INTERLEUKIN -2 (IL-2) AND GAMMA INTERFERON (IFN γ) OF LYMPHOCYTE CULTURE SUPERNATANT IN IRON DEFICIENCY ANEMIA PATIENTS WITH INFECTION

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Abstract

Iron is an essential nutrient for every living cells because of it role as molecule for transport of oxygen, as well as DNA synthesis through synthesis of ribonucleotide reductase. Iron deficiency anemia patients, especially pregnant women and children are more susceptible to infection because of deterioration of their immune response. This was supported by findings of decreased in phagocytic activities of white blood cells and T-cell lymphocyte proliferation impairment. Iron deficiency anemia (IDA) patients also affect working capacities hence diminishing working outcomes. Although the underlying mechanism of immune defect in iron deficiency anemia is not clearly understood, multifactor events considered play their contributing roles such as abnormality of ribonucleotide reductase enzym, impairment of T-cell proliferation and activities, altered cytokine production of IL-2 and IFNγ.

The study was done to asses the relationship of IL-2 and gamma IFN with infection in IDA patients on lymphocyte culture supernatant of IDA patients. Study was conducted on cross-sectional analytic design. Sixty-four iron deficiency anemia patients treated in Sanglah General Teaching Hospital were recruited, and 31 (48.4%) out of 64 IDA patients were man and 33 (51.6%) women, have been selected for the study. This study found 17 (26.7%) IDA patients with infection, aged 38 ± 14.48 years and 47 (73.3%) IDA patients without infection, with age average of 40.5 ± 14.4 years. All variables of data characteristics examined did not indicate any statistical significant difference between group of IDA patients with infection and those without infection. The average level of hemoglobin between the two groups did not differ statistically. Similar result was obtained if samples were differentiated into severe (Hb< 7g/dl) and mild anemia. The study also revealed that there were no differences of cytokine level observed between older and younger age (upper and below 44.5 years) in IDA patients with infection and without infection. Furthermore, no differences of cytokine level were found based on gender between IDA male 10.9 (8.60 – 12,65) (pg/l) patients and IDA female patients 10.6 (7.50 – 13.43) (pg/l) with Z -0.490, p =0.624. Nevertheless, significant differences were noted between supernatant of IL-2 and IFNγ in IDA patients with infection when compared to IDA patients without infection (Z= - 2.509, p= 0.012 for supernatant IL-2; and Z= -2.569, p= 0.010 for supernatant IFNγ).

The study conclusion is that level of IL-2 and IFNγ from lymphocyte culture supernatant of patient suffered from IDA with infection is significantly lower when compared to IDA patient without infection. It therefore summarized that lower level of IL-2 and gamma IFN in patients suffered from iron deficiency impaired their immune

response to certain infections therefore this findings support the theory that IDA patients more susceptible to get infected.

Key words : IDA with infection, Supernatant IL-2 and IFNγ.

Introduction

Iron deficiency anemia (IDA) is anemia caused by decreased level of iron in the body resulting in depleted iron reserve that is not sufficient for normal erythropoesis process. The most common cause of IDA is deficiency iron loss due to chronic bleeding.

Study of etiology of iron deficiency anemia for 3,5 years on 122 IDA patients revealed that ankylostomiasis, hemorrhoid, peptic ulcer and malignancy as a prominent causes. The study carried out in 2003 at Sanglah Hospital/Department of Internal Medicine, 14% of IDA cases were found with infections.[1] Iron deficiency can lead to increase morbidity especially for babies and pregnant woman due to impairment of immune response such as decreased killing activities of neutrophil, defect in T cell proliferation.[2,3]

The relation of iron deficiency with infections is difficult to study in humans, either by observational or non-interventional studies. As such, studies on the relation of IDA with infections tend to give inconsistent results. Several observational studies done on human subjects showed increase of infection cases in the subjects with IDA. On the contrary, other studies have found opposite results in which iron deficiency was even protective against infections.[4,5]

Many other studies on humans and experimental animals have shown significant decrease of the levels of certain cytokines in iron deficiency state. The study by Bergman *et al.*[6] on adult subjects with IDA showed a lower level of IL-2 than in normal subjects, but the levels of other cytokines such as IL-6, IL-10, and TNFα were not significantly different from those of normal subjects. A study in Malawi, Africa found IDA was associated with lower production of IFNγ and IL-8 as compared with subjects having normal iron state. In this study a strong correlation was also found between IDA and the level of IL6-producing lymphocytes.[7] Similarly, another study done in Paris on 53 patients with IDA and 28 controls with normal iron state found significant different levels of IL-2 in the two groups.[8]

