ISSN 2436-5173 (Online)

Metallomics Research

SPECIAL ISSUE

Zinc in Biology and Medicine

Vol. 2 / No. 1
March 2022

JSBRTE
Japan Society for
Biomedical Research on Trace Elements

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collaboration with Japanese society for Zinc Nutritional Therapy

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https://metallomicsresearch.brte.org/

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Metallomics Research 2022 (1) #MR202111

Review https://metallomicsresearch.brte.org

Zinc is essential not just for the surgery but for the periods before and after surgery

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Abstract

Zinc deficiency is associated with delayed bone healing, skin fragility, and susceptibility to infection due to immunodepression, therefore it has a significant impact on surgical outcomes. The author first became interested in the mechanism of bone healing in a case where an ankle fracture in a dialysis patient did not heal after three operations, and a zinc wave was later found in electron microscopy and electron probe X-ray microanalysis of frozen sections of the ossified area of the yellow ligament. Subsequently, shoulder injections to a rheumatoid arthritis patient caused pyogenic arthritis, suggesting that low zinc levels in rheumatoid arthritis patients resulted in weak skin and a low skin tenderness threshold. Later analysis showed that patients with zinc levels below 50 μg/dL died early due to infections, suggesting that low zinc levels are also related to poor immunity.

Two groups were compared after 2 months and after 6 months of supplementation with 34 mg/day of zinc to examine how much zinc should be supplemented by the time of joint replacement surgery. There was no significant difference in zinc levels in the two groups at 1 month before surgery, but at 7 days before surgery and 3 days after surgery, in the first group zinc levels were significantly lower, skin necrosis occurred in three cases, and skin healing was delayed in four cases.

If there is concern about the patient's preoperative condition, it is recommended that zinc levels be measured and that adequate zinc supplementation be performed before surgery.

Key words: zinc, immunity, arthroplasty, objective face scale, rheumatoid arthritis, nutritional support team

Statements about COI: The author declares no conflict of interest associated with this manuscript.

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Received: December 28, 2021
Accepted: February 7, 2022
Released online: March 31, 2022

1. Introduction

The human body contains about 2 grams of zinc, an essential trace metal, and the hippocampus and cerebral cortex in the brain contain high concentrations of nearly 100 ppm. Other organs in the human body that contain large amounts of zinc are bones (about 60%) and muscles (about 30%). Therefore, zinc seems to be an important element for orthopedic surgeons who deal with bones and muscles. Furthermore, since there is a negative correlation between grip strength and the ratio of copper to zinc levels [1], the need to maintain high zinc levels to prevent skeletal muscle weakness is now well known to many doctors.

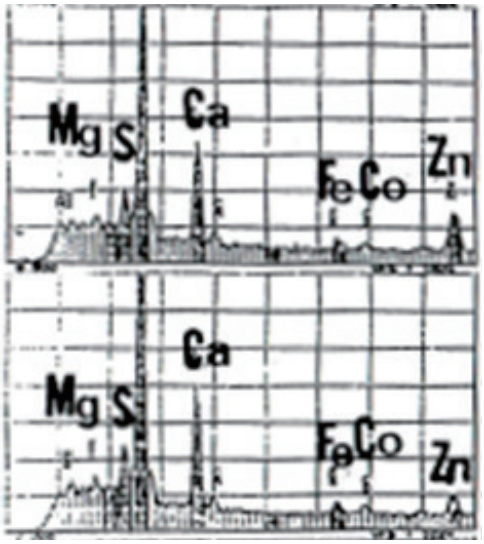

Fig. 1. Electron microscopic micro-Xray analysis of frozen sections of lumbar ligamentum flavum to detect zinc waves.

Recently, it has become clear that zinc is also involved in immunity to prevent infections [2-5]. Since bones are intractable when infected, zinc is an important element. Zinc levels in preoperative patients are important for orthopedic surgeons, but I will discuss why other surgeons should also pay attention to preoperative and postoperative zinc levels.

2. Zinc and Ossification

The ossification and bone repair mechanisms are important in orthopedic surgery. Early in my career I saw another doctor operate three times on a dialysis patient with an ankle joint fracture. The third operation included bone grafting from the pelvis and plate fixation, but the bone did not heal. This is the reason why I became interested in the ossification mechanism. It is now clear that in patients with severe renal failure, such as those on dialysis, intestinal absorption of zinc is poor and serum zinc levels are low [6], and that low serum zinc levels worsen osteoblast function [7].

In 1993, Nakamura reported that serum osteocalcin level significantly increased from 15.3±2.2 to 24.2±2.3 (p<0.01) after 6 months of zinc administration [8]. The increase in serum zinc levels increases ALP, IGF, and osteocalcin, which are favorable for bone formation.

In the 1980s, research on ossification of the posterior longitudinal ligament and yellow ligament of the spine began. I was also interested in the microstructure of endochondral ossification because of the poor bone healing in dialysis patients, and began to examine the ossified area of the end of the bone stem in mice and the ossified area of the lumbar yellow ligament in humans using an electron microscope [9]. In 1991, when frozen sections of human yellow ligament ossification were placed on a carbon grid and subjected to electron microscopy and electron probe X-ray microanalysis, zinc waves were detected (**Fig. 1**). Zinc waves were found in the tide mark of the ossification of the ligamentum flavum in the area of the mesenchymal cell osteoblasts. At that time, zinc in the ligamentous ossification area coexisted with calcium, magnesium, iron, and cobalt, and was thought to be derived from matrix vesicles.

According to Arizumi, in osteoblasts on a high-zinc diet, the Golgi and endoplasmic reticulum around the nucleus are well developed, as seen electron microscopically [10]. This is consistent with an increase in zinc in this region. The involvement of zinc in ossification is evident from the fact that Xia in 2002 found by atom microscopy many osteoblasts in the tibial metaphysis of rats on a high-zinc diet [11].

3. Skin fragility, immunocompromise and serum zinc level in rheumatoid arthritis patients

Working at a hospital in Nagano Prefecture, I have had the opportunity to see many patients with rheumatoid arthritis. For the first time in my career as a doctor I saw a case of a hospitalized rheumatoid arthritis patient, Case I, who developed pyogenic arthritis, even though my supervisor asked me to give him a shoulder joint injection and I did so with care. When I went to apologize to my supervisor, he said, “Oh, it happens all the time.” When I paid attention to the patient's general condition to find

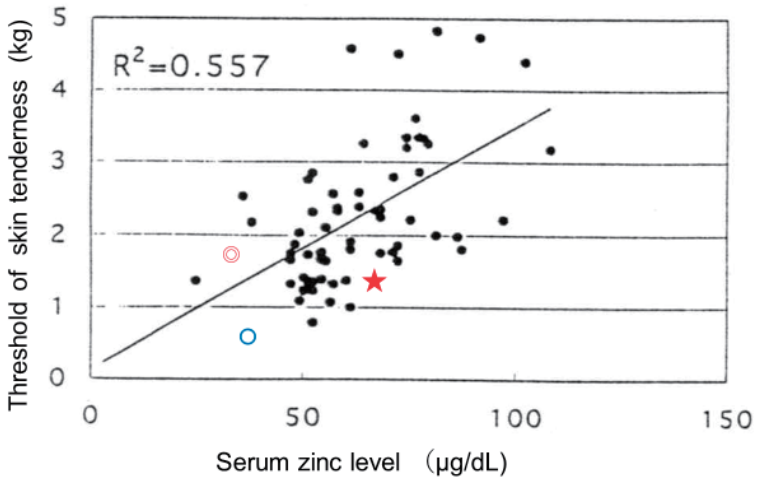

Fig. 2. Relationship between serum zinc level and mean skin tenderness threshold of both lower legs in rheumatoid arthritis patients. "red double circle": case 4; "blue circle": case 2; "red star": rheumatoid arthritis patient with diabetes between left TKA and right TKA

out what was causing the frequent infections in rheumatoid arthritis, I noticed that the patient's skin was as thin as cellophane. Furthermore, the epidermis sometimes peeled off easily with minor external force, and the patient's arms and lower legs were sometimes wrapped in cotton bandages to protect the skin. Based on my clinical experience of using zinc oxide ointment to treat dermatitis in babies with weak skin and rashes, I wondered if serum zinc levels were low in patients with fragile skin. First, I measured the zinc level of the patient with pyogenic shoulder arthritis. The zinc level was 33 μg/dL, which is very low ("red double circle" in **Fig. 2**: case 2 in **Table 1**) [12].

Thinking that the zinc levels of other rheumatoid arthritis patients with fragile skin might also be low, I measured the zinc levels of 66 patients (as volunteers) during the period from March 1998 to August 1999. The mean value was 63 μg/dL, which is a similar level to the mean value of 66 μg/dL (54-86 μg/dL) [13] in RA patients published by Kawate (2018) and much lower than the mean serum zinc level of 86 μg/dL found in 113 RA patients in 1975 by Kennedy et al. [14] The 26 patients with low serum zinc levels are shown in **Table 1**.

Reasons for the low zinc levels in Japanese rheumatoid arthritis patients may be that the zinc content in Japanese food is decreasing, and the absorption in the small intestine is impaired as a result of the shortening of intestinal villi due to heavy medicine use. A third possible cause is the increased use of electronic devices, less sleep time, and a stressed society, which is thought to have increased wear and tear on the liver, brain, and other parts of the body.

At the same time as measuring zinc levels in rheumatoid arthritis patients, I measured the mean skin tenderness thresholds of both forearms and both lower legs as criteria for skin fragility. The mean values of the skin tenderness thresholds of both lower legs were more strongly correlated to serum zinc levels, so they were graphed, and it was found that the patients whose lower leg skin was more vulnerable to pain had lower zinc levels (**Fig. 2**).

Table 2 sorts patients into Group A with zinc levels of less than 70 and Group B with zinc levels of 70 or more, based on Harrison's Internal Medicine, 17th edition [18] which defines zinc levels of less than 70 μg/dL (12 μmol/L) as zinc deficiency. The zinc level in group A was 52.5±9.3 μg/dL and that in group B was 81.0±10.6 μg/dL.

In the case of a 65-year-old woman, Case II (Case 4 in **Table 1**, indicated by a blue circle in the graph in **Fig. 2**), MRSA and Streptococcus pneumoniae were detected in her sputum and pharynx while she was waiting for artificial joint surgery. In April 1999, she developed skin fragility and skin rash on her thighs and lower legs. The serum zinc level was 37 μg/dL. She had an objective face scale of 17. Serum copper level was 91 μg/dL. The mean skin tenderness threshold on both lower legs was 0.63 kg, much lower than 4.02±1.84 kg in 62 healthy subjects. She was treated with oral polaprezinc (34 mg of zinc per day), but died of hematemesis caused by gastric ulcer 4 months after the zinc level was tested.

Case I (Case 2 in **Table 1**, indicated with a red double circle in **Fig. 2**), the 71-year-old patient [15] who had pyogenic shoulder arthritis, and the first patient I measured zinc levels for, was cured of the infection with intravenous antibiotics. The objective face scale was 18. Eventually, after 3 months of polaprezinc (34 mg of zinc per day) and 2 consecutive weeks of intravenous

Table 1. | Subjective face scale in rheumatoid arthritis patients with low serum zinc levels and cause of death in 13 patients

case	serum zinc	age	sex	face scale	cause of death	case	serum zinc	age	sex	face scale	alive or dead
	(μg/dL)						(μg/dL)				10 years later
1	25	64	F	17	pneumoia	14	51	49	F	10	alive
2	33	71	F	18	rupture of intestinse	15	51	76	F	13	alive
3	36	62	F	16	pneumonia	16	52	76	F	12	alive
4	37	65	F	17	bleeding from a stomach ulcer	17	52	69	F	10	alive
5	38	56	F	16	pneumoia	18	52	58	F	10	alive
6	47	50	F	12	pyelonephritis	19	52	68	F	11	alive
7	47	71	F	13	pneumonia	20	52	78	M	15	alive
8	47	56	F	14	pneumonia	21	54	72	F	11	alive
9	48	69	F	14	pneumonia	22	54	69	F	10	alive
10	49	59	F	13	pneumonia	23	54	63	F	10	alive
11	49	72	F	14	pneumonia	24	55	43	F	8	alive
12	50	51	F	13	pneumonia	25	55	61	F	10	alive
13	50	68	F	11	sepsis due to diabetes	26	56	78	F	9	alive
Avg.	42.8	63		14.5		Adv.	53.1	66		10.7	
SD	8	7.3		2.1		SD	1.7	11		1.8	

Table 2. | Skin tenderness threshold of group A with serum zinc level less than 70 and group B with serum zinc level more than 70 μg/dL

	Group A	Group B	Mann Whiteney U test
Zinc (μg/dL)	52.3 ± 9.3	81.0 ± 10.6	$p < 0.001$
GOT (IU/L)	20.2 ± 7.5	22.7 ± 10.6	N.S.
LDH (IU/L)	223 ± 54	210 ± 48	N.S.
CRP (mg/dL)	1.80 ± 1.80	2.00 ± 2.23	N.S.
Threshold for tenderness			
of forearm skin (kg)	1.68 ± 0.67	2.50 ± 0.96	$p < 0.01$
Threshold for tenderness			
of lower leg skin (kg)	1.75 ± 0.67	2.75 ± 0.98	$p < 0.001$

Aminofluid®, the zinc level did not increase. The patient had no appetite and chronic diarrhea, but neither upper nor lower gastrointestinal fibers revealed any abnormality, and during hospitalization, the gastrointestinal tract ruptured. She suffered three ruptures of the digestive tract within one year (four in total) and died.

The pathological findings were not severe inflammation, just perforation, similar to a leaky gut [16]. The inability in this patient to increase the zinc level from 33 μg/dL with a dose of 34 mg of polaprezinc led me to believe that a higher amount than 34 mg of zinc per day is a minimum dose necessary to increase low zinc levels.

Zinc deficiency is associated with an increased risk of gastrointestinal infections, adverse effects on the structure and function of the gastrointestinal tract, and impaired immune function [17,18,19].

As for the function and development of Paneth cells, which have a great influence on the formation of the intestinal microbiota, it has become clear that if zinc, especially ZIP7, does not work, intestinal stem cells are lost in the intestinal crypt and Paneth cell development is impaired. Even if there are no major findings on endoscopies [20], it is essential to measure zinc levels in patients with chronic diarrhea.

In 1998, the effects of zinc had not yet become widely known, and with my desperate guidance, I managed to convince the patient to take 34 mg of zinc in the morning and evening. In spite of taking 34 mg daily, four patients died within one year of taking zinc (cases 1, 2, 4, and 13 in **Table 1**). The number of patients who died 1 to 10 years after treatment and whose zinc level was less than 50 μg/dL at the time of initial diagnosis was 9 (cases 3, 5-12 in **Table 1**).

The causes of death in the 13 cases of rheumatoid arthritis with serum zinc levels below 50 μg/dL who died within 10 years were pneumonia in 9 patients, intestinal rupture in 1 patient, hematemesis of gastric ulcer in 1 patient, sepsis after diabetic foot necrosis in 1 patient, and pyelonephritis in 1 patient. There were 10 deaths due to infections out of 13 patients even after 34 mg of zinc was administered, and this was a time when many medical professionals protested that zinc administration was ineffective. Nevertheless, I did not want to lose the ability to administer zinc under the health insurance, so since 1998 I continued to submit the detailed descriptions of symptoms to the social insurance organization, stating the necessity of testing zinc levels and the necessity of sufficient zinc administration. Finally, in September 2011, it became possible to administer Promac® to patients with zinc deficiency in Nagano Prefecture.

All 13 patients with zinc levels below 50 μg/dL in **Table 1** were mentally depressed and had a mean objective face scale of 14.5 ± 2.1 [21,22]. The 13 patients with zinc levels of 51 μg/dL or higher had a mean of 10.7±1.8. Zinc is an antagonist of the N-methyl-D-aspartate (NMDA) glutamate receptor and exhibits antidepressant-like activity in rodent tests and models of depression. In a random sample of 100 female high school students, for every 10 μg/dL increase in serum zinc level, Beck's depression inventory (BDI) and hospital anxiety depression scale (HADS) decreased by 0.3 and 0.01 respectively ($p < 0.05$) [23]. These results suggest that serum zinc levels were associated with the Objective Face Scale.

From the physician's point of view, the five patients with zinc levels below 40 μg/dL (cases 1 to 5 in **Table 1**) gave the impression of being grumpy all the time, no matter when they were visited. Those with zinc levels below 50 μg/dL seemed not to be listening to me seriously, no matter how much I talked about the importance of zinc. I wondered if this was due to a decrease in zinc and an AGE (advanced glycation end-products) accumulation in the brain [24]. It could also be due to decreased microglial function.

The reason why I am describing an objective face scale is as follows. Before the consultation, I ask the patient to fill out the face scale questionnaire. Even if the physician thinks that the symptoms have improved and the patient's facial expression has improved, many patients continue to insist that the current situation is unsatisfactory and that the symptoms are still bad, so there is no change in the face scale. Since then, I have been using the face scale from the time the patient enters the examination room to the time they sit down as an objective face scale in their chart. This is because I believe that if there is "white-coat hypertension," there should be a "white-coat face scale," and that it is important to get a sense of the patient's true facial condition before they face the doctor.

Of the 53 patients with rheumatoid arthritis whose serum zinc levels were 50 μg/dL or higher who were tested between March 1998 and August 1999, only one died within 10 years despite continued zinc administration (Promac®: 32 mg of zinc per day).

The only deceased case was a 62-year-old female patient, Case III, ("red star" in **Fig. 2**) who had bilateral arthroplasties within one year; she had a 1999 zinc level of 64 μg/dL and a mean bilateral lower leg tenderness threshold of 1.42 kg. It was lower than the mean value of 2.50±0.96 kg in group B (serum zinc level >70) and lower than the mean value of 1.75±0.67 kg in group A (serum zinc level <70) (**Table 2**). I thought this was a great risk for surgery and at the pre-surgical meeting, I insisted that the zinc level be raised to 70 μg/dL or higher before surgery, but this was rejected, and the left knee joint arthroplasty was performed. Immediately after that, diabetes developed. According to the preliminary plan, the right arthroplasty, on the opposite side of the body, was scheduled one month after the surgery, but it was postponed to three months later due to the treatment of diabetes. The serum zinc level before the second surgery decreased to 32 μg/dL, and I rated the objective face scale as 16. The right knee arthroplasty was performed as usual and the surgery went well, but she had a stroke two days after the surgery and eventually died one month later.

By this time zinc administration was beginning to be shown to be effective in children with respiratory tract infections in double-blind, controlled trials [25]. The relationship between zinc and cerebral infarction is not clear, but the relationship between zinc and blood vessels, PPARγ, and PPARα has been reported [26], so it seems necessary to pay attention to zinc levels in patients who are prone to cerebral infarction. It has also been found that zinc inhibits phosphate-induced vascular calcification via TNF-α-induced protein 3-mediated inhibition of NF-κB [27]. Also, with regard to diabetes mellitus, it was clear in 2015 that zinc levels were low [28,29], but the pre-surgical meeting at the time dismissed my insistence on waiting until we increased

the zinc level to 70 μg/dL.

Since insulin granules of pancreatic β-cells decrease in zinc-deficient states [30] and zinc has actually been observed in insulin-containing granules of pancreatic β-cells [31], I believe that surgery should be postponed until zinc levels recover, if zinc levels are low in patients with diabetes mellitus.

In 2006, Kurasawa reported the results of zinc level measurement in local residents in Nagano Prefecture. The mean serum zinc level of 341 adults (mean age 54.8 years) was 78.9±11.6 μg/dL [32].

In 2007, a double-blind study of elderly patients in 33 nursing homes in Boston reported a significant reduction in pneumonia infection rate (about half), fewer days of illness, fewer days of antibiotic use, and lower all-cause mortality [33] in elderly patients with serum zinc levels in the normal range.

After three years of experience at the Marunouchi Hospital, I became convinced that patients with 1) weak skin, 2) poor objective face scale, 3) chronic diarrhea, and 4) diabetes mellitus should be tested and low zinc levels should be improved to at least 70 μg/dL before surgery. Therefore, I conducted a study between 2006 and 2008 at Shinonoi General Hospital, Minami-Nagano Medical Center, my next place of work, to determine 1) what symptoms would improve if the patient's zinc level was raised to 70 μg/dL and 2) what amount of zinc would be needed to raise the serum zinc level to 70 μg/dL.

4. Improvement in clinical symptoms by zinc administration

Serum zinc levels were measured in 312 patients with rheumatoid arthritis who visited the Minami Nagano Medical Center between April 2001 and October 2004. Fluctuations in serum zinc levels, the presence or absence of improvement in 21 subjective symptoms, and a disease activity index of 28 were investigated. The results showed that 228 patients (73.1%) had levels below 70 μg/dL. Of the 228 cases, 90 were treated with 34 mg of polaprezinc daily zinc dose [34]. Of these, 81 patients had been on the medication for more than 6 months, and during that period they took the same prescription of the drug used to treat rheumatoid arthritis.

For comparative purposes, the patients were divided into three groups: Group 1: those whose serum zinc levels increased after 6 months of polaprezinc, Group 2: those whose serum zinc levels did not change after 6 months of polaprezinc, and Group 3: those who did not receive polaprezinc. As a statistical check, a paired test was performed for each item before and after polaprezinc administration. There were 64 cases in group 1; 17 cases in group 2; and 19 cases in group 3.

In group 1, the average zinc serum level increased from 56.1 μg/dL to 86.3 μg/dL during the first 6 months of zinc administration. In group 2, zinc serum levels had decreased from 59.5 μg/dL to 56.1 μg/dL. The ratio of males to females was approximately 1:3 in each group. In terms of age, there was little difference among the three groups. There was also little difference in the duration of the disease.

In the first group with increased serum levels of zinc, the CRP level significantly improved from 1.82 mg/dL to 1.22 mg/dL. The total cholesterol level also increased significantly from 189 mg/dL to 198 mg/dL. In the second group where there was no change in serum levels of zinc, there was also no change in values other than zinc. In the third group, which did not receive polaprezinc medication, no changes were observed in any values (**Fig. 3**).

The main subjective symptoms of the first group were significantly better than those of the second group, including swollen joints (39.3%>20.0%), pale complexion (41.2%>14.3%), susceptibility to colds (66.7%>50%), mental instability (100%>33.3%), and dermal abscess (63.6%>50%). In the Zinc non-medication group no subjective symptoms improved.

DAS28 (Disease Activity Score 28) was calculated using four factors: A. Number of painful joints, B. Number of swollen joints, D. Visual analog scale, and E. CRP, which was used instead of erythrocyte sedimentation rate (C). By comparing the DAS28 of each patient at two different time points, improvement or response can be defined.

In the first group, 28.1% had "good" DAS28 and 29.7% were "moderate". In group 2, there were no cases judged to be "good" and 29.4% were "moderate". In group 3, where polaprezinc was not administered, only 5.6% were "moderate", and 94.4% had "no symptom improvement". In other words, DAS28 was improved by administration of zinc and increase in serum zinc level (**Fig. 4**).

However, the zinc levels of about 20% of the patients did not increase even after zinc administration, and it was necessary to examine whether a dosage of 68 mg or more, instead of 34 mg, was necessary to improve symptoms within 6 months in the rheumatoid arthritis patients with zinc deficiency. However, due to insurance reasons and drug costs, we could not perform a significant difference test between zinc doses of 34 mg, 68 mg, and 100 mg.

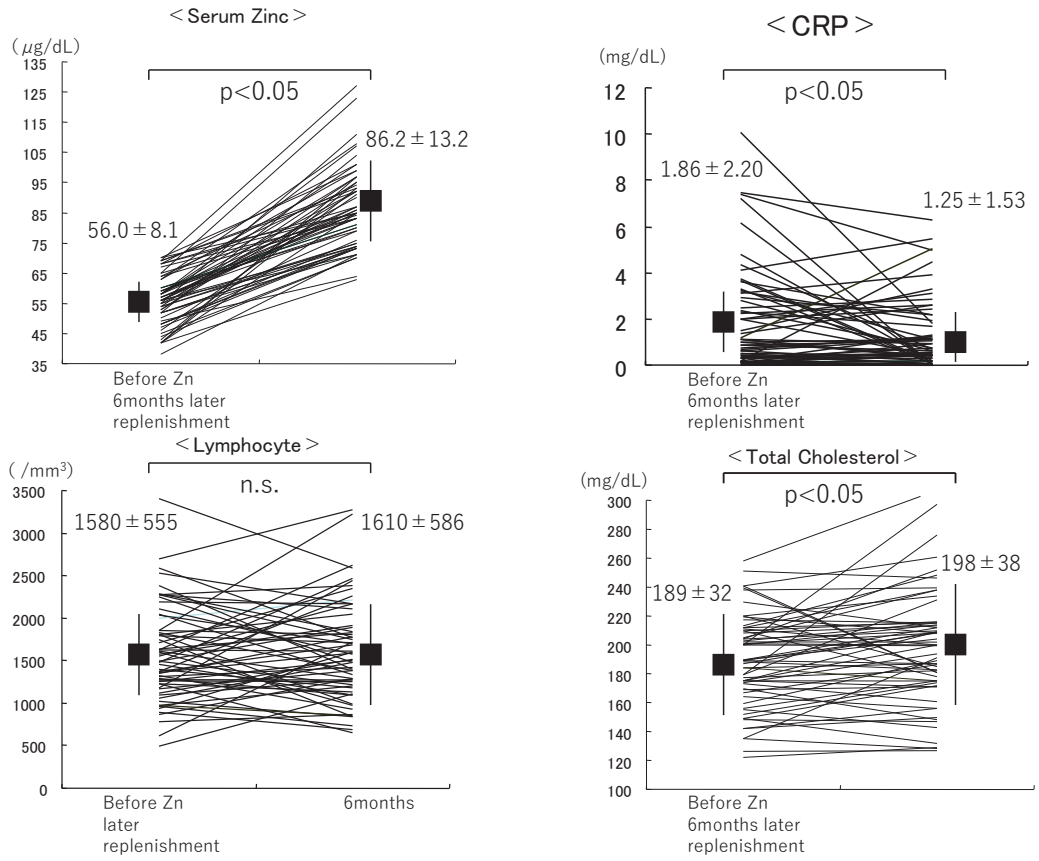

Fig. 3. | Change in CRP and cholesterol level in increased zinc group

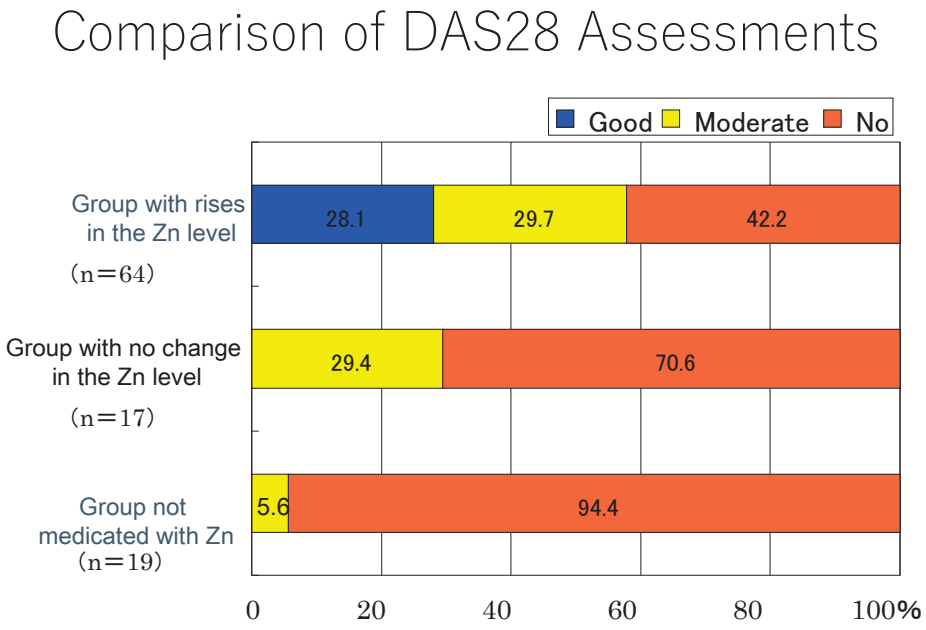

Fig. 4. | DAS28 is more likely to be improved in the group with increased zinc level

Later, in 2019, we reported that we were able to improve the sense of smell in patients with olfactory deafferentation after 2 years and 8 months of treatment with 34 mg of zinc followed by 3 months of treatment with 68 mg of zinc. There were three cases of olfactory disorders treated [35].

In addition, patients with neuropathic pain experienced relief after 9 months, 4 months, 3 months, 8 months, and 1 month of treatment with zinc doses of 100 mg or more per day. Since patients cannot tolerate their own neuropathic pain for a long period of time, I would argue that the attending physician should increase the zinc dose to the required amount needed by the patient.

Unless patients are on dialysis, the zinc dose should be increased so that the patient can reach a zinc level of 100 μg/dL to improve the symptoms quickly. I believe that the saving of a near-infected prosthesis was the result of raising the serum level to 108 μg/dL, and I attribute the case of a widespread pressure ulcer where Pseudomonas aeruginosa was detected all the time in local culture but the wound closed to increasing the serum level to 110 μg/dL. A case of sleepless neuropathic pain that improved from a VAS of 98 to 6 resulted from an increase in serum zinc levels from 61 μg/dL to 106 μg/dL. I aim to achieve a zinc level of 100 μg/dL or higher in cases that I judge to be difficult to treat.

5. Preoperative zinc administration and postoperative course

In 2002, I experienced a case of a patient who was referred from another physician to our department. The patient was treated with zinc only for a few days before surgery and did not have a good postoperative course.

The patient, Case IV, was diagnosed with rheumatoid arthritis in 1996 and had been treated with methotrexate 4 mg/week, and was first seen by a doctor in our department in August 2001, diagnosed as Steinbrocker stage IV class III. Because of the presence of a large geode (cystic bone destruction) and the side effects of 10 different medications, we offered to measure her zinc level and bone mass at the preoperative meeting. The zinc level was 55 μg/dl just before the surgery, so we suggested postponing the procedure until the zinc level increased, but the patient did not want to postpone. I performed the left knee arthroplasty with only 5 days of Promac® (34 mg zinc/day) supplementation; bone grafting and arthroplasty was performed on the geode, but immediately after, the patient developed left peroneal nerve palsy(**Fig.5**). She had a loss of muscle strength in the left tibialis anterior, left extensor hallucis longus, and left extensor digitorum longus muscles (manual muscle test was 0), and painful sensory loss between the first and second toes was observed, but tactile sensation was maintained. After 3 weeks of treatment with 2 tablets of Promac® (daily zinc dose 34 mg: the amount allowed by insurance), Tinel's sign was observed at the neck of the fibula.

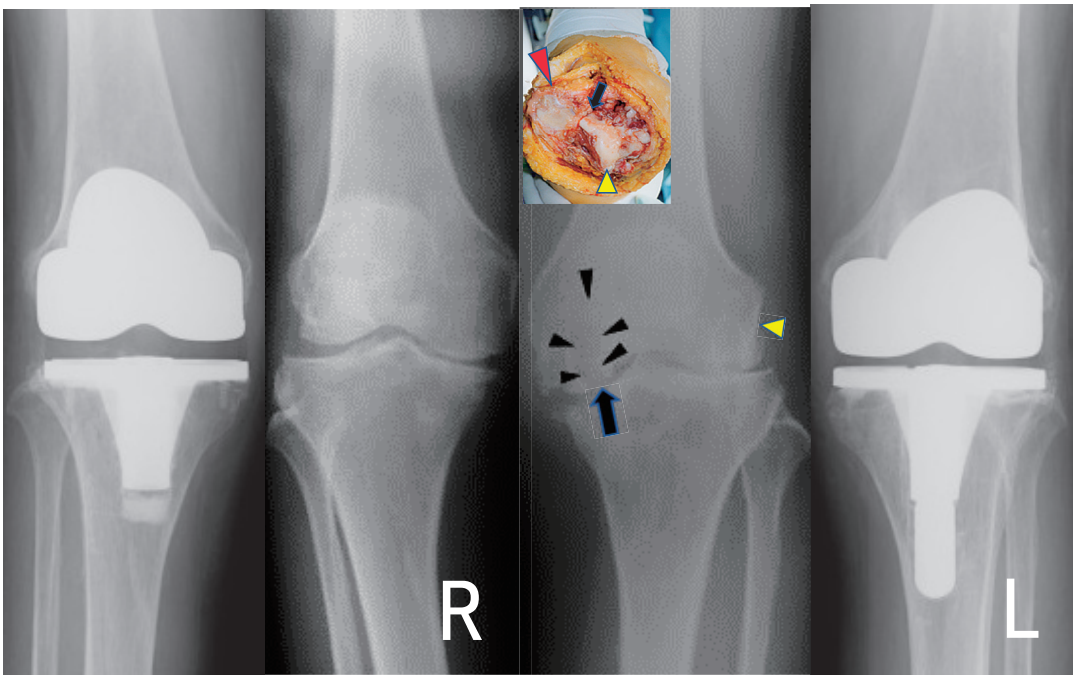

Fig. 5. Comparison of radiographs taken before and 10 years after surgery. Arrow heads: geode of left femur; arrows: hole leading from the cartilage of the femur to the geode; yellow arrow: left femur; red arrow: left patella

Postoperatively, there was skin fusion failure, and it was possible to remove stitches after 21 days. Three Promac® tablets (51 mg zinc/day) were continued, and muscle strength was completely restored after four months [36]. Since it usually takes about 6 months, we thought that zinc supplementation might have some effect on the peripheral nerves. Zinc was also observed in the synaptic vesicles of neurons in 1999 [37]. It is possible that the zinc worked to improve the damaged nerves.

In 2016, it was found that the expression of RANKL in synovial fibroblasts is mainly involved in the formation of osteoclasts and erosions in inflammatory arthritis [38], so the large femoral defect and the bone erosion on the tibial side in this case seemed to be caused by the action of RANKL in fibroblasts.

In this case with low zinc levels and poor osteoimmunity [39], I believe that excessive activation of the immune system inhibited bone formation and promoted bone resorption, resulting in disruption of bone homeostasis and inflammatory bone destruction.

Five months after zinc supplementation, she reported that the taste of cola improved. In other words, the patient had not been aware of any preoperative taste disturbance, despite a serum zinc level of 55 μg/dl.

Thereafter, zinc supplementation was continued and the right knee arthroplasty was successfully completed without peroneal nerve palsy or delayed skin fusion. After the right hip replacement autologous bone graft was performed. The left hip replacement was also successfully performed.

The patient was tranfered to another doctor, she stopped taking zinc and the serum level dropped to 58 μg/dL. Six months later, she suffered a fragile pubic fracture and was hospitalized; her osteocalcin level reached 6. A dosage of 34mg intake zinc had allowed completion of four arthroplasties (**Fig.6**), hand surgery and brain surgery [40]. The patient is still able to walk 19 years after the first surgery, keeping zinc levels above 80 μg/dl, and has not had a fragility fracture since 2010. The objective face scale improved from 14 at the initial visit to 3 after the final surgery. There is a striking contrast with Case III which I believe is due to osteoimmunity resulting from zinc treatment.

Based on the post-operative experience of the first prosthesis in this case, we reviewed the case zinc values of patients with rheumatoid arthritis who had prosthesis surgery over the period 2003-2010. Patients who were referred from other doctors and supplemented with 34 mg of zinc per day for 2 months prior to surgery were group 1, and patients who were treated at our hospital and supplemented with 34 mg of zinc per day for 6 months prior to surgery were group 2. The first group consisted of 67 patients with a mean age of 65.7±8.9 years, and the second group consisted of 24 patients with a mean age of 68.3±7.7 years, so there was no significant difference in age.

