

Comparison of *in-vitro* and *in-vivo* response to fetal hemoglobin production and γ -mRNA expression by hydroxyurea in Hemoglobinopathies

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BACKGROUND: Hydroxyurea, which induces Fetal hemoglobin (HbF) synthesis, is the only drug widely used in different hemoglobinopathies; however, the response is very variable. We compared the efficacy of hydroxyurea *in-vitro* in erythroid cultures and *in-vivo* in the same patients with different hemoglobinopathies to induce HbF production and enhance γ -messenger RNA expression.

MATERIALS AND METHODS: A total of 24-patients with different Hemoglobinopathies were given hydroxyurea and their response was studied *in-vivo* and *in-vitro* on mononuclear cells collected from them simultaneously.

RESULTS: A total of 57.7% of patients (responders) showed no further crisis or transfusion requirements after hydroxyurea therapy with a mean increase in fetal cells (F-cells) of $63.8 \pm 59.1\%$ and γ -mRNA expression of $205.5 \pm 120.8\%$. *In-vitro* results also showed a mean increase in F-cells of $27.2 \pm 24.7\%$ and γ -mRNA expression of $119.6\% \pm 65.4\%$ among the treated cells. Nearly 19.0% of the partial-responders reduced their transfusion requirements by 50% with a mean increase in F-cells of $61.2 \pm 25.0\%$ and $28.4 \pm 25.3\%$ and γ -mRNA-expression of $21.0\% \pm 1.4\%$ and $80.0\% \pm 14.1\%$ *in-vivo* and *in-vitro* respectively. The non-responders (15.3%) showed no change in their clinical status and there was no significant increase in F-cells levels and γ -mRNA expression *in-vivo* or *in-vitro*.

CONCLUSION: Thus, this method may help to predict the *in-vivo* response to hydroxyurea therapy; however, a much larger study is required.

Key words: 2 phase liquid erythroid cell culture, hydroxyurea, sickle cell disease, β -thalassemia syndromes, γ -mRNA expression

Introduction

Inherited disorders of hemoglobin, such as the structural Hemoglobinopathies, and the thalassemia syndromes are common, affecting around 7% of the global population with significant clinical conditions.^[1] In India, the prevalence of sickle cell carriers ranges from 2% to 34% in different population groups^[2,3] whereas that of β -thalassemia ranges from 1% to 17%.^[4,5] On the other hand, Hemoglobin E (HbE) is generally found in the eastern and north eastern region of the country with a frequency of 3-10% and 4-51% respectively.^[6]

Although, sickle cell disease has a milder clinical presentation than in Africans among tribal groups in India, many patients particularly from the non-tribal groups suffer from severe forms of the disease leading to repeated vaso-occlusive crisis, severe anemia, and end organ damage.^[7,8] So far, mainly supportive measures have been used for decreasing the severity of symptoms. Few children with the severe forms of β -thalassemia receive regular blood transfusions and adequate iron chelation, the mainstay of treatment, due to the high-cost involved for optimum management.^[5] Thus, there was a need for an efficacious drug with low

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toxicity, which could reduce the clinical symptoms of these Hemoglobinopathies in a cost-effective way.

Hydroxyurea reduces the frequency of vaso-occlusive crisis and blood transfusion requirements in sickle cell anemia patients^[8,9] as well as the transfusion requirements in some β -thalassemia patients.^[10,11] As there are no clear clinical or genetic factors, which can predict the response of these patients to hydroxyurea therapy, patients who are non-responders also get exposed to the drug. Thus, this study thus investigated whether the response to hydroxyurea in erythroid cultures of patients with different Hemoglobinopathies to increase fetal hemoglobin (HbF) and γ -gene expression *in-vitro* correlates simultaneously with the *in-vivo* response to hydroxyurea among the same patients.

