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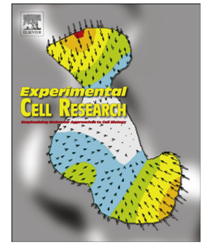

Research Article

Novel aberrant genetic and epigenetic events in Friedreich's ataxia

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ABSTRACT

It is generally accepted that Friedreich's ataxia (FRDA) is caused by a deficiency in frataxin expression, a mitochondrial protein involved in iron homeostasis, which mainly affects the brain, dorsal root ganglia of the spinal cord, heart and in certain cases the pancreas. However, there is little knowledge as to other possible genes that may be affected in this disorder, and which can contribute to its complexity. In the current study we compared human periodontal ligament cells gene expression of healthy individuals and FRDA patients. The expression of active-caspase 3, as well as other apoptosis-related genes, was increased in the FRDA cells. Furthermore, iron–sulphur cluster genes, as well as oxidative stress-related genes were overexpressed in FRDA. Moreover, brain-derived neurotrophic factor, neuregulin 1 and miR-132 were all upregulated. These three genes are capable of regulating the expression of each other. Interestingly, when the cells from FRDA patients were co-cultured in the presence of idebenone and deferiprone, caspase expression decreased while antioxidant gene expression, as well as frataxin expression, increased. Regarding epigenetic mechanisms, the frataxin gene was hypermethylated, compared to the healthy counterparts, in the upstream GAA repetitive region. Of the three DNA methyltransferases, DNMT1 but not DNMT3's gene expression was higher in FRDA cells. In conclusion, our data show that FRDA cells present altered expression of genes related to cell cycle, oxidative stress and iron homeostasis which may be implicated in the increased apoptotic levels. Also, the altered expression is in a certain degree normalized in the presence of idebenone and deferiprone.

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Abbreviations: FRDA, Friedreich's ataxia; FXN, frataxin; ISC, iron–sulphur cluster; hPDL, human periodontal ligament; CASP3, active caspase-3; DNMT, DNA methyltransferase; CpG, cytosine phosphate group guanine; TSS, transcription start site; UP, upstream GAA repetitive region; miRNA, microRNA; IDE, idebenone; ROS, reactive oxygen species; DFP, deferiprone; WT, wild type; AZA, 5-Aza-2′-deoxycytidine; p21, cyclin-dependent kinase inhibitor 1A; CCND1, cyclin D1; GLB1, galactosidase-beta 1; SOD, superoxide dismutase; ISCU, Iron–Sulphur Cluster assembly enzyme; LYRM4, LYR motif containing 4; GLRX5, glutaredoxin 5; BDNF, Brain-derived neurotrophic factor; NRG1, neuregulin 1; MSP, methylation specific PCR; SEM, standard error of the mean.

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Introduction

Friedreich's ataxia (FRDA) is the most prevalent autosomal recessive ataxic disease. This pathology is caused by a GAA-triplet repeat expansion in the first intron of the frataxin (FXN) gene [1], which impedes transcription elongation [2]. As a result, FXN levels are low, causing numerous alterations in mitochondrial iron metabolism, biosynthesis of the iron–sulphur cluster (ISC), as well as in oxidative stress regulation [3–6]. The most susceptible tissues are the large sensory neurons of the dorsal root ganglia in the spinal cord, the heart and pancreas [7].

There is little information as to the specific mechanisms by which frataxin is regulated, and silenced in the case of FRDA, as well as how frataxin silencing can affect the expression of other genes. It is known that there is a complex interaction between genetic and epigenetic factors that are responsible for the pathophysiology of neurodegenerative diseases. DNA methylation is the most well characterized epigenetic mechanism, in which a methyl group, catalyzed by DNA methyltransferases (DNMTs), is added onto cytosines almost exclusively in CpG dinucleotides of somatic cells. These CpGs are found densely clustered within sequences known as CpG islands. DNA methylation takes place, on the one hand in gene regulatory regions, such as promoter sequences blocking the interaction between transcription factors and the promoter, which subsequently inhibits gene transcription. On the other hand, DNA methylation is known to occur throughout the genome with a crucial role in genomic stability, DNA repair, imprinting and heterochromatinization [8]. In the case of the frataxin gene, its methylation pattern around the transcription start site (TSS), in the promoter, and in the upstream GAA repetitive region (UP) have been previously described in post-mortem human heart, brain and in blood and buccal FRDA cells [9,10]. Another epigenetic mechanism are microRNAs (miRNAs) defined as small non-coding RNAs of 19–22 nucleotides in length, which participates in gene expression regulation [8]. In FRDA, two miRNAs have been described to be especially relevant [11], miR-132 and miR212.

It has been previously described in our group that human periodontal ligament (hPDL) cells express several stem cell and neural crest markers [12]. These cells, when isolated from FRDA patients, express low frataxin levels compared to healthy controls, and increased levels of apoptotic markers [13]. In this work, we analyze if these expression levels remain constant throughout the passages. Furthermore, genes related to the ISC, oxidative stress and cell cycle are analyzed. The epigenetic regulation of the frataxin gene is also studied by DNMTs.

There are currently few therapeutic options to treat FRDA, although many experimental approaches are in development [14]. Two of the most widely used medications are idebenone (IDE) and deferiprone (DFP). IDE is an analog of the CoQ antioxidant which penetrates mitochondrion membrane more efficiently than CoQ, is reduced by ROS and releases electrons to the respiratory chain. IDE treatment has been shown to reduce heart size in FRDA patients with hypertrophic cardiomyopathy [15], although this effect remains controversial. DFP is used in iron-related diseases, removing iron excess and redistributing it between overloaded and depleted compartments [16]. Though both of these drugs are in the market, their pre-clinical study in