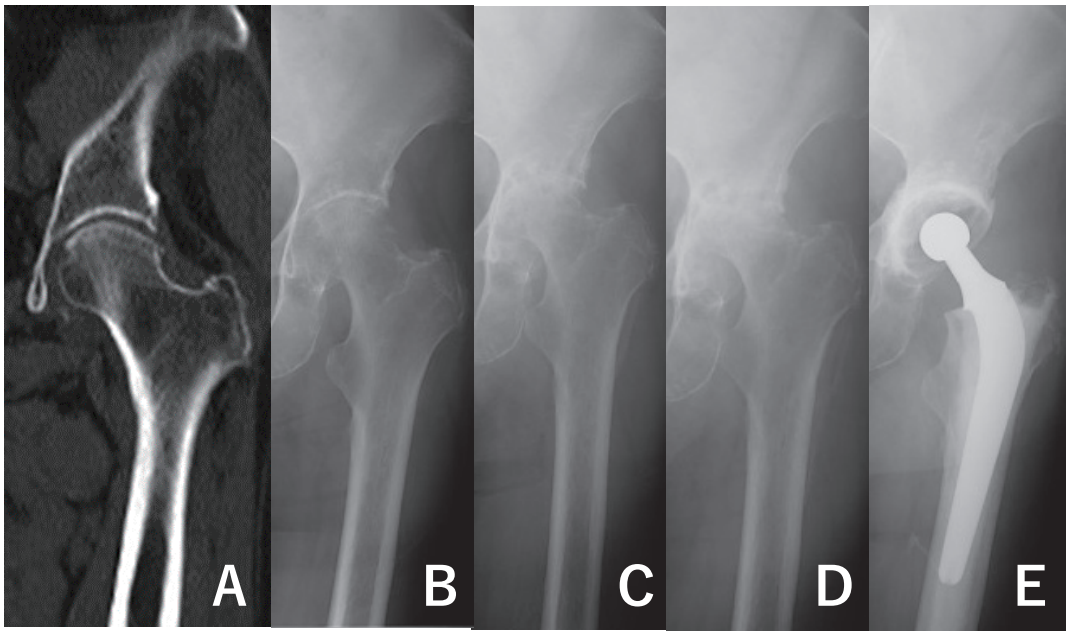

Fig. 6. The last arthroplasty of left hip joint. Preoperative CT, preoperative radiographs and postoperative radiographs of the left hip joint
A: CT of the right hip joint in December 2004; B: X-P in August 2005; C: X-P in June 2006; D: X-P in August 2006; E: Postoperative X-P in October 2006
As can be seen in A, B, C, and D, the joint gap narrowed as the years progressed

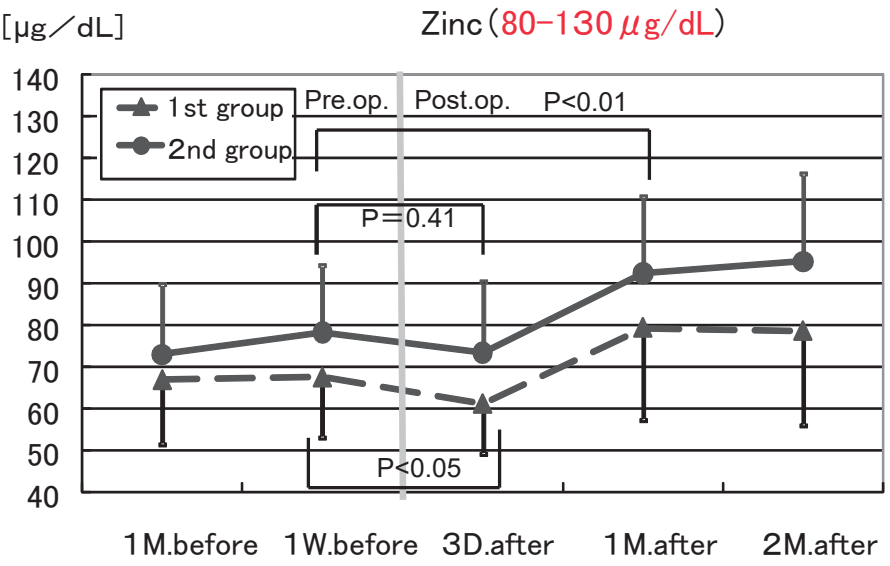

Fig. 7. Postoperative zinc levels were more stable in the 6-month preoperative zinc supplementation group than in the 2-month preoperative zinc supplementation group

One month before the surgery, the mean zinc level was slightly higher in the second group, but the difference was not significant at p=0.12. However, one week before the surgery, the level was higher in group 2 with p<0.01 (**Fig. 7**). One month after the decision to have surgery, patients are anxious. In the first group, there were four cases in which zinc levels dropped by more than 10 μg/dL just before the surgery compared to one month before, despite zinc supplementation. The range of decline was 22, 15, 14, and 13 μg/dL. In the second group, there was only one case that dropped 14 μg/dL from one month to one week before. The reason for this was thought to be that the patients were able to endure mental stress after 6 months of medication, as considered from the results of the previous study [23,41].

The postoperative wound condition was as follows: 3 cases of skin necrosis and 4 cases of delayed skin healing among 67 patients in the first group. In group 2, there were no cases of skin necrosis or delayed skin healing. Nishida showed that zinc released by mast cells is involved in the production of IL-6 during the inflammatory phase of wound healing [42]. It can be said that high serum zinc levels were effective for wound healing.

Considering the fact that the copper level did not drop below the reference level in any of the cases with a high dose of zinc, the safety margin of zinc in the preoperative and postoperative periods is much wider than expected.

The following are cases from the first group of patients who received zinc supplementation for two months.

Case V: The patient had severe signs of infection from 3 days after surgery, and in order to eliminate the risk, she was forced to use double the amount of zinc, 64 mg, until 2 weeks after surgery, when she had to use Aminofluid®, and then 34 mg of zinc per day, so she was placed in Group 1.

The patient was a 74-year-old woman with a history of ovarian cyst and intestinal obstruction; she had a history of pus from the toe two months after left toeplasty in 1993, and pus from the right upper arm and right sternoclavicular joint in the same year, both of which were detected to be Staphylococcus aureus; she underwent right knee arthroplasty by another doctor in 1993, which resulted in yellow exudate and delayed healing. In 1994, she had another abscess from the left fifth toe, and Staphylococcus aureus was detected by bacterial culture.

In 2007, she wanted to have left knee arthroplasty, so zinc supplementation was started 2 months before, and her serum zinc level was 75 μg/dL 1 month before. The zinc test was outsourced to SRL, and the report came back two days before the surgery that the zinc level had been 137 μg/dL one week before the surgery. I expected the zinc level to be 80 μg/dL, but the level was so much higher that I suspected contamination, but I gave up on retesting because the results would not be available until after the surgery. Therefore, the data of zinc level one week before the surgery was left blank. The preoperative VAS of pain was 64. The preoperative objective face scale was 14, which was a matter of concern.

The surgery was performed as usual, and the zinc level 3 days after surgery was 58 μg/dL, although the result came to us 10 days after surgery as reported by SRL. Five days after surgery, the blisters broke and the CRP was 7.61 (**Fig. 8A**). Thereafter, the skin rapidly became necrotic, and 8 days after surgery, the patient had a fever of 38.2°C. The zinc level 8 days postoperatively was 108 μg/dL (**Fig. 8B**). Pus from the ulcer area was submitted for bacterial culture on three occasions, but no bacteria were detected. During the course of the disease, the patient was treated according to the postoperative infection of the prosthesis [43,44].

From 5 to 14 days after surgery, the patient was treated with a combination of polaprezinc (68 mg of zinc per day) and an intravenous infusion of a vitamin, sugar, electrolyte, and amino acid preparation (Bfluid®) containing 2.5 μmol (0.7 mg) zinc, followed by 34 mg of zinc per day, and the serum zinc level was maintained at 107 μg/dL after one month. The wound was completely closed at 83 days postoperatively (**Fig. 8H**) [45]. Two months after surgery, the patient's face scale improved to 2. Preoperatively, the patient was very anxious and refused to have her face photographed even though I wanted to do so. After the surgery, the patient was smiling so it was easy for me to take her picture. I would really like to have a comparison of the pre- and post-operative facial photos, but more than 90% of the patients refused to have their pre-operative photos taken, so there are no comparison photos.

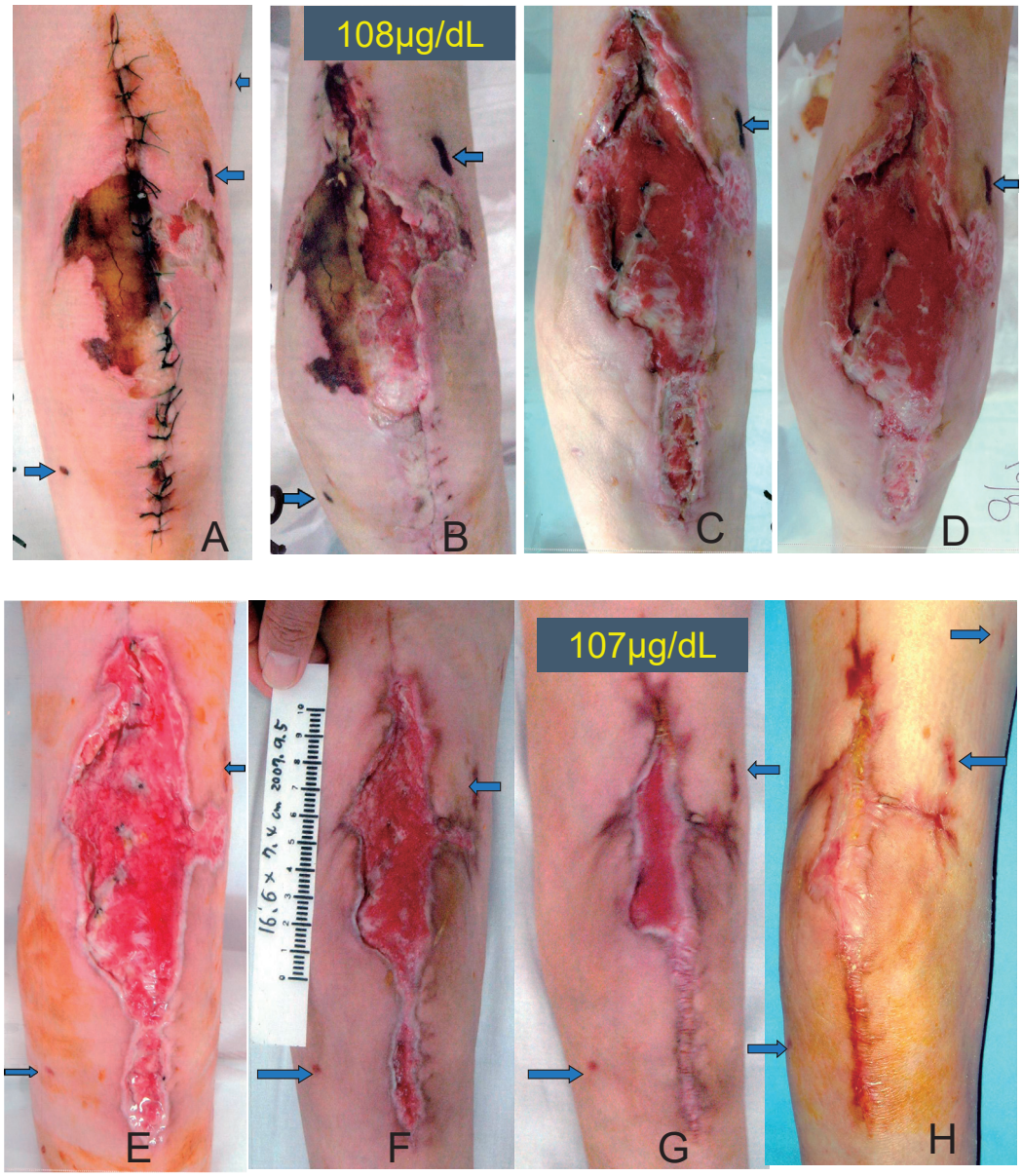

Fig. 8 A-H. Change in skin condition after knee replacement surgery. Arrows show skin necrosis site. A: 5d.aft.surg. B: 8d.aft.surg. H: Wound completely closed 83 days after surgery

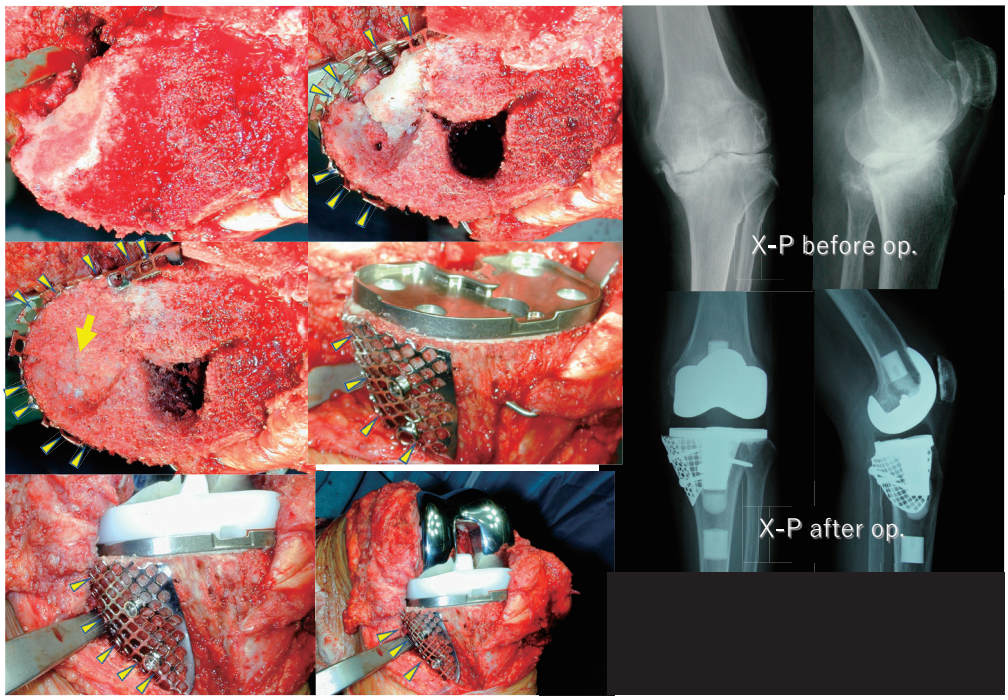

Fig. 9. | Knee joint prosthesis after impaction bone graft covered with mesh

The serum zinc level was 75 μg/dL one month before the surgery, so we were confident to start zinc supplementation two months before the surgery. However, considering the history of four Staphylococcus aureus infections, the preoperative objective face scale of 14, and the VAS of pain of 64, we thought that the patient was at significant risk and should have been treated with zinc for more than six months before the surgery. As a final result, the right knee was able to flex 100 degrees after surgery, and the patient and her family were grateful.

This case, which was very difficult to treat, made the hospital laboratory aware of the dangers that can occur if zinc testing is not performed in the hospital. Sinotest's Acuras Auto Zn® was introduced to the Minami-Nagano Medical Center in April 2008. Since then, it has been very useful for us to be able to immediately retest the zinc in case of any contamination such as red blood cell destruction.

It has long been known that zinc acts on skin metabolism and promotes wound repair [46,47]. We have continued to supplement 34 mg of zinc after surgery in all cases, and the patients have been followed for up to 18 years after joint replacement. In all postoperative patients, including those who had large bone blocks grafted, those who had impaction bone grafting with rim mesh (**Fig. 9**), [45] and dialysis patients, there were no patients with crushed bones or diabetes mellitus after 18 years. When postmenopausal osteoporosis was treated with zinc preparations, bone density was reported to be higher as serum zinc was satisfied [48]. There were no cases of skin necrosis. In cases of skin necrosis, the necrosis could spread and develop into pyogenic arthritis and pyogenic osteomyelitis of the bone joints. In orthopedic surgery, it is very important to prevent infection of bones and joints.

Vikbladh was the first to report a decrease in serum zinc levels in infectious diseases in 1951 [49], but when I became an orthopedic surgeon in 1983, the detailed mechanism was not yet known. Only a few surgeons recognized that zinc was related to infections. Therefore, no orthopedic surgeon would measure zinc levels before operating on a patient. Later papers advocating the importance of zinc levels in surgery were published by Shoji et al. [50], Ono [36], Kaido et al. [51], Yan et al. [52] and others.

In 2006, a prospective study of 80 total hip arthroplasties was published, suggesting an association between delayed wound healing and preoperative serum zinc levels [53].

6. A study of patients from other departments who had problems before and after surgery

During my 11 years of experience as the chief of a nutrition support team (NST) in a 433-bed hospital from April 2005 to March 2016, I have had many problems with zinc levels among the 30 referrals per week. Among them, I would like to show you the patients who left a lasting impression on me after surgery.

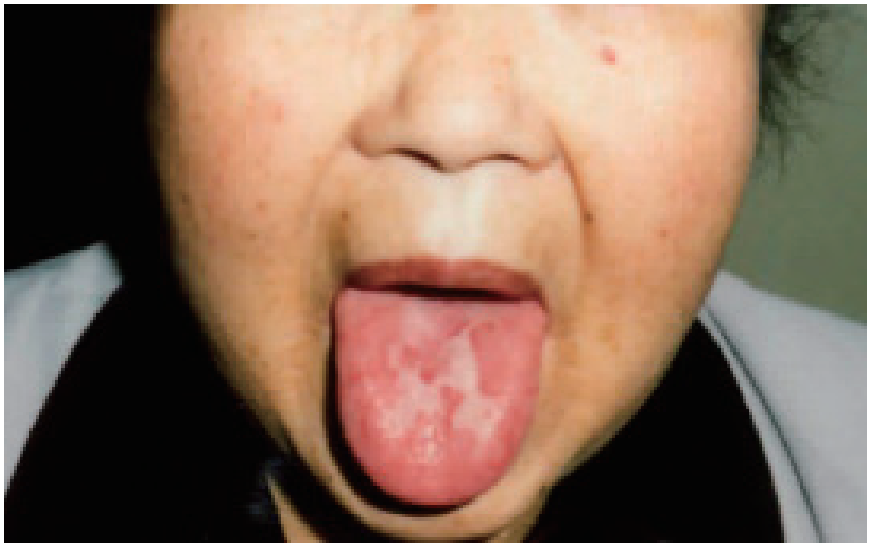

Fig. 10. | Grade 3 oral mucosal disorder of tongue

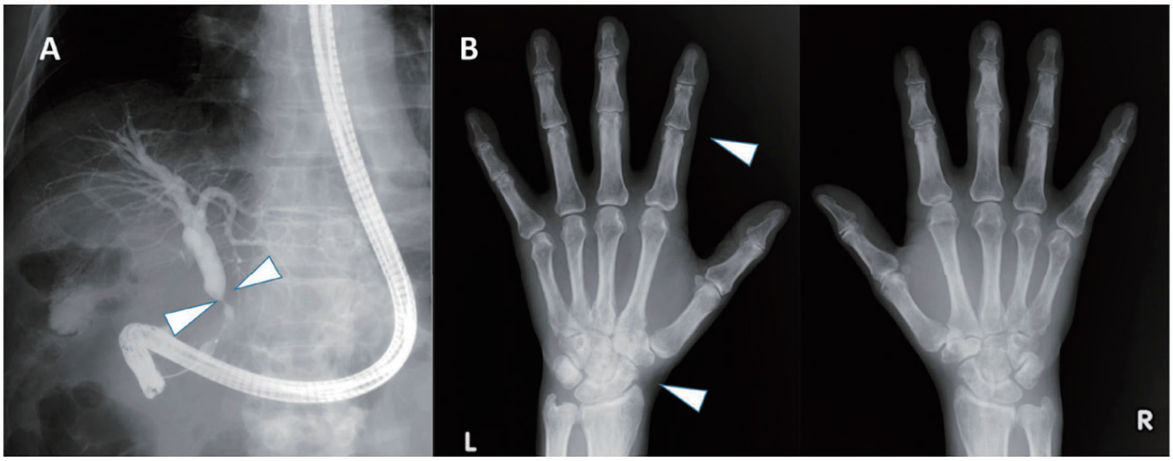

Fig. 11. | Hand pain after pancreatoduodenectomy, but no bone ulcer

The patient, Case VI, came to see me after thoracic surgery. The skin of the chest did not heal, the zinc level was 54 μg/dL, and the patient had grade 3 oral mucosal disorder (**Fig. 10**). She had an objective face scale of 12. After 58 days of treatment with 3 Promac® tablets (51 mg zinc), the sutures were closed and the moderate grade 3 oral mucosal ulcerations were healed when the zinc level reached 84 μg/dL. The objective face scale was 7. The patient was encouraged to swallow Promac® after keeping it in her mouth for about 1 minute so that it could spread to the oral mucosa.

A postoperative abdominal surgery patient also had problems.

Case VII: The patient was a 67-year-old male post-surgical patient. He underwent pancreaticoduodenectomy for cholangiocarcinoma, and anticancer drugs were used from the postoperative period until one year and two months after the surgery was completed. One year and eight months after surgery, CRP was 0.08 and CT and PET showed no metastasis. Immediately thereafter, the patient developed fever for 3 days, with swelling of the hands and wrist joint pain (**Fig. 11**).

The main complaint was stiffness of the hands. Other symptoms were severe general malaise, difficulty walking, decreased right grip strength, insomnia, and loss of appetite. The patient's right grip strength was 90 mmHg, which was very low. He could only complete the 30-second chair stand test [54,55] 3 times. The patient's first words were, “I'm going to die if you don't do something”. We suspected rheumatoid arthritis because of the RF quantification of 58 and CRP of 1.65, but the anti-CCP

antibody was 35.3 and MMP-3 was 65.5 at this point. Furthermore, joint echocardiography showed no synovitis, and radiographs showed no erosions, so rheumatoid arthritis was ruled out. The only abnormal value was the serum zinc level (21.3 μg/dL) [56], which was the lowest zinc level I had ever experienced.

Polaprezinc (51 mg of zinc per day) was started, and after 11 days, the patient's right grip strength recovered to 220 mmHg and CRP improved to 0.15. Thereafter, 68 mg of zinc daily was administered until 10 weeks later, and the patient recovered to CRP 0.06, right grip strength 280 mmHg, and zinc level increased to 70.1 μg/dL. The objective face scale was 11. This patient was very noncompliant. He stopped taking zinc after each symptom improvement, did not see the doctor for several months, and then only when the symptoms reappeared.

Three years and six months after surgery (September 2018), he was taking 100 mg of zinc (Nobelzin®: Zinc acetate hydrate preparation) daily and had a zinc level of 89.5 μg/dL, anti-CCP antibody of 33.8, and MMP-3 of 75.4, which was not bad. His grip strength improved to 300 mmHg on the right side. He had been insomniac, but now he could sleep well and his appetite was recovered. His fatigue and feeling of wanting to die disappeared.

On the day of his visit, I spent an hour explaining to him and his family that a decrease in zinc level would cause Th17 of T lymphocytes to function poorly, resulting in runaway immunity, but he did not understand it at all.

He did not take zinc internally for 8 months and was seen again 4 years and 4 months after surgery due to general fatigue. His serum zinc level had dropped to 47.2 μg/dL and his anti-CCP antibody had doubled to 184. His MMP-3 was 80.1, and joint echocardiography showed no synovitis, so no arthritis was occurring.

The patient's zinc level was originally low at 21.3 μg/dL, suggesting low intestinal absorption, but when the zinc level rose to 89.5 μg/dL and his symptoms improved, he stopped taking zinc. I showed him my book. After I explained it in detail, his understanding gradually deepened. From the time he entered the examination room to the time he sat down in the chair, his objective face scale was 10.

If the grip strength is less than 200 mmHg and the lower limb muscle strength is less than 20 times in the 30-second chair stand test, the preoperative zinc level should be measured, and if it is low, zinc supplementation is necessary.

Some urological postoperative patients also had problems.

An 81-year-old male patient, Case VIII, who had undergone left nephrostomy for left hydronephrosis was referred to NST 13 days after surgery. His medical history included retroperitoneal tumor, left hydronephrosis, left pyothorax, and sepsis. Thirteen days after surgery, the serum zinc level was 54 μg/dL as shown in the left picture. The objective face scale was 15. Polaprezinc 1.5 g (51 mg of zinc per day) was started, and the zinc level rose by 13 μg/dL to 67 μg/dL after 14 days (**Fig. 12**). At that point, the stomatitis and glossitis were cured. The face scale was 9, probably due to the absence of pain in the mouth. With zinc supplementation, the patient's main complaint of not being able to eat any food disappeared, and he was very happy to be able to eat eel.

At the same time, many patients in the cardiology department were also cured of post-catheterization stomatitis by zinc administration (34 mg of zinc per day). Furthermore, the Cu/Zn ratio increases with the progression of liver metastasis and gastric cancer, and is useful for estimating malignancy and prognosis [57]. Therefore, it is recommended that zinc levels be measured in patients with severe disease, even after surgery for cancer.

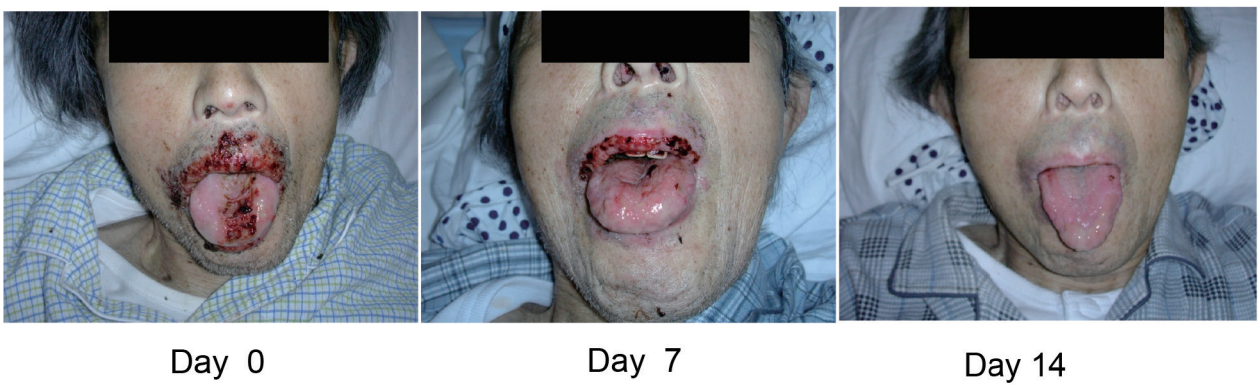

Fig. 12. | Postoperative tongue disorder in a patient with urological disease, healed after 14 days of zinc supplementation

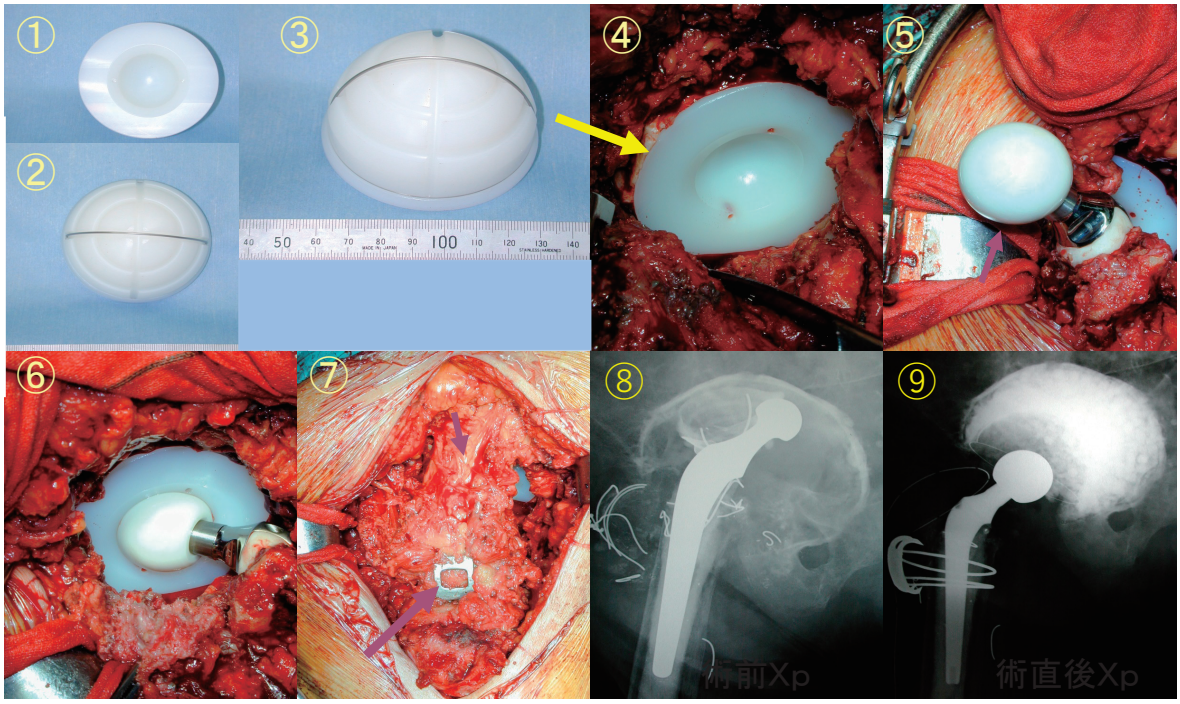

Fig. 13. Revision Total hip arthroplasty (THA) with large cup in a dialysis patient
1, 2, 3: Special order large socket (Φ73 X 68 mm) was fixed by 160g bone cementing. 4: Socket fixation
5: Zirconia ball diameter was 32 mm. 6: Stem was fixed by cement-in-cement. 7: Bone atrophy was marked.
8: Before revision THA 9: After revision THA

A patient, Case IX, 23 years after starting hemodialysis had continued shoulder edema, and the joint prosthesis operated by another orthopedic surgeon dislocated 20 years after surgery, rendering the patient unable to walk. She was scheduled for reoperation, but was referred to NST every two weeks because of shoulder edema and drainage of more than 100 cc. The zinc level was 58.1 μg/dL. She was always in a wheelchair. She had an objective face scale of 12. After 9 years of zinc supplementation, her zinc level reached 78 μg/dL and her shoulder joint edema disappeared. The objective face scale improved to 7. Our surgical team performed bone grafting using three cryopreserved femoral heads, followed by special order large socket (φ73 x 68 mm) hip replacement in 2009 (**Fig. 13**). The grafted bone was still attached two years later [58]. The patient recovered to be able to drive a car by herself.

Suzuki (2015) also reported that osteocalcin was decreased, serum Ca was decreased, and serum PTH was increased in the zinc-deficient diet group of rats, inducing bone fragility [59]. In the case of dialysis patients, it is important not to lower copper levels too much in order to maintain blood vessels, and the fact that zinc levels only increased from 58.1 μg/dL to 78.8 μg/dL in order not to lower copper levels too much by administering polaprezinc (34 mg of zinc per day) may have led to a favorable prognosis.

According to Nishime in 2020, it is safe to administer zinc to patients who are not on dialysis and raise their serum zinc level to 250 μg/dL, but copper is only calculated to drop to 75 μg/dL. However, if a patient on dialysis is given zinc to raise the zinc level to 100 μg/dL, the copper level will drop to 52 μg/dL, causing vascular fragility, which is dangerous [60]. For serum zinc levels in dialysis patients, it is preferable to use cocoa in combination with zinc tablets while measuring copper levels and reducing the amount of zinc tablets when copper levels become low. If the serum zinc level increases, ALP, IGF, and osteocalcin also increase, which is beneficial for bone formation.

There is a report from vascular surgeons on the surgical outcomes of the zinc level group above 60 μg/dL and the group below 60 μg/dL. In clinical outcomes after subungual bypass surgery for critical lower limb ischemia, hypozinchemia below 60 μg/dL worsened limb salvage rate, amputation-free survival rate, and wound healing rate [61]. It is gratifying to see increasing reports of the dangers of hypozinchemia in surgery at other institutions.

Another patient, Case X, is a 77-year-old with rheumatoid arthritis who had been experiencing joint edema and other doctors were thinking of performing arthroscopic surgery. The patient had been experiencing diarrhea for a long time, and the

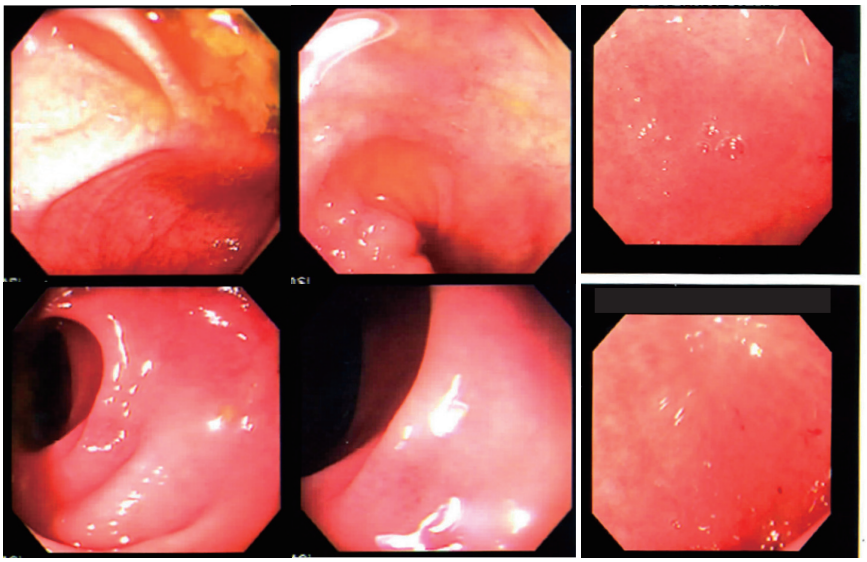

Fig. 14. Neither upper gastrointestinal endoscopy and colonfiberscopy, revealed any abnormal findings.

gastroenterologist performed both upper gastrointestinal endoscopy and colonfiberscopy, but found no abnormalities anywhere (**Fig. 14**). In our department, we found a zinc level of 36 μg/dL.

The patient was admitted to the hospital for 8 weeks and treated daily with 34 mg of polaprezinc and intravenous Aminofluid® to increase the zinc level to 53 μg/dL, and the diarrhea was cured. Finally, when the serum zinc level was raised to more than 70 μg/dL, the arthroedema healed and arthroscopy was not performed.

Zinc is clearly effective in the treatment of diarrhea in children [62,63,64]. Since even 5 mg zinc is effective in children after 30 days of administration, it seems that even a 77-year-old rheumatoid arthritis patient with a very poor absorption rate could be improved in 8 weeks.

Furthermore, in my personal experience, over the past 30 years from 1991 to 2021, there have been more than 1,000 patients with zinc levels below 78 μg/dL who have been treated with zinc doses. Of the zinc-treated patients, only one 79-year-old with liver cancer (who complained beer did not taste good any more) was found to have cancer after 3 years of zinc treatment. According to the National Cancer Institute, the probability of dying from cancer in the entire population was 26.7% for men and 17.8% for women in 2019 data. The cancer mortality rate of 0.1% in zinc-treated patients is clearly lower than this, so I would even say that it is beneficial rather than safe. According to Prasad et al. zinc supplementation should have beneficial effects on cancer by decreasing angiogenesis and the induction of inflammatory cytokines while increasing apoptosis in cancer cells [65], and these effects will need to be further investigated. I believe that zinc is safe as long as the patient is properly tested for copper at 6-month intervals.

These serum zinc level measurements and associated clinical studies were approved by the Ethics Committee and Clinical Research Review Committee in February 1998, March 2001, and July 2016. Furthermore, the study was approved by the Ethics Committee and Clinical Research Review Committee of the hospital in 2019 under approval number R-20.

Conclusion

Before any surgery, I recommend that patients with poor face scale, obvious skin fragility, chronic diarrhea, diabetes, or weakened immune system should have their zinc levels measured and raised before the surgery. However, in elderly patients aged 65 years or more with serum zinc levels below 60 μg/dL, even 3 months of supplementation with 30 mg/day of zinc failed to increase serum zinc levels above 70 μg/dL [66]. Therefore, if we want to raise the serum zinc level of preoperative patients from below 60 to above 80 μg/dL as soon as possible, we should consider administering 50, 75, or 100 mg of zinc per day using Nobelzin®.

Acknowledgments

I would like to thank Masaaki Maruyama, MD. PhD, Kazuhiko Yoneda, MD, and Teruo Shimano MD. of Shinonoi General Hospital for assisting me in the joint surgery.

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Review https://metallomicsresearch.brte.org

Overview of the zinc absorption mechanism for improving zinc nutrition

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Abstract

Zinc is an essential trace element with various physiological functions; it is a structural, catalytic, and signaling component of proteins. Owing to its wide range of functions, zinc deficiency causes various symptoms, such as taste disorders, dermatitis, hair loss, decreased appetite, growth disorders, and gonad dysfunction. The global prevalence of zinc deficiency is estimated to be about 25%; thus, its prevention is important for human health. Approximately 2–3 g of zinc is present in the adult human body. Systemic zinc homeostasis is regulated by the zinc transporters ZIP4 and ZNT1, which play major roles in regulating the absorption of food-derived zinc, primarily in the duodenum and jejunum. ZIP4 is expressed on the apical membrane of intestinal epithelial cells and allows divalent zinc ions to enter cells from the lumen. Zinc in enterocytes is subsequently transported by ZNT1 on the basolateral membrane into the portal vein, where it binds to albumin and α2-macroglobulin. In turn, zinc regulates the expression of ZIP4 and ZNT1. This review briefly describes the mechanism of dietary zinc absorption, focusing on zinc in foods and the transporters involved in zinc absorption in the intestinal tract. Moreover, we discuss the potential of dietary components to increase the efficiency of zinc absorption in the intestinal tract via zinc transporters and improve zinc nutrition.

