ENTOMOLOGICAL SURVEILLANCE FOR DETECTION RIFT VALLEY FEVER VIRUS ACTIVITY IN THE RISK AREAS IN EGYPT
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ABSTRACT
In the present study, mosquitoes were collected and tested for the presence of Rift Valley Fever (RVF) virus by RT-PCR using specific primers to fragment of the M-segment of RVFV. The mosquitoes were collected through June to November of the year 2009. Mosquitoes collected from previously infected governorates during last outbreak, Sharkia, Dakahlia, Kafr-El Sheick and Aswan governorates was negative for amplification of RVFV M-segment., these results may reflect the low levels of circulating RVF virus typical in interepizootic periods in the surveyed areas or the virus may be not present in these areas.

KEY WORDS: Mosquitoes, RT-PCR, RVF, Survey.

1. INTRODUCTION

Rift Valley fever (RVF) is one of the most serious transboundary animal diseases and is caused by a member of the Phlebovirus genus, one of the five genera in the family Bunyaviridae. RVF virus (RVFV) is transmitted by mosquitoes, particularly those belonging to the Culex, Anopheles and Aedes genera [1, 2]. Transmission is mostly horizontal, but a vertical mode has been described for some Aedes species [3, 4] where the RVFV is present in the eggs of Aedes mosquitoes which breed in isolated depressions called dambos often found in grassland areas. When dambos are flooded during periods of extensive and widespread rainfall, these eggs hatch and the subsequent adult mosquitoes are capable of transmitting the virus to domestic animals including sheep, goats, cattle, camels, and buffalos [5]. These depressions are also good breeding habitats for Culex and Anopheles mosquitoes’ species. When Aedes mosquitoes infect domestic animals with RVFV, viral amplification occurs in these vertebrate hosts resulting in a RVF outbreak. Blood feeding on these infected animals by other competent vectors, including Culex and Anopheles mosquitoes, can increase the range of the original outbreak due to insect flight or even wind-borne dispersal [6]. Cases of RVF in Human are typically caused by direct contact with the fluids from infected animals after spontaneous abortion or slaughtering of viremic animals. When an epizootic occurs in animals, it is easily transmitted to humans leading to an epidemic [7].

RVF virus was first identified in 1931 in the Rift Valley of Kenya [8]. Egypt has suffered several epidemics of RVF and consequent animal losses and human infection. In 1977, RVF disease was identified for the first time in Egypt and caused abortion in pregnant domestic animals. Some depressions are also good breeding habitats for Culex and Anopheles mosquitoes’ species. When Aedes
ruminants and deaths in young lambs [9]. After that epidemic, the next documented RVF outbreak was in early 1981 [10]. After 12 years of absence, the RVF disease was again noted in human patients and domestic ruminants in late May 1993 at Aswan Governorate [11]. RVF outbreaks occurred again in Egypt between April and August 1997 in Aswan and Assiut governorates where infected cattle and sheep exhibited high fever, icterus, bloody diarrhea and abortion [12]. The purpose of the current study is to assess RVFV activity in the risk areas in Egypt during the summer and autumn seasons (2009) by assessing infection rates in wild caught mosquitoes.

2. MATERIALS AND METHODS

2.1. Insect collection and identification.
A total of 3840 mosquitoes were captured in the field using CDC - miniature light traps. Mosquitos’ surveillance was conducted in the Dakahlia, Sharkia, Kafr-El shick and Aswan governorates and trapping performed at different seasons of the year. Trapping was performed as described by the John W. Hock Company (2004). The captured mosquitoes were transported dry to the laboratory and identified according to the species level using morphological keys [13]. After identification, female mosquitoes were grouped into 410 pools (each pool contains 5-10 mosquitoes of one species and sex), according to mosquitoes species, month of collection, location, feeding status. A PCR assay was used to detect RVFV in these pools of mosquitoes.