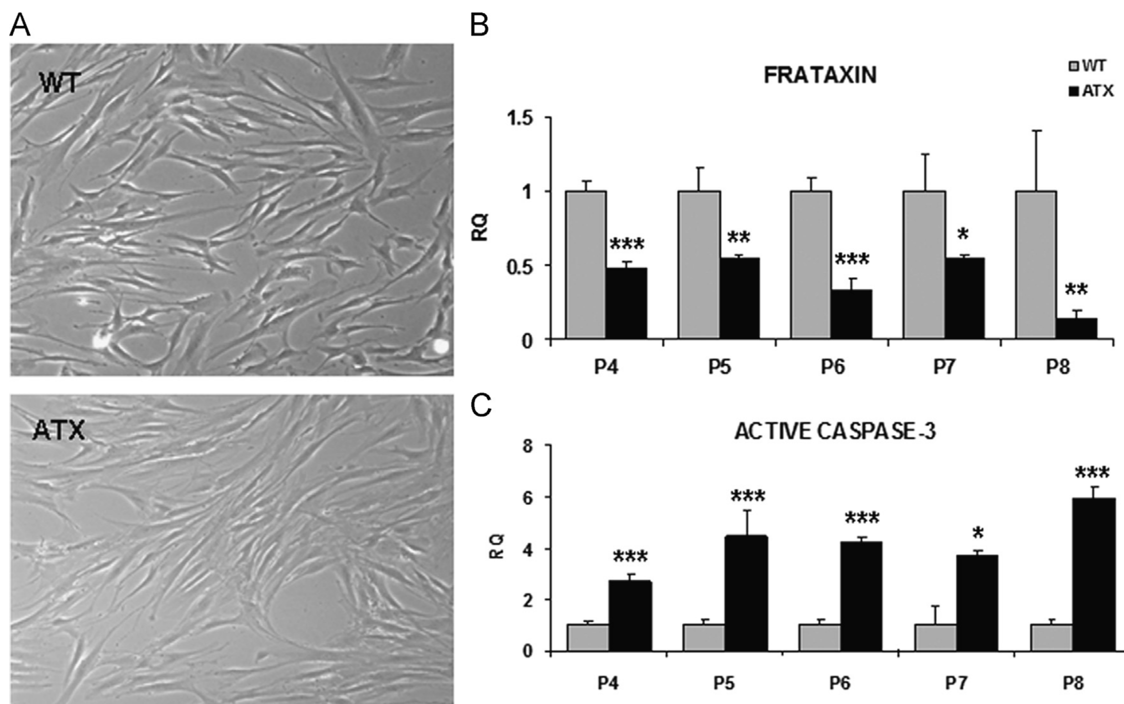

Fig. 1 – Human periodontal ligament cultured cells isolated from healthy and FRDA patients and qPCR analysis of FXN and CASP3 genes throughout culture passages. (A) hPDL culture cells from healthy individual and from FRDA patient. Gene expression analysis of frataxin (B) and active caspase-3 (C) in periodontal ligament cells of healthy (WT, gray bars) and FRDA patients (FRDA, black bars) from passages 4 to 8. In each case the mean (+SEM) of gene expression is shown as determined from three different individuals in each group. The cells from healthy individuals are considered as control (value=1). *Y*-axis indicates the relative quantity of the genes analyzed (RQ). $^{***}p<0.001$, $^{**}p<0.005$ and $^{*}p<0.05$.

cultured cells is little to non-existent, requiring further studies to be performed as to how they act upon the target cells both individually as well as taken together.

The results of this work increase our knowledge as to how, besides frataxin, other molecules are affected in FRDA, as well as indicate how the combined therapeutic effect of idebenone and deferiprone can compensate these alterations. Furthermore, new information as to how frataxin may be silenced is also included in this study.

Results

Low frataxin and high active caspase-3 expression is sustained throughout culture passages

Quantification of frataxin (FXN) and active caspase-3 (CASP3) gene expression were carried out by real time qPCR comparing FRDA and healthy hPDL cells in culture. The cells were isolated and the culture was maintained up to passage (P) 8 (Fig. 1A). As a result, FRDA cells expressed significantly lower levels of frataxin gene in the analyzed passages, from 4 to 8, which was maintained throughout culture passages (Fig. 1B). On the other hand, CASP3 presented higher levels of expression in FRDA cells compared to control (Fig. 1C). Previous studies in our group with hPDL cells have shown the same result at passage 3, corroborating our results [13].

Frataxin gene is unmethylated at the Transcription Start Site (TSS) in FRDA patients but is hypermethylated in the upstream GAA repetitive region in periodontal ligament stem cells

DNA methylation of the frataxin gene was assessed both in healthy and FRDA hPDL cells, as it is known that in this disorder there is an alteration in gene expression as well as heterochromatinization that causes the deficient elongation of FXN transcription in this region. Two regions were analyzed by MSP, both containing CpG islands: TSS (including exon 1), whose CpG sites correspond to numbers 9–25 of the frataxin promoter assay previously published in Evans-Galea MV et al. [10]; and immediately upstream of the GAA repetitive region (UP) located in intron 1, where CpG sites correspond to the 4 CpGs immediately preceding number 1 of the frataxin UP [10] (Fig. 2A). As a result, the TSS regions in FRDA and WT cells were unmethylated (data not shown). However, the UP region in FRDA cells showed a clear hypermethylation *versus* a heterogeneous methylation in WT cells (Fig. 2B). This was observed in all the passages analyzed. Our results are in agreement with previous methylation studies described in other human tissues [9,10]. As the methylation pattern is the same in all the passages regardless being from FRDA or WT cells, hPDL cells at passage 4 from healthy and FRDA patients were treated in culture with the methylation inhibitor 5-Aza-2′-deoxycytidine (AZA), a cytosine analog that is incorporated into genomic DNA and traps DNMTs, inhibiting them and provoking DNA demethylation. AZA treatment