Key words: zinc, absorption, trace element, zinc transporter

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

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Received: December 30, 2021
Accepted: February 14, 2022
Released online: March 31, 2022

1. Introduction

Zinc is the second most abundantly distributed essential trace element in the body at a level of approximately 2–3 g. About 60% of total zinc in the body is stored in skeletal muscles; ~30% in bones; ~5% in the liver and skin, and the rest is widely distributed throughout the body, including the brain and kidneys [1]. Based on bioinformatics research, approximately 2,800 human proteins are potentially zinc-binding proteins *in vivo* [2]. Zinc functions as an important cofactor for the activity of as many as 300 zinc-containing enzymes, including alcohol dehydrogenase, carbonic anhydrase, and superoxide dismutase [3,4]. Zinc enzymes are present in all six major classes of enzymes: oxidoreductases,

transferases, hydrolases, lyases, isomerases, and ligases. In addition, zinc is essential for certain protein structures, including zinc finger transcription factors and the insulin hexamer. Moreover, zinc functions as a signaling mediator through changes in the concentration of zinc ions inside and outside of the cell in response to various stimuli [1, 5]. Because zinc has such a wide range of functions, zinc deficiency can cause a variety of symptoms, including taste disorders, dermatitis, hair loss, growth retardation, anorexia, gonad dysfunction, decreased immunity, and delayed wound healing [6].

Recently, zinc deficiency has been reported in both developing countries and developed countries [7-9], including Japan, and it is estimated that approximately 20–30% of the Japanese population is zinc deficient [10]. Under these circumstances, a zinc preparation for hypozincemia was approved for the first time in Japan in March 2017, and the Japanese Society of Clinical Nutrition issued Japan's Practical Guideline for Zinc Deficiency 2018 [11].

There has been accumulating interest in efficient prevention of zinc deficiency. Here, we discuss zinc content in foods, the mechanism of intestinal zinc absorption, factors affecting zinc absorption, and foods that can help increase zinc absorption and prevent zinc deficiency.

2. Nutritional status of zinc in Japan

2.1. Zinc consumption in Japan

Zinc deficiency is common in Japan and the recommended daily allowance (RDA) of zinc in adult males and females, based on intake standards of the United States and Canada, is 11 mg and 8 mg, respectively [12]. For pregnant and lactating women, an additional dose of 2 mg and 4 mg is recommended, respectively, as serum zinc levels decrease as the pregnancy period progresses. However, the actual intake of zinc is approximately 9 mg for adult males in their 20s to 70s, and for females in numerous age groups, intake does not reach the recommended level, especially for those in their 20s to 30s, which is only approximately 7–7.5 mg [13]. Furthermore, it has been reported that approximately 60–70% of men and women over 20 years in Japan consume less than the recommended amount of zinc [14]. Pregnant women and lactating mothers also consume only 7.4 mg and 8.0 mg of zinc, respectively, which raises concerns about insufficient zinc intake and deficiency. Breast milk, especially colostrum, generally contains much higher concentrations of zinc than found in serum [15], and the demand for zinc in newborns is thought to be high; thus, it is important for nursing mothers to obtain sufficient zinc.

2.2. Zinc deficiency in Japan

According to the analysis of serum zinc concentration data in the Japanese population, serum zinc concentrations decrease as people age, and the percentage of people over 60 years with low serum zinc levels is reported to be as high as ~40% [16]. A study evaluating zinc nutrition by measuring zinc concentration in Japanese hair reported that the risk of zinc deficiency is particularly high in children aged 0–4 years, suggesting that ~40% are zinc deficient [17]. While the rate of zinc deficiency tends to increase with age, the rate decreases in men over 80 and women over 90, suggesting that people with longevity may be zinc sufficient [17]. This suggests that zinc is an essential nutrient for achieving a long and healthy life.

Serum zinc levels are commonly used as an indicator of zinc nutrition; however, serum zinc is only approximately 0.1% of the total zinc in the body and is known to exhibit diurnal variations; it tends to be higher in the morning and lower in the afternoon and it can be affected by stress and hormonal status [16]. Therefore, it is necessary to perform measurements early in the morning on an empty stomach. In addition to serum zinc levels, other biomarkers for zinc have been reported, such as the zinc concentration in hair and urine and plasma alkaline phosphatase activity [18,19]. It is hoped that future studies will develop noninvasive, simpler, and more sensitive bioindicators for zinc nutrition.

2.3. Causes of zinc deficiency

Zinc deficiency may be caused by insufficient zinc intake, factors inhibiting zinc absorption such as phytate and polyphosphate, consumption of medicines that inhibit zinc absorption or increase zinc excretion, or congenital zinc deficiency. Congenital zinc deficiency is known as acrodermatitis enteropathica (AE) [20,21], which is caused by reduced intestinal zinc absorption, and transient neonatal zinc deficiency [22-25], which is caused by low zinc concentration in breast milk.

In Japan's super-aged society (defined as >20% of the population over 65 years old), several people suffer from chronic diseases and consume multiple medications. Zinc deficiency may increase the risk of undernutrition due to taste disorders and anorexia, and delay the healing of bedsores; therefore, prevention of zinc deficiency is important.

3. Zinc in food

3.1. Food items having high zinc content

The absorption rate of zinc in the gastrointestinal tract varies depending on the amount consumed. Although zinc absorption is approximately 90% in zinc-deficient diets [26,27], it is typically 30% under normal conditions [28]. Therefore, sufficient zinc intake and improvement in absorption efficiency are important for maintaining good zinc nutrition. Zinc is primarily found in animal foods and is known to have a higher bioavailability efficiency than plant foods. Oysters are particularly rich in zinc, with one serving (three oysters) containing approximately 8.4 mg, which is most of the RDA for adults [12,29]. In addition, meat, especially liver, is rich in zinc (**Table 1**). Among plant foods, soybeans and seeds are relatively high in zinc. The Japanese population has a high zinc intake derived from staple cereals, particularly rice [13].

3.2. Factors inhibiting zinc absorption

Plant foods are one of the major sources of zinc. However, plant foods, especially the starch layer and germ of cereals, endosperm, and cotyledons of legume seeds also contain a large amount of phytic acid (myo-inositol hexaphosphate), which inhibits zinc absorption; thus, the bioavailability of their zinc may not be high [30]. Phytic acid is negatively charged under physiological conditions, indicating its potential to form complexes with positively charged multivalent cations, especially iron, zinc, magnesium, and calcium. Given that these complexes are soluble under acidic conditions in the stomach and precipitate at neutral pH in the intestine, these minerals in the gastrointestinal tract are poorly absorbed and are excreted in the feces [31]. Therefore, decreasing phytic acid is expected to improve mineral absorption. Phytase, which hydrolyzes phytic acid, is found in the small intestine; however, its activity in the human intestine is much lower than in that of sheep and pigs, which are able to degrade phytate with their own intestinal phytase [31]. Moreover, phytase of plant or microbial origin is widely applied to reduce phytate content in foods to improve the bioavailability of minerals and trace elements during food processing and preparation, predominantly during soaking, malting, germination, fermentation, and bread making [30,32]. Therefore, the use of fermented foods, such as natto (fermented soybeans), miso (fermented soybean paste), and germinated brown rice, among other plant-based

Table 1. | Zinc content in foods

Food	Zinc concentration (mg/100 g) *1	Zinc content per dish (mg) *2
Oysters (raw)	14.0	8.4 (3 pieces 60 g)
Pork liver (raw)	6.9	4.8 (1 meal 70 g)
Beef chuck eye roll (red meat)	5.6	3.9 (1 meal 70 g)
Scallop (raw)	2.7	2.7 (1 piece 100 g)
Beef liver (raw)	3.8	2.7 (1 meal 70 g)
Grilled eel	2.7	2.2 (1/2eel 80 g)
Snow crab (boiled)	3.1	1.2 (2 legs 40 g)
Rice (brown rice)	0.8	1.2 (1 bowl 150 g)
Rice (polished rice)	0.6	0.9 (1 bowl 150 g)
Natto	1.9	0.8 (1 pack 40 g)
Whole egg	3.6	0.7 (1 piece 60 g)
Egg yolk	1.1	0.7 (1 piece 20 g)
Processed cheese	3.2	0.6 (1 piece 20 g)
Yogurt	0.4	0.4 (1 meal 100 g)
Cashew nuts	5.4	0.4 (5 grains 8 g)
Firm tofu	0.6	0.4 (1 meal 70 g)
Cocoa	7.0	0.4 (1tbsp 6 g)
Soybean (boiled)	1.9	0.4 (1 meal 20 g)
Almond	3.6	0.2 (5 grains 6 g)

*1 Calculated using the data in Standard Tables of Food Composition in Japan 2020 (Eighth revised edition).

*2 Calculated based on the standard amount of food for one meal.

foods, is considered efficient in terms of mineral nutrition.

A knockout strain of the *SPDT* gene in rice, a novel SULTR-like phosphorus distribution transporter, reduced phosphorus and phytate in brown de-husked rice by 20–30%, while yield, seed germination, and seedling vigor were not affected [33]. This is expected to improve the bioavailability of minerals in grains.

Several factors inhibit the absorption of zinc in food additives. Phytic acid, as mentioned above, is used to prevent discoloration, oxidation, and pH adjustment of foods via its metal-chelating effect. Polyphosphates, used as binding agents and emulsifiers, and sodium ethylenediaminetetraacetate, used as an antioxidant, have zinc chelating effects [34]. Consuming a well-balanced diet of various foods may reduce the effect of these factors on zinc absorption; however, care should be taken to avoid an extremely unbalanced diet, which may lead to zinc deficiency.

3.3. Zinc-based food additives

In Japan, zinc gluconate and zinc sulfate are designated as food additives [35]. In 1983, the use of zinc gluconate for nutritional enhancement was limited to "breast milk substitute foods." However, it was later approved for use in "food for specified health use" and "food with nutrient function claims," with the amount of zinc contained in the recommended daily intake not exceeding 15 mg; zinc gluconate could also be used in "food with special dietary use" with permission or approval (limited to those for sick people). Thus, zinc gluconate can also be used in comprehensive nutritional foods as a meal replacement for sick people who may be at risk of zinc deficiency [35].

Zinc sulfate is approved only for use in "breast milk substitutes" for nutritional enhancement, and for use as a manufacturing agent in the production of "sparkling liquor," such as beer. The addition of zinc sulfate to foaming liquors maintains good yeast nutrition during the fermentation process; since most zinc ions are consumed by yeast, zinc sulfate has little effect on the amount of zinc in the product after manufacture [35]. Zinc gluconate, zinc sulfate, zinc acetate, zinc carbonate, zinc chloride, and zinc oxide are regarded as generally recognized as safe substances in the U.S., and are recognized as compounds that can be added to foods in the EU [36,37]. They are also added to supplements, candies, and beverages.

4. Mechanisms of zinc absorption in the intestine

Zinc ingested from food is primarily absorbed in the duodenum and jejunum, bound to albumin and α2-macroglobulin in the portal vein, adsorbed into the liver, and subsequently distributed to peripheral tissues. Zinc that is not absorbed into the gastrointestinal tract or contained in gastrointestinal secretions and sloughing mucosal cells is excreted in the feces [1]. Although the excretion of zinc in urine is minor, the amount excreted in urine is reduced in cases of zinc deficiency and increased in some diseases [38].

Zinc homeostasis is regulated by several proteins, including zinc transporters and metallothioneins. Zinc transporters include the ZRT, IRT-like protein (ZIP) and Zn transporter (ZNT) families. Fourteen members of the ZIP family and 10 members of the ZNT family have been identified in humans; however, ZNT9 is thought to have no zinc transport function [1]. In general, ZIP proteins import divalent zinc ions into the cytosol of the cell from the extracellular space or intracellular compartments, whereas ZNT proteins export divalent zinc ions from the cytosol into the extracellular space or intracellular compartments.

The zinc transporter ZIP4 is expressed on the apical membrane of intestinal epithelial cells, and ZNT1 is expressed on the basolateral membrane of intestinal epithelial cells; both play a critical role in intestinal zinc absorption. ZIP4 transports zinc from the lumen into enterocytes, and ZNT1 exports zinc into the portal blood. These transporters regulate the absorption of dietary zinc in the gastrointestinal tract (**Fig. 1**). In 2002, *ZIP4* was identified as the causative gene of AE, a rare autosomal recessive genetic disorder of zinc deficiency, demonstrating the importance of ZIP4 [20,21]. More than 30 mutations or unclassified variants of *ZIP4* have been reported [39]. Analysis of intestinal-specific knockout mice confirmed that ZIP4 plays an essential role in zinc absorption in the mammalian intestine [40]. The expression of ZIP4 is regulated by zinc levels in a post-transcriptional and post-translational manner. During zinc deficiency, *ZIP4* mRNA is stabilized [41], whereas the ZIP4 protein accumulates on the cell surface and functions in the uptake of food-derived zinc [41,42]. Under conditions of zinc sufficiency, ZIP4 is endocytosed and rapidly degraded by both the lysosomal and proteasomal pathway [41,43] (**Fig. 2**). Potential zinc-sensing elements and specific motifs for ZIP4 endocytosis have been proposed in the ZIP4 sequence [44-49].

ZNT1 is expressed in various tissues; in the intestinal tract, it is expressed in the basolateral membrane and is thought to function in the transport of zinc from the intestinal epithelial lining to the bloodstream [50]. Unlike ZIP4, *ZNT1* transcription

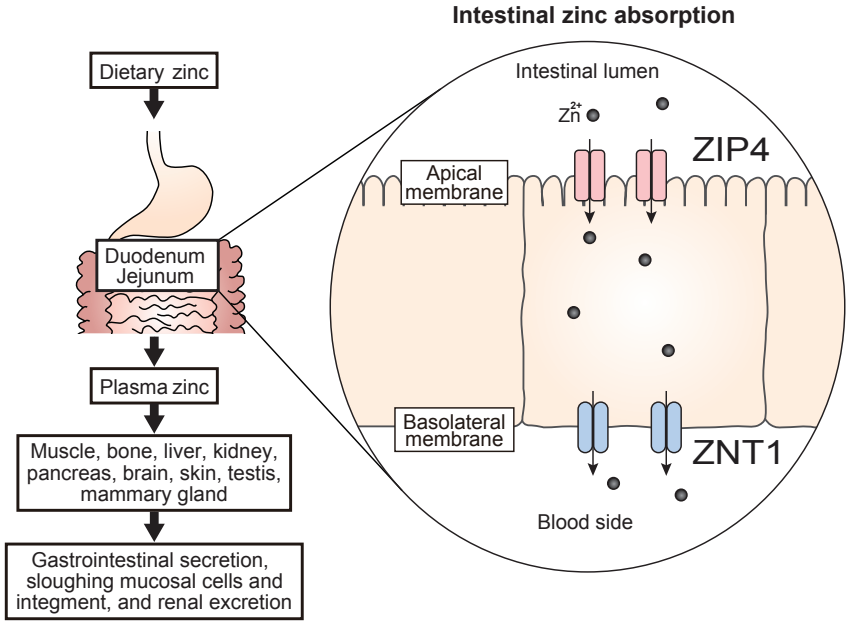

Fig. 1. **Dietary zinc absorption mechanisms.**
Dietary zinc is primarily absorbed by the duodenum and jejunum. Divalent zinc ions released from food are taken up intracellularly via ZIP4, which accumulates on the luminal side of intestinal epithelial cells. Zinc is transported into the blood via ZNT1, which is present on the basolateral membrane. Zinc in plasma is bound to albumin and α2-macroglobulin and delivered to the whole body. Zinc that is not absorbed by the gastrointestinal tract or contained in detached intestinal cells, pancreatic juice, or bile, is excreted in feces. Zinc is also excreted in urine and sweat.

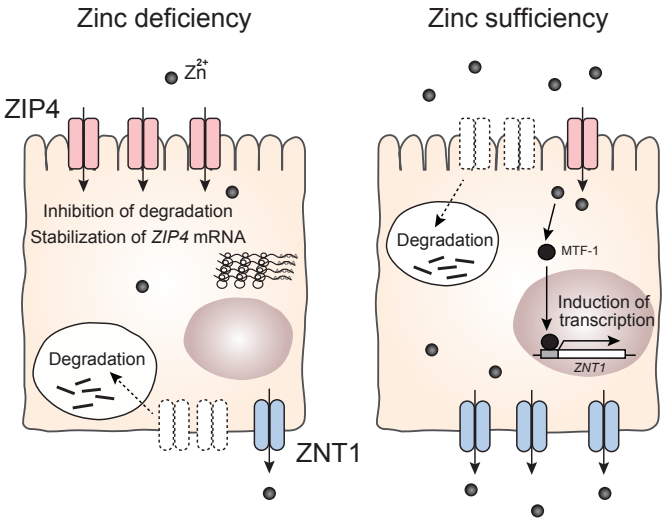

Fig. 2. **Regulation of ZIP4 and ZNT1 expression according to zinc levels.**
ZIP4 expression is regulated by dietary zinc in a post-transcriptional, and post-translational manner. Under conditions of zinc deficiency, ZIP4 accumulates on the apical membrane through mRNA stabilization and inhibition of protein degradation. When there is sufficient zinc, ZIP4 is rapidly endocytosed and undergoes ubiquitin-mediated degradation. In contrast, ZNT1 undergoes endocytosis and intracellular degradation during zinc deficiency, whereas it accumulates on the basolateral membrane under zinc-sufficient conditions, regulated by metal response element binding transcription factor 1 (MTF-1). MTF-1 senses cytoplasmic zinc concentration in enterocytes and regulates ZNT1 expression.

is induced by MTF-1, a metal response element binding transcription factor 1, during zinc sufficiency [51,52]. Under conditions of zinc deficiency, ZNT1 expression is also regulated by zinc levels; however, surface ZNT1 is endocytosed and degraded (**Fig. 2**). Thus, ZIP4 and ZNT1 function cooperatively in zinc absorption in the intestine.

In addition, ZIP5 and ZIP14 are expressed on the basolateral membrane of intestinal epithelial cells and are thought to transport zinc from the blood to intestinal epithelial cells [41,53,54]. In contrast to ZIP4, *ZIP5* mRNA levels do not change in response to zinc treatment. However, the translation of ZIP5 is zinc-responsive. When zinc is deficient, *ZIP5* mRNA translation is arrested; when zinc is adequate, *ZIP5* mRNA is translated, and the protein accumulates in the basolateral membrane [41]. Some zinc transporters may be involved in the regulation of zinc homeostasis *in vivo*; however, their specific roles in zinc absorption have not been elucidated [29,55].

Moreover, the systemic hormone hepcidin, which is produced in response to iron loading and regulates iron homeostasis, appears to attenuate ZNT1 expression and influence its export from small intestinal epithelial cells [56].

As zinc exists as a divalent ion, its absorption mechanism in the gastrointestinal tract is different from that of iron and copper. Iron and copper ions present in food are reduced by their respective reductases before uptake into intestinal epithelial cells by their respective transporters, DMT1 and CTR1. Therefore, the absorption of iron and copper is markedly affected by the activity of reductases [57,58]. Alternatively, zinc does not undergo redox reactions and is absorbed in the form of a divalent cation. Thus, the presence of zinc transporters may be an important factor in determining the amount of zinc absorbed by the gastrointestinal tract.

5. Food factors enhance zinc absorption efficiency by targeting zinc transporters

Given that zinc absorption is approximately 30%, and the absorption rate decreases with increasing zinc intake, improving intestinal zinc absorption may be an effective strategy to prevent zinc deficiency. Based on the hypothesis that food factors that increase the expression of ZIP4 have a positive effect on zinc absorption, we established a screening method for dietary components that would enable an increase in ZIP4 expression and cellular zinc levels. We examined more than 400 food extracts, and found several soybean extracts were effective in promoting the expression of ZIP4 [59]. Detailed analysis of the mechanism of increased

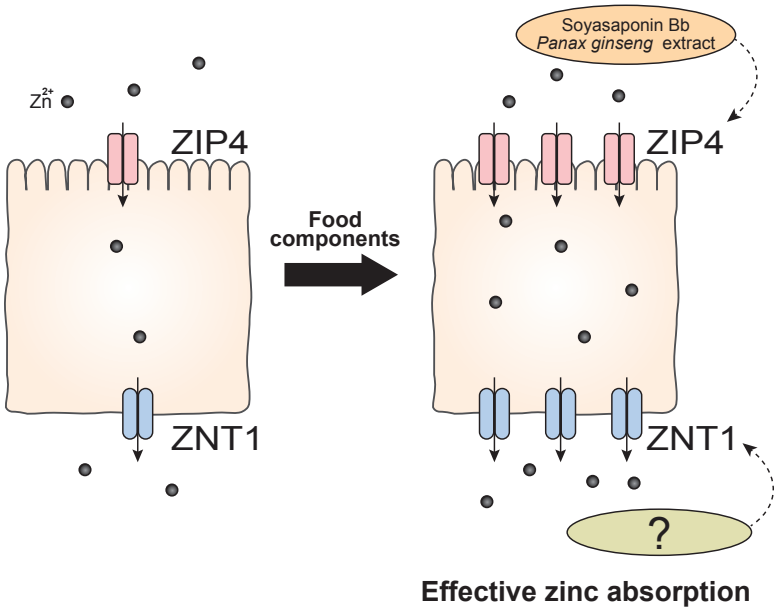

Fig. 3. **Effective zinc absorption by food factors enhancing expression of zinc transporters.** Improving the efficiency of intestinal zinc absorption can be an effective strategy to prevent zinc deficiency and improve zinc nutrition through the daily diet. Food-derived factors with the ability to increase ZIP4 or ZNT1 transporters may increase intestinal zinc absorption. Soyasaponin Bb and *Panax ginseng* extract are food factors that increase ZIP4 expression and cellular zinc levels. Soyasaponin Bb inhibits ZIP4 endocytosis and increases surface ZIP4 abundance. *P. ginseng* extract increases *ZIP4* mRNA levels. Although food factors that increase ZNT1 have not yet been reported, ZIP4 and other zinc transporters, such as ZNT1, promote zinc absorption.

ZIP4 expression by these extracts suggests that they inhibit ZIP4 endocytosis and subsequently increase the amount of ZIP4 present on the cell surface, which consequently contributes to increased cellular zinc levels. Furthermore, the active factor responsible for these effects was found to be soyasaponin Bb [59]. Moreover, it was recently reported that an extract of *Panax ginseng* induces the mRNA expression of *ZIP4*, which has been shown to promote zinc uptake into cells [60].

If food-derived factors that promote zinc absorption by targeting the intestinal zinc absorption mechanism, specifically by targeting ZIP4 and ZNT1, are identified, these factors may be effective in preventing zinc deficiency through the daily diet (**Fig. 3**).

Conclusion

Here, we reviewed zinc in foods, its absorption mechanisms in the intestinal tract, factors that affect its absorption, and discussed food factors that contribute to efficient absorption. The molecular mechanisms controlling zinc absorption remain unclear, and it is necessary to elucidate the mechanisms of regulation of zinc transporters whose expression is regulated by zinc levels. In addition, the zinc trafficking process in enterocytes needs to be investigated, as well as the crosstalk between zinc and other minerals in intestinal absorption. Clarifying these points will provide new insights into improving zinc nutrition and contribute to the maintenance and improvement of human health.

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Review https://metallomicsresearch.brte.org

ZIP6-centered zinc regulatory and malignant characteristics of breast cancer cells

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Abstract

Zinc (Zn) is an essential trace element for numerous biological events in mammals. Zn functions as a signaling mediator, leading to the regulation of physiological cell actions and, therefore, has therapeutic potential. Recent breast cancer research has shown that Zn transporters contribute to malignancy processes; thus, elucidating the roles of Zn and Zn transporters in breast cancer may lead to the development of novel strategies for breast cancer diagnosis and therapy. The Zn transporter ZIP6 mediates the acquisition of malignant phenotypes such as hypoxic resistance and epithelial-mesenchymal transition (EMT), which determine breast tumor grade and prognosis. ZIP6 expression contributes to the efficacy of anticancer therapy through Zn-induced autophagy. The maintenance of breast cancer stem-like cells requires Zn modulation through the cooperative function of ZIP6 and ZIP7. These findings suggest that the ZIP6-mediated Zn network is a potent driving force toward malignancy. In this review, we summarize recent progress in understanding the emerging roles of Zn and ZIP6 in the regulation of malignant characteristics related to hypoxic adaptive response, drug therapy, and stemness. We also discuss the possibility and future challenges of innovative breast cancer therapies using ZIP6 and Zn-related molecules.

Key words: Zinc, zinc transporter, breast cancer, hypoxia, Bcl-2, breast cancer stem-like cell

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

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Received: December 30, 2021
Accepted: February 18, 2022
Released online: March 31, 2022

Introduction

Breast cancer is the most commonly occurring cancer in women, with both morbidity and mortality increasing on a yearly basis [1,2]. Recent breast cancer studies have elucidated the molecular mechanisms of its pathological conditions, and evidence-based theoretical treatment protocols including pharmacotherapy using molecular target drugs are selected according to the molecular features of breast cancer cells [3,4]. However, breast cancer cells acquire malignant phenotypes aggressively during treatment, even in the early stages of growth, causing metastasis, recurrence, and resistance to therapy [5,6]. Thus, the range of current therapies is limited, and little is known

about the regulatory machinery of breast cancer malignant transformation.

In recent years, a growing body of evidence has indicated that zinc (Zn) and Zn transporters are closely related to the malignant processes of breast cancer, presenting an attractive potential approach for novel therapeutic strategies [7-11]. As a trace element, Zn is essential for human survival; intracellular Zn^{2+} levels are spatiotemporally regulated by ZRT IRT-like proteins (ZIPs; SLC39A, 14 members) to facilitate the influx of Zn into the cytosol, and zinc transporters (ZnT; SLC30A, 9 members) mediate Zn efflux from the cytosol [12,13]. These transporters contribute to maintaining Zn homeostasis within cells and throughout the body by strictly managing the cellular Zn balance according to the distributions of their molecular species in a tissue-specific manner [8-13]. Zn has been shown to target molecules selectively as an intracellular and extracellular signaling mediator, such that the Zn signaling pathway spatiotemporally regulates cell functions and Zn transporter aberrations are linked to the onset and progression of diseases such as cancers [7-14], Alzheimer's disease [15], diabetes [16-18], and other pathological processes [19-24] (**Figure 1**). Interestingly, zinc transporters in cancers have been reported from various fields including clinical analysis as well as molecular and structural biology, and only 5 ZnTs and almost all ZIP transporters are involved in a number of cancers [25]. Patterns of ZIP dysregulation differ among cancers, and indicate the complicated roles of Zn in various cancer types including prostate cancer [26,43], pancreatic cancer [27-29,44,59], hepatocellular carcinoma [30,45], lung cancer [31], glioma [32], oral squamous [33], nasopharyngeal [34], ovarian cancer [35,60], colorectal cancer [46], esophageal cancer [36,47], breast cancer [37-42,49-52,54-57], gastric adenocarcinoma [48], neuroblastoma [53], and bladder cancer [58], as well as in phenotypes associated with tumorigenesis, metastasis, and therapeutic resistance (**Table 1**). In metastatic cancers, ZIP14 is upregulated in skeletal muscle, promoting cachexia [61].

Zn levels in the breast tissue and serum of patients with breast cancer are higher and lower, respectively, than those of healthy subjects [62-65]. Both clinical and *in vitro* studies have demonstrated the potential roles of ZIP6 in estrogen receptor (ER)-positive breast cancer in about 70% of breast cancer patients [37-42], of ZIP7 in ER antagonist tamoxifen-resistant breast cancer [49-52],

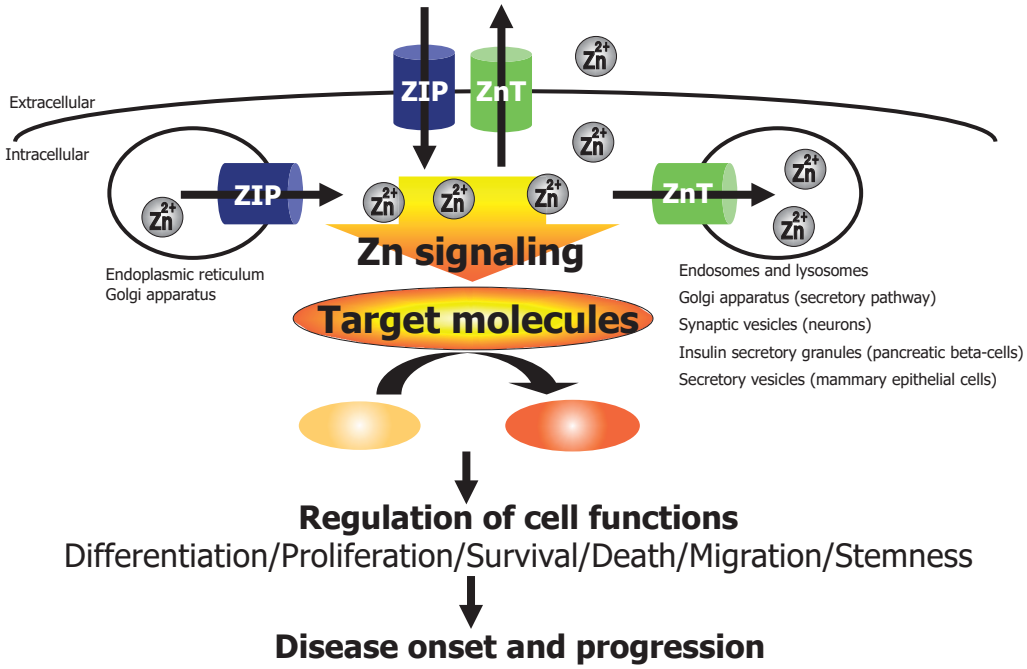

Figure 1. **Zn transporters contribute to cell functions and pathological processes.**
Zn modulates the intracellular signaling pathway, thereby determining the final outputs of various physiological reactions such as cell differentiation, proliferation, survival, and migration. Disorder of Zn transporters led to specific diseases.

Table 1. | ZIP transporters and cancers.

Gene name	Protein name	Type of cancer cells	Functional activity in cancer cells	References
SLA39a1	ZIP1	Prostate	Expression down-regulated	26
SLA39a2	ZIP2	Prostate	Expression down-regulated	26
SLA39a3	ZIP3	Prostate	Expression down-regulated	26
		Pancreatic	Expression down-regulated	27
SLA39a4	ZIP4	Pancreatic	Expression up-regulated	28
		Pancreatic	Expression up-regulated in cancer cell-derived exosomes	29
		Hepatocellular	Expression up-regulated	30
		Lung	Expression up-regulated	31
		Glioma	Expression up-regulated	32
		Oral squamous	Expression up-regulated	33
		Nasopharyngeal	Expression up-regulated	34
		Ovarian	Expression up-regulated	35
SLA39a5	ZIP5	Esophageal	Expression up-regulated	36
SLA39a6	ZIP6	Metastatic ER+breast	Expression up-regulated	37,67
		ER+breast	Expression down-regulated	38-42
		Prostate	Expression up-regulated	43
		Pancreatic	Expression up-regulated	44
		Hepatocellular	Expression up-regulated	45
		Colorectal	Expression up-regulated	46
		Esophageal squamous-cell	Expression up-regulated	47
		Gastric	Expression up-regulated	48
SLA39a7	ZIP7	Anti-hormone resistance in ER+breast	Expression up-regulated	49-52,92
SLA39a8	ZIP8	Neuroblastoma	Proliferation inhibited by ZIP8 knockdown	53
SLA39a9	ZIP9	Prostate, breast	Apoptosis induced by ZIP9 activation	54
SLA39a10	ZIP10	Metastatic breast	Expression up-regulated	55-57
SLA39a11	ZIP11	Renal cell	ZIP11 variant as risk factor	58
		Bladder	ZIP11 variant as risk factor	58
		Pancreatic	Expression up-regulated	59
SLA39a13	ZIP13	Ovarian	Expression up-regulated	60
SLA39a14	ZIP14	Cachexia in metastatic cancer	Expression up-regulated in skeletal muscle	61

and of ZIP10 in breast cancer invasion and metastasis [55-57]. In particular, ZIP6 was identified in human breast cancer MCF-7 cells expressing substantial levels of ER [66], and the ZIP6-centered Zn network was found to have important implications for ER-positive breast cancer [8,11,37-42,67]. Decreased expression of ZIP6 in MCF-7 in response to stimuli in the environment surrounding cancer cells such as high glucose concentration and hypoxia induces epithelial-mesenchymal transition (EMT), promoting stemness and resistance to cell death [8,11,39]. In the clinical setting, patients with low ZIP6 expression in primary breast cancer sites have worse prognoses than those with high ZIP6 expression, suggesting that ZIP6 is negatively associated with prognosis [40]. Therefore, the cell culture model of ZIP6-knockdown MCF-7 mimics highly malignant breast cancer and is a potential tool for the investigation of breast cancer progression [8,11,39].

In this review, we summarize current progress in our understanding of the role of the main ZIP6 and ZIP6-related Zn transporters, focusing on breast cancer cell behaviors in hypoxia as a crucial factor in the tumor microenvironment, as well as on hormone therapy and breast cancer stem-like cells, which directly impact malignant progression and prognosis. We also discuss Zn transporters and Zn-targeted molecules as candidate markers for the malignant phenotype of breast cancer, which may represent a promising target for novel treatment strategies.

ZIP6 and hypoxic adaptive response of breast cancer cells

Hypoxia occurs in internal solid tumor structures, including breast cancer, as a result of rapid tumor cell growth [68]. This hypoxic region has been reported to contribute to tumor plasticity and heterogeneity, and to promote more aggressive phenotypes such as angiogenesis, invasion, EMT, stemness, and resistance to chemotherapy and radiotherapy [69-71]. To clarify the hypoxic profile in breast cancer cells, we cultured MCF-7 in three dimensions using a soft agar colony formation assay [8,11]. MCF-7 formed colonies, and colony interior was confirmed to be hypoxic according to strong red fluorescence of the hypoxia-sensing probe LOX-1. The specific membrane-permeable Zn chelator *N,N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN)

significantly inhibited colony formation, and this inhibition was rescued by increasing the level of intracellular free Zn using zinc ionophore pyrithion. These findings suggest that intracellular Zn is required for survival under hypoxia and tumorigenic potential. Hypoxia-inducible factor 1α (HIF-1α) is a pivotal regulator in the process of hypoxic adaptive response [72,73]. MCF-7 cultured under hypoxic conditions showed higher viability than normal cells, depending on the activity of HIF-1α, and HIF-1α activity was positively correlated with intracellular Zn levels under hypoxic conditions. These data suggest that Zn-mediated HIF-1α activity promotes MCF-7 cell survival. Although the mechanisms underlying Zn-mediated HIF-1α activity in MCF-7 remains unknown, HIF-1α protein requires Zn to maintain its structure [74]. The knockdown of ZIP6, which plays an important role in maintaining intracellular Zn homeostasis in MCF-7, indicated that the disruption of intracellular Zn homeostasis, implying the presence of other zinc transporters, leads to increased intracellular free Zn concentration and higher survival rates under hypoxic conditions through apoptosis inhibition and EMT induction [39]. A zebrafish embryo study showed that a downstream factor of ZIP6 regulates signal transducer and activator of transcription 3 (STAT3) expression, which is linked to EMT [37]. ZIP6 expression levels were found to be decreased in high-grade primary tumor sites among patients with breast cancer [8,11,41], and low ZIP6 expression levels at primary breast cancer sites are correlated with shorter relapse-free survival periods [40]. Both experimental findings and clinical observations indicate that ZIP6 downregulation is deeply involved in the malignancy phenotype, suggesting that ZIP6 levels in primary breast tumors are potential biomarkers of cancer grade and poor prognosis in patients with breast cancer (**Figure 2**) [8,11,40,41]. Further analysis of the ZIP6 downregulation-switched zinc network, including the involvement of other Zn transporters and the behavior of intracellular and extracellular Zn in malignant progression under hypoxia, is expected to indicate future directions for the development of breast cancer diagnosis and treatment.