2.2. Detection of RVF virus in collected mosquitoes pools using RT-PCR.

2.2.1. RNA extraction.
Total RNA was extracted from individual insects using RNEasy kit according to manufacturer's instructions (QIAGEN).

2.2.2. One-step RT-PCR on RNA assay for RVFV on RNA samples.
A total PCR reaction volume of 20 μl contained 12 μl of reaction mix provided with the kit (Qiagen kits) and 8 μl of RNA. The PCR primers for this assay (RVS.AAAGGAACAATGGACTCTGGTC, RVAs.CACTTTTACTACCATGTC, CTCCAAT, RVP. AAAGCTTTGATATCTCAGTGCCCAA) anneal to G₂ glycoprotein region within the M segment of the tripartite negative-sense single stranded RNA genome of RVFV [14]. RT-PCR of RVFV with 5’ nuclease probe detection involved reverse transcription at 50 °C for 30 min, initial denaturation at 95°C for 15 min, and 45 cycles with 95°C for 5 sec and 57 °C for 35 sec. fluorescence was read at the combined annealing extension step at 57 °C. PCR was performed using an ABI 7500. The ≥ 95% detection limits of the RT-PCR were determined by probit using the Statgraph plus 5.0 software package (statistical Graph Inc) According to [14].

2.3. Reverse transcriptase reaction for detecting RNA quality by testing mosquito RNA by insecticide resistance primer.
Because all field-caught mosquitoes were negative for RVFV, it was important to demonstrate that RNA extracts contained amplifiable targets, otherwise, negative results could be due to poor quality RNA. To verify the quality of RNA in extracts, transcripts of two mosquitoes genes involved in insecticide resistance (ace-1 and para) were amplified from a subset of the mosquitos’ pools. Total RNA was extracted from five mosquitos’ samples using the guanidinium thiocyanate phenol-chloroform method. RNA was reversibly transcribed into single-stranded cDNA using the Superscript II reverse transcriptase (Life Technologies, France). The reaction was primed with a mixture of 45 μl of Master mix and 5 μl of samples of RNA mosquitoes. The PCR conditions
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were 30 min at 50°C and 15 min at 95°C then 1 min at 94°C, 2 min at 48°C and 2 min at 72°C for 40 cycles followed by 10 min at 72°C. DNA fragments were separated by electrophoresis on 1.5% agarose gels and were visualised by ethidium bromide staining under UV light. The procedures were carried out according to [15,16].

3. RESULTS

3.1. Entomological results.
A total of 3840 mosquitoes, belonging to 4 genera and 7 species, were collected from Dakahlia, Sharkia, Kafr-El shick and Aswan governorates at different seasons of the year (Table 1). The predominant species at Sharkia was Culex pipiens (85.71%), followed by Culex antennatus (10.31%), Anopheles pharoensis (2.87%) and lastly Culex theileria, Aedes dentatus and Culiceda (of each 0.48%). At Dakahlyia, the most abundant species was Culex antennatus (96.92%), followed by Cx. Pipiens and Anopheles pharoensis (1.53%). At Kafr- El Shick, the Culex antennatus was the most abundant species (59.09%), followed by Cx. Perexiguus (18.18%), Cx. Pipiens (13.63%) and lastly Anopheles pharoensis (9.09%). The most frequent species at Aswan was Cx. Pipiens (50%), followed by Cx. Antennatus and Anopheles pharoensis (of each 25%). These results indicate that the Culex genus was more abundant than Anophelus and Aedes genera. A total of 410 monospecific pools were constituted regarding to species, and site of collection. Individual mosquitoes were then submitted to RT-PCR assay for RVFV detection.

3.2. RT-PCR amplification using RVFV specific primer set.
A reverse transcriptase polymerase chain reaction (RT-PCR) was applied to detect Rift Valley Fever Virus (RVFV) in Culex pipiens mosquitoes pools collected during the period from July to November 2009 from Sharkia, Dakahlyia, Kafr- El Shick and Aswan governorates of Egypt.

