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# The Ratio of Heinz Body Formation in Different Hemoglobin Zurich Subjects

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# THE RATIO OF HEINZ BODY FORMATION IN DIFFERENT HEMOGLOBIN ZURICH SUBJECTS

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THE RATIO OF HEINZ BODY FORMATION IN DIFFERENT HEMOGLOBIN ZURICH PATIENTS

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I dedicate this thesis to my fiance, Xiao Ming, and to my parents. Without their love and support, this research would not have been possible.

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Hemoglobin Zurich is a hemoglobin anomaly that results when one amino acid (histidine) is substituted by arginine at position 63 in the beta chain of hemoglobin molecules [$\beta$ 63 His---Arg]. When Hemoglobin Zurich individuals are exposed to sulfonamide medication, their hemoglobins denature and subsequently form Heinz bodies which attach to the surface of the plasma membrane.

Four Hemoglobin Zurich family members were the subjects of the current study. They included a splenectomized female subject, non-splenectomized female and male subjects, and a non-splenectomized female member without Hemoglobin Zurich as the control. The results collaborate that splenectomy increases the number of erythrocytes containing Heinz bodies in peripheral blood. The menstrual cycle apparently has no statistical affect on the increased ratio of Heinz body-containing erythrocytes to normal erythrocytes.

# INTRODUCTION

Upon the administration of anti-microbial sulfonamides, patients with Hemoglobin Zurich (Hb Zurich) exhibit severe erythrocytic hemolysis. The abnormal hemoglobin was discovered in Zurich, Switzerland, in 1960 by Hitzig et al. [1]. Subsequently, other cases were reported in Maryland [2] and Japan [3]. Hb Zurich is the result of a specific genetic mutation [$\beta$63 His--Arg] in which there is a single amino acid substitution in the beta chain of the hemoglobin molecule [4]. The side chain of the distal arginine attaches itself to the propionate of the heme, leaving the heme pocket open, allowing sulfonamides access to the iron [5]. Denatured hemoglobins bind tightly to the erythrocytic membrane, often forming dense aggregates on the inner membrane surface termed Heinz bodies. Hemoglobin is denatured by being oxidized initially to methemoglobin. Minor structural rearrangements can lead to formation of a reversible hemichrome which under appropriate conditions can revert back to methemoglobin. Otherwise, the reversible hemichromes denature further to form irreversible hemichromes which eventually aggregate to form Heinz bodies. The rate of hemoglobin denaturation in Hemoglobin Zurich patients is greater than in normal individuals [6].

The abnormal hemoglobin employed in the current study was detected initially by Dr. Alfred Kraus, University of Tennessee Medical School, in 1965. Since Hemoglobin Zurich in this family has not been previously reported in the literature, a summary of the original case report follows. A female patient, approximately two months pregnant, developed a urinary tract infection during the latter part of January, 1965, and was treated with sulfadyne, eight tablets daily, four days continuously. On day four, she

became jaundiced and then developed aching in the neck, nausea, spots in front of her eyes, and dark urine. On admission to the Baptist Memorial Hospital, Memphis, Tennessee, she was found to have a temperature of 100°F and continued with the low grade fever for the next several days. Anemia and autohemolysis tests were performed. The urine was essentially normal. Initial blood analysis resulted in the following:

total protein 6.3;

albumin 4.3;

alkaline phosphatase, 3.0 King-Armscrong units;

serine glutamine pyruvate transaminase, 21;

cholesterol, 174;

bilirubin total, 6.2 mg%, direct, 0.2 mg%;

direct Coombs test, negative.

Because of these unusual findings, the following additional laboratory determinations were performed:

rapid screening for glucose-6 phosphate dehydrogenase deficiency, normal;

lactic acid dehydrogenase electrophoresis, normal;

two varieties of hemoglobin: one with the mobility of hemoglobin A and the other with the mobility of hemoglobin S;

sickle cell preparation, negative;

fetal hemoglobin, 3.5%.

Heinz body studies were initiated three days after the blood had been obtained from the patient. The blood was exposed to acetylphenylhydrazine and stained with brilliant cresyl blue. Numerous Heinz bodies were present in each cell. Peptide mapping and amino acid analysis of the hemoglobin resulted in its identification as Hemoglobin Zurich. Because of severe

anemia occurring in the second month of pregnancy, the patient was given several units of packed erythrocytes, and a splenectomy was performed. The spleen was found to be enlarged and estimated to be about three times normal size. The pathologist reported that the spleen weighed 150 grams. Microscopic examination showed no specific abnormalities other than some nucleated red cells in the splenic pulp. The rest of the family members were screened for the abnormal hemoglobin with the results shown in the pedigree chart, Figure 1.

