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Comparison between *Spinacia Oleracea* and FeSO4 as a Source of Iron to Treated Anemia in Mice

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Abstract :

In this study thirty healthy male mice (Swiss albino aged of eight week) were used and divided in five groups. Three groups were feed with bread made of flour containing spinach (three different percentage 10%, 20%, and 30% respectively) as source of iron, compare with group number four which was feed with bread made of flour fortified with premix (consist of FeSO4, Folic acid and Maize starch). It is add according to the information given by the supplier (200g/ 1000 kg flour). Group number five was the control (feed with bread made of unfortified flour).

Same food was used during the time of the experiment (fourteen days). The study was designed to make anemia in all groups by injection with phenylhydrazine . Samples of blood were taken by bleeding through heart puncture to measure the hemoglobin (Hb) and the packed cell volume (PCV).

At the end of the experiment, mice in all the groups were then sacrificed and slides from the tissues of heart, kidney, liver and spleen had been made to measure the histological effects of these sources of iron. Results showed that there were significant differences in weights, (Hb) and (PCV) between mice feed with bread containing (10%, 20% and 30%) spinach and that containing premix and the control. In the same time slides of harts, kidneys, livers and spleens did not showed any detectable effect.

Introduction:

Suffering of the peoples in many cases of anemia resulting from a decrease of some elements in food is considered to be iron which is the most important of these elements and that will lead to what is known as iron deficiency anemia (1).

This will cause the decrease in the level of each of hemoglobin (Hb), the packed cell volume (P C V), and also may lead to the reduction of the stock of iron in liver, and reduce of the size of red blood cell. (2). The persons who suffer from iron deficiency anemia had been estimated by more than2000 million (3, 4).

Generally the proportion of those who suffer iron deficiency anemia in the world is estimated at about 14% (5). This was attributed to environmental factors or food, which lead to the proliferation of these cases (6).

In 1996 the food conference which has been held in the capital of the Philippines under the title of: (Manila Declaration on food fortification). It has been emphasized on the use of economic and scientific method and a law to regulate the addendum basic micronutrient in food, including iron.

(FAO) confirmed the necessity of selecting material which may be appropriate to transfer the added micronutrient to the beneficiary (7) Many countries, such as Oman, Brazil, Bolivia adopted programmer to fortified food with different micronutrient such as iodine, iron and vitamin A. UNICEF confirmed that the success of a programmer of fortification requires consolidation and the existence of clear laws as well as the intervention of government and cooperation of relevant international organizations (8).

The relying level, of iron and other micronutrient had been identified according to the objective of the addendum, as well different fortified product, according to the country. In Morocco adopted salt as carriers for iron in the food of children. In India fortified rice used to feed children, and in other case they mixed iron and iodine with salt (in south India). Fortified wheat flour was used to feed women in Thailand (9, 10).

The strengthening of wheat flour with iron and folic acid believed to be the most marketing in the world. The fortification of wheat flour with iron continues to increase, as the proportion of fortified of food increase. The percentage of fortified wheat flour of total production reached 18%

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in 2004, but the percentage reached 27% in 2007 (11). Ferrous sulfate considered to be the best chemical source to be used in different fortified programmed. It is normally added as powder, but attempts were made later to give this material in form of capsules in some countries such as Kuwait (12).

At the same time, amid mounting calls from all interested organizations health matters by adding iron to strengthen food to treated iron deficiency anemia, It should be emphasized that the amount of iron added must be within certain limits because excess will have a negative impact and may caused group of damage to health, for example, cancer and heart diseases and some other diseases such as hepatitis, (13, 14, 15, 16, 17, 18.).

At the present time there are a shafting to the use balanced food with suitable proportion of iron such spinach instead of chemical material which can caused some side effect. The researchers reported different rates of iron in spinach but all agreed about the possibility of the use of spinach as a source of iron in food (19, 20,21,22,23,24,).

The absorption of iron from spinach can be affected by the present of some material in the same meal. The absorption may decrease in the presence of tea, phytic acid, oxalic acid and adiposity in women and children, on the other hand the present of ascorbic acid enhance the absorption of iron (25, 26, 27,28,29,30, 31). Spinach also contain some other materials such antioxidant, vitamin A, and it has been found that spinach reduce ischemic brain damage (32, 33, 34).

Materials and Methods:

Thirty healthy male mice were used. They had been divided into five groups each total of six mice in each cage.

