The effect of demethylation agent decitabine on microRNA-34 expression and cellular viability in Burkitt Lymphoma cells

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BACKGROUND

Burkitt Lymphoma (BL) is an aggressive non-Hodgkin lymphoma subtype derived from a mature B lymphocyte. This tumor is most common in children and is characterized by MYC chromosome translocation resulting in MYC over expression. MYC is a transcription factor that activates genes related to several cellular processes and regulates microRNAs (miRNAs) expression. miRNAs are a small noncoding RNAs that mediate post-transcriptional regulation. miR-34 family members are direct p53 targets, and induce apoptosis and cell-cycle arrest. In mammalians, miR-34 family comprises miR-34a, miR-34b and miR-34c being identified as tumor suppressor miRNAs. Epigenetic silencing by aberrant methylation on promoter gene has been demonstrated to be associated with the transcriptional inactivation of tumor suppressor genes such as miR34-b/c in many human malignancies. Given that DNA methylation is a reversible process, demethylation agents are been investigated about their successful of reversing the DNA hypermethylation status to restore the sensitivity of cancer cells to the antitumor drugs. In this study, our proposal was to evaluate the effects of decitabine on methylation and expression of miR-34b in BL cell lines with different *TP53* status.

OBJECTIVES

- To identify the methylation status of miR-34b in Burkitt Lymphoma cell lines: Ramos and Raji that shows mutated TP53 and in the P493-6 cell line, wild-type TP53;
- To quantify the relative expression levels of miR-34b and DNA methyltransferase 1 (DNMT1) after treatment with DNA demethylating agent;
- To evaluate the effects of the DNA demethylating agent on proliferation, cell cycle and induction of cell death in BL cells line;
- To evaluate miR-34b targets involved in the regulation of cell death (BCL2 and MYC) and in the cell cycle (CDK6) after treatment with DNA demethylating agent.

MATERIAL AND METHODS

- BL cell lines: Ramos, Raji (*TP53* mutant) and P493-6 (*TP53* wild type);
- Methylation-specific PCR (MSP) assay to identify the methylation status of miR-34b;
- BL cell lines incubation with 5-aza-2-deoxycytidine (Decitabina) (Sigma);
- Quantification of miR-34b and mRNA P16 levels by Real Time PCR (RT PCR);
- Protein expression analysis by Western Blotting (WB);
- Proliferation, viability and cell cycle analysis by MTT assay, trypan blue exclusion assay and flow cytometry;
- Statistical analysis was performed using the Anova test and p<0.05 was considered statistically significant.

RESULTS

Figure 1: miR-34b methylation status. Amplified samples by methylation-specific PCR (MSP) were submitted to electrophoretic run in agarose gel 2%. It's observed an amplification of the specific methylation reactions (M) in all cell lines: Ramos, Raji, BL41, SUDHL4 and P493-6. In Ramos and BL41 cell lines was also observed an amplification of the specific unmethylated reactions (U).

