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Effects of Quercetin in transcriptional and post-transcriptional regulation of fetal hemoglobin

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Abstract - *Sickle Cell anemia (SCA) is a hereditary hemoglobinopathy with formation of hemoglobin S, associated with severe health outcomes. Currently, induction of fetal hemoglobin (HbF) is one of the most promising therapeutic strategies. Here we aimed to assess the potential of the natural compound Quercetin, in transcriptional expression of globin and HbF regulatory/silencing genes.*

In this study, the K562 cell line was used as an SCA model. Cells were exposed to Quercetin at final concentrations of 0.2 and 20 μM, and Hydroxyurea (25 μg/mL) was used as a positive control. Cell viability and proliferation were assessed through trypan blue exclusion assay. Transcriptional expression was performed by RT-qPCR using specific primers. Significant differences were analyzed using a t-test.

No cytotoxic effects were observed following exposure to Quercetin. Transcriptional analysis demonstrated that Quercetin affects mRNA levels of HbF regulatory/silencing genes with associated downregulation of BCL11A, MYB, KLF1 and HBB and upregulation of HBG and BGLT3, as well as alterations in the expression of miRNAs involved in HbF post-transcriptional regulation.

Our results sustain Quercetin potential as an HbF inducer with associated upregulation of HbF-activators and decreased expression of HbF-inhibitors. These data support the need for further studies in order to confirm the potential of this compound as a new therapeutic option for β-hemoglobinopathies in the future.

Keywords— *sickle cell disease, reactivation of fetal hemoglobin, Quercetin, flavonoid, Hydroxyurea, HbF regulatory/silencing genes*

I. INTRODUCTION

Sickle Cell anemia (SCA) is a hereditary hemoglobinopathy with autosomal recessive transmission caused by a mutation in the β-globin gene, giving rise to hemoglobin S (HbS). When deoxygenated, HbS polymerizes in the red blood cell, giving it a sickle shape and making it rigid and fragile[1].

Currently, therapies that induce fetal hemoglobin (HbF) are promising, such as hydroxyurea (HU). However, due to high costs for underdeveloped countries and its adverse side effects, it is important to develop new compounds[2].

Thus, the discovery of new HbF inducers with fewer side effects, affordable and easily accessible, such as natural compounds (e.g. Quercetin) is crucial. Still, further studies are needed in order to understand its mechanism of action and its therapeutic potential for SCD treatment.

Several regulatory proteins and miRNAs that control the expression levels of HbF participate in the hemoglobin switch. The genes subject to regulation are the adult forms of hemoglobins (*HBA* and *HBB*) and the fetal forms (*HBG1* and *HBG2*), the regulatory genes being *MYB*, *KLF1*, *BCL11A* and *BGLT3,* which encode transcription factors, among others[3].

The expression patterns of related genes such as globins (*HBA*, *HBB*, *HBG1* and *HBG2*), HbF silencing genes (*MYB*, *KLF1*, *BCL11A*), HbF activators (*BGLT3*), and also the expression levels of regulatory miRNAs (miR-486-3p, miR-34a-5p, miR-210-5p, miR-32-5p and miR-96-5p) were thus analyzed upon incubation of K562 cells with Quercetin.

The main purpose of this work was thus to evaluate the in vitro effects of Quercetin in the reactivation of HbF (*HBG1* and *HBG2* genes) by analyzing the transcriptional and post-transcriptional expression of globin and HbF regulatory/silencing genes.

II. MATERIALS & METHODS

A. K562 cell culture and reagents

In the present study, the K562 cell line was used as an SCD model, since it has been proposed as an *in vitro* model to study the molecular mechanisms regulating human embryonic and fetal globin expression. The cells were defrosted under sterile conditions and passaged with RPMI 1640 culture medium (Catalog Number: L0495-500, Biowest) containing L-Glutamine and 25mM Hepes and supplemented with 10% (v/v) fetal bovine serum (FBS) and with 500 μL of antibiotic (200 mM L-Glutamine, 10000 U penicillin and 10 mg/mL streptomycin) (Product Number: G6784, Sigma-Aldrich). Phosphate buffered saline (PBS) (Catalog Number: 51226, Lonza) was used for cell passages and the cell pellet was resuspended in new medium.