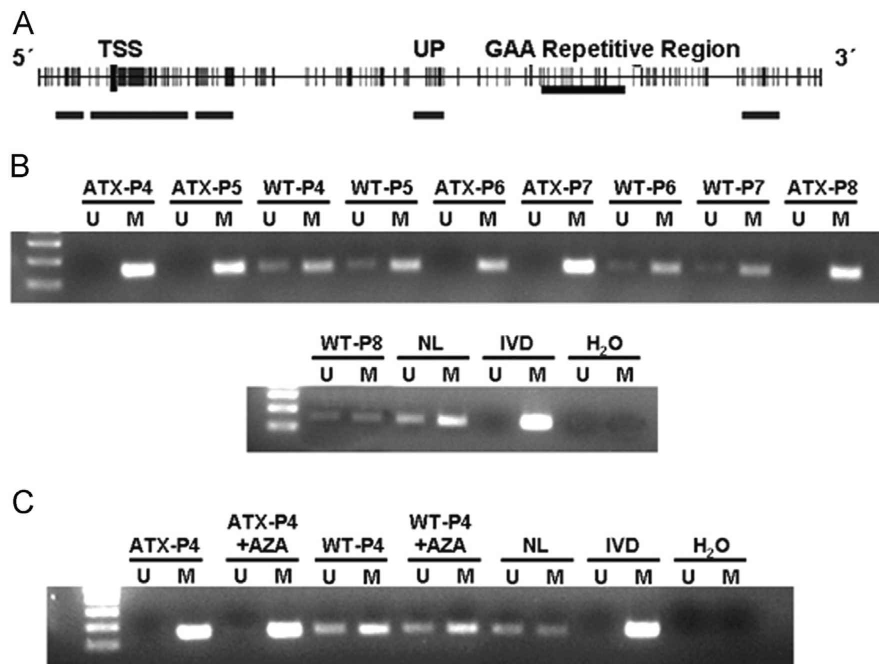

Fig. 2 – Frataxin gene CpG island report and methylation study by MSP in hPDL cells from healthy and FRDA patients. **(A) Representative description of CpG islands in FXN gene. Each CpG is represented by a vertical line; the transcription start site (TSS) and the upstream GAA repetitive region (intron 1) (UP) CpG islands, by solid horizontal lines; GAA repetitive region by a curly bracket. (B) Methylation analysis of FXN gene in the UP region. The MSP shows a hypermethylation in the UP region of FXN gene in FRDA cells ($n=3$) while a heterogeneous methylation in WT cells ($n=3$). (C) Methylation pattern of FXN gene in the UP region after AZA treatment in hPDL cultured cells. At passage 4, hPDL cells were treated with the DNA methylation inhibitor, AZA, and no changes occurred in the UP region neither in FRDA nor in WT cells. In the MSPs: PCR product is 147 bp; Methylation (M) and unmethylation (U); passage (P); healthy lymphocytes (NL); commercial *in vitro* methylated DNA (IVD); master mix without bisulfite modified DNA (H_2O).**

in culture cells did not cause any change in the hypermethylated UP region neither in FRDA nor in WT cells (Fig. 2C). Also, frataxin expression was not altered in the presence of AZA (data not shown).

The expression of cell cycle and epigenetic-related genes are altered in hPDL cells from FRDA patients

Since the hPDL cells isolated from FRDA patients presented increased levels of apoptosis marker active caspase-3, the expression of cell cycle-related genes were analyzed, as cell cycle and apoptosis are related. Specifically at passage 4, p53, cyclin-dependent kinase inhibitor 1A (p21), cyclin D1 (CCND1) and galactosidase-beta 1 (GLB1) were analyzed. As a result, the cells from FRDA patients expressed significantly higher levels of all the analyzed genes ($p<0.001$) (Fig. 3A).

Of the analyzed genes, p53 is capable of activating DNA methyltransferase 1 (DNMT1) under stress situations. DNMTs are responsible for DNA genome methylation [17]. Since p53 was upregulated in cells from FRDA patients, we analyzed if DNMTs expression may also be increased. As a result, DNMT1, but not the other DNMTs, was overexpressed compared to cells isolated from healthy individuals ($p<0.001$) (Fig. 3B). This indicated a possible correlation between apoptosis, cell cycle arrest and gene silencing in FRDA.

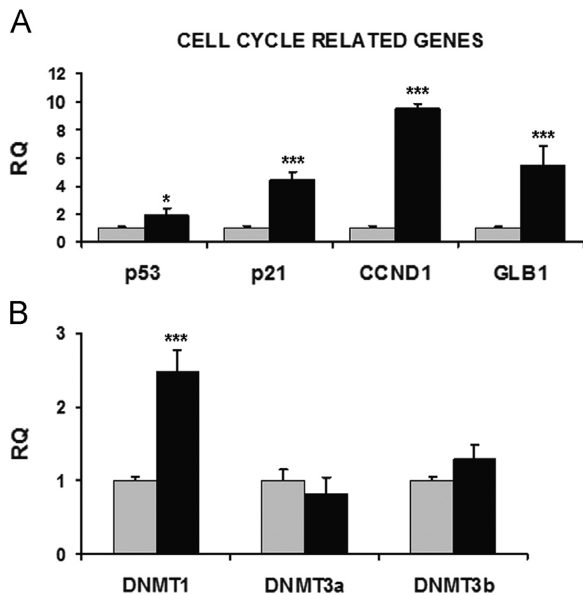

Fig. 3 – Relative quantification expression analysis of cell cycle relative genes and DNMTs in hPDL from healthy and FRDA patients. (A) Analysis of p53, p21, CCND1 and GLB1 genes expression in periodontal ligament cells of healthy (WT, gray bars) and FRDA patients (FRDA, black bars) in P4 shows a significant increase of mRNAs levels in FRDA cells. (B) Analysis of DNMTs genes expression in periodontal ligament cells of healthy (WT, gray bars) and FRDA patients (FRDA, black bars). In each case the mean (+SEM) of gene expression is shown as determined from three different individuals in each group. The cells from healthy individuals are considered as control (value=1). *Y*-axis indicates the relative quantity of the genes analyzed (RQ). $^{***}p<0.001$ and $^{*}p<0.05$.

Superoxide dismutases genes and iron–sulphur cluster components are overexpressed in cells isolated from FRDA patients

Previous works have shown that cells isolated from FRDA patients are susceptible to oxidative stress, contributing to cell death [13]. Subsequently, superoxide dismutases (SODs) expression was analyzed in the cells isolated from FRDA. As a result, the expression of all three SODs were significantly upregulated in the cells from FRDA patients compared to healthy individuals ($p<0.05$) (Fig. 4A). Furthermore, since frataxin is involved in the biosynthesis of the iron –sulphur cluster, its low expression in FRDA may also affect the expression of the other components of the cluster [18,19]. As a result, the expression of Iron–Sulphur Cluster assembly enzyme (ISCU), LYR motif containing 4 (LYRM4) and glutaredoxin 5 (GLRX5) mRNAs were significantly higher in cells from FRDA patients ($p<0.005$), possibly as a compensatory mechanism due to the lack of frataxin (Fig. 4B).

Altered expression of miRNAs miR-886 and miR-132 in cells isolated from FRDA patients

Among a group of miRNAs previously studied in blood from FRDA patients, miR-886 was upregulated while miR-132 was downregulated compared to unaffected individuals [11]. However in the present work, using hPDL cells we observed the opposite result, being miR-886 downregulated and miR-132 upregulated ($p<0.001$) (Fig. 5A). The DNA methylation status of miR-886 was unaltered, being heterogeneous in cells from FRDA and healthy individuals (Fig. 5B). Also, AZA treatment did not change that methylation pattern (Fig. 5B). As for miR-132, this gene was unmethylated in both FRDA and WT cells (Fig. 5C). Another micro-RNA, miR-212, which works in tandem with miR-132, was also unmethylated in cells from FRDA patients (Fig. 5C).

