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Can irritable bowel syndrome be detected by ultrasound?

Yohei Okawa*

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SUMMARY Functional gastrointestinal disease is one in which gastrointestinal symptoms persist chronically or recurrently. This disease is challenging because it does not have an organic cause that can be detected in routine laboratory tests. Among them, the symptoms of irritable bowel syndrome (IBS), which is a type of functional gastrointestinal tract disease, include abnormal bowel movements associated with abdominal pain. However, no specific test has been established to definitively diagnose these diseases, including IBS. The traditional Rome IV diagnostic criteria are used to diagnose IBS by assessing subjective symptoms. However, it has been suggested that IBS is difficult to diagnose using the Rome IV criteria among unconscious or cognitively impaired patients. It is recommended that abdominal ultrasonography be used to assess IBS with diarrhea and constipation. Previously, constipation among elderly people who ingested food orally was objectively assessed by ultrasound, and colonic fecal distribution patterns were classified in constipated patients and healthy people. Objective visualization of the large intestine was used to assess constipation. Therefore, fecal retention among adults and elderly individuals was reported using ultrasonography. It was suggested that stool retention could be confirmed by observing the hyperechoic region of the rectum. Strong hyperechoic regions with acoustic shadows in the rectum indicate the presence of hard stools, thus enabling medical workers to identify constipation. In the future, ultrasonography may be useful for diagnosing IBS in unconscious patients or those with cognitive decline.

Keywords functional gastrointestinal disease, gastrointestinal symptoms, constipation

1. Introduction

Functional gastrointestinal disease is a disease in which gastrointestinal symptoms persist chronically or recurrently. There are no organic lesions on clinical examination, and the symptoms are due to functional abnormalities (*1*). Types of functional gastrointestinal disorders include irritable bowel syndrome (IBS), functional abdominal distension, functional constipation, functional diarrhea, and unspecified functional bowel disease (*1-3*). Symptoms of IBS, which is a typical functional gastrointestinal disorder, include abdominal pain, abdominal discomfort, and associated bowel abnormalities (*3*). However, objective examination that can make a definitive diagnosis has not been established for these disease groups, including IBS. Therefore, we suggest that a novel, objective method for diagnosing IBS should be developed based on various views of articles.

2. Diagnosis of IBS

Recently, Rome IV diagnostic criteria have been used as diagnostic criteria for IBS by assessing subjective symptoms (*1-4*). The Rome IV diagnostic criteria can distinguish functional bowel disease with chronic symptoms from transient gastrointestinal symptoms. The criteria include abdominal pain and defecation disorders occurring more than 6 months before diagnosis and for more than 3 days in the last 3 months (*3,4*). Defecation disorder subtypes can be classified into IBS with diarrhea (IBS-D) and IBS with constipation (IBS-C); these subtypes are considered useful in clinical practice. Stool shapes range from watery to hard stools, and it has been suggested that transit time through the gastrointestinal tract is reflected by stool shape (*5,6*). On the other hand, it has also been reported that the defecation frequency and transit time of IBS patients are often the same as those of healthy subjects (*7,8*). This classification method includes that in addition to diarrhea type and constipation type, there are patients who are judged as having mixed IBS (IBS-M) with both characteristics and those with unsubtyped IBS (IBS-U), which does not include all criteria (*2-4*). However, this diagnosis is limited to those patients who have a clear awareness. Rome IV criteria are evaluated based on

the patient's subjective symptoms, which are based on abdominal pain (*4*). This fact suggests that it is difficult to use the Rome IV criteria to diagnose IBS in patients that are unconscious or have cognitive decline.

A previous study suggested that it is difficult to completely exclude organic diseases by diagnosing symptoms using only the Rome criteria (*9*). On the other hand, the study also suggested that the Rome criteria can be used to exclude unnecessary examinations and that other diseases can be significantly narrowed down (*9*). However, many physicians do not remember the diagnostic criteria for IBS; it has been reported that physicians use diagnostic criteria in only 4% of clinical practice. In addition, there is not enough evidence that the Rome criteria can be used to diagnose unconscious patients or those with cognitive decline because the Rome criteria evaluate only subjective symptoms. Additionally, many epidemiological studies have indicated that the Rome IV criteria are not sufficient. However, several epidemiological studies have used the Rome II criteria, which are effective for diagnosing IBS. Additionally, the Rome III criteria were developed in statistical studies of epidemiological data based on the Rome II criteria. Furthermore, a number of studies have suggested that the home environment, psychosocial factors, gastrointestinal motility abnormalities, visceral hypersensitivity, intestinal bacteria, and brain-intestinal correlations have been thoroughly examined (*4,10*). However, the Rome criteria are based only on the patient's subjective symptoms, such as abdominal pain or defecation. Therefore, there is not enough evidence that the Rome criteria can be used to diagnose unconscious patients or those with cognitive decline.

3. Subjective symptoms can be evaluated by questionnaires

The diagnosis of IBS is made by Rome IV diagnostic criteria (*4*) and other questionnaires. However, there are various factors related to the onset of IBS, so several questionnaires are used to evaluate the relationship between daily living conditions, quality of life (QOL), psychological conditions and abdominal symptoms (*11-14*). The evaluation of QOL using the SF-36 is clinically useful because it can be compared with that of patients with other diseases. In addition, the IBS-QOL, which is an IBS-specific QOL survey, is effective in assessing the effects of treatment (*15*). SCL-90-R, HDRS (*16*), EPQ, and DSSI (*11*) are also available. The symptoms of IBS patients can be evaluated by using a combination of these questionnaires. In particular, these questionnaires might reveal mental problems, such as anxiety and depression.

4. Effects of colonoscopy and colonography as objective methods of evaluation

The diagnosis of IBS is based on the Rome IV diagnostic criteria (*2,4*) and other questionnaires (*17,18*). However, it is difficult to subjectively evaluate patients who are unconscious or have reduced cognitive function. Therefore, it is important to use objective evaluation methods. There are few epidemiological studies on the effectiveness of colonoscopy and colonography in the diagnosis of IBS. A previous study suggested that pain during colonoscopy was significantly more severe in IBS patients than in non-IBS patients (*19*). Furthermore, another previous study of colorectal angiography also suggested that IBS patients showed significant spasm compared with those without abdominal symptoms (*20*). Although the use of routine colonoscopy and colon radiography in the current diagnosis of IBS is not required, evidence-based objective methods of evaluation may be necessary for exclusion diagnosis, especially for patients with signs of IBS.

5. Abdominal ultrasound may be used to objectively and noninvasively evaluate IBS

Although there are few epidemiological studies on the usefulness of endoscopic imaging examinations on anything other than the large intestine in the diagnosis of IBS, there are articles on ultrasound examination and upper gastrointestinal endoscopy (*21*). In a previous study on ultrasound, it was reported that the contractile movement of the gallbladder was higher in the IBS group than in the control group in both the fasted state and after diet loading (*22,23*). It has been suggested that IBS is associated with cholecystectomy, but the small number of cases and its direct association with IBS symptoms are unknown. In addition, a previous study in Japan evaluated colonic motility by abdominal ultrasonography and reported that colonic contraction in the IBS group was enhanced by observing the sigmoid colon on an empty stomach. In the postmeal observation of the sigmoid colon, 9 IBS cases diagnosed by Rome II criteria and 4 controls were compared. In IBS-C, segmental movement was enhanced. On the other hand, in IBS-D, enhanced transport of intestinal contents to the anus was observed (*24*). Based on these reports, ultrasonic examination can be considered a noninvasive examination and is expected to be useful for the evaluation of intestinal motility. However, since there are few research reports, future verification is expected.

Furthermore, very few reports have diagnosed IBS using ultrasound. Among them, some previous studies have confirmed the characteristic changes in the gastrointestinal tract of IBS by using ultrasound. In a previous study, ultrasound was used to investigate the gastric emptying rate (GER) and antral motility of 76 IBS children who met the Rome III diagnostic criteria. The GER was significantly reduced in the IBS group exposed to stressful events. This result indicates that in IBS patients, stress causes more damage to the

GER and antral motility than in healthy individuals. In this ultrasound study, the GER and pyloric motility slowed gastric emptying and impaired pyloric sinus motility in all four IBS subtype children. However, a clear relationship between gastrointestinal motility abnormalities and symptoms has not been shown (*25*). In addition, a previous study investigating whether transvaginal ultrasound was useful in diagnosing IBS reported that the intestinal wall of the sigmoid colon was thickened in approximately 27 patients with a history of IBS (*26*). However, transvaginal ultrasound is a test for women only, and it is a difficult method for people living a general healthy social life.

Thus, studies on IBS diagnosis using ultrasound have not been sufficiently conducted. Several transvaginal and transrectal research methods have been reported (*25-28*), all of which are painful for patients and difficult to use in home care and medical facilities.

In recent years, it has become possible to easily and noninvasively inspect constipation by applying a small ultrasonic wave percutaneously to the abdominal wall (*29*). In particular, elderly people with impaired cognitive motor function cannot complain of subjective symptoms, so it is an important advance to easily test for constipation and diarrhea with such a small ultrasound procedure.

6. Is it possible for nurses to objectively evaluate IBS by using ultrasound?

Ultrasound can visualize information in the body in real time by simply applying a probe to the abdomen. In addition, unlike X-ray abdominal radiography, there is no exposure, and it can be repeated (Table 1). Furthermore, in recent years, the development of pocket-sized ultrasound devices has rapidly advanced (*29*), and it is becoming possible to use ultrasound not only in the examination room but also at home or at the bedside (*30,31*).

In a previous study, constipation among elderly people who ingested food orally was evaluated objectively by ultrasound, and the fecal distribution pattern of the large intestine was classified in patients with constipation (*29,30*). Objective visualization of the large intestine was used to evaluate constipation. For this reason, fecal retention was reported using ultrasonography in adults and elderly individuals, and Japanese nurses used ultrasound as a physical assessment tool to assess constipation (*31*). It has been suggested that stool retention can be confirmed by observing the hyperechoic region of the rectum.

Furthermore, it has been reported that a hyperechoic region is also found in the rectum of patients with functional constipation (*30-32*). The strong hyperechoic region with acoustic shadow in the rectum indicates the presence of hard stool and has been suggested to be able to identify IBS.

However, previous studies evaluated only constipation patients and did not adequately examine diarrhea in enterally fed elderly people with impaired cognitive motor function. In addition, ultrasound has been used to diagnose enteritis in the digestive tract. In the case of infectious enteritis and ischemic colitis, specific echo findings are recognized, and ultrasound can be used to diagnose the disease. Thus, while the effectiveness of ultrasound has been shown for specific diseases, it has not been verified for diarrhea in elderly individuals.

In Japan, the 2016 medical fee revision (*33*) established a new "urination independence guidance fee", which includes "remaining urine measurement" using ultrasound and "urination diary" as requirements. Ultrasound has become a new assessment method for which general nurses should learn and develop educational programs for nurses (*34-39*). From the above, if it becomes possible for a nurse to predict diarrhea by using ultrasound, they can change the content of meals according to the individuality of the elderly patient and provide excretory supplies (incontinence pants, diapers, pads, *etc.*). Drug administration will make the daily life of patients safer and easier. Therefore, future research should examine these subjects.

7. Limitation

Rome IV diagnostic criteria based on subjective symptoms are used to diagnose IBS. This approach is limited to those who can communicate and complain on their own. On the other hand, ultrasonic waves can be a tool for diagnosing IBS by an objective index. To date, it has been reported by several previous studies that constipation and normal stool can be confirmed percutaneously easily and noninvasively. However, it is difficult to evaluate abdominal pain, which is essential for IBS diagnosis, by ultrasound. In the future, further visualization of defecation disorders and indicators that can objectively evaluate abdominal symptoms, such as abdominal pain, should be verified.

Table 1. Examination of intestinal colon

Examination	Evaluate contents	Real time	Invasion
Abdominal X-ray	Distribution of stool (volume) Colonic transit time (movement)	No	Radiation exposure
CT imaging	Distribution of stool (volume and quality)	No	Radiation exposure
MRI scan	Distribution of stool (volume and quality)	No	Take up too much time; Noise exposure
Ultrasound imaging (US)	Distribution of stool (volume, quality and movement)	Yes	Non-invasive

8. Conclusion

The traditional Rome IV diagnostic criteria are used to diagnose IBS by assessing subjective symptoms. The Rome IV diagnostic criteria can distinguish between functional bowel disease with chronic symptoms and transient gastrointestinal symptoms. The Rome IV criteria assess whether abdominal pain and defecation disorders occurred more than 6 months before diagnosis and whether these symptoms persisted for more than 3 days in the last 3 months. The defecation disorder subtypes can be divided into IBS-D and IBS-C, but this diagnosis is used only in patients with clear awareness. The Rome IV criteria are used to assess subjective symptoms in patients who have abdominal pain or bowel movements, suggesting that diagnosing IBS using the Rome IV criteria among unconscious patients or cognitively impaired patients is difficult.

It is recommended that abdominal ultrasonography be used to assess IBS. In a previous study, constipation among elderly people who ingested food orally was objectively assessed by ultrasound, and the pattern of fecal distribution in the large intestine was classified in patients with constipation. Objective visualization of the large intestine was used to assess constipation. Therefore, fecal retention among adults and elderly individuals was reported using ultrasonography. Thus, it was suggested that stool retention could be confirmed by observing the hyperechoic region of the rectum. Furthermore, hyperechoic regions have also been reported in the rectum of patients with functional constipation. Furthermore, strong hyperechoic regions with acoustic shadows in the rectum indicate the presence of hard stools, thus enabling medical workers to identify constipation. In the future, ultrasonography may be useful for diagnosing IBS in unconscious patients or those with cognitive decline.

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References

1. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology. 2006; 130:1480-1491.
2. Drossman DA. Functional gastrointestinal disorders: History, pathophysiology, clinical features and Rome IV. Gastroenterology. 2016; S0016-5085(16)00223-7.
3. Drossman DA. The functional gastrointestinal disorders and the Rome III process. Gastroenterology. 2006; 130:1377-1390.
4. Drossman DA, Hasler WL. Rome IV-functional GI disorders: Disorders of gut-brain interaction. Gastroenterology. 2016; 150:1257-1261.
5. Degen LP, Phillips SF. How well does stool form reflect colonic transit? Gut. 1996; 39:109-113.
6. Törnblom H, Van Oudenhove L, Sadik R, Abrahamsson H, Tack J, Simrén M. Colonic transit time and IBS symptoms: What's the link? Am J Gastroenterol. 2012; 107:754-760.
7. Ragnarsson G, Bodemar G. Division of the irritable bowel syndrome into subgroups on the basis of daily recorded symptoms in two outpatients samples. Scand J Gastroenterol. 1999; 34:993-1000.
8. Saad RJ, Rao SS, Koch KL, Kuo B, Parkman HP, McCallum RW, Sitrin MD, Wilding GE, Semler JR, Chey WD. Do stool form and frequency correlate with whole-gut and colonic transit? Results from a multicenter study in constipated individuals and healthy controls. Am J Gastroenterol. 2010; 105:403-411.
9. Jellema P, van der Windt DA, Schellevis FG, van der Horst HE. Systematic review: Accuracy of symptom-based criteria for diagnosis of irritable bowel syndrome in primary care. Aliment Pharmacol Ther. 2009; 30:695-706.
10. Mayer EA, Savidge T, Shulman RJ. Brain-gut microbiome interactions and functional bowel disorders. Gastroenterology. 2014; 146:1500-1512.
11. Boyce PM, Koloski NA, Talley NJ. Irritable bowel syndrome according to varying diagnostic criteria: Are the new Rome II criteria unnecessarily restrictive for research and practice? Am J Gastroenterol. 2000; 95:3176-3183.
12. Walter SA, Kjellström L, Talley NJ, Andreasson AN, Nyhlin H, Agréus L. Prospective diary evaluation of unexplained abdominal pain and bowel dysfunction: A population-based colonoscopy study. Dig Dis Sci. 2011; 56:1444-1451.
13. Robinson A, Lee V, Kennedy A, Middleton L, Rogers A, Thompson DG, Reeves D. A randomised controlled trial of self-help interventions in patients with a primary care diagnosis of irritable bowel syndrome. Gut. 2006; 55:643-648.
14. Andrae DA, Patrick DL, Drossman DA, Covington PS. Evaluation of the irritable bowel syndrome quality of life (IBS-QOL) questionnaire in diarrheal-predominant irritable bowel syndrome patients. Health Qual Life Outcomes. 2013; 11:208.
15. Heitkemper MM, Jarrett ME, Levy RL, Cain KC, Burr RL, Feld A, Barney P, Weisman P. Self-management for women with irritable bowel syndrome. Clin Gastroenterol Hepatol. 2004; 2:585-596.
16. Guthrie E, Creed F, Fernandes L, Ratcliffe J, Van Der Jagt J, Martin J, Howlett S, Read N, Barlow J, Thompson D, Tomenson B. Cluster analysis of symptoms and health seeking behaviour differentiates subgroups of patients with severe irritable bowel syndrome. Gut. 2003; 52:1616-1622.
17. Ghoshal UC, Gwee KA, Chen M, *et al.* Development, translation and validation of enhanced Asian Rome III questionnaires for diagnosis of functional bowel diseases in major asian languages: A Rome Foundation-Asian neurogastroenterology and motility association working

team report. J Neurogastroenterol Motil. 2015; 21:83-92.
18. Kanazawa M, Nakajima S, Oshima T, Whitehead WE, Sperber AD, Palsson OS, Drossman DA, Miwa H, Fukudo S. Validity and reliability of the Japanese version of the Rome III diagnostic questionnaire for irritable bowel syndrome and functional dyspepsia. J Neurogastroenterol Motil. 2015; 21:537-544.
19. Kim ES, Cheon JH, Park JJ, Moon CM, Hong SP, Kim TI, Kim WH. Colonoscopy as an adjunctive method for the diagnosis of irritable bowel syndrome: Focus on pain perception. J Gastroenterol Hepatol. 2010; 25:1232-1238.
20. Lanng C, Mortensen D, Friis M, Wallin L, Kay L, Boesby S, Jørgensen T. Gastrointestinal dysfunction in a community sample of subjects with symptoms of irritable bowel syndrome. Digestion. 2003; 67:14-19.
21. Zhao Y, Zou D, Wang R, *et al.* Dyspepsia and irritable bowel syndrome in China: A population-based endoscopy study of prevalence and impact. Aliment Pharmacol Ther. 2010; 32:562-572.
22. Guliter S, Yilmaz S, Yakaryilmaz F, Keles H. Evaluation of gallbladder motility in patients with irritable bowel syndrome. Swiss Med Wkly. 2005; 135:407-411.
23. Güçlü M, Pourbagher A, Serin E, Kul K, Ozer B, Cosar A, Içer MO, Gür G, Boyacioglu S. Ultrasonographic evaluation of gallbladder functions in patients with irritable bowel syndrome. J Gastroenterol Hepatol. 2006; 21:1309-1312.
24. Kusunoki H, Kamada T, Sato M, Haruma K, Hata J. Ultrasonographic assessment of sigmoid colon in patients with irritable bowel syndrome. Nihon Rinsho. 2006; 64:1461-1466.
25. Devanarayana NM, Rajindrajith S, Bandara C, Shashiprabha G, Benninga MA. Ultrasonographic assessment of liquid gastric emptying and antral motility according to the subtypes of irritable bowel syndrome in children. J Pediatr Gastroenterol Nutr. 2013; 56:443-448.
26. Crade M, Pham V. Ultrasound examination of the sigmoid colon: Possible new diagnostic tool for irritable bowel syndrome. Ultrasound Obstet Gynecol. 2006; 27:206-209.
27. Awad RA, Martin J, Cal y Major M, Noguera JL, Ramos R, Amezcua C, Camacho S, Santiago R, Ramirez JL, Castro J. Transrectal ultrasonography: Relationship with anorectal manometry, electromyography and sensitivity tests in irritable bowel syndrome. Int J Colorectal Dis. 1998; 13:82-87.
28. O'Connor OJ, McSweeney SE, McWilliams S, O'Neill S, Shanahan F, Quigley EM, Maher MM. Role of radiologic imaging in irritable bowel syndrome: Evidence-based review. Radiology. 2012; 262:485-494.
29. Yabunaka K, Matsumoto M, Yoshida M, Tanaka S, Miura Y, Tsutaoka T, Handa M, Nakagami G, Sugama J, Okada S, Sanada H. Assessment of rectal feces storage condition by a point-of-care pocket-size ultrasound device for healthy adult subjects: A preliminary study. Drug Discov Ther. 2018; 12:42-46.
30. Tanaka S, Yabunaka K, Matsumoto M, Tamai N, Noguchi H, Yoshida M, Nakagami G, Sugama J, Sanada H. Fecal distribution changes using colorectal ultrasonography in older people with physical and cognitive impairment living in long-term care facilities: A longitudinal observational study. Healthcare (Basel). 2018; 6:55.
31. Matsumoto M, Tanaka S, Yabunaka K, Yoshida M, Miura Y, Tsutaoka T, Handa M, Nakagami G, Sugama J, Okada S, Sanada H. Ultrasonographic evaluation of changes over time in one defecation cycle in adults with functional constipation: A report of two cases. Drug Discov Ther. 2018; 12:304-308.
32. Yabunaka K, Nakagami G, Tabata K, Sugama J, Matsumoto M, Kido Y, Iuchi T, Sanada H. Constipation in the elderly in a Japanese long-term medical facility: An ultrasonographic investigation. Drug Discov Ther. 2018; 12:233-238.
33. Ministry of Health, Labor and Welfare Insurance Bureau. Outline of Medical Fee Revision in 2016. Japanese Version. (in Japnese)
34. Yamada T, Minami T, Soni NJ, Hiraoka E, Takahashi H, Okubo T, Sato J. Skills acquisition for novice learners after a point-of-care ultrasound course: Does clinical rank matter? BMC Med Educ. 2018; 18:202.
35. Selim AA, Ramadan FH, El-Gueneidy MM, Gaafer MM. Using Objective Structured Clinical Examination (OSCE) in undergraduate psychiatric nursing education: Is it reliable and valid? Nurse Educ Today. 2012; 32:283-288.
36. Smith V, Muldoon K, Biesty L. The Objective Structured Clinical Examination (OSCE) as a strategy for assessing clinical competence in midwifery education in Ireland: A critical review. Nurse Educ Pract. 2012; 12:242-247.
37. Cawthorn TR, Nickel C, O'Reilly M, Kafka H, Tam JW, Jackson LC, Sanfilippo AJ, Johri AM. Development and evaluation of methodologies for teaching focused cardiac ultrasound skills to medical students. J Am Soc Echocardiogr. 2014; 27:302-309.
38. Duff B, Massey D, Gooch R, Wallis M. The impact of a multimodal education strategy (the DeTER program) on nurses' recognition and response to deteriorating patients. Nurse Educ Pract. 2018; 31:130-135.
39. Wanjiku GW, Bell G, Wachira B. Assessing a novel point-of-care ultrasound training program for rural healthcare providers in Kenya. BMC Health Serv Res. 2018; 18:607.

