease in catalase and GPx activities (H_2O_2 detoxifying enzyme). Statistically significant differences ($p < 0.05$) compared with the control value.

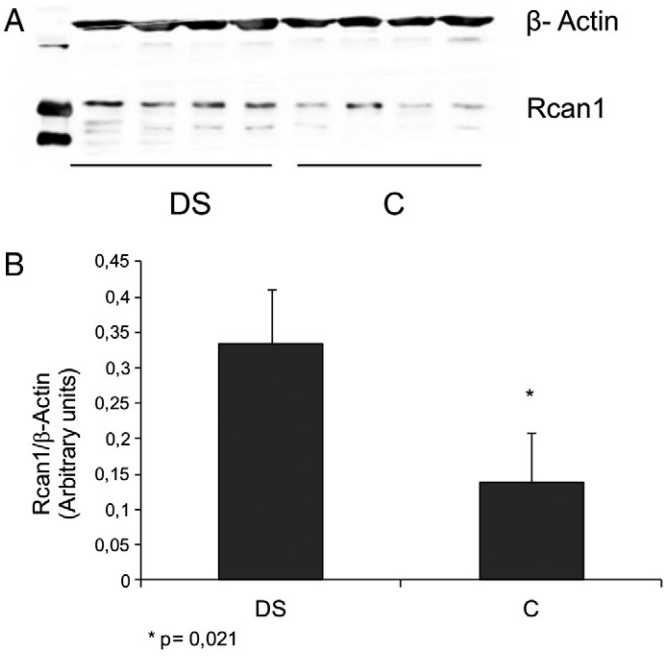

Fig. 8. (A) A representative experiment is shown for the determination of Rcan1 protein levels by Western blot, using fibroblasts 24 h after culture, from four different controls and four different patients. (B) Quantification of Western blot of Rcan1. Graph bars show mean ± SD of a total of three experiments. Statistically significant differences ($p < 0.05$) compared with the control value.

between cell proliferation rate and telomere length in fibroblasts from DS fetuses. This probably could clarify this clear tendency shown here where cell lines from DS patients show shorter telomeres and lower proliferation rate.

Trisomy 21 has long been known as a redox imbalance-related disease. The over-expression of copper–zinc superoxide dismutase (*SOD1*), located in chromosome 21, is a landmark of the disease. The in vivo pro-oxidant state in DS patients remains a conspicuous observation both in vivo and in vitro [4–8,10]. However, the exact source of this oxidative stress remains elusive, although mitochondria are obvious, and one of the main candidate sources. In fact, we have previously described considerable morphological, metabolic, and DNA changes in mitochondria in DS (for a review see Pallardó et al.) [48] and other authors [49–51].

As previously underscored a number of reports have emphasized the importance of the imbalance in the [Cu/ZnSOD / (catalase + GPx)] activity in DS [9,10] and here, we show a similar imbalance in our DS cell model fibroblasts from human fetuses. Indeed, a considerable change in the mRNA of *SOD1* (Fig. 4A) expression and a pronounced increase in the protein levels for Cu/ZnSOD (Fig. 4B and C) were found. These results correlated with the increased activity of Cu/ZnSOD (Fig. 6A). On the other hand, a significant decrease in the *GPX* gene expression, protein levels, and activity was also detected (Fig. 5). However, no changes were found in catalase expression (Fig. 5). The variations in the gene expression and protein levels produced a considerable impact in the enzymatic activities of the antioxidant enzymes. Thus, when we calculated the [Cu/ZnSOD / (catalase + GPx)] activity ratio (Fig. 7) it was significantly increased in fibroblasts from DS fetuses when compared with their control counterparts. We suggest that this imbalance in the [Cu/ZnSOD / (catalase + GPx)] activity ratio gives rise to an oxidative stress, producing high levels of hydrogen peroxide (Fig. 1C).

In keeping with this, the analysis of the GSSG/GSH ratio, a recognized marker of oxidative stress, showed a significant increase (Fig. 1D). In addition, the levels of oxidized proteins (Fig. 2B) in fibroblasts from DS were higher than in control cells demonstrating that a clear oxidative stress in these cells took place.