ZIP6 and pharmacotherapy in breast cancer

At least 70% of breast cancers are female hormone ER-positive and thus benefit from endocrine treatments including ER antagonist tamoxifen and aromatase inhibitors, which block estrogen production [75]. However, more than 25 % of all breast cancer patients show endocrine resistance, and even after 5 years of tamoxifen use, one in three patients with endocrine treatments still recur within 15 years; thus, the elucidation of endocrine-resistant mechanisms is an urgent clinical challenge [75,76]. High expression of Bcl-2, an anti-apoptotic molecule that regulates programmed cell death, has been reported to cause endocrine

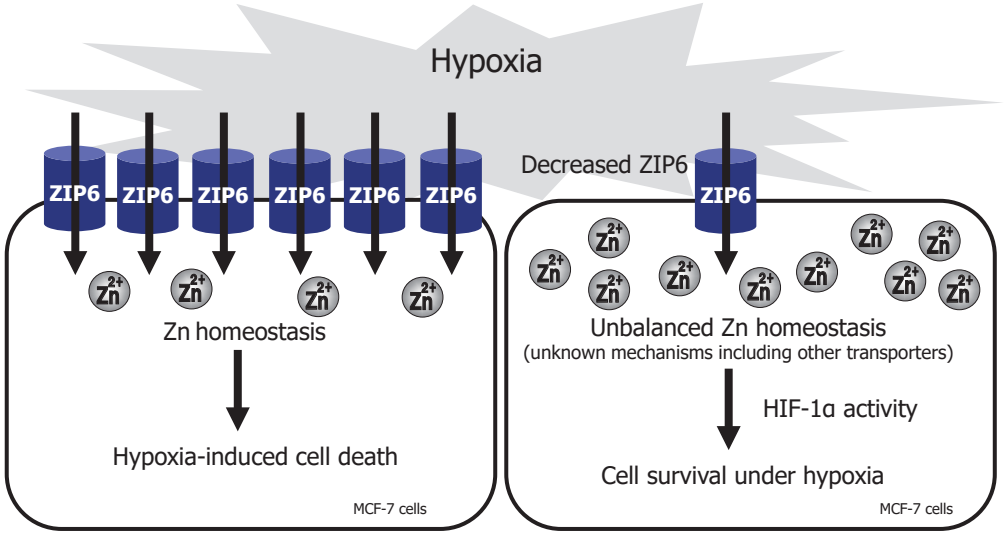

Figure 2. ZIP6 plays an important regulatory role under hypoxic stress.
ZIP6-downregulation disturbs intracellular Zn homeostasis, leading to increased cell survival against hypoxic stress in MCF-7 cells. ZIP6 is a key molecule for adaptation to hypoxic stress, which is implicated in malignant progression.

therapy resistance in breast cancer, and efficient inhibition of Bcl-2 function is expected to be a promising strategy to prevent endocrine resistance [76,77]. Great effort has been made to investigate the sensitivity of genetic or pharmacological inhibition of Bcl-2 in breast cancer [78]. YC137, a novel small molecule-specific inhibitor of Bcl-2, induces apoptosis in breast cancer cells that express high levels of Bcl-2, but does not attack normally functioning cells including hematopoietic progenitors, peripheral blood mononuclear cells, small intestine epithelial cells, and myoblasts [79]. Intriguingly, we found that the cytotoxicity of YC137 against estrogen-dependent human breast cancer cell, MCF-7, was markedly enhanced in combination with Zn chloride ($ZnCl_2$). Treatment with $ZnCl_2$ increased the concentration of labile Zn in MCF-7, and Zn supplementation triggered a switch from apoptotic cell death by YC137 (type I programmed cell death) to non-apoptotic cell death with autophagy (type II programmed cell death). Autophagy, in which autophagy-related protein-mediated autophagosomes form around misfolded proteins and damaged organelles to induce their degradation, is crucial for maintaining cell homeostasis and controlling cell death according to stress condition and breast cancer development stage [80,81]. Autophagy can be induced, and plays a cytoprotective or pro-survival role under most cancer therapies, whereas the collapse and excess of autophagy contributes to cytotoxicity in breast cancer cells [82]. During treatment with tamoxifen in MCF-7 cells, Zn accumulates in autophagosomes, resulting in autophagosome-mediated cell death [83]. Recent studies have demonstrated that Zn transporters ZIP4, ZIP8, ZIP14, ZnT2, ZnT4, and ZnT10, are located in endosomes, lysosomes, and autolysosomes, and have suggested that Zn contributes to autophagy [84,85]. The enhancement of YC137 cytotoxicity in combination with $ZnCl_2$ was abolished in ZIP6-knockdown MCF-7 cells, indicating that Zn supplied by ZIP6 is required for increased sensitivity of YC137. Studies on the roles of Zn in breast cancer therapy have recently begun, and will need to take into account the relationship between ZIP6 and autophagy. The combination of YC137 and $ZnCl_2$ does not induce damage to normal mammary gland cell model, human non-tumorigenic breast epithelial MCF-10A cells. As ZIP6 downregulation is involved in malignant progression in breast cancer [8,11,40,41], we propose that the combination of YC137 and $ZnCl_2$ may be an effective cancer-specific anticancer method for preventing the generation of therapeutic resistance, especially in early breast cancer, where ZIP6 expression is high. Future studies are required to elucidate the *in vivo* response to this treatment and to determine the appropriate dosing regimen with Zn supplement as adjuvant.

ZIP6/ZIP7 and breast cancer stem-like cells (BCSCs)

BCSCs are a distinct group of breast cancer-initiating cells with the capacity for self-renewal and differentiation; they hide within tumor tissue or throughout in the body [86,87]. BCSCs contribute to the cellular origin, tumor maintenance, and progression of breast cancer [88]. Clinically, BCSCs are considered to be responsible for the development of treatment resistance and cancer recurrence due to their relative resistance to radiation, cytotoxic chemotherapy, and molecular targeted therapy [86-88]. However, the molecular mechanisms that regulate BCSC maintenance remain poorly understood, and evaluation is required to improve the clinical outcomes of patients with breast cancer [86-88].

Among three-dimensional *in vitro* cell culture models, the spheroid culture method has been widely used as a powerful tool to maintain and grow cells selectively with cancer stem cell characteristics [88]. When MCF-7 cells were sphere-cultured in serum-free medium under non-adherent conditions, they formed spherical aggregates (spheres) and showed high expression of stem cell markers, self-renewal ability, and high tumorigenicity *in vivo*, all of which are characteristics of BCSCs [89]. In a spheroid culture assay using MCF-7 cells, treatment with TPEN revealed strong inhibition of sphere-forming capacity and nuclear expression of PR domain zinc finger protein 14 (PRDM14), a cancer stem cell transcription factor essential for the maintenance of BCSCs [90]. This inhibition was neutralized by the addition of Zn through zinc ionophores, suggesting that intracellular Zn-dependent PRDM14 expression in nuclear is essential for sphere-forming capacity. ZIP6 knockdown in MCF-7 cells resulted in significantly higher sphere formation efficiency compared to control cells. The high Zn-dependent PRDM14-mediated sphere-forming capacity of ZIP6-knockdown MCF-7 cells was significantly suppressed by functional inhibition of the zinc transporter ZIP7, which transports Zn from the endoplasmic reticulum to the cytoplasm. ZIP7, which directly drives pro-survival signaling pathways such as AKT, mTOR, and MAPK for tumorigenesis [91], is increased in endocrine-resistant breast cancer [50]. Phosphorylated ZIP7, which is the Zn-transporting form, may be a good candidate marker of aggressive cancer [92]. ZIP7 expression is also strongly associated with poor outcomes in clinical breast cancer samples [93]. BCSCs are increased in ZIP7 activity against ZIP6, suggesting that ZIP6 and ZIP7 cooperatively regulate BCSC maintenance via Zn-dependent regulation of PRDM14 expression (**Figure 3**). PRDM14 is not expressed in normal tissue stem cells, whereas PRDM14 is highly expressed in BCSCs

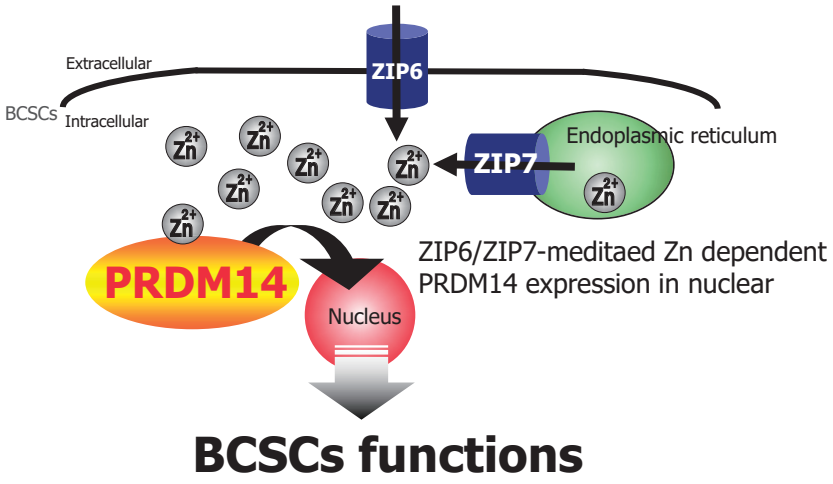

Figure 3. ZIP6/ZIP7 contribute to the maintenance of breast cancer stem-like cell (BCSC) functions.
ZIP6/ZIP7-mediated Zn is suggested to regulate BCSC maintenance through PRDM14 expression in nuclear. This is the first report of a biological role for Zn transporters in BCSCs.

as an essential transcription factor [90]. Survival analysis of breast cancer patients at stage II or III revealed that patients with high PRDM14 expression had worse prognoses than those with low or undetectable PRDM14 expression [90]. These findings suggest that PRDM14 is a promising target for the development of definitive therapeutic strategies. However, it is difficult to target the PRDM14 molecule using antibody and small-molecule therapeutics, because PRDM14 is expressed and activated in the nucleus [94]. The elucidation of Zn-mediated BCSCs through PRDM14 could dramatically expand the development of new molecularly targeted therapies for BCSCs.

Conclusion and perspective

ZIP6 is a key molecule determining the fate of ER-positive breast cancer cells, and its expression is controlled by the surrounding environment and malignant progression (**Figure 4**). A deeper understanding of the Zn regulatory network originating from ZIP6 will help to clarify the malignant mechanism of breast cancer and promote the development of novel strategies involving Zn-related molecules. The role of Zn biochemistry at the molecular level in therapeutic strategies, and the biology of the target molecules of Zn signaling and mechanisms regulating ZIP6 gene expression, require further study. Interestingly, the construction of ZIP6 includes a PrP-like amino acid sequence in the extracellular NH_2-terminal region, and two members of the LIV-1 subfamily (ZIP5 and 10) to which ZIP6 belongs have been reported to be involved in etiology of prion disease via processing [12,95-98]. The trafficking of ZIP6 to the plasma membrane is regulated by processing on the endoplasmic reticulum [12,37]; therefore, ZIP6 processing regulation may have important implications in breast cancer physiology, although the relationship between Zn status and processing must be considered [12]. The relationships between Zn transported via ZIP6 and Zn reservoirs (intracellular metallothionein or glutathione), the effects of other metals on Zn functions, and the roles of ZIPs other than ZIP6/ZIP7 and ZnTs in the cellular/extracellular Zn network of breast cancer also require further investigation. ZIP6 has been reported to function in conjunction with its close homolog ZIP10, which is located on the plasm membrane [55,56,99,100]. Zn transported via ZIP6 and ZIP10 was found to interact with NCAM1, which promotes cell detachment through the activation of glycogen synthase kinase 3 (GSK-3), which allows cells to migrate and become metastatic [100]. This finding is consistent with the high levels of ZIP6 found in patients with ER-positive breast cancer and lymph node metastasis [67]. A recent study also demonstrated that *ZIP10* mRNA expression was significantly higher in the breast cancer tissues of patients with lymph node metastasis than in

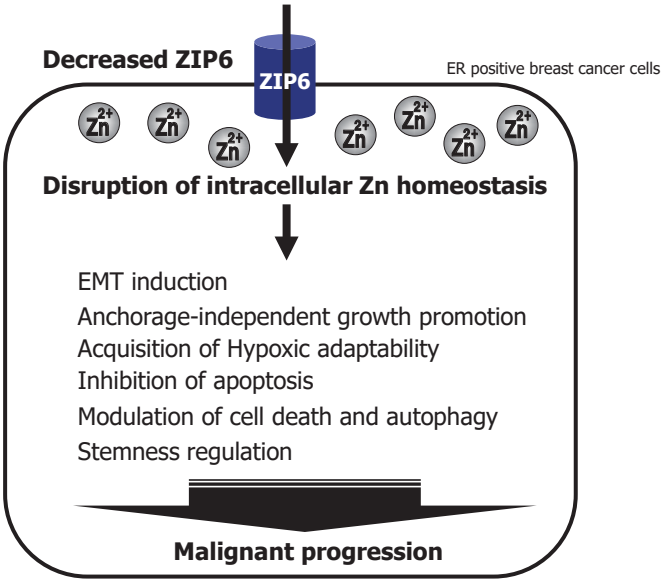

Figure 4. ZIP6 is a potent driving force of malignancy.
ZIP6 plays a key role in the fate of ER-positive breast cancer cells. An understanding of the Zn regulatory network originating from ZIP6 is anticipated to lead to promising therapeutic strategies, although the precise mechanisms remain unclear.

those without metastasis [57]. These data suggest that the role of ZIP6 differs between metastatic foci and primary tumors, and that ZIP6 and ZIP10 may cooperate with transporter functions during cell migration, although further studies are needed to clarify the involvement of a ZIP6 and ZIP10 heteromer [8,11,99,100]. For therapeutic applications, it is necessary to consider the regulation of ZIP6-mediated Zn signaling in a breast cancer phenotype-specific manner, in conjunction with drug delivery system research. The discovery and development of inhibitors and activators of ZIP6 and other Zn transporters may reveal promising anticancer therapies. A ZIP7 inhibitor (NVS-ZP7-4) was recently identified as the first reported ZIP-specific small molecule inhibitor using a phenotype-based high-throughput screening system that targeted the Notch pathway, although its function in breast cancer and *in vivo* remain poorly understood [101].

Finally, a further understanding of the role Zn in breast cancer is anticipated to reveal crucial molecular mechanisms that will facilitate the development of precise diagnostic and effective therapeutic strategies.

Acknowledgement

This work was supported in part by Japan Society for the Promotion of Science (JSPS) KAKENHI grants (nos. 24790099, 15K07955, 18K06676, and 21K06567 for T. T.-N.), the Naito Foundation (T.T.-N.), a Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan (to C. M.), and the Takeda Science Foundation (no. J20ZZ00356 to I.N.). We thank Ms. Chinami Ikushima, Dr. Reiko Yutani, and Dr. Haruki Torii for their helpful support.

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Metallomics Research 2022 (1) #MR202114

Review https://metallomicsresearch.brte.org

Novel insight into the role of zinc in the pathogenesis of chronic liver diseases

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Abstract

Zinc (Zn) homeostasis is largely regulated by the liver, at the same time, Zn is necessary for the maintenance of normal liver function. Therefore, Zn deficiency results in the impairment of hepatocyte function, leading to chronic liver injuries such as hepatic inflammation, fibrosis and steatosis. Numerous metabolic abnormalities, including impaired glucose tolerance, dyslipidemia, hepatic encephalopathy, and sarcopenia, are also associated with these chronic liver injuries. Zn supplementation can recover these chronic liver injuries and related metabolic disorders. Recent advances in molecular biological techniques have enabled us to elucidate the putative mechanisms by which chronic liver disorders evoke varieties of metabolic abnormalities derived from Zn deficiency. This review focuses on the most recent discoveries regarding the role of Zn deficiency in chronic liver diseases, including chronic hepatitis, liver cirrhosis, nonalcoholic fatty liver disease, and autoimmune liver diseases. Moreover, we would like to verify Zn supplementation on these chronic liver diseases.

Key words: zinc, chronic liver disease, hepatic activity, hepatic fibrosis, hepatic steatosis, metabolic abnormalities

Statements about COI: The authors have no conflicts of interest to declare.

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Received: December 31, 2021
Accepted: February 22, 2022
Released online: April 12, 2022

Abbreviations:

ALT: alanine aminotransferase, BCAA: branched-chain amino acid, CLD: chronic liver disease, DAA: direct-acting antiviral agents, ECM; extracellular matrix, ER: endoplasmic reticulum, HBV: hepatitis B virus, HCC: hepatocellular carcinoma, HCV: hepatitis C virus, HDL-C: high-density lipoprotein cholesterol, HOMA-IR: homeostasis model for assessment of insulin resistance, IFN: interferon, IGF-1: insulin-like growth factor-1, LDL-C: low-density lipoprotein cholesterol, miRNA: microRNA, MMP-1: matrix metalloproteinase-1, MT: metallothionein, NAFLD: nonalcoholic fatty liver disease, NASH: nonalcoholic steatohepatitis; PPAR-α: peroxisome

proliferator-activated receptor-α, T2DM: type 2 diabetes mellitus, TG: triglyceride, TIMP-1: tissue inhibitor of metalloprotease, TGF-β: transforming growth factor-β, Zn: zinc

Introduction

Zinc (Zn) is an essential trace element which participates in wide range of biological functions, including cell differentiation and proliferation. Approximately 10% of the human proteome are recognized as Zn-containing proteins. So far, more than 300 enzymes that contain Zn ions within their catalytic domains have been identified [1,2]. Zn also regulates intracellular signaling in both innate and adaptive immune systems [3,4]. Moreover, the anti-inflammatory, anti-oxidant and anti-apoptotic properties of Zn have been fully established [5-7].

The liver turns out to be the main organ responsible for the maintenance of Zn homeostasis [8-10]. Zn homeostasis is primarily regulated by metallothionein (MT) and two types of Zn transporters: Zn transporters (ZnTs) and Zrt- and Irt-like proteins (ZIPs). These Zn transporter families participate in the absorption, excretion, transportation and intracellular storage of Zn [11]. The impairments of these Zn regulation processes eventually lead to Zn deficiency.

Zn deficiency is frequently observed in patients with chronic liver diseases (CLDs) such as chronic viral hepatitis, nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease and especially liver cirrhosis [12-17]. Zn deficiency results in the exacerbation of fibrosis and steatosis in the liver, accompanying a variety of metabolic abnormalities including insulin resistance, dyslipidemia, iron overload, hepatic encephalopathy, and even sarcopenia [13,14,16,18]. Hence, Zn supplementation may be a potential therapeutic strategy to improve these metabolic abnormalities as well as the associated liver diseases [12,16,18]. Indeed, some clinical guidelines specially advocate the clinical significance of Zn supplementation in the management of patients with liver cirrhosis [19,20].

Zn status is ordinarily monitored by serum Zn concentration, because serum Zn concentration is proportional to hepatic Zn content [8,9]. However, the definition of Zn deficiency remained indefinite. The clinical diagnosis of Zn deficiency has been currently established in Japan as less than 60 μg/dL [21].

Recent advances in molecular biological techniques have provided many pieces of novel evidence on the roles of zinc in the pathogenesis of CLDs. These novel techniques have enabled us to investigate the close interactions between the liver and other organs such as the brain, lung, kidney, pancreas, gut and muscles. The impairment of other organs due to Zn deficiency affects liver function.

This review article highlights the current understanding of Zn metabolism in the pathogenesis of CLDs. In addition, we also discuss the clinical efficacy of Zn supplementation in the management of these metabolic abnormalities as well as in the associated liver diseases.

The effects of Zn supplementation in CLDs

1. Antiviral effects of Zn on hepatotropic viruses

A large amount of evidence has accumulated showing that Zn possesses a variety of direct and indirect antiviral properties in relation to numerous viruses [22]. Zn has even been shown to exhibit antiviral effects on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection [23]. Thus, lower serum Zn levels may predict an unfavorable prognosis in patients with severe corona virus disease 2019 (COVID-19) [24].

Similarly, Zn plays crucial roles in the inhibition of hepatitis C virus (HCV) replication [22,25]. It is well established that persistent HCV infection evokes oxidative stress and subsequently inflammation and fibrosis in the liver, leading to chronic liver damage such as chronic hepatitis, liver cirrhosis, and ultimately hepatocellular carcinoma (HCC) [26]. Read et al. revealed that zinc sulfate (ZnSO4) could inhibit HCV replication *in vitro* [27]. The authors speculated that MTs induced by Zn possessed antiviral activities either directly or indirectly. Moreover, they also documented a single-nucleotide polymorphism of interferon-lambda 3 (IFN-λ3), which is recognized as an anti-viral and pro-inflammatory cytokine, potentially participating in the initiation of hepatic MT through increased systemic Zn levels [28]. In a previous randomized controlled trial (RCT), the combination treatment of Zn with IFN significantly improved the rate of viral clearance in patients with chronic hepatitis C [29], although other RCTs did not confirm the antiviral effects of Zn in such patients [30,31]. Therefore, it may make sense that the eradication of HCV by treatment with direct-acting antiviral agents (DAAs) resulted in the increased serum Zn levels in patients with

HCV-related CLD [32,33].

Hepatitis B virus (HBV) infection often causes serious public health problems throughout the world, especially in East Asia and Africa [34]. Mother–to-child transmission is the most common cause of persistent HBV infection. Individuals who are persistently infected with HBV also develop chronic liver disease, including chronic hepatitis and liver cirrhosis, and HCC [26]. No evidence that Zn impedes the replication of HBV has been provided. However, zinc ion supplementation was sufficient to initiate assembly of the HBV capsid protein *in vitro* [35]. In addition, Zn-saturated lactoferrin significantly inhibited the amplification of HBV-DNA in a dose-dependent manner, although lactoferrin by itself did not affect HBV-DNA copies at all [36]. Lactoferrin caused a larger conformational change if it was saturated with zinc. Such a conformational change might exhibit antiviral effects on HBV.

In a clinical study, serum Zn status at entry became a useful predictor for the outcome of IFN treatment [37], which is widely used as a treatment for chronic hepatitis B. However, a RCT did not provide the evidence that combination treatment of Zn with IFN affected the load of HBV DNA in such patients [38]. On the other hand, similar to results in patients with chronic hepatitis C who achieved the clearance of HCV (sustained virological response: SVR) by treatment with DAAs, patients with persistent HBV infection had elevated hepatic Zn contents after treatment with nucleic acid analogs [39].

2. Verification of Zn supplementation on hepatic inflammation in CLDs

It is well recognized that serum Zn concentrations are inversely correlated with serum aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) levels or histological activities in patients with chronic hepatitis [37,40,41], or those with NAFLD [42], as Zn has beneficial properties to inhibit inflammation in the liver. Therefore, we previously confirmed the administration of polaprezinc, a complex of zinc and L-carnosine, resulted in the significant improvement of serum ALT levels in patients with HCV-related CLD [43], which was approximately consistent with the results obtained by other studies in such patients [44,45] and even in patients with NAFLD [46] (**Table 1**). The improvement of serum ALT levels in patients with HCV-related CLD by Zn supplementation may be attributed to an indirect effect rather than a direct action on HCV. However, other studies did not confirm the improvement of serum ALT levels by additional polaprezinc supplementation to the IFN-based treatment in patients with HCV-related CLD [30,31]. In the future, the anti-inflammatory effects of other Zn compounds should be investigated in patients with CLD.

However, it was of interest that a positive correlation between serum Zn and ALT levels were observed in obese and young females, indicating that serum ALT levels were increased due to the higher Zn levels in such individuals [47]. Bai et al. also demonstrated that dietary Zn was correlated with serum ALT levels in adolescents. Especially, young females seemed to be susceptible to elevated ALT levels by higher Zn intake [48]. Taking these results into consideration, higher intake of Zn may be deleterious to the liver.

The role of a Zn transport in the pathogenesis of liver injury has been explored. Su et al. revealed that knockout of ZnT8, which is an important Zn transporter and was highly expressed in pancreatic islets, resulted in the attenuation of acetaminophen-induced liver injury through the upregulation of hepatic Zn and MT [49]. The data elucidated by their study provided novel insights on the organ-organ interactions between the liver and pancreas.

3. Verification of Zn supplementation on hepatic fibrosis in CLDs

Hepatic fibrosis is widely accepted as the final stage of chronic liver injury, and is mediated by hepatic stellate cell activation and extracellular matrix (ECM) secretion and deposition [50]. The activation of hepatic stellate cells is mediated by the release of transforming growth factor-β (TGF-β) from Kupffer cells. The ECM in the normal liver mainly consists of type I and type III collagens [51]. As the stage of hepatic fibrosis progresses, these collagens are increased due to the inactivation of matrix metalloproteinase-1 (MMP-1) [52], which is a Zn-containing endopeptidase, and plays a crucial role in the degradation of ECM. In addition, serum Zn concentrations were gradually decreased in proportion to the severity of hepatic fibrosis in patients with HCV-related CLD [40,53,54] and those with NAFLD [55,56]. Taken together, these data may indicate that the state of Zn deficiency results in the impairment of MMP-1 activity, leading to the promotion of hepatic fibrosis. Kang et al. confirmed that zinc regulated the synthesis of collagen in hepatic stellate cells through the inhibition of TGF-β signaling *in vitro* [57]. Interestingly, the authors also revealed that a decrease in MT synthesis by Zn deficiency caused apoptosis of hepatic stellate cells

Table 1. | Verification of zinc supplementation on hepatic inflammation, fibrosis and steatosis in patients with CLDs

Reference	Study design	Assigned paients	Formulation	Dosage and duration	Outcomes
Inflammation					
Himoto et al. [43]	retrospective study (end of point vs. base line)	HCV-related CLD (n=14)	polaprezinc	225mg, 6 months	decrease in serum ALT level decrease in serum ferritin level no effect on load of HCV RNA
Murakami et al. [44]	RCT (Zn+ IFN-based treatment vs. IFN-based treatment)	chronic hepatitis C (n=11)	polaprezinc	150mg, 48 weeks	decrease in serum ALT level
Matsuoka et al. [45]	RCT (Zn group vs. untreated group)	HCV-related CLD (n=62)	polaprezinc	150mg, 3 years	decrease in serum ALT level
Fathi et al. [46]	RCT (Zn+calorie-restriction vs. calorie-restriction)	NAFLD (n=25)	zinc gluconate	220mg, 12 weeks	decrease in serum ALT and γ-GT level decrease in waist circumferance
Suzuki et al. [30]	RCT (Zn+ IFN-based treatment vs. IFN-based treatment)	chronic hepatitis C genotype 2 (n=41)			no effect on serum ALT level no effect on load of HCV eradication
Kim et al. [31]	RCT (Zn+ IFN-based treatment vs. IFN-based treatment)	chronic hepatitis C (n=16)	polaprezinc	150mg, 48 weeks	no effect on serum ALT level no effect on load of HCV eradication
Fibrosis					
Takahashi et al. [67]	case control study (Zn-responder group, Zn-nonresponder group vs. untreated group)	liver cirrhosis (n=17)	polaprezinc	150mg, 24 weeks	decrease in serum TIMP-1 level
Moriya et al. [68]	case control study (Zn-responder group vs. Zn-nonresponder group)	AIH (n=27)	polaprezinc	150mg, 2 years	decrease in serum procollagen type III and type IV collagen 7S levels
Attallah et al. [69]	RCT (Zn+DAA vs DAA)	chronic hepatitis C (n=147)	zinc sulfate	440mg, 12 or 24 weeks	improvement of liver stiffness (genotype CC) improvement of SVR (genotype CT/TT)
Steatosis					
Fathi et al. [46]	RCT (Zn+calorie-restriction vs. calorie-restriction)	NAFLD (n=25)	zinc gluconate	220mg, 12 weeks	no effect on hepatic steatosis

γGT; γ-glutamyl transferase, AIH: autoimmune hepatitis, SVR: sustained virological response

in an *in vitro* experiment [58]. By contrast, the administration of Zn turned out to suppress the activity of prolyl-hydroxylase, which acts as an enzyme for collagen synthesis. [59] (**Figure 1**).

Several lines of novel evidence on the beneficial effects of Zn compounds have been provided by experimental animal models of hepatic fibrosis. The administration of zinc sulfate significantly inhibited hepatic fibrosis induced by bile duct ligation through the selective alleviation of M1 macrophages [60]. Surprisingly, the polarization of M1 macrophages was correlated with the Notch 1 signaling, which might participate in the process of hepatic fibrosis [61]. Another study elucidated the inhibitory effect of Zn on hepatic fibrosis induced by bile duct ligation through the enhancement of MMP-13 synthesis [62]. Supplementation with polaprezinc improved hepatic fibrosis through the attenuation of tissue inhibitor of metalloproteinase-1 (TIMP-1) in an experimental model of nonalcoholic steatohepatitis (NASH) [63] and in a thioaccetamide-induced model of hepatic fibrosis [64]. Polaprezinc also inhibited the synthesis of fibrotic markers such as collagen I, fibronectin and α-smooth muscle actin (α-SMA) in human hepatic stellate cells via the attenuation of human hepatic stellate cells' proliferation and migration [65]. In addition, the anti-fibrotic action of supplementation with zinc oxide nanoparticles via the alleviation of oxidative stress was confirmed in

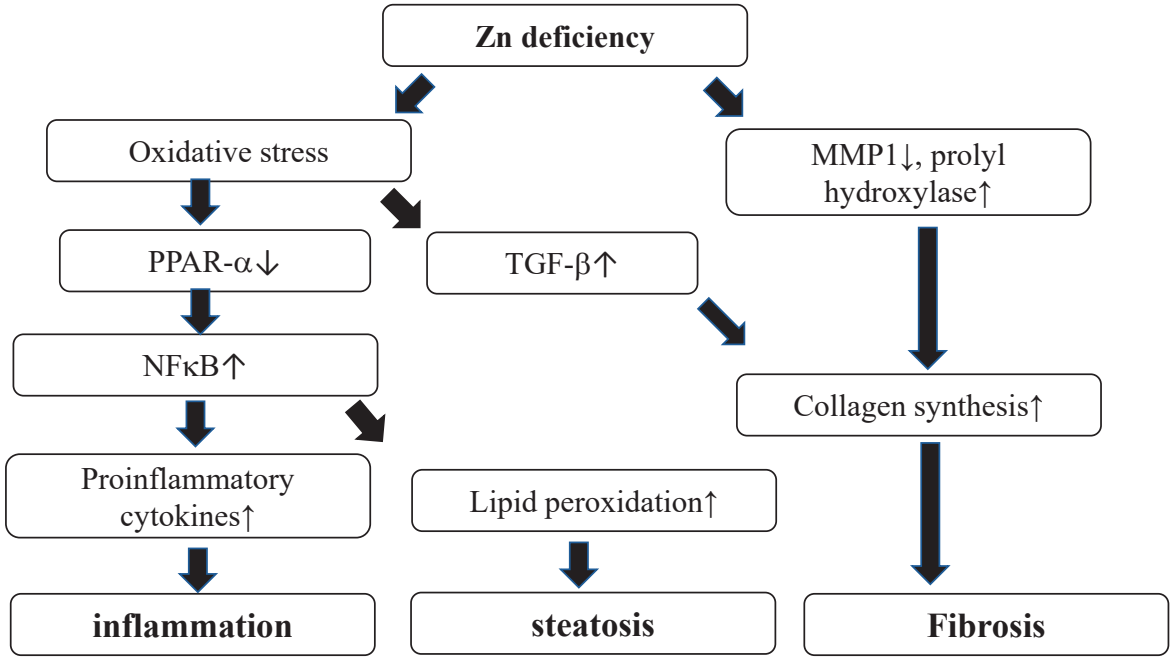

Figure 1. | Putative mechanisms by which Zn deficiency causes liver damage in patients with CLDs.

a thioacetamide-induced hepatic fibrosis model [66].

There have been a few human studies to investigate the effect of Zn supplementation on hepatic fibrosis. The administration of polaprezinc resulted in the suppression of serum TIMP-1 concentrations in patients with liver cirrhosis with Zn elevation [68]. Likewise, serum procollagen type III and type IV collagen-7S levels were significantly decreased by treatment with polaprezinc in autoimmune hepatitis patients with Zn elevation [68]. It was of interest that additional zinc sulfate administration significantly improved hepatic fibrosis in patients with HCV-related CLD who possessed interleukin28 (IL28) rs12979860 polymorphism CC genotype and received a DAA treatment [69] (**Table 1**).

4. Verification of Zn supplementation on hepatic steatosis in CLDs

Hepatic steatosis is characterized by an abundance of lipid droplets in hepatocytes. Zn participates in the activation of peroxisome proliferator-activated receptor-α (PPAR-α), a regulator of lipid homeostasis [70]. Zn deficiency thus results in the attenuation of PPAR-α activity, and subsequently promotes lipid peroxidation, finally leading to the exacerbation of hepatic steatosis (**Figure 1**). Indeed, dietary Zn deficiency exacerbated ethanol-induced hepatic steatosis in mice [71]. In human studies, serum Zn levels were gradually reduced in HCV-related CLD patients as the grade of hepatic steatosis, which was one of the common histological features in those patients [72], became more progressive from mild to severe [73,74]. Moreover, we elucidated the close correlation between insulin resistance and the grade of hepatic steatosis in such patients. Surprisingly, no correlation between serum Zn levels and the grade of hepatic steatosis in patients with NAFLD was found [42]. However, these studies were conducted with small sample sizes. A large-scaled multi-centered trial should be performed to verify the relationship between serum Zn levels and grades of hepatic steatosis in patients with HCV-related CLD and those with NAFLD.

The favorable effects of Zn compounds on hepatic steatosis were exhibited in several animal studies. Alcohol-induced steatosis was reversed by the administration of zinc sulfate in mice via activated PPAR-α and hepatocyte nuclear factor-4α [75]. Interestingly, the activation of PPAR-α induced by zinc sulfate supplementation also caused lipophagy [76]. Xu et al. provided the data that Zn supplementation resulted in the alleviation of hepatic steatosis in lipid disturbance rabbits, accompanied by decreased triglyceride (TG) and increased high-density lipoprotein cholesterol (HDL-C) levels [77]. Type 2 diabetes mellitus (T2DM)-induced hepatic

steatosis in mice was also improved by the treatment with zinc sulfate through the promotion of nuclear factor-erythroid 2-related factor 2 (Nrf2)-metallothionein pathway [78]. The administration of zinc sulfate [79-82], zinc glycine [83], or zinc oxide nanoparticles [84] reversed hepatic steatosis in both *in vitro* and *in vivo* experimental animal models of NAFLD. However, polaprezinc did not alter the grade of hepatic steatosis in a mouse model of NASH [63].

Unfortunately, there have been few studies that have verified the efficacy of Zn compounds on hepatic steatosis in human clinical trials. Fathi et al. revealed that the administration of zinc gluconate (30mg of Zn) did not affect the grade of hepatic steatosis after 12 weeks in overweight/obese patients with NAFLD who adhered to a calorie-restricted diet [46] (**Table 1**). Instead, the authors confirmed that insulin resistance was improved by the same treatment in those patients [85]. When the evidence that Zn deficiency resulted in the exacerbation of hepatic steatosis in patients with NAFLD is provided, Zn supplementation may be promising for the attenuation of hepatic steatosis in such patients. In the near future, the optimal dose of Zn supplementation, the types of Zn compounds and the duration of Zn supplementation in those patients are urgent matters for researchers to address.

5. Verification of Zn supplementation on impaired glucose metabolism in CLDs

It is fully established that Zn is indispensable for the synthesis, storage, and release of insulin, indicating that Zn plays crucial roles in glucose metabolism [86]. In addition, Zn has been considered to show insulin-like action by activating the Akt/PKB signaling pathway [87]. Accordingly, Zn deficiency induces insulin resistance and ultimately leads to the occurrence of T2DM. Zn is stored in the endoplasmic reticulum (ER) of cells, and it is essential for maintaining the homeostatic function of this organelle. Hence, Zn depletion results in the activation of ER-localized chaperon BiP, and the impairment of protein folding in the ER, known as ER stress [88]. This response is closely linked to the different processes involved in the development of insulin resistance and T2DM [89]. The downregulation of hepatic MT expression induced by Zn deficiency has been proposed as the putative mechanism by which Zn depletion initiates ER stress [90]. A recent study suggested that impairment of the Zn transporter, ZIP7 might be responsible for insulin resistance in patients with T2DM [91].

We previously documented that serum Zn concentrations were inversely correlated with the homeostasis model for the assessment of insulin resistance (HOMA-IR) values, the hallmark for insulin resistance in patients with HCV-related CLD, indicating that insulin resistance may derived from Zn deficiency in those patients [54,73]. The inverse correlation was also confirmed in NAFLD patients [55]. In addition, our study elucidated an inverse correlation between the serum Zn level and the insulin-like growth factor (IGF-1)/IGF-binding protein-3 (IGFBP-3) ratio, which is a surrogate for circulating free IGF-1 level in patients with HCV-related CLD [92] (**Figure 2**).

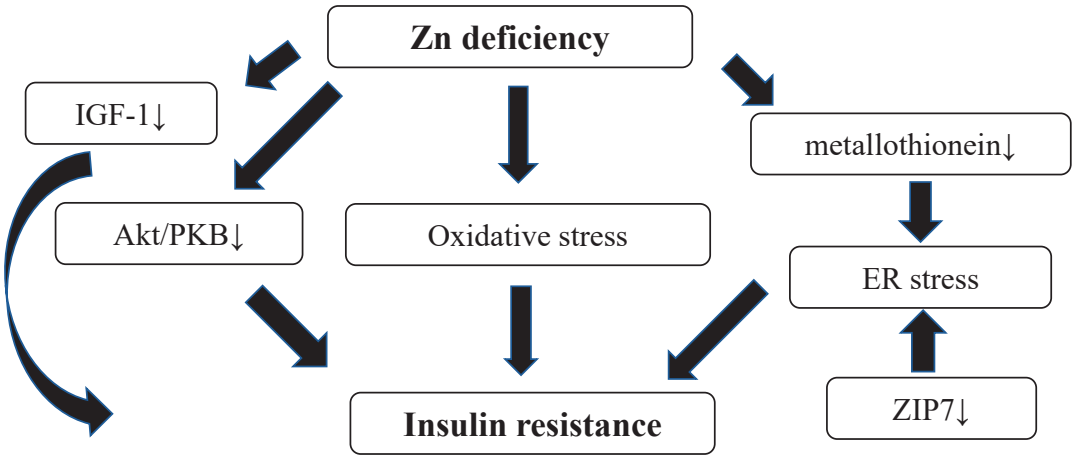

Figure 2. | Putative mechanisms by which Zn deficiency evokes insulin resistance in patients with CLDs.