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Original Article

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Effects of the linagliptin, dipeptidyl peptidase-4 inhibitor, on bone fragility induced by type 2 diabetes mellitus in obese mice

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SUMMARY Recently, it has been suggested that glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), which play important roles in the homeostasis of glucose metabolism, could be involved in the regulation of bone metabolism. Inhibitors of dipeptidyl peptidase 4 (DPP-4), an enzyme that degrades GIP and GLP-1, are widely used clinically as a therapeutic agent for diabetes. However, the effects of DPP-4 inhibitors on bone metabolism remain unclear. In this study, we investigated the effects of linagliptin, a DPP-4 inhibitor, on bone fragility induced by type 2 diabetes mellitus (T2DM). Non-diabetic mice were used as controls, and T2DM mice were administered linagliptin orally on a daily basis for 12 weeks. In T2DM mice, decreased bone mineral density was observed in the lower limb bones along with low serum osteocalcin levels and high serum tartrate-resistant acid phosphatase-5b (TRAP) levels. In contrast, the decreased serum osteocalcin levels and increased serum TRAP levels observed in T2DM mice were significantly suppressed after the administration of linagliptin 30 mg/kg. Bone histomorphometric analysis revealed a reduced osteoid volume and osteoblast surface with an increase in the eroded surface and number of osteoclasts in T2DM mice. This decreased bone formation and increased bone resorption observed in the T2DM mice were suppressed and trabecular bone volume increased following the administration of 30 mg/kg linagliptin. Collectively, these findings suggest that linagliptin may improve the microstructure of trabecular bone by inhibiting both a decrease in bone formation and an increase in bone resorption induced by T2DM.

Keywords Dipeptidyl peptidase-4 inhibitor, type 2 diabetes mellitus, bone fragility

1. Introduction

The bone is a metabolically active organ that undergoes continuous remodeling due to bone resorption by osteoclasts and bone formation by osteoblasts (*1*). Under healthy conditions, the balance between bone formation and bone resorption remains consistently uniform; thus, bone strength and bone density are maintained. Certain pathological states and drugs affect normal bone remodeling, which can induce skeletal disorders including osteopenia or osteoporosis (*2*).

Diabetes mellitus increases bone fragility by affecting bone metabolism, resulting in secondary osteoporosis, which increases the risk of fractures in patients (*3-7*). Furthermore, it has been revealed that several oral hypoglycemic agents affect bone metabolism (*8*). Long-term users of thiazolidinedione, an insulin sensitizer, have been reported to be at a significantly increased risk of fractures (*9-12*). Previous investigations have indicated that suppressed bone formation (*13*) and enhanced bone resorption (*14*) are the mechanisms underlying thiazolidine-induced bone fragility. Recently, it has been suggested that the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), which play an important role in the homeostasis of glucose metabolism, may be involved in the regulation of bone formation (*15*) and bone resorption (*16*). Currently, GLP-1 receptor agonists, which enhance incretin action, and inhibitors of dipeptidyl peptidase 4 (DPP-4), an enzyme that degrades GIP and GLP-1, are extensively used clinically as therapeutic agents in type 2 diabetes mellitus (T2DM). However, the effects of GLP-1 receptor agonists and DPP-4 inhibitors on bone metabolism have not been clarified. In this study, we

investigated the effects of linagliptin, a DPP-4 inhibitor, on bone fragility induced by T2DM in obese mice.

2. Materials and Methods

2.1. Animals

Five-week-old male obese type 2 diabetic mice (BKS.Cg-+ *Lepr^{db}*/+ *Lepr^{db}*/*Jcl*) and age-matched non-diabetic mice (BKS.Cg-*m* +/ + *Lepr^{db}*/*Jcl*) were purchased from CLEA Japan Inc. (Tokyo, Japan). The animals were housed at 22 ± 2°C and 55 ± 5% humidity on a 12-h light-dark cycle with *ad libitum* access to standard chow (MF; Oriental Yeast Co., Tokyo, Japan) and water. All procedures were approved by the Animal Research Committee of Niigata University of Pharmacy and Applied Life Sciences according to the Japanese Government Animal Protection and Management Law and the Japanese Government Notification on Feeding and Safekeeping of Animals.

2.2. Drugs

Commercially available linagliptin (Nippon Boehringer Ingelheim Co., Ltd., Tokyo, Japan) agents was obtained and suspended in 0.2% carboxymethylcellulose sodium solution (CMC-Na; Sigma-Aldrich, St. Louis, MO, USA).

2.3. Experimental procedure

The animals were randomly divided into four groups (10 animals per group): [1] non-diabetic mice (non-DM), [2] T2DM mice treated with vehicle (0.2% CMC-Na) (T2DM), [3] T2DM mice treated with 3 mg/kg linagliptin (Lina 3), and [4] T2DM mice treated with 30 mg/kg linagliptin (Lina 30). The drug doses were selected based on a previous report (*17*) relevant to the effective doses of linagliptin with regard to glucose metabolism in obese mice. The treatments were administered *via* oral gavage at a volume of 0.1 mL/10 g of body weight once daily for 12 weeks. Blood samples from the tail vein were collected to measure blood glucose levels by using a blood glucose measuring device (FreeStyle Freedom; Nipro Co., Ltd., Osaka, Japan). All animals were euthanized under CO_2 anesthesia 24 h after the final drug was administered. The femur and tibia were dissected, and soft tissue was removed.

2.4. Bone strength analysis

Bone strength of the femoral mid-diaphysis was evaluated *via* a three-point bending method using a mechanical testing machine (EZ-S; Shimadzu, Tokyo, Japan). The femur was positioned on two supports placed 10 mm apart. The bending load was vertically applied to the mid-diaphysis with a crosshead speed of 1.0 mm/min until fracture. The load deformation curves were calculated using operation software (Trapezium X; Shimadzu, Tokyo, Japan), and the maximum load, breaking energy, and stiffness were directly calculated from the load deformation curve.

2.5. Bone mineral density measurements

The bone mineral density (BMD) of the whole femur and tibia was measured using quantitative computed tomography (LaTheta LCT-100; Aloka, Tokyo, Japan) with a pixel size of 250 × 250 μm and slice thickness of 1 mm. Cortical, trabecular, and total BMD values were calculated using LaTheta software (ver. 1.31; Aloka, Tokyo, Japan).

2.6. Serum biochemical markers

Serum levels of osteocalcin, a bone formation marker, were measured using the osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA). Furthermore, serum levels of tartrate-resistant acid phosphatase-5b (TRAP), a bone resorption marker, were measured using the TRAP assay (Immunodiagnostic Systems Ltd., Tyne & Wear, UK).

2.7. Bone histomorphometry

Non-decalcified specimens from the proximal tibia metaphysis were prepared according to the following method. The tibia was fixed with 70% ethanol for 7 days, stained with Villanueva Bone Stain (basic fuchsin, fast green, orange G, and azure II; Merck, Darmstadt, Germany) in 70% methanol for 7 days, and embedded in methyl methacrylate resin. The resin blocks were then sliced to 5-μm thickness on a microtome (Leica RM2255; Leica Inc., Nussloch, Germany). All bone histomorphometric parameters were measured in the secondary spongiosa region. To exclude the primary spongiosa, the measurement region was 0.11.2 mm distal to the lowest point of the growth plate and 0.1 mm from the lateral cortex.

Bone histomorphometric measurements were performed using a semiautomatic image analyzing system (Histometry RT CAMERA; System Supply, Nagano, Japan) at ×400 magnification. The bone structural parameters evaluated included bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). The bone formation parameters included the osteoid surface per bone surface (OS/BS), osteoid volume per bone volume (OV/BV), and osteoblast surface per bone surface (Ob.S/BS). Bone resorption parameters included the eroded surface per bone surface (ES/BS), osteoclast surface per bone surface (Oc.S/BS), and osteoclast number per bone surface (N.Oc/BS). Standard bone histomorphometric

nomenclature, symbols, and units were based on those described in the report of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (*18*).

2.8. Statistical analysis

Data are presented as mean ± standard error (SE) values. Differences between groups were analyzed by one-way analysis of variance followed by Tukey-Kramer multiple comparisons. $p < 0.05$ was considered significant.

3. Results

3.1. Body weight and blood glucose levels

During the experimental period, body weight and blood glucose levels in the T2DM group were significantly higher than those in the non-DM group (Figure 1). There were no significant differences in mean body weight among the T2DM, Lina 3, and Lina 30 groups during the treatment period of 12 weeks. At 12 weeks after treatment, the mean blood glucose levels in the Lina 3 and Lina 30 groups decreased by approximately 8% and 22%, respectively, compared with that in the T2DM group. However, intergroup differences were not significant.

3.2. Bone strength properties

The following parameters of the femoral mid-diaphysis significantly decreased in the T2DM group compared with those in the non-DM group: maximum load (26%), breaking energy (28%), and stiffness (22%) (Table 1). There were no significant differences in the bone strength parameters in the Lina 3 and Lina 30 groups compared to those in the T2DM group.

3.3. BMD

Compared to the non-DM group, the T2DM group exhibited significantly decreased cortical BMD (10%, 14%), trabecular BMD (22%, 27%), and total BMD (19%, 22%) of the whole femur and tibia, respectively

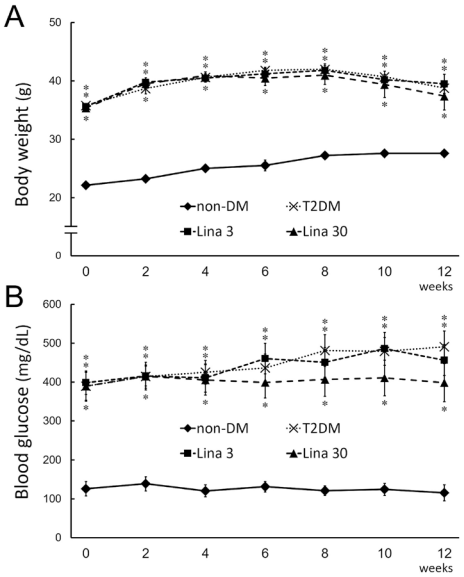

Figure 1. Body weight (A) and blood glucose levels (B). non-DM: non-diabetic mice, T2DM: type 2 diabetic mice treated with vehicle (0.2% CMC-Na), Lina 3: T2DM mice treated with 3 mg/kg linagliptin, Lina 30: T2DM mice treated with 30 mg/kg linagliptin. Data represent the mean ± SE ($n = 10$). $^{*}p < 0.05$ *vs.* the non-DM group.

Table 1. Bone strength properties of the femoral mid-diaphysis

	non-DM	T2DM	Lina 3	Lina 30
Maximum load (N)	18.1 ± 0.79	13.4 ± 0.45*	13.9 ± 0.34*	14.5 ± 0.41*
Breaking energy (N.mm)	4.80 ± 0.38	3.47 ± 0.37*	3.68 ± 0.28*	4.00 ± 0.25*
Stiffness (N/mm)	60.1 ± 4.51	46.9 ± 3.31*	48.7 ± 3.03*	52.2 ± 3.57*

non-DM: non-diabetic mice, T2DM: type 2 diabetic mice treated with vehicle (0.2% CMC-Na), Lina 3: T2DM mice treated with 3 mg/kg linagliptin, Lina 30: T2DM mice treated with 30 mg/kg linagliptin. Data represent the mean ± SE ($n = 10$). $^{*}p < 0.05$ *vs.* the non-DM group.

Table 2. BMD of whole femur and tibia

	non-DM	T2DM	Lina 3	Lina 30
Whole femur				
Cortical BMD (mg/cm^3)	974 ± 13.7	871 ± 12.8*	881 ± 13.4*	886 ± 12.3*
Trabecular BMD (mg/cm^3)	623 ± 14.9	483 ± 13.5*	499 ± 13.9*	511 ± 15.5*
Total BMD (mg/cm^3)	917 ± 13.2	745 ± 15.4*	754 ± 13.0*	778 ± 12.5*
Whole tibia				
Cortical BMD (mg/cm^3)	998 ± 10.1	855 ± 7.86*	860 ± 11.9*	868 ± 7.50*
Trabecular BMD (mg/cm^3)	664 ± 7.63	487 ± 16.0*	434 ± 17.8*	512 ± 16.1*
Total BMD (mg/cm^3)	898 ± 12.9	786 ± 8.96*	787 ± 11.6*	799 ± 14.4*

BMD: bone mineral density, non-DM: non-diabetic mice, T2DM: type 2 diabetic mice treated with vehicle (0.2% CMC-Na), Lina 3: T2DM mice treated with 3 mg/kg linagliptin, Lina 30: T2DM mice treated with 30 mg/kg linagliptin. Data represent the mean ± SE ($n = 10$). $^{*}p < 0.05$ *vs.* the non-DM group.

(Table 2). On the other hand, no significant differences in BMD were observed after treatment with linagliptin compared to that in the T2DM group.

3.4. Serum biochemical markers

Serum osteocalcin levels were significantly lower (45%) and serum TRAP levels were significantly higher (70%) in the T2DM group than in the non-DM group (Figure 2). No differences were observed both serum osteocalcin levels and serum TRAP levels in the Lina 3 group compared with the T2DM group. However, in the Lina 30 group, serum osteocalcin levels significantly increased (55%), whereas serum TRAP levels significantly decreased (38%) compared with the T2DM group values.

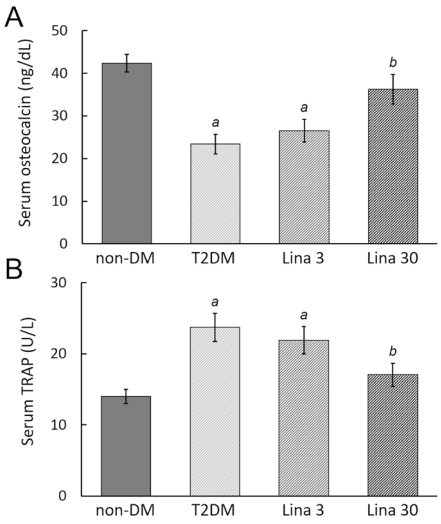

Figure 2. Serum biochemical markers (A: osteocalcin, B: TRAP). non-DM: non-diabetic mice, T2DM: type 2 diabetic mice treated with vehicle (0.2% CMC-Na), Lina 3: T2DM mice treated with 3 mg/kg linagliptin, Lina 30: T2DM mice treated with 30 mg/kg linagliptin, TRAP: tartrate-resistant acid phosphatase-5b. Data represent the mean ± SE (n = 10). [a]$p < 0.01$ *vs.* the non-DM group, [b]$p < 0.01$ *vs.* the T2DM group.

3.5. Bone histomorphometric evaluation

Bone formation and bone resorption parameters are shown in Figure 3. Compared to those in the non-DM group, in the T2DM mice, the bone formation parameters OV/BV, OS/BS, and Ob.S/BS significantly decreased by approximately 39%, 59%, and 79%, respectively, whereas ES/BS, Oc.S/BS, and N.Oc/BS significantly increased by approximately 94%, 96%, and 84%, respectively. However, OV/BV, OS/BS, and Ob.S/BS (44%, 81%, and 70%, respectively) were significantly increased in the Lina 30 group compared with the T2DM groups. Moreover, ES/BS, Oc.S/BS, and N.Oc/BS (40%, 33%, and 34%, respectively) were significantly decreased in the Lina 30 group compared with those in the T2DM group. Trabecular bone structural parameters are shown in Figure 4. In the T2DM mice showed significantly reduced BV/TV (55%), Tb.Th (35%), and Tb.N (47%) relative to those in the non-DM group. Furthermore, the values of these bone structural parameters in the Lina 30 group were significantly higher than those in the T2DM group. Figure 5 shows typical microphotographs of the proximal tibia metaphysis using Villanueva Bone Stain. These images confirmed the marked decrease in bone volume, trabecular thickness, and osteoid volume and an increased eroded surface in the T2DM group compared with the non-DM group. In contrast, the Lina 30 group showed markedly increased bone volume, osteoid volume, and trabecular thickness compared with the T2DM group.

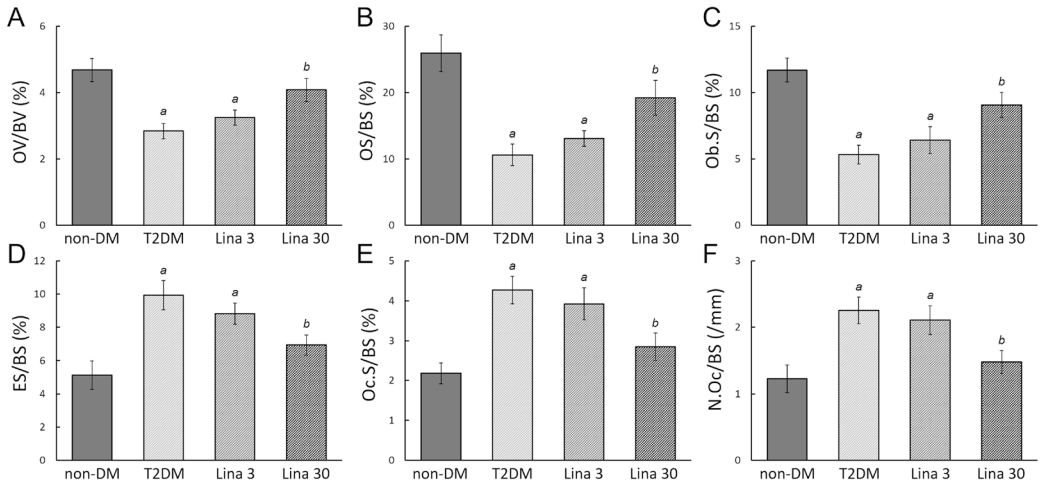

Figure 3. Bone formation parameters (A: osteoid volume [OV/BV], B: osteoid surface [OS/BS], C: osteoblast surface [Ob.S/BS], D: eroded surface [ES/BS], E: osteoclast surface [Oc.S/BS], F: osteoclast number [N.Oc/BS]) according to bone histomorphometry of the proximal tibia metaphysis. non-DM: non-diabetic mice, T2DM: type 2 diabetic mice treated with vehicle (0.2% CMC-Na), Lina 3: T2DM mice treated with 3 mg/kg linagliptin, Lina 30: T2DM mice treated with 30 mg/kg linagliptin. Data represent the mean ± SE ($n = 10$). [a]$p < 0.01$ *vs.* the non-DM group, [b]$p < 0.01$ *vs.* the T2DM group.

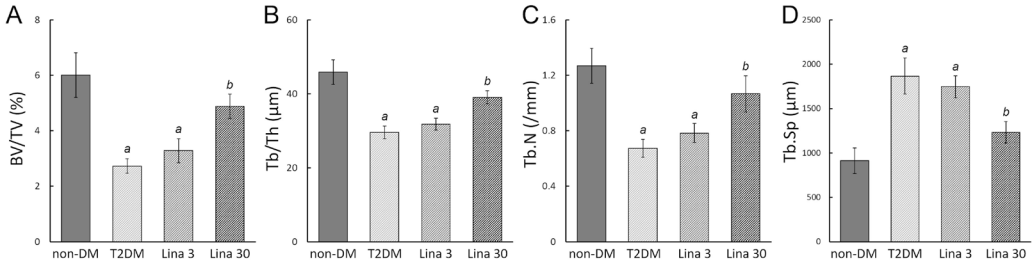

Figure 4. Trabecular bone structural parameters (A: bone volume per tissue volume [BV/TV], B: trabecular thickness [Tb.Th], C: trabecular number [Tb.N], D: trabecular separation [Tb.Sp]) according to the bone histomorphometry of the proximal tibia metaphysis. non-DM: non-diabetic mice, T2DM: type 2 diabetic mice treated with vehicle (0.2% CMC-Na), Lina 3: T2DM mice treated with 3 mg/kg linagliptin, Lina 30: T2DM mice treated with 30 mg/kg linagliptin. Data represent the mean ± SE ($n = 10$). $^{a}p < 0.05$ *vs.* the non-DM group, $^{b}p < 0.05$ *vs.* the T2DM group.

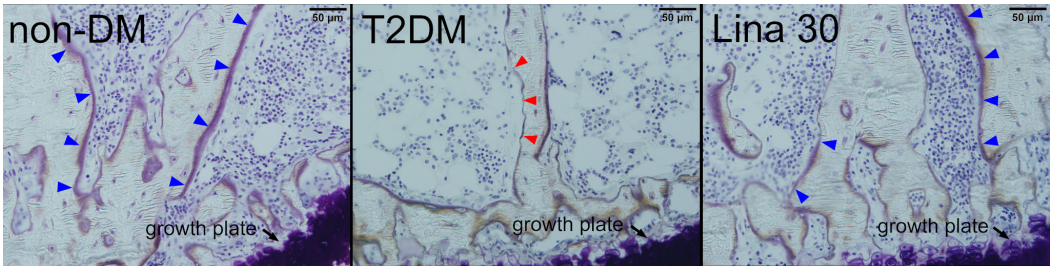

Figure 5. Typical micrographs of slices assessed by bone histomorphometry (Villanueva Bone Stain). The osteoid surface and eroded surface are indicated by blue and red arrows, respectively. non-DM: non-diabetic mice, T2DM: type 2 diabetic mice treated with vehicle (0.2% CMC-Na), Lina 30: T2DM mice treated with 30 mg/kg linagliptin.

4. Discussion

GIP and GLP-1 are produced by K cells mainly present in the upper small intestine and L cells predominantly present in the lower small intestine, respectively. They are secreted into the blood following dietary intake, and promote insulin secretion from pancreatic β-cells and suppress postprandial blood glucose elevation (*19*). A previous study demonstrated that the actions of GIP and GLP-1 are reduced in patients with T2DM compared to those in healthy individuals (*20,21*). In addition to their roles in glucose metabolism, GIP and GLP-1 have various physiological actions. GIP directly acts on adipocytes, promotes the uptake of glucose and fatty acids, and induces fat accumulation (*22*). GLP-1 reportedly delays gastric emptying in the gastrointestinal tract (*23*) and suppresses appetite *via* the central nervous system (*24*). In addition to these actions, GIP and GLP-1 may be involved in the regulation of bone metabolism (*15,16*). DPP-4 inhibitors inactivate the incretin-degrading enzyme DPP-4 and exert antidiabetic effects by increasing the plasma concentrations of both GIP and GLP-1 (*25*). Several clinical studies have assessed incretin-related drug use and the risk of fracture. A meta-analysis of randomized controlled trials revealed a reduced risk of fracture associated with the use of GLP-1 receptor agonists (*26*) and DPP-4 inhibitors (*27*) in the treatment of T2DM patients. Conversely, a retrospective cohort study showed that even long-term use of DPP-4 inhibitors failed to affect the risk of fracture (*28*). However, the mechanism by which increased incretin action through DPP-4 inhibitors affects bone metabolism remains unclear.