A study on mice with IDA carried out by Kuvibidilla *et al*.[9] showed a decrease of IFN γ and IL-12 by 64% and 66% respectively, in which the level of these cytokines had positive correlation with iron status indicator (r=0.68). Another researcher proved that suppressed production of IFN γ in mice with lipo-polysaccharide tolerance caused macrophages dysfunction that subsequently impaired cellular immunity.[10] According to the above findings, it is understandable that the relation between pro-inflammation interleukin and iron deficiency remains unclear. The production of IL-2 and IFN γ as pro-inflammation interleukin and CMI parameters is assumed to decrease in IDA cases. Based on that, it is necessary to carry out a study to assess the level of IL-2 and IFN γ in human IDA patients.

Materials and Methods

This study was carried out using descriptive analytical cross sectional study to find out the mean difference of IL-2 and IFN γ levels in both IDA patients with infection and those without infection. The accessible population was all IDA patients who visited the Sanglah Hospital and several doctors' private practices in Denpasar. The study subjects were patients with IDA (intended samples) selected from the accessible population that met the inclusion and exclusion criteria by using *consecutive sampling technique*. The *actual study subjects* were patients with IDA who had confirmed to involve in this study by signing an informed consent. The criteria for IDA using combination of hypochromic – microcyter anemia with level of ferritin serum less than 20 ug/l. Exclusion criteria of study were: patients who suffered from protein energy malnutrition, patients who received immunosuppressive medicine, such as steroid and chemotherapy, minimum in the last one month, patients with renal failure, genetic disorders, and malignancies, patients who did not consent to involve in the study. Patients who finally consent to join the study were informed about the study aims. Materials of the study consisted of supernatant of lymphocytes culture for IL-2 and IFN γ concentrations examination as well as list of questionnaires for obtaining data on age, gender, body weight, infections suffered, use of immunosuppressive medicines, malnutrition, malignancy and genetic disorder, and IDA diagnosis.

All collected data were first assessed before taken for analysis. The normality test of *Kolmogorov-Smirnov* (*Shapiro-Wilk* for small samples) was done to assess whether the data were normally distributed or not. Descriptive statistics was used to illustrate patient's characteristics and frequency distribution of various variables. U Mann-Whitney test was used to evaluate the average difference of IL-2 and IFN γ concentrations in IDA with and without infection. The above statistic analysis used $p < 0.05$ as the significance standard, by means of statistic software *SPSS for window version 15.0* .

Results

There were 64 IDA patients in this study, 31 patients males (48.4%) and 33 females (51.6%) with age average of 40.5 ± 14.4 years. A number of 17 (26.7%) were IDA patients with infection and the remainder 47 (73.3%) were without infection. Type of infection mostly lung infection then followed by urinary infection, gastroenteritis and viral disease. All variables of data characteristics examined did not indicate any statistical significant difference between group of IDA patients with infection and those without infection. The average level of hemoglobin between the two groups did not differ statistically. Similar result was obtained if samples were differentiated into severe (Hb< 7g/dl) and mild anemia. Basic characteristics of IDA patients according to presence of infection are described in table 1.

Based on gender there were no significant differences showed between IDA male patients and IDA female patients as shown on table 2.

Table 1 Basic Characteristics of IDA Patient

Characteristics	IDA with Infection Means ± SD	IDA without Infection Means ± SD	P value
Age (years)	38±14.48	41±14.54	0.59
Sex (M/F)	8/9	23/24	0.89
Leukocyte (K/µl)	9.7±3.91	8.0±3.66	0.17
Hemoglobine (g/dl)	6.5±1.82	6.6±1.91	0.81
MCV (fl)	65.8±7.81	81.9±8.65	0.95
MCH (pg)	19.3±2.57	19.2±4.06	0.88
MCHC (%)	29.1±1.78	28.0±4.10	0.58
Thrombocyte (K/µl)	400.7±132.40	418.5±178.6	0.73
SI (µg/l)	12.1±3.95	13.5±7.18	0.34
Transferin Saturation (%)	4.28±1.90	3.51±2.03	0.22
Ferritin (µg/l)	10.5±8.26	8.6±7.50	0.39
AST (IU/l)	22.2±10.10	23.14±8.23	0.81
ALT (IU/l)	16.0±11.10	18.6±10.3	0.59
BUN (mg/dl)	10.7±4.84	16.2±6.46	0.05
SC (mg/dl)	0.99±0.20	0.78±0.31	0.07