All groups of mice were injected with phenylhydrazine to enhance anemia (35). The dose was (60mg/kg). Two mice of each group were scarified for the purpose of measuring the percentage of hemoglobin (Hb) and the packed cell volume (PCV).

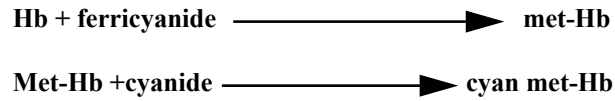
The rested mice in each group were fed as flow. Bread prepared from flour containing three percentage of spinach (10%, 20% and 30%) was used to feed the first three groups of mice. Spinach was obtained from the local market. It had been dried under the sun light and used as a source of iron by mixing with wheat flour which also was obtained from the local flour mill. The fourth group was feed with bread made of flour fortified with premix.

Premix is consisting of FeSO4, Folic acid and Maize starch. It is add according to the information given by the supplier (200g/ 1000 kg flour). The fifth group was feed with food free of both premix and spinach (control).

The period of study was 14 days Analysis had been made for the flour before and after fortified with premix. Animal's weights had been measured at the beginning of the experiment, and at the end of the trial period. Blood, was drawing on the heart (heart puncture) for the purpose of measuring the percentage of (Hb) and (PCV).

The standard way of measuring (Hb) (Drabkins method) was employed. The principle of this test is base on the treated of blood with the Drabkin's reagent which contains potassium ferricyanide, potassium cyanide and mono potassium phosphate.

In an alkaline medium, potassium ferricyanide oxidizes hemoglobin and its derivatives to methemoglobin. Subsequent reaction with potassium cyanide produces the more stable cyanmethemoglobin which has a maximum absorbance at 540 nm. Color intensity is proportional to total hemoglobin concentration.



The working solution was prepared by mixing 1 volume of concentrated Drabkin s reagent with 49 volume distilled water.

Whole blood collected in EDTA. Samples 20ul mixed with 5ml of the working solution. Assay has been done using optical path 1cm, wavelength 540nm and working solution used to zero adjustment.

Standard (cyanmethaemoglobin solution) 5ml without any addition treated in the same way as the samples. The calculation has been done according to this equation:

$$\text{Result} = \frac{\text{OD sample}}{\text{OD standard}} \times 1.5 \text{ g. dL}^{-}$$

The packed cell volume (PCV) was measure by using the poetic pipeline length 7.5 cm in diameter about internal one millimeter and filled two-thirds of a tube and blood in a manner capillarity and then one of the parties dam with artificial mud and put poetic pipeline in a centrifuge (Microcentrifuge) for 5 minutes, then the line between plasma and blood measure in particular ruler.

After that animals had been sacrificed for the purpose of collecting samples for the liver, spleen, kidney and heart. Those organs put in a container containing physiological saline solution for the purpose of cleared from the remnants of the fatty textile surrounding it, and then kept in test tubes containing bone solution for a period ranging between 48-60 hours for the purpose of stabilization.

Then transferred to test pipelines containing alcohol by 70 percent for the keeping of until the time of their use, and then transferred to a container tubes containing ethanol by 90% for 6 hours.

Organs had been then transferred to other test pipelines containing 99 percent of alcohol for 6 hours.

Then the substance of the Xylol for two hour then poured in a waxy container, cut off to thick chips to about 5 micron by using the manually orbit microtome.

These segments have been put in a warm water bath 45 degree Celsius and heat for some second then plastered with glass chip at an angle on uptrend to start the stage of the use of staining. Staining is used to demonstration of iron in tissue section.

There are two type of iron are found in tissue section, ferric ion by far the most common, is demonstration by Perls, Prussian blue reaction, while ferrous salts are demonstrated by Tiran's reaction.

Quincke, s reaction will demonstrate both ferric and ferrous iron, by converting ferric salt ferrous, both then being demonstrated. Prussian reaction, this method will demonstrate ferric iron which is demonstrated by the Tirmann, s method. Sections had been placed in xylol, then down to water followed be rinsed in distilled water.

Sections, then transfer to Perls, s solution for 15 minutes and then rinse in distilled water, then had been counterstained in 1 percent neutral red, then rinse in tap water. Sections then dehydrate, clear and mount in DPX. This stain is normally used to detect the excess of iron in the form of hemosidren.

Other slides had been stain by the use of natural day of Eosin and Hematotoxilin which they are normally used to detect the effect on the cells of the tissues.