In this study, we used T2DM mice, exhibiting obesity and hyperglycemia induced by marked overeating due to a deficiency of the leptin receptor (*29*). The T2DM mice in this study exhibited significant hyperglycemia that persisted throughout the 12-week experiment. Further, daily administration of linagliptin reduced the mean blood glucose levels in the T2DM mice in a dose-dependent manner, but not significantly. The results of the weak hypoglycemic effect of linagliptin on T2DM mice are consistent with those reported previously in non-diabetic mice (*30*), streptozotocin-induced diabetic mice (*31,32*), and T2DM mice (*33,34*). Importantly, the administration of linagliptin significantly increased serum active GLP-1 concentration in mice without exerting a clear hypoglycemic effect (*30,32*). Furthermore, the T2DM mice showed significantly decreased bone strength and BMD in the lower limb bones, with a significant decrease in serum osteocalcin levels and a significant increase in serum TRAP levels. These results indicate that bone fragility in T2DM mice is caused by decreased bone formation and increased bone resorption. Linagliptin administration did not significant affect the decreased bone strength and BMD in T2DM mice. However,

it significantly suppressed both decreased serum osteocalcin levels and increased serum TRAP levels in T2DM mice. Based on biochemical marker analysis, it can be suggested that linagliptin may suppress both decreased bone formation and increased bone resorption induced by T2DM. However, although the biochemical indices of bone metabolism are relatively sensitive indicators of bone turnover, they do not reflect changes in bone density and microstructure (*35*). Bone histomorphometry is a method used to study histology and quantitatively evaluate the bone remodeling process. Consequently, a combination of bone histomorphometry and biochemical analysis will serve as a powerful tool to assess the changes in bone morphology and metabolism. Therefore, in this study, bone histomorphometry was performed to assess changes in bone remodeling and microstructure attributable to linagliptin administration. T2DM mice showed significantly decreased BV/TV, Tb.Th, and Tb.N and significantly increased Tb.Sp of the proximal tibial trabecular bone, exhibiting rarefaction of trabecular bone owing to the onset of T2DM. In contrast, the deterioration of bone microstructural parameters observed in the T2DM mice improved following linagliptin administration. Furthermore, these mice showed significantly decreased OV/BV, OS/BS, and Ob.S/BS. Notably, OS/BS and OV/BV are indicators of the ratio of uncalcified bone volume, whereas Ob.S/BS is considered a marker of osteoblast activity (*36*). Furthermore, the T2DM mice showed significantly increased ES/BS, which indicates the ratio of the bone surface where bone resorption by osteoclasts was induced. This increase in ES/BS was accompanied by an increase in Oc.S/BS and N.Oc/BS, indicating the ability of osteoclast differentiation. Conversely, linagliptin administration significantly suppressed the decreased OV/BV, OS/BS, and Ob.S/BS as well as increased ES/BS, Oc.S/BS, and N.Oc/BS in T2DM mice. These bone histological findings revealed that linagliptin ameliorates the deterioration of the trabecular bone microstructure by suppressing both decreased bone formation and increased bone resorption caused by T2DM. The mechanisms by which GIP and GLP-1 affect osteoblast and osteoclast functions remain unknown. In a previous study, it was reported that GIP receptors are also present in osteoblasts and that GIP suppresses the apoptosis of osteoblasts (*15*). In addition, the presence of GIP receptors in osteoclasts has been revealed, and GIP has been shown to directly suppress bone resorption activity increased by the parathyroid hormone (*16*). In fact, GIP receptor-deficient mice exhibit a significant decrease in the bone formation rate and an increase in the number of osteoclasts (*15*). In contrast, GIP-overexpressing transgenic mice showed significantly higher BMD, demonstrating an increase in serum osteocalcin levels and a decrease in the levels of the bone resorption marker pyridinoline (*37*). Furthermore, it has been reported that compared to wild-type mice, GLP-1 receptor-deficient mice exhibit reduced bone strength and decreased BMD, accompanied by suppression of bone formation and enhancement of bone resorption (*38*). These previous reports and our findings in this study indicate that an increase in GIP and GLP-1 may be responsible for both enhanced bone formation and suppressed bone resorption induced by linagliptin administration. To our knowledge, this is the first study to provide data supporting the phenomenon of linagliptin-induced enhanced bone formation and suppressed bone resorption from the perspective of bone histomorphology.

In conclusion, in this study, we examined the effects of linagliptin on bone fragility in obese T2DM mice. Linagliptin administration suppressed both decreased serum osteocalcin levels and increased serum TRAP levels in T2DM mice. In addition, in terms of histomorphological changes, linagliptin suppressed both decreased bone formation by osteoblasts and increased bone resorption by osteoclasts due to T2DM. Furthermore, linagliptin ameliorated rarefaction in the trabecular bone microstructure in T2DM mice. Based on the above results, this study showed that linagliptin improves trabecular bone microstructure by suppressing both decreased bone formation and increased bone resorption induced by T2DM. These novel findings pertaining to the effects of DPP-4 inhibitors on bone metabolism regulation will be a powerful tool for selecting optimal oral hypoglycemic agents based on the consideration of not only glucose metabolism but also bone fragility in patients with T2DM.

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Conflict of Interest: The authors have no conflict of interest to disclose.

References

1. Raisz LG. Physiology and pathophysiology of bone remodeling. Clin Chem. 1999; 45:1353-1358.
2. Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. Science. 2000; 289:1508-1514.
3. Heilmeier U, Patsch JM. Diabetes and bone. Semin Musculoskelet Radiol. 2016; 20:300-304.
4. Hothersall EJ, Livingstone SJ, Looker HC, Ahmed SF, Cleland S, Leese GP, Lindsay RS, McKnight J, Pearson D, Philip S, Wild SH, Colhoun HM. Contemporary risk of hip fracture in type 1 and type 2 diabetes: a national registry study from Scotland. J Bone Miner Res. 2014; 29:1054-1060.
5. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 2007; 166:495-505.
6. Melton LJ 3rd, Leibson CL, Achenbach SJ, Therneau

TM, Khosla S. Fracture risk in type 2 diabetes: update of a population-based study. J Bone Miner Res. 2008; 23:1334-1342.
7. Weber DR, Haynes K, Leonard MB, Willi SM, Denburg MR. Type 1 diabetes is associated with an increased risk of fracture across the life span: a population-based cohort study using The Health Improvement Network (THIN). Diabetes Care. 2015; 38:1913-1920.
8. Watts NB. Adverse bone effects of medications used to treat non-skeletal disorders. Osteoporos Int. 2017; 28:2741-2746.
9. Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, Kravitz BG, Lachin JM, O'Neill MC, Zinman B, Viberti G. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. N Engl J Med. 2006; 355:2427-2443.
10. Yaturu S, Bryant B, Jain SK. Thiazolidinedione treatment decreases bone mineral density in type 2 diabetic men. Diabetes Care. 2007; 30:1574-1576.
11. Meier C, Kraenzlin ME, Bodmer M, Jick SS, Jick H, Meier CR. Use of thiazolidinediones and fracture risk. Arch Intern Med. 2008; 168:820-825.
12. Nissen SE, Nicholls SJ, Wolski K, Nesto R, Kupfer S, Perez A, Jure H, De Larochellière R, Staniloae CS, Mavromatis K, Saw J, Hu B, Lincoff AM, Tuzcu EM. Comparison of pioglitazone *vs* glimepiride on progression of coronary atherosclerosis in patients with type 2 diabetes: the PERISCOPE randomized controlled trial. JAMA. 2008; 299:1561-1573.
13. Lecka-Czernik B, Gubrij I, Moerman EJ, Kajkenova O, Lipschitz DA, Manolagas SC, Jilka RL. Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARgamma 2. J Cell Biochem. 1999; 74:357-371.
14. Kanda J, Izumo N, Kobayashi Y, Onodera K, Shimakura T, Yamamoto N, Takahashi HE, Wakabayashi H. Effect of the antidiabetic agent pioglitazone on bone metabolism in rats. J Pharmacol Sci. 2017; 135:22-28.
15. Tsukiyama K, Yamada Y, Yamada C, Harada N, Kawasaki Y, Ogura M, Bessho K, Li M, Amizuka N, Sato M, Udagawa N, Takahashi N, Tanaka K, Oiso Y, Seino Y. Gastric inhibitory polypeptide as an endogenous factor promoting new bone formation after food ingestion. Mol Endocrinol. 2006; 20:1644-1651.
16. Zhong Q, Itokawa T, Sridhar S, Ding KH, Xie D, Kang B, Bollag WB, Bollag RJ, Hamrick M, Insogna K, Isales CM. Effects of glucose-dependent insulinotropic peptide on osteoclast function. Am J Physiol Endocrinol Metab. 2007; 292:E543-E548.
17. Kern M, Klöting N, Niessen HG, Thomas L, Stiller D, Mark M, Klein T, Blüher M. Linagliptin improves insulin sensitivity and hepatic steatosis in diet-induced obesity. PLoS One. 2012; 7:e38744.
18. Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res. 2013; 28:2-17.
19. Gautier JF, Choukem SP, Girard J. Physiology of incretins (GIP and GLP-1) and abnormalities in type 2 diabetes. Diabetes Metab. 2008; 34:S65-S72.
20. Nauck M, Stöckmann F, Ebert R, Creutzfeldt W. Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. Diabetologia. 1986; 29:46-52.
21. Holst JJ, Knop FK, Vilsbøll T, Krarup T, Madsbad S. Loss of incretin effect is a specific, important, and early characteristic of type 2 diabetes. Diabetes Care. 2011; 34:S251-S257.
22. Miyawaki K, Yamada Y, Ban N, *et al.* Inhibition of gastric inhibitory polypeptide signaling prevents obesity. Nat Med. 2002; 8:738-742.
23. Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. Nat Med. 1996; 2:1254-1258.
24. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature. 1996; 379:69-72.
25. Eto T, Inoue S, Kadowaki T. Effects of once-daily teneligliptin on 24-h blood glucose control and safety in Japanese patients with type 2 diabetes mellitus: a 4-week, randomized, double-blind, placebo-controlled trial. Diabetes Obes Metab. 2012; 14:1040-1046.
26. Su B, Sheng H, Zhang M, Bu L, Yang P, Li L, Li F, Sheng C, Han Y, Qu S, Wang J. Risk of bone fractures associated with glucagon-like peptide-1 receptor agonists' treatment: a meta-analysis of randomized controlled trials. Endocrine. 2015; 48:107-115.
27. Monami M, Dicembrini I, Antenore A, Mannucci E. Dipeptidyl peptidase-4 inhibitors and bone fractures: a meta-analysis of randomized clinical trials. Diabetes Care. 2011; 34:2474-2476.
28. Driessen JH, van den Bergh JP, van Onzenoort HA, Henry RM, Leufkens HG, de Vries F. Long-term use of dipeptidyl peptidase-4 inhibitors and risk of fracture: A retrospective population-based cohort study. Diabetes Obes Metab. 2017; 19:421-428.
29. Guest PC, Rahmoune H. Characterization of the *db/db* mouse model of type 2 diabetes. Methods Mol Biol. 2019; 1916:195-201.
30. Terawaki Y, Nomiyama T, Kawanami T, Hamaguchi Y, Takahashi H, Tanaka T, Murase K, Nagaishi R, Tanabe M, Yanase T. Dipeptidyl peptidase-4 inhibitor linagliptin attenuates neointima formation after vascular injury. Cardiovasc Diabetol. 2014; 13:154.
31. Ide M, Sonoda N, Inoue T, Kimura S, Minami Y, Makimura H, Hayashida E, Hyodo F, Yamato M, Takayanagi R, Inoguchi T. The dipeptidyl peptidase-4 inhibitor, linagliptin, improves cognitive impairment in streptozotocin-induced diabetic mice by inhibiting oxidative stress and microglial activation. PLoS One. 2020; 15:e0228750.
32. Zhang Y, Fava GE, Wu M, Htun W, Klein T, Fonseca VA, Wu H. Effects of linagliptin on pancreatic α cells of type 1 diabetic mice. J Endocr Soc. 2017; 1:1224-1234.
33. Takahashi H, Nomiyama T, Terawaki Y, Horikawa T, Kawanami T, Hamaguchi Y, Tanaka T, Motonaga R, Fukuda T, Tanabe M, Yanase T. Combined treatment with DPP-4 inhibitor linagliptin and SGLT2 inhibitor empagliflozin attenuates neointima formation after vascular injury in diabetic mice. Biochem Biophys Rep. 2019; 18:100640.
34. Michurina SV, Ishenko IJ, Klimontov VV, Archipov SA, Myakina NE, Cherepanova MA, Zavjalov EL, Koncevaya GV, Konenkov VI. Linagliptin alleviates fatty liver disease in diabetic *db/db* mice. World J Diabetes. 2016; 7:534-546.

35. Vidal B, Pinto A, Galvão MJ, Santos AR, Rodrigues A, Cascão R, Abdulghani S, Caetano-Lopes J, Ferreira A, Fonseca JE, Canhao H. Bone histomorphometry revisited. Acta Reumatol Port. 2012; 37:294-300.
36. Recker RR, Kimmel DB, Dempster D, Weinstein RS, Wronski TJ, Burr DB. Issues in modern bone histomorphometry. Bone. 2011; 49:955-964.
37. Xie D, Zhong Q, Ding KH, Cheng H, Williams S, Correa D, Bollag WB, Bollag RJ, Insogna K, Troiano N, Coady C, Hamrick M, Isales CM. Glucose-dependent insulinotropic peptide-overexpressing transgenic mice have increased bone mass. Bone. 2007; 40:1352-1360.
38. Yamada C, Yamada Y, Tsukiyama K, Yamada K, Udagawa N, Takahashi N, Tanaka K, Drucker DJ, Seino Y, Inagaki N. The murine glucagon-like peptide-1 receptor is essential for control of bone resorption. Endocrinology. 2008; 149:574-549.

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Original Article

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Haemophagocytic lymphohistiocytosis in human immunodeficiency virus: a systematic review of literature

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SUMMARY Diagnosis and management of hemophagocytic lymphohistiocytosis (HLH) in patients with human immunodeficiency virus (HIV) infection are scarcely described in the published literature. The aim of this systematic review was to delineate the triggers of HLH in patients with HIV and understand the role of steroids in the management. We conducted a comprehensive search of English medical literature *via* the Medline / PubMed database using different synonyms of "HIV" AND "HLH". The review was registered in PROSPERO (CRD42018099987). The titles and abstracts of the 185 articles between January 1986 and April 2018 were reviewed. The final analysis was done from 42 articles with 52 patients. HLH was associated with malignancy in 17 patients, while infection was found in 25 patients. No cause was identified in eight patients, out of which four had acute HIV infection. Death was reported in 21 patients. Presence of either malignancy (p = 0.051) or opportunistic infection (p = 0.69) was not associated with increased chances of death by univariate analysis. A total of 26 patients were treated with steroids, while etoposide was used in only four patients. Administration of steroids as a treatment of HLH was associated with more chances of death (p = 0.048). Malignancy and Opportunistic infections are important triggers for HLH in patients with HIV. Acute HIV by itself can act as a trigger for HLH. Evidence on the use of steroids as a treatment of HLH in patients with HIV is not convincing.

Keywords infections, malignancy, steroids

1. Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a hyper-immune condition characterized primarily by fever, bi-cytopenia, hyper-ferritinemia, and hemophagocytosis (*1*). HLH is usually triggered by malignancy, infection, drugs or auto-immune conditions. With the increase in knowledge and understanding of the disease, there is an increase in the number of reported cases of HLH. HLH in human immunodeficiency virus (HIV) infected patients is a rarely described condition in published literature. It is postulated that HLH in HIV patients can be either be due to a co-existent malignancy/ infection or due to uncontrolled replication of HIV itself (*1*). The diagnosis and management of HLH in HIV patients are usually made according to the HLH 2004 criteria and management protocol (*2*). It is tricky to diagnose HLH in HIV infected patients as there is a vast overlap in the clinical and laboratory manifestations of HLH and advanced HIV disease. The treatment of HLH is immunosuppressive therapy (steroids, etoposide and cyclosporine) (*3*). The decision to administer immunosuppressive therapy in an already immunocompromised patient (HIV) is a difficult decision to make. The aim of this systematic review (SR) was to delineate the number of reported cases of HLH, their triggering factors, treatment and outcome in patients with HIV.

2. Methodology

2.1. Search strategy

We first searched for any existing SR on HIV-HLH. Although narrative reviews were present, no SR was identified. We conducted a comprehensive search of English medical literature between January 1986 and April 2018 *via* the Medline/PubMed database. We used ("HIV" OR "human immunodeficiency virus" OR "AIDS" OR "acquired immune deficiency syndrome")

AND ("HLH" OR "haemophagocytic syndrome" OR "hemophagocytic syndrome" OR "haemophagocytic lymphohistiocytosis" OR "hemophogocytic lymphohistiocytosis" OR "hemophagocytosis" OR "haemophagocytosis") as the search terms.

2.2. Inclusion and exclusion criteria

All case studies and case series (prospective or retrospective), where individual patient data were available were included. Those patients where at-least four of the eight HLH criteria were fulfilled were included. Those articles where any of the individual patient data of the following parameters were not present in the paper were excluded: age, sex, details of antiretroviral therapy, CD4 count, the trigger for HLH (malignancy/infection/others), details of anti-HLH treatment (steroids/etoposide) and outcome (discharged/death).

2.3. Study selection

The titles and abstracts of the 185 articles were reviewed independently by two authors (FF and NG) to find cases with both HIV and HLH (Figure 1). In case of any disagreement on study selection between the two authors, the third author (AM) was consulted. Nineteen articles were excluded because they were not in English. A total of 48 articles were excluded because the patient did not have either HIV or HLH or both. Seventeen articles were excluded because they were non-case studies (narrative reviews/perspectives). A total of 101 articles were included for analysis. Full articles were not available for ten articles. Out of the 91 remaining articles, 49 articles were excluded because they did not meet or did not mention the fulfilment of the HLH 2004 diagnostic criteria or did not meet the inclusion criteria. Individual patient data were then extracted from 42 articles (number of patients: 52) (Figure 1) (*4-45*).

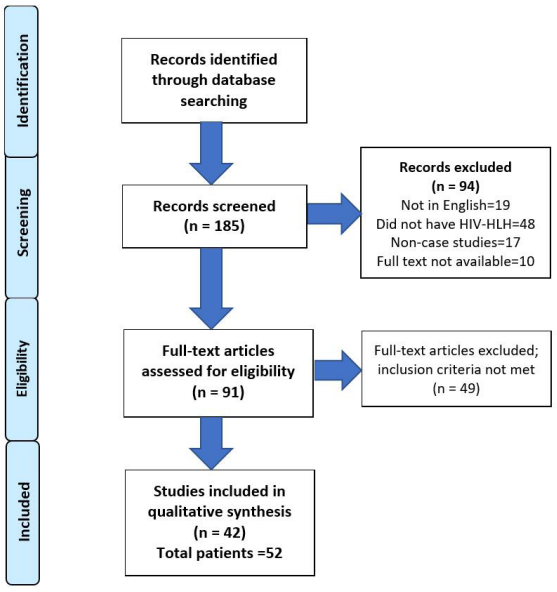

Figure 1. PRISMA flow diagram of the literature search and eligibility for cases with HIV and HLH.

2.4. Data extraction

The following study and patient characteristics were extracted on a pre-designed spreadsheet: age, sex, acute/chronic HIV, treatment-naive/experienced, the regimen of anti-retroviral therapy (ART), the trigger for HLH (malignancy/infection/others), the details of criteria fulfilled, anti-HLH treatment (steroids/etoposide) and the outcome (discharged/death).

2.5. Statistical analysis

Continuous data were presented as mean ± standard deviation (for normally distributed variables) or median and interquartile range when standard deviation was more than 50% of the mean (extremes of data). The frequency of categorical variables was expressed in numbers and percentage. All analyses were done using STATA version 13. PROSPERO registration number: CRD42018099987.

3. Results

A total of 52 cases of HIV and HLH were reviewed. Out of these, 42 (80.8%) were male. The mean age was 38.2 +/– 14.2 years. The median CD4 count at the time of diagnosis of HIV with HLH was 41/µL (IQR: 8-94/µL). Only 17 patients were on antiretroviral therapy (ART) at the time of diagnosis. Of these, a total of seven patients were virologically suppressed at the time of diagnosis of HLH. A total of 26 patients were treated with steroids, while etoposide was used in only four patients (*6-8,8,11-14,16,18,20,23,24,27,30-37,41,44*). Death was reported in 21 patients (*6,12-15,20,27,29,31,36-41,44*). Steroids, when given as a part of HLH management, was associated with increased mortality ($p = 0.048$).

The details of the fulfilment of HLH 2004 criteria is summarized in Table 1 and 2. HLH was associated with malignancy in 17 patients (*6-8,11,12,15,29-31,35-37,39,44*). Infection was associated with HLH in 25 patients (*4,9,10,13,14,17-20,24,26,28,32,33,38,41-43*). In two patients, both malignancy and infection were attributed as the cause for HLH (Table 3) (*34,40*). Some studies reported the frequency of infections or malignancies in patients with HIV and HLH, but they were excluded from the analysis as individual patient data were not available in these studies (*47,48*). When the data from these studies were also combined in the analysis, out of 174 cases with HIV and HLH, 85 had malignancy, 72 had infections, six had both and no cause was identified in 11 patients (*47,48*). Presence of

either malignancy ($p = 0.051$) or opportunistic infection ($p = 0.69$) was not associated with increased chances of death.

No cause was identified in eight patients, out of which four had acute HIV infection (*5,21-23*). All eight patients had a significantly high viral load. Only one patient was on ART at the time of diagnosis (*27*). This patient was diagnosed with HLH as a manifestation of immune reconstitution inflammatory syndrome (IRIS) and died soon after presentation. Rest of the seven patients were started on antiretroviral treatment. Three patients received adjunctive steroids also (*16,23,27*). All seven of these patients improved.

Table 1. Mean/Median of laboratory parameters in patients with HIV & HLH

Parameters	Number[#]	Mean/Median	SD/IQR
Ferritin[*]	43	15,000	4,392-31,435
Haemoglobin	52	8.12	2.68
Total leucocyte count[*]	37	2,540	1150-3750
Platelet count[*]	51	41,000	12,000-80,000
Triglyceride levels[*]	33	312	257-431
Fibrinogen levels[*]	14	168	90-273

[#]Number of patients where the data was available. [*]Median with interquartile range.