Heinz bodies were first described in 1890 by Robert Heinz [7, 8]. They are insoluble, oxidatively denatured hemoglobin which reflects the presence of a metabolic derangement or abnormality in the primary structure of hemoglobin [9]. The principles of their formation and increased hemolysis are: 1) deficiency in one of the reducing systems of blood, e.g. glucose-6-phosphate dehydrogenase deficiency; 2) presence of an unstable hemoglobin, such as Hemoglobin Shepherk's Bush, Hemoglobin Gun Hill, Hemoglobin Philly and Hemoglobin Zurich; and 3) exposure to certain chemicals and drugs, e.g. sulfonamide medication inducing hemolytic anemia in people who have a reducing system deficiency and no unstable hemoglobin [10]. Since the spleen removes aged or defective blood cells and platelets from the blood and acts as a blood filter, the rate of removal of erythrocytes containing Heinz bodies after a splenectomy will be slower [11]. A study in which erythrocytes containing Heinz bodies were injected into premature infants and adults with and without a splenectomy reported that the rate of removal was decreased after splenectomy [12].

The purpose of this time-course study is to compare the ratio of intra-erythrocytic Heinz bodies of Hemoglobin Zurich members of the same family utilizing two parameters: splenectomized and non-splenectomized

individuals. The course of the study was five weeks.

