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Anti Anaemic Potential of Ferrous Phosphate Nanoparticle Loaded Iron on Phenylhydrazine Induced Anaemia in Rats

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ABSTRACT

The anti anaemic potential of the ferrous phosphate nanoparticles on the phenylhydrazine hydrochloride induced anaemia in rats was investigated. Anaemia was induced with phenylhydrazine hydrochloride at a dose of 30 mg/kg body weight by subcutaneous administration. Treatment of anaemia was done with the oral administration of ferrous phosphate nanoparticles drug, synthesized through controlled hydrolysis method. The nanoparticles with significant morphological and surface characteristics were subjected for *in vivo* evaluation, to know the bioavailability values. The acute toxicity test carried out for the ferrous phosphate nanoparticle drug of 30mg/kg showed no abnormalities with body weights and pre terminal deaths, comparing with the normal rats. *In vivo* evaluation showed that the ferrous phosphate nanoparticles administered with the dosage of 30 mg/kg body weight revealed significant increase ($p<0.05$) in the haematological parameters in rats. The increase in haemoglobin (Hb), red blood cells (RBC), packed cell volume (PCV) and ferritin protein level was observed in the rats from the day 9, even after the maintanence dose of phenylhydrazine at an interval of 3 days. The results unveils the anti anaemic potential of the ferrous phosphate nanoparticles, which was found to be appreciably high, compared to the commercially used standard ferrous sulphate powders. This study therefore, shows that the utilization of ferrous phosphate nanoparticle direct uptake as a drug has the anti anaemic potential, which can be exploited for iron deficiency in future.

Keywords: Anaemia, Ferrous phosphate, Phenylhydrazine hydrochloride, Haematology

INTRODUCTION

Many infants, childrens and women of childbearing age, particularly in the poorer countries of the developing world, are iron deficient. About half of these iron deficient individuals develop iron deficiency anaemia (IDA), the most advanced form of the disease, which has several major negative impacts on health and contributes substantially to the risk of early death and disability[1].

The incidence of anaemia is higher in the world especially in the developing countries were due to the presence of many aggravating factors such as poor nutrition, high prevalence of blood parasites example, plasmodium, trypanosomes and helminthes infestation. It is also known that women are susceptible to anaemia during pregnancy due to high demand from the developing feotus[2,3].

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Anaemia could also be induced by using some toxic chemicals. Phenylhydrazine hydrochloride is such a type of toxic nitrogen based compound. Exposure to phenylhydrazine may cause damage to red blood cells, potentially resulting in anaemia and consequential secondary involvement of other tissues, such as the spleen and liver. Phenylhydrazine is used for the induction of haemolytic anaemia and the study of its mechanism in many species such as rabbit, rat, mouse, chicken, goldfish etc.[4].

The elimination of iron deficiency however has not proved easy. Dietary diversification is hindered by the difficulty in achieving behavioural changes, as well as by the predominance of plant-based diets in developing countries, which are deficient in the more bioavailable haeme form of iron. The choice of suitable iron compounds is often a compromise between reasonable cost, bioavailability and the acceptance of any sensory changes. Water soluble iron compounds were found to cause adverse organoleptic changes and poorly soluble Fe compounds although more stable tend to have low bioavailability[5].

As a result of the fact that anaemia is very common and the incidence is likely to increase in future[6], there is a need to prevent it or seek for more cost effective and better treatment strategies. Ferrous phosphate is a poorly soluble iron compound, with high biological impact and unknown absorption values because of its bulk size. The scientific opinion of the european food safety authority indicates the importance of ferrous phosphate compounds as a food additive and its unrevealed nature of absorption and acidic solubility[7]. Most researchers have worked under the assumption that improvement in bioavailability of some specific compounds, comes from improvement in apparent solubility and there are no much investigation found on the direct nanoparticles uptake, which play a vital role in enhancing the bioavailability.

The present investigation was therefore, planned with an objective of finding the anti aneamic potential of the ferrous phosphate nanoparticle direct uptake as a drug, synthesized through controlled hydrolysis method.

