Relationship between oxidative stress, ferritin and insulin resistance in sickle cell disease

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Abstract. – Background: Sickle cell disease (SCD) is a hereditary hemoglobinopathy characterized by hemolytic anemia. The oxidative phenomena play a significant role in its pathophysiology. Blood transfusions are a therapeutic mainstay in SCD and repeated transfusions can result in iron overload. There is little direct information available to confirm the correlation between the oxidative stress, iron overload and insulin resistance in SCD patients.

Objective: To investigate the relationship between iron overload, the disorders of antioxidants and insulin levels in blood of SCD patients and their matched controls.

Methods: The antioxidant activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as the malondialdehyde (MDA, the membrane lipid peroxidation products) and carbonyl contents (the oxidative products of proteins) were estimated spectrophotometrically in erythrocytes of patients and control subjects of matched sex and ages. In addition, fasting blood glucose (FBG), ferritin and insulin levels were estimated in the sera of the same subjects.

Results: The mean activity values of SOD, CAT and GSH-Px were significantly decreased, whereas the average values of MDA and carbonyl contents were significantly increased in erythrocytes of SCD patients in comparison to the corresponding values of the control subjects. The average levels of FBS, ferritin, insulin and homoeostasis model assessment of insulin resistance (HOMA-IR) were significantly elevated in the sera of SCD patients as compared to the controls. In addition, both serum ferritin, and oxidative products (expressed as MDA and carbonyl levels) were significantly correlated with blood glucose, insulin level, and HOMA-IR.

Conclusion: These findings may explain the role of elevated ferritin and oxidative products (i.e. MDA & carbonyl contents) in the development of insulin resistance and high glucose levels in SCD patients.

Key Words: Sickle cell, Ferritin, Antioxidants, Insulin resistance.

Introduction

Sickle cell disease (SCD) is characterized by micro-vascular vaso-occlusion, severe anemia, vasculopathy, and both acute and chronic multi-organ injury. The abnormal sickle hemoglobin (HbS) arises from a single point mutation in the -globin gene that encodes synthesis of beta globin of the adult hemoglobin (HbA)1. Sickled Hb is associated with steady state increases in plasma cell-free hemoglobin and overproduction of reactive oxygen species (ROS)1. This disorder is predominantly found in Africa, Southeast Asia, Central and Southern parts of India, and the eastern area of Saudi Arabia2,3.

The generation of reactive oxygen species (ROS) is a steady-state cellular event in respiring cells. Their production can be grossly amplified in response to a variety of pathophysiological conditions such as inflammation, immunologic disorders, hypoxia, etc.4. These ROS cause oxidative stress which often leads to damage of cellular macromolecules; e.g. DNA, protein, and lipids, potentially leading to cellular apoptosis5.

A number of major cellular defense mechanisms exist to neutralize and combat the damaging effects of these ROS. One of these cellular defense mechanisms is the enzymatic system which includes superoxide dismutase, catalase, and glutathione peroxidase6. Some previous studies stated that the activities of SOD, CAT and GSH-Px were reduced,7-9 while others reported that the activities of both
SOD and GSH-Px were increased\(^{10-12}\) in erythrocytes of SCD patients. A recent study has shown that the total antioxidant capacity was reduced in serum of SCA patients compared with healthy controls\(^{13}\). Various studies have shown that human sickle RBCs exhibit increased levels of thiobarbituric acid reactive substances (TBARS)\(^{14,15}\).

Insulin resistance is a condition in which insulin level is slightly elevated, which at the same time, has no effect on muscle and adipose cells leading to the elevation of blood glucose levels. Thus, the pancreas is stimulated to release more insulin to compensate for insulin resistance\(^{16}\). There is evidence that oxidative stress leads to insulin resistance and tissue damage and pathogenesis of late diabetic complications\(^{17,18}\).

Matthews et al.,\(^{19}\) have proposed that the homeostasis model assessment for insulin resistance (HOMA-IR) is related to the steady-state fasting blood glucose (FBG) and insulin concentration. Thus, HOMA-IR was calculated according to the following equation:

\[
\text{HOMA-IR} = \frac{\text{FBG (mg/dl)} \times \text{insulin level (µU/ml)}}{22.5}
\]

Where:
- HOMA-IR = Homeostasis Model Assessment for Insulin Resistance
- FBS = Fasting Blood Glucose Level

Ferritin, one of the key proteins regulating iron homeostasis, is used as a biomarker to evaluate iron deficiency, insulin resistance and metabolic syndrome\(^{20,21}\). It has been reported that serum ferritin levels were significantly increased in sickle cell anaemia, and \(\beta\)-thalassaemia\(^{22,23}\).