The beneficial effects of Zn on impaired glucose tolerance have been revealed in several animal studies. Co-supplementation of zinc sulfate and sodium selenite resulted in the improvement of HOMA-IR values in a rat model of NAFLD [79]. In another rat model of NAFLD, zinc sulfate administration and strength exercise increased insulin signaling activity and improved hepatic steatosis [82]. In contrast, Yu et al. documented that zinc sulfate did not affect glucose metabolism, although it significantly alleviated hepatic steatosis in diabetic mice [78]. In a human study, the administration of zinc gluconate (30mg of Zn) for 12 weeks resulted in the improvement of insulin resistance in overweight/obese patients with NAFLD [85] (**Table 2**).

6. Verification of Zn supplementation on impaired lipid metabolism in CLDs

Numerous studies have demonstrated that Zn is involved in lipid metabolism [93]. Indeed, Zn deficiency exacerbates hepatic lipid metabolism. A Zn-deficient diet significantly elevated plasma cholesterol and triglyceride (TG) levels in low-density lipoprotein (LDL)-receptor-deficient mice by way of increases in very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) fractions [94]. Marginal Zn deficiency reduced plasma HDL-C and altered fatty acid profiles in healthy men [95]. Zn deficiency resulted in dysregulation of plasma concentrations of ω3 fatty acids concentration in HCV-related patients with heavy drinking habits [96].

Favorable outcomes of Zn supplementation on impaired lipid metabolism have been revealed in animal studies and *in vitro* studies. High-dose Zn potentially initiated the upregulation of stearoyl-CoA desaturase-1 (SCD-1) expression in hepatocytes and thereby facilitated fatty acid synthesis [97]. In an animal study of NAFLD, co-administration of zinc sulfate and sodium selenite improved the LDL-C/HDL-C ratio and the TG/HDL-C ratio [79]. Zn supplementation caused a decrease in serum TG levels and an increase in serum HDL-C levels in a rabbit model of NAFLD, although it did not alter the serum LDL-C level in the animals [77]. In contrast, few clinical studies investigating the efficacy of Zn treatment on impaired lipid metabolism have been conducted. The administration of zinc gluconate (30mg of Zn) for 12 weeks led to the improvement of serum LDL-C and TG levels in overweight/obese patients with NAFLD [85] (**Table 2**).

Table 2. | Verification of zinc supplementation on metabolic abnormalities in patients with CLDs

Reference	Study design	Assigned paients	Formulation	Dosage and duration	Outcomes
Impaired glucose and lipid metabolism					
Fathi et al. [85]	RCT (Zn+calorie-restriction vs. calorie-restriction)	NAFLD (n=29)	zinc gluconate	220mg, 12 weeks	decrease in HOMA-IR value decrease in serum LDL-C and TG levels decrease in HbA1c
Hyperammonemia					
Hayashi et al. [105]	double blind RCT (Zn+BCAA vs. BCAA)	liver cirrhosis (n=19)	zinc sulfate	200mg or 600mg, 5-6 months	decrease in blood ammonia level no effect on serum Alb level
Takuma et al. [106]	RCT (Zn+standard treatment vs. standard treatment)	liver cirrhosis (n=39)	polaprezinc	225mg, 6 months	decreased in blood ammonia level improvement of hepatic encepalopathy grade increase in serum Alb level
Mousa et al. [107]	double blind RCT (Zn+vitamin A,C,E +lactulose vs. lactulose)	liver cirrhosis (n=31)	zinc gluconate	175mg, 3 months	decrease in blood ammonia level improvement of minimal hepatic encepalopathy decreased in serum ALT level
Ozeki et al. [108]	retrospective study (end of point vs. base line)	liver cirrhosis (n=60)	zinc acetate	200mg, 3 months	decrease in blood ammonia level
Katayama et al. [109]	double blind RCT (Zn vs. placebo)	liver cirrhosis (n=7)	zinc acetate	150mg, 3 months	decrease in blood ammonia level (not significant)
Horiguchi et al. [110]	retrospective study (end of point vs. base line)	liver cirrhosis (n=12)	zinc acetate	50mg, 3 months	no effect on blood ammonia level no effect on sarcopenia

HbA1c; hemoglobin A1c, Alb; albumin

7. Verification of Zn supplementation on hyperammonemia

Hepatic encephalopathy is a serious neuropsychiatric complication of liver disease which derives from fulminant hepatitis or decompensated liver cirrhosis [98]. The accumulation of ammonia is considered as a cause of the disease. Organs which largely participate in ammonia metabolism are the liver and the muscle. In the liver, ornithine transcarbamylase converts ammonia to urea [99]. On the other hand, glutamine synthetase metabolizes ammonia to glutamic acid in the muscle [100]. These enzymes require Zn as a cofactor for the ammonia metabolism. Therefore, Zn deficiency results in a marked increase in blood ammonia level, leading to hepatic encephalopathy [101].

Riggio et al. first confirmed that Zn supplementation reduced plasma ammonia level in cirrhosis rats [102]. Several human studies elucidated the efficacy of Zn supplementation on hepatic encephalopathy [103,104]. Additional administration of zinc sulfate [105], polaprezinc [106], or zinc gluconate [107] to the standard treatment for hepatic encephalopathy showed more favorable effects than the standard treatment alone. The administration of zinc acetate also reduced the blood ammonia level in patients with liver cirrhosis [108]. However, some studies did not confirm the effect of Zn supplementation on the improvement of blood ammonia levels in cirrhotic patients [109,110] (**Table 2**).

8. Verification of Zn supplementation on sarcopenia in CLDs

Sarcopenia is well defined as skeletal muscle volume loss and low muscle strength [111], and it is frequently observed in patients with liver cirrhosis [112]. Zn deficiency was also associated with sarcopenia in patients with CLDs [113,114]. Sarcopenia in such patients might be caused by an indirect rather than a direct effect of Zn deficiency. Zn supplementation is recommended as one of the nutritional interventions in CLD patients with sarcopenia [18]. Unfortunately, no evidence that Zn supplementation reverses skeletal muscle volume in patients with CLD has been provided. The involvement of Zn deficiency in sarcopenia is conflicting [110]. Further examinations should be performed to clarify the effect of Zn on sarcopenia in those patients.

Branched-chain amino acids (BCAAs) are also recognized as a candidate for the treatment of sarcopenia in cirrhotic patients. It is of interest that the BCAA supplementation resulted in the elevation of serum Zn levels as well as the increase in muscle mass volumes in such patients [115]. As one of the reasons for Zn elevation by treatment with BCAA, the authors speculated that the BCAA contained sachet contained 52mg of Zn. Another reason might be attributed to an increase in serum albumin level by BCAA supplementation. The combined treatment of Zn with BCAA will be promising in cirrhotic patients with sarcopenia [116], because these agents are effective to decrease plasma ammonia levels which induce the synthesis of myostatin in patients with hepatic encephalopathy [117].

It is of noteworthy that deletion of the Zn transporter, Zip14, caused the impaired function of intestinal barrier and the subsequent leakage of endotoxin into the systemic circulation. Endotoxemia induced the activation of transcription factors including NF-κB and Mef2c in the skeletal muscle of Zip14 knockout mice, finally leading to the skeletal muscle wasting [118].

9. Verification of Zn supplementation on gut dysbiosis in CLDs

Gut dysbiosis, which causes a variety of critical complications including endotoxemia and hepatic encephalopathy, was frequently observed in patients with liver cirrhosis. It appears to be responsible for small intestinal bacterial overgrowth and/or an increase in intestinal permeability, known as “leaky gut” [119]. Endotoxinemia due to increased intestinal permeability has been also implicated in the development of NASH [120].

Zn deficiency may be associated with gut dysbiosis in patients with CLDs. Zhong et al. documented that Zn deficiency was involved in the dysfunction of intestinal barrier in a mouse model of alcohol-induced steatohepatitis [121]. Later, the authors revealed that Zn regulated bactericidal activity of Paneth cells in mice of alcohol-induced steatohepatitis and that dietary Zn deficiency caused dysfunction of Paneth cells [122]. Another study revealed that the administration of zinc sulfate preserved intestinal barrier function and improved endotoxinemia in rats of alcohol-induced steatohepatitis [123]. It is of interest that the Zn transporter, ZIP14 is involved in maintenance of intestinal tight junctions [124].

10. Inhibitory effects of Zn supplementation on hepatocarcinogenesis in CLDs

Zn is considered to be involved in antioxidant defense, DNA repair and activation of transcriptional factors for cancer prevention [125]. Therefore, Zn deficiency leads to increased occurrence of malignant diseases. Recent studies revealed that

lower serum Zn concentration might become an indicator for the development of HCC [126,127]. Another study documented that lower serum Zn levels in HCC patients who underwent initial hepatectomy might predict unfavorable prognosis [128]. Similarly, early HCC patients who treated curatively and were Zn deficient showed worse overall survival [129].

Several studies have provided the evidence that Zn supplementation, including zinc sulfate [130] and polaprezinc [65], suppressed the proliferation of HCC *in vitro*. These Zn compounds exerted remarkable anti-tumor effects by inducing cell cycle arrest and apoptosis of HCC cells. In a RCT study, the administration of polaprezinc produced more favorable outcomes in patients with chronic hepatitis C by inhibiting cumulative incidence of HCC [131]. Likewise, a retrospective study revealed that supplementation of zinc sulfate resulted in the maintenance of favorable hepatic reserve and the prevention of HCC development in patients with CLDs [132].

microRNAs (miRNAs), which are small, single stranded non-coding RNAs of 19-25 nucleotide in length, negatively regulate gene expression via translational inhibition or messenger RNA (mRNA) degradation [133]. miRNAs play essential roles in diverse biological processes, including cell differentiation, proliferation, migration, and survival [134]. A recent study identified some miRNAs which promoted the development of esophageal cancer in Zn-deficient rats [135]. miRNAs which regulate the development of CLDs in Zn-deficient state should be also explored.

Conclusion

Recent advances in various types of molecular biological technologies have led to novel evidence that Zn deficiency participates in the metabolic abnormalities as well as the pathogenesis of CLD. Many studies have elucidated the favorable outcomes of Zn supplementation, both in terms of the improvement of metabolic abnormalities and histological recovery in the liver, using experimental animal model of CLDs. However, the beneficial therapeutic effects of Zn supplementation on humans have not been fully established in clinical trials. The evidence levels in the human studies on the efficacy of Zn supplementation remain low. The dosage and duration of Zn administration, and the type of Zn compound administration should be investigated in patients with CLD in order to optimize the potential effects of Zn supplementation. Further prospective multicenter cohort studies should be conducted to verify the usefulness of Zn supplementation in CLD patients.

Acknowledgements: This study has no financial support.

Author Contributions: TH wrote the manuscript. TM made critical revisions.

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Review https://metallomicsresearch.brte.org

Role of zinc in microglial phenotypes

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Summary

Microglia are resident immune cells of the central nervous system (CNS) that continuously survey the local microenvironment by extending and withdrawing their cellular processes in the resting state. When activated by tissue injury or other signals, microglia retract their processes and transform into an activated amoeboid morphology. These activated cells are known to polarize into M1 pro-inflammatory or M2 anti-inflammatory phenotypes during neuropathological conditions, including stroke, which suggests that this polarization might play a role in the development and progression of brain disorders. Furthermore, zinc homeostasis in the CNS is integral to normal CNS function, such as learning and memory. Although the effects of zinc on microglial activation are not well known, recent studies have demonstrated that zinc affects microglial activation as well as neuronal function. In this review, we discuss in detail the effects of extracellular and intracellular zinc levels on microglial activation and the M1 and M2 microglial phenotypes. Extracellular zinc might act as a novel trigger for the microglial morphological changes via a zinc-induced microglial activation signaling pathway, where intracellular zinc accumulation via Zrt-Irt-like protein 1 is the initial step. Additionally, extracellular zinc might promote the inflammatory M1 phenotype, while increased intracellular free zinc levels in interleukin-4-induced M2a microglia might negatively regulate arginase-1 expression. The zinc-promoted M1 phenotype is involved in post-ischemic cognitive decline and suppression of astrocytic engulfing activity, whereas zinc-modulated arginase-1 expression might regulate the phagocytic activity of M2a microglia.

Key words: zinc, microglial activation, M1/M2 polarization

Statements about COI: The author declares no conflict of interest associated with this manuscript.

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Received: January 13, 2022
Accepted: February 25, 2022
Released online: April 5, 2022

Introduction

Microglia, a type of glial cell derived from the embryonic yolk sac, are resident innate immune cells of the central nervous system (CNS) [1-3]. Under normal physiological conditions, they are ubiquitously distributed in the mature CNS and exist in a resting state characterized by a ramified morphology with highly motile, active, and long cellular processes [2]. Two-photon microscopy studies have demonstrated that resting microglia survey their microenvironment with highly motile processes in

the healthy brain [4, 5], supporting the proposition that microglia are involved in the maintenance of brain homeostasis as well as host defense [6, 7]. Conversely, in response to neuronal injury or pathogen-derived molecules, resting microglia transform into an activated state comprising an amoeboid morphology, increased proliferation, and release of several types of mediators [8-11]. Furthermore, activated microglia under pathological conditions, such as brain ischemia and Alzheimer's disease, can exert either detrimental or protective effects, suggesting that these cells may acquire opposing phenotypes, termed M1 and M2 activation states [12-14]. However, the concept of M1/M2 microglial activation remains controversial. The M1 phenotype is a pro-inflammatory and cytotoxic phenotype, which is activated mainly by pathogens and pro-inflammatory factors (including lipopolysaccharide [LPS], interferon gamma, and tumor necrosis factor-alpha [TNF-α]) and produces pro-inflammatory cytokines, such as interleukin (IL)-1, IL-1β, and IL-6 [15]. Conversely, the M2 phenotype is an alternative activation state involved in the fine-tuning of inflammation, tissue remodeling, and repair [15]. Therefore, understanding the regulation of the polarization and function of these phenotypes may facilitate the development of effective therapeutic strategies for brain disorders.

Zinc is the second most abundant essential trace element in the brain at an estimated concentration of 150 mM, which is 10-fold of that of the serum zinc levels [16]. Zinc homeostasis in the brain is tightly regulated by primarily two families of transporters, Zrt-Irt-like proteins (ZIPs) and zinc transporters, along with zinc-bound proteins. Accumulating evidence indicates that zinc homeostasis in the brain contributes to normal brain functions, such as learning and memory [17-19]. Conversely, dysregulated zinc homeostasis has been implicated in the pathogenesis of a wide range of neurological diseases. However, the effects of zinc on microglial activation are not well known. In this review, we discuss the effects of extracellular and intracellular zinc levels on microglial activation and the M1 and M2 microglial phenotypes.

Role of zinc in microglial morphological changes

Resting microglia retract their processes and transition into an activated amoeboid morphology within several hours in response to numerous neuropathological conditions, leading to a dramatic change in appearance [20]. In the adult mammalian brain, zinc is concentrated in the synaptic vesicles within a specific subset of glutamatergic neurons in the hippocampus and cerebral cortex [21] and released into the extracellular space in an impulse- and calcium-dependent manner to modulate neurotransmission [17-19]. However, under pathological conditions, including transient brain ischemia and hypoglycemia, massive amounts of vesicular zinc are released into the extracellular space and accumulate in the postsynaptic neurons, resulting in neuronal cell death [22, 23]. Accordingly, treatment with zinc chelators has been shown to attenuate neuronal cell death in animal models of brain disorders. These findings suggest that the dysregulation of extracellular zinc release promotes brain injury [23-26]. In line with these findings, our group demonstrated that ischemia-induced morphological changes in microglia were blocked in mice receiving intraventricular calcium ethylenediaminetetraacetic acid (CaEDTA), an extracellular zinc chelator [27]. Additionally, the addition of 15-60 μM $ZnCl_2$ to microglial cultures caused the retraction of the microglial processes and transition to the round amoeboid morphology, with the changes reaching nearly maximal stage within 2 h [27, 28]. Therefore, the release of massive amounts of extracellular zinc might act as a signal that triggers morphological changes in microglia (**Fig. 1**). However, extracellular actual concentration of zinc released in the brain under pathological conditions remains uncertain. Ischemia-induced zinc release has been calculated to raise extracellular zinc concentrations up to 300 μM. Direct measures of extracellular free zinc during ischemia have identified elevations to only the nanomolar range [22]. On the other hand, treatment of microglial cultures with 120 μM $ZnCl_2$ led to cell death [28], indicating that the range of extracellular zinc concentration which induces microglial morphological changes is limited. Therefore, it is necessary to determine the concentration of extracellular zinc that promotes microglial morphological changes in brain under pathological conditions.

Increasing evidence has shown that extracellular zinc is taken up into the cytosol of neurons via Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [29], L-type voltage-gated Ca^{2+} channels [29, 30], N-methyl-D-aspartate (NMDA) receptors [31], and transporters [32]. Moreover, excessive amounts of extracellular zinc have been reported to induce glial cell death [33] and affect normal astrocytic functions [34]. However, the glial transmembrane routes of zinc are not fully understood. Recently, the ZIP family, which consists of 14 members in the humans and mice, have been investigated for their role in zinc uptake in glial cells because of their expression in various glial cells, including microglia [28, 35], astrocytes [36, 37], and oligodendrocytes [38]. Accordingly, the studies have shown that ZIP1, which is localized on the plasma membrane, is highly expressed in mouse microglia [28], while nickel (a competitive inhibitor of ZIP1) and ZIP1 knockdown decrease zinc uptake

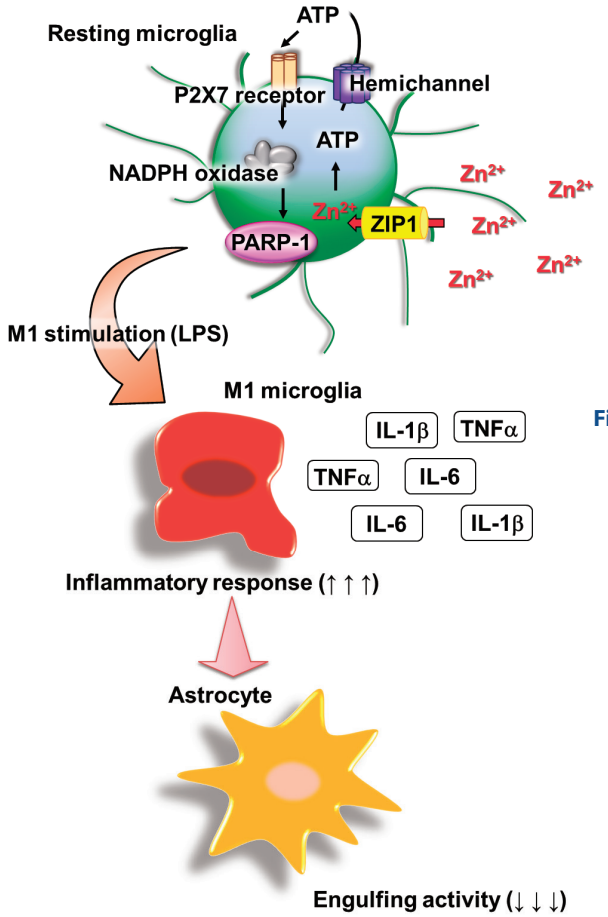

Fig. 1. **Promotion of pro-inflammatory M1 phenotype of microglia by extracellular zinc**
Extracellular zinc triggers microglial morphological changes via the zinc-induced microglial activation signaling pathway (ZIP1-mediated zinc uptake induces release of ATP, followed by P2X7 receptor activation and the sequential activation of NADPH oxidase and PARP-1). This pathway mediates the aggravation of pro-inflammatory M1 phenotype of microglia in response to M1 stimuli, including LPS. These aggravated microglia then suppress astrocytic engulfing activity and produce pro-inflammatory cytokines. ZIP1, Zrt-Irt-like protein 1; PARP-1, poly(ADP-ribose) polymerase; LPS, lipopolysaccharide; IL-, interleukin-; TNF-α, tumor necrosis factor-alpha.

by microglia [35]. Additionally, intracellular zinc chelation by *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) suppresses zinc-induced morphological changes in microglia [28]. This indicates that ZIP1 is the major transporter responsible for zinc uptake in microglia, and ZIP1-mediated zinc transport may be the first step in the microglial morphological changes.

After brain disorders, such as brain ischemia and trauma, ATP is released from the damaged and dying cells into the extracellular space, where it serves as a signal for microglial activation and chemotaxis. Furthermore, microglia release ATP in response to stimuli such as LPS, lysophosphatidic acid, amyloid β, and hypotonic stress [39-42]. Microglial ATP release is primarily mediated by hemichannels, vesicular nucleotide transporter-dependent exocytosis, or cystic fibrosis transmembrane conductance regulators [43-45]. Our earlier study showed that zinc stimulation induced an increase in extracellular ATP in primary microglia culture, and this ATP increase was inhibited by a non-specific hemichannel inhibitor, carbenoxolone (CBX) [28]. Furthermore, the pretreatment of microglia with CBX suppresses zinc-induced morphological changes [28]. Additionally, we revealed that a P2X7 receptor-selective antagonist, oxATP, abolished zinc-induced microglial morphological changes. Conversely, exposure to ATP or a relatively highly selective P2X7 receptor agonist, BzATP, transformed microglia into its activated amoeboid form [28]. Thus, hemichannels might be a potential pathway for ATP release in zinc-stimulated microglia, and this hemichannel-mediated ATP release is involved in the subsequent cascade of microglial morphological changes via autocrine and/or paracrine activation of P2X7 receptors.

The activation of P2X7 receptors in many types of brain cells stimulates NADPH oxidase activity, a major source of reactive oxygen species (ROS) [41, 46, 47]. Microglial ROS production by NADPH oxidase promotes microglial activation [48]. The exposure of microglia to zinc induces an increase in ROS levels, whereas the inhibition or genetic disruption of NADPH oxidase blocks zinc-induced increase in ROS levels and morphological changes [27]. Additionally, the zinc-induced increase in microglial ROS levels is suppressed by the inhibition of intracellular zinc accumulation, hemichannel-mediated ATP release, and P2X7 receptor activation in microglia after zinc stimulation [28]. Therefore, a necessary step for zinc-induced morphological changes in microglia might involve the activation of NADPH oxidase, which lies downstream of intracellular zinc accumulation and

P2X7 receptor activation via hemichannel-mediated ATP release.

ROS acts as a secondary messenger in many cell types. Oxidative DNA damage modulates the activation of microglia via poly(ADP-ribose) polymerase (PARP)-1 [49-51], which is an ADP-ribosylating enzyme involved in various DNA and RNA metabolic processes, including DNA repair. When activated by oxidative DNA damage, PARP-1 contributes to the regulation of the expression of inflammation-associated genes, such as cyclooxygenase-2, and inducible nitric oxide synthase (iNOS) [52, 53]. Conversely, PARP-1 inhibitors suppress the morphological changes in microglia that are induced by treatment with TNF-α, amyloid β, S100B, and NMDA [54-57]. In our earlier study, PARP-1 activation led to the transition to the amoeboid form in microglia treated with zinc [27]. However, the zinc-induced PARP-1 activation and morphological changes were suppressed by the inhibition of NADPH oxidase and PARP-1 [27], indicating that zinc-induced morphological changes in microglia are mediated by the sequential activation of NADPH oxidase and PARP-1. Furthermore, we demonstrated that zinc-induced PARP-1 activation was abolished when microglia were pretreated with a hemichannel inhibitor and P2X7 receptor antagonist [28].

Therefore, these findings suggest that extracellular zinc acts a novel trigger for microglial morphological changes, which are initiated by intracellular zinc accumulation via ZIP1. Furthermore, these morphological changes are mediated by ATP release through hemichannels and autocrine/paracrine activation of P2X7 receptors, and followed by the sequential activation of NADPH oxidase and PARP-1 (zinc-induced microglial activation signaling pathway) (**Fig. 1**). To our knowledge, there are no reports showing the aforementioned intracellular signaling pathway induced by other stimuli. It is expected to determine whether the zinc-induced microglial activation signaling pathway is unique.

Zinc and M1 activation of microglia

Although the concept of M1/M2 activation of microglia remains controversial, increased expression of pro-inflammatory mediators and M1 cell surface markers have been demonstrated by numerous studies on animal models and patients with brain disorders, including stroke, Alzheimer's disease, amyloid lateral sclerosis, and Parkinson's disease [58-65]. These findings imply that an excess accumulation of pro-inflammatory mediators, such as IL-1β, IL-6, and TNF-α, caused by the chronic activation of M1 microglia might lead to neuronal damage. Additionally, studies have shown that conditioned media collected from ischemic neurons prime microglial polarization toward the M1 phenotype [58], while the microenvironment associated with spinal cord injuries leads to the upregulation of the M1 phenotype and downregulation of the M2 phenotype [66]. Thus, an endogenous soluble factor that regulates microglial M1/M2 polarization might exist in the brain under pathological conditions.

Since resting microglia rapidly transform into an amoeboid morphology and exhibit an M1 phenotype in response to the corresponding M1 stimuli [67], we focused on extracellularly released zinc following transient brain ischemia as a modulator of microglial polarization. In our earlier study of LPS-induced M1 polarization of microglia, the pretreatment of microglia with $ZnCl_2$ resulted in a dose-dependent increase in iNOS expression and IL-1β, IL-6, and TNF-α secretion [68]. However, the effects of zinc pretreatment on microglia were suppressed by treatment with TPEN, a cell-permeable zinc chelator; Trolox, a radical scavenger; and A438079, a P2X7 receptor antagonist [68]. This implies the involvement of a zinc-induced microglial activation signaling pathway that leads to the transformation of ramified microglia into the amoeboid form. Moreover, we found that intra-cerebroventricular pre-injection with CaEDTA attenuated ischemia-induced pro-inflammatory cytokine expression and M1 polarization of microglia in the hippocampus as well as protected against post-ischemic cognitive decline [68]. Therefore, extracellular zinc may be an endogenous factor involved in promoting the inflammatory M1 phenotype of microglia arising in response to M1 stimuli (**Fig. 1**). Moreover, considering that zinc plays an important role in brain functions, interventions targeting zinc-induced microglial activation signaling pathway may be effective strategy for preventing brain dysfunction following ischemia. Therefore, it is necessary to clarify the precise mechanism underlying zinc-promoted M1 phenotype in future studies. Furthermore, since vesicular zinc has been shown to be in the glutamatergic neurons in the several brain regions other than the hippocampus, it is expected to address regional differences of zinc-promoted M1 phenotype.

Effect of zinc-promoted microglial M1 activation on astrocytic function

Astrocytes play an important role in normal physiological brain function by providing neurons with structural, metabolic, and trophic support [69, 70]. Microglia, on the other hand are responsible for the clearance of dead cells and debris that accumulate in the affected region after brain damage. Recently, studies have demonstrated that astrocytes could also engulf

dead cells and small axonal or myelin debris to restore impaired neuronal neural circuits and attenuate the inflammatory impact of damaged neuronal cells [71-73]. Therefore, astrocytes can also function as phagocytes together with microglia under pathological conditions. However, in a rodent model of transient brain ischemia, phagocytic astrocytes exhibited an ischemic spatiotemporal pattern distinct from that of microglial cells [71]. Furthermore, attenuation of the engulfing activity of cultured astrocytes occurred under sub-lethal oxidative stress [74]. This indicates that astrocytic engulfing activity is impaired or limited by the disruption of the local neural microenvironment. Recently, Hamada et al. revealed that astrocytic engulfing activity was suppressed by conditioned medium derived from zinc-pretreated M1 microglia [75]. Other studies have shown that the chronic or aggravated inflammatory M1 phenotype of microglia exacerbates brain injury [58, 76] and that extracellular zinc released after brain ischemia promotes this M1 microglial phenotype in the hippocampus [68]. Therefore, zinc might be involved in the suppression of astrocytic engulfing activity by promoting the M1 phenotype and disrupting the microenvironment (**Fig. 1**). Conversely, recent accumulating evidence indicates that astrocytes can affect microglial function (for example, microglial neuroinflammation is inhibited by exosomes derived from astrocytes) [77]. However, further studies are needed to determine whether astrocytes modulate zinc-induced promotion of M1 activation.

Zinc and M2 activation of microglia

In contrast to the M1 activation of microglia, the M2 activation state is subdivided into the M2a, M2b, and M2c subtypes. Although these subtypes have some biochemical overlaps, they have different activation mechanisms and function [78]. M2a activation is induced by IL-4, which can induce CD206 expression and is associated with the upregulation of anti-inflammatory mediators, such as arginase-1 [79]. Arginase-1 prevents NO production by competing with iNOS for the substrate L-arginine. These responses of M2a microglia contribute to the attenuation of brain damage caused by excessive inflammation and to the promotion of tissue remodeling and repair [80, 81]. In contrast, excessive arginase-1 activity has been reported to cause endothelial dysfunction in traumatic brain injury [82] and retinopathy [83] models. In support of these findings, studies in mouse models of Alzheimer's disease have revealed accumulation of arginase-1 in the subiculum and CA1 regions of the hippocampus, the major areas of amyloid β deposition and neurodegeneration. Furthermore, the pharmacological inhibition of arginase activity protected the model mice from Alzheimer's disease-like pathologies [84]. Thus, arginase-1 might exert either beneficial or detrimental effects depending on the degree of its expression.

In the brain cells, including neurons and glial cells, most zinc binds to proteins such as enzymes, signaling molecules, and transcription factors, which in turn are involved in maintaining the efficient performance of the brain cells. Consequently, intracellular free zinc is considered to be excessively low under normal physiological conditions in the CNS [85]. Additionally, transient changes in intracellular free zinc concentration have been reported to play an important role in signal transduction and cell function [86-88]. Moreover, free zinc concentration in neurons and astrocytes is shown to increase in response to several pathophysiological stimuli, such as hypoosmolality [89, 90], glucocorticoids [91], and oxidative stress [36, 92], and this increase in intracellular free zinc is mediated by zinc influx from the extracellular space via zinc importers or by release from cytosolic zinc-binding proteins (intracellular zinc release) [93]. In our earlier study, when microglia were treated with IL-4, intracellular free zinc concentration increased after the induction of arginase-1 expression, whereas the induction of arginase-1 expression was prevented by an intracellular zinc chelator (and not an extracellular zinc chelator) [94]. Additionally, chelation of intracellular zinc resulted in a dramatic increase in both mRNA levels and enzymatic activity of arginase-1 in IL-4-induced M2a microglia. Therefore, IL-4 might induce intracellular zinc fluctuation in M2a microglia through intracellular zinc release, and the increased intracellular zinc level may in turn act as a negative regulator that prevents excessive expression of arginase-1 in M2a microglia [94].

In addition to regulating arginase-1 expression, M2a microglia can phagocytose cell debris to promote reconstruction of the extracellular matrix, tissue repair, and neuronal survival [78]. However, the overexpression of recombinant murine IL-4 in the hippocampus of a mouse model of Alzheimer's disease led to an increase in the "M2-like" microglial phenotype, along with downregulation of microglial phagocytosis [95]. Chelation of intracellular zinc suppressed IL-4-induced phagocytic activity in microglia, which was then reversed by L-arginine supplementation [94]. L-arginine, a substrate for arginase-1, is an important factor that promotes phagocytosis in peripheral immune cells [96-98]. Therefore, these findings suggest that microglial intracellular zinc release after IL-4 stimulation may play an important role as a negative regulator of arginase-1 expression, and this negative regulation of arginase-1 expression may be essential for maintaining the normal phagocytic activity of M2a microglia (**Fig. 2**).

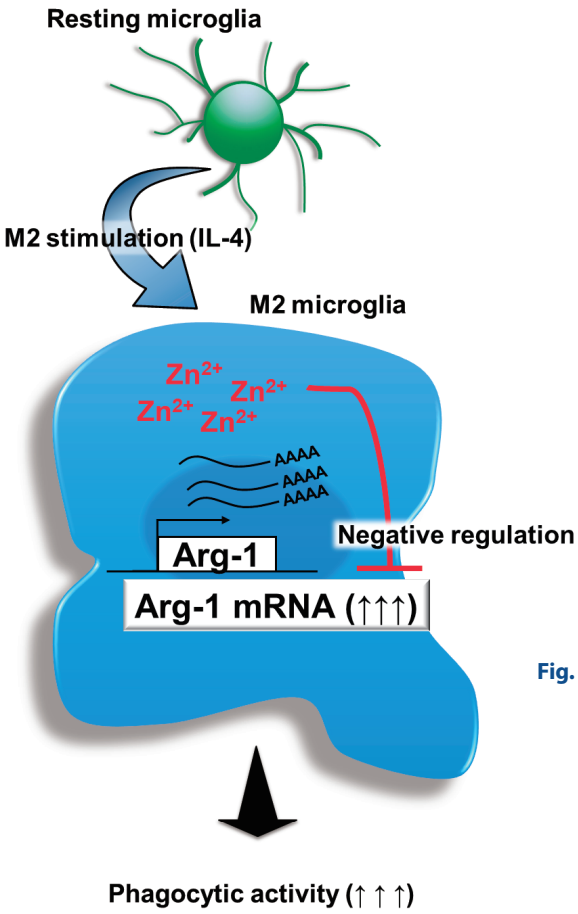

Fig. 2. **Regulation of M2a microglial function by accumulation of intracellular free zinc**
Intracellular zinc accumulated in IL-4-induced M2a microglia acts as a negative regulator to prevent the excessive expression of arginase-1. This regulation contributes to the maintenance of the phagocytic activity of M2a microglia. IL-4, interleukin-4.

However, it is unknown how much concentration of zinc is released in microglia after IL-4 stimulation. In future, it is necessary to determine the concentration of intracellular free zinc negatively regulates excess arginase-1 expression in M2a microglia, and what mechanism underlying the negative regulation of intracellular zinc-induced arginase-1 expression. Considering that M2 microglia are subdivided into subtypes, including the IL-4-induced M2a, immune complex-induced M2b, and IL-10/transforming growth factor-beta-induced M2c, studies examining the effect of zinc on the induction of the other subtypes of M2 microglia are required.

Conclusion

This review highlighted studies investigating the role of zinc in microglial phenotypes. A brief description of the main findings follows. Extracellular zinc triggers morphological changes in microglia via the intracellular zinc signaling cascade and promotes the inflammatory M1 phenotype. M1 microglia are involved in post-ischemic cognitive decline and suppression of the engulfing activity of astrocytes. In case of the M2 phenotype, M2a microglia activation is induced by IL-4. Furthermore, the increase in intracellular free zinc in M2a microglia after IL-4 stimulation acts as a negative regulator of arginase-1 expression, which then contributes to the regulation of the phagocytic activity of M2a microglia. Therefore, zinc plays different roles in microglia depending on their activation state. Numerous studies have revealed that the balance between the M1 and M2 phenotypes is disrupted during chronic inflammation conditions, such as those associated with ischemia, traumatic brain injury, Parkinson's disease, and Alzheimer's disease [58, 99-101]. Moreover, alterations in zinc homeostasis in the brain are involved in the pathophysiological progression of these disorders [24, 102-104]. All these discussed findings suggest that zinc has an important role in the microglial phenotypes and that the disruption of zinc homeostasis in the brain is one of the major causes of imbalance in the M1/M2 phenotype. Therefore, more advanced studies investigating the role of zinc in the microglial phenotypes under pathophysiological conditions are required for clarifying these findings and developing an effective strategy for the prevention and alleviation of brain disorders.

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Roles of zinc signaling in mammalian reproduction

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Summary

Zinc is a critical trace element that is important for cellular function in both female and male reproductive organs. Zinc imbalance and/or altered zinc signaling causes multiple disorders in the reproductive process, including oogenesis, spermatogenesis, fertilization, and embryogenesis. Extracellular and intracellular dynamics of zinc ions are regulated by cell-specific transporters, i.e., Zrt-, Irt-related protein (ZIP) or zinc transporter (ZnT), which respectively transport zinc ions in or out of the cytoplasm through biological membranes. The expression and function of these transporters vary among cell types. The elucidation of the mechanisms underlying zinc homeostasis and zinc dynamics in reproductive function will lead to better infertility treatments for humans as well as the improvement of livestock production. In this review, we discuss the essential roles of zinc signaling in the key events in mammalian reproduction, with a focus on the period from gametogenesis to embryonic development.

