Clinoptilolite supported feeding reduces excessive iron in thalassemia rat model created with iron loading

Durdane Serap Kuruca, Kadriye Akgun Dar, Ayesegul Kapucu, Dilsad Ozerkan

1Istanbul University Faculty of Medicine, Department of Physiology, Istanbul, Turkey
2Istanbul University Faculty of Science, Department of Biology, Istanbul, Turkey
3Istinye University, Health Sciences Faculty, Department of Physiotherapy and rehabilitation, Istanbul, Turkey

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Abstract

There is no regulatory mechanism for the removal of iron that accumulates in the body. Thalassemia patients are most affected by iron overload. The method often used in the treatment of these patients is iron chelation therapy, which involves removing excess iron from the bloodstream, but it is insufficient. Nutritional supplements and herbal remedies are complementary tools that may help improve the health of an individual with iron load. Here, we tried to develop a method that affecting intestinal absorption in an animal model with clinoptilolite feeding to reduce the iron load in the circulation. 32 rats were divided into 4 groups as control, Cli, Iron, Cli+Iron. Iron and Cli+Iron groups received iron at a dose of 250 mg/kg/day for 10 days, and Cli and Cli+Iron groups were fed a diet with 50% clinoptilolite for one month. Histological preparation and Fe²⁺, Cu²⁺, Zn²⁺ measurements were done in all tissues. Consequently, clinoptilolite significantly lowered the iron level in the stomach. On the contrary, iron absorption was increased in the small intestine, but iron transportation to the blood was decreased by clinoptilolite. The iron levels of clinoptilolite groups (Cli and Cli+Iron) were reduced in the tissues of heart, lung, liver, kidney, and spleen because of the different level of iron necessities of each tissue compared to the group with iron overload. The clinoptilolite could preserve organs against iron toxicity by enhancing the absorption of iron in the small intestine but lowering the iron level in blood. Even though there was no detailed information regarding the mechanism of reduction iron overload by the clinoptilolite, serum and chyme could help to make some helpful inferences to elucidate the mechanism of iron chelation.

Keywords: Clinoptilolite, Iron overload, heavy metal accumulation, atomic absorption spectrophotometry

Introduction

The thalassemia types are a group of anemias that result from inherited defects in the production of hemoglobin. The anemia is treated by frequent erythrocyte transfusions. This therapy results in the accumulation of iron overload that is exacerbated by the breakdown products of hemoglobin and the increased iron uptake but ineffective erythrocyte production. As a result, organ damage and even death may occur. In patients with thalassemia, regular blood transfusions are performed to maintain normal hemoglobin levels in the blood. Even in non-transfusion thalassemia intermedia patients, iron load is 2.5 g per year [1]; in those who are transfused, this load is doubled and primarily liver, heart and endocrine glands are affected.