The pictures of the textile chip were took use a digital camera under microscope strongly zooming were 10x and 40xr respectively.

The collected data were subjected for analysis by using statistic program SPSS version 19.s. The values of the investigated parameters were given in terms of mean+_ standard error, and differences between mean were assessed by analysis of variance (ANOVA), Least Significant Difference according to Duncan test.

Results and Discussion:

This study designated to measure the difference between the effect of spinach when it used as a source of iron and the chemical source (premix).

The percentage of iron in the flour before and after fortification with premix had been measured and it was zero and 20 ppm respectively. On the other hand the percentage of Hb and PCV had been determined after two days of injection with phenylhydrazine and compare with control. The values of Hb and PCV were 11 and 37 respectively, but the values of the control were 13 and 40.

These values indicate that phenylhydrazine made anemia in the mice which were injected with it. Results in table (1), are indicating the weight of mice's under study before and after starting the experiment. There was a significant difference between the control group itself and the other groups which were feed with food contain spinach in all concentration and also between the control and the premix. On the other hand we cannot find a significant difference between the weights of mice's which were feed with food contain 10%, 20% and 30% spinach respectively.

There was clear difference between groups T2andT3 and the group of the premix. The significant increase of weight of the spinach groups indicate that this type of food rich with micro nutrient necessary for a normal growth (36). On the other hand we cannot find a significant difference between spinach groups them self.

Table (1) Weights of mice's before and after experiment

groups		We. Before experiment Mean ± SE*	We. after experiment Mean ± SE*
control		20.5 ± 0.64[a]	[c] 24.00 ± 0.33
Premix		20.o ± o.41[a]	[b]28.00 ± 0.86
spinach	T1	2o.2 ± 0.85[a]	[ab]29.00 ± 0.51
	T2	20.5 ± 0.64[a]	[a]29.70 ± 0.30
	T3	20.5 ± 0.64[a]	[a]29.80 ±0.42

**Different letters: Significant difference (P ≤ 0.05) between means of column*

Table (2) summarized the results of the percentage of hemoglobin and the packed cell volume. There are clear significant differences between the control group and the other groups of mice in both values, the (PCV and Hb).

From the same table we found that there are a liner increase in the values of PCV and Hb as the percentage of spinach increase.

Also there is a significant difference between T3 and the other groups T1 and T2. On the other hand, there was a significant difference between T3 and premix in the both value of Hb and PCV.

The values of Hb and PCV of the control group are less than 13 and 40 respectively which were classified in the region of anemia, but the treated animals have the values of Hb and PCV out of the classification of anemia (37).

Table (2) the percentage hemoglobin (Hb) and the packed cell volume (PCV).

groups		Concentration in percentage	Mean ± SE* PCV	Mean ± SE* Hb
control		00.00	0.9±^{c}37.5	^{c}12± 0.40
premix			1.8±^{ab}47.83	0.6±^{ab}15.16
spinach	T1	10	^{b}46.5 ± 0.6	^{b}14.7 ± 0.21
	T2	20	^{ab}48.0 ±0.9	^{ab}15.6 ± 0.3
	T3	30	0.8±^{a}50.50	0.4±^{a}16.0

**Different letters: Significant difference (P ≤ 0.05) between means of column*

The difference was considered significant when the probability value was equal or less than (0.05).

The use of a chemical source of iron with a certain level can be causing some histological damage to some organ such as liver, spleen and kidney (16, 17, and 18).

In this study slides for the tissue of harts, kidney, liver and spleen had been made and studied. There are no effects for the food containing spinach on the hart of all groups as shown in figure (1).

Sections of the kidneys of mice feed with bread contain spinach T1K, T2K and T3K indicate that there are no effect on the tubuli renales and glomerulus but we found very few degeneration but the overall effects were negligible.