Table 1 Indicates number and species abundance of mosquitoes during the period from July to November 2009 at different location of Sharkia, Dakahlyia, Kafr-El Sheick and Aswan governorates.

<table>
<thead>
<tr>
<th>Governorate</th>
<th>Species</th>
<th>Total Number</th>
<th>Species Abundance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sharkia</td>
<td>CX. Pipiens</td>
<td>216</td>
<td>85.6%</td>
</tr>
<tr>
<td></td>
<td>CX. antennatus</td>
<td>26</td>
<td>10.31%</td>
</tr>
<tr>
<td></td>
<td>CX. perxigus</td>
<td>2</td>
<td>0.79%</td>
</tr>
<tr>
<td></td>
<td>CX. theileria</td>
<td>1</td>
<td>0.48%</td>
</tr>
<tr>
<td></td>
<td>Anoph. Pharoensis</td>
<td>5</td>
<td>2.87%</td>
</tr>
<tr>
<td></td>
<td>Aedes. Det</td>
<td>1</td>
<td>0.48%</td>
</tr>
<tr>
<td></td>
<td>Culiceda</td>
<td>1</td>
<td>0.48%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>252</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>CX. pipiens</td>
<td>2</td>
<td>1.53%</td>
</tr>
<tr>
<td></td>
<td>CX. antennatus</td>
<td>126</td>
<td>96.92%</td>
</tr>
<tr>
<td>Dakahlyia</td>
<td>Anoph. pharoensis</td>
<td>2</td>
<td>1.53%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>130</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>CX. pipiens</td>
<td>3</td>
<td>13.63%</td>
</tr>
<tr>
<td></td>
<td>CX. antennatus</td>
<td>13</td>
<td>59.09%</td>
</tr>
<tr>
<td>Kafr-El Shick</td>
<td>CX. perxigus</td>
<td>4</td>
<td>18.18%</td>
</tr>
<tr>
<td></td>
<td>Anoph. pharoensis</td>
<td>2</td>
<td>9.09%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>22</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>CX. pipiens</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>CX. antennatus</td>
<td>1</td>
<td>25%</td>
</tr>
<tr>
<td>Aswan</td>
<td>Anoph. pharoensis</td>
<td>1</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Abundance = number of individuals of one species/ total number of mosquitoes collected (approximated to one decimal).
The RT-PCR approach is a fast, efficient alternative to the more time consuming and cumbersome conventional virus isolation laboratory procedure [17]. Figure (1&2), shows the amplification plot and report of RT-PCR analysis, which indicated that all collected mosquitoes pools were negative for RT-PCR for RVF virus. The blue and brown lines indicate the experimental positive control (RVFV RNA) which was used at different dilution (1.10 and 1.100).

Figure (1) The real-time detection of the specific PCR products by fluorescence (y axis) (F1 detection wavelength or F1/F2 ratio) dependent on the PCR cycle number (x axis)

Fig. 1 The real-time detection of the specific PCR products by fluorescence (y axis) (F1 detection wavelength or F1/F2 ratio) dependent on the PCR cycle number (x axis)

Fig. 2 RT-PCR result for detection of RVF virus in mosquitoes pools were collected during the period from July 2009 to November 2009 from Sharkia, Dakahllya, Kafr- El Shick and Aswan governorates. Reverse transcriptase reactions were done on extracted RNA of mosquitoes samples by using mosquitoes insecticide resistance primers for check on the quality of RNA extracted step. Figure (3) clearly shows that field caught mosquitoes contain the expected 194 bp amplification product for ace-1. Figure (4) shows the same RNA pools but with a second target amplified.

Again, all field caught mosquitoes produce a band of expected size (481-510 bp) for para. In both experiments, the bands generated by the field mosquitoes is the same size as that produced by Laboratory Cx.pipiens DNA and Laboratory Cx.pipiens RNA which used as controls positive.