Iron chelation therapy aims to prevent harmful iron accumulation and remove excess iron in patients with thalassemia, but this treatment is not always successful. Many clinical investigations have addressed pharmacokinetic efficacy of some effective nutraceuticals for β-thalassemia. Supplementation diet including functional food such as vitamins, vegetables, fruits are shown to have beneficial effects in a lot of studies. Curcumin significantly reduced iron overload in iron-loaded rats’ liver and kidney [2]. It has been determined that green tea extract blocks dietary iron absorption and chelating [3-6]. Administration of nutraceuticals to patients with thalassemia in addition to the regular application of iron chelation therapy can significantly reduce organ damage and dysfunction. However, using these foods that have antioxidant effects is not sufficient alone. In addition, the body does not have a physiological mechanism that can reduce the excess iron load by itself. Nowadays, it is known that chelation therapy applied in
patients with thalassemia is insufficient to reduce iron overload. Zeolites are aluminum silicates with crystalline hydration. They were formed millions of years ago by the chemical reaction of ash and lava that emerged from the mixture of volcanic eruptions with lake or sea waters [7]. This means the zeolites have crystal lattice and inorganic structure which makes them minerals. As porous materials, zeolites are known as selective ion exchangers, adsorbents, and molecular sieves. That is why the zeolites are utilized in various areas, from industrial applications to medical uses [8]. For example, some zeolites, including clinoptilolite, F-type and W-type, have the property of administering hemodialysis in ammonia ion exchange systems where a major challenge in developing a hemodialysis system is the removing of ammonia from a recirculating dialysate stream [9]. Also, much effort has been made to improve the adsorption efficiency of zeolites against toxins in various environments [10-12]. It has also been suggested that the ability of zeolite (FAU13 × and FERCP914C) to decrease ROS accumulation in extracorporeal cycles in patients with chronic renal failure undergoing dialysis is as beneficial as antioxidant supplementation (i.e. vitamin E) and leads to reduce mortality [13]. Several studies have investigated whether zeolites can be used as food supplements in poultry houses and farms to improve their performance and efficiency by removing heavy metals from animals' bodies. A group of scientists used zeolites as dietary supplements for dairy cows and observed that long-term feeding of zeolites increased milk yield and that zeolites had no significant negative effect on cows liver function and hematological parameters [14]. On the other hand, a South Korean scientist postulated and corrected the hypothesis of using zeolites as an effective nutraceutical to lower contaminants in poultry and pig manure [15]. However, even after these experiments and studies on animals, people still thought zeolites to be carcinogenic substances because of erionite [16, 17], a species in the zeolite family. However, there are some contradictory studies, and their results break the taboos of people regarding the use of zeolites in medicine. Zeolites other than erionite are recognized as GRAS (Generally Considered Safe) by the US Food and Drug Administration. These studies highlight the detoxifying, anti-inflammatory, antioxidant, and anticancer effects on the human body as well as the enhancing effects of clinoptilolite use as a supplementary micronutrient for humans on their own immune systems [18, 19].

Natural zeolites have been used since the 1920s. Some natural zeolites were thought to be effective in diarrhea according to physicochemical results, and translational biochemical and microbiological studies and clinical trials were conducted. Natural clinoptilolite-Enterex, which is an anti-diarrheal effector, was introduced to the market after the standards in accordance with the Cuban Pharmaceutical Quality Agency and started to be used as a new anti-diarrhea drug [20]. A clinoptilolite named Absorbatox™ effect was evaluated in a randomized, double-blind, placebo-controlled pilot clinical trial including 23 participants against NSAID-induced gastric mucosal erosion. Patients receiving 1500 mg of Absorbatox orally three times a day were compared with placebo control patients and it was shown that Absorbatox binds to hydrogen ions and biologically active amines, resulting in gastroprotection [21]. US troops have been using zeolites in the form of Ca-zeolite as coagulants in Iraq and Afghanistan for many years to treat life-threatening injuries. Therefore, it is inevitable to develop zeolite-based nanoparticles to achieve hemostasis [22]. Various experiments have been conducted investigating the biological and chemical effects of natural zeolites on humans and animals. In many of these studies, it was aimed to benefit from the adsorption and ion exchange properties of clinoptilolite. However, the effects of natural zeolites in reducing iron load in vivo are still controversial. Therefore, in our study, we planned to use the thalassemia rat model created by iron loading and to investigate the effectiveness of Clinoptilolite obtained from Manisa-Gördes/Turkey on this model.

Material and Methods

Animals

This study with rats was performed after taking permission numbered 2011/94 from Istanbul University Animal Experiments Local Ethics Committee. 32 adult male Wistar albino rats weighing 200-250 g were used. The subjects were divided into 4 experimental groups which were the control group (n=7, Group 1), clinoptilolite-supported feeding group (n=7, Group 2), iron-overloaded group, (n=9, Group 3), clinoptilolite-supported feeding group with iron overload (n=8, Group 4). The rats in Group 3 and 4 received iron (Ferro III hydroxide polymaltose) by gavage at a dose of 250 mg/kg/day for 10 days, and Group 2 and 4 were fed a diet 50% clinoptilolite for one month as mentioned before [23, 24].

Chemicals

Clinoptilolite used in this experiment is (Ca, K+, Na+, Mg4Al4Si8O24H2O, and has a 7.0-8.0 pH value. The clinoptilolites’ mean particle size is <40 μm, and their active pore diameter is 4Å. Also, SiO2/Al2O3 ratio in the particles is 5.4-6.0. Clinoptilolite used in our study was provided by Rota Mining Corporation, TURKEY.