As Brain-derived neurotrophic factor (BDNF) upregulates miR-132 expression in the retina [20] as well as in cultured cortical neurons [21], we determined BDNF mRNA expression in hPDL cells. In cells from FRDA patients, BDNF mRNA was overexpressed compared to WT cells ($p<0.001$) (Fig. 5D), where it could be interpreted that a relation between BDNF and miR-132 may exist in hPDL cells as it is described in other tissues. Neuregulin 1 (NRG1) is upregulated by BDNF [22,23], and its expression was detected to also be upregulated in FRDA-isolated cells ($p<0.001$) (Fig. 5D). As BDNF plays a role in anti-apoptosis mechanisms [24], it is possible that its upregulation, as well as of NRG1, may be a counterbalance method due to the increased oxidative stress and cell cycle arrest detected in hPDL cells from FRDA patients.

Idebenone (IDE) and deferiprone (DFP) produce changes in oxidative-related genes, frataxin and active-caspase-3 mRNAs from FRDA patients

IDE and DFP are two commonly-used medications to treat FRDA. However, there is very little information concerning their effect *in vitro* in cells isolated from patients. To this end, hPDL cells from FRDA patients were cultured in the presence of IDE and DFP for 24 h at different concentrations (Fig. 6). In the case of IDE, increasing concentrations significantly decreased active caspase-3 levels ($p<0.05$) (Fig. 6A). DFP, on the other hand, did not alter caspase-3 expression in any of the concentrations used

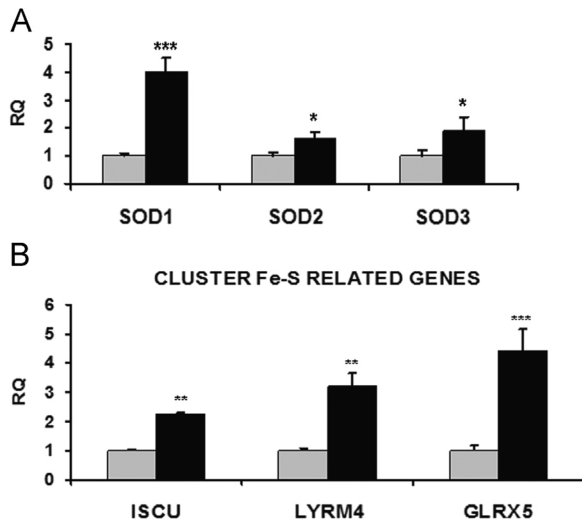

Fig. 4 – Comparison of relative quantification expression of superoxide dismutases (SODs) and Cluster Fe–S (ISC) related genes between FRDA and WT cells. (A) Analysis of SOD1, SOD2 and SOD3 mRNA levels in hPDL cells of healthy (WT, gray bars) and FRDA patients (FRDA, black bars). SODs genes are significantly upregulated in FRDA *versus* WT cells. (B) Relative quantification of ISCU, LYRM4 and GLRX5 mRNAs in hPDL cells of healthy (WT, gray bars) and FRDA patients (FRDA, black bars). The transcriptional levels of the three ISC implicated genes are significantly increased in FRDA compared to WT cells. In each case the mean (+SEM) of gene expression is shown as determined from three different individuals in each group. The cells from healthy individuals are considered as control (value=1). *Y*-axis indicates the relative quantity of the genes analyzed (RQ). $^{***}p<0.001$, $^{**}p<0.005$, $^{*}p<0.05$.

(25–150 μM, data not shown). Furthermore, frataxin expression was analyzed (Fig. 6B). As a result, IDE and DFP by themselves did not alter frataxin expression. However, the combined effect significantly increased frataxin levels ($p<0.05$). As for antioxidant-related genes, SOD2 and catalase expression were upregulated, mainly due to IDE ($p<0.05$) (Figs.6C and D, respectively), while no significant changes were observed in SOD1 expression (data not shown).

Discussion

In order to better understand the complex pathology that comprises FRDA, in this study we compared hPDL from patients and healthy individuals, analyzing frataxin expression, its methylation pattern, as well as how this affects the expression of other related genes. Interestingly, our cellular model derives from the neural crest progenitors and express neural crest stem properties [12], the same progenitors that also originate dorsal root ganglia neurons. Therefore it may represent an appropriate cellular model to understand the physiopathology of FRDA in the nervous system, as well as the therapeutic effect in neural derived cells. A recent work from our group has shown that FXN mRNA expression is downregulated in these cells and active-caspase3 is overexpressed [13], at a low culture passage. In the current work, we demonstrate that this is maintained throughout the passages. Moreover, other groups have shown that there is a distinct methylation pattern in the frataxin gene at the promoter and upstream repetitive region in post-mortem human heart, brain, blood and buccal FRDA cells [9,10]. Our results corroborate these reports, being unmethylated in the promoter sequence of both populations, and hypermethylated and heterogeneously methylated in the upstream repetitive region of FRDA and healthy samples, respectively. Thus, the methylation pattern of the frataxin gene is the same in cells from different tissue sources. Furthermore, frataxin deficiency in FRDA patients results from the blockage of the transition from initiation to an efficient elongation of frataxin transcription due to the hypermethylation of the upstream GAA repetitive region as well as an altered enrichment of different histone modifications associated with heterochromatin-like structures [25].

Cells isolated from FRDA patients suffer from oxidative stress [13], which also increases p53 transcriptional activity [26]. To this extent, p53-mediated cell survival, proliferation and cell death is affected, which also affects the expression of other genes that are downstream. Of these, p21 gene is one of the targets and its induction is remarkably sensitive even at very low p53 protein levels, indicating an effective cell cycle arrest caused by a moderate damage or oxidative stress [27]. Our present work in hPDL cells from FRDA patients shows a significant increase in p53 transcriptional levels which is accompanied by the upregulation of p21, indicating a block in cell cycle progression. Moreover, cyclin D1 (CCND1) is also significantly upregulated in FRDA. This gene regulates the progression of the cell cycle at the G1-S checkpoint, and is involved in DNA damage response and repair [28] as well as chromosome stability [29]. Our results indicate that there is an arrest in cell cycle progression, possibly due to DNA repair mechanisms. Overexpression of GLB1, which is implicated in senescence [30], corroborates that a permanent cell cycle arrest could be taking place in cells isolated from FRDA patients. Moreover, high levels of active caspase-3 in hPDL cells from FRDA patients evidence that an apoptotic process may also be occurring. Overall, the expression of these genes indicates that the cell cycle of hPDL cells from FRDA patients is affected, either entering cellular senescence where they could alter the tissue microenvironment, or apoptosis which leads to cell death in FRDA. Observation of both processes has also been reported in a previous studies of p53, corroborating our results [31].