But the glutathione system is not the only antioxidant mechanism that is affected. Our results showed that the glutathionylated proteins were increased in DS fibroblasts (Fig. 2A). Although oxidative stress can contribute to this increase in glutathionylated proteins, the detoxifying systems involved in the de-glutathionylation of the oxidized proteins may be affected. Fig. 3 shows that the thioredoxin and glutaredoxin systems were also decreased in DS fetal fibroblasts. Results showed that both the *TXN1* gene expression and the protein levels were markedly decreased (Fig. 3A and C). Glutaredoxin 1 gene expression and the protein levels were also decreased although to a lesser extent (Fig. 3B and D). Thus, a distorted antioxidant scenario emerges in cells from DS fetuses. The decrease in the gene expression and protein levels of the glutaredoxin and thioredoxin systems partially explains the increase in glutathionylated proteins. It has been previously reported [52] that a decrease in the glutaredoxin system impairs mitochondrial activity due to the increase of glutathionylated proteins of the electronic transport chain.

In this preliminary study we describe for the first time the contribution of thioredoxin and glutaredoxin systems to the imbalance of the antioxidant system in DS, that explains, at least in part, the increased levels of different free radical species in DS and the increase in oxidized proteins. Trx1 has been described as one of the most important antioxidant enzymes. Furthermore, its down-regulation produces cell proliferation inhibition due to its importance in DNA synthesis [53], thus, it contributes to the defects in the antioxidant shield and also to the low proliferation capability of DS fibroblasts.

Numerous reports consistently underscore the low incidence of solid tumors in DS patients [32–35]. It has been proposed that this is due to the increased expression of the Down Syndrome candidate region-1 (Dscr1/RCAN1) that is located in chromosome 21 [36]. This gene encodes a protein that is able to suppress the vascular endothelial growth factor (VEGF) [37–42], thereby decreasing cell proliferation. Recently, Dean Nižetić and Júrgen Groet wrote an impressive and challenging review on this matter [54].

Baek et al. [55] proposed the calcineurin signaling pathway and its regulators DSCR1 and DYRK1A as the mechanism for the reduced cancer incidence in DS. Fig. 1A shows impairment in cell proliferation in fibroblasts from fetal DS individuals. We determinate the protein levels of Rcan1 in order to explain this early decrease in the proliferative capability of those cell lines from DS fetuses. As previously reported in other cell types from DS patients [56,57], the expression of the different isoforms of Rcan1 detected by the antibody we used (a synthetic peptide corresponding to amino acids 235–252 of human Rcan1) was increased in fibroblasts from DS fetuses (Fig. 8). Ermark and Davies [58] suggested that a prolonged elevation of RCAN1-1L levels, one of the isoforms of RCAN1, is associated with the type of neurodegeneration observed in several diseases, including Alzheimer's disease and DS. Here we show that even in a very early step of human development (fetal fibroblasts) these changes are present in DS. Rcan1 is also involved in the regulation of oxidative stress, at least in neurons [59]. Interestingly, the altered expression of *nebula*, the *RCAN1* homologue of *Drosophila melanogaster*, caused severe learning defects [60].

In addition to the mitochondrial localization of *nebula*, its altered expression caused the increase of ROS levels and mitochondria number as well as the reduction of ATP levels and mitochondrial DNA content [61]. The oxidative damage by H_2O_2 was lessened in primary neurons from *Rcan1*$^{-/-}$ mouse [56]. Moreover, the sensitivity to oxidative stress was increased when *RCAN1-1S* was overexpressed [56].

Our preliminary results clearly indicate that the alteration of not only *SOD1* gene expression and Cu/ZnSOD protein levels, but also different antioxidant enzymes, such as thioredoxin 1, contributes to the poor proliferative capability of tissues in DS mediated by telomeric attrition, and the increased expression of Rcan1, which partially explains the progeroid phenotype of the patients.

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