Abstract: In vitro and in vivo studies have provided evidence for neuroprotective properties of Erythropoietin in neurodegenerative disorders. Although the magnitude of effect is still controversial, very recent findings point to neuronal protection in the central nervous system by Erythropoietins. Erythropoietin is a powerful growth factor which enhances cellular size and ultimately increases the number of mitochondria. Friedreich Ataxia (FA), an inherited neurodegenerative disorder is caused by a loss of function mutation in the first intron on chromosome 9. FA patients therefore suffer a marked reduction of Frataxin, a mitochondrial protein which is involved in mitochondrial iron homeostasis and/or assembly of iron-sulfur (FeS) proteins and heme synthesis. Mitochondrial dysfunction results in a deleterious energy deficit especially in tissues highly dependent on oxidative phosphorylation such as neurons, muscle cells or pancreatic insular cells. Beneficial effects of recombinant human Erythropoietin (rhuEPO) may derive from an increase in Frataxin levels through currently unknown post-transcriptional and/or post-translational mechanisms. Moreover, additional effects via BDNF and through mitochondrial iron chelation may complete the spectrum of rhuEPOs actions in FA and may be part of its beneficial treatment effects. However, there are clear limitations to chronic rhuEPO treatment. Apart from hematopoietic side effects, tumor growth may be enhanced by rhuEPO application. In this review we provide an overview of studies using rhuEPO in FA and discuss potential beneficial effects of Erythropoietin in FA.

Keywords: Friedreich ataxia, erythropoietin, neuroprotection, mitochondria, iron metabolism.

INTRODUCTION

Friedreich Ataxia (FA) is the most common inherited ataxia. FA is characterized by multiple symptoms including progressive spinocerebellar ataxia, diabetes mellitus and hypertrophic cardiomyopathy [1]. It is caused by a GAA-trinucleotide expansion in the Frataxin gene located on chromosome locus 9q13, which results in a reduced expression of Frataxin, a small mitochondrial protein [2]. Frataxin’s exact physiological function of is unknown, but it may be involved in mitochondrial iron homeostasis and/or assembly of iron-sulfur (FeS) proteins and heme synthesis [3]. Intramitochondrial iron accumulation has been postulated to initiate the production of hydroxyl radicals by the Fenton reaction, leading to inactivation of FeS enzymes, lipid peroxidation and damage to nucleic acids, proteins and ultimately resulting in cell death. Intramitochondrial iron accumulation is found in heart, liver, nervous system and spleen of FA patients [4]. Moreover, there is a reduction of mitochondrial DNA, the FeS cluster-containing subunits of the mitochondrial electron transport chain (complex I-III) and of the enzyme aconitase.

Although there is not an effective treatment for FA yet, the improved understanding of the role of Frataxin has led to the consideration of antioxidants and iron chelators as potential therapeutic agents. These drugs may have a potential to reduce some clinical features of FA, although they cannot cure the disease itself. Substances like hemin, butyric acid, 3-nitropropionic acid or cisplatin that result in an increase in Frataxin levels have been considered a further potential approach to FA treatment [5-7].

ERYTHROPOIETIN

Erythropoietin has received considerable attention because it was found to exert neuroprotective effects by a still poorly understood mechanism [8]. It has been known for a long time that Erythropoietin signaling plays a key role in bone marrow erythrocyte proliferation and hemoglobin synthesis. In response to low oxygen or ischemic events, Hypoxia Inducible Factor (HIF) regulates the expression of a number of critical hypoxia-inducible genes including that for Erythropoietin [9]. The actions of Erythropoietin in neuroprotection, independent of changes in erythrocyte synthesis, are supposed to be due to several different mechanisms [10]. First, Erythropoietin can act within the in vivo context to reverse vasospasm [11, 12], protect vascular endothelial cells [13], modulate inflammation [14] and recruit stem cells [15]. Second, Erythropoietin can act directly on neurons. It is supposed to attenuate the
production of damaging molecules such as ROS or glutamate-stimulated excitotoxicity [16, 17]. Receptor-initiated cell signaling by Erythropoietin occurs via multiple molecular cascades, which differ in importance depending on the specific tissue or cell type, as well as the type of injury [18]. Most tissue-protective responses are initiated by the phosphorylation of JAK-2 [18]. Additionally, Erythropoietin exerts strong neurotrophic and antiapoptotic effects via the currently not well defined ß-common-receptor (ßcR) in the nervous system [19]. How specific pathways can be selectively activated by Erythropoietin in the central nervous system has to be enlightened.

ERYTHROPOIETIN IN FA

Recently, a number of in vitro, in vivo as well as human studies have investigated the role of recombinant human Erythropoietin (rhuEPO) as a potential symptomatic disease modifying substance in FA. These studies have been triggered by the observation that rhuEPO increases Frataxin levels in cell cultures of subjects that suffer from renal disease (unpublished observation).