**Objectives**

Erythrocytes are considered as critical targets for cell damage by ROS as a model of the biological membranes\(^{24}\). The prevalence of sickle cell anaemia is high in the Eastern region of Saudi Arabia. Subsequently, the aim of this study was to evaluate the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as the levels of carbonyl and MDA in RBCs of SCD patients and control subjects. The plasma ferritin and insulin levels would also estimate in the plasma of SCD patients and control subjects. In addition, the present study would investigate relationship between insulin resistance and both oxidative stress and ferritin levels.

**Subjects and Methods**

**Subjects**

The subjects in this study are from Al-Ahsa City, Saudi Arabia, comprised of the following: Sickle cell patients (SCD), 51 patients were selected from the Hematology Clinic at King Fahd Hospital; while their age and gender matched controls (N=50) were randomly selected from those attending other clinics or accompanying patients and free from any blood diseases (as proved by revision of their records).

All subjects did not have any medicine for at least a month before taking the blood sample. The study was performed in the steady state, i.e., free of acute clinical illness.

Exclusion criteria include:
- No history or evidence of cardiovascular, hepatic, renal, or gastrointestinal disease or the presence of chronic infectious illnesses (including HIV infection), or inflammation and no blood transfusion within 3 months of study.

Written informed consent was obtained from all participants enrolled in the study. The study was approved by College of Medicine, King Faisal University, Al-Ahsa in collaboration with the King Fahd Hospital, Saudi Arabia.

Fasting blood samples were withdrawn from the subjects under study inpatient King Fahd hospital. After an overnight fasting from 10 PM, the subjects were weighed (in kilograms) using an electronic scale (Seca 770, Hamburg, Germany) and height was measured (in centimeters) measured using a Harpenden stadiometer.

**Materials and Methods**

Most of parameters under study were measured by using kits of high quality.

**Blood Sample**

Fasting blood samples were freshly withdrawn and one part is distributed into heparin tubes and other part was kept for clotting from both patients and volunteers and immediately transferred from the King Fahd Hospital to our laboratory at the College of Medicine, King Faisal University in an icebox.
Each sample was centrifuged at 4000 rpm and the serum was kept at −80°C for determining of insulin and ferritin levels, but RBC was taken to measure other parameters immediately.

Biochemical Analyses

Preparation of Blood Samples:
Normal and sickle RBCs were lysed with ice-cold water and the clear lysate obtained after spinning down the cell debris at 8500 g for 10 min at 4°C was used for the assays.

1. Determination of hemoglobin (Hb %): Hb was estimated spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) by using kit obtained from Biodiagnostic, Cairo, Egypt according to the method of Baure. The values are expressed as g/dL.

2. Estimation of blood glucose: Blood glucose concentration was estimated spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) through application of method described by Marks by using enzymatic test kit (glucose oxidase) supplied by Biodiagnostic, Cairo, Egypt. The results were expressed as mg/dL.

3. Determination of malondialdehyde: Malondialdehyde (MDA), an end product of lipid peroxidation of erythrocytes, can react with thiobarbituric acid (TBA) to form a colored complex called thiobarbituric acid reactive substance (TBARS), which was assayed spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) by using a diagnostic kit supplied by Biodiagnostic, Cairo, Egypt, by using the method described by Stocks et al. The results were expressed as nmol/gHb.

4. Carbonyl derivative: Reactive carbonyl derivative content – as a marker of protein oxidation was measured spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) by the method described by Levine et al. by using a diagnostic kit supplied by Biodiagnostic, Cairo, Egypt. The results were expressed as nmol/gHb.

5. Antioxidant enzymes: The activities of three antioxidant enzymes in erythrocytes were evaluated, as follows:

- **Catalase activity (CAT; EC 1.11.1.6)**: CAT activity was measured spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) using a standard CAT assay kit Biodiagnostic, Cairo, Egypt, through following the decomposition rate of H₂O₂ at 240 nm according to the method of Aebi. The results were expressed as U/g Hb.

- **Superoxide dismutase (SOD) activity (SOD; EC 1.15.1.1)**: The assay of Halliwell and Gutteridge was used to estimate the total SOD activity was performed spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) in RBCs hemolysate by using test kit obtained from SpinReact Biodiagnostic, Cairo, Egypt. The results were expressed as U/g Hb.

- **Glutathione peroxidase (GSH-Px; EC 1.1.1.9)**: The activity of GSH-Px in erythrocytes was estimated spectrophotometrically (Boeco S-20 Spectrophotometer, Hamburg, Germany) by using the method described by Paglia and Valentine by using a diagnostic kit provided by Biodiagnostic, Cairo, Egypt. The results were expressed as mU/g Hb.