Materials and Methods

Out of 24 patients (8 males and 16 females; age 5-30 years) with different Hemoglobinopathies (severe sickle cell anemia patients – 6; severe sickle- β -thalassemia patients – 2, these patients had more than 5 episodes of painful vaso-occlusive crisis and occasional blood transfusion requirements; β -thalassemia intermedia patients – 5, who presented after 2 years of age and required occasional blood transfusions initially however, later they became transfusion dependent; β -thalassemia major patients – 5, who were on regular blood transfusion; severe HbE- β -thalassemia patients – 6, who had 6-12 transfusion requirements annually) were included. Informed consent was taken from the patients and parents of pediatric patients and the Ethical Committee of the National Institute of Immunohematology approved these studies.

In-vivo studies

Hydroxyurea (Cytodrox, Cipla Ltd, Mumbai) was started at a dose of 10-15 mg/kg/day^[9,10] after initial blood collection for *in-vitro* studies, hematological investigations like complete blood count, hemoglobin analysis on the Variant Hemoglobin Testing System (BioRad Laboratories, Inc., Hercules, CA, USA) and quantitative estimation of fetal cells (F-cells) by flow cytometry using a monoclonal HbF antibody (BD (Becton Dickinson

and Company) Immunocytometry Systems, San Jose, CA, USA).^[12] Molecular analysis such as confirmation of the HbS (sickle hemoglobin) and HbE mutations and characterization of β -thalassemia mutations were carried out as described earlier.^[13] Xmn I polymorphism analysis was carried out by PCR (polymerase chain reaction) and restriction enzyme digestion.^[14] Absolute quantification of gamma (γ)-globin mRNA transcripts was carried out by real time PCR using Taqman probes and the 7900 High Temperature Fast Real-Time PCR System (Applied Biosystems, NJ, USA) as described earlier.^[15] The expression of the housekeeping gene β -actin was used as control in each sample. Patients were assessed clinically and laboratory investigations carried out monthly for 24 months to evaluate their status after starting hydroxyurea therapy. The numbers of remaining capsules of hydroxyurea were counted during each follow-up to evaluate therapeutic compliance and the hematological status was monitored to rule-out cytopenia. At the end of 24 months the patients' response to hydroxyurea therapy was evaluated by the reduction in clinical severity of the disease in case of sickle cell disease patients and reduction or cessation of transfusion requirements in β -thalassemia and HbE- β -thalassemia patients. After starting hydroxyurea therapy, the β -thalassemia patients were transfused only if their hemoglobin dropped below 7.5 g/dl. Partial responders of hydroxyurea therapy among the β -thalassemia were those patients whose transfusion requirement reduced by 50% whereas non-responders were those patients who did not show any reduction in transfusion requirements after 1 year of hydroxyurea therapy.

In-vitro studies

In-vitro studies were performed using the two phase liquid culture technique.^[16,17] Mononuclear cells, collected from peripheral blood of all patients before starting hydroxyurea were cultured in phase-I medium containing serum free StemSpan medium (StemCell Technologies Inc.), 50 ng/ml of stem cell factor (SCF), 25 ng/ml of interleukin-3 (IL3), 0.01% bovine serum albumin (BSA) and Cyclosporin A (1 μ g/ml) (Sigma-Aldrich Co. USA) for 7 days at 37°C and 5% CO₂. Non-adherent cells after day-7 were further cultured in phase-II medium consisting of StemSpan medium, 2 U/ml of human recombinant erythropoietin,

50 ng/ml SCF and 10^{-7} M Dexamethazone (Sigma-Aldrich Co. USA). The culture was then bifurcated with a cell concentration of less than 1×10^6 /ml and hydroxyurea ($1.5 \text{ mM}/1 \times 10^6$ cells) was added to one of the cultures between days 6 and 8 of phase-II. After 10-12 days the cells were collected for F-cells estimation and γ -mRNA expression as described above.