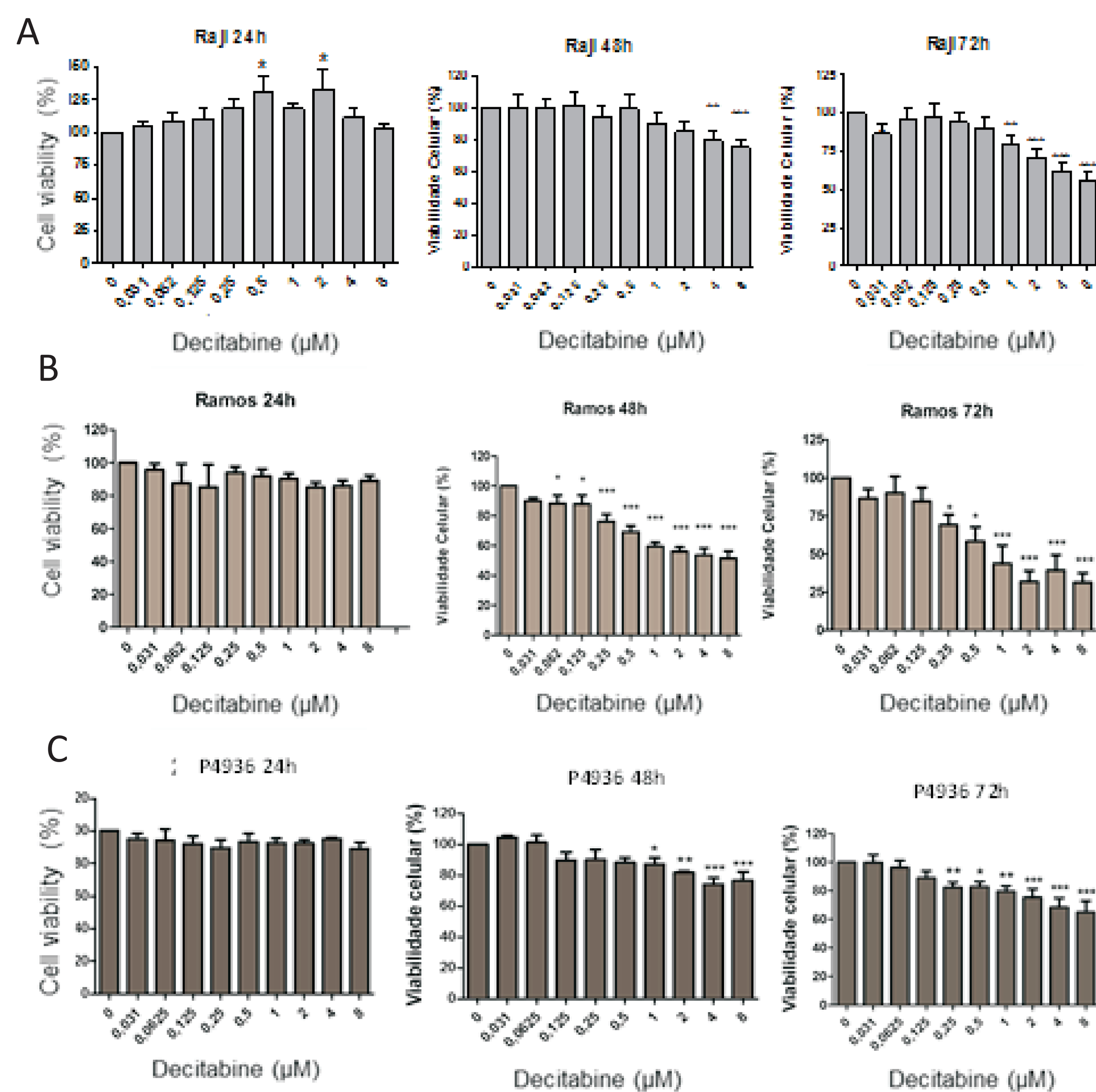

Figure 2: Analysis of the percentage of viable cells after decitabine treatment. The percentage of viable cells (y axis) of each cell line was analysed by MTT assay for 24, 48 and 72 hours after treatment with different decitabine concentrations (x axis). Raji cells (A), Ramos cells (B) and P493-6 cells (C). Representative graphics of the mean and standard error of 3 independent experiments were shown.

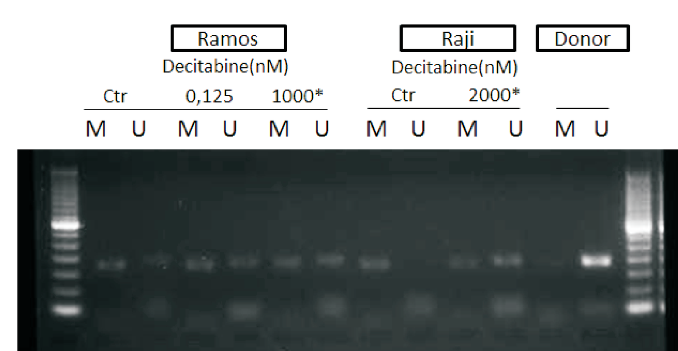

Figure 3: Analysis of methylation status by MSP after decitabine treatment. The Ramos cell line was treated with 0,125 nM and 1 µM of decitabine and Raji cells was treated with 2µM. The plasma DNA sample from a healthy donor was used as a positive control of unmethylated reaction. The PCR-MSP products was submitted to electrophoretic run in agarose gel 2%.