Tabel 2
Profile of cytokine of Iron Deficiency Anemia (IDA) patients based on gender

Cytokine	Iron Deficiency anemia		Z value	P value
	Male (n=31) Median (Interquartile)	Female (n=33) Median (Interquartile)		
Supernatan IL-2 (pg/l)	10.9 (8.60 – 12.65)	10.6 (7.50 – 13.43)	-0.490	0.624
Supernatan IFNγ (pg/l)	26.3 (14.50 –43.00)	31.3 (17.50 –43.50)	-0.336	0.707

Tabel 3
Profile of cytokine of Iron Deficiency Anemia (IDA) patients based on median ages.

Cytokine	Iron Deficiency anemia		Z value	P value
	Age < 44.5 (n=39) Median Interquartile	Age > 44.5 (n=25) Median Interquartile		
Supernatan IL-2 (pg/l)	10.5 (9.5-13.4)	11.2 (7.9-13.1)	-0.186	0.85
Supernatan IFNγ (pg/l)	39.0 (21.3-48.3)	19.8 (2.2-40.3)	-2.408	0.06

Table 4.
Difference of Median Interquartile of Cytokine of Supernatant between IDA Patient with Infection and IDA Patient without Infection

Supernatant Cytokine	Iron Deficiency Anemia		Z value	P value
	With Infection (n=17) Median Interquartile	With Infection (n=17) Median Interquartile		
Interleukin-2 (pg/l)	9.7 (7.90–11.10)	11.4 (9.80–14.30)	- 2.509	0.012
IFNγ (pg/l)	20.4 (11.20–30.45)	39.2 (18.80–48.30)	- 2.569	0.010

As we aware of, old ages influence immune response as well as cytokine production. But on table 3 can be seen that there were no differences of cytokine level observed between older and younger age (upper and below 44.5 years).

Statistic test applied was the non-parametric one because the non-normal distribution of IL-2 and IFNγ considering that Kolmogorov Smirnov test gave insignificant results (IL-2 Z= 3.320; $p<0.001$ and IFNγ Z= 2.857; $p<0.001$) and still did not distribute normally even though a logarithm transformation has been applied to both variables. Hence, non parametric test of Mann Whitney U was used since this test was accurate as a substitute for other inadequate parametric tests that did not meet analysis requirements due to efficiency capacity close to 95% with an increase of n value.

The cytokine concentration difference examined from supernatant lymphocytes culture (Mann Whitney U test) of IDA group without infection is shown on table 4.3. It showed that IL-2 and IFNγ concentrations of the two groups were statistically significant difference with Z= -2.509 ; p= 0.012 and Z= -2.569 ; p= 0.010 respectively.

Discussion

Diagnosis of Iron Deficiency Anemia

Basically the diagnosis procedures of iron deficiency anemia consist of three components: (1) diagnosis of the anemia; (2) diagnosis of the iron deficiency, and (3) determination of the etiologic factors of the iron deficiency.[11,12] Staining of iron contained in bone marrow aspirate by the *Prussian blue* or *Pearls' staining* method comprises a diagnostic measure to determine the body's iron depot. Absence of stained iron as hemosiderin dots in cytoplasm of reticuloendothelial cells confirms the existence of iron deficiency, with no other lab examinations required.[13] However, examination of bone marrow's iron is a highly inpractical and invasive procedure; thus causing most patients tend to reject it.

In this study the diagnosis of iron deficiency anemia was based on finding of hypochromic, microcytic red blood cells and ferritin serum level < 20 ug/l. According to the *National Health and Nutrition Evaluation Survey II,* the use of two or more indicators of the iron status is highly recommended for the diagnosis of iron deficiency anemia. This study also noted that single low serum ferritin level comprised one important indicator for the diagnosis of iron deficiency anemia, besides *iron depleted state*.[14] A correlation exists between the level of serum ferritin and the level of body's iron reserve; thus iron deficiency is the only cause of low level of serum ferritin.[11,15,16] The weakness of serum ferritin examination for the diagnosis of iron deficiency anemia exists in the fact that the level of serum ferritin increases in infectious diseases, chronic inflammations, malignancies, and certain liver diseases. In spite of such conditions, however, the correlation of serum ferritin level with body's iron reserve still exists, though consequently with a higher value. The level of serum ferritin still has its high diagnostic value in the confirmation of iron deficiency anemia, provided the above abnormal conditions are taken into account in making interpretation. However, using serum ferritin level as a single indicator for diagnosis of iron deficiency anemia in the tropical areas tends to give such a result categorized as *under estimate,* due to several compounding factors. Hence, the use of combined several parameters is highly recommended.[17]