Flow cytometry

For F-cells estimation, the cultured cells were washed twice and blocked with 2% BSA and stained with cell surface markers like anti-human-CD45 (leukocyte common antigen) tagged with perCP (Peridinin Chlorophyll Protein Complex) ($10 \mu\text{l}$) and anti-human-CD71 (transferrin receptor) tagged with Phycoerythrin ($10 \mu\text{l}$) and incubated in the dark for 30 min. After washing, the fluorescently labeled cells were fixed, permeabilized and stained with anti-HbF-FITC (Fluorescein isothiocyanate) as mentioned earlier.^[12] Cultured cells, which were CD45 negative were gated (10,000 cells) and used for flow cytometric analysis to quantitate the number of F-cells.

Statistical analysis

Student *t*-test was used to compare the findings of *in-vitro* studies with and without hydroxyurea and of patients before and after hydroxyurea therapy. A $P < 0.01$ was considered statistically significant.

Results

Clinical and hematological response to hydroxyurea therapy in-vivo

After hydroxyurea therapy, 8 patients, which include 6 sickle cell anemia patients and 2 sickle- β -thalassemia patients had no further episodes of vaso-occlusive crisis, need for blood transfusions, infections, stroke or acute chest syndrome and they had a feeling of general well-being after therapy. The mean hematological parameters of these 8 patients also showed a significant increase in Hb, MCV (Mean corpuscular volume), HbF, and F-cells ($P < 0.001$) [Table 1] after hydroxyurea therapy. The γ -mRNA expression carried out in 2 sickle- β -thalassemia patients also showed a 390% and 255% increase after hydroxyurea therapy [Table 2].

5 β -thalassemia intermedia patients who presented

after 2 years of age and required blood transfusions every 20-25 days thereafter responded by not having any further transfusion requirements after 3-6 months of hydroxyurea therapy and maintained a Hb level of 8-10 g/dl. They showed a significant increase in mean Hb, MCV, HbF, and F-cells ($P < 0.001$) after hydroxyurea therapy [Table 1].

3 of the 5 β -thalassemia major patients, showed a reduction in their transfusion requirements from 12 to 15 transfusions per year to 7-8 transfusions per year. Their Hb levels remained between 7 and 8 g/dl for 45-60 days without transfusion. An increase was seen in HbF and F-cells after hydroxyurea therapy. However, 2 non-responders failed to show any change in their transfusion requirements or hematological parameters after 1 year of therapy. The mean increase in HbF and F-cells was not sufficient to maintain a stable Hb level without transfusion. A total of 2 patients (1 partial-responder and 1 non-responder) had two episodes of neutropenia because of which the hydroxyurea was discontinued until the counts recovered. No other side-effect was observed.

2 of the 6 HbE- β -thalassemia patients requiring 12-15 transfusions per year, responded to hydroxyurea therapy with discontinuation of transfusion requirements and a significant increase in mean Hb and MCV ($P < 0.01$) and HbF and F-cells ($P < 0.001$). Two patients were partial-responders with increase only in HbF and F-cells and two patients were non-responders [Table 1]. Table 3 shows the mean percent increase in F-cells, CD71 + F-cells and γ -mRNA expression seen among the responders, partial-responders, and non-responders of hydroxyurea therapy both *in-vitro* and *in-vivo*. The difference in the percent increase in F-cells seen in responders and partial-responders was not statistically significant ($P < 0.1$). However, the difference in the increase in γ -mRNA expression seen in responders and partial-responders was statistically significant ($P < 0.01$).

Response to hydroxyurea therapy in-vitro

The erythroid cells of the patients were cultured for an average of 18.5 ± 4 days. Erythroblasts increased dramatically from day 1 to 4 with a decrease in cell volume from days 5 to 7. Orthochromatic normoblasts

Table 1: Hematological parameters of patients with sickle cell anemia, sickle- β -thalassemia, β -thalassemia intermedia, β -thalassemia major and HbE- β -thalassemia before and after hydroxyurea therapy