Fig. 3 Gel image of reverse transcriptase reaction done on extracted RNA of mosquitoes samples by using mosquitoes organophosphorus resistance primer (Ace 1) for showing the quality of RNA extracted.

Fig. 4 Gel image of reverse transcriptase reaction done on extracted RNA of mosquitoes samples by using mosquitoes pyrethroid resistance primer (Kdr mutations) for showing the quality of RNA extracted.

4. DISCUSSION

RVF is a common zoonotic disease transmitted between animal and man through mosquitoes. Mosquitoes perpetuate transmission either by vertical (transovarial) or by horizontal transmission to vertebrates which then infect other blood-feeding mosquitoes. The
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Relative importance of vertical versus horizontal transmission in maintaining virus transmission or in RVF outbreaks remains speculative. Because of this, entomological surveillance is necessary to assess RVF virus activity in wild mosquitoes. Mosquitoes’ surveillance was conducted in Nile Delta and Aswan during the summer and autumn seasons 2009, and focused on areas where RVF outbreaks were previously detected by using different epidemiological and biotechnological tools.

From the entomological surveillance during the period of study and areas under investigations, the Cx.pipiens was more abundant in Sharkia and Aswan governorates but Cx. Antennatus was more abundant in Dakahlyia and Kafr-El Shick. The difference of the mosquitoes species collected in this survey depends on the difference on biology and ecology behavior of each mosquito’s species. During flooding, Culex mosquitoes have the highest abundance and breed in dambos.

The mosquito’s density was recorded in July, August and peaked during September, followed by decline in October at Nile Delta (Sharkia, Dakahlyia and Kafr-El Shick governorates). The summer season is predicted to pose the highest risk of large mosquitoes populations in Sharkia, Dakahlyia and Kafr-El Shick. This risk is associated with the rice growing season. Conversely, the highest mosquitoes density in Aswan was recorded in November, perhaps due to increased humidity and warm temperature. High humidity or flooded rice fields, coupled with warm temperatures, act as risk factors for RVF virus transmission and maintenance in these governorates. The high humidity with warm temperature or flooded water rice field with warming temperature affected on the biology of vectors, by increasing feeding frequency and egg production, and by decreasing the length of the development cycle and the extrinsic incubation period lead to increase vector density and increase vector capacity to transmit the virus [18].

RT-PCR is a rapid, sensitive and specific tool for detecting arthropod-borne viruses [19]. Although RVFV was not detected in wild caught mosquitoes, these data do not necessarily prove that the study areas are free of infection. Egypt is currently experiencing an inter-epizootic period; consequently, circulating virus levels may be very low in the mosquitoes’ population. This scenario is consistent with [20], where in an outbreak situation, RVFV was isolated from sixteen-abortion foeti and one dead calf in Madagascar, but analysis of 11,371 mosquitoes (61% Culex antennatus) collected at the same localities failed to isolate the virus. Thus, even in areas with high levels of RVFV transmission, entomologic surveys may sometimes fail to detect the viral load in insects.

Taken together, it is recommended that seroprevalence studies of animal and mosquitoes are necessary to obtain epidemiological data about RVFV in Egypt. Also, existing surveillance systems must be alert for outbreaks in neighboring countries and the possibility of sporadic or flareup infections during inter-epizootic period.