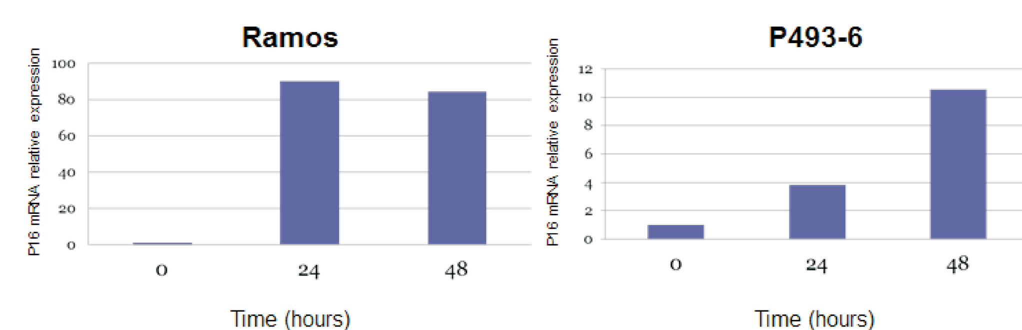

Figure 4: P16 quantitative expression analysis in Ramos and P493-6 cell lines. The mRNA expression analysis was done by Real Time PCR. The y axis represents the mRNA relative expression values and the x axis the incubation time of the Ramos (A) and Raji (B) cell lines with decitabine. TBP gene was used as endogenous control.

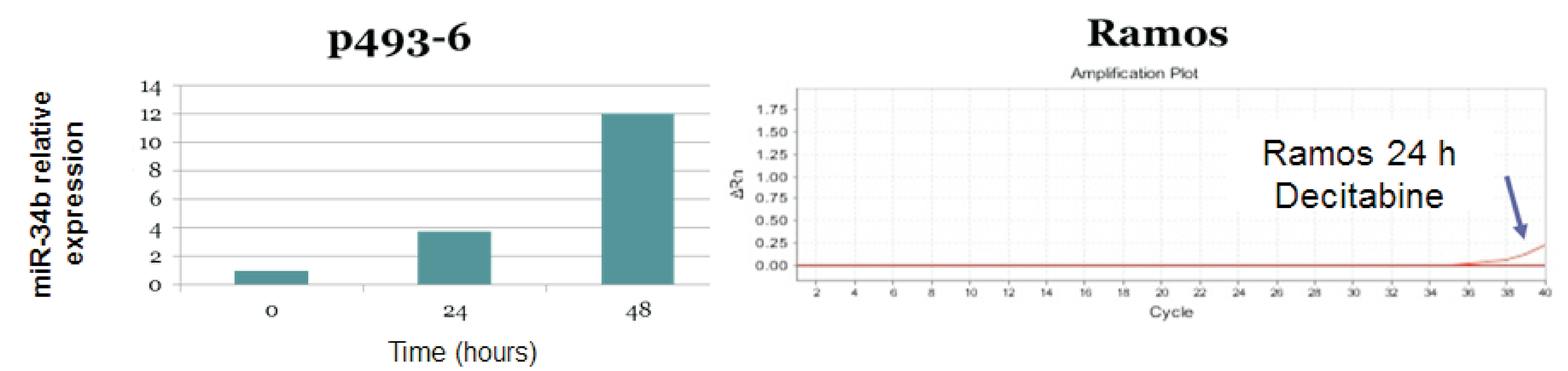

Figure 5: The miR-34b expression analysis in Ramos and P493-6 cell lines. Real Time PCR assay was perfomed in P493-6 and Ramos cell lines. The quantitative analysis of miR-34b in P493-6 was performed for 24 and 48 hours decitabine treatment (left graph). The right curve shows a increase of miR-34b in Ramos cell line treated with decitabine for 24 hours.