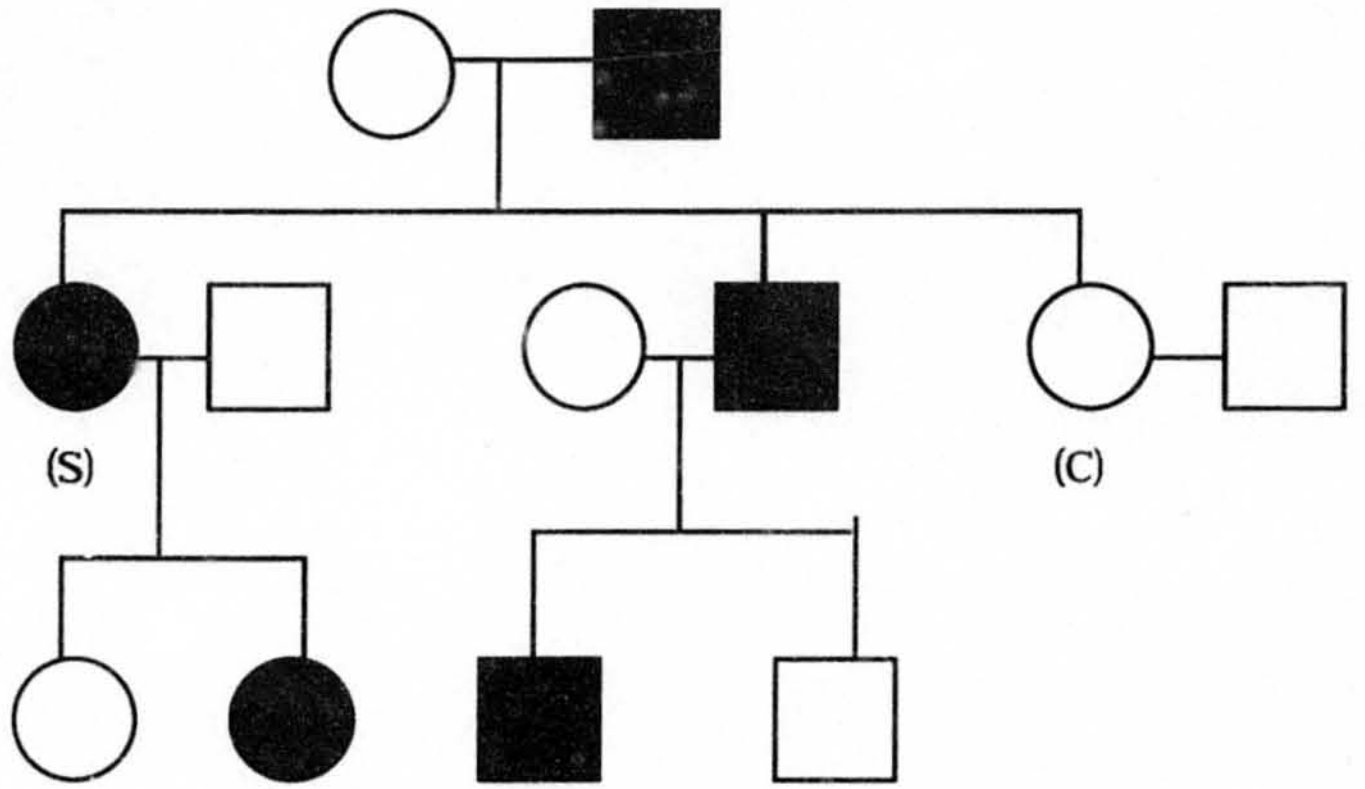


Figure 1. Pedigree chart of the Hemoglobin Zurich family. Abbreviations: S= Splenectomized female Hemoglobin Zurich subject; F= Non-splenectomized female Hemoglobin Zurich subject; M= Non-splenectomized male Hemoglobin Zurich subject; C= Female control.

## MATERIALS AND METHODS

Fresh Hemoglobin Zurich blood samples were acquired from the menostasic female subject (S), who was splenectomized in 1965; a non-splenectomized Hemoglobin Zurich female, (F), who is non-menostasic and the daughter of the subject ; a non-Hemoglobin Zurich, non-menostasic female, (C), the sister of S, who was one of the control subjects in this study; and a nephew of the individuals S and C, who was the second control, (Figure 1). Five to 10 ml of blood were drawn on Monday from each person for five continuous weeks by one of the following health facilities: The Medical Center at Bowling Green, Bowling Green Medical Clinic and the Student Health Clinic at Western Kentucky University, Bowling Green, Kentucky. EDTA was the anticoagulant.

The erythrocytes were stained with crystal violet obtained from Matheson Coleman and Bell Division, Norwood, Ohio. The blood was examined immediately after drawn, no more than four hours later, with one exception; the male subject's blood drawn on the fourth week was examined two weeks later.

Heinz bodies were stained with crystal violet according to the method of Beutler [12] with the following modification: crystal violet was dissolved in 0.9% (w/v) sodium chloride solution instead of 0.73% (w/v) sodium chloride solution. Crystal violet, 1 g, was added to 50 ml of 0.9% sodium chloride solution, shaken five minutes and filtered through miracloth, lot 901777, Calbiochem Corporation, La Jolla, California. The filtrate was mixed with 50 ml of 0.9% sodium chloride solution.

The erythrocytic pipette was inserted into the edge of the blood, and the blood was allowed to enter to the 0.5 mark on the pipette. The tip of the

pipette was immediately placed in the crystal violet solution until the fluid level reached the 1.01 mark. The pipette was shaken vigorously, and the erythrocytes were stained for five minutes.

The cover slip was placed in position over the counting area of a hemacytometer. The pipette was shaken again. The tip was placed exactly at the junction between the cover slip and the chamber. The diluted blood was drawn under the cover slip by capillary action. There were no bubbles present under the cover slip or fluid present in the moats.

The erythrocytes with and without Heinz bodies were counted in eight of the 25 groups of 16 small squares on the hemacytometer. The groups were randomly selected. All erythrocytes were counted which touched the upper and left boundary lines of the squares but not the cells touching the lower and the right boundary lines. The erythrocytes were observed employing a Nikon microscope (43X) from Nikon Inc. Instrument Division, Nippon Kogaka, K.K. The method of statistics that employed in this time-course study was the t test.

# RESULTS

Five weeks of data (Figure 2) illustrated that the female subject with a splenectomy had the highest ratio of Heinz body-containing erythrocytes to normal erythrocytes. In the fourth week, the non-splenectomized female Hb Zurich subject had the same ratio as the splenectomized Hb Zurich female subject because of menses. The non-splenectomized female and male subjects had an intermediate ratio, and the control had the lowest ratio. Table 1 and Figure 3 contain the mean ratios of the entire five weeks and standard deviations. Compared to the mean ratio of the control, the ratio of the female subject with a splenectomy had the highest significant difference. The female and the male subjects had highly significant differences as well (Table 2). Since the female subject with a splenectomy had the highest ratio of Heinz body-containing erythrocytes to normal erythrocytes and the highest significant difference among the three Hemoglobin Zurich subjects, a comparison of the splenectomized subject and the non-splenectomized subject was made. The results are also shown in Table 2. The ratio of erythrocytes with Heinz bodies of the splenectomized female subject and the non-splenectomized female subject still had a significant difference. Thus, the splenectomy had an affect on the ratio of Heinz body-containing erythrocytes to normal erythrocytes. After a splenectomy, the ratio increases. In order to determine if the male and the female subjects had a different ratio, the Heinz body content of erythrocytes from both of the non-splenectomized male and female subjects were studied. There was apparently no significant difference (Table 2 ).