Fe2+, Cu2+, Zn2+ Measurement

Atomic absorption spectroscopy is a technique used to determine the concentration of a metal element in a liquid solution. This technique is based on the principle of measuring the amount absorbed based on the absorption of UV and visible light by free atoms from the ground state to the excited state [25]. The tissue specimens were weighed and put into metal-free glass tubes for digestion. Tissues were kept at -18°C until use. Firstly, the samples were digested with 2 ml of concentrated nitric acid at 100°C in the furnace for 1 hand. 2 ml of HClO₄ (Perchloric acid) (60%) was supplemented to the cooled substances. The substances were digested at 120°C till the substances were reduced to one-half its original total volume. Digested substances were diluted with deionized water to 10 ml. Last dilutions of the samples were mixed in a shaker for 15 min just before measurement. Fe2+, Cu2+, Zn2+ levels were measured by flame atomic absorption spectrophotometer (Shimadzu AA-680). Results were calculated as µg/g wet weight. The blood was then allowed to coagulate and centrifuged for 15 min at 3000 rpm to extract the serum. The blood serum was aliquoted into Eppendorf tubes and stored at -80°C for analysis.
of trace elements. Blood collection and serum separation were performed in a dust-free environment. Fe\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\) levels were determined by using flame atomic absorption spectrometry (Shimadzu AA-680).

**Tissue sample collection and histological slide preparation**

The rats were decapitated at the end of the experiment. Their stomachs, small intestines, large intestines, livers, spleens, kidneys, heart, and lungs were removed, fixed with 10% neutral formalin, and then embedded in paraffin. Four-micrometer paraffin tissue sections were mounted on the slides which were then deparaffinized. The sections were stained with hematoxylin and eosin (H&E) and Prussian Blue [26]. The slides were microscopically examined and captured using Kameram 390CU Imaging system (Micro System Ltd. Turkey).

**Statistical Analysis**

First, whether the data show a normal distribution or not was evaluated primarily through the histogram graph. The normality test was carried out because of the graphics that seemed contrary to the normal distribution. Results were analyzed non-parametric student’s t test with the Graphahad Prism statistical program (version 5.0). Experimental groups were compared with respect to using Bonferroni’s multiple comparison test by applying the one-way variance analysis. All results were expressed as means±SD and p≤0.05 was regarded as significant.

**Results**

**Histological examination of tissues obtained from all groups**

**Small Intestine**

The most significant alteration observed from the examination of small intestine section was morphological changes of villi in the Iron group. These changes were an increase in number of villi and shortening of them. Also, there were invaginations in epithelial tissue which was on the luminal side of small intestine. There was very little reaction in the sections which were stained with Prussian blue. The villi’s structure from clinoptilolite group was slightly changed; in contrast, there were some significant changes such as increase in number and lengthening of the villi from the Cli+Iron group; therefore, it has been observed that the villi were in a tight order as small intestine’s luminal surface was enlarged, and there were lymph nodes as well. Furthermore, the Prussian blue reaction in the Cli+Iron group was more severe than the reaction in the Iron group (Figure 1a).

**Large Intestine**

In this study, the large intestine was examined in three sections as LI1; LI2; LI3. In the control group, normal intestinal histology was observed in all three sections. The epithelium had striated margins and the crypts were smooth.

The sections were stained darker in the group given clinoptilolite, the number of blood vessels and leukocytes increased compared to the control. Connective tissue was increased in all parts of the intestine, mostly in the LI3 region. Leukocyte infiltration was present in all departments, mostly in the LI3 region (Figure 1b).

In the iron treated group, In the LI1 region, the epithelium was fragmented in patches and the integrity of the striated margin was disrupted, but it was visible. The crypts were corrupted. An increase in the number of blood vessels was observed. An increase in connective tissue was determined, but this increase was less than in the zeolite group. The fragmentation in the apical of epithelial cells in the LI2 region was greater than that of LI1.

The amount of connective tissue in the LI2 region increased more with the LI1 region than the group given zeolite. The brush border could not be seen in the LI2 region, the increase in leukocyte was observed the most in this group. While examining the sections, leukocyte infiltration was detected in 3-4 areas in the LI3 region, one of which was quite large. The eosinophil count was increased.