6. Estimation of serum insulin: The immunoassay for determination of serum insulin level was performed using a standard insulin assay kit (Roche Diagnostic GmbH, D-68298 Mannheim, Germany) according to the method of Anderson et al. by using Elecsys and Cobas Immunoassay Autoanalyzer.

7. Estimation of serum ferritin: The serum ferritin levels were estimated by immunoassay method described by Hussain et al. using a standard ferritin assay kit (Roche Diagnostic GmbH, D-68298 Mannheim, Germany) by using Elecsys and Cobas Immunoassay Autoanalyzer.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software version 12. (SPSS Inc. Chicago IL, USA). Continuous data were expressed using mean, ±SD, while frequency data were expressed in proportions and percent. Log transformation was carried out for non linear variables as revealed using Kolmogrov-Smirnov test. Chi-square, Student “t” test with Levene’s test homology for equal variances and Mann-Whitney test were applied as appropriate. Pearson correlation coefficient was measured.
Table I. Basic characteristics of the included sickle cell disease patients and their controls.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sickle patients (N = 51)</th>
<th>Controls (N = 50)</th>
<th>Total (N = 101)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Males</td>
<td>39 (76.5)</td>
<td>39 (78.0)</td>
<td>78 (77.2)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>12 (23.5)</td>
<td>11 (22.0)</td>
<td>23 (22.8)</td>
</tr>
<tr>
<td>Residence</td>
<td>Urban</td>
<td>22 (43.1)</td>
<td>32 (64.0)</td>
<td>54 (53.5)</td>
</tr>
<tr>
<td></td>
<td>Rural</td>
<td>29 (56.9)</td>
<td>18 (36.0)</td>
<td>47 (46.5)</td>
</tr>
<tr>
<td>Age in years (mean ± SD)</td>
<td>22.9 ± 4.9</td>
<td>21.7 ± 1.7</td>
<td>22.2 ± 3.6</td>
<td>1.718**, 0.089</td>
</tr>
<tr>
<td>Body weight in Kg (mean ± SD)</td>
<td>63.4 ± 20.3</td>
<td>71.0 ± 11.6</td>
<td>67.2 ± 16.9</td>
<td>2.090**, 0.040*</td>
</tr>
<tr>
<td>Height in cm (mean ± SD)</td>
<td>161.1 ± 19.4</td>
<td>167.5 ± 10.1</td>
<td>164.3 ± 15.7</td>
<td>1.874**, 0.064</td>
</tr>
<tr>
<td>Body mass index! (mean ± SD)</td>
<td>22.6 ± 4.5</td>
<td>25.6 ± 4.6</td>
<td>24.1 ± 4.8</td>
<td>2.988**, 0.004*</td>
</tr>
<tr>
<td>Hemoglobin in (mean ± SD)</td>
<td>10.5 ± 1.7</td>
<td>13.0 ± 1.3</td>
<td>11.8 ± 1.9</td>
<td>8.185**, 0.001*</td>
</tr>
</tbody>
</table>

SD = standard deviation. †Body mass index = weight in kg/height in meter². *Statistically significant. †Chi-square and **t-test for independent samples.

Results

Table I displays the basic characteristics of both sickle cell disease patients and their controls. Age and gender distribution showed no significant difference between the two groups, sickle cell patients were resided more in the rural region.

Body weight, body mass index (BMI) and hemoglobin levels were disproportionately higher among controls compared to sickle cell patients.

Table II demonstrates the level of blood glucose, insulin level and serum ferritin in fasting blood samples of the included sickle cell patients and their controls. All the three parameters were significantly higher among sickle cell patients compared to their age and gender matched controls. Ferritin level showed skewed distribution among sickle cell patients, for cases; a mean ± SD = 2.67±0.32 and for controls: 1.94±0.04, t = 90.82 (P = 0.0001).

Table III depicts the levels of some antioxidants markers in blood cells from sickle cell patients and their controls. The anti-oxidant activities were significantly higher among controls compared to sickle cell patients. Glutathione peroxidase level showed a skewed distribution among controls, for cases; a mean ± SD = 1.68±0.06 and for controls: 2.24±0.03, t = –58.98 (P = 0.0001).

Table IV displays that the levels of Malondialdehyde and carbonyl were significantly higher in the red blood cells of sickle cell disease patients compared to their controls.