Patient	Type	Hb (g/dl)		MCV (fl)		HbF (%)		F-cells (%)		Clinical response
		Baseline	24 months	Baseline	24 months	Baseline	24 months	Baseline	24 months	
1	SS	9.5	10.9	64.3	74.2	17.5	21.6	57.0	65.0	Responder
2	SS	8.6	10.1	95.4	96.7	10.5	15.9	42.0	50.0	Responder
3	SS	9.6	10.8	94.0	100.3	24.9	28.2	64.0	86.0	Responder
4	SS	11.4	13.6	59.4	74.0	12.4	21.0	44.0	68.0	Responder
5	SS	9.5	10.9	81.7	89.8	20.9	27.8	78.0	87.0	Responder
6	SS	8.9	11.4	85.8	87.9	24.1	30.7	30.0	72.0	Responder
7	S β -thal	8.5	12.5	66.4	94.0	6.0	22.5	24.0	65.0	Responder
8	S β -thal	7.0	11.1	67.8	73.9	25.1	29.7	61.0	75.0	Responder
9	β -TI	8.1	9.9	70.8	73.6	40.1	86.3	45.0	99.0	Responder
10	β -TI	5.8	8.5	80.2	95.3	80.2	95.3	93.0	99.0	Responder
11	β -TI	8.4	10.3	72.4	77.9	30.5	99.5	36.0	98.0	Responder
12	β -TI	7.8	9.4	81.2	86.3	62.0	91.0	78.0	99.0	Responder
13	β -TI	7.3	8.6	82.3	98.9	42.1	96.3	66.0	95.0	Responder
14	β -TM	7.4	8.5	78.4	86.3	57.2	98.5	61.0	95.0	Partial-responder
15	β -TM	7.0	7.5	87.2	88.6	7.3	29.3	28.0	46.0	Partial-responder
16	β -TM	7.9	7.9	79.0	81.6	15.2	15.9	30.0	34.0	Non-responder
17	β -TM	8.3	7.5	85.0	92.3	3.4	21.8	8.0	39.0	Partial-responder
18	β -TM	7.1	6.4	79.5	81.0	15.6	23.5	21.0	23.5	Non-responder
19	E β -thal	7.6	7.9	81.2	94.8	25.1	54.0	52.0	72.0	Responder
20	E β -thal	8.0	9.1	60.2	71.8	20.9	37.9	37.0	68.0	Responder
21	E β -thal	7.1	6.5	75.8	81.6	33.6	29.1	72.0	60.0	Non-responder
22	E β -thal	7.0	7.2	68.6	75.2	7.7	19.2	45.0	54.0	Partial-responder
23	E β -thal	8.1	7.8	81.8	79.0	2.4	6.8	16.0	20.0	Non-responder
24	E β -thal	7.7	7.8	90.6	93.1	13.6	29.0	45.0	78.0	Partial-responder

SS: Sickle homozygous, S β -thal: Sickle- β thalassemia, β -TI: β thalassemia intermedia, β -TM: β thalassemia major, E β -thal: HbE- β -thalassemia, Hb: Hemoglobin, MCV: Mean corpuscular volume, F-cells: Fetal cells; HbE: Hemoglobin E

representing the late erythroblastic cells stage rarely lead to enucleation under the culture conditions. Hydroxyurea added on day 7 ± 1 in one of the duplicate sets of culture to evaluate the F-cells response was titrated and a dose of $1.5 \text{ mM}/1 \times 10^6$ cells was found to be optimum for the cells. A concentration above 1.5 mM reduced the cell number and the expression of CD71. Figure 1 shows the flow cytometric data of cultured cells showing co-positivity for F-cells and CD71 before and after addition of hydroxyurea.

The percent increase in F-cells, F-cells + CD71 and γ -mRNA expression of individual patient's cultured erythroid cells after treating with hydroxyurea is shown in Table 2. The difference in F-cells + CD71 and γ -mRNA expression seen among the responders and partial-responders was not statistically significant [Table 3]. 4 non-responders *in-vivo*, did not show any increase in F-cells in their cultured cells treated with hydroxyurea. Only 1 of the 4 non-responders (β -thalassemia major patient) showed an increase in F-cells + CD71 by 1% after hydroxyurea treatment, whereas the other 3 patients did not show any increase. No increase was found in the γ -mRNA expression carried out in 2 of the 4 non-responders' cultured erythroid cells after hydroxyurea treatment.