During the five-week study, the non-splenectomized Hb Zurich female subject was observed to have a monthly jaundice, which was hypothesized

to possibly be associated with the menstrual cycle. In the fourth week of the study, the non-splenectomized female subject and the control started their menses on the same day which coincided with the same day blood samples were drawn. Figure 5 shows that both ratios increased. The change between the mean ratio of the first three weeks and ratio of the fourth week in the non-splenectomized Hb Zurich female and the female control were almost the same value (Table 3). The data in Table 3 indicate that the menstrual cycle had no specific affect on the increased ratio of Heinz body-containing erythrocytes to normal erythrocytes in the Hemoglobin Zurich subject since the female control had an increased ratio during the menstrual cycle as well (Figure 5).

The male subject's blood sample drawn in the second week which had been stored at 4°C, was examined again the fifth week. The ratio of erythrocytes with Heinz bodies of stored blood was the same as fresh blood. The three females' blood that was drawn and had been stored at 4°C for 4 months was re-analyzed. All the ratios of the two female subjects and the control were much higher than originally recorded. Table 4 and Figure 4 contain the results of this experiment.

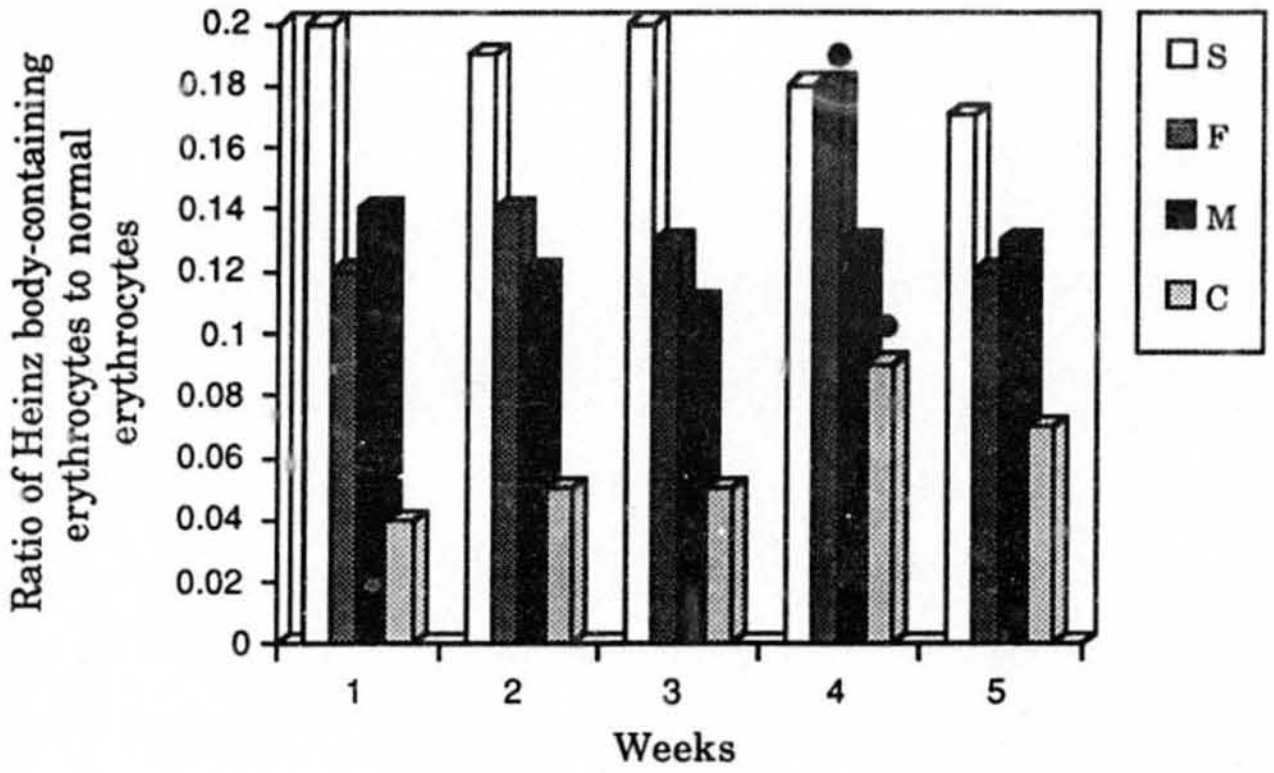


Figure 2. Five-week ratio of Heinz body-containing erythrocytes to normal erythrocytes in the splenectomized subject, the non-splenectomized subjects and the female control (● Menses)