MATERIALS AND METHODS

Synthesis of ferrous phosphate nanoparticles

The ferrous phosphate nanoparticles were synthesised by controlled hydrolysis method. To the aqueous solution of 0.5 M sodium phosphate, 0.5 M of ferrous sulphate solution was added drop wise, to obtain the stochiometric concentration of the precursors. The solution developed was kept at room temperature, pH – 6.2 under vigorous stirring for 40 minutes. The final product was dried at 80ºC and subjected for characterization. The morphological, spectroscopic and the surface characters of the nanoparticles were studied using Atomic force microscopy and BET analysis.

Animal Experiments

Female wistar rats weighing between 170 and 190 g were used for this study. The animals will be obtained from animal house, IRT, Perundurai medical College, Erode, TamilNadu, India. On arrival, the animals were randomly grouped in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2°C and relative humidity of 30-70%. A 12:12 light:day cycle was followed. All the animals were allowed to free access to water and fed with standard commercials pelleted rat chaw (M/s. Hindustan Lever Ltd., Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional animal ethics committee (688/2/C-CPCSEA) of Nandha College of Pharmacy, Erode, Tamilnadu, India and were accordance with the guidelines of the IAEC. Approval was obtained from the IAEC, NCP, Erode (Proposal No: NCP/IAEC/No-30/2012-13).

Acute toxicity studies

Acute toxicity of a drug was determined by the calculation of LD_{50}, i.e., the dose that will kill 50% of animals of a particular species. An approximate LD_{50} values were determined as the pilot study using the 'staircase method'. Three doses were chosen for the test, according to the iron level needed for the body weight of the rat model. In our study, for the estimation of LD_{50}, ferrous phosphate nanoparticle drug in three doses of 10 mg, 20 mg and 30 mg were given orally to three groups containing six animals in a group. The animals were observed for 15 days for any toxic symptoms, for the case of iron overload. The body weight, body weight changes and preterminal mortality rate were analysed for the observed rats, to calculate the LD_{50} values.

Induction of anaemia

This was done as described by *Harris and Kugler*[8] *(1971)*. Briefly, anaemia was induced in all the rats except the control rats, by subcutaneous administration of 2.5 % neutralised Phenylhydrazine Hydrochloride (Fisher Scientific company, USA) at a dose of 30 mg/kg body weight with a two maintenance dose of 10 mg/kg at the interval of 3 days.

The animals were randomly distributed into the following four groups containing six animals in a group.

Group 1 – Non Anaemic rats control
Group 2 - Anaemic control
Group 3 - Anaemic rats treated with ferrous phosphate nanoparticle drug,
Group 4 - Anaemic rats treated with commercially available oral drug.

Blood Collection and analysis

The experiment in rats with anaemia induced using phenylhydrazine was performed for 3 weeks. On the day of establishment of anemia, the weight, PCV, RBC, Hb and ferritin levels were taken in all the groups. Anaemia was allowed to establish in 24 hours[9]. Measurement of PCV, RBC, Hb and ferritin levels were taken day after day for the first 3 days, then on day 6, 9, 12, 15, 18 and 21.

The rats were given with standard diets and the drugs were administered orally, after the diagnosis of anaemia, at a level of 30 mg body weight for the group 3 and 40 mg body weight for group 4. About 4.0 ml of blood was collected from each rat and transferred into a clean glass bottles with EDTA (haematological tests) and without EDTA (for serum) by sinus puncture.

Red Blood Cell (RBC) counts were done by haemocytometer. Haemoglobin (Hb) estimation and Packed Cell Volume (PCV) were determined by cyanomethanoglobin method using a spectrophotometer and microhaematocrit, respectively. The ferritin protein level was determined by immunoturbidimetric method.

Statistical analysis

The mean and standard deviation (SD) of the data was calculated. The results were analyzed by one way analysis of variance (ANOVA) and where applicable, least significant difference (LSD) was used to determine significant results. The differences between groups were considered significant at $P < 0.05$ (5%) by Tukeys HSD test.

RESULTS

The results of the synthesized ferrous phosphate nanoparticles subjected for characterization showed a spherical shaped morphology with a particle size range of 80 – 110 nm (**Figure 1**). The surface characteristics of the particles were found to be advantageous with high surface area of 220 m^2/g, which may produce high absorption properties, resulting in high bioavailability values.