ERYTHROPOIETIN IN VITRO STUDIES

In in vitro studies primary lymphocytes from FA patients, primary human cardiac cells and P19 cells (neuronal type) were incubated using different concentrations of rhuEPO. Measurements applying Western Blot technique revealed that rhuEPO increases Frataxin levels in a dose dependent manner in primary lymphocytes from FA patients [20]. These results have been replicated by Acquaviva and co-workers recently [21]. In line with our findings (non-published data) they did not detect an increase in mRNA expression using real time PCR. Therefore the increase in Frataxin protein in response to rhuEPO is probably attributable to posttranscriptional and/or posttranslational mechanisms.

ERYTHROPOIETIN IN VIVO STUDIES

As rhuEPO is an approved drug that has been safely administered at hematopoietic doses for more than twenty years, and animal models in FA have their shortcomings, we decided to bypass preclinical models and performed an investigator-initiated “proof-of-concept” study, which was approved by the local Ethics Committee.

STUDIES OF ERYTHROPOIETIN IN FA PATIENTS

In a first open label single site “proof of concept” study in twelve genetically proven FA patients the primary outcome measure was a persistent up-regulation of Frataxin levels over 8 weeks. Two patients did not reach the study end-point. The remaining 10 FA patients received 5000 IU of rhuEPO (Neorecormon, Roche, Vienna, Austria) 3 times a week subcutaneously over an 8 week time period. Frataxin levels were measured from whole blood samples by ELISA. Other blood parameters such as iron, ferritin, transferrin, hemoglobin and hematocrit levels were monitored using routine laboratory methods. Neurological state was assessed using the Scale for the Assessment and Rating of Ataxia (SARA).

A stable increase of Frataxin levels was shown in 8 FA patients during the 8 weeks rhuEPO treatment. The Frataxin increase compared to their individual baseline ranged from 15 to 63% (mean 27%). Two patients (1 female, 1 male) showed no increase in their Frataxin levels during the 8 weeks rhuEPO treatment. Increase in Frataxin is shown in Fig. (1).

![Fig. (1). Increase in Frataxin levels after 8 weeks of rhuEPO treatment.](image)

Treatment with rhuEPO showed a persistent and significant increase in Frataxin levels in 7 out of 10 FA patients after 2 and 8 weeks (P<0.01). One patient was not evaluable for technical reasons. In two FA patients Frataxin did not increase.

"Proof of concept" phase

Extension phase

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Fig. (2). Study design rhuEPO in FA.
A major neurological improvement was not suspected after 8 weeks treatment. Still, the severity of ataxia measured by the scale for the assessment and rating of ataxia (SARA) [22] at baseline and after 8 weeks showed a 6% improvement in the whole study population. After exclusion of those patients who did not show a stable increase in Frataxin levels during the rhuEPO treatment, a 11.5% improvement in SARA was obtained.

Markers for oxidative stress such as 8-Hydroxydeoxyguanosine (8-OHdG) and Peroxides were measured at baseline and week 8. 8-Hydroxydeoxyguanosine (8-OHdG) is a measure for oxidative damage to chromosomal and mitochondrial DNA inside the cell. An ELISA kit (8-OHdG Check, Japan Institute for the Control of Aging, Fukuroi, Japan) was used to check the level of 8-OHdG in urine. After 8 weeks rhuEPO treatment a highly significant reduction of urine 8-OHdG has been detected. This reduction was also found in FA patients without an increase in Frataxin levels. Peroxides: The "Peroxide-activity" assay (POX ACT; Tatzber KEG, Klosterneuburg, Austria) was used to measure Peroxide concentrations. Serum Peroxide concentrations were measured at baseline and after 8 weeks of rhuEPO treatment. Again serum Peroxide levels at week 8 compared to baseline revealed reduction in rhuEPO treated FA patients.

In addition to an increase of Frataxin levels indicators for oxidative stress such as 8-OHdG and Peroxides were significantly reduced in rhuEPO treated FA patients after eight weeks. If the reduction in oxidative stress parameters reflect a direct or an indirect effect due to rhuEPO’s neuroprotective properties remains to be elucidated [23].

HEMATOPOIETIC (SIDE-)EFFECTS OF rhuEPO IN FA

During the eight weeks period the raise in hematocrit (Hct) remained stable in female patients (mean Hct baseline vs week 8: 0.4 ± 0.03 vs 0.4 ± 0.04), whereas 2 out of 7 male patients showed an increase to the upper reference range (mean Hct baseline vs week 8: 0.45 ± 0.01 vs 0.53 ± 0.03). Serum ferritin and transferrin saturation decreased significantly.