6. REFERENCES

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BIOCHEMICAL EFFECT OF ERYTHROPOIETIN IN IRON OVERLOADING
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ABSTRACT
This study aimed to follow the effect of Erythropoietin hormone in experimental iron overloading induced in male albino rats. Was studied revealed that: I- iron overload, led to significant increase in serum iron, ferritin, Triiodothyronine (T3), Thyroxin (T4), Thyroid Stimulating Hormone (TSH), Parathyroid hormone (PT), Hemoglobin (Hb), Red blood cells (RBCs), Glycosylated hemoglobin (HbA1C). However, there were significant decrease in Total iron binding capacity (TIBC), Free-Triiodothyronine (fT3), free Thyroxine (fT4), Glucose–6-phosphate dehydrogenase (G6PD), Bands of Hemoglobin electrophoresis (HbA2 and Hbs). II- injection of Erythropoietin hormone lead to significant increase in TIBC, G6PD, Hb, RBCs and HbA1 (after 2 and 4 weeks), but occur significant decrease in: serum iron level, ferritin, HbA2 (after 2 and 4 weeks) and Hbs (after 2 weeks but not after 4 weeks). PTH, were without significant increase as compared with control group. III- injection of erythropoietin hormone with iron led to significant increase in serum iron, ferritin, total iron binding capacity, PTH, Hb, RBCs, HbA1 (only after 2 and 4 weeks), but occur significant decrease in: G6PD, HbA2 and Hbs (after 2 weeks but not after 4 weeks).

KEY WORDS: Erythropoietin hormone, Ferritin, Glycosylated hemoglobin, Iron, Thyroxin.

1. INTRODUCTION
The value of iron in maintaining growth and survival is offset by its potential to catalyze formation of highly reactive free radicals, which can damage cellular components [1]. Since the human body has no effective mechanism for excreting iron, cells must tightly regulate uptake and store iron safely, in order to prevent detrimental effects of free iron [2]. Recombinant human erythropoietin was studied in a controlled trial of patients with anemia refractory to oral iron treatment [3]. EPO can be used as erythropoiesis-stimulating agent and, therefore, is part of a finely-tuned feedback circuit that controls red blood cell levels. The aim of the present work was to investigate the effect of intra muscular iron therapy alone or interaction recombinant human erythropoietin in cases of iron overload, effects of iron overload on hemoglobin variants by using HB-electrophoresis and biochemical changes of body organs.

2. MATERIALS AND METHODS
2.1. Experimental animals:
Forty male albinos rats, 4–6 weeks old and of body weight range 100-150 gm were obtained from laboratory animals' research center, Faculty of Veterinary Medicine, Benha University. The rats were randomly classified into four groups:
Group I: (Control group) Comprised of (10) rats, were fed ordinary diet and not receive drugs injected with saline 1ml/kg body weight

Group II: (Iron overload group) Included (10) rats, were fed on normal diet and injected with three times (10 mg/kg body weight) of ferric hydroxide poly maltose complex "I.M".

Group III: (Erythropoietin) Comprised (10) rats administered dose injected with erythropoietin as (Epotin 4000 IU as Alpha recombinant human erythropoietin) "sc" per week (100 - IU/kg body weight), once weekly for 8 weeks.

Group IV: (Erythropoietin and Iron) Included (10) rats injected with dose of erythropoietin (100 IU/kg body weight), and also (10 mg/kg body weight) ferric hydroxide poly maltose complex "I.M" one weekly for 8 weeks.

Blood samples collected 4 times each 2 weeks. Clear serum used freshly for determination the following parameters Iron profile iron [4], TIBC, [5]. Ferritin), thyroid profile ( T3, FT3, T4, FT4, TSH by radioimmunoassay according to [6, 7], PTH [8], G-6PD, Meanwhile, EDTA samples used for determinations of Hb, RBCS Hb-Electrophoresis, by [8].

2.2. Statistical analysis:
Data were expressed as mean ±S.E. and were statistically analyzed by [9].

3. RESULTS AND DISCUSSION

Iron is used in treatment of anemia but overload leads to toxicity and cell death via free radical formation and lipid peroxidation, the oxidative stress from iron overload might cause osteoporosis free radical injury and cancer in the kidneys, liver, brain, femoral bone and neural effects in this study we concerned with hemoglobin (Hb) variants.