Discussion

Many pharmacological agents have been studied to enhance the production of HbF in various *in-vivo* and *in-vitro* systems. Use of these drugs on non-human primates are a pre-requisite for clinical trials in patients, however, they are too expensive and time consuming for large scale screening of these compounds. Hence, attempts have been made to mimic the *in-vivo* condition *in-vitro* by various experimental systems using either immortalized cell lines or isolation and culture of erythroid progenitor cells from the bone marrow or peripheral blood. Fibach *et al.* in 1989 established primary erythroid cultures by isolating erythroid progenitor cells from bone marrow, cord blood or peripheral blood, which when grown *in-vitro* resembles more closely the *in-vivo* situation.^[18] Fibach in 1998 studied many pharmacological inducers of HbF like hydroxyurea and 5-azacytidine using peripheral blood of sickle cell anemia patients with this technique.^[16] Treatment with hydroxyurea resulted in reduction in cell number, increase in MCV, MCH and HbF. It was further suggested that this technique could be used to predict the responders of hydroxyurea.

Table 2: Comparison of the percent increase in F-cells, CD71+F-cells and γ -mRNA expression of cultured erythroid cells (*in-vitro*) with the *in-vivo* percent increase in F-cells and mRNA expression in few patients before and after hydroxyurea therapy

Patient	Type	Mutation	Xmn I Polymorphism	Alpha Thalassemia	<i>In-vitro</i>			<i>In-vivo</i>		Clinical response
					% increase in F-cells	% increase in CD71+F-cells	% increase in γ -mRNA	% increase in F-cells	% increase in γ -mRNA	
1	SS	HbS homozygous	+/+	$\alpha^{3.7}/\alpha^{3.7}$	28	6.5	–	14	–	Responder
2	SS	HbS homozygous	+/+	$\alpha\alpha/\alpha\alpha$	23	12.1	66.6	19	–	Responder
3	SS	HbS homozygous	+/+	$\alpha\alpha/\alpha\alpha$	8	5	193.5	34	–	Responder
4	SS	HbS homozygous	+/+	$\alpha^{3.7}/\alpha^{3.7}$	20	36	75	55	–	Responder
5	SS	HbS homozygous	+/+	$\alpha^{3.7}/\alpha\alpha$	12	5.5	78	12	–	Responder
6	SS	HbS homozygous	+/+	$\alpha^{3.7}/\alpha\alpha$	43	23.3	84	140	–	Responder
7	S β -thal	HbS+IVS-1-5 (G→C)	+/-	$\alpha\alpha/\alpha\alpha$	6	15	–	170	390	Responder
8	S β -thal	HbS+XD 15 (G→A)	+/-	$\alpha^{3.7}/\alpha\alpha$	15	17	160	23	255	Responder
9	β -TI	IVS-5 (G→C)	+/+	$\alpha^{3.7}/\alpha\alpha$	5	12	–	120	204	Responder
10	β -TI	IVS 1-5 (G→C) Homo	+/+	$\alpha\alpha/\alpha\alpha$	10	5	–	6	–	Responder
11	β -TI	IVS2-837 (T→G) +Asian Indian Inversion	+/+	$\alpha\alpha/\alpha\alpha$	6	27	–	175	218	Responder
12	β -TI	IVS 1-1 (G→T) Homo	+/+	$\alpha\alpha/\alpha\alpha$	21	78	–	27	–	Responder
13	β -TI	IVS1-5 (G→T) +619 del	-/-	$\alpha\alpha/\alpha\alpha$	3	40	–	41	200	Responder
14	β -TM	IVS 1-1 (G→T) Homozygous	+/+	$\alpha\alpha/\alpha\alpha$	15	28	–	62	–	Partial-responder
15	β -TM	IVS 1-1 (G→T) +FS 41/42	+/-	$\alpha\alpha/\alpha\alpha$	3.4	25	–	64	–	Partial-responder
16	β -TM	IVS1-5 (G→T) Homozygous	+/-	$\alpha\alpha/\alpha\alpha$	0	0	0	13	3.5	Non-responder
17	β -TM	IVS1-5 (G→T) +FS41/42 (-CTTT)	-/-	$\alpha\alpha/\alpha\alpha$	2.5	9	–	87	20	Partial-responder
18	β -TM	IVS1-5 (G→T) Homozygous	-/-	$\alpha\alpha/\alpha\alpha$	0	1	–	12	2.3.0	Non-responder
19	E β -thal	HbE+FS 41/42 (-CTTT)	+/-	$\alpha\alpha/\alpha\alpha$	7	47	200	38	100	Responder
20	E β -thal	HbE+CD 15 (G→A)	+/+	$\alpha\alpha/\alpha\alpha$	8	79	100	84	72	Responder
21	E β -thal	HbE+IVS 1-5 (G→C)	+/-	$\alpha\alpha/\alpha\alpha$	0	0	–	0	–	Non-responder
22	E β -thal	HbE+IVS 1-5 (G→C)	+/-	$\alpha\alpha/\alpha\alpha$	3.7	7	70	20	–	Partial-responder
23	E β -thal	HbE+IVS 1-5 (G→C)	+/-	$\alpha^{3.7}/\alpha\alpha$	0	0	0	23	–	Non-responder
24	E β -thal	HbE+FS 41/42 (-CTTT)	+/-	$\alpha\alpha/\alpha\alpha$	3	28	90	73	22	Partial-responder