At the same time, p53 is related to DNMT1, which propagates the existing methylation marks across successive cell divisions, maintaining the methylation pattern and genomic integrity. In one of the signaling pathways that regulates DNMT1 mRNA transcription, p53 binds to DNMT1 promoter in absence of any stress and preventing its transcription, which is released under situations of DNA damage, leading to an overexpression in DNMT1 mRNA levels [17]. Our analysis in DNMTs expression shows a significantly difference in the mRNA levels in DNMT1 between FRDA and healthy hPDL cells, being higher in ataxia, indicating that it could be involved in repairing DNA damage [32]. Furthermore, DNMT1 also functions regulating heterochromatin formation, thus helping in the FXN gene UP GAA repetitive sequence to be compacted by methylation as we see in MSP experiments, and as a consequence being involved in transcriptional stop elongation. Thus, evidence shows that DNMT1 in hPDL not only is involved in DNA methylation, but also in repairing DNA, being both systems very complex. On the other hand,

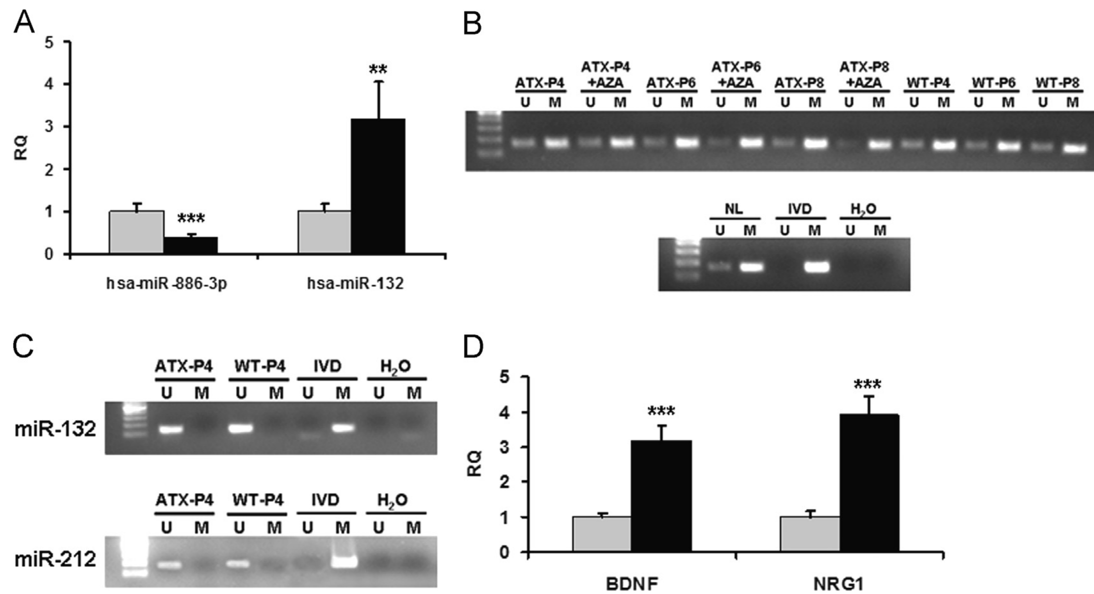

Fig. 5 – MicroRNAs expression and methylation study in hPDL cells. Characterization of BDNF and NRG1 mRNAs levels in hPDL cells. (A) miR-886-3p and miR-132 optimized assay expression analysis. Both miRNAs are differently expressed in hPDL cells of healthy (WT, gray bars) *versus* FRDA patients (FRDA, black bars), where miR-132 is highly over transcribed while miR-886-3p is downregulated in FRDA cells. (B) miR-886-3p promoter methylation pattern. CpG island in the promoter sequence of miR-886-3p was analyzed by MSP in hPDL both in WT and FRDA cells (product size is 153 bp). In all passages and in both cell types (data from P5 and P7 not shown), miR-886-3p was heterogeneously methylated. AZA treatment in FRDA cells did not change that methylated status. (C) Tandem miR-132 and miR-212 promoters methylation analysis. CpG islands methylation in the promoters sequences of those miRNAs were defined by MSP approach and the figure shows unmethylation in hPDL from WT and FRDA cells. Product sizes are 131 and 157 bp respectively. (D) qPCR analysis of DBNF and NRG1 mRNAs. BDNF and NRG1 genes are upregulated in hPDL cells from FRDA patients (FRDA, black bars) *versus* healthy (WT, gray bars). In each case the mean (+SEM) of gene expression is shown as determined from three different individuals in each group.The cells from healthy individuals are considered as control (value=1). *Y*-axis indicates the relative quantity of the genes analyzed (RQ). $^{***}p<0.001$, $^{**}p<0.005$. In the MSP studies, methylation (M) and unmethylation (U); passage (P); healthy lymphocytes (NL); commercial *in vitro* methylated DNA (IVD); master mix without bisulfite modified DNA (H_2O).

DNMT3a y DNMT3b, which mainly participate in methylating *de novo* DNA, have no significant variation in mRNAs levels in both FRDA and WT cells.

In FRDA, as well as in other neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease (AD) and Amyotrophic Lateral Sclerosis (ALS), oxidative stress is linked to neurodegeneration and cell death [33]. With the cells isolated from FRDA patients being susceptible to oxidative stress, as well as the DNA damage and apoptosis increase detected in our studies which in turn causes more oxidative stress, it is not surprising to detect an increase in the expression of antioxidant systems such as superoxide dismutases (SOD1, SOD2 and SOD3). This upregulation in hPDL cells from FRDA patients may accelerate the redox signaling to help compensate the oxidative imbalance that the cells suffer. Moreover, it has been recently described that SOD1 regulates the expression at the transcriptional level of a large set of oxidative response genes, by association with the promoters that are known to provide resistance to oxidative stress, DNA damage repair and relief of replication stress [34]. From this new functional point of view, SOD1 mRNA upregulation in FRDA may indicate a cellular demand in oxidative stress regulation trying to maintain the homeostasis.

As FXN participates in the biosynthesis of the iron–sulphur cluster (ISC), its low expression in FRDA induces low levels of this cluster [18,19]. In this work, we analyzed if mRNAs expression of ISCU and LYRM4, as proteins that form the cluster, and GLRX5 that participates in its biosynthesis are aberrantly expressed in FRDA patients. As a result, all of the three genes were overexpressed. Furthermore, other works have shown that mutations in human genes involved in the biosynthesis of ISC are identified as disease-causing genes, such as ISCU [35], LYRM4 [36] y GLRX5 [37]. This can be interpreted as that the upregulation of these genes in hPDL cells from FRDA patients may be an instrument to compensate the low FXN mRNA levels and produce more ISC. However, despite the increased expression of ISC mRNAs, the proteins would be degraded since it is not possible to form the cluster, as shown in a previous report [18].