Cell culture was performed aiming at a density of $1x10^5$ cells/mL, and subcultures were performed every 3 days and maintained in a humidified atmosphere of 5% (v/v) CO2 at 37ºC. Prior to each subculture, confluence, morphology, proliferation and cell viability were observed.

B. Incubation of Cells with Quercetin

Quercetin was (CAS Number: 117-39-5, Sigma-Aldrich), dissolved in 50 μL of dimethyl sulfoxide (DMSO)[4] to a stock solution of 1.3 M[1]. The final Quercetin solutions were used at 0.2 and 20 μM concentrations.

K562 cells were exposed to Quercetin and to a positive control consisting of 25 μg/mL Hydroxyurea (HU) (HYDREA®, USP), the compound proved to induce HbF. In addition, a negative control consisting of culture medium supplemented with DMSO was used.

Cells were incubated in a humidified environment at 37°C with 5% (v/v) CO_2 for 24 hours (treatment exposure time), and then harvested in order to assess proliferation, cell viability, and analyze transcriptional and post-transcriptional results.

C. Analysis of cell viability and proliferation

In order to evaluate cytotoxic effects after 24h of exposure, the trypan blue exclusion assay was used in order to determine the number of viable cells present in a cell suspension. A cell suspension solution containing 0.4% trypan blue (CAS Number: 72-57-1, Sigma-Aldrich) was added.

After visualizing the cells in Newbauer's chamber on an inverted phase-contrast microscope, the cell number was counted, and the proliferation rate and percentage of cell viability were calculated.

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D. Extraction, quantification and conversion of total RNA to complementary DNA (cDNA)

Total RNA was extracted from the K562 cell pellet obtained after treatment with the indicated solutions and stored at -80ºC. The extracted RNA was then quantified using the NanoDrop-ONE equipment (Thermo Scientific).

Thereafter, 2.0 µg of total RNA was used to be converted into cDNA. The "SuperSciptTM IV First-Strand Synthesis System" kit (Catalog Number: 18091050, Invitrogen™, Thermo Fisher Scientific Inc.) was used for this reaction along with random hexamer primers in a final volume of 20 µL, following the manufacturer's instructions.

Lastly, the Bio-Rad iCyler® thermal cycler was used, with the following conditions: 25ºC for 10 minutes (hybridization of primers or annealing), 37ºC for 30 minutes (cDNA synthesis from total RNA by RT), 95ºC for 5 minutes and stop at 4ºC. For cDNA synthesis of the miRNAs study we used the miRCURY LNA RT Kit (Catalog Number: 339340, Qiagen) with the reagents 5x Reaction Buffer, Nuclease free water, Enzyme mix and UniSP6 Spike-in.

E. Amplification of cDNA by real-time quantitative PCR (RT-qPCR)

Real-time quantitative PCR (RT-qPCR) was performed on the CFX Connect™ Real-Time PCR Detection System (Bio-rad) using the iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad). Triplicates were performed for each biological replicate, in order to increase the statistical power of the Cq value obtained in each sample.

In the present study, gene expression was compared between the samples. For this, the data obtained in the CFX Manager software was collected and analyzed through the relative quantification of RNA by RT-qPCR. Through this it is possible to determine the relative difference between the expression of the reference gene and the expression of the gene under study. Using the ΔΔCq method, the relative difference between gene expressions was calculated and the GAPDH gene (Glyceraldehyde 3-phosphatedehydrogenase), our reference gene, was used to normalize the data.

F. Statistical analysis

The statistical analysis of the data obtained from the gene expression level in the K562 cell line was performed using Microsoft Excel 365 software. Significant differences between treatments and concentrations of the same t-test, with p-value < 0.05 were considered as statistically significant values.