In this first single site open label pilot “proof of concept” study there was a stable increase of Frataxin levels during 8 weeks treatment with rhuEPO and a marked apparently independent reduction of indicators for oxidative stress. These findings, despite all limitations of an open label trial, warranted a preplanned investigation of rhuEPO in a six months open label follow-up study (see Fig. 2). In consequence FA patients with stable Frataxin upregulation in the initial “proof of concept” study were invited to participate in an open-label six months extended treatment phase. Since there were no data on long-term rhuEPO application in FA patients, the study protocol allowed dose adjustments after the initial proof of concept phase in case of safety concerns. Eight out of ten FA patients participating in the original two months proof-of-concept study and stable up-regulation of Frataxin entered and finished the 6 months follow-up study. Two patients did not give their informed consent for the follow-up study.

Long-term treatment with rhuEPO over 6 months in eight FA patients revealed a significant clinical improvement using two different ataxia rating scales. Using Friedreichs Ataxia Rating Scale (FARS) [24] an improvement of about 8 points in clinical scores has been detected (see Fig. 3A). A second ataxia rating scale, SARA, also revealed clinical improvement (see also Fig. 3B) [22, 25]. Changes in locomotion and speech made the greatest contribution to the overall change in score in both ataxia rating scales. Self assessment by FA patients applying quality of life instruments such as the SF36 revealed no significant improvement in the Physical Component Score (PCS) whereas the Mental Component Score (MCS) revealed a significant improvement between baseline and study endpoint.

Oxidative stress parameters such as urine 8-OHdG were markedly reduced after two and six months of rhuEPO
treatment. Peroxide measurements were also found significantly reduced. Measurements of oxidative stress parameters (8-OHdG, Peroxides) are shown in Figs. (4, 5).

**Fig. (4). Oxidative stress parameter 8-OHdG pre-treatment and after 6 months.** Urine 8-OHdG levels decreased significantly between pre-treatment and study endpoint (P = 0.012, mean ± SD pre-treatment 22.79 ± 13 ng/mg creatinin versus study endpoint 5.52 ± 1.33ng/mg creatinin).

**Fig. (5). Peroxide levels in FA patients pre-treatment and after 6 months rhuEPO treatment.** Serum Peroxides were significantly reduced after 6 months (P = 0.028, mean ± SD pre-treatment 136.91 ± 57.33µM versus 6 months 40.12 ± 36.45µM).

Four out of eight FA patients had hematocrit increase beyond the pre-defined values and underwent phlebotomies. While Hct values remained within normal ranges in 2/3 female patients, the remaining patient underwent phlebotomy after 2, 3 and 5 months. Within the male study population recurrent phlebotomy was necessary in 3/5 patients. Hematopoietic response (side effects) to rhuEPO treatment over six months is shown in Fig. (6).

**THE POSSIBLE IMPACT OF rhuEPO TREATMENT IN FA**

The actions of Erythropoietin in neuroprotection, independent of changes in erythrocyte synthesis, are not entirely clear. Still, Erythropoietin is supposed to attenuate the production of damaging molecules such as radical oxygen species or glutamate-stimulated excitotoxicity.

**Fig. (6). Hematopoietic response during rhuEPO long-term treatment.** Four out of eight FRDA patients had an increase of their hemoglobin (Hb) levels above the upper limit of normal (> 16 for females, >18.5 for males) and required repeated phlebotomies (1 female, 3 males).

[26, 27]. Established markers of oxidative stress such as urine 8-OHdG, a marker for oxidative mitochondrial and nuclear DNA damage, and serum Peroxide levels remained significantly reduced in FA patients after a six months treatment period [28]. Interestingly, a substantial reduction of indicators for oxidative stress was also found in patients who did not show a rise in Frataxin levels. The observed potency of Erythropoietin to reduce oxidative stress in all FA patients, besides an increase in Frataxin levels, may be at least as important and necessary for a symptomatic effect of rhuEPO treatment. Other agents such as Idebenone did not rescue from oxidative stress and 8-OHdG remained unchanged in a phase II trial [29]. The mechanism by which Erythropoietin exerts protection against oxidative stress apart from an increase of Frataxin is currently unclear.

Animal studies revealed increased brain parenchymal BDNF levels and neurogenesis in the Hippocampus as soon as 24 hours following controlled cortical Erythropoietin infusions [30]. Increased BDNF levels appear then to result in an increase of mTOR (LEE) and enhance the efficiency of translational control and mRNA transport / translation [31] possibly resulting in changes of cellular volume. Therefore Erythropoietin may, together with well-known positive effects of activated mTOR on the biogenesis of mitochondria and the increase in glucose transporter production and
translocation, restore the cellular energy homeostasis and induce translation, which ensures proper cell growth and thus increase in cell mass (see also Fig. 7).