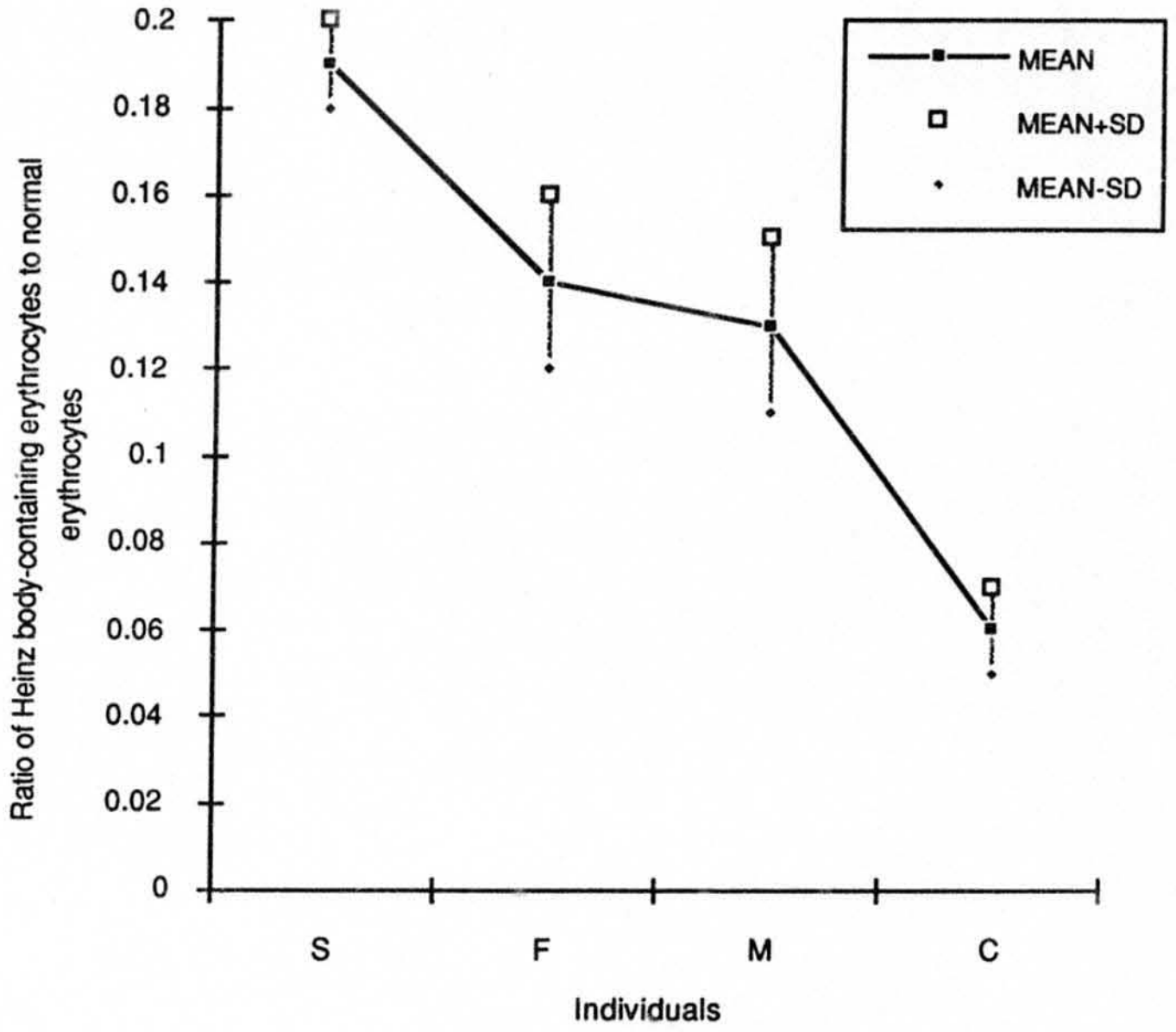


Figure 3. Mean ratio of the Heinz body-containing erythrocytes to normal erythrocytes and standard deviation in subjects and the control during five weeks. Abbreviations: S= Splenectomized Hb Zurich female; F= Non-splenectomized Hb Zurich female; M= Non-splenectomized Hb Zurich male; C= Female control.