Another very important epigenetic mechanism in gene transcription regulation are miRNAs. In this work, we show that miR-886-3p is significantly down regulated in hPDL FRDA cells. This result differs from that observed in a previous work, where miR-886-3p was overexpressed in individuals with FRDA [11]. This may be partly due to the fact that the tissue source (peripheral blood) differed from the one used in our work (PDL cells). It has been demonstrated that miR-886-3p is suppressed in a wide variety of cancer cells, and its down regulation in early stages of cancer activates the PKR/eIF2a pathway which provokes cell death [38]. Another study also proposes that miR-886-3p acts as a tumor suppression gene in acute myeloid leukemia and when it is unmethylated, patients have better outcome [39]. The lower

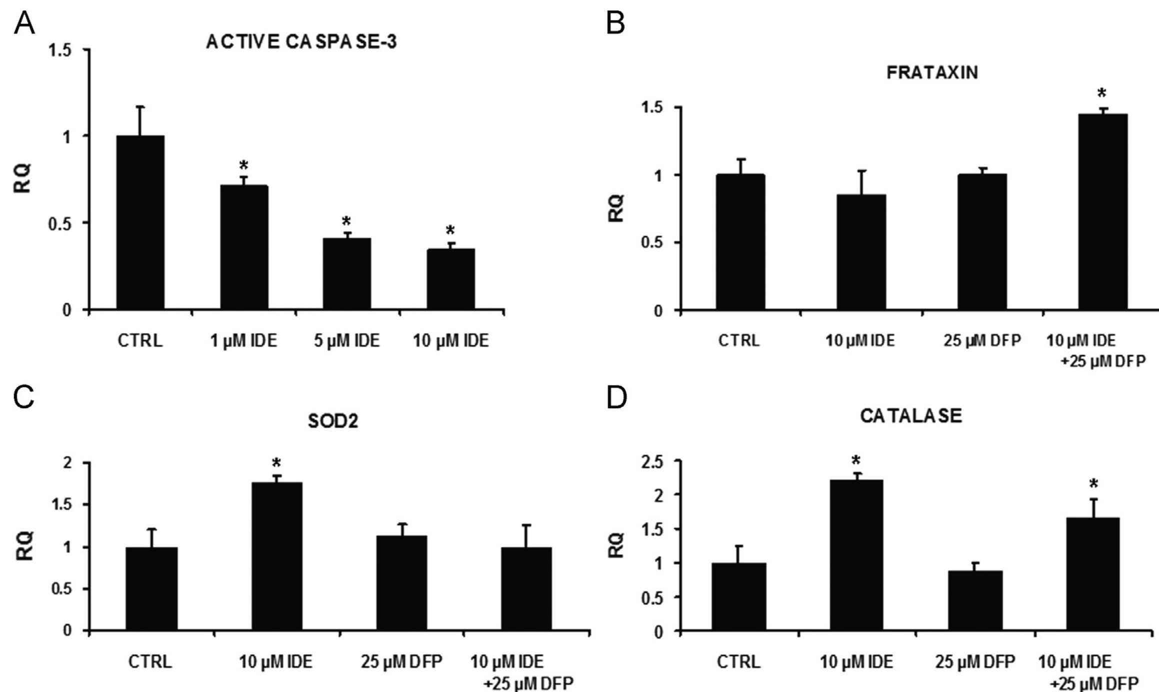

Fig. 6 – Treatment of periodontal ligament cultured cells from FRDA patients with idebenone (IDE) and deferiprone (DFP). (A) Analysis of CASP3 mRNA expression by qPCR after different IDE concentrations. Three different IDE concentrations were applied in hPDL FRDA culture cells: 1 μM, 5 μM and 10 μM. Comparing with the FRDA control (CTRL), CASP3 is significantly decreasing with higher IDE concentrations. (B) FXN mRNA expression after 10 μM IDE and/or 25 μM DFP treatments. FRDA cells showed a significantly higher frataxin transcriptional expression after the combination of IDE and DFP but nor with the individual IDE or DFP treatment compared with CTRL. (C) SOD2 mRNA expression after 10 μM IDE and/or 25 μM DFP treatments. SOD2 gene is overexpressed in FRDA cells *versus* CTRL, when 10 μM IDE is applied in the cell culture, but not in the other conditions. (D) Catalase mRNA expression after 10 μM IDE and/or 25 μM DFP treatments. Catalase mRNA is upregulated when cells are treated not only with 10 μM of the antioxidant IDE, but also in combination with 25 μM DFP related to CTRL. In each case the mean (+SEM) of gene expression is shown as determined from three different individuals in each group. *Y*-axis indicates the relative quantity of the genes analyzed (RQ). Holm–Sidak method: $^{*}p<0.05$.

miR-886-3p expression in hPDL from FRDA patients could be related to an apoptotic/antiproliferative pathway as CASP3 and stress response related genes are elevated in those cells. The miRNA miR-886-3p is monoallelicaly methylated in 75% of healthy individuals ($n=20$) while the remaining 25% is biallelic hypomethylated [39]. In this work we show in hPDL cells that miR-886-3p is heterogeneously methylated both in FRDA and WT cells.

As our group has recently described, hPDL cells contains stem cells that originate from the neural crest, and in the present work we have analyzed miR-132 expression in those cells. miR-132 is enriched in the central nervous system and is one of the most studied for its implication in neurite extension and synapse formation; is a key regulator of neuronal plasticity, maturation and development and its deregulation is associated with several neurological disorders such as Alzheimer's and Huntington diseases [40,41]. Our results show significantly higher levels miR-132 transcriptional activation in hPDL from FRDA patients compared to healthy individuals, and is divergent with low miR-132 expression levels described in FRDA lymphoblast cell line and peripheral blood samples [11]. Furthermore, miR-132 is known to be upregulated in human acute monocytic leukemia cells after exposure to lipopolysaccharide (LPS), indicating its participation in the innate immune response and its role as an endotoxin-responsive gene [42]. It is also upregulated in biopsies from patients with inflammatory bowel disease [43], showing its role in inflammation. Overall, heart tissue isolated from FRDA patients autopsies showed a clear evidence of inflammatory processes, which was also detected in the brains of FRDA mouse models [44]. These processes would elevate miR-132 levels, as we have observed in this study.