The obtained data in table proved that
1) Serum iron concentration was increased very highly significantly within the experimental period after (2, 4, 8) weeks within iron overload group. This data in agreement with [10]. Results revealed that iron-induced liver injury was reflected by significant changes in the liver function indices, hyperammonemia and reduced serum urea level. A significant deposition of iron in liver was associated with enhanced oxidative stress. But in case of (EPO) group serum iron concentration value decrease less than control this results similar to results that discussed by [11]. Careful evaluation of the subject's iron stores is necessary to utilize erythropoietin most effectively. Even patients with "normal" iron stores may respond poorly to erythropoietin therapy. The result is dampened red cell production relative to that seen with abundant iron stores. But in case of (EPO+Fe) group serum iron concentration was increased significantly after 2 and 4 weeks and no increase after 8 weeks and this in agreement with [12, 13] shown that IV iron is effective when administered in conjunction with relatively low dose epoetin therapy (2,000 IU/week) in patients with progressive renal insufficiency, causing a rise in Hb from 9.7 to 11.05 g/dL.

2) Serum ferritin concentration in (IO) group showed significant increase after 2 week and after 8 weeks but after 4 weeks highly significant increase this results in agreement with [14]. But in case of (EPO) group no significant increase after 2 weeks But proved significant decrease after 4 and after 8 weeks this results in accordance with [15]. Although the extent of iron overload differs among these patients, in these cases, the increase in serum ferritin is secondary to an increase in systemic iron [16], effective iron delivery system. Serum ferritin, which is believed to be iron-poor, carries much less iron than this. But in case of (EPO+Fe) group serum ferritin showed that significant increase after 2, 4 and 8 weeks. Ferritin plays an important role in the storage of intracellular iron, and has been the
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subjective of extensive recent reviews [17].

3) Serum TIBC concentration showed that in (IO) group significantly decreased after 2, 4 and 8 weeks. Other investigators suggest that [18] serum iron and total iron binding capacity (TIBC) decrease (the latter decreasing less than the other parameters). In order to differentiate functional iron deficiency from inflammation serial measurements of TSAT and SF are required. But in case of (EPO) group showed that highly significant increase after 2, 4 and 8 weeks [19]. But in case of (EPO+Fe) group, results showed that highly significant increase after 2, 4 and 8 weeks [20] dramatically demonstrated the interplay between iron and erythropoietin. This increase reflected the enhanced erythropoiesis produced by erythropoietin supplementation.

4) Hemoglobin (Hb) concentration in (iron overload) group, significant increase after (2, 4 and 8) weeks [21]. Hemoglobin increased by approximately 1 g/dl. This increase reflected the enhanced erythropoiesis produced by erythropoietin supplementation. The elevated hemoglobin level persisted for as long as 24 days after the initial infusion. But in case of (EPO) group it was proved that, significant increase after 4 and 8 weeks and no significant increase after 2 weeks, in turn, EPO may provide a protective environment for these cells. Oral iron might be as effective as IV iron in raising Hb in pre-dialysis patients. But in case of (EPO+Fe) group proved that, highly significant increase after 2 and 4 weeks and very highly significant increase after 8 weeks. Iron-deficiency anemia, stimulation of erythropoiesis, hypoxia, and dyserythropoietic disorders, all these conditions have been shown to fully repress hepcidin gene expression

6) Serum PTH concentration in (IO) group significant increase after 2, 4 and 8 weeks [25]. Increased levels of PTH in serum correlated positively with ferritin in 210 French patients with liver iron overload syndromes. But in (EPO) group no significant increase after 2, 4 and 8 weeks. But in (EPO+Fe) group significant increase after 2, 4 and 8 weeks [10] were observed in iron-overloaded rats with a reduction of femoral bone mineral density, i.e. reabsorption of calcium from the proximal tubular epithelial cells of the kidney might be affected and urinary discharge of calcium might be elevated.