Key words: Zinc, Zinc spark, oogenesis, spermatogenesis, fertilization

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

1. Introduction

Zinc is an essential trace mineral that is involved in many cellular processes such as cell proliferation, immune function, antioxidant defense, gene expression, and RNA polymerase activity [1-3]. Since 1963, when zinc deficiency was first reported to be associated with hypogonadism and dwarfism [4], the importance of zinc has received much attention due to the decrease in female and male reproductive functions caused by zinc deficiency. Most recently, it appears that the cytokine storm in SARS-CoV-2 infection (COVID-19 disease) may induce a depletion of zinc and increased oxidative stress in reproductive tissues, and this possibility is especially relevant to the fertility of affected couples [5].

Approximately 30%–40% of cellular zinc is present in the nucleus, with ~50% in the cytoplasm and organelles; the remaining is localized in the cell membrane [6]. The spatiotemporal zinc dynamics are tightly regulated through zinc transporters, which contains two of the solute carrier protein families; SLC30A (Zinc transporter, ZnT) and SLC39A (Zrt-, Irt-related protein, ZIP), to provide crucial cellular signaling [7, 8]. The influx and efflux of zinc ions are regulated by ZIP and ZnT, respectively (**Fig. 1A**) [9-11]. In mammals, a total of 23 zinc transporters have been

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Received: December 30, 2021
Accepted: February 28, 2022
Released online: April 5, 2022

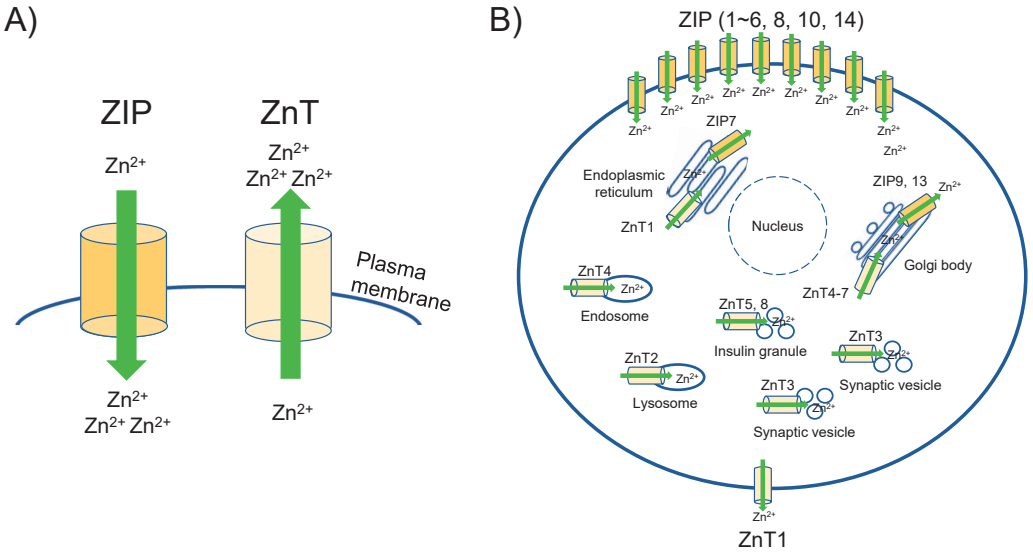

Fig. 1. **Direction of zinc transport and subcellular localization of ZIP and ZnT transporters.**
A: Zinc transporters are localized to various intracellular compartments and the plasma membrane. *Green arrows* indicate the direction of zinc by ZIP and ZnT, respectively. ZIP functions in zinc influx into the cytoplasm, while ZnT moves zinc in the opposite direction.
B: The localization of ZIP and ZnT transporters is shown referring to the information available in many reports.

identified: ZIP1–14 and ZnT1–9 (**Fig. 1B**) [12-14]. These zinc transporters are expressed at various locations in the cells and tissues [15, 16]. Although the detailed mechanisms of cellular zinc dynamics are not fully understood, recent studies have highlighted zinc homeostasis and its function in essential reproductive events. In this review, we discuss the relationship between zinc and mammalian reproduction, including oogenesis, spermatogenesis, fertilization, and embryo development.

2. Zinc signaling in oogenesis

2.1. Ovarian function

In the ovary, follicular development is controlled by multiple factors and physiological events [17]. The regulation of primordial follicle dormancy and activation is important for reproductive sustainability [18]. For example, the primordial follicles remain in a resting and dormant state until they are "activated" to begin follicle growth, and the follicle activation involves a complex interaction between germ cells and somatic factors to ensure the steady maintenance of the growing follicle pool. There is no information to date on the role of zinc in the early follicular or germ cell development in mammals. However, in the early and late germ cell development of *Caenorhabditis elegans*, zinc deficiency has been reported to cause reduced fertility due to impaired oocyte development [19, 20], indicating that zinc plays an important role in early and late germ cell development in *C. elegans*. In mammals, the involvement of zinc in early oocyte meiosis needs to be investigated in detail.

2.2. Oocyte maturation

A divalent ion, i.e., Ca^{2+}, has the most important role in gametogenesis and fertilization [21-23]. Most mammalian oocytes are arrested at the prophase of the first meiosis, which is also called the germinal vesicle (GV) stage because these oocytes possess a GV. After a surge of luteinizing hormone (LH), GV oocytes resume meiosis and progress to the metaphase of the second meiosis (MII) through GV breakdown (GVBD). Oocytes that reach the MII stage are arrested again just before fertilization. The Mos/Mitogen-activated protein kinase (MAPK) pathway regulates the arrest at the first meiosis [24]. Several research groups revealed that an optimal concentration of zinc in oocytes was required for the arrest through a component of the Mos/MAPK pathway called 'cytostatic factor (CSF)' [25-27]. Treatment with a zinc chelator, i.e., N, N, N′, N′-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), can induce meiotic resumption from the GV stage [27]. Kong *et al*. also reported that meiotic resumption induced by TPEN can be inhibited by an injection of Mos short interfering (si)RNA or treatment with cycloheximide, a potent inhibitor

of protein synthesis [27]. Taking the above findings together, it appears that at least in immature (GV) oocytes, zinc signaling has an inhibitory role in meiotic resumption via a suppression of CSF activity (**Fig. 2**).

After the resumption of meiosis from the GV stage, a large fluctuation of the level of zinc occurs [26]. It is reported that TPEN treatment throughout the first meiosis resulted in a failure of progression to asymmetrical division (the extrusion of the first polar body) and in arrest at telophase I [26]. An injection of nondegradable cyclin B1, which is a component of maturation promoting factor (MPF), partially rescued the arrest of zinc-insufficient oocytes, enabling them to enter MII [28]. MPF is also thought to be another component of CSF, suggesting that zinc may have different roles at each meiotic stage via the regulation of CSF activity.

The results of an *in vivo* study using a zinc-deficient diet revealed that zinc plays an important role in follicle development and ovulation. Feeding a zinc-deficient diet for 10 days completely blocked ovulation and compromised cumulus expansion *in vivo* [29]. A more acute 3-day treatment with a zinc-deficient diet did not block ovulation but did increase the number of oocytes trapped in luteinizing follicles. Moreover, 23% of ovulated oocytes did not reach MII due to severe spindle defects [29]. These observations correspond to the results from oocytes treated with TPEN [26-28, 30]. Together these findings indicate that zinc signaling has an essential role in the meiotic progression of oocytes both *in vitro* and *in vivo.*

The complete understanding of zinc-mediated effects in cumulus oocyte complexes (COCs) during the periovulatory period is lacking. The lack of cumulus cell expansion is known to be due to an almost complete suppression of phospho-Sma- and Mad-related protein 2/3 (SMAD2/3) signaling. The chelation of zinc with TPEN causes an acute increase in steroidogenic transcripts (such as *Cyp11a1* and *Star* mRNA) and an increase in progesterone accumulation in the culture medium [29, 31]. TPEN is also known to abolish SMAD2/3 signaling in COCs. The signaling through the zinc-binding SMAD (mothers against decapentaplegic homolog) transcriptional pathway is known to inhibit progesterone production [32-34], and TPEN treatment potently suppresses SMAD2/3 phosphorylation in COCs [29]. These results have shown that suppression of SMAD2/3 signaling also leads to other defects including a complete failure of cumulus expansion as seen in the TPEN-treated COCs.

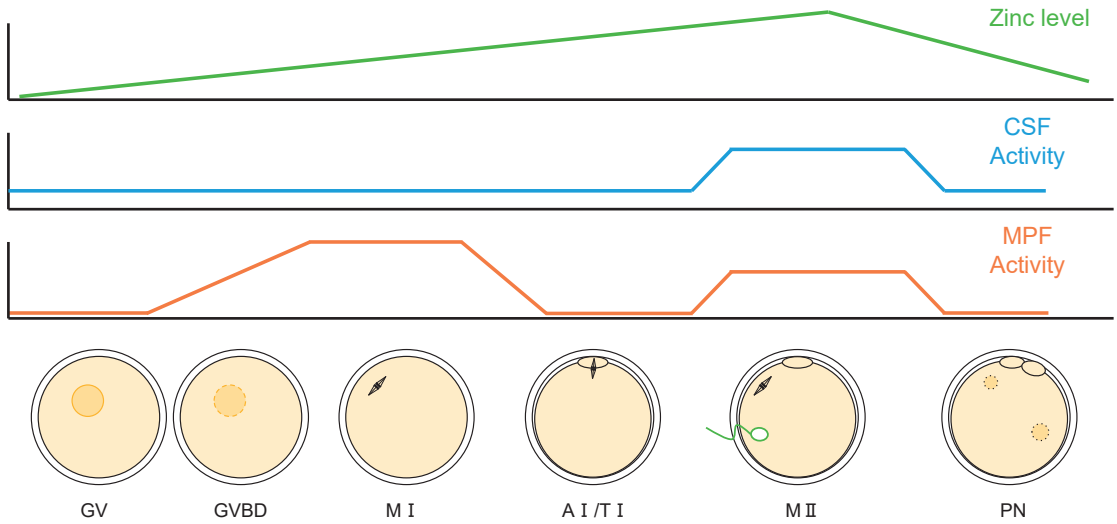

Fig. 2. **The proposed model of the relationship between zinc and CSF activity in the regulation of the dynamics of oocyte maturation.**
Zinc acts as a switch to regulate CSF during the establishment, maintenance, and arrest of MII.
Upper panel: the approximate relative zinc levels in GV oocytes, MII, and early embryos.
Subsequent panels: the dynamics of key cellular activities during oocyte maturation and fertilization.
In this model, as intracellular zinc increases, the MPF activity also increases, inducing the resumption of meiosis; at the AI/TI stage, when intracellular zinc exceeds a certain level, CSF is activated, leading to CSF-mediated MII arrest. After maturation, zinc sparks cause a decrease in intracellular zinc, which leads to reduced CFS and MPF activity in fertilization. AI/TI: anaphase I/telophase I, GV: germinal vesicle, GVBD: GV breakdown, MI: metaphase I, MII: metaphase II, PN: pronucleus.

2.3. The accumulation of zinc during oocyte maturation

Studies using zinc-selective indicators such as Fluozin-3 AM or Zinc BY1 have shown that zinc is accumulated by the MII phase before 'zinc spark' described below [30, 35-40]. Kim *et al*. (2011) revealed distinct regions of high zinc concentration that formed a polarized and hemispherical pattern [35]. The polarization of the zinc-enriched compartments mirrored that of the cortical granules (CGs), which are exocytic vesicles that participate in the cortical reaction, which involves the hardening of the zona pellucida to establish a block to polyspermy at fertilization [41]. These vesicles undergo dynamic movement during oocyte maturation and exocytosis at the time of fertilization [42].

3. Zinc signaling in spermatogenesis

Zinc also has critical roles in spermatogenesis, sperm maturation, and capacitation. The experimental restriction of zinc intake in adult men for 24–40 weeks was shown to result in oligospermia [43]. This was caused by a decrease in testosterone levels with impaired Leydig cell function, which was subsequently restored after 2–32 months of zinc supplementation. The administration of a severe zinc-deficient diet to rats resulted in decreased weight of the testis and paragonadal gonads (seminal vesicles and prostate) and increased abnormal spermatozoa with flagella that were shorter by ~25% compared to controls, which is associated with modulated fatty acid composition and interrupting essential fatty acid metabolism [44]. Zinc deficiency was also observed to cause testicular atrophy accompanied by the loss of spermatozoa and spermatocytes [45], increased apoptotic degeneration [46], and high rates of oxidative damage to lipids, proteins, and DNA in the testes of male rats [47]. Marginal zinc deficiency also sensitized the prostate to oxidative stress in rats [48]. These results indicate that zinc plays an important role in male fertility, regulating multiple cellular processes.

Zinc is present mainly in Leydig cells, late differentiating spermatocytes, and spermatozoa in testicular tissues [49]. The developing spermatocytes contain high levels of zinc since zinc is required for DNA condensation and meiosis [50]. The chromatin structure of a spermatozoon is completely different from that of somatic cells, since each spermatozoon needs to transport the haploid genome to eggs. The zinc-dependent chromatin stability model has been proposed as the chromatin structure is packed in order to be extremely resistant to DNA damage in the sperm and rapidly decondensed to make the DNA available in the ooplasm after fertilization [51]. Thus, zinc dynamics are responsible for fertilization competency in sperm cells.

The prostate gland, the seminal fluid, and ejaculated sperm have much higher zinc content than testicular tissues [6], indicating that sperm accumulate zinc as they are transported through a seminal duct. The zinc concentration in semen is positively correlated with the sperm count and normal sperm morphology, and poor zinc nutrition may be associated with a low quality of sperm and male infertility [52]. It is pointed out that reduced seminal zinc levels associated with cigarette smoking is an important risk factor for poor sperm quality and idiopathic male infertility [53, 54]. Semen, which contains a high concentration of zinc, plays an important role in preventing premature sperm fertilization and exerts antioxidant activity; low concentrations of zinc may be a prerequisite for successful acrosomal exocytosis [55].

Few studies have examined how zinc is transported into the maturing gametes during spermatogenesis. In mice, different types of ZIP and ZnT are expressed in the testes at each stage from spermatogonia to spermatogenesis [49]. Investigations using that murine model have shown that ZIP5 imports zinc into Sertoli cells and spermatocytes, augmented by ZIP10 in primary spermatocytes and ZIP8 in secondary spermatocytes [49]. Round spermatids express ZIP6, ZIP8, and ZIP10, whereas elongating spermatids express ZIP1 and ZIP6. ZIP14 was detected in undifferentiated spermatogonia and Leydig cells. ZnT1 is dominantly expressed in Sertoli cells [56], indicating its important role in the export of zinc from Sertoli cells to the developing germ cells. In addition, the expressions of ZIP6 and ZIP10 were greatly reduced in testes of mice fed a zinc-deficient diet [49], suggesting that intracellular zinc dynamics activate zinc signaling through the expression of stage-specific transporters.

4. Zinc signaling in fertilization and embryogenesis

4.1. Fertilization and oocyte activation

In most mammalian species, oocytes are arrested at the MII stage before ovulation [57-59]. 'Oocyte activation' requires the progressive initiation of several events: cortical granule exocytosis, the inactivation of CSF, the resumption of meiosis and exit from MII arrest, the extrusion of the second polar body, and the formation of the pronucleus (PN) [59-61]. The completion of these events ensures the initiation of early embryo development [62]. In all species studied to date, oocyte activation requires

a fertilization-associated increase in the intracellular concentration of Ca^{2+} [63]. While oocyte activation in many invertebrates and vertebrates is triggered by a transient increase in calcium ions [64], oocyte activation in mammalian species causes a repeated Ca^{2+} rise and fall, i.e., calcium oscillation [65-71].

These Ca^{2+} oscillations play an important role in triggering key events in oocyte activation, such as cortical granule exocytosis, the resumption of meiosis, and exit from MII arrest. Although it has been known for several decades that Ca^{2+} is an essential trigger for egg activation, the mechanism by which sperm triggers an intracellular Ca^{2+} release has not been established. In addition, the details of the mechanism underlying the Ca^{2+} release at fertilization in vertebrates remain to be fully clarified, especially regarding the role of the interaction between the sperm and oocyte in this process [72, 73]. To solve these problems, it is necessary to clarify the fertilization mechanism at the molecular level.

Three major hypotheses have been proposed regarding the mechanism of Ca^{2+} release by sperm during fertilization: the membrane receptor hypothesis, the Ca^{2+} conduit hypothesis, and the sperm factor hypothesis [61, 64, 74-76]. The sperm factor hypothesis, which is widely supported at present, can be explained by the finding that an injection of mammalian sperm extract induces Ca^{2+} responses and oocyte activation events similar to those in spontaneous fertilization. An investigation of the 'sperm factor' activity in the above-mentioned mammalian sperm extract revealed that this activity appeared to be based on a sperm-specific phospholipase C (PLC) with distinctive properties such as enhanced Ca^{2+} sensitivity compared to the known PLC isoforms [68, 77-79]. In 2002, the sperm-specific PLC named PLCζ was discovered [80]. PLCζ appeared to have all the expected properties of an endogenous agent of oocyte activation. An important structural difference between PLCζ and other PLC isoforms is that PLCζ is smaller than all of the previously identified PLC isoforms and lacks a Pleckstrin-homology (PH) domain. It is known that upon the release of sperm-derived phospholipase C zeta (PLCζ) into the ooplasm, this enzyme hydrolyzes phosphatidyl inositol 4, 5-biphosphate (PIP_2) to produce inositol 1, 4, 5-trisphosphate (IP_3) and diacyl glycerol (DG) [81]. IP_3 binds IP_3 receptor (IP_3R), which is distributed along the endoplasmic reticulum (ER; the main Ca^{2+} store of the cell), gating the receptor and inducing periodic intracellular Ca^{2+} increases [82-85] (**Fig. 3**).

PLCζ was subsequently identified in a number of mammalian species [86-89] and other animal species [90-92], and it was demonstrated that a microinjection of PLCζ complementary cRNA induced Ca^{2+} oscillations in mouse oocytes comparable to those seen during natural fertilization in mice. PLCζ is localized in the anterior and posterior somatic membrane regions [93].

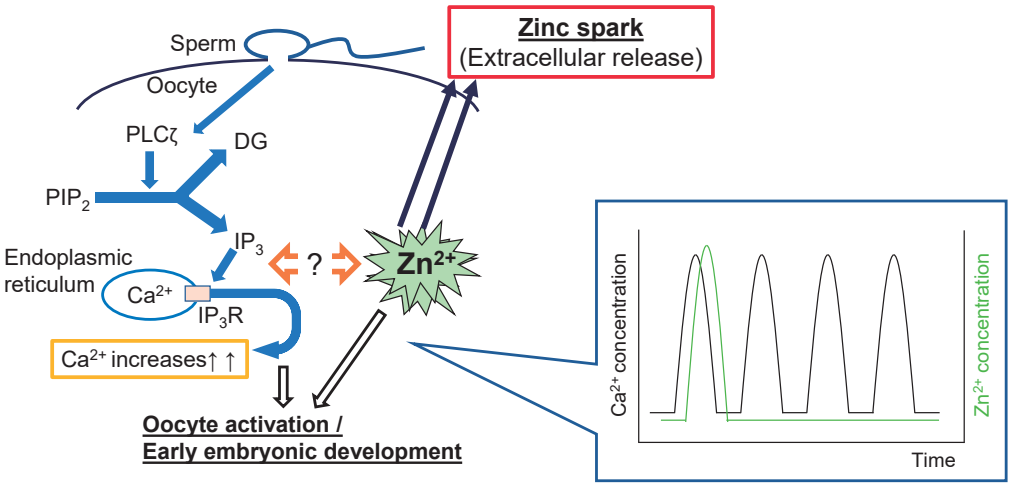

Fig. 3. **Calcium and zinc-dependent mechanism of fertilization in mammals (hypothesis).**
PLCζ is released from the sperm into the ooplasm. This enzyme hydrolyzes PIP_2 to produce IP_3 and DG. IP_3 binds IP_3R on the ER, stimulating Ca^{2+} release. This series of events causes calcium oscillations, with Ca^{2+} repeatedly rising and falling. Zinc into the oocyte is released to the extracellular environment following fertilization and egg activation. This phenomenon is termed a 'zinc spark'. The zinc spark occurs immediately after the first Ca^{2+} rise. Ca^{2+} oscillation is necessary for mammalian fertilization, and the zinc signal is also important for fertilization. DG: diacylglycerol, ER: endoplasmic reticulum, IP3: inositol 1, 4, 5-trisphosphate, PIP2: phosphatidyl inositol 4, 5-biphosphate, PLCζ: phospholipase C zeta.

Nakai *et al*. clarified that in pig sperm, PLCζ was present in the sperm tail in addition to its expected localization in the sperm head [94]. Thus, the localization of PLCζ in sperm may differ among animal species. Taking the above-mentioned observations together, the consensus is that PLCζ is likely to be the trigger of egg activation and embryo development — at least in mammals and probably in many other vertebrate species.

4.2. The discovery of the 'Zinc spark'

The mechanism of fertilization had been discussed mainly in terms of calcium ions, until recently, when it was shown that meiotic resumption can be artificially triggered with TPEN, and the meiotic resumption can be inhibited by overloading the oocyte with zinc ionophores [30, 95]. In mice, TPEN treatment was also shown to be sufficient to activate MII-stopped oocytes injected with "inactivated" sperm heads that did not cause intracellular calcium oscillations, resulting in live births after embryo transfer [95]. Full-term development thus seems not to be dependent on Ca^{2+} release during MII exit.

It remains unclear whether zinc removal is an essential physiological phenomenon in fertilization. The fertilization of a mature, zinc-enriched mouse egg triggers the transient ejection of zinc into the extracellular milieu in a series of coordinated events termed the 'zinc spark' [35, 42]. It was also demonstrated that the zinc spark occurred immediately after the first Ca^{2+} rise (**Fig. 3**). The zinc spark was also observed in human and bovine eggs following fertilization and egg activation [39, 40], indicating that the zinc spark is highly conserved (at least in several mammalian species). These results have established that zinc is an important regulator of meiosis from GV to MII [27, 28]. Bernhardt *et al*. proposed a model in which zinc was responsible for a concentration-dependent regulation of meiosis through the CSF component EMI2, a zinc-binding protein, and they noted that zinc sparks could ensure the rapid and efficient inactivation of EMI2 [30]. However, it is known that EMI2 inactivation results from a Ca^{2+}-dependent mechanism [96], and zinc sparking does not occur when Ca^{2+} is chelated [39].

Although several studies have described a zinc spark, the significance of the zinc spark remains unclear. Zinc ions must accumulate on the oocyte in order to spark. The above-mentioned investigations revealed that immature oocytes (e.g., GV) in mouse ovaries cannot produce a zinc spark, indicating that an acute accumulation of zinc during meiotic maturation is important [26, 39]. It was also reported that zinc was accumulated in MII oocytes [97]. Kong *et al*. reported that *Zip6* and *Zip10* were highly expressed in mouse oocytes [36]. It has thus been proposed that the influx of zinc in oocytes is regulated by ZIPs (**Fig. 4**). Zinc spark profiles revealed that zygotes that developed into blastocysts released more zinc than those that failed to develop, and the rate of embryo development and the total cell number were higher [98]. The amount of zinc ions at the zinc sparks may therefore serve as an early biomarker of zygote quality in mouse models.

4.3. Embryo development and zinc

Although the importance of zinc in fertilization is clear from the above-cited reports, it remains unclear how zinc functions in embryo development. Kong *et al*. observed that zinc was present in early mouse embryos [38]. In contrast to the rise in total zinc levels that occurs during meiotic maturation, this transition metal remained constant throughout early embryonic development,

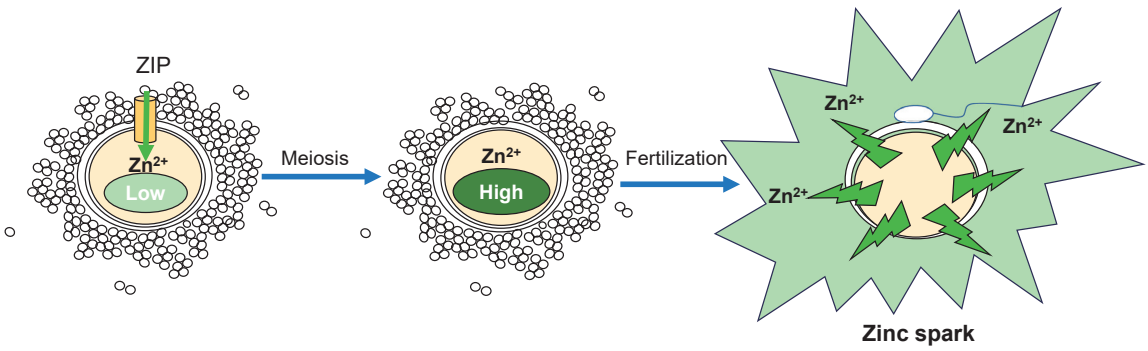

Fig. 4. **Zinc dynamics in oogenesis and fertilization.**
The schematic illustrates the zinc flux regulated by ZIP. Zinc flows into oocytes with low zinc levels, and meiosis is restarted, followed by an increase in the zinc concentration in the oocyte. After fertilization, the zinc-enriched oocyte causes a zinc spark.

and its level was similar to the levels observed in the GV oocyte [38], suggesting the continued functional significance of this metal pool in the preimplantation embryo [26]. When TPEN, a chelator of zinc ions, was added to the early mouse embryo, the embryo development was arrested. In zebrafish embryos, zinc influxes specifically through the ZIP6/ZIP10 heteromer into cells to trigger mitosis [99]. These observations have suggested that the preimplantation embryo requires tight zinc regulation and homeostasis for the initial mitotic divisions of life many species.

5. Conclusions

Zinc is essential for both female and male reproductive tissues to create good-quality embryos. Although further studies are required to understand the relationships between the intracellular/extracellular dynamics of zinc and reproductive events, an inadequate intake of zinc is most likely to be relevant to female and male infertility or subfertility. A better understanding of zinc biology will help improve the reproductive performance of domestic animals and increase the success rate of human assisted reproductive technology.

Funding

This study was supported by Japan's Ministry of Education, Culture, Sports, Science and Technology (MEXT)-Supported Program for the Private University Research Branding Project (2016–2019) and by grants from the Japan Society for the Promotion of Science (JSPS) KAKENHI (nos. 21H02384 and 20H05373 to JI). This study was also supported by the Center for Human and Animal Symbiosis Science, Azabu University.

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Metallomics Research 2022 (1) #MR202203

Review https://metallomicsresearch.brte.org

The usefulness of zinc administration and the importance of measuring serum zinc concentrations

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Abstract

Zinc is an essential trace element that plays various and pivotal roles in the human body. Therefore, zinc homeostasis is tightly regulated by ion channels and zinc transporters, including the Zrt-Irt-like protein (ZIP) and zinc transporter (ZnT) families. Disrupting this zinc homeostasis often causes zinc deficiency in various diseases (e.g., liver cirrhosis, chronic renal diseases, and inflammatory bowel diseases) and contributes to the underlying pathological conditions. Although the amount of zinc in the serum accounts for only approximately 0.1% of the total zinc in the body, this serum concentration is usually measured and analyzed in these diseases. Measuring the serum concentration is the only way to assess pathological conditions and evaluate the effect of zinc treatment when specific symptoms are not observed. For liver diseases, zinc concentrations decrease with the progression of fibrosis in patients with chronic liver diseases (CLDs); thus, many investigators treat these patients with zinc preparations. Supplementation with zinc for not only a short time but also a long time was effective, and the zinc serum concentration is a useful index for achieving good prognosis. Several studies revealed that it is important to maintain a serum zinc concentration of more than70 μg/dl after zinc supplementation, and a quantity of zinc of approximately 90 mg/day is needed in patients with liver cirrhosis.

Key words: Zinc, HCC, liver function, zinc concentrations

Statements about COI: The author declares no conflict of interest associated with this manuscript.

Funding: nothing to report.

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Received: April 10, 2022
Accepted: May 27, 2022
Released online: June 13, 2022

Abbreviations used in this paper:

ZIP; Zrt-Irt-like protein, ZnT; zinc transporter, CLD; chronic liver diseases, ZF; zinc finger, TFs; transcription factors, SOD; superoxide dismutase, OTC; ornithine transcarbamylase, GDH; glutamate dehydrogenase, HCC; hepatocellular carcinoma

Introduction

Zinc is an essential trace element that has been reported to play various and pivotal roles in the human body. It acts not only as a cofactor for the functions of over 300 enzymes but also as an essential component for zinc finger (ZF)-containing transcription factors (TFs), copper/zinc superoxide dismutase (SOD) and various proteins involved in DNA repair [1-3]. Notably, in the

liver, zinc is needed for the activation of many enzymes, such as ornithine transcarbamylase (OTC) and glutamate dehydrogenase (GDH), which are utilized in the urea cycle or glutamine synthetase cycle. To fulfill this crucial role, zinc homeostasis is tightly regulated by ion channels and zinc transporters, including the Zrt-Irt-like protein (ZIP) and zinc transporter (ZnT) families [4, 5]. Put simply, the amount of zinc in the body is adjusted via absorption in the digestive tract and excretion in feces. Disrupting this zinc homeostasis often causes zinc deficiency in various diseases (e.g., liver cirrhosis, chronic renal diseases, and inflammatory bowel diseases, **Figure 1**), which contributes to the underlying pathological conditions [6-9]. Zinc deficiency was observed even in half of healthy volunteers, which might show that intake of zinc is not enough in this modern life.

Although the amount of zinc in the serum accounts for only approximately 0.1% of the total zinc in the body, this serum concentration is usually measured and analyzed in many cases. Another zinc index is needed to accurately evaluate total zinc in the body, and some attempts have been made, such as alkaline phosphatase and OTC. In current clinical settings, however, the serum zinc concentration is the only way to assess total zinc in the body. In an animal study, the serum zinc concentrations correlated with the zinc content in hepatic tissue [10]. The serum zinc concentration correlated with the pathologies of specific diseases in many studies [11]. Therefore, the serum zinc concentration may be a useful index for assessing pathological conditions and evaluating the effect of treatments. Furthermore, determining how to adjust systemic serum zinc concentrations through dietary supplementation may provide useful information for the management of specific diseases. The aim of this review is to summarize the evidence in the most recent literature with a focus on the serum zinc concentration and chronic liver diseases (CLDs). Moreover, this review also discusses the relationships between zinc supplementation and the improvement of pathological conditions.

Mechanisms of zinc deficiency in chronic liver diseases

Vallee RL et al. first reported in 1956 the occurrence of marked hypozincemia in patients with severe cirrhosis [12]. This hypozincemia was confirmed by many investigators, and three reasons have been proposed [13, 14]. First, patients with CLD have an inadequate dietary intake of zinc [15]. Second, absorption of zinc is also impaired in these patients because of portal

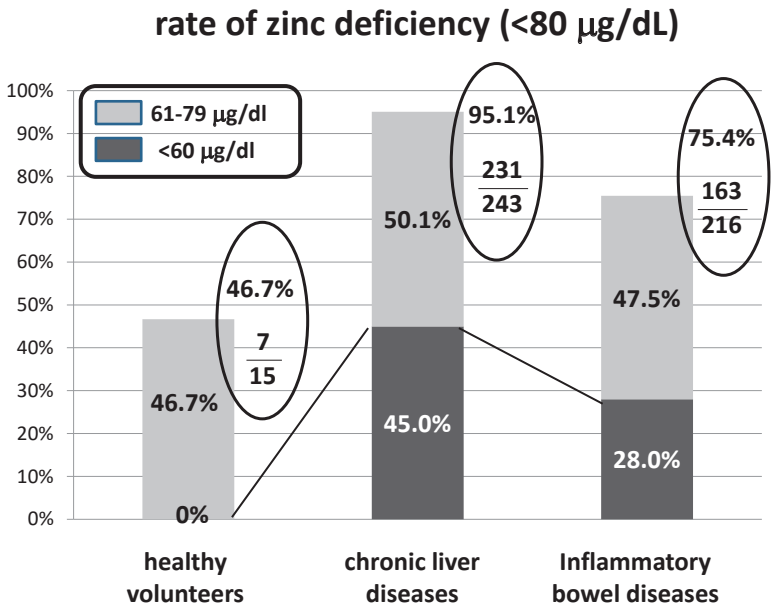

Figure 1. Rate of zinc deficiency (<80 μg/dl) including latent zinc deficiency (60-79μg/dl) in healthy volunteers and patients with various diseases

This data was obtained by single center, retrospective cohort study. Those who had health checkup were recruited in this study as healthy volunteers if they had no diseases. Serum zinc concentrations were examined among patients with chronic liver diseases and inflammatory bowel diseases on their first visit to our hospital or when they were diagnosed on the first time. Gray bar displays the ratio of latent zinc deficiency. Chronic liver diseases; chronic hepatitis (164 cases) and liver cirrhosis (79 cases), Inflammatory bowel diseases; ulcerative colitis (208 cases) and Crohn's diseases (8 cases).

hypertension [16]. Third, urinary excretion of zinc is increased, and greater excretion was observed in patients taking diuretics [14, 17]. The serum zinc concentration gradually decreased with the progression of CLD by these mechanisms and this fact was reported by Iwata K [18]. This is consistent with our results (**Figure 2** and **Table 1**). As described above, zinc homeostasis is tightly regulated by many ion channels and zinc transporters, but it cannot be maintained in patients with CLD. When the zinc concentration decreases, the expression of ZIP4 usually increases. Additionally, its intracellular localization is changed to absorb a large amount of zinc and augment the amount of zinc in the body [19]. It is unknown whether these mechanisms are disrupted or whether zinc absorption is interrupted more than the regulation of zinc transporters. The zinc concentration ultimately decreases with the progression of fibrosis in patients with CLD.

Effect of zinc supplementation

As zinc helps to activate some enzymes in the urea cycle, many investigators have focused on improving hepatic encephalopathy through the administration of supplemental zinc. Reding et al. first reported that oral zinc supplementation improved hepatic encephalopathy in 22 cirrhotic patients in a double-blind randomized trial [20]. The effect of taking 600 mg zinc acetate per day for 7 days was evaluated in that study. Katayama et al. also showed the importance of zinc supplementation; in that study,

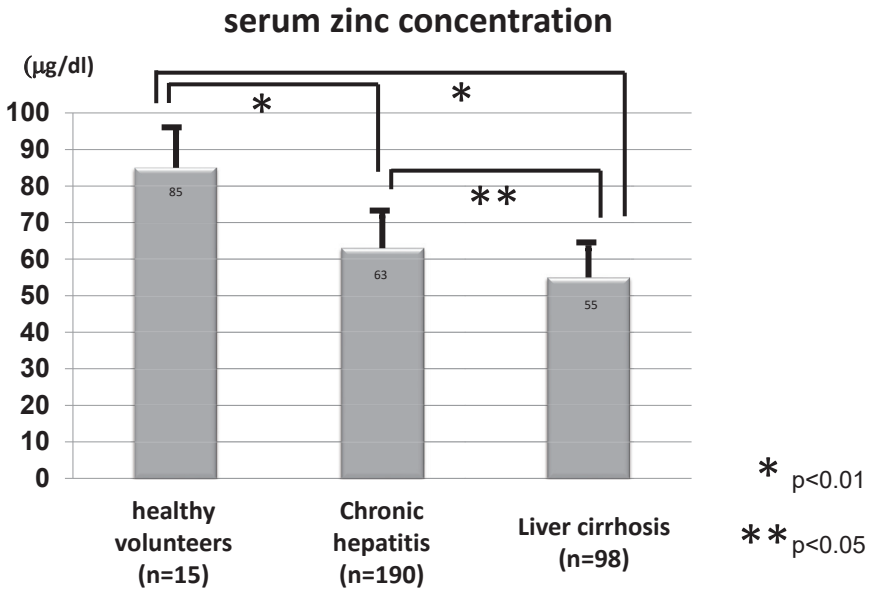

Figure 2. **Serum zinc concentrations in healthy volunteers, and patients with CLD or liver cirrhosis**
This data was obtained by single center, retrospective cohort study. Clinical profiles were shown in the **Table 1**. Some patients were diagnosed by liver biopsy, but others were clinically judged by the criteria proposed by Enomoto H [26]. Serum zinc concentrations decreased with the progression of fibrosis.