FRDA patients suffer cardiomyopathy and have cardiac hypertrophy, being the main cause of death heart failure [45]. It is very interesting that a recent work describes miR-132 participation in regulating cardiac hypertrophy and autophagy in cardiomyocytes, as miR-132 expression is upregulated in cardiomyocytes during hypertrophy both *in vitro* and *in vivo* in rats, and miR-132 is necessary and sufficient for cardiomyocyte hypertrophy *in vitro* [46]. Taken all together, our result in miR-132 overexpression in hPDL from FRDA patients is contributing to the disease and this knowledge may help to find other molecular targets that could be identified as biomarkers in those patients, opening new insights in the research for other therapies in FRDA.

Brain-derived neurotrophic factor (BDNF) has a role in neural growth, survival and neurogenesis, and after different types of injuries, BDNF can activate compensatory mechanisms that palliate the detrimental effects of stress, injury or disease [24]. We analyzed the possible relation between BDNF and miR-132 in

hPDL cells, since there are previous studies that describe BDNF like a miR-132 expression inductor. They show that BDNF upregulates miR-132 expression in cultured cortical neurons [21], and in primary retinal cultures where axon branching is promoted [20]. We also showed that BDNF is overexpressed in FRDA compare to WT cells, corroborating an upregulation in both BDNF and miR-132 transcripts in hPDL cells from FRDA patients. In these cells, where the homeostasis is broken and they are vulnerable to oxidative stress [13], we detect in this work that there is an imbalance in the aberrant expression of cell cycle, DNA damage and oxidative stress related genes. BDNF upregulation in hPDL cells from FRDA patients may be a mechanism to prevent neuronal damage caused by oxidative stress, in order to return the cells to a more stable state. Furthermore, there is evidence that endogenous BDNF signaling promotes a stage-dependent release of neuregulin 1 (NRG1), a growth and differentiation factor, from axons to Schwann cells [22] with a critical role in peripheral nerve development and spinal cord microglial activation in chronic pain and in ALS; as well as in cardiac development and can stimulate proliferation and survival of different cell types [47]. A complex interrelation between BDNF and NRG1 has also been described in another neurodegenerative model, specifically in experimental autoimmune encephalomyelitis [23]. In this disorder, BDNF overexpression induces NRG1 expression, similar to what we have observed in FRDA. Thus, our work indicates that BDNF may be regulating NRG1 expression, similar to that observed in other diseases, as a method to avoid cell death.

IDE and DFP treatments change gene expression levels in hPDL cultured cells. IDE decreases apoptosis marker CASP3 and may improve cell survival, and its combined effect with DFP produced a significant upregulation in FXN mRNA. Further studies are needed to explain which molecular mechanisms are involved in this effect on frataxin expression, which opens a new approach both to understand better the disease as well as search for new therapeutic tools. As an antioxidant, 10 μM IDE provokes a significant upregulation in SOD2 mRNA and this result is in correlation with previous studies both in other neurodegenerative and cancer diseases, in which different antioxidants promote SOD2 expression increase with a potential neuroprotection when submitted to cellular stress [48,49]. SOD1 expression did not significantly change in the presence of IDE or DFP (data not shown), possibly due to the fact that in FRDA there is two-fold more expression of SOD1 with respect to SOD2, which may be sufficient for its oxidative stress response, as it has been recently shown that SOD1 is capable of acting as a transcription factor to regulate other oxidative stress-related genes [34]. Furthermore, IDE alone or combined with DFP upregulates catalase mRNA in FRDA cells compared with untreated cells, indicating a major cell capacity in response to oxidative damage and improving this pathway. Previous works had resulted in controversial results using IDE [14], however our studies indicate that this antioxidant is capable of increasing cell survival in FRDA, although further studies are required.

In the present work, we show that frataxin absence is associated with significant gene expression changes in FRDA patients. Interestingly, we found overexpression of cell death-related genes, cell cycle arrest and oxidative related genes, as well as DNMT1, supporting the notion that DNA repair and epigenetic mechanisms are implicated in the development of FRDA disease. Furthermore, ISC involved genes that are responsible for the iron–sulphur metabolism are also deregulated in FRDA, promoting the characteristic imbalance that the patients present. Thus, frataxin deficiency is not the only affected factor in this disorder, many other genes implicated in different roles seem to be connected with this gene. Understanding how these genes are connected with frataxin can help us to understand better this complex disease, and therefore discover more effective therapeutic approaches for the patients.

Material and methods

Periodontal-ligament-derived stem cell isolation and culture

Periodontal ligament cells from three FRDA patients and three healthy individuals used in this work were taken from the cells collection inscribed in the National Biobank Register with reference C.0002622 (Ministerio de Economía y competitividad). The cell pellet was washed and centrifuged twice, and counted in a Neubauer chamber before placing in culture. The culture medium used was animal-origin-free mesenchymal stem cell medium (StemCells Technologies), and the cells were placed in a cell culture flask. The medium was changed 48 h after the initial plating and afterwards every 3–4 days. After 7–10 days, when the culture was almost confluent, the cells were detached using TrypLE Select animal-origin-free stable trypsin replacement (Gibco) and replated at a concentration of 50.000 cells/flask. For the experiments, cells from passages 4–8 were used.

5-Aza-2′-deoxycytidine (AZA) treatment of cultured cells

For *in vitro* passive DNA demethylation, culture cells were treated in fresh medium with 5-Aza-2′-deoxycytidine (AZA) (Sigma) to a final concentration of 5 μM for 3 consecutive days. Culture medium was changed every 24 h. Control cells without AZA were growth with fresh medium.

Idebenone (IDE) and deferiprone (DFP) treatments of culture cells

FRDA culture cells were treated in fresh medium with IDE (Enzo, Life Sciences), an analog of the coenzyme Q antioxidant, for 1 day at different concentrations to analyze CASP3 mRNA levels: 1 μM, 5 μM y 10 μM. Afterwards, because CASP3 mRNA levels were lower with 10 μM IDE, this concentration was used to treat FRDA culture cells for the other analyzed genes alone or in combination with 25 μM of the chelating agent DFP (ApoPharma Inc), for 1 day.

Nucleic acid extraction

Genomic DNA was isolated from cultured cells by the phenol-chloroform standard method. Total RNA was isolated by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions.