Subjects with sickle cell disease were also significantly showed a higher HOMA-IR index versus the controls. Carbonyl level displayed skewed distribution among SCD cases: for cases;

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Fasting blood sugar (mmol/L)</th>
<th>Insulin (µ/ml)</th>
<th>Ferritin level (ng /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (no.)</td>
<td>50</td>
<td>49</td>
<td>47</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4.10 ± 0.49</td>
<td>16.70 ± 2.49</td>
<td>86.90 ± 8.50</td>
</tr>
<tr>
<td>Sickle cell (no.)</td>
<td>49</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4.30 ± 0.50</td>
<td>27.59 ± 4.29</td>
<td>467.4 ± 34.0</td>
</tr>
<tr>
<td>t-test and P value</td>
<td>2.52, 0.013*</td>
<td>15.38, 0.001*</td>
<td>73.40†, 0.001*</td>
</tr>
</tbody>
</table>

SD = Standard Deviation. *Statistically significant. †t-test with unequal Levene’s test for variances.
Relationship between oxidative stress, ferritin and insulin resistance in sickle cell disease

Discussion

Oxidants and Antioxidants in SCD

The role of oxidant damage to red cells in sickle cell anemia has been of interest in recent years. The present study showed that the activities of SOD, CAT, and GSH-Px were significantly decreased in the SC subjects as compared with the control normal subject group (Table III). The deficiency of the activities of these enzymes may be attributed to the high production of ROS in these patients which may destroy these antioxidant enzymes[1,33,34]. Furthermore, the excess production of malondialdehyde (MDA), have additional toxic effects leading to alterations of the proteins, modifications of amino-acid side chain, and lipids structure. These alterations may result in a partial or complete loss of protein functionality including antioxidant enzymes and protein receptors[35].

Recently, it has been reported that nitric oxide (NO) reacts with superoxide at a rate three times faster than the reaction of superoxide with superoxide dismutase (SOD)[36], which may cause a decrease in SOD activity which is the actual case (Table IV).

Table III. Superoxide dismutase, catalase and glutathione peroxidase levels revealed form blood samples in sickle cell patients and their controls.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Superoxide dismutase % inhibition/g Hb</th>
<th>Catalase (µ/g Hb)</th>
<th>Glutathione peroxidase (mg µ/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (no.)</td>
<td>46</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.30 ± 0.71</td>
<td>5.30 ± 0.81</td>
<td>176.30 ± 12.71</td>
</tr>
<tr>
<td>Sickle cell (no.)</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>3.17 ± 0.49</td>
<td>3.46 ± 0.49</td>
<td>48.50 ± 6.2</td>
</tr>
<tr>
<td>t-test and P value</td>
<td>16.71, 0.001*</td>
<td>10.38, 0.001*</td>
<td>60.70†, 0.001*</td>
</tr>
</tbody>
</table>

SD = Standard Deviation. *Statistically significant. †t-test with unequal Levene’s test for variances.

Table IV. Levels of Malondialdehyde, and Carbonyl contents in red blood cells and the level of insulin resistance (HOMA-IR) in both sickle cell cases and their controls.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Malondialdehyde (nmol/g Hb)</th>
<th>Carbonyl (µmol/g Hb)</th>
<th>HOMA-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (no.)</td>
<td>51</td>
<td>48</td>
<td>49</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.84 ± 0.15</td>
<td>5.10 ± 0.54</td>
<td>3.00 ± 0.60</td>
</tr>
<tr>
<td>Sickle cell (no.)</td>
<td>51</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5.05 ± 0.98</td>
<td>35.52 ± 3.30</td>
<td>5.30 ± 1.00</td>
</tr>
<tr>
<td>t-test and P value</td>
<td>30.05†, 0.001*</td>
<td>43.98†, 0.001*</td>
<td>13.54, 0.001*</td>
</tr>
</tbody>
</table>

SD = standard deviation. HOMA-IR = [fasting blood sugar in mmol/L *Insulin in µ/ml]/22.5. *Statistically significant. †t-test with unequal Levene test for variances.
Excess production of ROS may also have a serious adverse effect on cell membrane of RBCs resulting in protein and lipid peroxidation enhancing production of carbonyl and MDA concentrations which is the actual case (Table IV)²¹,³⁸.

GSH is an essential cofactor for GSH-Px activity. It has been reported that GSH concentration was decreased in erythrocytes of sickle cell disease (SCD) individuals due to the excess production of ROS which consume GSH leading to the reduction in the activity of GSH-Px³⁷.

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**Figure 1.** Insulin resistance (HOMA-IR) in relation to serum ferritin level among sickle cases and controls.