Table 2. HLH criteria in patients with HIV & HLH

HLH 2004 diagnostic criteria	Frequency
Hyperferritenemia	42/43
Anaemia	40/52
Fever	52/52
Leucopenia	22/30
Thrombocytopenia	47/51
Hyper TG	24/33
BM showing phagocytosis	39/45
Low NK	2/5
Splenomegaly	42/46
Hypofibrinogenemia	6/15
Increased CD25	10/11

4. Discussion

HLH in HIV, although not common, is well reported and is associated with increased mortality. In a study by Grateau *et al.*, HLH was reported in 0.6% of the patients with HIV (*46*). In a series of patients with infection-associated HLH, concomitant HIV was present in 50% of the patients (*47*). Patients with HIV are predisposed to HLH because of the hyper-inflammatory response due to the increased cytokines (*48*). Increased cytokine levels may be triggered by the opportunistic infection or malignancy or acute HIV itself (*48*). Managing HLH in the setting of HIV infection could be very challenging as treatment focuses on immunosuppression (with steroids, etoposide, *etc.*) which may complicate the course of illness in an already immunocompromised host. The current SR was done to study the profile of patients with the aim of determining the common causes associated with HLH in HIV and their outcome.

Following our search strategy, a total of 52 individual cases were analyzed. HLH was more commonly reported with infections (48%) than malignancy (33%). There was no difference in the mortality between the two groups.

No cause could be identified in 8 cases (19%); however, 4 out of these 8 had acute HIV, and all 8 had a high HIV viral load. Acute HIV is known to be associated with high levels of viraemia. HIV viraemia is associated with changes in cytokine levels (*49*). Presence of viraemia and consequent cytokine changes may have some association with the occurrence of HLH. Unlike HLH due to opportunistic infections or malignancy in HIV, HLH in patients with acute HIV had good prognosis (*21*). Most patients recovered with ART alone (*50*). The fact that viraemia may be associated with HLH and ART may have some role in the treatment of patients with HIV and HLH is suggested by the fact that only 7 out of 52 patients were virologically suppressed. Also, according to the study by Fardet *et*

Table 3. Triggers for HLH in patients with HIV

Trigger	This study	Fardet *et al.*(*48*)	Lerolle *et al.*(*47*)
Number	52	43	19
Malignancy			
Lymphoma	11	16	
Kaposi Sarcoma	6	3	
Multicentric Castleman disease		5	
Infections			
Fungal	Histoplasmosis-15, penicilliosis-1, invasive candidiasis-1, invasive aspergillosis-1	Histoplasmosis-1	
Parasitic	Leishmaniasis-1, Toxoplasmosis-2	Toxoplasmosis-2	Toxoplasmosis-2
Bacterial	Bartonellosis-1	Typhoid-1 *Pseudomonas aeruginosa*-1	*Escherichia coli*-1 *Legionella*-1
Viral	Cytomegalovirus-2, Epstein-Barr virus-1	Cytomegalovirus-2	Cytomegalovirus-1 Herpes Simplex virus 2-1
Tuberculosis	1	8	4
Combined	2		7

al., the mortality of HLH in patients with HIV was considerably higher in the pre-ART era (1981-1996) compared to the ART era (*48*). In our SR, only three patients were from the pre-ART era, one of whom died.

The diagnosis of HLH was made using the HLH 2004 criteria. According to this protocol, to make a diagnosis of HLH, five out of eight criteria should be fulfilled. Since many studies made a diagnosis of HLH prior to the protocol being published, details of individual criteria were often not quoted. Besides, CD25 levels and NK cell activity are resource-intensive, and many studies did not mention it. For this reason, we took those cases that were diagnosed as HLH and had the details of at-least four positive criteria. The most consistent clinical finding was fever (100%), followed by splenomegaly (91%). Amongst the laboratory parameters, hyper-ferritinemia (98%) and thrombocytopenia (92%) were most commonly reported. Bone marrow results were available for 45 cases out of which 39 (86%) patients showed evidence of hemophagocytosis. CD25 values were available only for 11 cases, but 10 (91%) showed increased expression.

The therapy for secondary HLH aims at treating the underlying cause and suppressing the hyper-inflammatory response with immunosuppressants such as corticosteroids (*51*). Mortality was reported in 40% of the patients. Although the consensus statement of experts of histiocyte society recommends steroids for treatment of HLH in patients with HIV, we found that use of steroids in patients with HIV and HLH was associated with higher mortality (*3*). There is a need for large scale prospective studies to understand the role of steroids in the treatment of HLH.

Limitations: Individual patient data were not available in certain large series which were eventually excluded from the analysis. Some series were excluded because they weren't in the English language or their full text could not be accessed. We used four criteria instead of five criteria to make the diagnosis of HLH to increase the sensitivity.

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References

1. Yildiz H, Van Den Neste E, Defour JP, Danse E, Yombi JC. Adult haemophagocytic lymphohistiocytosis: a review. QJM. 2020.
2. Henter JI, Horne A, Aricó M, Egeler RM, Filipovich AH, Imashuku S, Ladisch S, McClain K, Webb D, Winiarski J, Janka G. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007; 48:124-131.
3. La Rosée P, Horne A, Hines M, *et al.* Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. Blood. 2019; 133:2465-2477.
4. Loganantharaj N, Oliver B, Smith T, Jetly R, Engel L, Sanne S. Hemophagocytic lymphohistiocytosis in an HIV-positive patient with concomitant disseminated histoplasmosis. Int J STD AIDS. 2018; 29:925-928.
5. Adachi E, Koibuchi T, Imai K, Kikuchi T, Shimizu S, Koga M, Nakamura H, Iwamoto A, Fujii T. Hemophagocytic syndrome in an acute human immunodeficiency virus infection. Intern Med. 2013; 52:629-632.
6. Khagi S, Danilova O, Rauwerdink C. Hemophagocytic syndrome in a patient with human immunodeficiency virus, Epstein-Barr viremia, and newly diagnosed Hodgkin lymphoma. Clin Adv Hematol Oncol HO. 2012; 10:260-262.
7. Flew SJ, Radcliffe KW. Haemophagocytic lymphohistiocytosis complicating Hodgkin's lymphoma in an HIV-positive individual. Int J STD AIDS. 2010; 21:601-603.
8. Ramon I, Libert M, Guillaume MP, Corazza F, Karmali R. Recurrent haemophagocytic syndrome in an HIV-infected patient. Acta Clin Belg. 2010; 65:276-278.
9. Patel KK, Patel AK, Sarda P, Shah BA, Ranjan R. Immune reconstitution visceral leishmaniasis presented as hemophagocytic syndrome in a patient with AIDS from a nonendemic area: a case report. J Int Assoc Physicians AIDS Care (Chic). 2009; 8:217-220.
10. De Lavaissière M, Manceron V, Bourée P, Garçon L, Bisaro F, Delfraissy JF, Lambotte O, Goujard C. Reconstitution inflammatory syndrome related to histoplasmosis, with a hemophagocytic syndrome in HIV infection. J Infect. 2009; 58:245-247.
11. Azevedo L, Gerivaz R, Simões J, Germano I. The challenging diagnosis of haemophagocytic lymphohistiocytosis in an HIV-infected patient. BMJ Case Rep. 2015; 2015:bcr2015211817.
12. Uemura M, Huynh R, Kuo A, Antelo F, Deiss R, Yeh J. Hemophagocytic lymphohistiocytosis complicating T-cell lymphoma in a patient with HIV infection. Case Rep Hematol. 2013; 2013:687260.
13. Townsend JL, Shanbhag S, Hancock J, Bowman K, Nijhawan AE. Histoplasmosis-induced hemophagocytic syndrome: A case series and review of the literature. Open Forum Infect Dis. 2015; 2:ofv055.
14. Thoden J, Rieg S, Venhoff N, Wennekes V, Schmitt-Graeff A, Wagner D, Kern WV. Fatal hemophagocytic syndrome in a patient with a previously well-controlled asymptomatic HIV infection after EBV reactivation. J Infect. 2012; 64:110-112.
15. Price B, Lines J, Lewis D, Holland N. Haemophagocytic lymphohistiocytosis: A fulminant syndrome associated with multiorgan failure and high mortality that frequently masquerades as sepsis and shock. South Afr Med J Suid-Afr Tydskr Vir Geneeskd. 2014; 104:401-406.
16. Usman M, Thapa SD, Hadid H, Yessayan LT. HIV infection presenting proliferation of CD8+ T lymphocyte and hemophagocytic lymphohistiocytosis. Int J STD AIDS. 2016; 27:411-413.
17. Subedee A, Van Sickels N. Hemophagocytic syndrome in the setting of AIDS and disseminated histoplasmosis: Case report and a review of literature. J Int Assoc Provid AIDS Care. 2015; 14:391-397.
18. Le Joncour A, Bidegain F, Ziol M, Galicier L, Oksenhendler E, Mechai F, Boutboul D, Bouchaud O. Hemophagocytic lymphohistiocytosis associated with

Bartonella henselae infection in an HIV-infected patient. Clin Infect Dis. 2016; 62:804-806.
19. Castelli AA, Rosenthal DG, Bender Ignacio R, Chu HY. Hemophagocytic Lymphohistiocytosis secondary to human immunodeficiency virus-associated histoplasmosis. Open Forum Infect Dis. 2015; 2:ofv140.
20. Bhatia S, Bauer F, Bilgrami SA. Candidiasis-associated hemophagocytic lymphohistiocytosis in a patient infected with human immunodeficiency virus. Clin Infect Dis Off Publ Infect Dis Soc Am. 2003; 37:e161-6.
21. Park KH, Yu HS, Jung SI, Shin DH, Shin JH. Acute human immunodeficiency virus syndrome presenting with hemophagocytic lymphohistiocytosis. Yonsei Med J. 2008; 49:325-328.
22. Manji F, Wilson E, Mahe E, Gill J, Conly J. Acute HIV infection presenting as hemophagocytic lymphohistiocytosis: case report and review of the literature. BMC Infect Dis. 2017; 17:633.
23. Miyahara H, Korematsu S, Nagakura T, Iwata A, Suenobu S, Izumi T. Hemophagocytic lymphohistiocytosis in a human immunodeficiency virus-positive homosexual high school student. Pediatr Int Off J Jpn Pediatr Soc. 2007; 49:997-999.
24. Ocon AJ, Bhatt BD, Miller C, Peredo RA. Safe usage of anakinra and dexamethasone to treat refractory hemophagocytic lymphohistiocytosis secondary to acute disseminated histoplasmosis in a patient with HIV/AIDS. BMJ Case Rep. 2017; 2017:bcr2017221264.
25. Fitzgerald BP, Wojciechowski AL, Bajwa RPS. Efficacy of prompt initiation of antiretroviral therapy in the treatment of hemophagocytic lymphohistiocytosis triggered by uncontrolled human immunodeficiency virus. Case Rep Crit Care. 2017; 2017:8630609.
26. Ohkuma K, Saraya T, Sada M, Kawai S. Evidence for cytomegalovirus-induced haemophagocytic syndrome in a young patient with AIDS. BMJ Case Rep. 2013; 2013:bcr2013200983.
27. Safdar SM, Rehman JU, Samman EM, Bahabri NM. Fatal hemophagocytic syndrome as a manifestation of immune reconstitution syndrome in a patient with acquired immunodeficiency syndrome. Saudi Med J. 2013; 34:861-864.
28. Pei SN, Lee CH, Liu JW. Hemophagocytic syndrome in a patient with acquired immunodeficiency syndrome and acute disseminated penicilliosis. Am J Trop Med Hyg. 2008; 78:11-13.
29. Koizumi Y, Imadome KI, Ota Y, *et al.* Dual threat of Epstein-Barr virus: an autopsy case report of HIV-positive plasmablastic lymphoma complicating EBV-associated hemophagocytic lymphohistiocytosis. J Clin Immunol. 2018; 38:478-483.
30. Shaikh H, Shaikh S, Kamran A, Mewawalla P. Cholestatic jaundice: a unique presentation leading to the diagnosis of HLH with Hodgkin lymphoma, HIV and EBV. BMJ Case Rep. 2018; 2018:bcr2018224424.
31. Bangaru S, Strickland A, Cavuoti D, Shah N. HHV-8-associated haemophagocytic lymphohistiocytosis in a patient with advanced AIDS. BMJ Case Rep. 2017; 2017:bcr2017222382.
32. Zanotti P, Chirico C, Gulletta M, Ardighieri L, Casari S, Roldan EQ, Izzo I, Pinsi G, Lorenzin G, Facchetti F, Castelli F, Focà E. Disseminated histoplasmosis as AIDS-presentation. Case report and comprehensive review of current literature. Mediterr J Hematol Infect Dis. 2018; 10:e2018040.
33. Asanad S, Cerk B, Ramirez V. Hemophagocytic lymphohistiocytosis (HLH) secondary to disseminated histoplasmosis in the setting of acquired immunodeficiency syndrome (AIDS). Med Mycol Case Rep. 2018; 20:15-17.
34. Zorzou MP, Chini M, Lioni A, Tsekes G, Nitsotolis T, Tierris I, Panagiotou N, Rontogianni D, Harhalakis N, Lazanas M. Successful treatment of immune reconstitution inflammatory syndrome-related hemophagocytic syndrome in an HIV patient with primary effusion lymphoma. Hematol Rep. 2016; 8:6581.
35. Uneda S, Murata S, Sonoki T, Matsuoka H, Nakakuma H. Successful treatment with liposomal doxorubicin for widespread Kaposi's sarcoma and human herpesvirus-8 related severe hemophagocytic syndrome in a patient with acquired immunodeficiency syndrome. Int J Hematol. 2009; 89:195-200.
36. Cuttelod M, Pascual A, Baur Chaubert AS, Cometta A, Osih R, Duchosal MA, Cavassini M. Hemophagocytic syndrome after highly active antiretroviral therapy initiation: a life-threatening event related to immune restoration inflammatory syndrome? AIDS. 2008; 22:549-551.
37. Yates JA, Zakai NA, Griffith RC, Wing EJ, Schiffman FJ. Multicentric Castleman disease, Kaposi sarcoma, hemophagocytic syndrome, and a novel HHV8-lymphoproliferative disorder. AIDS Read. 2007; 17:596-598, 601.
38. Guillaume M-P, Driessens N, Libert M, De Bels D, Corazza F, Karmali R. Hemophagocytic syndrome associated with extracerebral toxoplasmosis in an HIV-infected patient. Eur J Intern Med. 2006; 17:503-504.
39. Preciado MV, De Matteo E, Fallo A, Chabay P, Drelichman G, Grinstein S. EBV-associated Hodgkin's disease in an HIV-infected child presenting with a hemophagocytic syndrome. Leuk Lymphoma. 2001; 42:231-234.
40. Chiu SS, Chan GC, Loong F. Epstein-Barr virus (EBV) induced hemophagocytic syndrome followed by EBV associated T/NK lymphoma in a child with perinatal human immunodeficiency virus (HIV) infection. Med Pediatr Oncol. 2001; 36:326-328.
41. Dalle JH, Dollfus C, Leverger G, Landman-Parker J, Tabone MD, Adam M, Courpotin C, Lasfargues G. Syndrome d'activation macrophagique chez l'enfant infecté par le VIH. A propos de trois cas [Hemophagocytic syndrome in children infected by HIV. Apropos of 3 cases]. Arch Pediatr. 1995; 2:442-446.
42. Dalle JH, Dollfus C, Courpotin C, Tabone AM, Landman-Parker J, Leverger G, Lasfargues G. Human immunodeficiency virus-associated hemophagocytic syndrome in children. Pediatr Infect Dis J. 1994; 13:1159.
43. Blanche P, Robert F, Dupouy-Camet J, Sicard D. Toxoplasmosis-associated hemophagocytic syndrome in a patient with AIDS: diagnosis by the polymerase chain reaction. Clin Infect Dis Off Publ Infect Dis Soc Am. 1994; 19:989-990.
44. Gérard L, Oksenhendler E. Hodgkin's lymphoma as a cause of fever of unknown origin in HIV infection. AIDS Patient Care STDs. 2003; 17:495-459.
45. Chi S, Ikezoe T, Takeuchi A, Takaoka M, Yokoyama A. Recombinant human soluble thrombomodulin is active against hemophagocytic lymphohistiocytosis associated with acquired immunodeficiency syndrome. Int J Hematol. 2013; 98:615-619.
46. Grateau G, Bachmeyer C, Blanche P, Jouanne M, Tulliez

M, Galland C, Sicard D, Séréni D. Haemophagocytic syndrome in patients infected with the human immunodeficiency virus: nine cases and a review. J Infect. 1997; 34:219-225.
47. Lerolle N, Laanani M, Rivière S, Galicier L, Coppo P, Meynard JL, Molina JM, Azoulay E, Aumont C, Marzac C, Fardet L, Lambotte O. Diversity and combinations of infectious agents in 38 adults with an infection-triggered reactive haemophagocytic syndrome: a multicenter study. Clin Microbiol Infect. 2016; 22:268.e1-8.
48. Fardet L, Lambotte O, Meynard JL, Kamouh W, Galicier L, Marzac C, de Labarthe A, Cabane J, Lebbe C, Coppo P, Molina JM, Martinez V. Reactive haemophagocytic syndrome in 58 HIV-1-infected patients: clinical features, underlying diseases and prognosis. AIDS. 2010; 24:1299-1306.
49. Norris PJ, Pappalardo BL, Custer B, Spotts G, Hecht FM, Busch MP. Elevations in IL-10, TNF-alpha, and IFN-gamma from the earliest point of HIV type 1 infection. AIDS Res Hum Retroviruses. 2006; 22:757-762.
50. Castilletti C, Preziosi R, Bernardini G, Caterini A, Gomes V, Calcaterra S, Carletti F, Capobianchi MR, Armignacco O. Hemophagocytic syndrome in a patient with acute human immunodeficiency virus infection. Clin Infect Dis. 2004; 38:1792-1793.
51. Esteban YM, de Jong JLO, Tesher MS. An overview of hemophagocytic lymphohistiocytosis. Pediatr Ann. 2017; 46:e309-313.

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Original Article

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Factors associated with symptoms of depression among pregnant women with gestational diabetes mellitus in Japan

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SUMMARY The objective of this study was to explore the factors associated symptoms of depression among pregnant women with gestational diabetes mellitus (GDM) in Japan. This cross-sectional study was conducted at a hospital in Toyota, Japan, from January 2015 to June 2016. Pregnant women who visited the hospital and were diagnosed with GDM in the second trimester were enrolled. We analyzed depression symptoms using the Centers for Epidemiological Studies Depression Scale (CES-D) and considered related factors of depression symptoms, such as dietary intake and daily walking. Dietary intake during the past month was assessed using a brief self-administered diet history questionnaire, and daily walking was assessed using an accelerometer. The prevalence rate for GDM was 8.8%, and 25 pregnant women with GDM were analyzed. The CES-D was not significantly correlated with pre-pregnancy BMI, postprandial plasma glucose, hemoglobin A1c, and the number of steps walked. In contrast, a significant negative correlation was observed between the CES-D score and intake of fish with bones, simmered fish, pickles, green leaves, mushrooms, and green tea. Furthermore, a significant positive correlation was found between Coke® and CES-D scores. For nutrient intake, a significant negative correlation was found between the CES-D score and vitamin K, folate, and β-carotene levels. The present study suggests that depression symptoms among pregnant women with GDM in the second trimester may be associated with diet.

Keywords gestational diabetes mellitus, depressive symptom, nutrition

1. Introduction

The link between diabetes and depression is well known (*1*). The position statement of routine psychosocial care for individuals with diabetes was published by the American Diabetes Association (*2*). A review indicated that the odds ratio of depression in the diabetic group was twice than that of the nondiabetic group, and the prevalence of depression in women with diabetes was significantly higher than that in men with diabetes (*3*). A recent meta-analysis also revealed that the prevalence of depression was 34% in 134,332 females with type 2 diabetes mellitus (*4*), which was 1.48 times more than what is seen in males. Although the age-matched population composition was different, we must also pay attention to depression for women with gestational diabetes mellitus (GDM).

GDM is a major perinatal complication (*5*). In 2010, the International Association of Diabetes and Pregnancy Study Groups established universal criteria based on the Hyperglycemia and Adverse Pregnancy Outcome study in 2008 (*6*). The prevalence of GDM has been reported to be approximately 8% in Japan (*7*). Diet and exercise therapy should be attempted first, with drug therapy added if diet and exercise alone cannot sufficiently correct glucose tolerance. Summary and recommendations for dietetic treatment for GDM were provided by the International Workshop Conference on GDM sponsored by the American Diabetes Association (*8*). The American College of Obstetricians and Gynecologists recommends at least 30 min of moderate intensity physical activity on most, if not all, days of the week (*9*). In a previous study, we reported that daily walking improves postprandial plasma glucose (PPG) in women with GDM (*10*). However, few studies have examined depression and pregnant women with GDM and suggest only the aforementioned relationship (*11-16*). For our second step, we analyzed factors associated with depression symptoms using the Center for Epidemiological Studies Depression (CES-D) Scale

(*17,18*) among pregnant women with GDM. It was a novel approach to focus on effective nursing care for pregnant women with GDM.

2. Materials and Methods

2.1. Setting and participants

As part of the longitudinal study (*10*), this cross-sectional study was conducted at TOYOTA Memorial Hospital in Toyota, Japan, from January 2015 to June 2016. In the present study, we focused on the symptoms of depression experienced during the second trimester. Inclusion criteria were as follows: (*i*) pregnant women who had regular prenatal checkups at the hospital and were diagnosed with GDM in the second trimester, (*ii*) pregnant women who were over 20 years old, and (*iii*) pregnant women who could complete a questionnaire. Exclusion criteria were as follows: (*i*) pregnant women with type 1 or 2 diabetes; (*ii*) pregnant women who were used steroids; (*iii*) pregnant women who were admitted to the hospital; (iv) pregnant women who had a pregnancy complicated by fetal disorders; and (*v*) pregnant women who had regular exercise with high intensity.

2.2. Procedures

Pregnant women with GDM in the second trimester were recruited for this study while awaiting routine examination in an outpatient hospital room; questionnaires assessed depressive symptoms and dietary intake. Background information, including maternal age, gestational week, pre-pregnancy body mass index (BMI), maternal history, complications of pregnancy, and laboratory biochemical data were obtained from medical charts. Daily walking was measured using an accelerometer (Lifecorder EX; Suzuken Co Ltd, Nagoya, Japan) (*19*), as previously reported (*10*). Participants attached the accelerometer to the waistbands of their skirts or pants, as instructed at the time of recruitment by investigators. The accelerometers assessed daily walking for 8 weeks (at least > 4 weeks), excluding sleeping and bathing.