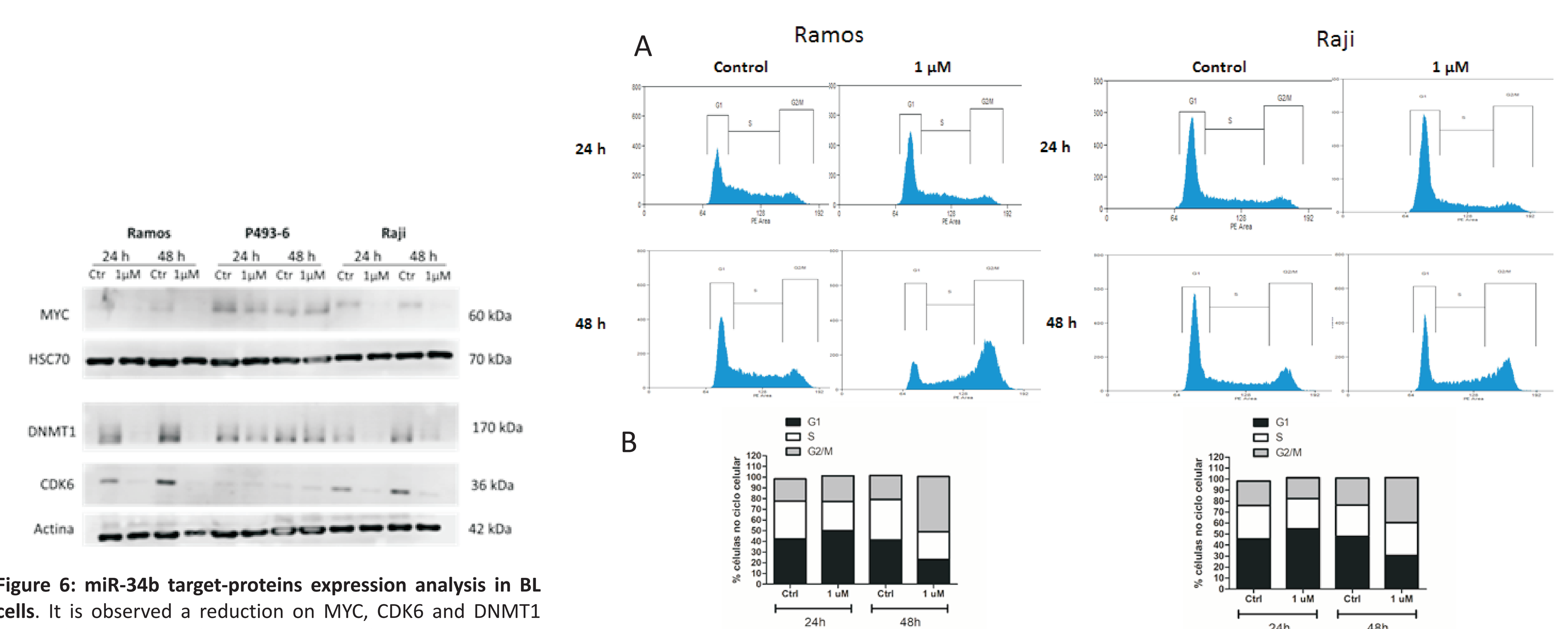

Figure 6: miR-34b target-proteins expression analysis in BL cells. It is observed a reduction on MYC, CDK6 and DNMT1 protein levels in treated samples compared to control samples in Raji and Ramos cell lines. There was no change in protein levels on MYC, CDK6 and DNMT1 in P493-6 cells. HSC70 and β-Actin were used as the endogenous control.