**Figure 2.** MDA levels in relation to serum ferritin in sickle cell cases and control.
The rise in the serum ferritin in sickle cell anemia patients of this study (Table II) may be due to the following reasons: (a) Excess free iron, due to the excess breakdown of Hb and the abnormal circulating mass of Hb in reticuloendo-

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**Figure 3.** Serum insulin in relation to MDA levels among sickle cell cases and their controls.

**Ferritin and Oxidative Stress**

Ferritin is a biomarker of iron stores and may serve to detoxify excess iron. Thus, serum-ferritin is frequently used as a first line blood test when iron overload is suspected.

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**Figure 4.** Carbonyl level in relation to serum insulin in sickle cell patients and their controls.
celial cells, exert a positive feedback on ferritin synthesis\textsuperscript{40}. (b) Our patients did not donate blood transfusion, but continuous severe anemia may predispose to excessive gastrointestinal absorption of iron from dietary iron intake, in particularly haem iron from red meat (which is the normal diet at eastern area of Saudi Arabia), which is known as one of the major determinants of body iron causing an increase in ferritin level\textsuperscript{41}. (c) Hyperinsulinemia (results from oxidative stress and excess ferritin as illustrated below) may increase the synthesis of ferritin genetically by enhancing the mRNA transcription of ferritin\textsuperscript{42}. (d) Ferritin levels may be increased in response to oxidative stress resulting from excess production of ROS\textsuperscript{43}.

**Oxidative Stress and Insulin Resistance**

Insulin resistance (IR) is broadly defined as a failure of target cells to respond to insulin leading to increase of insulin secretion- to compensate for the insulin resistance- and decreased insulin clearance\textsuperscript{44}. The molecular basis of oxidative stress of ROS-induced insulin resistance may be attributed to one of the following mechanisms: (a) Reactive oxygen species (ROS) may interfere with insulin signaling at various levels, impairing insulin uptake through a direct effect on insulin receptor function\textsuperscript{45,46} which in turn, leading to elevation of plasma insulin level which is the actual case (Table II). (b) ROS may reduce glucose uptake by inhibiting the translocation of GLUT4 (glucose transporter) to the plasma membrane leading to increase blood glucose concentration (Table II) which induces pancreatic secretion of insulin\textsuperscript{47}. Previous evidences suggested that antioxidants treatment might prevent ROS-induced impairment of insulin signaling and improve biomarkers of metabolic syndromes\textsuperscript{48} which confirm the correlation between insulin resistance and oxidative stress.

**Ferritin and Insulin Resistance**

Ferritin is a high molecular weight iron storage protein occurring mainly in the cells of the liver and reticuloendothelial system. Serum ferritin is also known to be a good indicator for hepatic iron content\textsuperscript{41}. It is known that one-half of the secreted insulin is extracted by the liver during the first pass through that organ. Therefore, the liver is exposed to twice as much insulin as is found in the systemic circulation\textsuperscript{49}. Thus, excess iron stores, in the form of ferritin, may induce insulin resistance through the reduction of hepatic extraction and metabolism of insulin\textsuperscript{50}. Thus, ferritin may interfere with insulin signaling in the

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**Figure 5.** Insulin levels in relation to serum in ferritin in sickle cell patients and their controls.

![Graph showing insulin levels in relation to serum ferritin](image)

$r = 0.836, p = 0.0001$
liver which may inhibit glucose uptake by the liver cells leading to the elevation of blood glucose which is the actual case (Table II). In addition, insulin promotes transcription of the glucokinase gene, resulting in an increase in liver enzyme protein and, therefore, of total glucokinase activity. Therefore, the insulin resistance by the effect of excess hepatic ferritin may cause a deficiency in hepatic glucokinase. This contributes to an inability of the patient to efficiently decrease blood glucose levels, which is the actual case (Table II)51. Previous studies have reported that decreasing iron stores through either iron depletion by phlebotomy52, or iron chelation by Desferoxamine53 would ameliorate insulin resistance and metabolic syndrome. Furthermore, it has also been found that the increased insulin sensitivity observed in vegetarians might be related to low-iron content in their diet54. These evidences illustrated the correlation between ferritin and insulin resistance.

**Conclusion**

The present results found that the abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid and protein peroxidation, and development of insulin resistance. Furthermore, our results show that elevated body iron stores-measured by serum ferritin levels-and the oxidative stress products-measured by MDA and carbonyl contents- confer a moderately increased risk of insulin resistance, HOMA-IR, and abnormal of glucose metabolism in SCD patients in Saudi Arabia.
In the future, it is better to conduct this study to investigate that these consequences of oxidative stress can promote the development of complications of diabetes mellitus.

In addition, it would investigate the improvements of the SCD complications by using some natural iron chelating agents.

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