Table 1. **Clinical profile of healthy volunteers and patients with chronic liver diseases**

	Healthy volunteers	Chronic hepatitis	Liver cirrhosis
the number of patients	15	190	98
mean age (years)	48.9 ± 10.4	66.4 ± 12.6	73.2 ± 9.3
gender (F/M)	11 / 4	62 / 128	18 / 80
Zn (μg/dl)	84.9 ± 10.8	63.2 ± 8.9	55.3 ± 8.6
T.Bil (mg/dl)	0.7 ± 0.6	1.2 ± 1.8	1.6 ± 1.8
Albumin (g/dl)	4.2 ± 0.4	4.0 ± 0.5	3.3 ± 0.8
PT activity (%)	98 ± 17	89 ± 10	72 ± 11
platlet count (10^4/ul)	23.2 ± 6.5	18.3 ± 8.5	12.8 ± 10.2

3 months of zinc acetate supplementation was shown to be effective and safe for the treatment of hyperammonemia in patients with cirrhosis [21]. A systematic review and meta-analysis of the use of zinc in patients with hepatic encephalopathy showed a significant improvement in psychological tests in those undergoing zinc therapy [22]. However, many of these studies have focused on short-term zinc administration, and few studies have explored the long-term effects of zinc administration in patients with CLD. We found that zinc administration improves liver function and decreases the cumulative incidence of HCC in patients with CLD over the course of long-term follow-up [6]. Taken together, supplementation with zinc for not only a short time but also a long time was effective for patients with CLD. The longer patients are treated with zinc, the more adverse events occur. The adverse drug reactions were reported to be digestive symptoms, such as abdominal discomfort, pain, and vomiting, and elevation of pancreatic enzymes (amylase, lipase), deficiency of cupper, pancytopenia, anemia, and neurological disorders [23]. The next question is how long zinc preparations need to be treated and whether it is possible to discontinue this treatment.

Serum zinc concentration as an evaluation after zinc supplementation

A zinc index is needed to accurately evaluate total zinc in the body after zinc supplementation, but the serum zinc concentration is currently the only way to assess total zinc in the body as described above. Subjects administered zinc demonstrated higher serum zinc concentrations at week 8 than subjects administered a placebo [23], and these levels decreased to the basal level after discontinuation of treatment (unpublished data). Continuous administration of zinc is needed to maintain higher serum zinc concentrations. However, is it necessary to do to achieve good clinical outcomes? There are almost no papers exploring the appropriate concentration of zinc, but Takamatsu reported that a serum zinc concentration of more than 72 μg/dl after zinc supplementation was needed for the suppression of fibrosis in 57 patients with CLD [24]. In our study, patients in the zinc-treated group were divided into 4 groups according to their zinc concentration at 6 months after the start of the zinc treatment, and we investigated the liver function and the cumulative incidence of hepatocellular carcinoma (HCC) in these groups. Finally, to obtain a better prognosis, patients with CLD must maintain a serum zinc concentration of more than 70 μg/dl after zinc supplementation [6]. Our results are almost consistent with Takamatsu's report, and both of these studies showed that it is important to increase and maintain a certain level of zinc for better prognosis.

The relationship between the dose of zinc administered and the serum zinc concentration

The final question was how much zinc is needed to reach the appropriate zinc concentration. Almost all investigators who have performed zinc analysis confirmed the importance of zinc administration in clinical settings, but the dose of zinc was quite different in these studies. Katayama used 150 mg/day of zinc to treat patients with liver cirrhosis and hyperammonemia. Nobelzin® capsules (50 mg) were administered after each meal three times a day, and each capsule contains 167.84 mg zinc acetate dehydrate including 50 mg of zinc (Nobelpharma Co., Ltd., Tokyo, Japan) [21]. In Himoto's study, they treated patients with CLD with polaprezinc (150-225 mg/day including 34-51 mg/day of zinc), and Takamatsu also used 150 mg/day of polaprezinc (34mg of zinc) [24, 25]. The minimum required quantities of zinc may depend on individual differences; thus, we focused on patients whose zinc administration doses were changed. Zinc sulfate contains an equivalent of 60-120 mg of zinc, while polaprezinc contains 17-51 mg of zinc. Some physicians began to treat patients with zinc sulfate, but this treatment was discontinued or the initial dose was reduced because of adverse events. Other physicians started polaprezinc but changed to zinc sulfate to increase the zinc concentration. In fifteen patients, zinc sulfate was changed to polaprezinc, and in nine patients, polaprezinc was changed to zinc sulfate. The clinical backgrounds of these patients are shown in **Table 2**. The serum zinc concentration increased to 62.0 μg/dl from 51.3 μg/dl after the polaprezinc therapy, and it remained low during the administration of polaprezinc (**Figure 3a**, left panel, mean period of the administration of polaprezinc was 9 months). It increased to more than 90 μg/dl after the change to zinc sulfate in these patients. The administration quantity of zinc increased from 37.6 mg to 98.6 mg (**Figure 3a**, right panel). This tendency was also observed in another 9 patients. The serum zinc concentration increased to more than 90 μg/dl after zinc sulfate therapy, and it was maintained during the administration of zinc sulfate (**Figure 3b**, left panel, mean period of administration was 26 months). The concentration decreased to less than 70 μg/dl after the change to polaprezinc in these patients. The administration quantity of zinc also decreased from 97.7 mg to 41.6 mg (**Figure 3b**, right panel). This finding shows that the zinc concentration decreased according to the quantity of zinc administration. This study revealed that most patients with liver cirrhosis needed to take more than 90 mg/day of zinc to reach a zinc concentration over 70 μg/dl. These findings suggest that 30-40 mg/day of zinc

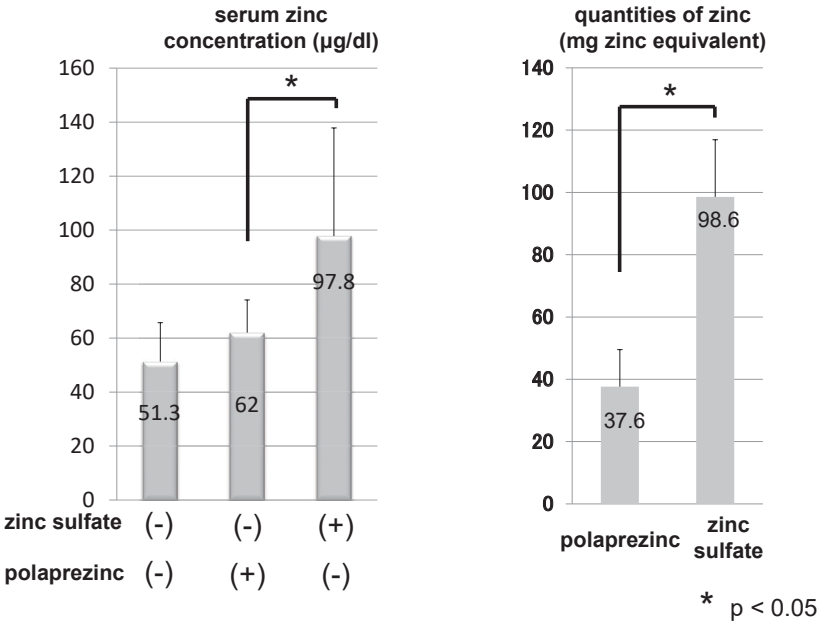

Figure 3a. **(Left panel) Serial changes in serum zinc concentrations before and after the administration of polaprezinc and after the exchange of polaprezinc to zinc sulfate (Right panel) Change in the administration quantity of zinc during the administration of polaprezinc or zinc sulfate**

Fifteen patients were administered polaprezinc. Then, it was discontinued, and they were treated with zinc sulfate.

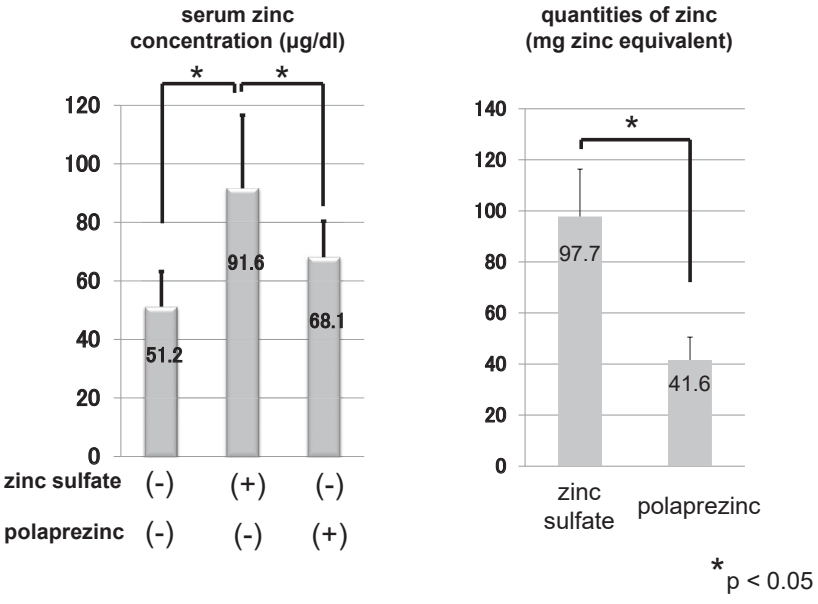

Figure 3b. **(Left panel) Serial changes in serum zinc concentrations before and after the administration of zinc sulfate and after the exchange of zinc sulfate to polaprezinc (Right panel) Change in the administration quantity of zinc during the administration of zinc sulfate or polaprezinc**

Nine patients were administered zinc sulfate. Then, it was discontinued and they were treated with polaprezinc.

Table 2. Clinical profiles of 24 patients with chronic liver diseases whose administration doses of zinc were changed

Fifteen patients were administered polaprezinc, and then, it was changed to zinc sulfate. Nine patients were administered zinc sulfate, and then, it was changed to polaprezinc.

	PZ→zinc sulfate	zinc sulfate→PZ
	Figure 3a	Figure 3b
the number of patients	15	9
mean age (years)	74.9 ± 5.4	75.6 ± 7.3
gender (F/M)	11 / 4	6 / 3
Zn (μg/dl)	51.3 ± 14.4	51.2 ± 12.0
T.Bil (mg/dl)	1.3 ± 0.7	1.2 ± 1.8
Albumin (g/dl)	3.1 ± 0.8	3.3 ± 0.8
PT activity (%)	68.4 ± 17.1	68.8 ± 10.2
platlet count (10^4/ul)	10.5 ± 4.5	11.8 ± 10.2
BTR (BCAA/tyrosine ratio)	3.49 ± 1.91	2.26 ± 0.61
Chronic hepatitis/liver cirrhosis	8 / 7	4 / 5

PZ: polaprezinc

in polaprezinc is not enough to attain optimal zinc levels in patients with CLD, especially in those with advanced fibrosis. Taken together, sufficient amount of zinc is needed to maintain a serum zinc concentration of more than 70 μg/dl.

Conclusion

This review article highlights the effect of zinc supplementation and the importance of the serum zinc concentration. Six years have passed since zinc preparation was approved for patients with zinc deficiency. The target recipients have recently been patients with certain diseases. In particular, patients with CLD can be treated with zinc; this treatment results in the improvement of pathological conditions, including hepatic encephalopathy and cancer development. We next focused on how to treat these conditions and who should be treated. The serum zinc concentration is one of the most useful and accurate indices to evaluate total zinc in the body and to assess the effects of zinc treatment. Many investigators have already confirmed that the administration of zinc is effective in improving pathological conditions in many diseases, but the ideal zinc concentration or administration dose is unclear. For the treatment of CLD, the zinc concentration should be more than 70 μg/dl to obtain good prognosis, and more than 90 mg/day of zinc should be administered to reach that concentration. In each disease, such as inflammatory bowel disease or chronic renal disease, the ideal concentration of zinc might be different and should be clarified in the future. In any case, we can treat zinc preparations in patients with zinc deficiency, which can improve pathological conditions. Zinc should be used appropriately based on numerous studies and firm evidences.

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A study on SARS-CoV-2 infected patients with measured serum zinc levels during home care

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Abstract

Background: COVID-19 is an infectious disease caused by the SARS-CoV-2 virus and causing pandemics around the world. Zinc is an essential trace element and important for maintaining immune function. Serum zinc level has been reported to be low in severe cases of COVID-19.

Patients and methods: Patients who were diagnosed with SARS-CoV-2 infection were requested to be examined by our hospital while waiting at home were included. Medical history was heard, body temperature and blood oxygen saturation was measured, blood was collected, and lung CT examination was performed.

Results: The mean age of 102 patients was 39.7 y/o. There were no cases of fever with a body temperature of 37.5 ℃ or higher. Mean serum zinc level was 79.1μg/dL. Comparing serum zinc levels with healthy individuals by age, the serum zinc levels were significantly lower in COVID-19 cases over 50 y/o. Pneumonia findings was found in 54 cases (52.9%). Patients with pneumonia were significantly older than those without pneumonia (48.3 vs 30.1 y/o). Serum zinc levels were significantly lower in patients with pneumonia than in patients without pneumonia (75.5 vs 83.2 μg/dL). Dysosmia and dysgeusia were seen in 36 cases (35.3%). There were significantly younger ages compared to those without dysosmia and dysgeusia (34.4 vs 42.6 y/o). There was no difference in serum zinc levels depending on the presence of dysosmia or dysgeusia.

Conclusion: Serum zinc levels were involved in the development of SARS-CoV-2 infection and pneumonia. The onset of dysosmia or dysgeusia was not associated with serum zinc levels.

Key words: SARS-CoV-2, COVID-19, Zinc, Pneumonia, Dysosmia, Dysgeusia

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

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Received: December 24, 2021
Accepted: February 17, 2022
Released online: April 1, 2022

Introduction

COVID-19 is a viral infection caused by SARS-CoV-2 virus that occurs in Wuhan, China and causes pandemics all over the world [1]. In Japan, as of November 2021, 1.7 million people were infected and 18000 people died. Vaccination is progressing and therapeutic drugs are being developed, but the current situation is that they have not yet converged.

Zinc is one of the essential trace elements and plays various important roles in the living body, especially for maintaining immune function and reported to have effect of blocking the

invasion and proliferation of pathogenic viruses [2,3]. It has been also pointed out that it may be effective in SARS-CoV-2 infection [4]. There are some reports investigating COVID-19 cases and serum zinc levels, in which zinc levels were low in severe cases and fatal cases, suggesting a link between COVID-19 and zinc [5,6,7,8,9,10].

One of the characteristics of COVID-19 is that dysosmia and dysgeunia often occur. However, there is only one report on zinc levels and dysosmia, and it is reported that it is not related to zinc concentration [11].

We had cases of SARS-CoV-2 infection who were on stay at home and were asked to undergo a test to determine the need for hospitalization. We have investigated the relationship between these patients and serum zinc levels.

Patients and methods

SARS-CoV-2 infectious disease is classified as type 2 infectious diseases in Japan, and infectious diseases in those categories require isolation. However, due to the increase in the number of infected cases, there was a situation where we had to wait at the hotel due to the shortage of hospital beds, and the number of cases waiting at home also increased. The Medical Coordination Headquarters, which coordinates the hospitalization of COVID-19 patients in Miyagi Prefecture, has asked medical institutions to inspect the cases that were judged to require severity determination among the cases waiting at home. The subjects of this study were cases diagnosed with SARS-CoV-2 infection between October 2020 and February 2021, and requested to assess the severity while waiting at home. Patients were planned to visit to our hospital from 10 am to 12 am. After arrival we asked the medical history, measured oxygen saturation and body temperature, collected blood, and performed lung CT examination. CT was read by a radiologist, and the case with the shadow characteristic of corona pneumonia was judged to have pneumonia. At the time of hearing the medical history, we also asked about the presence or absence of taste and smell abnormalities. Data from our staff (385 cases) were used to compare serum zinc levels as healthy individuals.

For statistical processing, Student's t-test and χ-square test were performed, and $p < 0.05$ was considered to be significantly different.

This paper has been approved by the Ethics Committee of the Sendai City Medical Center (2021-0052).

Results

A total of 102 patients were included in the study with a mean age of 39.7 ± 16.2 y/o (**Table 1**). The gender was 55 males and 47 females. Of these, 92 were symptomatic and 10 were asymptomatic. There were no cases in which the body temperature measured on the day of the examination was 37.5℃ or higher. Oxygen saturation was 96% or higher (mild) in 95 cases (93.1%), 94-95% (moderate I) in 1 case (1.0%), and 93% (moderate II) in 1 case (1.0%) and 5 unknown cases (4.9%). In symptomatic cases, the average time from onset to examination was 9.9 ± 4.7 days. At blood sampling, CRP, which reflects the degree of inflammation, was 0.68 ± 1.68 mg/dL, with a median of 0.15 mg/dL. The serum zinc level was 79.1 ± 12.7 μg/dL (reference value 80-130 μg/dL), which was the lower limit of the reference value. The relationship between serum zinc levels and age showed

Table 1. | Characteristics of SARS-CoV-2 infected patients

No of cases	102 cases
Age	39.7±16.2（34.5） y/o
M/F	M：55　F：47 cases
Symptom	yes：92　no：10 cases
Fever（＞37.5℃）	0 case
O_2 saturation ≥96%	95 cases（93.1%）
94-95%	1 case（1.0%）
93%	1 case（1.0%）
unclear	5 cases（4.9%）
Days from onset	9.9±4.7（10） days
CRP	0.68±1.68（0.15） mg/dL
Zn	79.1±12.7（80） μg/dL

mean±SD（median）

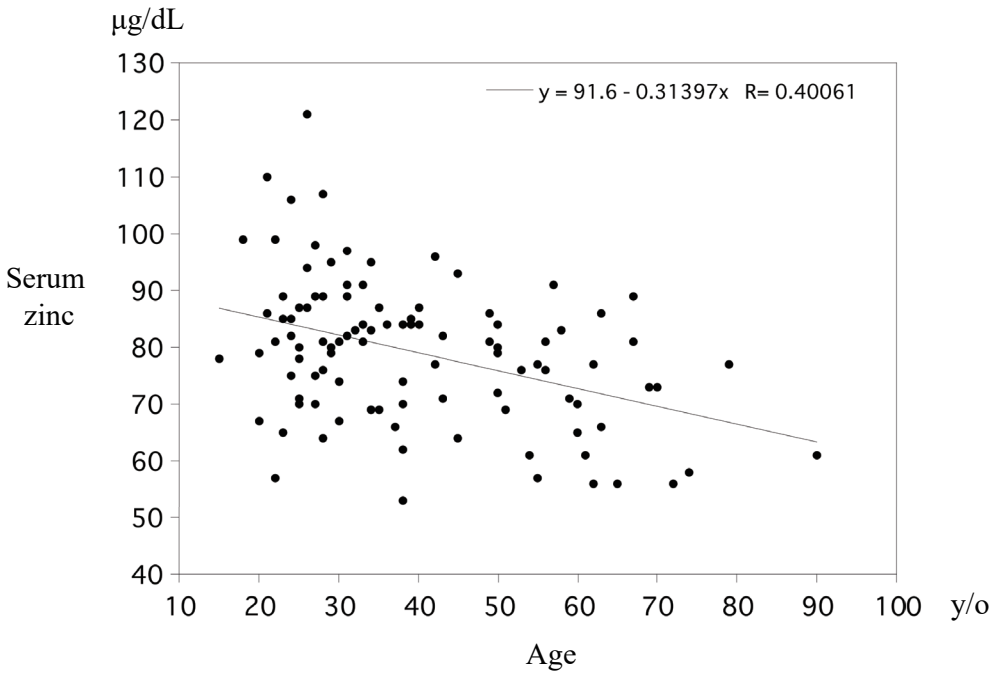

Fig. 1. | Relationship between serum zinc level and age in SARS-CoV-2 infected patients

Table 2. | Comparison of serum zinc levels between healthy control and SARS-CoV-2 infected patients by age

	Age	20s	30s	40s	50s	≥60	Total
Control	Serum zinc (μg/dL)	82.6 ± 11.2	82.5 ± 11.8	81.9 ± 11.7	86.4 ± 12.4	81.6 ± 13.0	82.9 ± 11.7
	n	147	114	58	53	13	385
SARS-CoV-2	Serum zinc (μg/dL)	84.3 ± 13.9	79.0 ± 10.8	82.1 ± 9.6	75.5 ± 19.0	69.1 ± 10.9	79.1 ± 12.7
	n	36	26	10	14	16	102
	p value	0.50	0.16	0.94	<0.001	0.01	0.007

mean ± SD

negative correlation (**Fig.1**). Comparing the serum zinc levels investigated by our hospital by age, there was no difference from the staff (healthy subjects) up to the age of 40s, but the serum zinc levels were significantly lower in the positive cases in the age group of 50s and over 60 y/o (**Table 2**). Regarding the relationship between serum zinc levels and CRP, zinc levels tended to be low in cases with high CRP, and all three cases with 4.0 mg /dL or higher were below the reference value (**Fig.2**).

In these patients, the number of cases showing pneumonia on CT was 54 (52.9%), which was more than half (**Table 3**). The relationship between pneumonia findings and age was 48.3 ± 16.0 y/o for those with pneumonia findings and 30.1 ± 9.7 y/o for those without pneumonia findings, which were significantly more common in the elderly (**Fig.3**). The frequency of pneumonia by age group showed a large difference: 7/36 (19.4%) in the 10-20 age group, 13/26 (50.0%) in the 30s group, 7/10 (70.0%) in the 40s group, 11/14 (78.6%) in the 50s group, and 16/16 (100.0%) in the over 60 y/o group. In terms of gender, 33 males (60.0%) and 21 females (44.7%) presented with pneumonia. Of the 10 asymptomatic cases, 8 had pneumonia findings. CT findings of a typical case without symptom was shown in **Fig.4**. The oxygen saturation was slightly lower in patients with pneumonia: 96% or more (mild disease) in 50 patients, 94-95% (moderate I) in 1 patient, and 93% (moderate II) in 1 patient, and 96% or more in all patients without pneumonia. There was no difference in the number of days elapsed from the onset depending on the presence or absence of pneumonia findings. CRP was 1.15 ± 2.18 mg/dL with pneumonia and 0.14 ± 0.44 mg/dL without pneumonia, showing a significant difference (p =0.002). Serum zinc levels were 75.5 ± 11.0 μg/dL with pneumonia findings and 83.2 ± 13.3 μg/dL without pneumonia findings, which were significantly lower in patients with pneumonia (p=0.002) (**Fig.5**). Even in cases

of younger than 40 y/o with low frequency of pneumonia findings, serum zinc levels were 77.4 ± 10.5 μg/dL with pneumonia (n = 20) and 84.3 ± 13.4 μg/dL without pneumonia (n = 42), which were significantly lower in patients with pneumonia (p = 0.03). In cases with a serum zinc level of 80 μg / dL or higher, 22 of 53 cases (41.5%) had pneumonia findings, but in cases of less than 80 μg / dL, 32 of 49 cases (65.3%) had pneumonia findings, which was significantly higher (p = 0.016).

There were 36 cases with dysosmia or dysgeusia in 35.3% of cases (**Table 4**). It was found in 18 of 55 males in 32.7% and in 18 of 47 females in 38.3%. The age was 34.4 ± 13.2 y/o with dysosmia or dysgeusia and 42.6 ± 17.0 y/o without dysosmia nor dysgeusia, which was significantly more common among young people (p = 0.008) (**Fig.6**). There was no difference in time from onset. CRP was 0.32 ± 0.96 mg/dL with dysosmia or dysgeusia and 0.87 ±1.95 mg/dL without dysosmia nor dysgeusia, which tended to be higher in cases without dysosmia nor dysgeusia (p = 0.06). The serum zinc level was 80.2 ± 14.8 μg/dL with dysosmia or dysgeusia, and 78.6 ± 11.4 μg/dL without dysosmia nor dysgeusia (**Fig.7**). The findings of pneumonia were 10/36 (27.8%) with dysosmia or dysgeusia and 44/66 (66.7%) without dysosmia nor dysgeusia, which was less common with dysosmia nor dysgeusia (p <0.001).

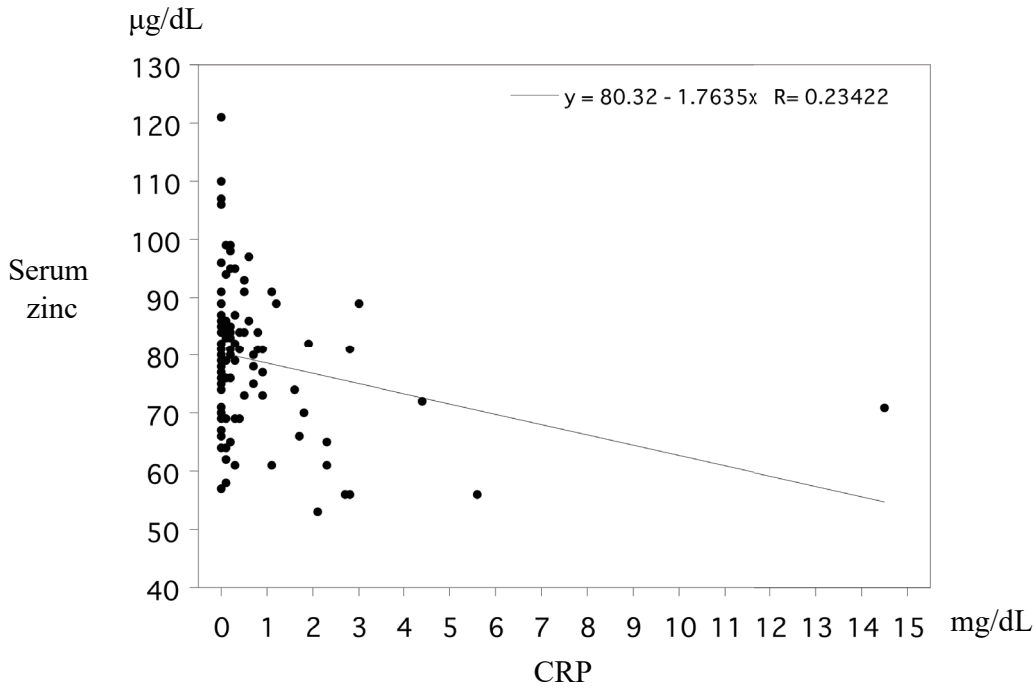

Fig. 2. | Relationship between serum zinc level and CRP in SARS-CoV-2 infected patients

Table 3. | Comparison between with and without pneumonia on CT examination

	Pneumonia (+)	Pneumonia (-)	p value
No of cases(%)	54 cases (52.9%)	48 cases (47.1%)	
Age	48.3 ± 16.0 (49.5) y/o	30.1 ± 9.7 (27.5) y/o	p<0.001
M/F	M:33 F:21 cases	M:22 F:26 cases	p=0.07
Symptom	yes：46 no：8 cases	yes：46 no：2 cases	p=0.12
O_2 saturation ≥96%	50 cases	45 cases	p=0.41
94-95%	1 case	0 case	
93%	1 case	0 case	
unclear	2 cases	3 cases	
Days from onset	9.2 ± 4.7 (9) days	10.5 ± 4.6 (10) days	p=0.21
CRP	1.15 ± 2.18 (0.5) mg/dL	0.14 ± 0.44 (0) mg/dL	p=0.002
Zn	75.5 ± 11.0 (76.5) μg/dL	83.2 ± 13.3 (83.5) μg/dL	p=0.002

mean ± SD（median）

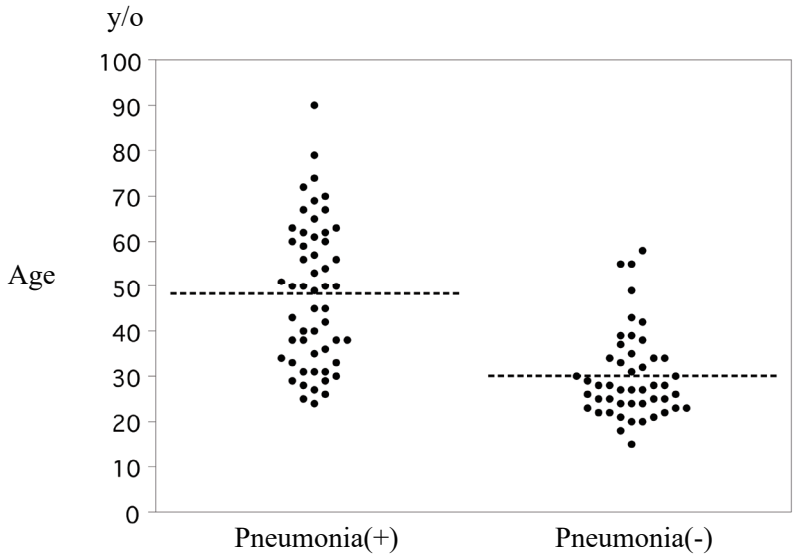

Fig. 3. Age of patients with or without pneumonia findings on CT examination

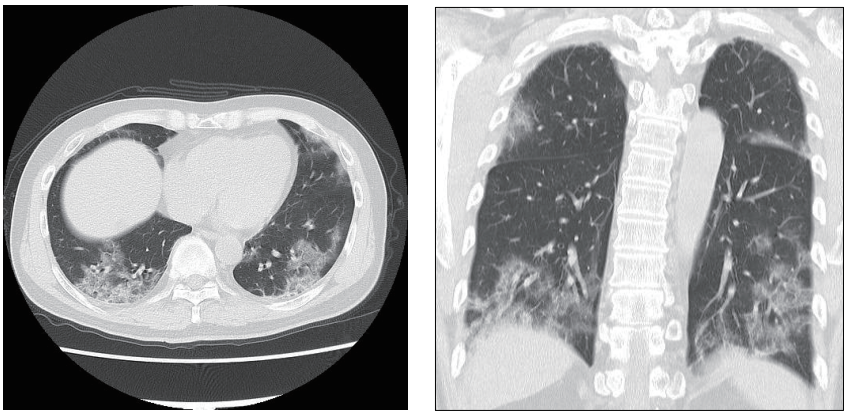

Fig. 4. CT findings of a COVID-19 patient without symptom.
60 y/o. Male. A close contact with COVID-19 patient and diagnosed as SARS-CoV-2 infection by PCR examination 6 days before a visit to our hospital. He is a smoker and under treatment for high blood pressure and diabetes mellitus. Body temperature 36.2°C, oxygen saturation 97%. He had no symptom of COVID-19. Serum Zinc 65μg/dL, CRP 2.3 mg /dL, D-dimer 1.43 μg/mL, white blood cell (WBC) count 5580/mm^3, lymphocyte count 1230 /mm^3. CT showed ground-glass opacity (GGO) in bilateral lungs.

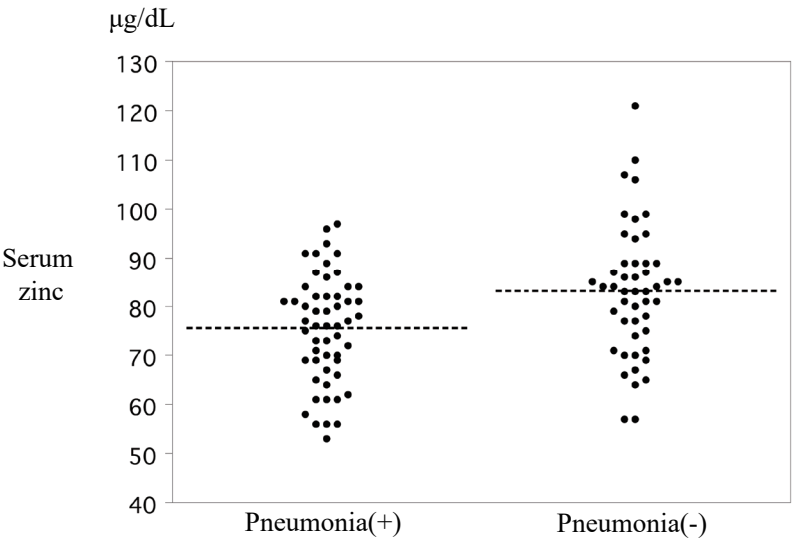

Fig. 5. Serum zinc levels of patients with or without pneumonia findings on CT examination

Table 4. | Comparison between with and without dysosmia/dysgeusia

	Dysosmia/Dysgeusia (+)	Dysosmia/Dysgeusia (-)	p value
No of cases (%)	36 cases (35.3%)	66 cases (64.7%)	
Age	34.4 ± 13.2 (28.5) y/o	42.6 ± 17.0 (39) y/o	p=0.008
M/F	M:18 F:18 cases	M:37 F:29 cases	p=0.56
Days from onset	10.1 ± 5.0 (10) days	9.7 ± 4.5 (10) days	p=0.66
CRP	0.32 ± 0.96 (0) mg/dL	0.87 ± 1.95 (0.25) mg/dL	p=0.06
Zn	80.2 ± 14.8 (80) μg/dL	78.6 ± 11.4 (80.5) μg/dL	p=0.56
Pneumonia (+)	10 cases (27.8%)	44 cases (66.7%)	p<0.001

mean ± SD (median)

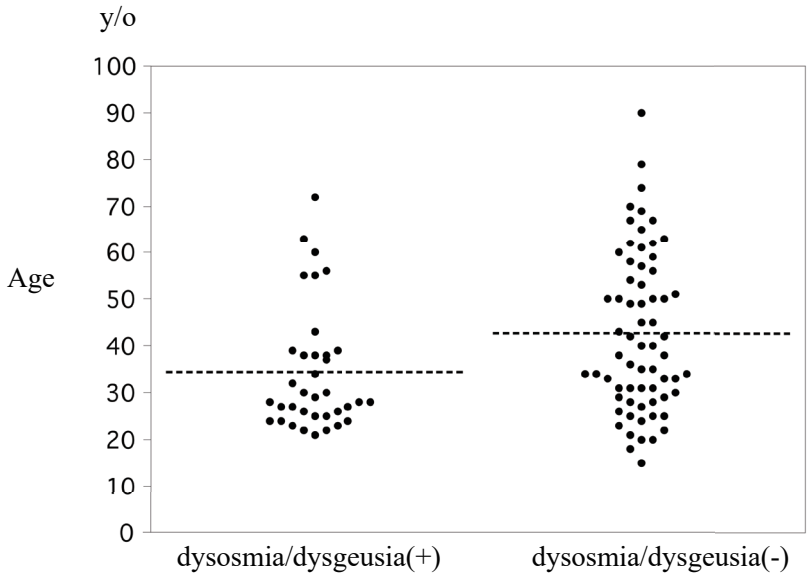

Fig. 6. | Age of patients with or without dysosmia/dysgeusia

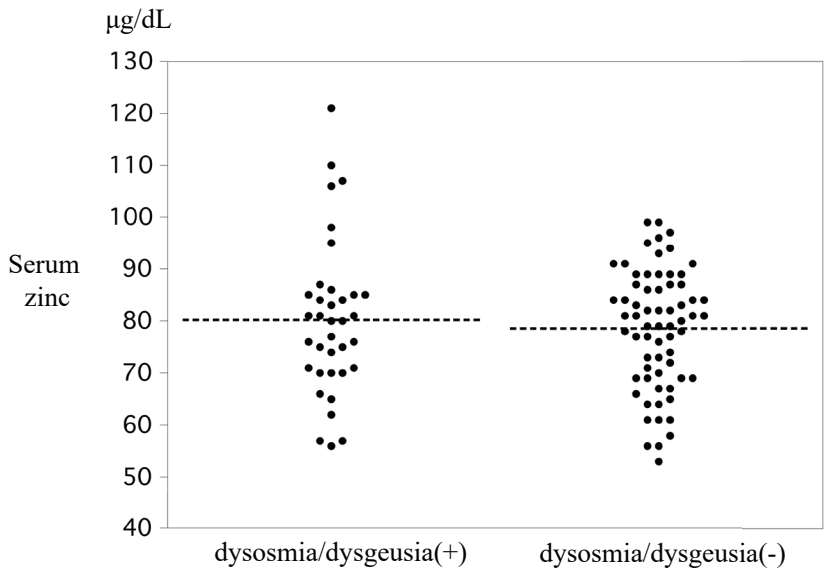

Fig. 7. | Serum zinc levels of patients with or without pneumonia findings on CT examination

Discussion

The 102 cases examined this time are cases in which SARS-CoV-2 infection was confirmed by PCR and were not treated such as oxygen inhalation during home medical treatment due to lack of hospital beds. There were no cases of fever with a body temperature of 37.5℃ or higher at the time of consultation. Oxygen saturation was also normal in 95 of the 97 measured cases (mild), not including severe cases. The average CRP, which indicates the degree of inflammation, was 0.68 ± 1.68 mg / dL, and the median was 0.15 mg / dL, and most of them were mildly ill patients. Lung CT examinations were also performed on these cases, and 52.9%, more than half, pointed out the findings of ground-glass opacity characteristic of COVID-19. Pneumonia was seen in 8 of the 10 asymptomatic patients. In a study of 104 passengers on the cruise ship Diamond Princess, where SARS-CoV-2 cluster infection occurred, 22 of 28 (79%) symptomatic cases had pneumonia, and 41 of 76 (54%) asymptomatic cases also had pneumonia on CT, and there was a discrepancy with clinical symptoms [12]. Therefore, it is considered that searching for the presence or absence of pneumonia by CT examination is useful for preventing respiratory failure and not missing the timing of starting treatment. When the cases with pneumonia images were examined by age in our case, pneumonia was observed in 19.4% of the cases under 30 y/o, but pneumonia findings were observed in all cases over 60 y/o. Careful follow-up is required because it has been reported that elderly people are more likely to become ill.