F-cells: Fetal cells; SS: Sickle homozygous; S β -thal: Sickle- β -thalassemia; β -TI: β -thalassemia intermedia; β -TM: β -thalassemia major; E β -thal: HbE- β thalassemia; CD: Codon; HbE: Hemoglobin E

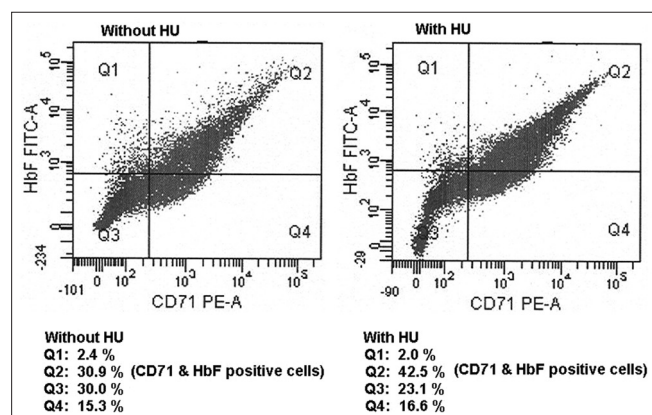
In 2002, Wojda *et al.* found that HbA and HbF content were regulated with the rate of proliferation during adult erythropoiesis and that there was no evidence for a HbF dominant population or switching during differentiation in adult cells.^[19] Wojda *et al.* in 2003 showed significant SCF mediated increase in HbF using progenitor cells

derived from donor cells.^[20] Mithramycin and rapamycin were shown to induce HbF production by up regulating γ -globin mRNA production in normal and thalassemia human erythroid precursor cells using the two phase liquid culture technique.^[21,22] Human erythroid progenitor cells isolated from 18 HbE- β -thalassemia patients

Table 3: Percent increase in F-cells, CD71+F-cells and γ -mRNA expression *in-vitro* and *in-vivo* among the responders, partial-responders and non-responders of hydroxyurea therapy

Response	<i>In-vitro</i>			<i>In-vivo</i>	
	% increase in F-cells mean \pm SD	% increase in CD71+F-cells mean \pm SD	% increase in γ mRNA expression mean \pm SD	% increase in F-cells mean \pm SD	% increase in γ mRNA expression mean \pm SD
Responders	14.3 \pm 11.0	27.2 \pm 24.7	119.6 \pm 65.4	63.8 \pm 59.1	205.5 \pm 120.8
Partial-responders	5.5 \pm 5.3	28.4 \pm 25.3	80.0 \pm 14.1	61.2 \pm 25.0	21 \pm 1.4
Non-responders	No increase	1.0	No increase	16.0 \pm 6.1	2.9 \pm 0.8
Responders versus partial-responders	$P<0.01$	$P<0.1$	$P<0.1$	$P<0.1$	$P<0.01$