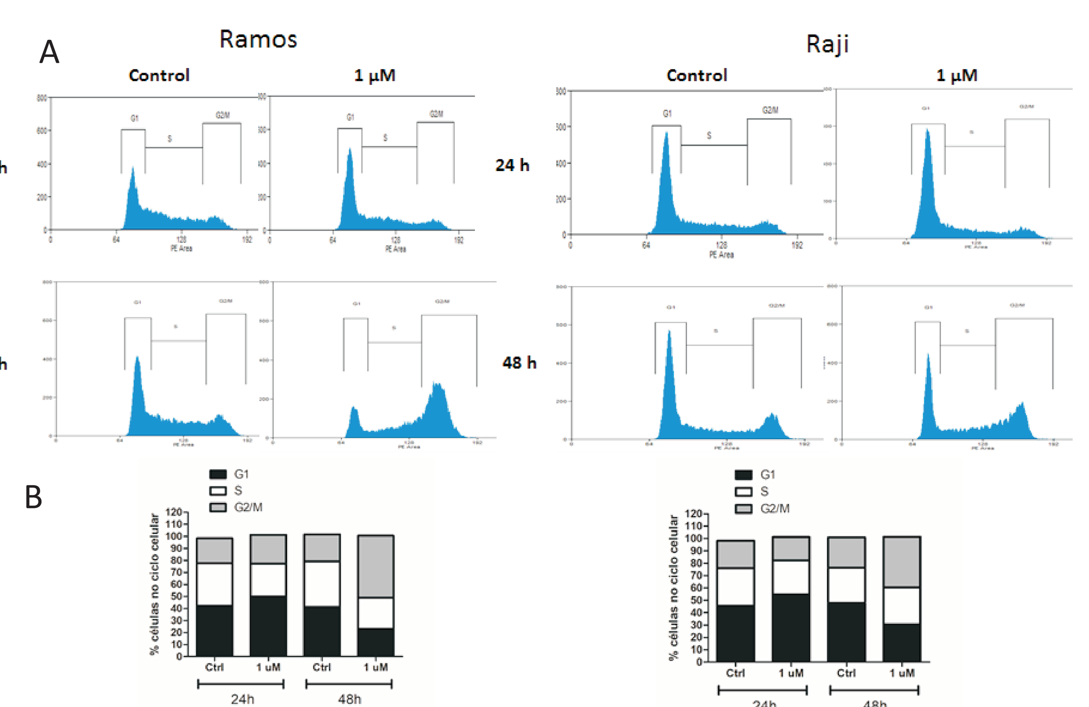

Figure 7: Cell cycle analysis on Ramos and Raji cell lines after decitabine treatment. The incubation times with decitabine were 24 and 48 hours. The analyzes was established on 10,000 events. (A) Representative cell cycle analysis from one experiment. In B the percentage of the mean of 3 independent experiments.