Bisulfite treatment of DNA and Methylation-Specific PCR (MSP)

1 μg of total DNA was bisulfite-modified using Epitect Bisulfite Kit (Qiagen), which consists in the conversion of all unmethylated, but not methylated cytosines to uracil. The MSP technique allows the identification of unmethylated and methylated alleles in genes by amplifying a certain DNA region with specific primer pairs, on the basis of sequence changes induced by the bisulfite treatment. In each PCR, unmethylated and methylated DNA controls were used. The following primers were designed using Methyl Primer Express Software (Applied Biosystems): Frataxin-transcription start site (FXN-TSS) methylated-sense-TAGTATGTGGATTTTCGGGC, methylated-antisense-TACAAATCGCATCGATATCG, unmethylated-sense-GAGTAGTATGTGGATTTTTGGGT, unmethylated-antisense-ACATACA AATCACATCAATATCA (155 bp); frataxin-upstream repetitive region (FXN-UP) methylated-sense-TAAAGGTGACGTTTATTTTGC, methylated-antisense- CCTTTCAAACCGTAACGTAA, unmethylated-sense-AGATAAAGGTGATGTTTATTTTGT, unmethylated-antisense-CCTCCT TTCAAACCATAACATAA (147 bp); hsa-miR-132 methylated-sense-TGTTGATTAACGTAGGCGTC, methylated-antisense-GAACTAACGT-CAAAACCGC, unmethylated-sense-GGTGTTGATTAATGTAGGTGTT, unmethylated-antisense-ACAAACTAACATCAAAACCAC (131 bp); hsa-miR-212 methylated-sense- TTGACGTTACGGTTTTTTGAC, methylated-antisense-AAAATACCGACGCGCTATC, unmethylated-sense-TTTTTGATGTTATGGTTTTTTGAT, unmethylated-antisense-CCA AAATACCAACACACTATCC (157 bp); hsa-miR-886 methylated-sense-CGTTAGTAGGACGTTTGGC, methylated-antisense-AACTAACTCC-GACCGAATA, unmethylated-sense-ATATGTTAGTAGGATGTTTQJ;GGT, unmethylated-antisense-AACTAACTCCAACCCAAATAAAA (153 bp).

In the case of FXN-TSS MSP product, the CpG sites analyzed correspond to numbers 9–25 of the frataxin promoter assay previously published in Evans-Galea MV et al. [10] and in FXN-UP MSP product, the CpGs sites correspond to the 4 CpGs immediately preceding number 1 of the frataxin UP assay mentioned in the same publication previously commented.

Reverse transcription and quantitative real-time PCR

For codified genes, 1 μg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 25 ng of cDNA was amplified by real-time PCR using Power SYBR Green Master mix (Applied Biosystems). For miRNAs, 10 ng of total RNA was reversed transcribed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and 1 μl of cDNA was amplified by real-time PCR using TaqMan Universal Master Mix II (Applied Biosystems). Three biological replicates both from FRDA and unaffected individuals were used. All the samples were run in triplicate using the StepOne Plus Real-Time PCR system (Applied Biosystems), and analyses were carried out in the StepOne Software using the Delta Ct method relative to Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) for codified genes, and RNU6B (RNA, U6 small nuclear 6, pseudogene) for miRNAs. Each PCR generated only the expected amplicon, as shown by the dissociation curve. The following primers for RT-PCR were designed using Primer3 Software: Iron-Sulphur Cluster assembly enzyme (ISCU): sense-CCAGCATGTGGTGACGTAAT, antisense-CCACAATCTTCCCCTTT TCA; LYR motif containing 4 (LYRM4) sense-GAGAGAGCAAGCGTTT-CAGC, antisense-GGCATCTCTTATCCTCCTGACA; glutaredoxin 5 (GLRX5) sense-CCTACAACGTGCTGGACGAC, antisense-TGGGCCAG TTGGAATAGTCT; tumor protein p53 (p53) sense-GTCTGGGCTTCTTGCATTCT, antisense- CTCCGTCATGTGCTGTGACT; cyclin-dependent kinase inhibitor 1A (p21) sense- GGAAGAC-CATGTGGACCTGT, antisense- AAGATGTAGAGCGGGCCTTT; cyclin D1 (CCND1) sense- CCCTCGGTGTCCTACTTCAA, antisense-TGGCATTTTGGAGAGGAAGT; galactosidase, beta 1 (GLB1) sense-ACTGAGGATGCAGTGtGCAG, antisense- CAATAGCGGCCAAGGT-TAAA; DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3a) sense- GATGACGAGCCAGAGTACGA, antisense- CATCCACCAAGACA-CAATGC; neuregulin 1 (NRG1) sense- CGGTGTGAAACCAGTTCTGA, antisense- TCAGCCAGTGATGCTTTGTT; GAPDH sense- TCTTC TTTTGCGTCGCCAG, antisense- AGCCCCAGCCTTCTCCA. The primers used were previously described [50] for DNA (cytosine-5-)-methyltransferase 1 (DNMT1) sense- TACCTGGACGACCCTGACCTC, antisense- CGTTGGCATCAAAGATGGACA and for DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3b) sense- GGCAAGTTCTCC-GAGGTCTCTG, antisense- TGGTACATG GCTTTTCGATAGGA. As for the miRNA assays, the following Taqman miRNA assays were used (Applied Biosystems): hsa-miR-886-3p, hsa-miR-132 y RNU6B. Also, the following primers were used taken from the PrimerBank webpage (http://pga.mgh.harvard.edu/primerbank/): frataxin (FXN) sense- AAGACTAGCAGAGGAAACGCT, antisense- ACCCAGTTTGA CAGTTAAGACAC; caspase 3, apoptosis-related cysteine peptidase (CASP3) sense- CATGGAAGCGAATCAATGGACT, antisense- CTGTAC CAGACCGAGATGTCA; superoxide dismutase 1, soluble (SOD1) sense-: AGGGCATCATCAATTTCGAGC, antisense- GCCCACCG TGTTTTCTGGA; superoxide dismutase 2, mitochondrial (SOD2) sense- AACCTCAGCCCTAACGGTG, antisense- AGCAGCAATTTG TAAGTGTCCC; superoxide dismutase 3, extracellular (SOD3) sense-ATGCTGGCGCTACTGTGTTC, antisense- ACTCCGCCGAGTCAGAGTT; catalase sense- TGGGATCTCGTTGGAAATAACAC, antisense- TCAG GACGTAGGCTCCAGAAG; brain-derived neurotrophic factor (BDNF) sense- TAACGGCGGCAGACAAAAAGA, antisense- TGCACTTGG TCTCGTAGAAGTAT.

Statistical analysis

Statistical significance between control and experimental groups was calculated with Sigmaplot v12.0 software, using *t*-test. For cells treated with IDE and/or DFP, One Way Analysis of Variance test was applied to confirm that a statistically significant difference exists and afterwards, a Holm–Sidak method was used to determine the differences between experimental and control samples. Values measured as mean (+SEM).

Conflict of interest

The authors state that there is no conflict of interest.

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