The research ethics committees of the Kyoto University School of Medicine (No. E2279) and the hospital approved the study procedures and protocol. The participants received explanations of the aim and contents in the study, the voluntary nature of their participation, the risks and benefits of their participation, and privacy considerations. Written informed consent was obtained from all participants.

2.3. Measurements

Depressive symptoms were assessed using a Japanese version (*20*) of the CES-D scale (*19*). It is a self-administered questionnaire consisting of 20 items. The participants indicated on a four-point scale how often they experienced each item in the past week from 0 (rarely) to 3 (most). The total score ranged from 0 to 60. Based on a validation study, a CES-D score of > 16 points is defined as depression.

Dietary intake during the past month was assessed using a brief self-administered diet history questionnaire (BDHQ), and the amount of daily intake for 50 foods and selected nutrients (*21,22*) was calculated from the BDHQ.

Daily walking was assessed using the accelerometer, which measured steps, PA-related intensity, and PA-related energy expenditure (PAEE), as previously reported (*10*).

2.4. Statistical analyses

The Spearman correlation coefficient was performed using Statistical Package Social Sciences version 25.0 (SPSS Inc., Chicago, IL, USA). Spearman correlation coefficients < 0.05 were considered statistically significant.

3. Results

3.1. Participants

During the 18-month recruitment period, 776 pregnant women in the first trimester had regular prenatal checkups at the hospital. Among the 716 pregnant women (92.3%) who underwent blood work, 245 pregnant women (34.2%) had PPG > 100 mg/dL in the first trimester or blood glucose > 140 mg/dL in a 50-g glucose challenge test in the second trimester. The 75-g oral glucose tolerance test (OGTT) was performed in 184 pregnant women (75.1%) to diagnose GDM based on the following standard criteria: blood glucose ≥ 92 mg/dL at fasting, ≥ 180 mg/dL at 60 min after loading, or ≥ 153 mg/dL at 120 min after loading. Clinical GDM was diagnosed in 63 patients. The prevalence rate of GDM in the hospital during the study period was 8.8%. An additional 10 pregnant women who had already been diagnosed with GDM with 75-g OGTT were referred to the hospital from nearby private clinics. Among the 73 pregnant women with GDM, 32 (43.8%) participated in the study. Seven pregnant women with GDM were excluded from the analysis due to the lack of CES-D data. Finally, we analyzed the data of 25 pregnant women with GDM. Figure 1 shows a flow diagram of the enrollment procedure.

The mean values (range) for age (years), weight before pregnancy (kg), BMI before pregnancy (kg/m^2), and gestational weeks at the beginning of the study for 25 women with GDM were 36.1 (29-42), 61.8 (39-99), 24.1 (16.7-36.8), and 22.4 (14-28), respectively. One woman had a pre-pregnant BMI of < 18 kg/m^2,

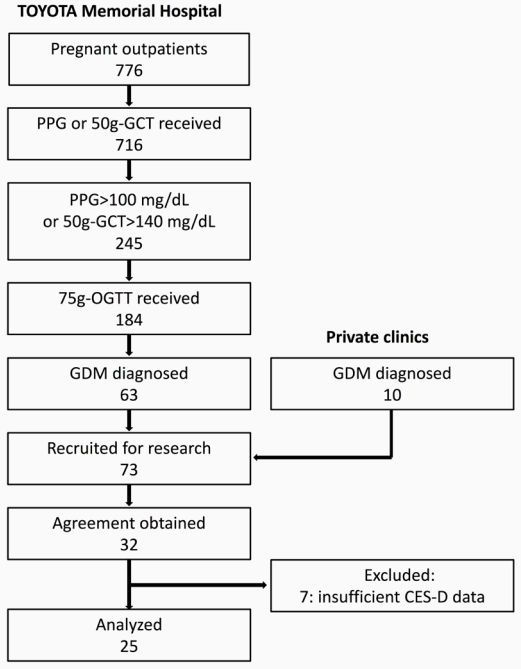

Figure 1. Flow diagram of the patients with gestational diabetes mellitus (GDM). Finally depressive condition in 25 women with GDM are analyzed.

and two had a BMI of > 30 kg/m^2. Twelve women were primiparas (Table 1). Of the 10 multiparas, one had GDM in prior pregnancies. None of the patients had symptoms of anemia. The PPG and hemoglobin A1c (HbA1c) values of the first day of walking measurement were 110 (69-173) and 5.4 (4.4-6.2), respectively. The number of steps walked/day was 6090 (2,947-10,772). The CES-D score was 9.3 (0-42), and the prevalence of depressive symptoms during the second trimester of pregnancy was 8.0%.

3.2. Relationship between CES-D score and pre-pregnant BMI, PPG, HbA1c, or walking step number

No significant correlation (r = – 0.169, p = 0.452; r = 0.236, p = 0.290; r = – 0.356, p = 0.114) was found between the CES-D score and pre-pregnant BMI, PPG, or HbA1c.

Our previous report (*10*) showed that daily walking is good for controlling PPG in pregnant women with GDM. On the other hand, no significant correlation (r = – 0.219, p = 0.294) was also observed between the CES-D score and the number of steps walked.

3.3. CES-D score relation to food intake and nutrient intake

Table 2 shows a significant negative correlation between the CES-D score and intake of fish with bone (g/day) (r = – 0.503, p = 0.017), simmered fish (g/day) (r = – 0.464, p = 0.030), pickles (green leaf) (g/day) (r = – 0.440, p = 0.041), pickles (other) (g/day) (r = – 0.468, p = 0.028), green leaf (g/day) (r = – 0.450, p = 0.036), mushrooms (g/day) (r = – 0.567, p = 0.006), and green tea (g/day) (r = – 0.433, p = 0.044). Furthermore, a significant correlation was found (r = 0.450, p = 0.036) between CES-D score and Coke® (g/day).

Table 3 shows a significant negative correlation between the CES-D score and vitamin K (μg/day) (r = – 0.496, p = 0.019), folate (μg/day) (r = – 0.465, p = 0.029), and β-carotene (μg/day) (r = – 0.451, p = 0.035).

4. Discussion

Although little is known about the relationship between depression and pregnant women with GDM, this may be the first study that reports that the diet of pregnant women with GDM can affect the presence of depressive symptoms. One strength of this study is that it is very useful for nutrition educated for pregnant women with GDM.

A review article (*23*) suggests that folate may be effective for treating depressive disorders. Patients diagnosed with major depressive disorder tend to have lower concentrations of folate in serum or red cells than healthy control subjects (*24,25*). A previous study showed that the co-administration of folic acid in female patients with depression substantially and significantly improved their response to fluoxetine (*26*). Low levels of folate and vitamin B12 have an effect on depressive symptoms (*27,28*). B-complex vitamins are coenzymes involved in homocysteine metabolism. In contrast, Watanabe *et al.* (*29*) reported no relationship between folate and homocysteine levels and depression in women in early pregnancy. Cho *et al.* (*30*) showed that the intake of multivitamins, including folic acid, was not associated with lower rates of depression during pregnancy. Our study found that CES-D scores were significantly correlated only with folate in B-complex vitamins. In food intake, green leaves and pickled green leaves were significantly correlated with CES-D scores; this was because green leaves include vitamin K and β-carotene. The association between deficiency in B-complex vitamins and depression in pregnancy is still unclear.

A previous observational study supported an association between low n-3 fatty acid intake from seafood and increased risk of high levels of depressive symptoms during pregnancy (*31*). Our study revealed that CES-D scores were significantly correlated with the intake of fish with bone and simmered fish, but no significant correlation was found with n-3 fatty acid intake. In addition to n-3 fatty acid intake, other researchers noted that zinc and iron intake, green tea intake, and intestinal environment improvement may also be useful for the alleviation of depressive symptoms (*32-35*). Our results also indicated that the CES-D scores were significantly correlated with green tea, mushroom, and pickle intake. No significant

Table 1. Participant characteristics and parameters

No.	Age (years)	Primipara(P)/ multipara(M)	History of GDM[a] at previous pregnancy	Weight when not pregnant	BMI[b] when not pregnant	Dietary intake (kcal/day)	CES-D[c]	At initiation of research					Gestational weight gain overall	Child birth		
								Gestational weeks	Body weight (kg)	PPG[d] (mg/dL)	HbA1c[e] (%)	Hb (g/dL)		Gestational weeks at delivery	Delivery mode	Birth weight (g)
1	35	M	-	62.0	25.8	2,403	0	28	67.2	108	5.4	12.9	6.0	39	NVD[g]	3,102
2	30	P		65.0	26.7	1,712	0	25	66.0	87	4.4	13.8	8.0	38	CS[i](elective)	2,950
3	40	M	-	66.0	27.5	1,520	0	27	66.0	111	6.2	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]
4	42	M	-	60.0	25.3	2,057	4	18	61.0	83	5.4	12.0	5.0	38	CS[i](elective)	3,030
5	40	M	-	73.0	25.3	1,358	5	25	79.0	90	5.1	11.9	ND[f]	40	NVD[g]	3,066
6	31	M	-	48.0	18.3	1,677	5	25	56.0	81	5.6	10.3	14.4	40	NVD[g]	4,124
7	42	P		67.0	24.6	1,210	6	22	65.0	113	5.1	13.2	-0.2	35	NVD[g]	1,764
8	37	P		56.0	24.1	2,235	6	14	62.8	123	5.8	10.9	13.0	41	NVD[g]	3,658
9	37	M	-	67.0	24.1	1,814	6	19	69.0	74	5.5	12.9	5.6	40	VD[h] (forceps)	3,562
10	42	M	-	49.0	18.7	1,810	7	24	53.4	102	5.3	11.2	6.8	38	NVD[g]	3,074
11	29	M	-	79.0	30.9	1,601	7	24	80.5	149	5.4	13.2	0.6	38	NVD[g]	2,988
12	40	P		48.0	20.5	1,552	8	19	50.0	128	5.3	14.0	9.8	41	NVD[g]	3,200
13	39	P		64.0	23.8	1,515	9	14	65.0	103	6.0	12.0	7.2	41	NVD[g]	2,946
14	38	P		71.0	29.6	1,204	9	26	77.5	173	5.2	13.0	6.8	34	CS[i](emergency)	1,554
15	40	P		54.0	19.1	1,881	9	21	56.8	90	5.1	12.5	ND[f]	ND[f]	ND[f]	ND[f]
16	32	P		66.0	26.1	1,125	10	16	60.0	156	5.4	11.6	8.6	41	NVD[g]	3,432
17	35	P		39.0	16.7	2,063	10	19	41.4	102	4.7	10.1	ND[f]	38	VD[h] (forceps)	2,752
18	35	P		58.0	21.0	1,503	10	27	63.2	127	5.3	11.2	7.0	40	NVD[g]	3,144
19	29	M	+	46.0	19.9	1,938	11	23	51.2	69	5.2	11.5	ND[f]	38	NVD[g]	3,012
20	41	P		70.0	25.1	1,682	11	27	59.2	97	5.8	12.5	3.2	37	CS[i](elective)	2,218
21	37	ND[f]		ND[f]	ND[f]	1,454	12	26	58.2	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]
22	36	ND[f]		ND[f]	ND[f]	ND[f]	14	26	43.0	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]
23	30	P		99.0	36.8	1,319	15	24	99.2	97	5.6	11.5	14.5	40	NVD[g]	3,746
24	30	ND[f]		ND[f]	ND[f]	ND[f]	17	17	49.0	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]	ND[f]
25	35	M	-	52.0	20.8	1,868	42	24	63.0	154	5.1	10.9	11.8	38	NVD[g]	2,966
mean	36.1			61.8	24.1	1,674	9.3	22.4	62.5	110	5.4	12.1	7.5	38.8		3,014
SD	4.3			12.8	4.6	326	7.9	4.2	12.3	28	0.4	1.1	4.1	1.9		597

[a]GDM, gestational diabetes mellitus; [b]BMI, body mass index; [c]CES-D, The Center for Epidemiologic Studies Depression Scale; [d]PPG, postprandial plasma glucose; [e]HbA1c, hemoglobin A1c; [f]ND, not detected; [g]NVD, normal vaginal delivery; [h]VD, vaginal delivery; [i]CS, Cesarean section.

Table 2. Food intake and spearman correlation coefficients between nutrient intake and CES-D[a] score

Food	Food intake (g/day) (mean ± SD[b])	*r*	*p*-value
Low-fat milk	75.4 ± 113.8	-0.191	0.393
Milk	52.4 ± 51.0	0.250	0.262
Chicken	33.0 ± 20.0	-0.046	0.840
Pork·Beef	37.1 ± 18.3	-0.323	0.143
Fish with bone	9.6 ± 26.5	-0.503	0.017*
Dried fish	14.9 ± 14.2	-0.311	0.158
Oily fish	21.0 ± 39.0	-0.030	0.895
Fish with little oile	17.0 ± 15.8	-0.331	0.132
Classification of the cooking style			
Raw fish	12.6 ± 15.3	0.035	0.879
Grilled fish	52.7 ± 62.0	-0.183	0.414
Simmered fish	35.0 ± 38.9	-0.464	0.030*
Fried fish	12.0 ± 17.1	-0.056	0.803
Egg	37.2 ± 25.8	-0.111	0.624
Tofu·Fried tofu	47.7 ± 32.8	-0.210	0.347
Natto	11.1 ± 10.4	-0.199	0.375
Pickles (green leaf)	4.7 ± 7.7	-0.440	0.041*
Pickles (other)	2.3 ± 3.9	-0.468	0.028*
Lettuce·Cabbage (low)	37.6 ± 24.2	-0.362	0.098
Green leaf	52.1 ± 44.7	-0.450	0.036*
Cabbage	41.3 ± 25.2	-0.415	0.055
Carrots·Pumpkin	24.4 ± 17.3	-0.296	0.181
Radish·Turnip	22.3 ± 21.7	-0.368	0.092
Root vegetables	44.3 ± 34.0	0.028	0.901
Tomato	39.0 ± 32.5	-0.017	0.942
Mushroom	11.2 ± 7.4	-0.567	0.006*
Seaweed	11.4 ± 10.2	-0.500	0.018
Green tea	65.6 ± 146.2	-0.433	0.044*
Tea·Oolong tea	65.6 ± 129.9	0.120	0.594
Coffee	49.6 ± 92.0	-0.197	0.379
Coke®	36.1 ± 53.0	0.450	0.036*

[a]CES-D, The Center for Epidemiologic Studies Depression Scale; [b]SD, standard deviation.

Table 3. Nutrient intake and spearman correlation coefficients between nutrient intake and CES-D[a] score

Nutrient	Nutrient intake (mean ± SD[b])	*r*	*p*-value
Protein (% of energy/day)	16.6 ± 4.2	0.079	0.721
Fat (% of energy/day)	30.8 ± 5.5	0.065	0.767
Carbohydrate (% of energy/day)	51.5 ± 8.4	0.059	0.791
Potassium (mg/day)	2,412.1 ± 811.4	-0.302	0.171
Calcium (mg/day)	542.7 ± 242.7	-0.238	0.286
Magnesium (mg/day)	227.1 ± 77.1	-0.352	0.108
Phosphorus (mg/day)	1,003.1 ± 379.4	-0.259	0.244
Iron (mg/day)	7.4 ± 2.8	-0.393	0.071
Zinc (mg/day)	7.8 ± 2.4	-0.309	0.162
Manganese (mg/day)	2.3 ± 0.9	-0.235	0.292
Vitamin D (μg/day)	13.7 ± 14.4	-0.297	0.179
Vitamin K (μg/day)	312.8 ± 169.9	-0.496	0.019*
Vitamin B1 (mg/day)	0.8 ± 0.2	-0.256	0.250
Vitamin B2 (mg/day)	1.2 ± 0.5	-0.222	0.321
Vitamin B6 (mg/day)	1.2 ± 0.4	-0.269	0.226
Vitamin B12 (μg/day)	9.3 ± 7.1	-0.238	0.287
Folate (μg/day)	319.4 ± 125.3	-0.465	0.029*
Vitamin C (mg/day)	111.0 ± 37.1	-0.382	0.080
Saturated fatty acids (g/day)	14.4 ± 5.7	0.120	0.596
cholesterol (mg/day)	368.3 ± 204.4	-0.222	0.320
n-3 fatty acid (g/day)	2.9 ± 1.6	-0.115	0.610
n-6 fatty acid (g/day)	10.8 ± 3.4	-0.069	0.762
α-carotene (μg/day)	463.3 ± 321.7	-0.303	0.170
β-carotene (μg/day)	3,876.8 ± 2191.0	-0.451	0.035*

[a]CES-D, The Center for Epidemiologic Studies Depression Scale; [b]SD, standard deviation.

correlation was found between zinc and iron intake. Our study also showed an association between high CES-D score and high Coke® intake. The effect of Coke® intake on depressive symptoms is unclear, and there is room for further consideration.

This study has several limitations. First, our sample size was too small and did not have adequate power. Second, since the study subjects participated on a voluntary basis, they may be healthier than the general population, causing a selection bias. Third, since the BDHQ queried diet history for the past month, recall bias was included. Fourth, we only investigated CES-D scores for depressive symptoms and could not examine the risk factors for temperament, character, and depressive episodes, including history of depression, lack of partner, lack of social support, poverty, family violence, and increased life stress (*36-38*).

In conclusion, the present study suggests that symptoms of depression in pregnant women with GDM in their second trimester may be associated with their diet. Future research should include studies with larger sample sizes. In addition, future longitudinal studies on the relationship between depressive symptoms and diet for pregnant women with GDM are required.

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References

1. Byrn MA, Penckofer S. Antenatal depression and gestational diabetes: a review of maternal and fetal outcomes. Nurs Womens Health. 2013; 17:22-33.
2. Young-Hyman D, de Groot M, Hill-Briggs F, Gonzalez JS, Hood K, Peyrot M. Psychosocial care for people with diabetes: a position statement of the American Diabetes Association. Diabetes Care. 2016; 39:2126-2140.
3. Anderson RJ, Freedland KE, Clouse RE, Lustman PJ. The prevalence of comorbid depression in adults with diabetes: a meta-analysis. Diabetes Care. 2001; 24:1069-1078.
4. Khaledi M, Haghighatdoost F, Feizi A, Aminorroaya A. The prevalence of comorbid depression in patients with type 2 diabetes: an updated systematic review and meta-analysis on huge number of observational studies. Acta Diabetol. 2019; 56:631-650.
5. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2009; 32 (Suppl 1):S62-S67.
6. HAPO Study Cooperative Research Group, Metzger BE, Lowe LP, *et al.* Hyperglycemia and adverse pregnancy outcomes. N Engl J Med. 2008; 358:1991-2002.
7. Morikawa M, Yamada T, Yamada T, Akaishi R, Nishida R, Cho K, Minakami H. Change in the number of patients after the adoption of IADPSG criteria for hyperglycemia during pregnancy in Japanese women. Diabetes Res Clin Pract. 2010; 90:339-342.
8. Metzger BE, Buchanan TA, Coustan DR, de Leiva A, Dunger DB, Hadden DR, Hod M, Kitzmiller JL, Kjos SL, Oats JN, Pettitt DJ, Sacks DA, Zoupas C. Summary and recommendations of the Fifth International Workshop-Conference on Gestational Diabetes Mellitus. Diabetes Care. 2007; 30:S251-60.
9. American College of Obstetricians and Gynecologists (ACOG) Physical activity and exercise during pregnancy and the postpartum period Committee opinion 650. Obstet Gynecol. 2015; 126:e135-e142.
10. Hayashi A, Oguchi H, Kozawa Y, Ban Y, Shinoda J, Suganuma N. Daily walking is effective for the management of pregnant women with gestational diabetes mellitus. J Obstet Gynaecol Res. 2018; 44:1731-1738.
11. Kozhimannil KB, Pereira MA, Harlow B. Association between diabetes and perinatal depression among low income mothers. J Am Med Assoc. 2009; 301:842-847.
12. Chazotte C, Freda MC, Elovitz M, Youchah J. Maternal depressive symptoms and maternal fetal attachment in gestational diabetes. J Womens Health. 1995; 4:375-380.
13. Katon JG, Russo J, Gavin AR, Melville JL, Katon WJ. Diabetes and depression in pregnancy: Is there an association? J Womens Health. 2011; 20:983-989.
14. Kim C, Brawarsky P, Jackson RA, Fuentes-Afflick E, Haas JS. Changes in health status experienced by women with gestational diabetes and pregnancy induced hypertensive disorders. J Womens Health. 2005; 14:729-736.
15. Langer N., Langer O. Emotional adjustment to diagnosis and intensified treatment of gestational diabetes. Obstet Gynecol. 1994; 84:329-334.
16. Mautner E, Greimel E, Trutnovsky G, Daghofer F, Egger JW, Lang U. Quality of life outcomes in pregnancy and postpartum complicated by hypertensive disorders, gestational diabetes, and preterm birth. J Psychosom Obstet Gynecol. 2009; 30:231-237.
17. Roberts RE, Vernon SW. The Center for Epidemiologic Studies Depression Scale: its use in a community sample. Am J Psychiatry. 1983; 140:41-6.
18. Shima S, Shikano T, Kitamura T. A new self-report depression scale. Psychiatry. 1985; 27:717-723. (Japanese).
19. Kumahara H, Schutz Y, Ayabe M, Yoshioka M, Yoshitake Y, Shindo M, Ishii K, Tanaka H. The use of uniaxial accelerometry for the assessment of physical-activity-related energy expenditure: a validation study against whole-body indirect calorimetry. Brit J Nutr. 2004; 91:235-243.
20. Radloff LS. The CES-D scale: a self-report depression scale for research in the general population. Appl Psychol Meas. 1977; 1:385-401.
21. Kobayashi S, Murakami K, Sasaki S, Naoko O Hirota N, Notsu A, Fukui M, Date C. Comparison of relative validity of food group intakes estimated by comprehensive and brief-type self-administered diet history questionnaires against 16 d dietary records in Japanese adults. Public Health Nutr. 2011; 14:1200-1211.
22. Kobayashi S, Honda S, Murakami K, Sasaki S, Okubo H,

Hirota N, Notsu A, Fukui M, Date C. Both comprehensive and brief self-administered diet history questionnaires satisfactorily rank nutrient intakes in Japanese adults. J Epidemiol. 2012; 22:151-159.
23. Taylor MJ, Carney SM, Goodwin GM, Geddes JR. Folate for depressive disorders: systematic review and meta-analysis of randomized controlled trials. J Psychopharmacol. 2004; 18:251-256.
24. Bjelland I, Tell GS, Vollset SE, Refsum H, Ueland PM. Folate, vitamin B12, homocysteine, and the MTHFR 677C→T polymorphism in anxiety and depression: The Hordaland Homocysteine Study. Arch Gen Psychiatry. 2003; 60:618-626.
25. Morris MS, Fava M, Jacques PF, Selhub J, Rosenberg IR. Depression and folate status in the US Population. Psychother Psychosom. 2003; 72:80-87.
26. Coppen A, Bailey J. Enhancement of the antidepressant action of fluoxetine by folic acid: a randomised, placebo controlled trial. J Affect Disord. 2000; 60:121-130.
27. Bjelland I, Ueland PM, Vollset SE. Folate and depression. Psychother Psychosom. 2003; 72:59-60.
28. Sánchez-Villegas A, Henríquez P, Bes-Rastrollo M, Doreste J. Mediterranean diet and depression. Public Health Nutr. 2006; 9:1104-1109.
29. Watanabe H, Suganuma N, Hayashi A, Hirowatari Y, Hirowatari T, Ohsawa M. No relation between folate and homocysteine levels and depression in early pregnant women. Biosci Trends. 2010; 4:344-350.
30. Cho YJ, Han JY, Choi JS, Ahn HK, Ryu HM, Kim HY, Yang JH, Nava-Ocampo AA, Koren G. Prenatal multivitamins containing folic acid do not decrease prevalence of depression among pregnant women. J Obstet Gynaecol. 2008; 28:482-484.
31. Golding J, Steer C, Emmett P, Davis JM, Hibbeln JR. High levels of depressive symptoms in pregnancy with low omega-3 fatty acid intake from fish. Epidemiology. 2009; 20:598-603.
32. Swardfager W, Herrmann N, McIntyre RS, Mazereeuw G, Goldberger K, Cha DS, Schwartz Y, Lanctôt KL. Potential roles of zinc in the pathophysiology and treatment of major depressive disorder. Neurosci Biobehav Rev. 2013; 37:911-929.
33. Yi S, Nanri A, Poudel-Tandukar K, Nonaka D, Matsushita Y, Hori A, Mizoue T. Association between serum ferritin concentrations and depressive symptoms in Japanese municipal employees. Psychiatry Res. 2011; 189:368-372.
34. Wakabayashi C, Numakawa T, Ninomiya M, Chiba S, Kunugi H. Behavioral and molecular evidence for psychotropic effects in L-theanine. Psychopharmacology (Berl). 2012; 219:1099-1109.
35. Aizawa E, Tsuji H, Asahara T, Takahashi T, Teraishi T, Yoshida S, Ota M , Koga N, Hattori K, Kunugi H. Possible association of Bifidobacterium and Lactobacillus in the gut microbiota of patients with major depressive disorder. J Affect Disord. 2016; 202:254-257.
36. Minatani M, Kita S, Ohashi Y, Kitamura T, Haruna M, Sakanashi K, Tanaka T. Temperament, character, and depressive symptoms during pregnancy: a study of a Japanese population. Depress Res Treat. 2013; 2013:140169.
37. Bonari L, Pinto N, Ahn E, Einarson A, Steiner M, Koren G. Perinatal risks of untreated depression during pregnancy. Can J Psychiatry. 2004; 49:726-735.
38. Kitamura T, Shima S, Sugawara M, Toda MA. Clinical and psychosocial correlates of antenatal depression: A review. Psychother Psychosom. 1996; 65:117-123.