7) Serum G6PD concentration iron overload group no significant decrease after (2,4) weeks and significant decrease after (8) weeks [26] results show that both in vitro (below 0.08 mM) and in vivo pharmacological levels of melatonin increased enzyme activity in erythrocytes. But in (EPO) group no significant increase after 2, 4 and 8 weeks Serum G6PD [27].
One of the most important antioxidant molecules that protects the SPMs is the reduced glutathione (GSH). The latter is produced in the pentose phosphate pathway that requires normal activity of glucose 6 phosphate dehydrogenase (G6PD). Deficiency of this enzyme leads to the deficiency of reduced GSH which is required to protect SPMs against lipid peroxidation and the consequent obstetric morbidity.

But in (EPO+Fe) group no significant decrease after (2) weeks and significant decrease after (2,8) weeks [28]. It was decreased in the presence of an allosteric inhibitor of G6PD, phospho (enol) pyruvate, and was not detected with G6PD of Leuconostoc mesenteroides, which does not possess the allosteric site.

8) blood HbA1 concentration was increased significant (In iron overload) group after (2) weeks and decrease significant after (4) weeks similar results reported by [16] HbF is gradually substituted by HbA in the peripheral blood, so that within the first two years of life, the characteristic hemoglobin phenotype of the adult with very low levels of HbF (less than 1%) is found . But in (EPO) group revealed that increased significant after (2, 4) weeks [29] whereas others are responsible for severe diseases such as sickle cell anemia. But in (EPO+Fe) group revealed that increased significant after (2, 4) weeks [30].

9) Blood HbA2 concentration. In (iron overload) group very highly significant decrease after 2 weeks and increase after 4 weeks this result in accordance to the [31] showed that iron anemia markedly reduced HbA2 levels, and these levels returned to higher values compatible with β-thalassemia detection after 12–16 weeks of treatment. But in (EPO) group revealed that very highly significant decrease after (2, 4) weeks Therefore, a careful determination of the HbA2 level is necessary to avoid diagnostic pitfalls [32].

But in (EPO+Fe) group revealed that very highly significant decrease after (2,4) weeks [19] have compared HbA2 values in β-thalassemia carriers with or without iron deficiency and observed no significant reduction of HbA2.

10) Blood HbS concentration In (iron overload) group no significant decrease or increase after (2,4) weeks [33]. But in (EPO+Fe) group revealed that in (thyroxin) group showed that [34] HbS and HbF values The HbS values and the interval between procedures had large ranges, reflecting the variability of clinical manifestations and clinical needs in different patients.

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The biochemical effect of erythropoietin in iron overload

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The aim of this study was to investigate the effects of iron overload and erythropoietin on the production of red blood cells and hemoglobin in rats. Following intramuscular injection of high doses of iron (30 mg/kg) into one group, high doses of erythropoietin were injected into the second group, and low doses of iron plus high doses of erythropoietin were injected into the third group. Blood samples were collected after 2, 4, and 8 weeks to measure the iron, ferritin, iron-binding capacity, thyroid-stimulating hormone, glucose-6-phosphate dehydrogenase, and types of hemoglobin (HbA1, HbA2, HbS).

Results showed that:
- Injection of high doses of iron through the muscle caused a significant increase in iron, ferritin, hemoglobin, and red blood cells. The increase was followed by a decrease in the second week, and then increased in the fourth week.
- Administration of high doses of erythropoietin under the skin caused a significant increase in iron-binding capacity and glucose-6-phosphate dehydrogenase, and red blood cells. The increase was followed by a decrease in the second and fourth weeks, while the thyroid-stimulating hormone remained unchanged compared to the control group.
- Administration of high doses of erythropoietin with low doses of iron caused a significant increase in iron, ferritin, hemoglobin, and red blood cells. The increase was followed by a decrease in the second and fourth weeks, while glucose-6-phosphate dehydrogenase and HbS decreased in the second week and increased in the fourth week compared to the control group.

It was concluded that:
- Using iron alone and using erythropoietin alone did not increase the levels of hemoglobin and red blood cells significantly, whereas the combination of iron and erythropoietin significantly improved the levels of hemoglobin and red blood cells and could be a beneficial tool in managing severe anemia.