Effect of herbo-mineral formulation EHb in experimental anaemia in rodents

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EHb—a herbo-mineral formulation of iron (ferrous form) produced a significantly higher and dose dependent increase in the haemoglobin level, as compared to Fefol (a non-complex-chelated iron preparation). Also, EHb did not produce any overt toxicity or gastric irritation at these dose levels. The results suggest that EHb can be of a better choice in the treatment of anaemia than any other commercially available chelated iron preparations.

EHb is a herbo-mineral formulation of Indian Herbs, Saharanpur, is used for treatment and prevention of anaemia. It is prepared by combining the dimeric-dibenzo-alpha-pyrone Fe (II) complex, isolated from Shilajit with a herbal extract containing small molecular weight gallo-tannoids. This combination strengthens the iron coordinating site (L, Str.1, 2. Fig. 1) of the herbo-mineral complex. The herbo-metallic complex is encaged in the micropores (100-500 Å diam.) of the contained humus. The complex entities represent resonance-stabilized, soft-spin nucleophiles, comprising metallo-organic oxygen radicals that act both as protector of biological membranes and modifier of biological responses at ambient energy states, like liquid crystals.

It is unique in that the active principle of Shilajit, the humato-ferrate complex is formulated to make the iron (ferrous form) in a highly bioavailable state. The amount of iron in the complex was calculated on the basis of the humato-ferrate moiety (= dimeric dibenzo-alpha-pyrone-DBP). The structures of the humato-ferrate (Fig. 1, str.1, 2) were established by comprehensive chromatographic and spectroscopic evidence.

The core dimeric DBP-humato ferrate (Fe^{3+}) complex has a molecular weight range of 450-550 Da and, therefore, readily absorbable. Since the slow release and re-absorption of the metal ion are an inherent property of the above humato ferrate complex, it releases the metal ion to metal hungry receptors and abstracts Fe^{3+} from loose metal ion areas. The DBP ligand that remains in the system, after the release of Fe^{2+} ion, promotes the immune function of the body.

All these attributes are well documented in humato-ferrate chelates. Additionally, it has a unique advantage that the humato-ferrate complex is different from the amino acid-Fe(OH) chelates and, therefore, does not suffer from the limitations of the latter type of chelates (i.e. metal ion irreversibility and static metal ion higher oxidation state).

Iron in ferric form (Fe^{3+}) as is the case with amino-acid chelates, has inherent difficulties of absorption while iron in ferrous form (Fe^{2+}) as is the case with EHb complex is quickly captured and absorbed by iron-hungry cells. Moreover, in EHb complex, the molar ratio of the ligands is more than that of metal ion hence free iron which is not absorbed, is again chelated with ligand(s) to prevent Fenton reaction and free radical induced damage. Thus EHb ensures maximum absorption of iron without toxicity.

Therefore, this study has been conducted to evaluate the efficacy of EHb in different doses given for different time periods and in comparison to Fefol capsule (Smithkline-Beecham) which contains dried ferrous sulphate (150 mg) and folic acid (0.5 mg) per capsule.

Fig. 1—Iron coordinating sites of herbo-mineral complex
Materials and Methods

Experiment 1—Albino rats (40) of either sex weighing 60-70 g body weight and maintained in colony cages under standard animal house conditions of 25° ± 2°C, with 12 hr L.D. period were used in the study.

Experiment 2—Albino rats (25) of either sex weighing 70-80 g in body weight were taken and maintained as in experiment 1.

Products and administration periods—In both the experiments the rats were kept on iron and protein deficient diets for 30 days and their haemoglobin levels were monitored. Following the above undernutrition, simulating the loss of blood during menstruation in ladies, blood was persistently shed (upto 1.5 ml/day) for three days or more from the retro-orbital plexus of the rats and haemoglobin was monitored until induction of anemia. When the haemoglobin level of rats fell below 8 g/dl, they were considered as anaemic.