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Brief Report

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Althaea officinalis improves wound healing in rats: a stereological study

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SUMMARY *Althaea officinalis* (AO) is reported to have the ability to activate fibroblasts as well as anti-inflammatory and antioxidative properties. Herein, we investigated the effects of this herbal medicine on wound healing in rat models by using stereological methods. In this experiment, 48 male Wistar rats were divided into four groups randomly (n = 12): the control group with no treatment, the gel-base treated group, 5% and 10% AO-gel treated groups. The treatments were administered every 24 hours. Wound closure rate, volume densities of collagen bundles, hair follicles, and vessels, vessel's length density and mean diameter, and fibroblast populations were estimated. Fibroblast populations, hair follicles, and mean diameter of vessels in the dermis of AO-treated groups were noticeably higher than those of control and base groups. Also, collagen bundles synthesis was significantly higher in the AO10%-treated group compared to the control and base groups. According to our research and previous studies, AO has the potential to be considered as an alternative medicine in wound healing treatment; however, further clinical investigations are suggested.

Keywords *Althaea officinalis*, wound healing, stereology, rat

1. Introduction

The skin is a bi-layer tissue that has the three roles, protection, sensation, and regulation, which could be compromised by injuries (*1*). The process of wound healing contains 5 stages of re-epithelialization, angiogenesis, activation and migration of fibroblasts, and endothelial cell proliferation (*2*). All of these stages are associated with inflammation and oxidation in the injured tissue (*1*). The release of growth factors from platelets and inflammatory cells like neutrophils leads to the initiation of the repair process immediately after the injury (*3,4*). Empowering the mechanisms involved in the process of wound closure and reducing scar formation are the main goals of wound treatment, specifically for skin wounds (*5*).

Nowadays, herbal medicines are used all around the world because of being efficient, safe, and also having fewer side effects and better compatibility with the human body (*6*). *Althaea officinalis* (AO), marshmallow, is a perennial plant from the Malvaceae family (*7*). This agent has been traditionally consumed in different cultures both as food and medicine. Recently, several studies have mentioned that the alcoholic, hydroalcoholic, and methanol extracts of this herb have considerable anti-inflammatory, antioxidative, and antibacterial effects (*7-11*). Besides, the aqueous extract of AO has great amounts of mucilage polysaccharides that stimulate the proliferative activity of fibroblasts and keratinocytes (*12*). AO extract has the potential to increase the release of cytokines that are responsible for apoptosis, and rise the gene expressions of molecules involved in creating the extracellular matrix and the bio-adhesive proteins (*13*).

In this study, we aimed to investigate the effects of hydroalcoholic extract of AO on wound healing in rat models by using histomorphometric and stereological evaluations.

2. Materials and Methods

2.1. Preparation of AO gel

AO was received from SIMR Co., Shiraz, Iran (Code: 3104). All plant organs were occupied as the herbal material for making the extract. We dried the material at room temperature for 5-6 days. Then, it was ground into powder. Thereafter, the powder was extracted with a mixture of ethanol: water (1:1, v/v) for 72 hours. The obtained product was then filtered and then evaporated to make a hydro-alcoholic extract (yield: 16.23%). We dissolved 5 mg of AO extract in 2 mL of ethanol (70%) in order to prepare 5% gel and then mixed the solution with 2% carboxymethylcellulose (CMC) (2 g/dL). The 10% gel was prepared with the same method by using 10 mg of AO. We also similarly used the vehicle for the control group without the AO component.

2.2. Animal models and procedures

In this experimental study, we used 48 male Wistar rats (200 ± 20 g) aged 2.5 months on average, which were kept in standard cages with sufficient food and water *ad libitum*. Animals were divided into 4 groups randomly (n = 12): the control group with no treatment, the gel-base treated group, 5%, and 10% AO gel treated groups. The study protocol was approved by the animal ethics committee of Shiraz University of Medical Sciences. In the beginning, on day 0, a 1 cm^2 circular full-thickness wound was created *via* excision of the skin. Under general anesthesia, wounds were created on the dorsal surface of each rat's neck. Every day, before applying the treatments, we irrigated the entire wound with normal saline. The control group received no treatment and the vehicle group received vehicle gel while the other groups were treated with 5% and 10% AO gels. According to a prior pilot study the end of the study was set as day 15. On the last day, we euthanized all the animals with a high dose of ether inhalation and took the skin samples from the wound site and transferred them into buffered formaldehyde (pH = 7.2) for making microscopic slides for stereological studies.

2.3. Stereological study

Every 4 days, we took a digital picture from the wound surface with a digital camera to measure the wound closure rate. A ruler was put next to the wound in each picture to measure the magnification on the monitor of computers. The "point grid" method was used to estimate the closure rate (*14*) by using the following formula: Wound closure rate (%) = ((area at visit 1 – area at each visit)/area at visit 1) ×100.

In a systematic random sampling manner, 8-13 pieces (each piece 1 mm^2) of each skin sample were obtained and were put in a cylindrical paraffin block. After Isotropic Uniformly Random (IUR) sectioning of the blocks with thicknesses of 4 µm and 20 µm (*14*), the slides were made and stained with hematoxylin and eosin (H&E; Figure 1).

Stereological parameters including volume densities of the collagen bundles and vessels, vessel's length density and mean diameter, and fibroblast populations were measured according to the study conducted by Ashkani-Esfahani *et al.* (*14*).

2.4. Statistical data analysis

Results were reported as mean and standard deviation (mean ± SD). SPSS statistical software (ver.19.0, IBM™, USA) was used to do statistical comparisons between the groups. The statistical analyses were carried out by employing Kruskal Wallis and Mann Whitney U tests. Furthermore, $p \leq 0.05$ was considered as statistically considerable.

3. Results and Discussion

3.1. Wound closure

The mean initial wound area in all four groups was 104.22 ± 7.26 mm^2 with no considerable contrast among the groups. Wound closure rates of 5%-AO (6.03%/day) and 10%-AO (5.94%/day) groups were noticeably higher than the control (3.14%/day) and gel-base treated (3.44%/day) groups ($p < 0.05$; Figure 2).

3.2. Fibroblast population

The numerical densities of the fibroblasts in the dermis of the AO-treated groups were noticeably higher than those of control and gel base groups. The numerical densities of the fibroblasts in 5% and 10% AO-treated groups were 112.8% ($p = 0.021$) and 45.3% ($p = 0.049$) higher than the controls, respectively, and 117.8% ($p = 0.018$) and 49.18% ($p = 0.027$) higher than the gel base group, respectively (Table 1).

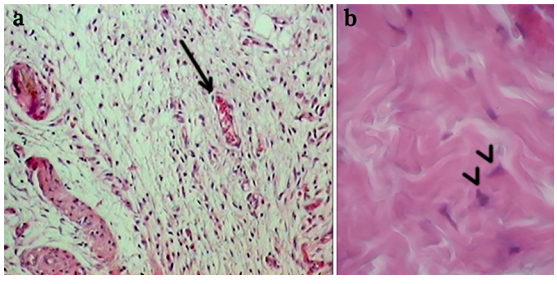

Figure 1. The effect of *Althaea officinalis* on the wound closure rate in rat models. The vessels (arrow) and collagen bundles were appreciated using 4 µm slices **(a)** while the fibroblasts (arrowheads) were counted using 20 µm slides **(b)**. Stereological methods were used to evaluate the skin tissues.

Table 1. Mean (SD) of the numerical density of the fibroblasts (×10^3 per mm^3), volume densities of the collagen bundles ($Vv_{collagen/dermis}$; %) and vessels ($Vv_{vessel/dermis}$; %), length density (mm/mm^3) and mean diameter (µm) of vessels in the dermis of the wounded rats treated with 5% AO and 10% AO gels, gel-base and untreated wounded group (Control).

Groups	Fibroblasts	Collagen bundles	Vessels			Hair Follicles
	Numerical density	Volume density	Volume density	Length density	Mean diameter	Volume density
Control	225.4 (21.1)	51.2% (4.8%)	0.8% (0.5%)	27.5 (8.9)	11.8 (1.8)	3.2% (1.4%)
5% AO	477.7 (70.1)*	78.3% (5.6%)	1.4% (0.8%)	27.6 (9.8)	24.3 (10.3)*	9.1% (4.1%)*
10% AO	328.5 (63.3)*	85.1% (5.3%)*	1.3% (0.4%)	28.2 (10.2)	21.3 (4.9)*	9.9% (2.7%)*
Gel-base	220.2 (53.1)	54.2% (5.2%)	0.9% (0.7%)	17.16 (7.3)	11.5 (2.1)	2.8% (1.1%)

*$p < 0.05$ in comparison with control and gel-base groups.

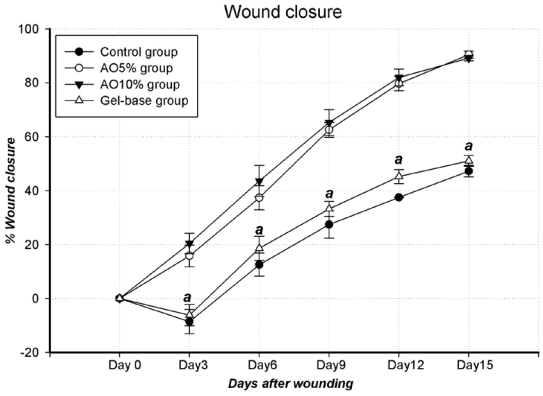

Figure 2. The effect of *Althaea officinalis* (AO) on the wound closure rate in rats of the control group, gel-base treated, 5%, and 10% AO-treated groups. Each point exhibits the mean ± SD of the twelve wounds. The "a" letter demonstrates considerable difference for the 5% AO and 10% AO treated rats compared to the control group and the base group ($p < 0.05$).

3.3. Volume densities of the collagen bundles and hair follicles

The volume densities of the collagen bundles were significantly higher by 66.2% ($p < 0.001$) and 57.1% ($p < 0.001$) in 10% AO-treated group in comparison to the control and base groups, respectively (Table 1). However, the collagen bundles' volume density in the 5% AO-treated group was not significantly higher in comparison to the control and base groups. The volume densities of the hair follicles in the 5% and 10% AO-treated groups were significantly higher in comparison to the base group ($p = 0.038$ and $p = 0.008$, respectively) and the control group ($p = 0.037$ and $p = 0.009$, respectively) (Table 1).

3.4. Volume density, length density, and diameter of the vessels

The length and volume densities of the vessels in the 5% and 10% AO-treated groups were not significantly higher in comparison to the gel base and control groups. However, there are considerable differences regarding the mean diameters of the vessels between the AO-treated groups, and the control and the gel base groups (Table 1).

Based on previous studies, we hypothesized that AO extract can be used as a treatment to accelerate the wound healing process. The results of this study revealed that hydroalcoholic gel-based AO extract has the potential to be an alternative treatment for wound healing. Our investigation demonstrated that AO extract increases the volume density of collagen fibers and the population of fibroblasts, besides it improves the process of vascularization. These results are consistent with previous reports which have mentioned various effects of the agent such as anti-inflammatory, antioxidant, and anti-microbial properties, as well as fibroblast proliferation-inducing effect (*7-11*). Böker *et al.* in their study showed that aqueous extract of AO has N-phenylpropenoyl-L-amino acids (NPA) which can stimulate keratinocytes and increases cellular activity of fibroblasts (*12*). Moreover, Benbassat *et al.* indicated that the ethanolic extract of AO has antioxidant activity that improves vascular endothelial function (*10*). Another study also mentioned the same property for aqueous extracts of AO, as it can stimulate tissue regeneration of epithelial cells (*13*). Our results highlighted that topical administration of AO extract improved fibroblast proliferation, collagen bundle synthesis, and re-vascularization in skin injuries. Based on previous studies and according to the present evaluations, it is revealed that AO improves dermal tissue reconstruction.

Our research shows that AO positively affects the processes of wound healing including angiogenesis, the proliferation of fibroblasts, and the accumulation of collagens. However, we suggest further experimental and clinical studies to evaluate the therapeutic properties of this herb and compare it with current treatments.

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References

1. Martin P. Wound healing – aiming for perfect skin regeneration. Science. 1997; 276:75-81.
2. Khoshneviszadeh M, Ashkani-Esfahani S, Namazi MR, Noorafshan A, Geramizadeh B, Miri R. Topical simvastatin enhances tissue regeneration in full-thickness skin wounds in rat models. Iran J Pharm Res. 2014; 13:263-269.
3. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. J Int Med Res. 2009; 37:1528-1542.
4. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev. 2003; 83:835-870.
5. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc. 2000; 5:40-46.
6. Gunjan M, Naing TW, Saini RS, Ahmad A, Naidu JR, Kumar I. Marketing trends & future prospects of herbal medicine in the treatment of various disease. World J Pharm Res. 2015; 4:132-155.
7. Ozturk S, Ercisli S. Antibacterial activity of aqueous and methanol extracts of *Althaea officinalis* and *Althaea cannabina* from Turkey. Pharm Biol. 2007; 45:235-240.
8. Sadighara P, Barin A, Jahed G, Farjadmand F. Assessment of antioxidant capacity and anti- inflammatory of alcoholic extraction of chamomile, morus, marshmallow, borage and rosemary. Knowled Health. 2013; 8:31-34.
9. Sadighara P, Gharibi S, Moghadam Jafari A, Jahed Khaniki G, Salari S. The antioxidant and flavonoids contents of *Althaea officinalis* L. flowers based on their color. Avicenna J Phytomed. 2012; 2:113-117.
10. Benbassat N, Yoncheva K, Hadjimitova V, Hristova N, Konstantinov S, Lambov N. Influence of the extraction solvent on antioxidant activity of *Althaea officinalis* L. root extracts. Cent Eur J Biol. 2014; 9:182-188.
11. Tešević V, Vajs V, Lekić S, Đorđević I, Novaković M, Vujisić L, Todosijević M. Lipid composition and antioxidant activities of the seed oil from three Mlvaceae species. Arch Biol Sci. 2012; 64:221-227.
12. Boeker I, Sendker J, Stark T, Kelber O, Fink C, Hensel A. Cytoprotective effects of aqueous extracts from Marshmallow roots (*Althaea officinalis* L.). Z Phytother. 2012; 33:6.
13. Deters A, Zippel J, Hellenbrand N, Pappai D, Possemeyer C, Hensel A. Aqueous extracts and polysaccharides from Marshmallow roots (*Althea officinalis* L.): cellular internalisation and stimulation of cell physiology of human epithelial cells *in vitro*. J Ethnopharmacol. 2010; 127:62-69.
14. Ashkani-Esfahani S, Zarifi F, Asgari Q, Samadnejad AZ, Rafiee S, Noorafshan A. Taurine improves the wound healing process in cutaneous leishmaniasis in mice model, based on stereological parameters. Adv Biomed Res. 2014; 3:204.

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Brief Report

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The *in vitro* and *in vivo* anti-virulence activities of *Cinnamomum bejolghota* by inhibiting type three secretion system effector proteins of Salmonella

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SUMMARY The bark of *Cinnamomum bejolghota* (Buch.-Ham.) Sweet (*C. bejolghota*) is widely used as medicine to treat bacterial diarrhea in Myanmar. We previously reported that the bark extract of *C. bejolghota* significantly inhibited secretion effector proteins of the type three secretion system (T3SS) in Salmonella. This study is designed to investigate the anti-virulence potential of the *C. bejolghota* bark extract against *Salmonella* Typhimuriumin *in vivo* and *in vitro* experiments. The results suggested that the polar fraction Fr.M_1 inhibited the secretion of effector proteins SipA, SipB, SipC and SipD without affecting bacteria growth and the translocation of SipC into MDA-MB-231 cells. In addition, Fr.M_1 alleviated inflammatory symptoms of mice in Salmonella-infected mouse model. Overall, the results provide evidence for medicinal usage of *C. bejolghota* bark to treat diarrhea in Myanmar.

Keywords *C. bejolghota*, T3SS effector protein, anti-virulence, *Salmonella* Typhimurium

1. Introduction

With the increasing of antibiotic resistance, many new targets for the development of non-traditional antibacterial drugs have been revealed, and the "anti-virulence strategy" is a promising avenue as an alternative (*1-3*). Most of the antibiotics target the key functions for bacterial survival and resistance to them is widespread. The "anti-virulence strategy" targets the virulence that not required for the survival of pathogens. Therefore, it is regarded as a promising target for the development of anti-virulence compounds (*4*). Among them, T3SS is a typically virulence mechanism employed by several gram-negative pathogens. Many medicinal plants have been reported with antibacterial and anti-infective activity, but the molecules from them showed weak antimicrobial activity, which means they may target virulence factors rather than bactericide (*5-7*).

C. bejolghota (Buch.-Ham.) Sweet, previously known as *C. obtusifolium* (Roxb.) Nees (*8*), is a large robust tree, distributed throughout the central and outer parts of eastern Himalayas and Burma. The stem barks were used locally as a substitute of cinnamon spice. The bark and its infusions have variety of local medicinal use for the treatment of headache, fever, urinary stone trouble, stomach disorder and diarrhea (*8,9*). More and more studies have confirmed that *C. bejolghota* has a wide range of pharmacological effects, including antihyperglycemic and anti-oxidative activity (*10*). Our recent results also indicated that *C. bejolghota* is one of the promising candidates as anti-virulence drug resource for treatment of Salmonella infection by inhibiting the effector proteins (*11*), which maybe the reason for traditional usage of *Cinnamomum* on the stomach disorder, gastroprotective activity and diarrhea (*12-15*). Therefore, to fulfill the speculation, we try to disclose the possible anti-virulence effectiveness of *C. bejolghota in vitro* and *in vivo* and the possible mechanism.

2. Materials and methods

2.1. Plant material and preparation of extracts and fractions

The *C. bejolghota* barks were collected from Kalaw Reserve Forest of Tanggyi Shan State (20°35'57.98N, 96°31'50.29E) in May 2017. It was authenticated by Yu Zhang and a voucher specimen of *C. bejolghota* bark (MB201705KLW009) was deposited in Kunming Institute of Botany, Chinese Academy of Sciences.

The barks (1.0 kg) were cut into pieces, powdered and extracted with 95% ethanol for three times. The ethanol extract was concentrated using a rotary evaporator *in vacuum* at 40°C to yield a residue. Then, the residue was dispersed in water and extracted with petroleum ether (PE), ethyl acetate (EA), and *n*-Butanol (Bu) to obtain PE, EA, Bu and water (W) extracts, respectively. Further, the Bu extract was dissolved in MeOH to obtain MeOH soluble fraction (Fr.M). Each extract was dissolved in DMSO to make 100 mg/mL stock solution. All the extracts (PE, EA, Fr.M and W) were screened for their inhibitory effects on the secretion of T3SS effector proteins of *S.* Typhimurium. The results suggested that Fr.M showed excellent anti-T3SS activity. Therefore, Fr.M was subjected to MPLC (RP-18 silica gel, 140 g) and eluted with gradient MeOH in water (H_2O, 30%, 50%, 70%, 90% and 100% MeOH) to obtain Fr.M_1-M_6. Then, Fr.M_1-M_6 was respectively dissolved in DMSO to make 100 mg/mL stock solutions for anti-T3SS screening.

2.2. Culture conditions of bacteria strain and cell line

Salmonella enterica serovar Typhimurium UK-1 χ8956 (*S.* Typhimurium) (*16*) was cultured in Luria-Bertani (LB) broth or on LB agar medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) with 0.2% arabinose at 28°C or 37°C overnight. The quantity of bacteria was counted by measuring the OD_{600}. Bacteria were harvested by centrifugation at 12,000 rpm for 5 min, then suspended in phosphate-buffered saline (PBS) or LB media and used for the next experiments.