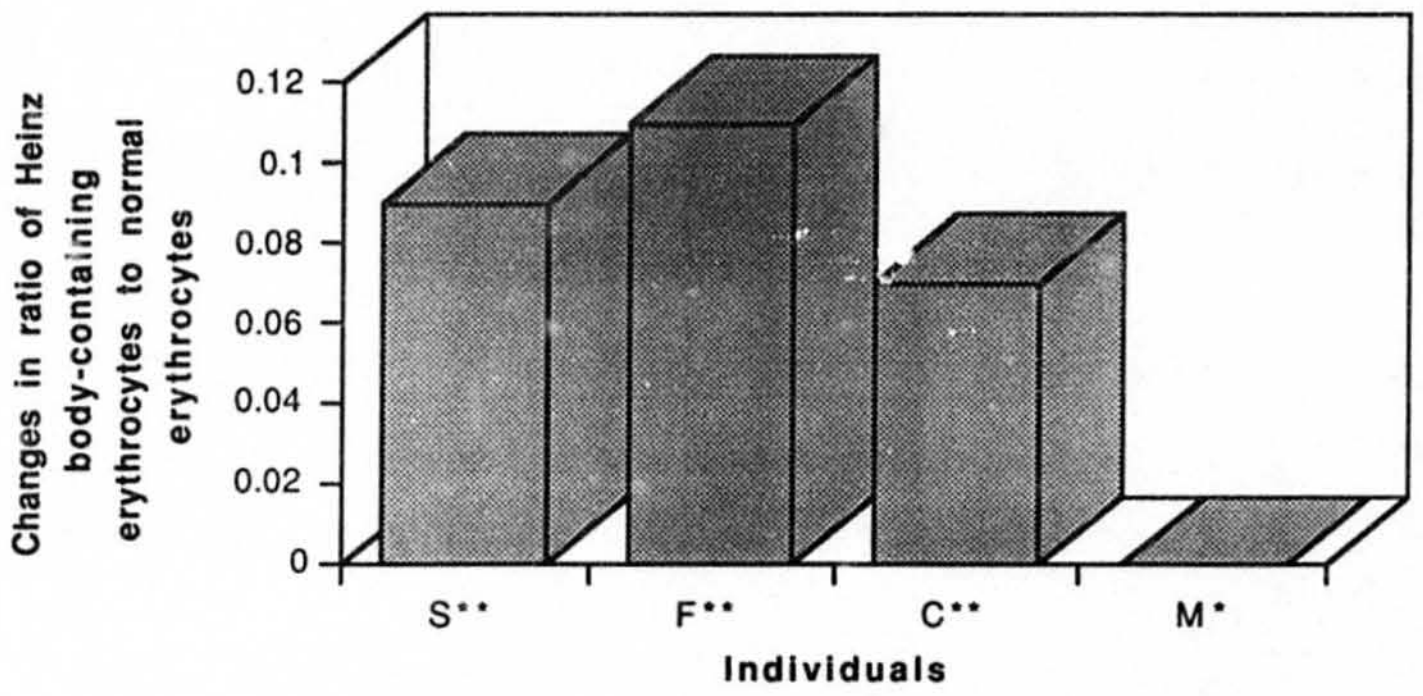


Figure 4. Changes in ratio of Heinz body-containing erythrocytes to normal erythrocytes in fresh and stored blood. ** Blood stored four months; * blood stored two weeks. Abbreviations: S= Splenectomized Hb Zurich female; F= Non-splenectomized Hb Zurich female; M= Non-splenectomized Hb Zurich male; C= Female control