In experiment-1, 40 anaemic rats were divided into eight equal groups. Group 1 and 5 were kept as untreated control. Two groups (groups 2 and 3) were subjected to three days of EHb administration at the dose level of 210 and 300 mg per kg body weight (containing 5.8 and 8.3 mg of elemental iron, respectively), orally as suspension. In group 4, the contents of one Fefol capsule per day were administered orally as suspension for three days.

The rats of group 6 and 7 were subjected to administration of EHb formulation at the dose level of 210 and 300 mg/kg respectively, as in group 2 and 3, but for 7 days. The rats of group 8 were administered one capsule of Fefol daily orally as suspension, for 7 days. Control animals received isovolumetric amounts of vehicle (0.3% CMC suspension) only. In another study LD 50 of EHb was found to be more than 2000 mg/kg body weight in albino mice. Therefore 1/10th of this dose (200 mg/kg body weight) was used, which is equivalent to 1/4th or 1/5th of the dose of commercially available and commonly used iron preparation, in terms of elemental iron therein. Each 210 mg dose contained around 10 mg of excipients for optimising shelf-life and palatability of the formulation. A higher dose of 300 mg/kg body weight) was also used.

In experiment-2, 25 anaemic rats were divided into five equal groups. EHb in four different doses viz 150, 200, 250 and 300 mg/kg body weight, respectively was administered orally as in experiment 1 but for 30 days to the rats of Groups A, B, C and D, respectively to assess the effect of different dosage. One group of rats served as untreated control and received isovolumetric amounts of vehicle only.

Evaluation of drug induced gastric irritation—At the end of the experimental period, all animals belonging to the various EHb groups described above were sacrificed after putting them under ether anaesthesia and then severing the neck vessels for complete bleeding. The abdomens of all the animals were opened and the stomachs were fixed in 1% formalin. These stomachs were then examined for any evidence of ulceration.

Haematological and biochemical estimations—Blood from all groups of animals was collected by retro-orbital puncture, for estimation of haematological and biochemical parameters. Whole blood haemoglobin content was assayed by cyanomethalomoglobin method using Chemkit (Ranbaxy Diagnostics). Protein levels were estimated in 7 days administration groups by method of Beers and Sizer. Catalase activity was estimated in 7 days administration groups by method of Beers and Sizer. The statistical significance was calculated using the Student’s t test method.

Results and Discussion

Experiment 1—The effect of 3 days and 7 days administration of EHb on the haemoglobin level in anaemic rats in experiment 1, is shown in Tables 1

<table>
<thead>
<tr>
<th>Group and treatment</th>
<th>Dose (mg/kg)</th>
<th>Haemoglobin (g/dl)</th>
<th>Change in haemoglobin over control (%)</th>
<th>Protein content (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr.1 Control</td>
<td>—</td>
<td>7.61 ± 0.46</td>
<td>7.61 ± 0.41</td>
<td>—</td>
</tr>
<tr>
<td>Gr.2 EHb</td>
<td>210 mg/kg body wt. (Iron – 5.8 mg)</td>
<td>7.60 ± 0.49</td>
<td>9.61 ± 0.76*</td>
<td>20.81</td>
</tr>
<tr>
<td>Gr.3 EHb</td>
<td>300 mg/kg body wt. (Iron – 8.3 mg)</td>
<td>7.59 ± 0.32</td>
<td>11.21 ± 0.84*</td>
<td>47.31</td>
</tr>
<tr>
<td>Gr.4 Fefol</td>
<td>Ferrous Sulphate 150 mg – capsule (Iron – 30 mg)</td>
<td>7.62 ± 0.18</td>
<td>8.09 ± 0.70</td>
<td>6.00</td>
</tr>
</tbody>
</table>

*P < 0.001 as compared to Group-1.
and 2. The normal haemoglobin level was 13.9 ± 0.4 g/dl (not shown in Table). Malnutrition and blood loss in rats produced a statistically significant fall in their haemoglobin level bringing it to less than 8 g/dl. EHb produced a significant and dose related augmentation of the haemoglobin level in anaemic rats. The reversal was evident within 3 days of administration which progressively increased to a maximum (54.16%) with higher dose for 7 days. There was no significant effect on the level of protein or catalase activity. On the other hand the commercial product Fefol capsules (containing ferrous sulphate equivalent to 30 mg of iron) did not increase the levels of haemoglobin to significant extent, following three days of treatment. However, Fefol capsule administration for 7 days produced significant (22.42%) increase in haemoglobin level though it was much less than the increase observed with both dosages of EHb formulation. EHb did not produce any overt toxicity at the two dose levels administered.