On-going stable and significant increase of the protein Frataxin throughout an extended treatment phase has been obtained. Although the exact biological significance and the mechanism of action of Frataxin is still unknown, it is well established that a Frataxin loss beyond 50% results in FA [48, 49]. Preliminary results of a single-dose study that aimed to determine the half-life of Frataxin in response to three different rhuEPO doses indicate for a comparable half-life between rhuEPO and Frataxin. Therefore, study protocols that favour stable Erythropoietin levels appear to be superior to intermittent treatment. Despite an increase of Frataxin in rhuEPO treated FA patients, a genuine “anti-ataxic” property of rhuEPO has not been shown until to date. Of note, a short duration treatment is unlikely to exhibit substantial clinical benefits due to slowing of disease progression. Thus, well-known additional effects of rhuEPO on energy metabolism, muscular activity and cognition might have positive influences on currently available clinical outcome measures.

Main concerns about Erythropoietin’s safety in the treatment of non-anaemic patients were due to its hematopoietic effects. These effects together with potential support to tumour growth are major shortcomings of Erythropoietin preparations and limit together with FA specific problems, such as cardiomyopathy, an extensive long-term use of conventional Erythropoietin. In parallel with hematopoietic effects marked changes in cellular iron metabolism and distribution take place. A reversible decrease in serum ferritin levels and a rise in serum transferrin occurs. Intra-mitochondrial iron accumulation in FA is postulated to play a major role in the production of hydroxyl radicals by Fenton chemistry, leading to inactivation of FeS enzymes, lipid peroxidation and damage to nucleic acids as well as proteins, finally resulting in cell death. In line with the concept of iron overload in FA, a 6 months open label study using the moderate iron chelator deferiprone (3-hydroxy-1,2-dimethylpyridin-4-one; DFP) has been performed. DFP reduced the iron content in the nucleus dentatus of the cerebellum and caused no apparent hematological or neurological side effects, but neurological improvement was only marginal [50]. In turn, the presence of increased levels of soluble transferrin receptors, an indicator for cytosolic iron deficiency, causes ongoing controversies about the benefit of cytosolic iron chelation in FA [51]. There is also some debate whether mitochondrial iron accumulation within mitochondria is the result or the cause of the oxidative stress which is responsible for mitochondrial damage. Studies with conditional knockout mouse models and cells of FA patients indicate that deficiencies in FeS enzymes precede iron accumulation. Thus, given Frataxin’s suggested role in mitochondrial iron metabolism, namely the regulation of mitochondrial iron export [52], in protecting aconitase (4Fe-4S)\textsuperscript{2+} clusters against disassembly and inactivation [53, 54], and its involvement in \textit{in vivo} production of heme and Fe-S clusters [55, 56], further studies about mitochondrial changes in iron utilisation and intracellular iron distribution and transport in
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FA and the potential role of rhuEPO in the latter are highly warranted. Still, in the last step of heme synthesis the mitochondrial enzyme ferrochelatase utilizes mitochondrial iron [57]. In principle, the changes in iron markers combined with an increase in hemoglobin levels during rhuEPO treatment may indicate for a reallocation of mitochondrial iron into heme. Whether rhuEPO treatment results in a substantial reduction of the reported mitochondrial iron overload in FA and the possible impact of this additional effect on mitochondrial function and survival remains to be enlightened.

CONCLUSION REMARKS

The actions of Erythropoietin in neuroprotection are supposed to be due to several different mechanisms mediated by the β-common-receptor (βcR). Erythropoietin may attenuate the production of damaging molecules such as ROS or glutamate-stimulated excitotoxicity. In FA it increases Frataxin levels through a currently unknown post-transcriptional and/or post-translational mechanisms. It may further interfere with mitochondrial iron homeostasis through iron intramitochondrial chelation and relocation via heme, and finally it may restore cellular energy homeostasis through biogenesis of mitochondria via BDNF. Clear limitations of chronic rhuEPO treatment are effects that derive from hematopoesis and its side-effects. Therefore, clinical studies on the efficiency of non-hematopoietic Erythropoietin formulations in FA may be of primary interest.

ACKNOWLEDGEMENTS

We thank M. Reindl who shared the tissue culture facility for sample preparation, and P. Chiba, W. Strobl, B. Gmeiner and S. Kapisotis for collection and routine analysis of blood samples.

We thank all FRDA patients and their families for their support.

This study was funded by a research grant from the RoFar-Foundation, Switzerland.

The following patents have been applied for (related to the use of rhuEPO for FRDA): A 1869/2004 and PCT/EP2005/011510.

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