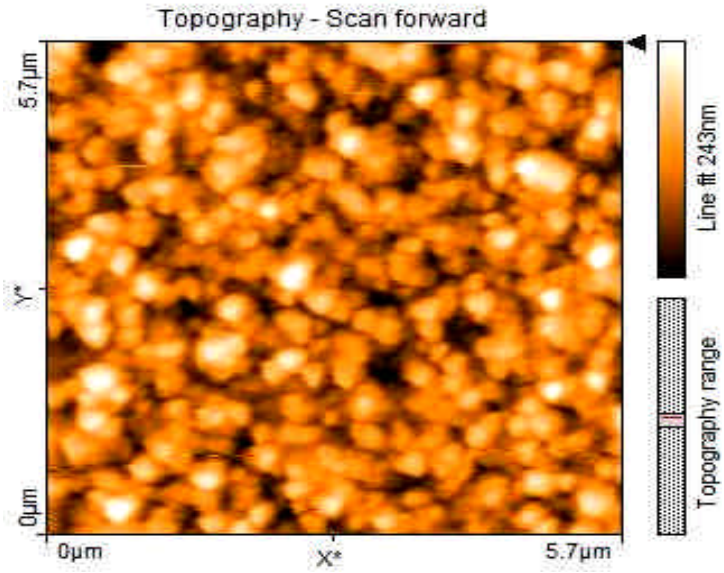

Figure 1: Morphological picture of ferrous phosphate nanoparticles analysed using AFM.

In toxicity studies the drug administered orally to rats upto the highest dose 30 mg/kg body wt. it does not produce any significant change in autonomic and behavioural responses during observation upto 8 hrs. The body weights were recorded on day 8 and 15. There were no gross necropsy findings during the study (**Table 1**). Also animals did not exhibit any toxic signs like restless, respiratory distress, convulsions, coma etc.

Table 2 and **3** shows the effect of ferrous phosphate nanoparticle drug on anaemia induced by phenylhydrazine hydrochloride. The reduction of PCV values by more than 50% of the baseline values in all rats at day 3, after phenyl hydrazine administration is an indication of anaemia. After anaemia has been induced in rats, daily oral treatment with the ferrous phosphate nanoparticle drug significantly increased the haematological parameters over the control. Remarkable anti anaemic activity was obtained with PCV of 50 – 58%, RBC count of 9 – 12 x 10^6 /µl, Hb value of 17 – 20 g/dl and the ferritin level of 3 – 5 mg/dl after treatment with the ferrous phosphate nanoparticle drug on day 9, compared with 26 % of PCV, 4 – 7 x 10^6 /µl of RBC, 11 – 13 g/dl of Hb and 2.5 – 3.5 mg/dl of ferritin value in the rats treated with phenylhydrazine hydrochloride treated animals at day 3.

Subsequent levels in the haematological parameters were observed at the day 18, even after the two maintanence dose of phenylhydrazine at day 6 and 12. This similar pattern of increase in PCV, RBC, Hb and ferritin levels were also observed from day 6 to day 21. The biochemical estimation for the ferritin protein level indicated a high level during the day 18 for the ferrous phosphate nanoparticle drug with 4-6 mg/dl in rats.

Table 1 : The Body weights, body weight changes and preterminal deaths of acute toxicity studies

Dose mg/kg b.wt.	Rat	Sex	Gross necropsy findings	Body weight (g) Weight initial	Weight 8th day	Weight change upto 8th day	Weight 15th day	Weight change upto 15th day	No. of dead / No.tested
30	**1**	Female	No abnormalities detected	152	180	28	192	40	**0/6**
	2	Female	No abnormalities detected	146	164	18	173	27	
	3	Female	No abnormalities detected	159	185	26	196	37	
	4	Female	No abnormalities detected	138	140	2	150	12	
	5	Female	No abnormalities detected	163	196	33	201	38	
	6	Female	No abnormalities detected	162	195	33	205	43	

Table 2: Anti anaemic property (PCV & RBC values) of ferrous phosphate nanoparticle drug against phenylhydrazine induced anaemia.