There was no gastric ulceration in any of the experimental groups and all rats showed healthy gastric mucosa following EHb administration.

Experiment 2—The effect of 30 days of EHb administration in different dosages on the haemoglobin level and protein content is shown in Table 3.

EHb treatment at different doses showed a significant reversal of the attenuated levels of Hb. However, the optimum response was obtained at 200 mg/kg dose of EHb for 30 days, after which an increase in dose did not produce any additional benefit. Iron preparations as inorganic compounds or amino-acid chelates are distinctly different entities as compared to iron preparation complexed by organic ligands due to difference in the physical, chemical and physiological properties. It is well established that inorganic salts like ferrous sulphate are poorly absorbed by the system as was evident from our present findings also.

With respect to the mechanism of absorption there are two kinds of iron in the diet-haem iron and non-haem iron utilising separate receptors on the mucosal cells. After the uptake of haem iron into the mucosal cells, the porphyrin ring is split by a special enzyme (haemoglobinase) within the cells and its iron released. As far as non-haem iron is concerned, receptors on the luminal side probably compete with

Table 2—Effect of 7 days EHb administration on haemoglobin level in anaemic rats
[Values are mean ± SE from 5 animals in each group]

<table>
<thead>
<tr>
<th>Group and treatment</th>
<th>Dose</th>
<th>Haemoglobin (g/dl)</th>
<th>Change in haemoglobin over control (%)</th>
<th>Protein content (g/dl)</th>
<th>Catalase activity (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr.5 Control</td>
<td>—</td>
<td>7.92 ± 0.36</td>
<td>—</td>
<td>5.25 ± 0.13</td>
<td>5.01 ± 0.50</td>
</tr>
<tr>
<td>Gr.6 EHb</td>
<td>210 mg/kg body wt. (Iron – 5.8 mg)</td>
<td>7.90 ± 0.19</td>
<td>10.02 ± 0.49**</td>
<td>26.19</td>
<td>5.96 ± 0.22</td>
</tr>
<tr>
<td>Gr.7 EHb</td>
<td>300 mg/kg body wt. (Iron – 8.3 mg)</td>
<td>7.84 ± 0.56</td>
<td>12.24 ± 0.91**</td>
<td>54.16</td>
<td>5.64 ± 0.31</td>
</tr>
<tr>
<td>Gr.8 Fefol</td>
<td>Ferrous sulphate 150 mg-capsule (Iron – 30 mg)</td>
<td>7.92 ± 0.83</td>
<td>9.72 ± 0.74*</td>
<td>22.42</td>
<td>6.02 ± 0.26</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.001 as compared to group 5 control.

Table 3—Effect of 30 days EHb administration of EHb on the haemoglobin level in anaemic rats
[Values are mean ± SE from 5 animals in each group]

<table>
<thead>
<tr>
<th>Group and treatment</th>
<th>Dose</th>
<th>Haemoglobin (g/dl)</th>
<th>Change in haemoglobin over control (%)</th>
<th>Protein content (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>8.02 ± 0.58</td>
<td>—</td>
<td>7.75 ± 0.46</td>
</tr>
<tr>
<td>Gr. A EHb</td>
<td>150</td>
<td>8.01 ± 0.62</td>
<td>11.98 ± 0.33**</td>
<td>49.36</td>
</tr>
<tr>
<td>Gr. B EHb</td>
<td>200</td>
<td>7.98 ± 0.74</td>
<td>12.99 ± 0.23**</td>
<td>62.17</td>
</tr>
<tr>
<td>Gr. C EHb</td>
<td>250</td>
<td>7.96 ± 0.45</td>
<td>12.51 ± 0.37**</td>
<td>56.18</td>
</tr>
<tr>
<td>Gr. D EHb</td>
<td>300</td>
<td>8.01 ± 0.26</td>
<td>12.75 ± 0.51**</td>
<td>59.17</td>
</tr>
</tbody>
</table>