Iron Deficiency Anemia and Infections

In this study iron deficiency anemia with concomitant infections was found in 17 subjects (26.7%) of 64 patients with confirmed IDA. In 2006, according to patient records in the Division of Hematology and Medical Oncology of the Department of Internal Medicine, Sanglah Hospital, 21 of 78 IDA patients were with infections. In a previous study carried out in 2003 at Sanglah Hospital/Department of Internal Medicine, 14% of IDA cases were found with infections.[1] In Malawi, Africa 26% of IDA cases was found with HIV, 24% with bacterial infections, and 20% with malaria.[7] Another study done in Israel on

680 IDA patients found a total of 5644 episodes of acute ear infections with an average of 8.3 episodes per patient; and 77.6% among 528 patients had simultaneous infections in both ears.[18]

The relation of iron deficiency with infections is difficult to study in humans, either by observational or non-interventional studies. In fact, iron deficiency can be regarded as a form of malnourishment that exists as intertwined social and health problems resulted from poverty. Moreover, it is against ethical consideration to delay administration of iron preparation to patients with IDA because this may worsen the patients' condition. As such, studies on the relation of IDA with infections tend to give inconsistent results.[5]

Several observational studies done on human subjects showed increase of infection cases in the subjects with IDA. A study, considered a pioneer of its kind until now, was done in London in 1928 to observe the effects of IDA on infection episodes. In this study it was found that about half of 541 subjects studied had significant lower episodes of infections in the group given iron preparation. Another study involving a greater number of subjects was done in Chicago in 1966 where more than 1000 babies with IDA were studied; the result showed a significant lower number of upper respiratory tract infections in those babies given iron preparation.[19] Higgs dan Wells[20] studied 31 patients with chronic musculocutaneous candidiasis, of which 23 had IDA, and found that 9 among 11 patients got better outcome after given iron preparation. A prospective clinical study on postsurgery patients showed a significant greater number of infection cases among those having low level of serum ferritin (228 cases) as compared with those (220 cases) who had normal level of serum ferritin.[5]

A study in Chile on 100 babies of which half of the babies given formula food containing iron while the other half were given formula food without iron. Morbidity data of the babies were recorded during weekly home visits. It was found that 30% of the babies given food without iron experienced illnesses, in contrast to only 5% of the babies given food with iron. No significant difference was noted between respiratory tract infection and gastrointestinal infection cases in the two groups; however, there might possibly be a slight difference as there were only 2 cases of anemia among 53 babies given iron-containing formula food, while there were 14 anemia cases among 47 babies given food without iron.[21]

On the contrary, other studies have found opposite results in which iron deficiency was even protective against infections. A study in Somalia, which has been very much cited, showed that iron deficiency state was even beneficial to the patients. In this study, randomly selected 137 adult patients with IDA were divided into two groups who were given iron and placebo tablets, respectively, for one month. In the group given iron (N = 66) 36 subjects experienced infection episodes as compared with only 3 subjects of the placebo group (N = 71) who experienced infection episodes. Although much criticized for its short time provided for *follow-up* and lack of *double blinded* approach, this study has proven that in a certain state iron administration may cause increase of certain infection episodes.[4] Another study by Oppenheimer in Papua New Guinea has shown that low level of serum iron in

babies protected them from getting malaria. The finding that low level of serum iron could protect the babies from malaria may be explained from the aspect that malaria parasites need iron of erythrocytes for completion of their life cycles.[22]

Different from the above two studies were a few other studies that found iron status had no influence on the susceptibility to infections. A study in Tanzania involving 800 babies who were given iron supplement for 24 weeks found no increase of malaria cases, but other types of infections were not recorded. A study in Chile found a similar result; administration of fortified iron preparation to babies up to one year of age did not result in increase of diarrhea or upper respiratory tract infections. However, it was found that infection cases occurred more frequently among babies with iron deficiency than those with normal iron status.[21]