In the group where iron and clinoptilolite were applied together, fragmentation in the apical of the intestinal epithelium was significantly reduced in all three regions compared to the iron-treated group. Crypta were seen to be smooth. Brush border integrity could be traced. The connective tissue was significantly reduced in the LI1 and LI2 regions compared to the iron given group but was slightly more in the LI3 group. The eosinophil count was lower than the iron-treated group (Table 1).
Table 1. Histological changes in the large intestine in all groups are summarized.

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<th>Disintegration in the apical epithelium</th>
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<th>Connective tissue</th>
<th>Number of blood vessels</th>
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<td>LI3</td>
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**Liver**

The Kupffer cells got visually more obvious and there was an increase in the number of them. Also, accumulation in the Cli+Iron group was less than the Iron group. On the other hand, the number and appearance of the Kupffer cells in the control and Cli groups were similar. In addition, there was no reaction in the liver sections stained by Prussian blue from any of the experimental groups and control group.

**Spleen**

According to the histological examinations, it has been observed that the red pulp areas of spleen tissue were stained by Prussian blue due to high intensity of iron in the control and the experimental groups.

**Stomach, Heart, Kidney and Lung**

There was histologically no difference between the control group and the experimental groups, and there was no reaction by Prussian blue.

**Measurement of The Elements with Atomic Absorption Spectrophotometry (AAS)**

The iron (Fe\(^{2+}\)), zinc (Zn\(^{2+}\)) and copper (Cu\(^{2+}\)) binding capacities of the groups were examined. The iron levels of clinoptilolite groups (Cli and Cli+Iron) were diminished in the tissues of heart, lung, liver, kidney, and spleen because of the several proportions of iron necessities of each tissue compared to the group with iron overload. The clinoptilolite have the ability of preserving organs against to iron toxicity by enhancing the absorption of iron in the small intestine but resulting in lower levels of iron in blood (Figure 2).

**Figure 2.** Amount of Fe\(^{2+}\) in the control and experimental groups. The bars represent the amount of Fe\(^{2+}\) measured by atomic absorption spectrometry for the control and experimental groups of each tissue section type (n=9 per groups). Data were shown as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001.

The clinoptilolite succeeded to absorb plenty of volume of free zinc from all tissues (Figure 3). Copper (Cu\(^{2+}\)) binding capacity were decreased in large intestine, spleen and heart tissues (Figure 4).

**Figure 3.** Level of Zn\(^{2+}\) in the control and experimental groups. The bars represent the amount of Zn\(^{2+}\) measured by atomic absorption spectrometry in each tissue section type of the control and experimental groups (n=9 per groups). The level of Zn\(^{2+}\) was measured as µg/g of the wet tissue. Data were shown as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001.
We observed that the iron amount is higher in Cli+Iron group than Iron group for stomach content. Following the analysis of serum levels, this situation has been reversed. Zn²⁺ and Cu²⁺ displayed different results separately. According to the Iron group, higher levels of Zn²⁺ were determined in the Cli+Iron group in both stomach content and serum. Contrary to this, Cu²⁺ content was established in reduced values (Figure 5).

Discussion

There is no regulatory mechanism for the removal of iron that accumulates in our body. The organism is programmed to protect the iron due to its benefits and therefore excess iron is stored in the body. When the capacity of these stores is exceeded, iron accumulates in a wide variety of organs and joints, such as the heart, liver, pancreas, thyroid gland and leads to the production of free oxygen radicals as a proxy. Free oxygen radicals that cannot be adequately detoxified by antioxidants are highly harmful and toxic and can cause damage to various organs leading to disorders like cirrhosis, cardiomyopathy, diabetes mellitus etc. [27].