Zinc is one of the essential trace elements contained in the body in about 2 g, and plays an important role in maintaining the intracellular function [13]. At the time of deficiency, various deficiency symptoms such as decreased immunity, susceptibility to infection, prolonged wound healing, and dysgeusia appear [14,15]. A survey of serum zinc levels of residents conducted in Japan has pointed out a decrease in serum zinc levels, especially in the elderly [16]. Recently, in viral respiratory infections, a study of 25 RCTs reported that zinc administration was effective in reducing severity and shortening the duration [17]. Razzaque et al. reported zinc has the effect of suppressing intracellular invasion and intracellular proliferation in SARS-CoV-2 infection, so zinc deficiency may increase the risk of aggravation in SARS-CoV-2 infection and administration of zinc may have a therapeutic effect [3]. However, there are few reports on the association between COVID-19 and zinc in clinical cases, and the association with dysgeusia is unclear.

The serum zinc level of the COVID-19 patients examined this time was 79.1 ± 12.7 μg/dL, which was about the same as the lower limit of the reference value (80-130 μg/dL). We have already reported zinc levels in 52 outpatients or inpatients, most of whom were mildly ill as in these cases, but the values were as low as 66.8 ± 12.1 μg/dL [10]. This may be due to the difference in the time of blood collection: in the previous report, blood was collected between 15:00 and 16:00, but in this study, blood was collected between 10:00 and 12:00, and the value was lower in the afternoon than in the morning due to diurnal variation [18]. An association was found between lower serum zinc and SARS-CoV-2 infection with increasing age. Comparing the blood sampling data of our hospital staff collected in the morning as the data of healthy subjects (n = 385, 82.9 ± 11.7 μg/dL), the value was significantly lower in the COVID-19 patients (79.1 ± 12.7 μg/dL). When compared by age group, there was no difference in serum zinc level between the COVID-19 patients and healthy subjects under 50 y/o, but serum zinc level of the COVID-19 patients was statistically lower compared to healthy subjects at 50 y/o or over. A comparison of zinc levels with and without pneumonia was significantly lower in patients with pneumonia (75.5 ± 11.0 vs. 83.2 ± 13.3 μg/dL). These results suggest a relationship between SARS-CoV2 infection, the presence or absence of pneumonia, and serum zinc levels.

Several reports of serum zinc levels in COVID-19 patients have been made in relation to aggravation and mortality. Yasui et al. measured serum zinc levels in 29 hospitalized patients, which were significantly lower in severe cases, 87.7 μg/dL in mild and moderately ill patients (n = 22) and 62.4 μg/dL in severe cases (n = 7) [5]. If the cut off value is 70 μg/dL, the proportion of cases below the cut off value is 14% for mild and moderate cases and 86% for severe cases, and it is possible to predict the severity of the disease. Gonzalez et al. reported in a review of 249 hospitalized patients that the serum zinc level in 21 patients who died was 49 μg/dL, significantly lower than the 62 μg/dL level in surviving patients, and that the mortality rate in patients with serum zinc levels of less than 50 μg/dL on admission was 21%, higher than the 5% mortality rate in patients with serum levels of 50 μg/dL or higher [6]. Jothimani et al. reported that the median serum zinc level in 47 hospitalized patients was 74.5 μg/dL, which was lower than that in healthy subjects (105.8 μg/dL), that the level of less than 80 μg/dL was found in 57.4% of patients with a mortality rate of 18.5%, and that there were no deaths in patients with a level of 80 μg/dL or higher [7]. Dubourg et al. measured the serum zinc levels of 275 inpatients, and divided the cases of death, ICU admission, and hospitalization for 10 days or more into poor outcome cases (n=75), and the others into good outcome cases (n=200). The serum zinc level of poor outcome cases

was 84.1 μg/dL, which was significantly lower than the good outcome cases of 100.7 μg/dL [8]. Skalny et al. treated inpatients (n = 150) as mild (mean SpO_2: 95.4%, body temperature 38.2℃, CRP: 4.5mg/dL), moderate (mean SpO_2: 94.8%, body temperature 38.1℃, CRP: 6.7mg/dL) and severe (mean SpO_2: 87.0%, body temperature 38.3℃, CRP: 16.1 mg/dL), 50 cases each and 44 healthy subjects were examined, and the serum zinc level was 96 μg/dL in normal subjects, 92 μg/dL in mild subjects, 90 μg/dL in moderate subjects, and 87 μg/dL in severe subjects, and the Cu/Zn ratio increased with increasing severity [9]. From the above reports, it is considered that there is a correlation between the severity and the serum zinc level, but it is unclear whether zinc deficiency aggravates the disease or zinc decreases due to the aggravation, and further investigation is required.

From the relationship between COVID-19 and serum zinc level, zinc administration is clinically used for treatment, and its effect is expected. Finzi et al. reported that high doses of zinc, 138-184 mg/d, were administered orally to 4 COVID-19 cases, and clinical symptoms such as oxygen saturation and fever resolution improved, and the following year, they reported improvement in 28 clinical cases [19,20]. Derwand et al. also reported that 141 outpatients with COVID-19 treated with 50 mg/d of zinc, 400 mg/d of hydoroxychloroquine, and 500 mg/d of azithromycine for 5 days had a low hospitalization rate of 2.8% compared with 15.4% from general data, and a mortality rate was 0.7% compared with the general data of 3.4%, indicating the effectiveness of early treatment of infection [21]. However, Thomas et al. conducted a randomized control trial of 214 outpatients infected with SARS-CoV-2 in four groups: a control group, a zinc 50 mg group, a vitamin C 8000 mg group, and a zinc and vitamin C group [22]. The efficacy of zinc administration was not confirmed, as no relief was obtained between the 4 groups. Furthermore, in an RCT of hydroroxychloroquine with or without zinc, no add-on effect of zinc was confirmed [23]. Based on the results of these RCTs, the NIH does not recommend the administration of zinc in its treatment guidelines for COVID-19 [24]. Thus, the efficacy of zinc administration as adjuvant therapy in COVID-19 patients is controversial, and the results of future RCTs will be expected [25,26,27,28,29].

SARS-CoV-2 is known to be transmitted by the binding of spike protein to ACE 2 on the cell membrane in COVID-19 patients [30,31]. It has been reported that its expression is abundant in the epithelial cells of the nasal mucosa in addition to the epithelium of the airways, lungs, and intestines, suggesting an association with olfaction disorders [32]. Yan et al. performed PCR on 1480 patients with influenza-like symptoms and found that among 102 COVID-19 positive patients, 68% had olfactory disturbance and 71% had gustatory disturbance. In contrast, 16% of COVID-19-negative patients had olfactory disturbances and 17% had gustatory disturbances, indicating a strong association between COVID-19 and olfactory and gustatory disturbances, making it useful for screening [33]. Regarding the frequency of olfactory dysfunction, meta-analysis of 10 papers reported that dysosmia was found in 52.7% of 1627 cases, but there was a difference in the diagnosis depending on whether a validated instrument was used or not, 36.6% in nonvalidated cases and 86.6% in validated cases [34]. In our hospital, 35.3% of the patients were diagnosed by a simple interview, which was about the same frequency as that diagnosed by nonvalidated instrument. It has been reported that there are many females by gender, but no difference was found in our cases [35]. In terms of age, Alsheri reported a higher frequency in younger patients: 25/44 (56.8%) under 20 years of age, 14/28 (50.0%) in the 20-35 age group, 18/53 (34.0%) in the 35-60 age group, and 19/73 (26.0%) in the 60+ age group [36]. In our case, the average age of cases with dysosmia or dysgeusia was significantly younger (34.4 vs. 42.6 y/o), and the frequency of occurrence by age was 46.8% under 40 y/o and 17.5% over 40 y/o. In the relationship between dysosmia/dysgeusia and the severity of the disease, fewer patients with dysosmia or dysgeusia had pneumonia on CT (27.8 vs. 66.7%) and lower CRP than those without (0.32 vs. 0.87 mg/dL, p=0.06). Talavera et al. reported that 146 of 576 cases (25.3%) had anosmia, but those with anosmia were less frequently managed by the ICU and had a lower mortality rate [37]. Similarly, Foster et al. reported that cases of anosmia were less hospitalized and less likely to enter the ICU [38].

There has been only one publication on zinc and olfactory disturbances in COVID-19 patients, by Abdelmaksoud et al [11]. They found no difference in serum zinc levels, 61 μg/dL in patients without anosmia (n=54) and 59 μg/dL in patients with anosmia (n=80). Similarly, in our study, there was no difference in serum zinc levels between subjects with and without anosmia, and no association was found. However, Abdelmaksoud et al. reported that treatment with 100 mg/d of zinc significantly shortened the time to improvement from a median of 18 days without zinc to a median of 7 days with zinc.

This paper has a limitation. COVID-19 patients were assigned to hospitalization, hotel stay, or home treatment by the medical task force, and the criteria for inclusion in the study may have changed depending on the occurrence of patients. In addition, the time elapsed since the onset of the disease is not constant, and because this is a one-time test, the relationship with the subsequent course of the disease is unclear. Systematic testing and changes in the overall course of the disease need to be examined in the future.

Conclusion

An assessment of outpatients with SARS-CoV2 infection was performed. Most of the cases were mild, but 52.9% showed pneumonia on CT. The frequency of pneumonia findings increased with age. The serum zinc level of the cases was lower than that of healthy subjects, especially in those over 50 y/o. Serum zinc levels in patients with pneumonia findings were significantly lower than in those without pneumonia. Dysosmia or dysgeusia was found in 35.3% of patients but was not associated with serum zinc. Further data collection on the role of zinc in COVID-19 is needed.

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Relationship between serum zinc levels/nutrition index parameters and pressure ulcer in hospitalized patients with malnutrition

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Summary

We performed a retrospective study of serum zinc levels and blood nutrient indices with malnutrition admitted to our hospital. The association between zinc levels and the onset of pressure ulcer was then investigated. The participants were 243 patients who were not administered zinc supplement as part of the nutrition support team intervention between January 2010 and March 2019. The mean zinc level was 59.7μg/dl, and 66 subjects (27.2%) had pressure ulcers. Significant positive correlations were found between serum zinc level and albumin level, and between serum zinc level and pre-albumin level. The subjects were divided into the following groups according to their serum zinc level: Normal (≥ 80μg/dl), latent zinc deficiency (60 ≤ Zn < 80μg/dl), and zinc deficiency (< 60μg/dl). The zinc deficiency group had significantly lower serum albumin levels and pre-albumin levels. The zinc deficiency group had no difference in the amount of energy intake and protein intake. This suggests that the zinc absorption control mechanism failed for some reason, which makes it difficult for the body to maintain zinc homeostasis, and that this may be related to increased excretion of zinc. Our logistic regression analysis designed to search for factors that cause pressure ulcer led to the extraction of serum zinc level and albumin level as independent factors. There is a high possibility that zinc deficiency and malnutrition are related to the onset of pressure ulcer and increased severity, respectively. We believe that future studies on pressure ulcer treatment should focus on appropriate nutrition management and zinc supplementation.

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Received: January 29, 2022
Accepted: May 23, 2022
Released online: June 1, 2022

Key words: Zinc, malnutrition, pressure ulcer, serum albumin level, Nutrition Support Team

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

Introduction

Zinc deficiency has recently become well known in Japan; however, in clinical settings, medical professionals are relatively unfamiliar with zinc deficiency. In cases where hospitalized patients become confined to beds because of malnutrition and illness, reduction in blood nutrient indices and the development

of pressure ulcers are sometimes observed. Although sufficient energy and protein supplements are considered effective treatments for pressure ulcers, there is no evidence indicating that zinc supplementation is effective [1, 2].

Zinc is an essential trace element in the body. Since it performs a wide variety of physiological actions, including nucleic acid synthesis, protein metabolism, immune functions, and blood cell differentiation, zinc deficiency causes a variety of complications. Recently, hospitalized patients tend to be increasingly advanced in age, and in cases in which appropriate nutrition management is not carried out for such patients, the resulting complications can have an effect on their vital prognosis and zinc deficiency complication can have an additional negative effect on their vital prognosis [3, 4].

At our hospital, we provide Nutrition Support Team (NST) intervention for hospitalized patients with malnutrition and for those in whom oral food intake is difficult. The NST makes bedside rounds, holds subsequent conferences, and provides feedback to the attending physician. Identifying that a hospitalized patient has zinc deficiency is important for the prevention of complications and to the improvement of the patient's vital prognosis. At our center, once NST intervention is determined to be necessary, the patient's serum zinc level and other blood nutrient indices are monitored. We previously reported on the relationship between nutritional indices such as serum zinc levels and pressure ulcers (grade II or higher) in 170 NST intervention, zinc-naïve patients from January 2010 to 2016, and found that factors associated with grade II or higher pressure ulcers were serum zinc levels, albumin levels, and total lymphocyte counts [5]. The results showed that the factors associated with pressure ulcers II and above were serum zinc level, albumin level, and total lymphocyte count. With the accumulation of subsequent cases, the number of cases to be examined was increased and the study was conducted again. In addition, a new item for serum zinc level in the diagnostic criteria for zinc deficiency was published in the Japanese Society of Clinical Nutrition "the Zinc Deficiency Clinical Practice Guidelines 2018" [6] and based on this, we reexamined the nutritional indices by group.

Here, we report on our retrospective study of serum zinc levels and blood tests related to nutrition performed on hospitalized patients who underwent NST intervention at our hospital, and we performed additional examination of how these values are related to pressure ulcers. In addition, we performed a survey of zinc deficiency among patients with malnutrition.

Methods

Although there were 284 patients at our hospital who underwent NST intervention during the 9-year and 3-month study period from January 2010 to March 2019, the subjects of this study were the 243 patients who were not administered the following drugs that can affect serum zinc levels: Polaprezinc® (promac granules or promac OD tablets: Zeria Pharmaceutical Co., Ltd. Tokyo, Japan) and Nobelzin® (Nobelpharma Co., Ltd. Tokuyo, Japan). Blood samples were taken at bedside (early morning, fasting) and the following were investigated: Serum zinc (Zn) level (measured using ACCURAS AUTO Zn: Shino-Test Corporation. Tokyo, Japan), serum albumin (Alb) level, serum pre-albumin (Pre-Alb) level, and total lymphocyte count (TLC). The following data was also obtained: Age, sex, amount of energy intake, amount of protein intake, and pressure ulcers (yes vs. no). Laboratory data were measured before the NST intervention during hospitalization. The actual energy intake and protein intake were consisted of eating meal volume (nurse visually confirmed the eating ratio), enteral nutritional supplement and infusion at the time of NST intervention. Basal energy expenditure was calculated by the Harris-Benedict estimation formula [7] (male: 66.47 + 13.75 × body weight(kg) + 5.0 × height(cm)-6.76 × age(years), female: 655.1 + 9.56 × body weight(kg) + 1.85 × height(cm) - 4.68 × age(years)). Theoretical requirement of energy was calculated by the Long formula [8] (Theoretical requirement of energy = Basal energy expenditure × activity factor × injury factor). The ratio of energy intake to theoretical requirement was defined as energy sufficiency rate. Theoretical requirement of protein was calculated by multiplying the underlying disease, coefficient according to the degree of stress and the ideal body weight based on the enteral nutrition guideline 3rd edition (edited by the Japanese Society for Parenteral and Enteral Nutrition) [9]. The ratio of protein intake to theoretical requirement was defined as protein sufficiency rate. Zinc sufficiency rate was not included in this study because it was difficult to retrospectively estimate the zinc intake in the diet. Statistical analyses were performed as follows: Two-group analysis was done using the student's t-test and the correlation between serum zinc level and each variable was investigated using Pearson's product moment correlation coefficient. Three-group analysis was done using One-way ANOVA and chi-square test. If a significant difference was observed after performing One-way ANOVA, the Tukey-Kramer method was performed. Logistic regression analysis was performed in order to identify factors related to pressure ulcers, with the stepwise method used to select the variables. Statistical processing was performed using SPSS Version 22.0 (IBM, Chicago, IL, USA) and Excel Statistics 2015 (SSRI, Tokyo, Japan). Significance

was set at $p < 0.05$.

This study was approved by the Ethics Committee of Saiseikai Wakayama Hospital (No.132,2021). Patients' consent was obtained using the opt-out method.

Results

The patients' background factors are shown in **Table 1**. There were 243 patients who were not administered either Polaprezinc® or Nobelzin® at the time of NST introduction. Overall, 145 patients were male and 98 were female. The mean age was 79.3 ± 9.6 years; BMI was 18.9 ± 3.8 kg; the energy sufficiency rate was 73.9 ± 0.3%; and protein sufficiency rate was 76.1 ± 0.3%, indicating obvious insufficiency. Pressure ulcers were observed in 66 patients (27.2%). The mean serum zinc level was 59.7 ± 18.3 μg/dl, indicating a low level. Mean serum albumin level was low, at 2.4 ± 0.5 g/dl, and mean serum pre-albumin level (rapid turnover protein) was low, at 12.2 ± 10.6 g/dl. TLC, which is a nutrient indicator that shows a decline in immune function, was 1310 ± 798/mm^3. Investigation of the correlation between serum zinc level and age, BMI, serum albumin level, serum pre-albumin level, and TLC showed that there was a significant positive correlation between serum zinc level and serum albumin level (**Figure 1**; $n = 243$, $p < .001$, $r = 0.303$) and that there was a significant positive correlation between serum zinc level and serum pre-albumin

Table 1. **Background factors.**
Values are expressed as the mean ± standard deviation. Energy/protein sufficiency rate; the ratio of energy/protein intake to theoretical requirement*. Abbreviations; TLC, total lymphocyte count.

Number of cases	243
Age (years old)	79.3 ± 9.6
Male/Female	145/98
Body mass index (kg/m^2)	18.9 ± 3.8
Energy intake (kcal/day)	1093 ± 448
Energy sufficiency rate (%)*	73.9 ± 0.3
Protein intake (g/day)	42.5 ± 17.4
Protein sufficiency rate (%)*	76.1±0.3
Pressure ulcer yes/no (%)	66/177 (27.2)
Zn (μg/dl)	59.7±18.3
Albumin (g/dl)	2.4 ± 0.5
Pre-Albumin (g/dl)	12.2 ± 10.6
TLC (/mm^3)	1310 ± 798

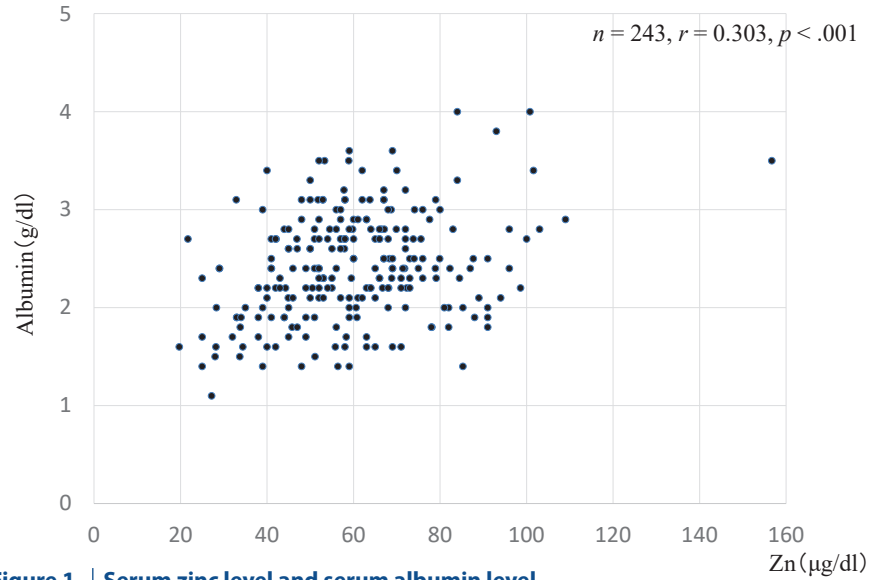

Figure 1. **Serum zinc level and serum albumin level.**
There was a significant positive correlation between serum zinc level and serum albumin level by pearson product-moment correlation's test ($p < .001$).

level (**Figure 2**; $n = 243$, $p < .001$, $r = 0.318$), which suggests that serum zinc level is related to nutritional status and early protein synthesis capacity.

Therefore, based on the serum zinc levels indicating zinc deficiency in the zinc deficiency diagnostic guidelines listed in the Zinc Deficiency Clinical Practice Guidelines 2018 [6], published by the Japanese Society of Clinical Nutrition, we divided the patients into the following groups: Normal group (Group A; at least 80 μg/dl: 30 subjects), latent zinc deficiency group (Group B; at least 60 μg/dl but under 80 μg/dl: 81 subjects), and zinc deficiency group (Group C; under 60 μg/dl: 132 subjects). We then examined the relationship between serum zinc level and serum albumin level, serum pre-albumin level, and TLC. As shown in **Figure 3**, There was a significant difference between serum zinc level and serum albumin level by one-way ANOVA (F (2, 238) = 4.15, p = .0169). The results indicated that compared to Groups B, Group C had significantly lower serum zinc level by Tukey-Kramer method (p = .0456). There was a significant difference between serum zinc level and pre-albumin level by one-way ANOVA

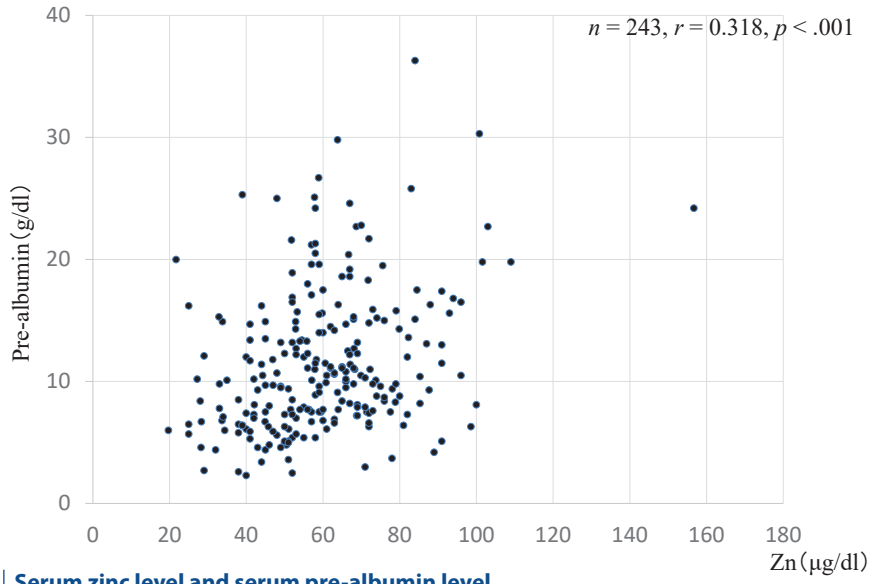

Figure 2. **Serum zinc level and serum pre-albumin level.**
There was a significant positive correlation between serum zinc level and serum pre-albumin level by pearson product-moment correlation's test ($p < .001$).

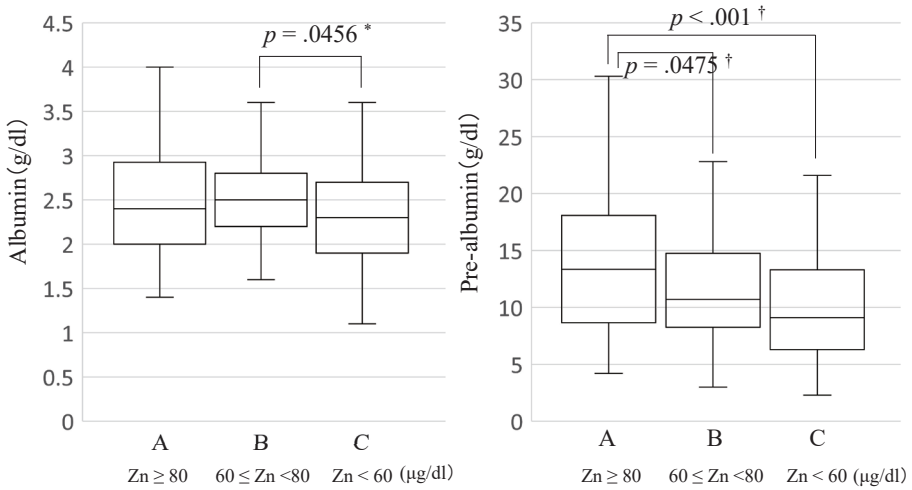

Figure 3. **Serum albumin level and serum pre-albumin level grouped by serum zinc level group.**
There was a significant difference between serum zinc level and serum albumin level by one-way ANOVA (left figure ; F (2, 238) = 4.15, p = .0169). The results indicated that compared to Groups B, Group C had significantly lower by Tukey-Kramer method*. There was a significant difference between serum zinc level and pre-albumin level by one-way ANOVA (right figure ; F (2, 239) = 8.04, $p < .001$). The results indicated that compared to Groups A, Group B and C had significantly lower by Tukey-Kramer method†.

(F (2, 239) = 8.04, p < .001). The results indicated that compared to Groups A, Group B and C had significantly lower serum zinc level by Tukey-Kramer method (p = .0475, p < .001, respectively). No significant differences were found between the three groups in terms of age, gender, body mass index, energy intake amount, energy sufficiency rate, protein intake amount, protein sufficiency rate or pressure ulcer yes/no by One-way ANOVA and chi-square test (**Table 2**). And no significant differences were found between the groups in terms of TLC.

Since the onset of pressure ulcers is problematic for hospitalized patients with malnutrition, we investigated the relationship between serum zinc level and pressure ulcer (yes vs. no). As a result, we found that the non-pressure ulcer group had a serum zinc level of 61.8 ± 18.3 μg/dl and the pressure ulcer group had a serum zinc level of 54.3 ± 17.4 μg/dl, indicating that the pressure ulcer group had significantly lower zinc levels (**Figure 4**; (t (241) = 2.88, p = .0043). We also performed logistic regression analysis with pressure ulcer (yes vs. no) as the dependent factor and age, sex, serum zinc level, serum albumin level, serum pre-albumin level, and TLC as independent factors in order to identify those factors that can cause pressure ulcers. The results were as follows: Univariate analysis indicated a significant relationship between serum zinc level (hazard ratio 0.98, 95% CI: 0.958-0.992; p =

Table 2. **Background factors by serum zinc level at the time of NST introduction.**
Three-group analysis was done using One-way ANOVA* and chi-square test†. Values are expressed as the mean ± standard deviation. Energy/protein sufficiency rate, the ratio of energy/protein intake to theoretical requirement. Abbreviations; NST, nutrition support team; BMI, body mass index

	Group A Zn ≥ 80	Group B 60 ≤ Zn <80	Group C Zn < 60 (μg/dl)	*p* value
Number of cases	30	81	132	
Age	75.9 ± 10.1	79.8 ± 7.7	79.9 ± 10.3	p = .1023*
Male/Female	20/10	55/26	70/62	p = .0703†
BMI (kg/m^2)	19.1 ± 3.4	19.3 ± 3.6	18.7 ± 4.0	p = .5128*
Energy intake (kcal/day)	1104 ± 392	1082 ± 437	1084 ± 472	p = .9651*
Energy sufficiency rate (%)	73.5 ± 24.7	72.3 ± 28.2	74.9 ± 32.0	p = .8234*
Protein intake (g/day)	39.8 ± 12.8	42.3 ± 16.7	43.3 ± 18.8	p = .6249*
Protein sufficiency rate (%)	67.9 ± 21.2	71.3 ± 28.3	80.8 ± 39.1	p = .0605*
Pressure ulcer yes/no (%)	3/27 (10.0)	23/58 (23.4)	40/92 (30.3)	p = .0748†

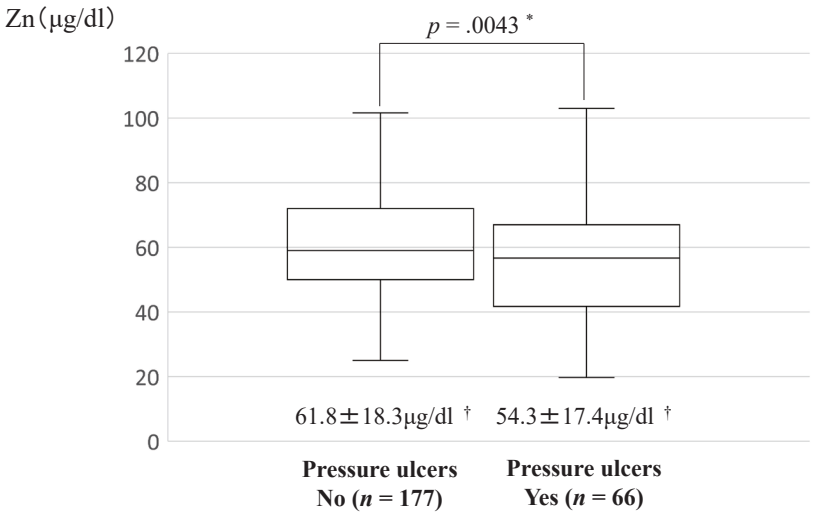

Figure 4. **Pressure ulcers(Yes/No) and serum zinc level.**
We found that the non-pressure ulcer group had a serum zinc level of 61.8±18.3 μg/dl and the pressure ulcer group had a serum zinc level of 54.3±17.4 μg/dl, indicating that the pressure ulcer group had significantly lower zinc levels by Student's T test* (t (241) = 2.88, p = .0043). Values are expressed as the mean ± standard deviation†.

Table 3. **Logistic regression analysis with pressure ulcer (Yes/No).**
Univariate analysis indicated a significant relationship between serum zinc level and serum albumin level.* Multivariate analysis that extracted serum zinc level and serum albumin level.†
Note. Logistic regression analysis was performed with the stepwise method used to select the variables. Female group was set for the baseline hazard (ratio = 1). Abbreviations; HR, hazard ratio; TLC, total lymphocyte count

		univariate HR (95%CI)	*p* value	multivariate HR (95%CI)	*p* value
Gender	Female	1			
	Male	1.57 (0.853-2.838)	$p = .149$		
Age		0.926 (0.972-1.032)	$p = .926$		
Zn		0.98 (0.958-0.992)	$p = .005$ *	0.98 (0.962-0.998)	$p = .031$†
Albumin		0.45 (0.256-0.799)	$p = .006$ *	0.54 (0.300-0.987)	$p = .045$†
Pre-albumin		0.98 (0.941-1.021)	$p = .336$		
TLC		1.00 (1.000-1.001)	$p = .122$		

.005) and serum albumin level (hazard ratio 0.45, 95% CI: 0.256-0.799; $p = .006$) and multivariate analysis that extracted serum zinc level (hazard ratio 0.98, 95% CI: 0.962-0.998; $p = .031$) and serum albumin level (hazard ratio 0.54, 95% CI: 0.300-0.987; $p = .045$), indicated that serum zinc level and serum albumin level are independent factors related to the formation of pressure ulcers (**Table 3**).

Discussion

Zinc is an essential trace element that performs a wide variety of physiological functions including nucleic acid synthesis, protein metabolism, immune functions, and blood cell differentiation. In our hospital, NST bedside rounds and team conferences attended by the hospital's staff are performed for hospitalized patients to provide feedback to the attending physician. Patients with malnutrition and particularly pressure sore complication have long been known to have complication of zinc deficiency. At our hospital, once the determination is made to start NST intervention, serum zinc levels are measured and the attending physicians of patients who are not administered zinc supplement are advised to administer oral zinc supplement and nutritional supplements containing zinc actively to their patients. In particular, patients with illnesses, such as malignant tumors who have undergone surgery and underwent anti-cancer drug treatment, cerebrovascular disease, cardiovascular disease, aspiration pneumonia become bedridden and suffer from complication of pressure ulcer; as a result, their treatment is delayed, and this further exacerbates their complications in a vicious cycle. Holding multidisciplinary in-hospital conferences in addition to the care provided by the attending physician is an important way to accurately identify malnutrition and zinc deficiency, and determine menus based on an appropriate nutrition management plan as soon as possible [10].

There were 284 patients at our hospital over the 9-year and 3-month study period, between January 2010 and March 2019, who underwent NST intervention. However, 243 of these patients who were not administered oral zinc supplement and whose serum zinc levels were monitored were the subjects of this study. The mean serum zinc level was low, at 59.7 ± 18.3 μg/dl, and 132 patients (more than half) had a serum zinc level below the cutoff point for zinc deficiency (under 60 μg/dl). The mean energy sufficiency rate at the time of NST intervention (actual energy intake vs. theoretical requirement of energy as calculated using the Harris-Benedict Equation) was 73.9% and the mean protein sufficiency rate (actual protein intake vs. theoretical requirement of protein) was 76.1%, indicating clear insufficiency. These data indicate that there were many cases of NST patients who had insufficient nutritional intake and complication of zinc deficiency. Investigation of the relationship between serum zinc level and various nutrient index parameters showed that there was a significant correlation with serum albumin level and serum pre-albumin level. Additionally, when the subjects were divided into three groups according to their serum zinc level, no inter-group difference was found in the background energy intake amounts or protein intake amounts, but in Group C (zinc deficiency group) serum albumin and serum pre-albumin levels were significantly lower than those of the other groups (serum albumin;

group B, serum pre-albumin; group A, respectively). Bate J et al. reported that patients with severely low serum zinc levels have low serum albumin levels and low pre-albumin (a rapid turnover protein) levels, and that zinc deficiency improved rapidly upon zinc supplement administration [11]. In their investigation of the relationship between blood zinc levels and serum albumin levels in 57 patients with chronic viral hepatitis, Takamatsu et al. reported that there was a significant positive correlation between blood zinc levels and serum albumin levels, and discussed the possibility that this may be due to increased urinary excretion of zinc in patients with liver cirrhosis, not for decreased absorption rates from food [12]. About 60 to 70 percent of albumin in the blood bonds weakly with the absorbed zinc, and an increase in the urinary excretion of zinc relates hypoalbuminemia and zinc deficiency can be treated with diuretics which is unrelated to absolute insufficiency in zinc intake [13, 14]. Regarding zinc absorption, Kambe et al. reported that ZIP4 and ZIP5, which are zinc transporters in tissues that are important to the internal storage of zinc (small intestine epithelial cells) are important to zinc homeostasis and for example, in cases of insufficient zinc levels, the amount of ZIP4 that is expressed increases and decomposition is suppressed [15, 16]. If a patient develops malnutrition, the absorption regulation mechanism for ingested zinc fails to function normally, which in turn may cause disruption of the maintenance of zinc homeostasis. We look forward to future research designed to identify the actual mechanism by which clinical administration of zinc is involved in this.

Pressure ulcer onset is an important complication experienced by hospitalized patients with malnutrition. In particular, in cases in which deep ulcers that form pockets occur, it is difficult to elevate serum albumin levels due to the outflow of exudates, which in turn makes it difficult to improve the patient's nutritional status. In many cases, this becomes the cause of additional ulcer infection. In our investigation of pressure ulcers and zinc, we performed logistic regression analysis in order to identify the factors that cause pressure ulcers. The results showed that serum zinc level and serum albumin level were both independent factors. However, although it is generally considered that protein intake and zinc intake decrease due to a decrease in food intake, it is considered that each deficiency may accelerate the other deficiency. This time, we have not examined the amount of zinc in the meal, and it is not clear, but it is a future examination issue.

Kurasawa et al. reported that even if the elevation of serum albumin level is insufficient, zinc supplement therapy improves pressures ulcers if serum zinc concentration is increased [17]. Furthermore, in the present study we also found that pressure ulcers and zinc deficiency were independently related [5]. Nishida et al. verified that the zinc transporter ZnT2 is the gene responsible for zinc accumulation in the secretion granules of mast cells and that the zinc secreted by mast cells is related to skin wound healing [18]. It has also been shown that the zinc released by mast cells is related to the production of IL-6 during the inflammatory phase of skin ulcer healing [18]. In their study using Zip13 knockout mice, Fukada et al. verified that in skin and other connective tissues Zip13 is the regulatory molecule for the BMP/TGF-β signal pathway [19, 20]. In this way, zinc is thought to play an important role in the healing process of the skin. Hence, we believe that it is important to provide zinc supplements in addition to appropriate nutritional management as part of the treatment of pressure ulcers.

Conclusion

Zinc is an essential trace element in the body. Although clinical physicians have an academic understanding of its importance, in actual clinical practice, appropriate zinc supplement administration is not done, as zinc deficiency is not suspected. In this study, we investigated the relationship of serum zinc level with nutrient index parameters and pressure ulcers in patients who qualify for NST intervention. In spite of the fact that the in-hospital physicians are aware of the existence of NST, few of them administer zinc supplement to their patients. A variety of previous studies reported the possibility that zinc administration to patients with zinc deficiency might improve their nutritional status [21]. Hence, it is necessary to check serum zinc concentration first when examining patients with malnutrition. We look forward to more vigorous activities that will raise awareness regarding zinc in the future. As additional important knowledge regarding zinc and its role in the body is being accumulated, we look forward to an increase in the understanding of zinc deficiency.

Acknowledgments

The authors would like to thank the NST staff at Saiseikai Wakayama Hospital and other hospital staff who provided valuable assistance.

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