Many studies on humans and animals have shown the presence of disorders of the cellular immunity and other nonspecific immunities in subjects with iron deficiency, but the relation of iron deficiency with infection remains unclear. Susceptibility to infections comprises a complex mechanism; it does not depend on one's iron status alone but also on other factors such as body condition, types of parasites/microorganisms, and environment. Among the other influencing factors are exposure to microorganisms, other nutritional deficiency, types of population (babies, children, females, males, elderly), duration and severity of the deficiency, type, dosage, and duration of iron therapy, and existence of other preconditional factors. It is clear that these concomitant factors greatly influence the degree of susceptibility to and severity of infections, regardless of the level of iron status. Iron deficiency influences susceptibity to certain types of infection, and duration and severity of the infections depend on the body condition and types of parasites, either extra or intra cellular microorganisms.[23]

Results of our study seem to agree with the theory that patients with IDA are prone to infections, as indicated by the finding of significant lower level of plasma and supernatant IL-2 and IFNγ in IDA patients with concomitant infections as compared with those without infections ($p<0.05$). As a response to infection, the body must activate its immunity mechanism, either natural or adaptive ones. For developing an optimal immunity response, especially the cellular immunity response, the body produces and activates the cytokines IL-2 and IFN γ.

Our study results also agree with that of a study done in China where 63 IDA patients with recurrent upper respiratory tract infections were examined for their levels of IL-2 and sIL-2R as well as for their subset lymphocyte T cells. In the latter study it was found that activation of IL-2 and percentage of CD3 and CD4 were significantly lower in children suffering from IDA with repeated upper respiratory infections as compared with a healthy control group.[24]

Many other studies on humans and experimental animals have shown significant decrease of the levels of certain cytokines in iron deficiency state. The study by Bergman *et al.* on adult subjects with IDA showed a lower level of IL-2 than in normal subjects, but the levels of other cytokines such as IL-6, IL-10, and TNFα were not significantly different from those of normal subjects.[6]

A study in Malawi, Africa found IDA was associated with lower production of IFNγ and IL-8 as compared with subjects having normal iron state. In this study a strong correlation was also found between IDA and the level of IL6-producing lymphocytes.[7] Similarly, another study done in Paris on 53 patients with IDA and 28 controls with normal iron state found significant different levels of IL-2 in the two groups.[8] An experimental study on iron deficient made animals also showed a lower level of IL-2.[25] Kuvibidila in his study on mice that were made iron deficient found a lower level of IL-12p40 and IFNγ in the group of mice with iron deficiency as compared with those mice given rations with normal iron supplement ($p< 0.05$). There was a positive correlation between the levels of cytokines and indicators of iron state (r 0,688, $p= <0.05$).[9] A similar result was also found in a controlled study on children in which a lower secretion of IL-2 was noted in children with iron deficiency as compared with the healthy control group.[26]

The mechanism of how iron deficiency can disturb cellular and nonspecific immunity responses is not yet clearly understood, but it is thought to be due to multifactorial features. These include decrease of activity of enzymes containing iron such as ribonucleotide reductase, myeloperoxidase, decrease of cytokines production, decrease of competent T cells, and probably disturbance of signals transduction. The exact phase of disturbance of signal transduction where iron plays a part is yet to be clarified, although previous studies have confirmed the influence of the disturbance in the activity of protein kinase C and its translocation on the plasma cells membrane and T cells of the spleen. These findings were seen in both human and animal study subjects. It is also known that binding of iron causes decreased production of mRNA needed for development of protein kinase C.[27] In the initial stage of T cells activation, disturbance occurs in the hydrolysis of *phosphatidylinositol 4,5-bisphosphate* (PIP2) by phospolipase C (an enzyme containing zinc), with its final result as *inositol 1,3,5-triphophate* (IP3) and *diacylglycerol* (DAG), which regulate activity of protein kinase C. Both protein kinase C and hydrolysis of phospholipid membrane are important for the initial process of transduction signal that can cause proliferation of T cells and activation of other functions. Thus, disturbance of protein kinase C and hydrolysis of phospholipid membrane result in disturbance of immune response in subjects with iron deficiency.[28]

Conclusions

From results and discussion described above, it can be concluded that concentration of IL-2 and IFN γ in supernatant of lymphocytes culture was significantly lower in IDA patients with infection than those without infection. Therefore IDA patients were more susceptible to certain infection because of impaired immune response due to low level of IL-2 and IFN γ.

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