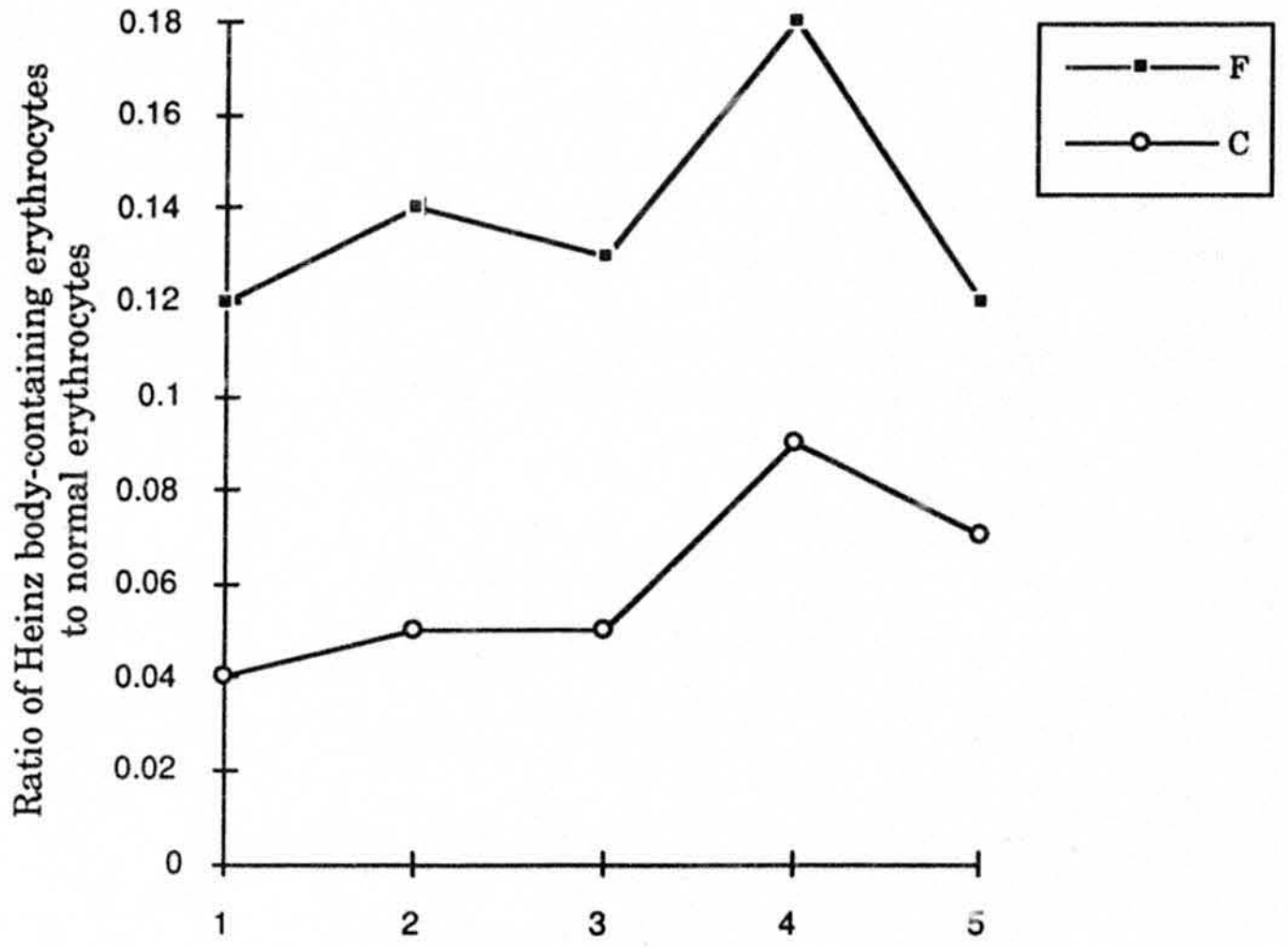


Figure 5. Comparison of five-week ratio of Heinz bodies of a non-splenectomized female with Hemoglobin Zurich (F) and the normal female control (C). (Menses started in the fourth weeks)

TABLE 1

Mean ratios of Heinz body-containing erythrocytes to normal erythrocytes and standard deviations in Hemoglobin Zurich (Hb Zurich) subjects and the control during five weeks

| Subjects | Mean* | Standard deviations |
|---|---|---|
| Splenectomized female Hb Z (S) | 0.19 | 0.01 |
| Non-splenectomized female Hb Z (F) | 0.14 | 0.02 |
| Non-splenectomized male Hb Z (M) | 0.13 | 0.01 |
| Non-splenectomized female control (C) | 0.06 | 0.02 |

*(n=5)

TABLE 2

t values and probability between the Hemoglobin Zurich subjects and the control

| Subjects | t values | Probability |
|---|---|---|
| Splenectomized female Hb Zurich vs control (S vs C) | 12.1490 | 0.001 |
| Splenectomized female Hb Zurich vs non-splenectomized female Hb Zurich (S vs F) | 3.9680 | 0.020 |
| Non-splenectomized female Hb Zurich vs control (F vs C) | 5.6022 | 0.010 |
| Non-splenectomized male Hb Zurich vs control (M vs C) | 6.7698 | 0.010 |
| Non-splenectomized female Hb Zurich vs non-splenectomized male Hb Zurich (F vs M) | 0.8137 | 0.500 |

TABLE 3

A comparison of the ratio of Heinz body-containing erythrocytes to normal erythrocytes between the non-splenectomized Hb Zurich female and the female control during their menstrual cycles

| Mean Ratios | F* | C** |
|---|---|---|
| First three weeks (average) | 0.130 | 0.045 |
| Fourth week | 0.180 | 0.090 |
| Difference | 0.050 | 0.045 |