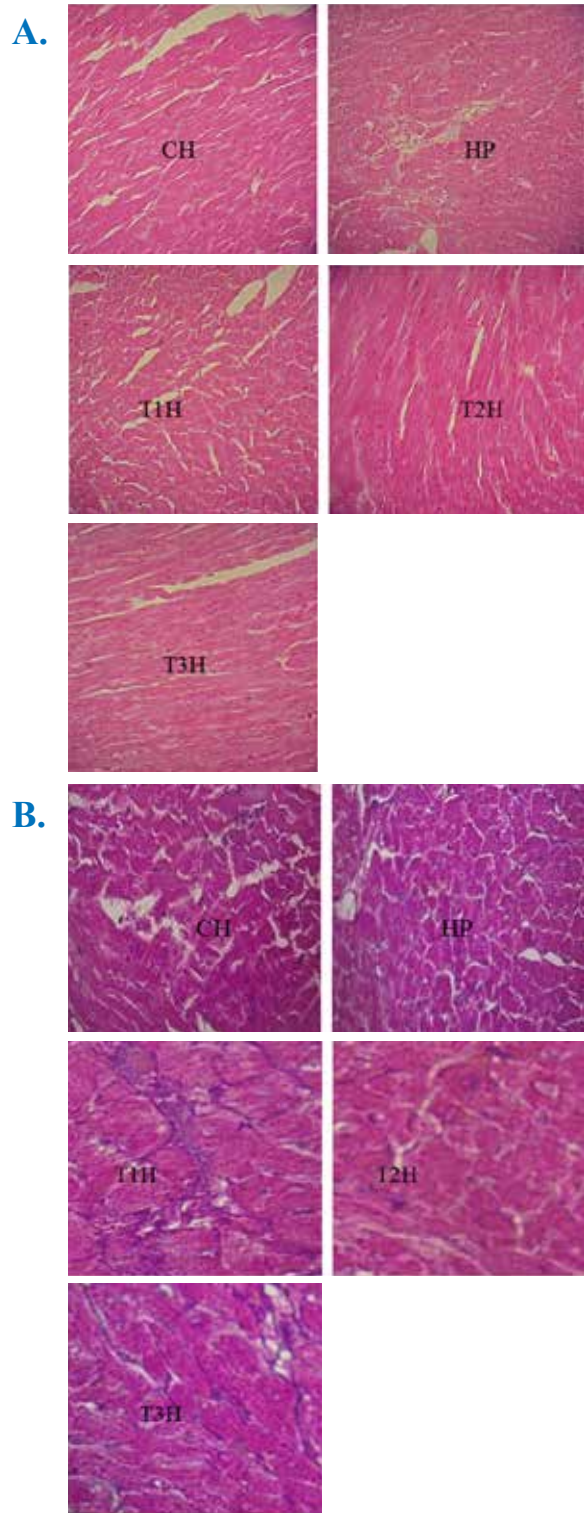


Figure (1) Sections of the heart of the control CH, treatment HP contain 20ppm iron, treatment T1H contain 10% spinach, treatment T2H contain 20% spinach and treatment T3H contain 30% spinach . Slides (A) stain with Perls Prussian to detect the excess of iron in the form of hemosidren , , but we can not find any precipitation of hemosidren. Slides B stain with hematoxylin to detect the effect on the cells of the tissues. 10x for A and 40x for B.