III. RESULTS

A. Transcriptional regulation of HBG1 and HBG2 and their regulatory genes

Data analysis of cell proliferation and viability (not shown) indicate that, although Quercetin induces a decrease of about 40% in the proliferation rate upon incubation at the 20 µM concentration (when compared to controls), no differences in cell viability were observed for none of the concentrations used in this study. These results indicate that Quercetin has no cytotoxic effects on the K562 cell line.

Regarding globins expression levels, it was observed that for the *HBA* gene, Quercetin at 20 µM, inhibited the expression levels of the *HBA* gene, with an opposite effect of that of HU **(Figure 1A)**. On the other hand, the results obtained with the HBB gene expression showed that both concentrations of Quercetin inhibited the transcription of this gene, also having a different effect to HU (not shown).

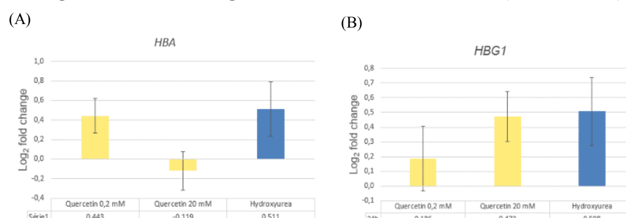

Figure 1. Quercetin effects on expression levels of HBA (A) and HBG1 (B) genes. RT-qPCR analysis of HBA and HBG1 expression levels normalised to GAPHD housekeeping gene relative to control cells.

In what refers to *HBG1* and *HBG2* genes, their expression levels are increased in the presence of both concentrations of Quercetin increased, having a similar effect as HU (Figure 1B).

Regarding HbF silencing genes (*MYB*, *KLF1* and *BCL11A*), Quercetin was found to decrease the expression levels of these genes at both concentrations, as shown for *MYB* and to increase the expression levels of the HbF activator gene (*BGLT3*). The results of this compound on *MYB* expression are identical to those of HU.

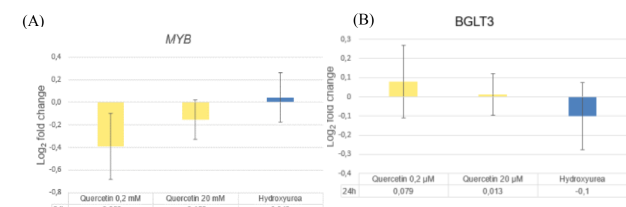

Figure 2. Quercetin effects on expression of *MYB* (A) and *BGLT3* (B) genes. RT-qPCR analysis of MYB and BGLT3 expression levels normalised to GAPHD housekeeping gene relative to control cells.

For the genes *KLF1* and *BCL11A*, the study compound had different results compared to HU (positive control). On the other hand, the results obtained for the *BGLT3* gene with this compound are identical to those obtained with HU. These results show that *KLF1* and *BCL11A* seem to act on the same HbF inhibition pathway, in agreement with studies reporting that *KLF1* is the main regulator of *BCL11A*, activating it[5].

In **Table I** it can be observed a summary of the variations in the expression levels of HbF regulatory genes in K562 cell extracts subjected to Quercetin or HU treatment.

Overall, transcriptional analysis reveals that exposure to Quercetin induces downregulation of *BCL11A*, *MYB*, *KLF1* and *HBB* and upregulation of *HBG* and *BGLT3* gene expression

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TABLE I. VARIATIONS IN EXPRESSION LEVELS OF HBF REGULATORY GENES IN K562 CELL EXTRACTS SUBJECT TO TREATMENT WITH QUERCETIN OR HU.