Human breast cancer cells MDA-MB-231 were cultured in RPMI-1640 medium (containing 10% fetal bovine serum) without antibiotics at 37°C and 5% CO_2.

2.3. Isolation and detection of T3SS effector proteins

The potential inhibitory effects of the different extracts and fractions against T3SS associated effector proteins were screened at the concentration of 100 μg/mL using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue and western blotting analysis as described in the previously published literature (*16,17*).

2.4. Cytotoxicity assay

MDA-MB-231 cells were seeded in 96-cell plates (Corning, USA) with a density of 1.2×10^5 per well and the cells were incubated overnight at 37°C with 5% CO_2. Each well was filled with 100 μL of medium with different concentrations of Fr.M_1 ranging from 6.25 to 100 μg/mL, and the DMSO control group was filled with 200 μL medium, and there are triplicates for each experiment. After that, the 96-well plates were incubated in a constant temperature incubator at 37°C with 5% CO_2 for 48 h. Ten μL CCK-8 reagent (Hanbio) was added to each well, then incubate for 4 h. The OD_{480} value was measured with a microplate reader, and the survival rate of co-cultured cells with extracts was calculated according to the following formula. Survival rate (%) = (OD_{480} of experimental group – OD_{480} of blank group)/(OD_{480} of control group – OD_{480} of blank group) × 100%.

2.5. Gentamicin protection assay

In order to test whether Fr.M_1 inhibited *S.* Typhimurium invasion into host cells, the gentamicin protection assay was carried out (*16,18*). Overnight cultures of the *S.* Typhimurium in LB medium (0.2% L-arabinose) in 25°C incubator were diluted 1:10 with fresh LB broth. Fr.M_1 was added at the indicated concentration of 100 μg and cultures were placed in 37°C/220 rpm for 4 h. MDA-MB-231 cells were seeded in 96-cell plates with a density of 1.2×10^5 per well and the cells were incubated overnight at the atmosphere of 37°C with 5% CO_2. Wash cells three times with PBS, and 200 μL cell culture medium was added to each well, then the plate was incubated at 37°C for 30 minutes. *S.* Typhimurium was added to MDA-MB-231 cells at the multiplicity of infection (MOI) of 20. After incubating for 1 h at 37°C, cell culture medium containing 100 μg/mL gentamicin was replaced to each well to kill noninvasive *S.* Typhimurium cells for 1 h at 37°C. Then, MDA-MB-231 cells were washed three times with PBS and 100 μL 1% TritonX-100 with 1 mM PMSF was added to each well to lyse cells. The colony forming units (CFU) of bacteria in the cell lysis solutions were counted by the method of plating 1:10, 1:100, 1:1,000 dilution in LB agar plate with 0.2% L-arabinose.

2.6. Detection of SipC in the invasion assay

MDA-MB-231cells were seeded in 60 mm flat-bottom plates, incubated for 16 h at 37°C, and 5% CO_2 in RPMI-1640 supplemented with 10% FBS. Then MDA-MB-231 cells were cultured for 30 min in RPMI-164 medium without FBS, and infected with *S.* Typhimurium at the MOI of 50 and 1,000. After the addition of Fr.M_1 to the final concentrations of 100 μg/mL, the co-culture of Salmonella-MDA-MB-231 cells were incubated for 15, 30 and 60 min at 37°C, respectively. The co-culture was centrifuged for 5 min at 12,000 g at 4°C. The cells were washed with PBS, and lysed with 1% Triton X-100 solution with PMSF at the final concentration of 1 mM. Cell lysate was collected and per 50 μL of lysate was mixed with 150 μL loading buffer. After 95°C denaturation, proteins SipC and GADPH in different groups were analyzed by western blotting. All experiments were performed in triplicates.

2.7. Measurement of bacterial growth

S. Typhimurium was cultured in LB broth with 0.2% L-arabinose at 28°C and then diluted at 1:200 in fresh LB broth and incubated for 4 h at 37°C with $Fr.M_1$ or DMSO. OD_{570} of the culture was measured every hour using a microplate reader (Bio-Rad 680, USA) and three replicates for each experiment.

2.8. Animal experiments

Kunming mice were purchased from the Experimental Animal Center of Shandong University. The mouse experiment is overseen by the Animal Welfare and Ethical Committee of Shandong University (Approval No, 20026). Mouse model for Salmonella infection was induced as described before (*19-21*). Mice were provided with water containing streptomycin (5 mg/mL) for two days before treated with free water and food. Drinking water was offered before Salmonella infection, followed by infection or sterile PBS treatments. Bacteria were successively incubated in a shaker at 25°C and 37°C to induce the production of T3SS effector proteins. Bacteria strains were washed three times with sterilized PBS before infection. By measuring the OD_{600} value, the CFU of the bacterial solution was determined to be properly concentration. Sixty 6-8 weeks old Kunming mice were divided into six groups, and every group included five males and five females. To make the infection model, each group mouse (n = 10) was infected with appropriately 3×10^8 of bacterial cells. Each mouse in groups A, B, C was respectively treated with 5 mg, 10 mg and 20 mg $Fr.M_1$ for 5 days at 12-h intervals every day. In each case, control groups received sterile PBS, the positive group was treated with streptomycin (5 mg/mL). The weight of the mice was measured daily for 10 days.

After 10-day post-infection, mice were sacrificed by cervical dislocation. The spleen, liver and kidney index of the Kunming mice were weighed and calculated.

To evaluate bacteria loads in spleen, liver and kidney tissue, all the samples were homogenized in cold PBS and serial dilutions of the homogenates were plated on LB plates under Salmonella-selective plating medium, followed by overnight incubation in 37°C.

For histopathological analysis, segments of kidney, liver and spleen were fixed and embedded in paraffin according to standard procedures (*22-24*). Cryosections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Pathological evaluation was performed by using microscope (ZEISS, Axio Observer A1m, Germany) in a proper manner.

2.9. Statistical analyses

Means and standard deviations were calculated and analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). The two-way analysis of variance (ANOVA) method was used in this software to evaluate the statistical significance between the different groups. p values of < 0.05 were considered statistically significance.

3. Results and Discussion

3.1. $Fr.M_1$ inhibit the secretion of effector proteins SipA, SipB, SipC and SipD of *S.* Typhimurium

C. bejolghota is a Myanmar medicinal plant, its bark soaking water or bark pasta made with water has profound potential for the treatment of severe diarrhea (*8*). Our recently screening results have suggested that the *C. bejolghota* bark extract showed inhibitory activity on the secretion of T3SS effectors of *S.* Typhimurium (*11*). In this study, the SDS-PAGE and western blotting results suggested that 100 μg/mL Fr.M showed significant anti-T3SS effects on the secretion of effector proteins SipA, SipB, SipC and SipD (Figure 1a). Further fractionation Fr.M by MPLC to obtain $Fr.M_1$ to M_6, and anti-T3SS results suggested that $Fr.M_1$ was the most active fraction (Figure 1b) inhibiting the secretion of effector proteins without an evident effect on the growth of bacteria (Figure 1c).

3.2. $Fr.M_1$ inhibited *S.* Typhimurium invasion into MDA-MB-231 cells

The cytotoxicity of $Fr.M_1$ was measured using the CCK-8 assay (*25*). 100 μg/mL $Fr.M_1$ showed no inhibitory effects on the growth of MDA-MB-231 cells (Figure 2a). Then we investigated the protective ability of $Fr.M_1$ by blocking *S.* Typhimurium into MDA-MB-231 cells. The gentamicin protection assay suggested that $Fr.M_1$ significantly reduced bacterial invasion into MDA-MB-231 cells compared to control group (Figure 2b). Meanwhile, the SipC level in MDA-MB-231 cells was detected by western blotting. The results suggested that $Fr.M_1$ reduced the SipC lever in MDA-MB-231 cells compared to control (Figure 2c), which suggested that $Fr.M_1$ can reduce *S.* Typhimurium invasion into MDA-MB-231 cells.

3.3. $Fr.M_1$ alleviated *S.* Typhimurium infection *in vivo*

In the infected experiment, 3 g/kg $Fr.M_1$ was orally administered to Kunming mice, but no death was observed after 10 days. To investigate the impact of $Fr.M_1$ treatment, the weight of Salmonella infected mice was measured daily for 10 days and the organ indexes were finally measured. As shown in Figure 3a, the weight loss of infected mice was significant after three days, whereas mice treated with $Fr.M_1$ and Streptomycin could reduce the degree of weight loss. The organ index is a sensitive indicator representing the effects of drugs on animal organs. An increased organ index indicates congestion, oedema or hypertrophy of the organs, *etc.*,

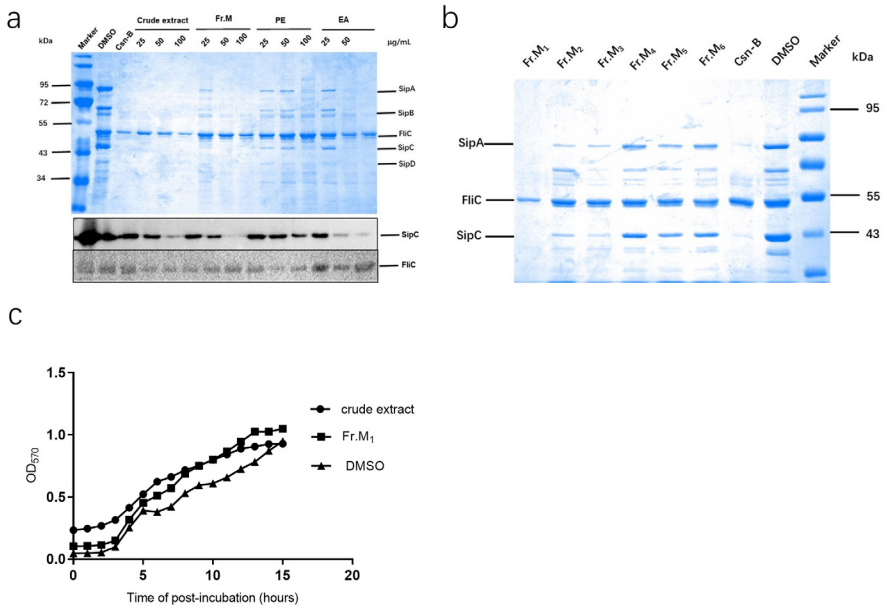

Figure 1. a) The screening results of different extract of *C. bejolghota* on the secretion of T3SS effector proteins SipA, SipB, SipC and SipD of *S.* Typhimurium (M, marker; cytosporone B, (Csn-B), positive control; DMSO, control); **b)** Fr.M_1 inhibited the secretion of effector proteins SipA, SipB, SipC and SipD of *S.* Typhimurium; **c)** *C. bejolghota* bark extract and Fr.M_1 did not significantly inhibit the bacterial growth at a concentration of 100 μg/mL.

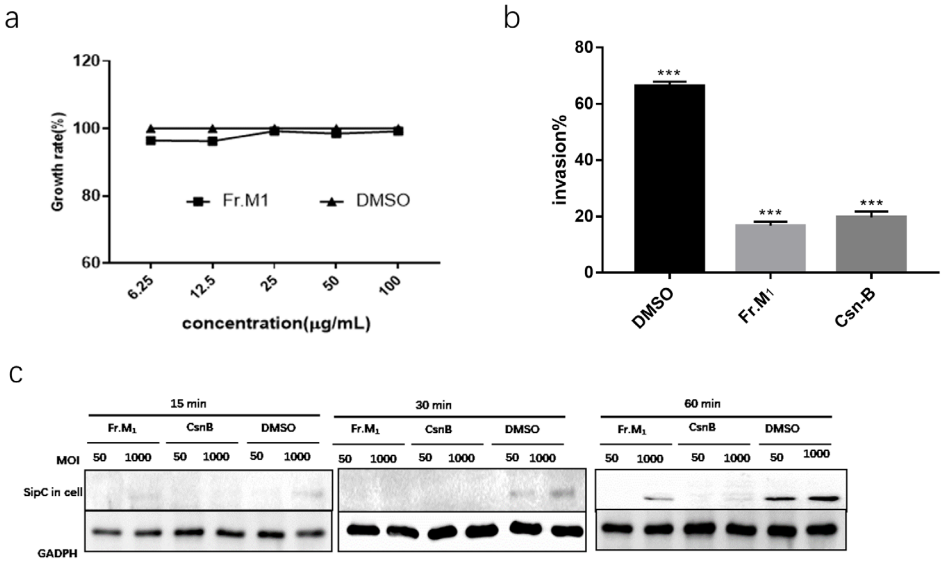

Figure 2. a) Fr.M_1 has no cytotoxic activity to MDA-MB-231 cells; **b)** Fr.M_1 inhibited S. Typhimurium invasion into MDA-MB-231 cells (The values represent percentages of invasion bacteria as related to the initial input, and error bars indicate standard deviations from the means. The levels of statistical significance is indicated as $^{***}p \leq 0.001$. The *p* value was calculated by comparing the value for the DMSO negative control.); **c)** Fr.M_1 significantly reduced the translocation of effector protein SipC into MDA-MB-231 cells compared to DMSO control.

while a decreased organ index indicates organ atrophy and other degenerative changes. The liver and kidney indexes of the infected mice were significantly higher than that of the Fr.M_1 treated mice, which indicated that Fr.M_1 treatment could reduce inflammation and edema of the organs (Figure 3b).

By observing the tissue sections of liver, spleen and kidney, we found that Salmonella infection generally caused organ congestion and edema, with a marked inflammatory cell infiltration around the vessels. Meantime, the pathological conditions of mice have improved after treatment with 20 mg/mouse Fr.M_1 (Figure 3c).

In recent years, many molecules from medicinal plants have been reported as T3SS inhibitors, such as cinnamaldehyde (*26*), paeonol (*27*), licoflavonol (*28*), thymol (*29*), epigallocatechin-3-gallate (*30*). The *in vitro* or *in vivo* bioassays suggested that Fr.M_1 was responsible for the anti-virulence activity of *C. bejolghota*. As a widely used medicinal and edible plant, *C. bejolghota*

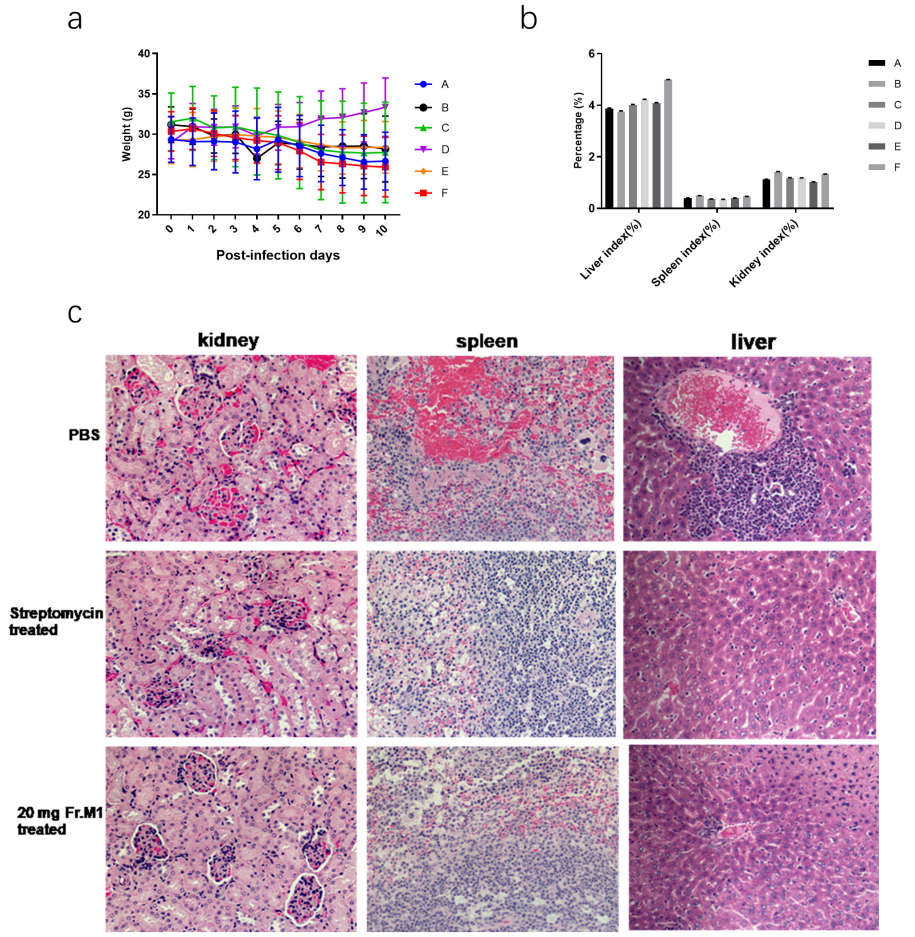

Figure 3. Protective effects of Fr.M$_1$ on *S.* Typhimurium-infected Kunming mice *in vivo*. a) the weight loss caused by Salmonella infection was alleviated; **b)** the organ indexes of liver, kidney and spleen are improved by Fr.M$_1$; **c)** Histopathology of the kidney, spleen and liver tissues were stained with hematoxylin and eosin (H&E) and observed by optical microscope (20 × 10).

also reported with diverse bioactivities such as anti-inflammation, antipyretic and analgesic activity (*31*) and antihyperglycemic property (*10*). The main constituents of *Cinnamomum* have been reported (*9,31,32*), and as one of the main constituents, cinnamaldehyde was reported to be an inhibitor of Salmonella T3SS by affecting the expression of key effector proteins, and reducing the translation of multiple virulence genes of the bacteria (*26*). Unlike cinnamaldehyde, Fr.M$_1$ was water soluble. Therefore, there must be undiscovered active ingredients in Fr.M$_1$ and need to be further explored.

Our study disclosed the polar fraction of *C. bejolghota* bark extract performed an excellent anti-virulent and anti-infectious property in *in vitro* and *in vivo* experiments. Therefore, we conclude that *C. bejolghota* bark could be used as an ideal material for the prevention and treatment of Salmonella caused infection or as supplementary food to be taken up by human or animals, and absorbed by the body together with a normal diet, which may become a new anti-virulence therapy.

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References

1. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov. 2015; 14:111-129.
2. Thabit AK, Crandon JL, Nicolau DP. Antimicrobial resistance: impact on clinical and economic outcomes and the need for new antimicrobials. Expert Opin Pharmacother. 2015; 16:159-177.
3. Muhlen S, Dersch P. Anti-virulence strategies to target bacterial infections. Curr Top Microbiol Immunol. 2016; 398:147-183.
4. Lewis K, Ausubel FM. Prospects for plant-derived antibacterials. Nat Biotechnol. 2006; 24:1504-1507.
5. Dickey SW, Cheung GYC, Otto M. Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. Nat Rev Drug Discov. 2017; 16:457-471.

6. Silva LN, Zimmer KR, Macedo AJ, Trentin DS. Plant natural products targeting bacterial virulence factors. Chem Rev. 2016; 116:9162-9236.
7. Wright GD. Opportunities for natural products in 21(st) century antibiotic discovery. Nat Prod Rep. 2017; 34:694-701.
8. DeFilipps RA, Krupnick GA. The medicinal plants of Myanmar. PhytoKeys. 2018; 102:1-341.
9. Gogoi B, Kakoti BB, Sharma N, Borah S. Pharmacognostic and preliminary phytochemical evaluation of *Cinnamomum bejolghota* (Buch.-Ham.) sweet bark. Indian J Nat Prod Resour 2016; 7:59-64.
10. Gogoi B, Kakoti BB, Borah S, Borah NS. Antihyperglycemic and *in vivo* antioxidative activity evaluation of *Cinnamomum bejolghota* (Buch.-Ham.) in streptozotocin induced diabetic rats: an ethnomedicinal plant in Assam. Asian Pac J Trop Med. 2014; 7S1:S427-434.
11. Li T, Zhang D, Oo TN, San MM, Mon AM, Hein PP, Wang Y, Lu C, Yang X. Investigation on the antibacterial and anti-T3SS activity of traditional Myanmar medicinal plants. Evid Based Complement Alternat Med. 2018; 2018:2812908.
12. Lee S, Gim H, Shim JH, Jung Kim H, Lee JR, Kim SC, Kwon YK, Ha KT, So I, Kim BJ. The traditional herbal medicine, Ge-Gen-Tang, inhibits pacemaker potentials by nitric oxide/cGMP dependent ATP-sensitive K(+) channels in cultured interstitial cells of Cajal from mouse small intestine. J Ethnopharmacol. 2015; 170:201-209.
13. Sujarwo W, Keim AP, Savo V, Guarrera PM, Caneva G. Ethnobotanical study of Loloh: Traditional herbal drinks from Bali (Indonesia). J Ethnopharmacol. 2015; 169:34-48.
14. Muhammad JS, Zaidi SF, Shaharyar S, Refaat A, Usmanghani K, Saiki I, Sugiyama T. Anti-inflammatory effect of cinnamaldehyde in *Helicobacter pylori* induced gastric inflammation. Biol Pharm Bull. 2015; 38:109-115.
15. Eswaran MB, Surendran S, Vijayakumar M, Ojha SK, Rawat AK, Rao Ch V. Gastroprotective activity of *Cinnamomum tamala* leaves on experimental gastric ulcers in rats. J Ethnopharmacol. 2010; 128:537-540.
16. Li J, Lv C, Sun W, Li Z, Han X, Li Y, Shen Y. Cytosporone B, an inhibitor of the type III secretion system of *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother. 2013; 57:2191-2198.
17. Hudson DL, Layton AN, Field TR, Bowen AJ, Wolf-Watz H, Elofsson M, Stevens MP, Galyov EE. Inhibition of type III secretion in *Salmonella enterica* serovar Typhimurium by small-molecule inhibitors. Antimicrob Agents Chemother. 2007; 51:2631-26355.
18. Negrea A, Bjur E, Ygberg SE, Elofsson M, Wolf-Watz H, Rhen M. Salicylidene acylhydrazides that affect type III protein secretion in *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother. 2007; 51:2867-2876.
19. Kaiser P, Diard M, Stecher B, Hardt WD. The streptomycin mouse model for *Salmonella diarrhea*: functional analysis of the microbiota, the pathogen's virulence factors, and the host's mucosal immune response. Immunol Rev. 2012; 245:56-83.
20. Hapfelmeier S, Hardt WD. A mouse model for *S.* Typhimurium-induced enterocolitis. Trends Microbiol. 2005; 13:497-503.
21. Tsolis RM, Xavier MN, Santos RL, Baumler AJ. How to become a top model: impact of animal experimentation on human salmonella disease research. Infect Immun. 2011; 79:1806-1814.
22. Greenfield EA. Harvesting tissue from immunized mice, rats, and hamsters. Cold Spring Harb Protoc. 2017; 2017:pdb prot100362.
23. Cardiff RD, Miller CH, Munn RJ. Mouse tissue fixation. Cold Spring Harb Protoc. 2014; 2014.
24. Robert D Cardiff 1 , Claramae H Miller 1 , Robert J. Munn Manual hematoxylin and eosin staining of mouse tissue sections. Cold Spring Harb Protoc. 2014; 2014:655-658.
25. Prabst K, Engelhardt H, Ringgeler S, Hubner H. Basic colorimetric proliferation assays: MTT, WST, and Resazurin. Methods Mol Biol. 2017; 1601:1-17.
26. Liu Y, Zhang Y, Zhou Y, Wang T, Deng X, Chu X, Zhou T. Cinnamaldehyde inhibits type three secretion system in *Salmonella enterica* serovar Typhimurium by affecting the expression of key effector proteins. Vet Microbiol. 2019; 239:108463.
27. Lv QH, Li SF, Wei HL, Wen ZM, Wang YL, Tang TZ, Wang JF, Xia LN, Deng XM. Identification of the natural product paeonol derived from peony bark as an inhibitor of the *Salmonella enterica* serovar Typhimurium type III secretion system. Appl Microbiol Biot. 2020; 104:1673-1682.
28. Guo ZX, Li XL, Li JF, Yang XF, Zhou Y, Lu CH, Shen YM. Licoflavonol is an inhibitor of the type three secretion system of *Salmonella enterica* serovar Typhimurium. Biochem Bioph Res Co. 2016; 477:998-1004.
29. Zhang Y, Liu Y, Qiu JZ, Luo ZQ, Deng XM. The herbal compound thymol protects mice from lethal infection by *Salmonella Typhimurium*. Front Microbiol. 2018; 9:1022.
30. Tsou LK, Yount JS, Hang HC. Epigallocatechin-3-gallate inhibits bacterial virulence and invasion of host cells. Bioorgan Med Chem. 2017; 25:2883-2887.
31. Baruah A, Nath SC. Taxonomic status and composition of stem bark oil of a variant of *Cinnamomum bejolghota* (Lauraceae) from Northeast India. Nord J Bot. 2001; 21:571-576.
32. Patil M, Choudhari AS, Pandita S, Islam MA, Raina P, Kaul-Ghanekar R. Cinnamaldehyde, cinnamic acid, and cinnamyl alcohol, the bioactives of *Cinnamomum cassia* exhibit HDAC8 inhibitory activity: an *in vitro* and *in silico* study. Pharmacogn Mag. 2017; 13:S645-S651.