Clinoptilolite draws interest as it binds selectively to specific heavy metal cations such as lead, cadmium, and nickel [28]. Clinoptilolite, which is among the natural zeolites, can be found very abundantly in nature, but not all clinoptilolite zeolites are suitable for medicinal purposes. Therefore, we tried to understand the effectiveness of clinoptilolite extracted from Manisa –Gordes region in this model in our country. Because the clinoptilolites used in the medical field are different in terms of particle size [29] and Si/Al ratio specific to the areas where they are extracted, and accordingly, their adsorption capacity changes [30]. Even though the mean particle size of clinoptilolite utilized in our experiment, 40 µm, indicates that clinoptilolite cannot pass from the intestinal lumen to the blood; as it is not small enough for passing through the cell membranes and intercellular matrix, we are not exactly sure whether the clinoptilolite which was taken orally was metabolized during its travel in the digestive tract of the rats. On the other hand, natural clinoptilolite in animals and humans is an auto bioregulator and its absorption depends on several events in which ion exchange occurs in body fluids. Heavy metals are removed by ion exchange because they own substantial affinity for the clinoptilolite frameworks and that the cations found in the framework are remarkably absorbed by organic materials in the organism [31].

Although an average of 20-25 mg of iron is taken per day with the diet, only 1-2 mg of it becomes absorbed [27]. If there is iron in the body and it is needed, it is absorbed from the small intestine. The duodenal cytochrome B (DcytB) enzyme found in enterocytes is involved in the conversion of Fe³⁺ to Fe²⁺ and is transported by a transmembrane protein, divalent metal transporter (DMT1). DMT1 is also responsible for the transport of other divalent metals, zinc, lead, copper, manganese. [32]. When iron homeostasis cannot be regulated, iron deficiency or iron overload occur, and progress of which is harmful to the body [33]. Iron overload anemias such as β-thalassemia, congenital dyserythropoietic, X-linked sideroblastic anemias and the mutations in DMT1 cause increased iron absorption in intestine and decrease the effect of erythropoiesis, respectively [34]. In the present study, we provide first evidence, iron overload was decreased in orally iron supplementation rats which were fed with natural clinoptilolite. The high iron level in
serum and the chyme of rats with iron supplementation was proved to cause iron deposition. Thus, it was shown diet with clinoptilolite was very effective to reduce the iron overload. In this study we gave Fe$^3+$ to rats orally and calculated the accumulation of Iron by two ways. During AAS measurements, it is possible to detect total iron (in Fe$^2+$ form) as the tissues are ruptured with nitric acid. In addition, since the proteins are not broken down with the Prussian Blue, it stains Fe$^3+$ bound to the proteins and the stored iron in the tissues is shown in this way. When we examine the tissues in detail; although staining with Prussian blue in the iron group was negative in all groups, iron in the stomach tissue was found to be quite high in AAS measurements. Although the iron absorption occurs in the duodenum, this can change due to severe Iron overload and iron deposits might be seen in gastric mucosa [35]. In our experiment the rats fed with the clinoptilolite-diet during the iron-overloading process, did not undergo any alteration in the level of any metal in the stomach tissues. In the Cli+Iron group, iron decreased in the stomach tissue and the increase in iron in the chyme of this group indicates that clinoptilolite succeed the retention of iron.

Although there is intense staining around the lumen of small intestine in iron and Cli+Iron groups; especially, the iron level of Cli+Iron group was significantly higher than control and iron groups by AAS . Also, the iron level of Cli group was higher than control group. These results in small intestine tissue showed that iron absorption increased in Cli and Cli+Iron groups and accumulated in the tissue but not observed in the blood. While Fe$^2+$ is high in serum of Iron group, Fe$^3+$ decreases in serum of Cli+Iron group. High serum Fe$^2+$ level resulting from iron overload in the Iron group decreases in intestinal absorption via the feedback route, and iron level in the small intestine tissue diminishes. Enterocytes work in conjunction with other cells that consume iron stores, such as erythroid precursors, or are involved in storing excess iron, such as hepatocytes, to regulate iron homeostasis [27]. According to the histological examination of the tissues, the increased level of invagination, the shortening in the length of villi and an increase in the number of villi showed that the absorption surface on the intestine was enlarged. The iron level in large intestine tissue of all other groups was smaller than control group. In all the experimental groups, we see that iron does not accumulate in the large intestine because it is not absorbed. In the small and large intestines, an increase in the lymphoid tissue and the number of eosinophils suggested that there was an increase in the immunological and allergic reactions. This could be the immunomodulatory impact of Clinoptilolite which leads to regulation of human homeostasis by intestine’s local detoxification feature [18].