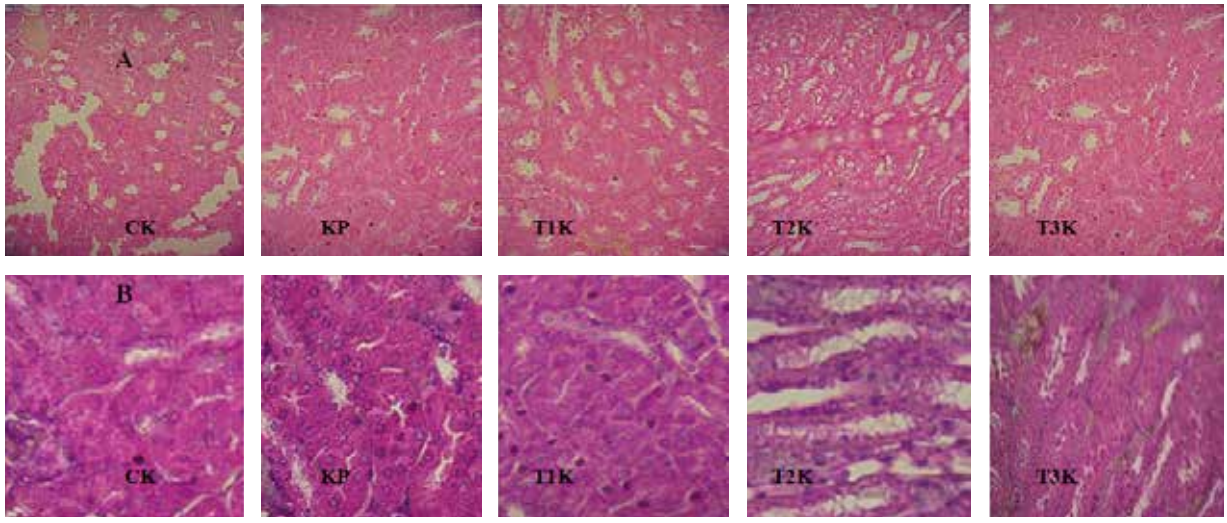


figure (2) Sections of the kidney of the control Ck, treatment KP contain 20ppm iron, treatment T1k contain 10% spinach, treatment T2k contain 20% spinach and treatment T3k contain 30% spinach. slides (A) stain with Perls Prussian to detect the excess of iron in the form of hemosidren , but we can not find any precipitation of hemosidren. Slides B stain with hematoxylin to detect the effect on the cells of the tissues. 10x for A and 40x for B

The liver sections of the mice's (figure 3), feed with both bread, contain premix and spinach did not show a clear histological effect

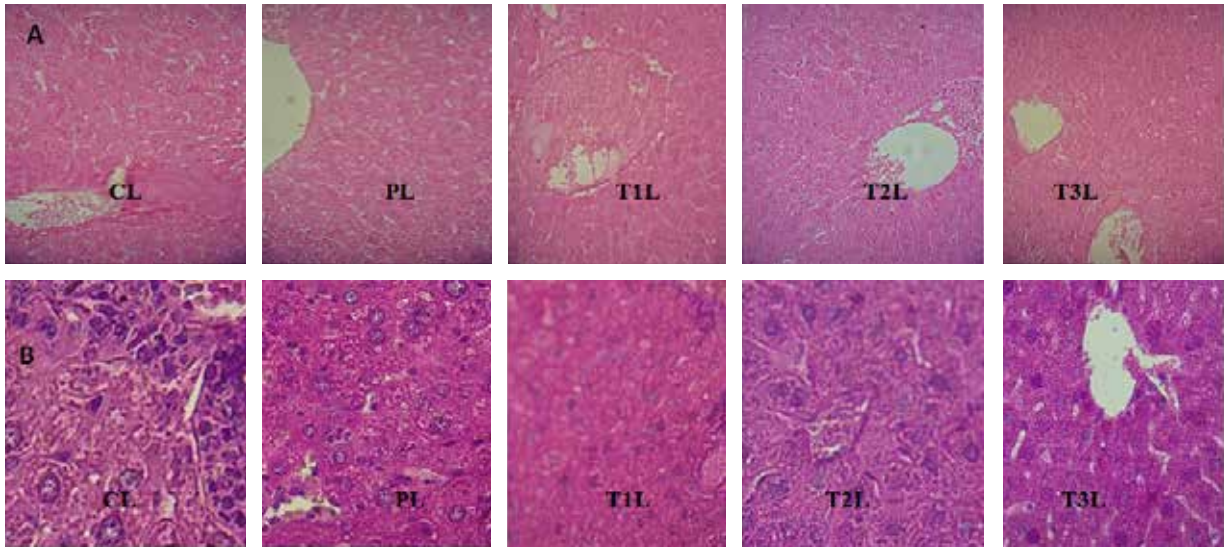


Figure (3) Sections of the liver of the control CL , treatment PL contain 20ppm iron, treatment T1L contain 10% spinach, treatment T2L contain 20% spinach and treatment T3L contain 30% spinach. slides (A) stain with Perls Prussian to detect the excess of iron in the form of hemosidren , but we can not find any precipitation of hemosidren. Slides B stain with hematoxylin to detect the effect on the cells of the tissues. 10x for A and 40x for B.

The sections of spleen figure 4 for all the samples do not show a significant effect on the tissue, only one slid of the group which was feed with food contain premix give an indication a precipitation of hemosidren.

This result give sport to what we believed about the addition of iron as a fortified nutrient must be in a low level and under a control.