**P<0.001 as compared to control.
complexing luminal ligands for the iron ions. It is probable that the iron can only be absorbed into the cells and pass the mucosal membrane in its ferrous form. Reducing substances, must therefore be present in the mucin layer of the mucosal cells for iron to be absorbed. About two-thirds of iron in the body is found in haemoglobin, the rest in myoglobin, various other iron-containing enzymes and the iron-transport protein viz., transferrin. Excess of iron is stored as ferritin and haemo-siderin, the latter is an insoluble product and occasionally involved in the systemically deleterious free radical (Fenton) reaction. The major portion of dietary iron is non-haem in nature and is present in the ferric i.e. Fe (III) state, while haem iron is present in ferrous i.e. Fe (II) state. Thus most of the dietary iron requires solubilisation and reduction to ferrous state to aid its absorption and utilisation. Therefore for better absorption of iron, the ferro-humus complex is combined with herbs containing reductones, in EHb formulation, which not only reduces Fe (III) to Fe (II), but helps in better absorption. Thus iron-organo assembly behaves similarly as transferrin. Iron from the assembly in EHb enters various cells of the body for synthesizing iron containing enzymes and proteins. Receptor-mediated endocytosis provides transferrin mimicking iron assembly to iron hungry cells. It enters the cytoplasm in a vacuole. The contents of the vacuole with acidic pH release iron from the iron assembly of EHb. Iron thus released chelates with various cellular constituents e.g. citrate, ATP and GTP. The iron free assembly is then ejected from the cells for recapture of iron from the free iron pool. Further, with administration of EHb, there would be no risk of post-Fenton reaction as the iron remains tenaciously bound to the assembly, till it is engulfed by iron hungry cells.

By forming fine colloidal suspension with water, the complex in EHb acts as potent and tenacious scavenger of both transition and fixed valency metal ions and is capable of penetrating into different cell types. The complex in EHb does not wilt or decay under the influence of free radicals, rather it interacts with free radicals to generate additional chelating functions (only in case of iron overload) that would sequester more metal ions. Thus EHb in small amounts is capable of tackling large amount of iron overload and modulates the iron content in the body with positive results.

The reducing property of the complex in EHb (through the polyphenol moieties) causes Fe$^{3+}$ to Fe$^{2+}$ reduction systemically resulting in mobilization and functionalization (e.g. attachment to haemoglobin) of systemic iron. This would occur not only under anaerobic condition but also in presence of oxygen and the attendant reactive oxygen species.

It is only the ferrous (Fe$^{2+}$) ion which is required to produce haemoglobin. When haemoglobin is metabolized into myoglobin, the Fe$^{2+}$ ion of haemoglobin is oxidised to Fe$^{3+}$ and remains loose in the system with the risk of undergoing Haber-Weiss (Fenton) reaction, producer of OH$^-$ (hydroxyl radical). Unlike amino-acid chelates, humato ferrate complex of EHb has the capability to abstract Fe$^{3+}$ from loose metal ion areas to form readily absorbable Fe$^{2+}$ complex and to present and slowly release the metal ion to metal hungry receptors.

The results of the present study also showed that herbo-mineral complex EHb was able to raise haemoglobin levels to significantly higher levels in shorter periods in comparison to non-complexed iron preparation.

EHb at the dose of 200 mg/kg body weight showed optimum (+ 62.17%) response indicating an increase of nearly 5 g/dl of haemoglobin in 30 days and bringing it close to the normal haemoglobin level of healthy rats. However higher dose of EHb (300 mg/kg body weight) showed better response in shorter period (3 days and 7 days) of administration.

References

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