An imbalance in iron level occurs when iron intake is consistently higher than normal iron loss. In high iron intake, excess iron is first stored in the reticuloendothelial system. When the capacity of these stores is exceeded, iron accumulates in various organs and joints such as heart, kidney lung, pancreas, thyroid gland. In our study, although the iron group was found to be high in tissues such as spleen, liver, heart, kidney and lung, the increase in stomach and liver was more significant. When tissues outside the gastrointestinal system are examined, we see that iron does not accumulate in the Cli and Cli + Iron groups. This is related to the low level of iron in the serum of these groups. It is related to the retention of excess iron by Kupffer cells. The same results were established in an experiment proved that iron aggregates diminished with clinoptilolite implement in Kupffer cells [36]. The iron level of spleen of Cli+Iron group was significantly lower than the iron level of Iron group. Also, the iron level of the spleen of Cli+Iron group was lower than the iron level of control group. Furthermore, the spleen, the iron deposit of body, produces the erythrocytes, and this could be correlated to the highest staining of the spleen tissues by Prussian blue in comparison with the level of staining in all the other organ tissues. In the other organs such as kidney, heart, and lung Cli+Iron application decreased iron levels when compared to Iron group. Even though there was no detailed information regarding the mechanism of reduction of iron overload by the clinoptilolite, the metal ion measurements by atomic absorption spectrophotometry and the histological examination on the tissue sections, serum and chyme could help to make some helpful inferences to elucidate the mechanism of iron chelation in our experiment.

In this study, besides iron accumulation, we wanted to understand the changes of levels of zinc and copper which are toxic in excessive levels and related to the mechanism of iron metabolism in the clinoptilolite implemented iron groups of rats. It was interesting that Zn increased especially in tissues with decreased Fe. It confirms that DMT1 is common in all tissues and increases in heterochromatosis [37]. The clinoptilolite treatment on the rats with iron-overload decreased the copper level of the serum. Whereas the iron and copper levels in the Cli group were not changed in comparison to the control group for the stomach tissues, the zinc level in the Cli group was significantly larger than the zinc level of the control group. In the Cli group, the clinoptilolite adsorbed the iron ions, which had their normal level in the body, during its travel in the digestive tract, and that is why the zinc ions could bind to the DMT1 receptors on the small intestine due to the paucity of iron ions, so the zinc level was increased. However, in the case of iron overload such as the Iron group, once the clinoptilolite-supported diet was given to the rats with iron-overload, the iron level was not decreased enough that the DMT1 receptors was competitively bound by the iron ions rather than the zinc ions. Therefore, in this case, the zinc level was decreased, but this was not statistically significant. Then, the zinc levels were increased because of its competitive binding to the DMT1 receptors because of the scarcity of iron ions.

The toxic metals like Cu and Zn are normally found in natural water because of industrial waste and some of them cannot be removed [38]. According to the ion exchange and adsorption features of Clinoptilolite; it is used to remove heavy metals from wastewaters. This mechanism is a very complex process, because of the variability of particle surface site affinity with metal ions [39]. So, removal capacity of heavy metal can differ due to this situation. For example, the maximum exchange capacity of Cu was observed more than Zn in the usage of different zeolites [40,41]. Our results represent that Clinoptilolite reduced the Zn levels in all tissues of iron-overloaded group. The opposite was determined for serum and stomach content. Zn adsorption may be affected by pH variability of tissues that leads degradation of crystal structure and competition of Zn ions by hydrogen ions. Also, lower pH is more effective for that [42]. On the other hand, Cu levels were diminished in large intestine, spleen lung, serum, and stomach content.
Conclusion

It can be concluded that Clinoptilolite are successful for decreasing iron overloading according to histochemical results and AAS measurements. So, it can be used as natural supplemental material in medicine, but more experimental research should be done to understand this mechanism.

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Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

All authors declare no financial support.

Ethical approval

This study with rats was performed after taking permission numbered 2011/94 from Istanbul University Animal Experiments Local Ethics Committee.

References