F-cells: Fetal cells

**Figure 1: Flow cytometric estimation of CD71 and fetal cells on cultured erythroid cells before and after addition of hydroxyurea**

were treated with 30 μ mol/l hydroxyurea *in-vitro* and a 30% increase in HbF was found in 4 patients, 20-30% increase in 9 patients and less than 20% in 5 patients.^[23] Jiang *et al.* in 2006 studied the expression of 5 protein coding genes, ALDH (aldehyde dehydrogenase) 8A1, HBS1 L, cMYB (myeloblastosis), AHI1 (Abelson helper integration site 1) and PDE7B (Phosphodiesterase 7B) on chromosome 6q23 by using erythroid cell culture technique and revealed a clear correlation of elevated HbF with low levels of cMYB expression and its involvement in regulation of HbF.^[17] Rigano *et al.* in 2010 studied 17 β -thalassemia intermedia patients *in-vivo* on hydroxyurea and confirmed that the erythroid cultures obtained from patients during treatment reproduced the observed *in-vivo* response.^[24]

In this study, the response to hydroxyurea is compared *in-vivo* as well as *in-vitro*. Our earlier study on the use of hydroxyurea among sickle cell disease patients in India has shown the drug to be very effective in reducing clinical crisis,^[11] however, only 60% of the β -thalassemia intermedia and 50% of HbE- β -thalassemia patients fully benefited from the drug.^[12,25] The *in-vitro* study on the effect of hydroxyurea on the cultured erythrocytes of

patients with different Hemoglobinopathies gives an idea about the parameters that may predict the response to hydroxyurea therapy among these patients.

Hydroxyurea was found to be beneficial both among adults and children with severe form of sickle cell disease^[8,26-28] and was further used in the treatment of patients with β -thalassemia and HbE- β -thalassemia where a good response was seen among a few β -thalassemia intermedia and HbE- β -thalassemia patients in whom many genetic and epigenetic factors are believed to play a role in predicting the response to hydroxyurea therapy.^[10,11,25,29-32] Watanapokasin *et al.* 2005 showed that 13 β -thalassemia/HbE- β -thalassemia patients treated with hydroxyurea *in-vivo* had HbF synthesis and γ -globin-mRNA that correlates with the *in-vitro* results suggesting that *in-vitro* testing may predict the *in-vivo* response to hydroxyurea.^[23] Bianchi *et al.* 2007 used 3 inducers of HbF from the biological materials, which included angelicin linear psoralens, resveratrol and rapamycin. A good correlation was found between *in-vitro* and *in-vivo* response to HbF production.^[33]

In our study, a significantly higher expression of γ -mRNA ($P < 0.01$) was seen among the cultures of responders treated with hydroxyurea as compared to γ -mRNA expression seen among partial-responders. The non-responders did not show any increase in F-cells or γ -mRNA expression *in-vitro*, however, they showed a 16.0 \pm 6.1% increase in F-cells but only a 2.9 \pm 0.8% increase in γ -mRNA expression *in-vivo* after therapy. Thus γ -mRNA expression was a better indicator of the response to hydroxyurea therapy both *in-vivo* and *in-vitro* as compared to the F-cells level as the F-cells showed an increase in most of the patients and their cultured erythrocytes treated with hydroxyurea, however, the percentage increase in F-cells levels differed among the responders and the non-responders.

This method of erythroid cell culture may be useful to compare the *in-vivo* response of hydroxyurea with *in-vitro* using γ -mRNA expression and the number of F-cells. However, a much larger study is required to confirm these results. This culture system could be further used to identify other pharmacological inducers of HbF production, which could be useful for non-responders of hydroxyurea and to study expression of other genes that may be involved in HbF production.

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