Treatment	PCV (%) Day 0	Day 3	Day 9	Day 18	RBC (x10^6 µl) Day 0	Day 3	Day 9	Day 18
Control	44±3.03	43.8±2.71	44.6±1.21	44.3±1.50	6.51±0.33	6.85±2.88[a]	6.95±0.36	7.18±0.31
Control induced	43.5±2.42	26.3±2.58[a]	26.5±1.87[a]	31.0±1.41[b]	6.45±0.24	4.81±0.14[a]	5.01±0.19[b]	5.36±0.17
Drug	42.8±2.78	26.5±2.07[a]	52.3±2.58	57±1.78	6.51±0.26	4.98±0.30[a]	8.76±0.39	9.48±0.40
Standard	42.6±2.16	25.1±2.63[a]	41.1±1.72	47.3±1.96	6.58±0.29	4.91±0.19[a]	6.66±0.34	7.73±0.32

a, b - indicates the significant variations in each homogenous subsets at p<0.05 level (5%) by Tukeys HSD test. Values are expressed in terms of ±SD.

Table 3: Anti anaemic property (Hb & ferritin values) of ferrous phosphate nanoparticle drug against phenylhydrazine induced anaemia

Treatment	Haemoglobin (g/dl)				Ferritin level (mg/dl)			
	Day 0	Day 3	Day 9	Day 18	Day 0	Day 3	Day 9	Day 18
Control	14.31±0.40	14.45±0.42	14.5±0.52	14.46±0.51	4.28±0.40	4.25±0.30	4.36±0.43	4.33±0.43
Control induced	14.41±0.49	12.96±0.61[a]	13.48±0.29	14.15±0.30	4.48±0.51	3.7±0.40	3.81±0.29	3.38±0.36[a]
Drug	15.20±0.35	12.73±0.51[a]	18.1±0.37	18.9±0.49	3.95±0.39	3.15±0.31[a]	4.48±0.44	5.66±0.35
Standard	13.96±0.28[a]	13.13±0.67[a]	16.03±0.23	17.36±0.36	4.28±0.27	3.05±0.20[a]	3.8±0.39 [a]	4.71±0.39

a - indicates the significant variations in each homogenous subsets at p<0.05 level (5%) by Tukeys HSD test. Values are expressed in terms of ±SD.

DISCUSSION

Anaemia was diagnosed by a more than 50% reduction in PCV values of all rat groups from the baseline values except the control group. Similarly, the PCV values and RBC counts significantly decreased from the baseline values 2 days after administration of phenyl hydrazine hydrochloride, indicating that the chemical could effectively induce anaemia in rats at the given dosage. Phenyl hydrazine hydrochloride has earlier been used to induce anaemia in rats (Bowman and Rand, 1980). Phenyl hydrazine was reported to induce the development of Henz bodies on RBC membranes after six days of exposure, which protect them against further destruction by the chemical[10,11].

The results of the haematological parameters indicate that the treatment with the ferrous phosphate nanoparticles direct uptake drug significantly elevates all the haematological parameters of the anaemic rats at the day 9. On the other hand the ferrous phosphate nanoparticles drug showed significantly high values on the parameters, when compared with the commercially used ferrous sulphate powders. Other investigators also reported that the extract of *Khaya senegalensis* stem bark has antianaemic activity in phenyl hydrazine induced anaemic rats[12]. Another report indicates that oral administration of *Tectona grandis* extract to rats, previously treated with phenyl hydrazine, increased the concentration of haemoglobin, red blood cells number, haematocrit and reticulocytes counts[13].

Earlier reports suggests that, decreasing the particle size of metallic Fe added to food & medicines, increases Fe absorption. The development and characterizing the nanoparticles of Fe, determines their bioavailability & potential toxicological effects and so these indications as well can serve as a good iron supplementation[14].

CONCLUSION

In the present investigation it was observed that, the ferrous phosphate nanoparticle, as an iron compound was found to produce an improved effect comparing to the standard. These increases in the haematological parameters can be correlated with the properties of the synthesized ferrous phosphate particles. The high surface area of the particles might be the responsible factor, leading to the high absorption of iron compounds in rats, thus resulting with high bioavailability values. The results, collectively demonstrates that the direct uptake of ferrous nanoparticles increases the bioavailability of iron in a superior way that found to produce a good increase in the haematological parameters.

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