Exposure Time	Treatment	Gene transcription levels
24 hours	Quercetin 0,2 µM	↑HBA, ↓HBB, ↑HBG, ↓MYB, ↓KLF1, ↓BCL11A, ↑BGLT3
	Quercetin 20 µM	↓HBA, ↓HBB, ↑HBG, ↓MYB, ↓KLF1, ↓BCL11A, ↑BGLT3
	HU 25 µg/mL	↑HBA, ↑HBB, ↑HBG, ↑MYB, ↑KLF1, ↓BCL11A, ↓BGLT3

.Postranscriptional regulation mediated by miRNAs

Several studies confirm that downregulation of miRNAs increases the clinical severity of sickle cell disease, and it can also be regulated in a way that increases the expression of HbF[6]. Thus, in the present study we analyzed the expression patterns of the following miRNAs involved in HbF regulation: miR-486-3p, miR-34a-5p, miR-210-5p, miR-32-5p and miR- 96-5p. The results obtained are summarized in table II, where the effect on miRNA expression levels are indicated as well a putative effect on induction of HbF gene expression levels. Synthesis of the effects of Quercetin on the expression of miRNAs and its potential effects on the induction of HbF.

TABLE II. SUMMARY OF THE EFFECTS OF QUERCETIN ON THE EXPRESSION OF MIRNAS AND ITS POTENTIAL EFFECTS ON THE INDUCTION OF HBF.

miRNA	Target gene(s)	Transcription	Effect on HbF
miR-486-3p	BCL11A	Quercetin increases	Induce
miR-34a-5p		Quercetin at 0,2 µM inhibits	Inhibit
miR-210-5p		Quercetin at 20 µM increases	Induce
miR-32-5p		Quercetin increases	Induce
miR-96-5p	HBG1 and HBG2		Induce

IV. DISCUSSION

In the present study the effects of the natural compound Quercetin on gene expression of globins (*HBA*, *HBB*, *HBG1* and *HBG2*), HbF regulatory genes (*MYB*, *KLF1*, *BCL11A* and *BGLT3*) and regulatory miRNAs (miR-486-3p, miR-34a-5p, miR-210-5p, miR-32-5p and miR-96-5p) were analyzed. Our transcriptional analysis reveals that exposure to Quercetin induces downregulation of BCL11A, MYB, KLF1 and HBB and upregulation of HBG and BGLT3 gene expression levels. Regarding miRNA profile they can be either associated with promotion or inhibition of HbF expression depending on the target genes they regulate with some of the results obtained, agreeing with a review by Adinew and colleagues that states that flavonoids can modulate the function of non-coding miRNAs[7], which in turn inhibit *MYB*, thus leading to increased HbF[6].

Overall, the results obtained indicate that Quercetin acts through different signaling pathways to hydroxyurea HU since it appeared to have different effects when compared to the effects obtained with HU. This hypothesis agrees with the hypothesis proposed by Pabuprapap and collaborators who state that Quercetin can induce HbF by signaling it through the NRF2/ARE115 pathway[8]. There are antioxidant responsive elements (ARE) in the promoter region of *HBG1* and *HBG2* genes, that respond to cellular stress by interacting with Nuclear factor-erythroid factor 2-related factor 2 (*NRF2*)[9]. Relevantly, NRF2 in addition to regulating HbF, also regulates antioxidant genes (*HMOX1* and *NQO1*) that reduce oxidative stress and decrease the pathophysiology of sickle cell disease[7].

Quercetin, an antioxidant stress polyphenol compound, induces *NQO1* gene expression through this signaling pathway, which consequently induces HbF. Our evidence that Quercetin potentially increases transcription of HbF-activating genes and inhibits transcription of HbF-inhibiting genes[10] further confirms that Quercetin may prove to be effective in the field of β-hemoglobinopathies treatment.

V. CONCLUSIONS

The results obtained indicate that Quercetin appears to induce HbF expression, either by increasing the transcription of γ-globin activating genes (*HBG* and *BGLT3*) or by inhibiting the expression of HbF inhibitors (*MYB* and *BCL11A*) or even by modulating the expression levels of candidate miRNAs. Thus, this preliminary study brings a new perspective of natural compounds as potential modulators of γ-globin gene expression. Further studies are needed in order to assess its potential in a future affordable and effective therapy for the treatment of SCD.

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