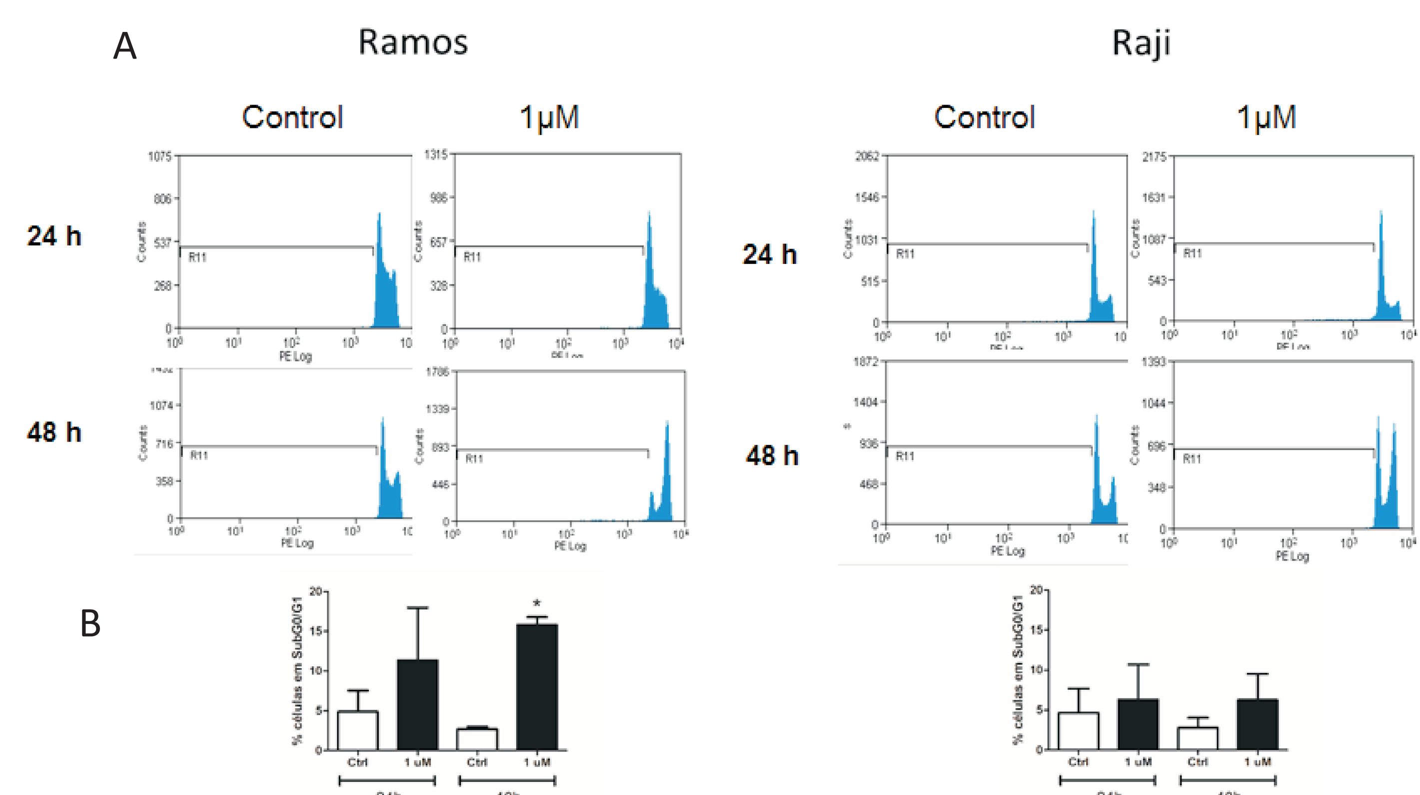

Figure 8: Cell fragmentation analysis on Ramos and Raji cells treated with 1 M Decitabine for 24 and 48 hours. It's observed an increase of cell fragmentation in the treated samples compared to the control samples in both cell lines. (A) Representative cell fragmentation analysis from one experiment. In (B) the percentage of the mean of 3 independent experiments.

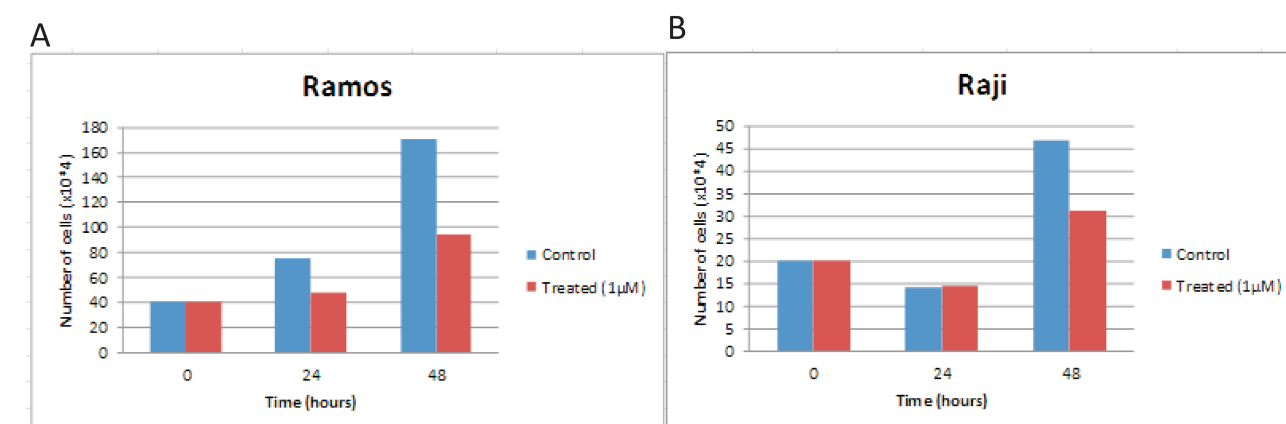

Figure 9: Number of viable cells after decitabine treatment. Number of viable cells was determined by trypan blue exclusion assay. The y axis represents the number of cells and the x axis represents the incubation time of Ramos (A) and Raji (B) cell lines with decitabine.

PARTIAL CONCLUSION

We can conclude that the miR-34b promotor gene is regulated by methylation and it is a reversible mechanism using a demethylation agent in BL cell lines. It was able to restore the miR-34b expression and downregulation of their targets. Our results may be helpful to a better understanding of the miR-34 role in the BL pathogenesis and have potential therapeutic implication.

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