*F: Non-splenectomized Hb Zurich female subject
**C: Female control

TABLE 4

The ratio of Heinz body-containing erythrocytes to normal erythrocytes of fresh and stored blood

| Sample | S* | F* | C* | M** |
|---|---|---|---|---|
| Fresh | 0.20 | 0.12 | 0.04 | 0.13 |
| Stored | 0.29 | 0.23 | 0.11 | 0.13 |
| Ratio differences between fresh and stored samples | 0.09 | 0.11 | 0.07 | 0 |

S= Splenectomized female Hemoglobin Zurich (Hb Z)
F= Non-splenectomized female Hb Z
M= Non-splenectomized male Hb Z
C= Non-splenectomized female control
* Blood was stored four months.
** Blood was stored two weeks.

# DISCUSSION

The results of this study indicate the existence of a significant difference in the ratio of Heinz bodies to erythrocytes between the three Hb Zurich subjects and the experimental female control. These results indicate that Hemoglobin Zurich is less stable than normal hemoglobin since Heinz bodies are denatured hemoglobin attached to the inner plasma membrane surface. The replacement of the distal histidine by arginine in Hb Zurich leaves a gap at the entrance to the heme pocket so that sulfonamides and other steric-related oxidants can bind to the heme-iron pocket [6]. Hb Zurich subjects are usually asymptomatic without anemia and severe jaundice unless they are administrated oxidative drugs, especially sulfonamides [3]. During the five-week period, all the subjects and the control were not taking any medication, but Hb Zurich individuals had the higher ratios of Heinz body-containing erythrocytes to normal erythrocytes. According to a study done by Ernesto et al. [7], the abnormal beta chains in Hb Zurich have an autoxidation rate exceeding those of the normal alpha chains. The difference in the rate of autoxidation between beta-normal and beta-Hemoglobin Zurich is about 25-fold. The alpha chain of both normal Hemoglobin and Zurich Hemoglobin have very similar rates of autoxidation [8]. Thus, even in the absence of administered sulfonamides, Hb Zurich subjects still contain more Heinz bodies in peripheral blood than in the control. Another reason may be that the rate of blood filtered by the spleen is constant; therefore, the number of Heinz body-containing erythrocytes should increase in peripheral blood when splenic filtration is saturated.

Also, Heinz body formation in cells can be induced by subjecting the cells to oxidative stresses. These stresses may arise as part of the body's normal defense mechanism against infection and disease. Firstly, the body temperature could be sufficiently raised to drastically reduce the stability of the hemoglobin and cause it to precipitate as Heinz bodies. Secondly, the oxidants, such as superoxide and $H_2O_2$ produced by phagocytic cells when destroying an infectious agent, could accelerate oxidation and denaturation of the unstable hemoglobin [10].

The ratio of Heinz body-containing erythrocytes to the normal erythrocytes of the splenectomized Hb Zurich subject was statistically significant compared to the other non-splenectomized individuals. The implication is that splenectomy has an effect on the increased number of erythrocytes containing Heinz bodies in peripheral blood. One of the most important functions of the spleen is to remove aged or defective blood cells and platelets from the blood. This function is lost after a splenectomy. Peripheral blood alterations occurring after splenectomy include prompt increase in the number of leucocytes, platelets, immature nucleated erythrocytes and target cells; a tendency toward thinness of the red cells; decreased osmotic fragility; and the appearance of Heinz bodies [11]. As previously described, the study in which erythrocytes containing denatured hemoglobin were injected intravenously in premature infants and patients following splenectomy, the rate of Heinz body disappearance was slower in splenectomized patients than in control subjects [12].

Both of the non-splenectomized male and female Hb Zurich subjects had a cyclic jaundice. However, it is clear that the cyclic jaundice is apparently not due to the menstrual cycle since it occurs in both the Hb Zurich male

and the female. The female control, who still undergoes menses, had an increased ratio of Heinz body-containing erythrocytes to normal erythrocytes during menses as well. The cyclic jaundice can not be explained at the present time.

In conclusion, Hb Zurich subjects had a higher ratio of Heinz body-containing erythrocytes, and the splenectomized subject had the highest ratio. The menstrual cycle had no effect on the increased ratio of Heinz body-containing erythrocytes to normal erythrocytes of Hb Zurich subjects. There may be a specific cycle of increased Heinz body-formation in peripheral blood for non-splenectomized Hb Zurich subjects.

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