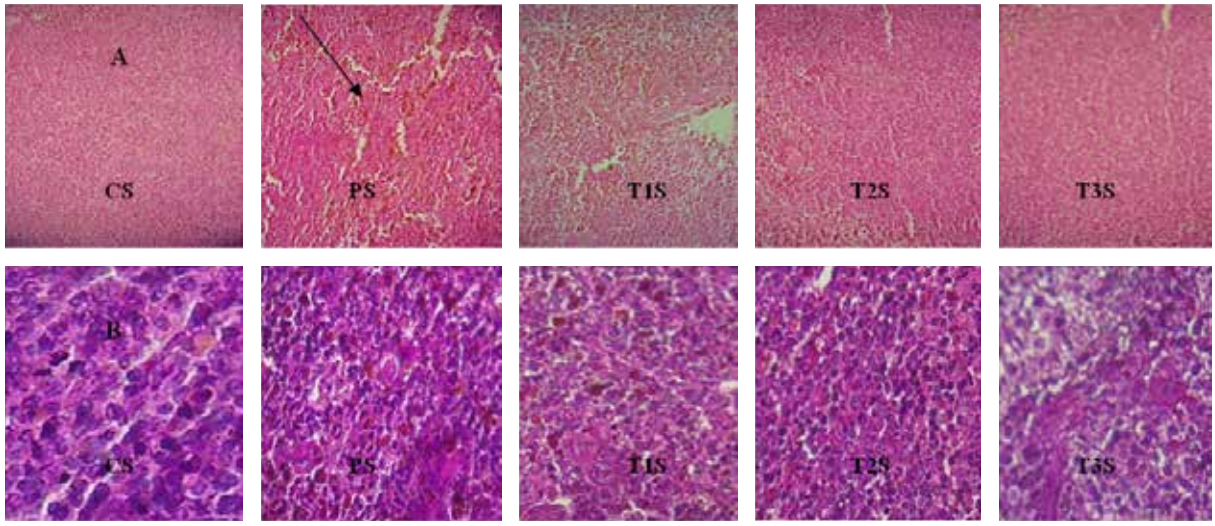


Figure (4) Sections of the spleen of the control CS, treatment PS contain 20 ppm iron, treatment T1S contain 10% spinach and treatment T2S contain 20% spinach . Slides (A) stain with Perls Prussian to detect the excess of iron in the form of hemosidren,.We find some precipitation of hemosidren on slide Ps (⟶) and that may lead to cues cancer . Slides (B) stain with hematoxylin to detect the effect on the cells of the tissues. 10x for A and 40x for B.

Generally all the slides of all organs did not show a reasonable difference between the control and the other groups.

On the other hand, as a response of the used phenylhydrazen to enhance an anemia we found some inflammatory cell.

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دراسه لمقارنة استخدام السبانخ Spinacia oleracea كمصدر للحديد مع كبريتات الحديدوز لمعالجة فقر الدم في الفأران

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الخلاصة:

في هذة الدراسة استخدم ثلاثون من ذكور الفاران نوع (Swiss albino) وبعمر ثمان اسابيع.قسمت الفاران الى خمس مجاميع بواقع ست فاران في كل مجموعة. استخدم في تغذية المجاميع الثلاث الاولى على الخبزالناتج من طحين الحاوي على نسب مختلفة من السبانخ (10% , 20% و30%) كمصدر للحديد. المجموعه الرابعه تم تغذيتها بخبز ناتج من نفس الطحين ولكن دعم بمادة ال(premix) الحاويه على كبريتات الحديدوز, حامض الفوليك و نشا الذره وحسب تعليمات المجهز (200 غم لكل 1000 كغم من الطحين). المجموعه الخامسه استخدمت كمجموعة سيطره (control) غذيت على خبز ناتج من نفس الطحين ولكن بدون اي اضافه. اسخدم نفس الغذاء المشار اليه حتى نهاية التجربه البالغه اربع عشرة يوم. صممت التجربه لاحداث فقر دم في اربع مجموات . استخدم الفينايلهايدزين في احداث فقر الدم لدى الفاران بعد ان تم حقنها لمدة يومين متتالين بمادة الفينايلهادزين . بعد يومين تم التضحية بفارين من كل مجموعة لغرض قياس الهيمغلوبين(Hb) وحجم مضغوط الخلايا (PCV) ومقارنت ذلك بقيمها بعد انتهاء التجربه. بعد نهاية التجربه اخذت نماذج الدم من قلب كل حيوان لقياس نسبة الهيموغلوبين وحجم مضغوط الخلايا, ومن ثم ضحي بكافة الحيونات وتم اعداد شرائح لا انسجة القلب, الكلية, الكبد و الطحال. اظهرت النتائج وجود فروقات معنوية بين اوزان الحيونات وكذلك في نسب الهيموغلوبين وحجم مضغوط بين مجموعة القياس والمجاميع الاخرى . فيما لم يلاحظ وجود تاثير ملموس في انسجه كل من القلب. الكلية, الكبد والطحال في كافة المجاميع.