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Case Report

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Lymphoma versus thymoma: A diagnostic challenge

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SUMMARY T-cell acute lymphoblastic lymphoma is a common hematological malignancy of childhood. It can involve the bone marrow, blood, or tissues like the thymus, lymph nodes as well as extra-nodal sites. Two aspects of the disease make early diagnosis critical - the clinically aggressive nature of the neoplasm, and availability of effective chemotherapy against the disease. Diagnosis is largely based on clinical suspicion and confirmation by histopathological examination of the affected tissue. However, biopsy results may not always be helpful in establishing the diagnosis. We describe the case of an 18-year old patient presenting with fever and an anterior mediastinal mass suspected to have a T-cell lymphoma where an initial biopsy from the mass had features of a thymoma. The patient was kept in close follow up for 2 months when there was a recurrence of symptoms and a repeat bone marrow evaluation revealed a T-cell lymphoma.

Keywords lymphoma, thymoma, fine-needle biopsy, mediastinal neoplasms

1. Introduction

T-cell acute lymphoblastic lymphoma (ALL) is a common hematological malignancy of childhood comprising of almost 15% of all acute childhood leukemias (*1*). The disease commonly affects lymph nodes, thymus or the bone marrow, presenting variably as an anterior mediastinal mass, pancytopenia or generalized lymphadenopathy. The diagnosis is usually straightforward, based on clinical features supported by histopathological evidence from the affected tissue. However, it is often thrown into doubt when there is a disagreement between histopathological findings and clinical features. We describe the case of an 18-year-old male suspected to have T-cell lymphoma clinically, with biopsy findings indicative of a lymphocyte rich thymoma where the patient was kept in close follow up with later biopsies confirming T-cell lymphoma.

2. Case report

An 18-year-old male presented with complaints of dry cough of six months duration with progressive shortness of breath, significant weight loss, and intermittent high-grade fevers for one month. Physical examination revealed left supraclavicular and axillary lymphadenopathy. Chest auscultation revealed reduced breath sounds over the left lower lung zones. Mediastinal widening with left pleural effusion was noted on chest radiogram. A subsequent contrast enhanced computerized tomography scan of the thorax evidenced a large homogenously enhancing structure encased by the mediastinal vasculature along with left pleural effusion and minimal pericardial effusion (Figure 1). The initial hemogram and reticulocyte count was within normal limits with the metabolic panel revealing hyperuricemia (12.8 mg/dL) and an elevated lactate dehydrogenase level of 1,212 U/L (Normal = 420 U/L). A provisional diagnosis of lymphoma was made and the patient was evaluated further. Thoracocentesis yielded exudative pleural fluid with no atypical cells. Fine needle aspiration of the supraclavicular lymph nodes was unrevealing. A biopsy of the mass was not feasible due to close proximity of mediastinal vessels. Hence, it was sampled by means of an ultrasound guided fine needle aspiration (FNA).

During the hospital course, the patient developed worsening respiratory distress and stridor due to increasing tracheal compression by the mediastinal mass. With the clinical suspicion of lymphoma, he was started on dexamethasone as a life-saving intervention despite a lack of histopathological evidence. His symptoms dramatically improved on steroids with diminution of lymphadenopathy, further supporting our suspicion of lymphoma.

The aspirate, however, revealed a focal meshwork of epithelioid cells staining positive for pancytokeratin with a predominant lymphoid background immunopositive for CD3 and terminal deoxynucleotidyl transferase (TdT) (Figure 2) consistent with the diagnosis of B1 subtype of

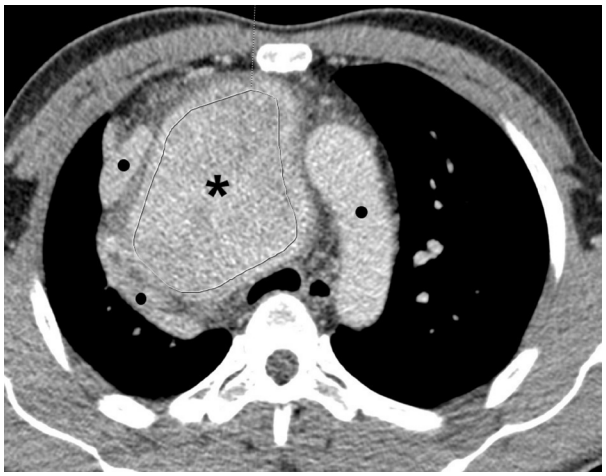

Figure 1. Contrast enhanced computerized tomography image of the thorax showing enhancing mediastinal mass (Asterix) encased by the mediastinal vasculature (Black ●).

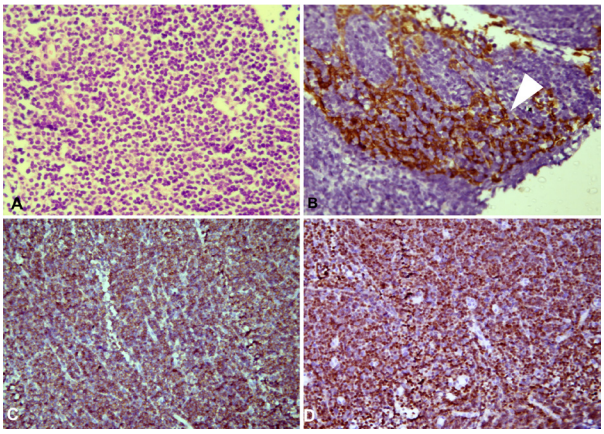

Figure 2. Histopathological and immunohistochemistry images from the aspirate of the anterior mediastinal mass. (A). Small to intermediate sized lymphoid cells (40× magnification). **(B)**. Focal meshwork of pan-cytokeratin positive epithelial cells (White arrowhead) (20× magnification). **(C)**. Immunohistochemistry showing background cells staining positive for CD3 (20× magnification). **(D)**. Immunohistochemistry showing background cells staining positive for Tdt (20× magnification).

thymoma as per the WHO classification. As this report was not compatible with our provisional diagnosis and clinical response to steroids, a bone marrow biopsy was done to look for evidence of occult hematologic malignancy. The bone marrow biopsy at this time was non-contributory. In light of clinical improvement but lack of evidence to support our diagnosis, it was decided to keep the patient on close follow up after discharge for further evidence of the natural progression of the disease. He was asymptomatic for a month after which he developed intermittent fevers and pancytopenia. The diagnosis was reached at this time by a repeat bone marrow biopsy which now revealed a diffuse infiltration by sheets of blasts immunopositive for TdT consistent with acute lymphoblastic lymphoma. He is now on regular follow up at the cancer clinic having gone into remission after multiple cycles of chemotherapy.

3. Discussion

This case illustrates the dilemma of differentiating a T-cell ALL from a lymphocyte-rich thymoma. It also emphasizes on the importance of clinical acumen during decision making in the absence of diagnostic evidence. In children, lymphoblastic tumors are the most common cause of an anterior mediastinal mass constituting nearly 45% of the cases. Non-Hodgkin's lymphoma presenting solely as a mediastinal mass is very rare with an incidence of 5%. Thymomas, on the other hand, are uncommon in children and young adults showing increasing incidence with age (*2*).

Diagnosis of an anterior mediastinal mass includes a concurrent consideration of the patient's clinical presentation, radiological and histopathologic evidence. Considering symptomatology, a short history of days to weeks with the presence of 'B' symptoms, significant lymphadenopathy and pleural effusion in males between 10 and 40 years suggests the diagnosis of lymphoblastic non-Hodgkin's lymphoma (LB-NHL) with moderate certainty. The presence of malignant cells in the pleural fluid and bone marrow makes the diagnosis certain. Thymomas on the other hand are more indolent, presenting with compressive symptoms in 20-30% of cases with pleural effusion and significant lymphadenopathy (2%) seen only rarely. In our case, the symptomatology and epidemiological characteristics of the disease placed a diagnosis of LB-NHL in good certainty (*3-5*).

Lymphoblastic lymphoma is diagnosed on FNA based on neoplastic cytomorphology with immunostaining for TdT and CD3, among other markers. Thymoma is a major non-neoplastic differential which is immunophenotypically similar. The presence of scattered cytokeratin positive thymic epithelial cells is diagnostic of lymphocyte-rich thymoma, which was the histopathologic diagnosis in our case (*6,7*). Diagnosis apart, FNA is also needed to characterize the type of lymphoma for the initiation of appropriate therapy.

The clinical presentation of our patient was confounded by the histopathologic picture. The lack of contributory evidence from the pleural fluid cytology and bone marrow biopsy made achieving the diagnosis harder still. Response to steroids made lymphoma a more probable scenario, though symptomatic thymomas have been reported to respond to steroids on multiple occasions (*8,9*). Considering the epidemiology, lack of evidence and the low rate of recurrence of a thymoma, allowing for further progression of the disease under close observation was the only feasible approach to attain the correct diagnosis leading to initiation of early and appropriate treatment.

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Conflict of Interest: The authors have no conflict of interest to disclose.

References

1. You MJ, Medeiros LJ, Hsi ED. T-lymphoblastic leukemia/lymphoma. Am J Clin Pathol. 2015; 144:411-422.
2. Mullen B, Richardson JD. Primary anterior mediastinal tumors in children and adults. Ann Thorac Surg. 1986; 42:338-345.
3. Carter BW, Marom EM, Detterbeck FC. Approaching the patient with an anterior mediastinal mass: a guide for clinicians. J Thorac Oncol. 2014; 9:S102-109.
4. Juanpere S, Cañete N, Ortuño P, Martínez S, Sanchez G, Bernado L. A diagnostic approach to the mediastinal masses. Insights Imaging. 2013; 4:29-52.
5. Ahmad U, Raja S. Lymph node metastases in thymic tumors: The more we know, the less we know. J Thorac Cardiovasc Surg. 2017; 154:e15-e16.
6. Li S, Juco J, Mann KP, Holden JT. Flow cytometry in the differential diagnosis of lymphocyte-rich thymoma from precursor T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma. Am J Clin Pathol. 2004; 121:268-274.
7. Young NA, Al-Saleem T. Diagnosis of lymphoma by fine-needle aspiration cytology using the revised European-American classification of lymphoid neoplasms. Cancer. 1999; 87:325-345.
8. Barratt S, Puthucheary ZA, Plummeridge M. Complete regression of a thymoma to glucocorticoids, commenced for palliation of symptoms. Eur J Cardiothorac Surg. 2007; 31:1142-1143.
9. Patel E, Juan G, Vaidya A, Thomas A, Taillon J, Milan S, Gross L, Shahzad S. Near complete resolution of invasive thymoma with corticosteroid therapy. Chest. 2015; 148:421A.

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Case Report

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Therapeutic potential of Chinese prescription Kangen-karyu for patient with lifestyle-induced metabolic syndrome

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SUMMARY We report a case of a 65-year-old patient with hypertension, dyslipidemia, type 2 diabetes, chronic kidney disease, and hyperuricemia, who showed an improvement in lifestyle-induced metabolic syndrome on the administration of 7.5 g of Kangen-karyu extract per day for 6 months. The levels of serum total cholesterol, low-density lipoprotein-cholesterol, and triglycerides were decreased. The systolic/diastolic blood pressure was decreased following administration. Other parameters such as estimated glomerular filtration rate, creatinine, uric acid, aspartate transaminase, alanine aminotransferase, γ-glutamyl transpeptidase, and creatine phosphokinase were improved by the administration of Kangen-karyu extract. At that time, the physical and subjective symptoms had partially disappeared. We present evidence supporting the use of Kangen-karyu extract against metabolic syndrome.

Keywords Chinese prescription, Kangen-karyu, lifestyle, metabolic syndrome, case report

1. Introduction

Lifestyle-induced metabolic disease is caused by an unhealthy lifestyle, which usually includes the combination of a poor diet, lack of exercise, environmental pollution, and excess stress. The most common causes of these lifestyle disorders are related to the diet – consuming unhealthy food uncontrolled eating, overconsumption of artificial sweeteners, processed foods, and junk foods. In addition, addictive habits like tobacco smoking, eating snacks, consumption of alcohol, irregular sleeping habits, stress, and modern-day urbanization have aggravated the situation. A subsequent milestone in the progression to metabolic disease is the development of metabolic syndrome, which is defined by having three or more of the following: increased waist circumference, high blood pressure, high triglycerides, low high-density lipoprotein (HDL)-cholesterol, and high fasting blood sugar. The final stage of this progression is the development of more severe metabolic disease such as diabetes, heart disease, obesity, fatty liver disease, and/or cancer (*1,2*).

In the treatment of metabolic syndrome, traditional Chinese medicine is an excellent example of alternative and complementary medicine with a long history, unique theory system, and variety of herbal remedies. Several randomized controlled trials have shown the curative effects of traditional Chinese medicine on metabolic syndrome, with some studies focusing on the independent components of metabolic syndrome (*3*). We chose Kangen-karyu (Guan-Yuan-Ke-Li in Chinese: a crude drug consisting of Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma, as shown in Table 1), a traditional Chinese prescription modified from Kan-shin No. 2 (Guan-xin No. 2 in Chinese) (*4*). It showed beneficial effects to improve signs of high-fructose diet-induced metabolic syndrome, such as hyperglycemia, hyperlipidemia, and hypertension, through the reduction of triglyceride and cholesterol levels by the regulation of hepatic sterol regulatory element-binding protein-1 (SREBP-1) expression, and also exhibited protective effects against high-cholesterol diet-induced hypercholesterolemia in rats (*5,6*). We also reported the beneficial effect of Kangen-karyu on dyslipidemia in a mouse model of type 2 diabetes (*7*). As Kangen-karyu has been clinically used as a treatment for cardiovascular disease, including angina pectoris and cerebrovascular diseases (*8,9*), the results of our previous

Table 1. Composition of Kangen-karyu

Common name	Botanical name	Family name
Salviae Miltiorrhizae Radix	*Salvia miltiorrhiza* Bunge	Labiatae
Cnidii Rhizoma	*Cnidium officinale* Makino	Umbelliferae
Paeoniae Radix	*Paeonia lactiflora* Pallas	Paeoniaceae
Carthami Flos	*Carthamus tinctorius* L.	Compositae
Aucklandiae Radix	*Aucklandia lappa* Dcne.	Compositae
Cyperi Rhizoma	*Cyperus rotundus* L.	Cyperaceae

study provide important evidence that this prescription ameliorates metabolic syndrome.

On the basis of the findings obtained from these fundamental studies, we administered Kangen-karyu to a patient with lifestyle-induced metabolic syndrome, and evaluated its treatment-based usefulness.

2. Case presentation

This study was conducted according to the ethical guidelines for epidemiological research set by the Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour, and Welfare. Ethical approval was obtained from the Clinical Research Ethics Committees of Shinseikai Toyama Hospital (Toyama, Japan). Written informed consent was obtained from the patient at the time of enrollment for the collection of clinical information and biosamples for archival and research purposes. A 65-year-old man with hypertension, dyslipidemia, type 2 diabetes, chronic kidney disease, and hyperuricemia was previously diagnosed with metabolic syndrome at Shinseikai Toyama Hospital. Subsequently, the patient modified his lifestyle and continued to receive existing treatments: an antihypertensive agent (micardis: 20 mg/day) and antilipidemic agent (livaro: 1 mg/day). However, he presented to our hospital on October 9, 2019, seeking to recover his functional level with herbal medicine. From the next day, Kangen-karyu extract (7.5 g/day) was administered three times a day before every meal until April 13, 2020.

Before Kangen-karyu extract administration, his initial anthopometric measurements included a body weight of 81.6 kg with a height of 173 cm, a body mass index (BMI) of 27.4 kg/m^2, and an abdominal circumference of 98.4 cm, which classified him as obese. His systolic/diastolic blood pressure was 130/86 mmHg (Table 2). Hemoglobin A1c (HbA1c) was 6.1%, showing poorly controlled blood glucose. The levels of serum lipids were as follows: total cholesterol: 216 mg/dL, low-density lipoprotein (LDL)-cholesterol: 118 mg/dL, HDL-cholesterol: 46 mg/dL, and triglycerides: 306 mg/dL, indicating hyperlipidemia (Table 3). In addition, the estimated glomerular filtration rate (eGFR) was 33.0 mL/min/1.73 m^2 based on the Modification of Diet in Renal Disease (MDRD) equation (*10*), and this corresponded to a serum creatinine level of 1.69 mg/dL. The serum uric acid level was 8.6 mg/dL, representing hyperuricemia derived from kidney disease.

Table 2. Physical characteristics on administration of Kangen-karyu for 6 months

Parameter	Pre	Post
Body weight (kg)	81.6	81.7
Height (cm)	173	173
BMI (kg/m^2)	27.4	27.2
Abdominal circumference (cm)	98.4	98.0
Systolic blood pressure (mmHg)	130	126
Diastolic blood pressure (mmHg)	86	70

Table 3. Laboratory data on administration of Kangen-karyu for 6 months

Parameter	Pre	Post
HbA1c (%)	6.1	6.5
Total cholesterol (mg/dL)	216	197
LDL-cholesterol (mg/dL)	118	108
HDL-cholesterol (mg/dL)	46	46
LDL-cholesterol/HDL-cholesterol	2.6	2.3
Triglycerides (mg/dL)	306	224
Uric acid (mg/dL)	8.6	7.7
Urea nitrogen (mg/dL)	21.7	20.8
Creatinine (mg/dL)	1.69	1.46
eGFR (mL/min/1.73 m^2)	33.0	38.7
AST (U/L)	26	18
ALT (U/L)	42	25
ALP (U/L)	215	202
LDH (U/L)	154	148
γ-GTP (U/L)	168	76
CPK (U/L)	197	112

The patient was an alcoholic, and alcohol consumption was assessed by questioning. The patient was asked about their average frequency (days per month) and amount (in mL) of alcoholic beverages ingested on a typical occasion or during a typical day, and categorized as a heavy consumer (≥ 30 g alcohol/day) according to the average daily alcohol consumption proposed by Agarwal (*11*). At that time, enzymes related to the hepatobiliary system and myocardial infarction were determined to assess their effects on the relationship between alcohol consumption and metabolic syndrome. Alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and creatine phosphokinase (CPK) were found to be poorly controlled. There were, however, no significant changes in the activities of aspartate transaminase (AST), alkaline phosphatase (ALP), or lactate dehydrogenase (LDH) (Table 3).

Assessment of somatic and subjective symptoms involved completing a series of questionaries at the beginning and end of the study. The symptom checklist included the following: dizziness and palpitation, stiff shoulders and headache, coldness of the limbs and fatigability, mental stress, sleeping disorder, tension of the stomach and abdomen, pain, numbness of the waist and body, dark circles around the eyes and lip symptoms, stains on the face, mottled skin, and tongue symptoms.

The change in each symptom was assessed with a 3-point rating scale: "marked improvement" was 5 points, "improvement" was 4 points, and "slight improvement" was 2 points. The assessment of global improvement rating of subjective symptoms simply involved the addition of points. At the same time, the tongue was evaluated based on factors such as the color, coating, and sublingual vein.

During the administration of Kangen-karyu extract, a physical examination was performed to evaluate its effect on metabolic syndrome. As shown in Table 2, BMI and abdominal circumference showed no changes on administration of the Kangen-karyu extract, but the systolic/diastolic blood pressure was decreased from 130/86 to 126/70 mmHg. The total cholesterol level had decreased from 216 to 197 mg/dL at the 6-month follow-up. The elevated levels of LDL-cholesterol and LDL-cholesterol/HDL-cholesterol were slightly reduced on treatment with Kangen-karyu extract during the follow-up period. Oral administration of Kangen-karyu extract significantly reduced the increased triglyceride level. Other parameters such as eGFR, creatinine, uric acid, AST, ALT, γ-GTP, and CPK were improved by the administration of Kangen-karyu extract, as shown in Table 3. At that time, somatic and subjective symptoms such as cold limbs, fatigue, insomnia, tension in the stomach and abdomen, gas in the bowels, pain and numbness of the lower back, pale complexion, and stains had improved. After 6 months, the score using the questionnaire had decreased from 46 to 36 at the follow-up. There was a slight improvement in the tongue coating on the administration of Kangen-karyu.

3. Discussion

Metabolic syndrome is defined as a disease state complicated by multiple metabolic diseases such as hyperlipidemia, hypertension, and diabetes. Even when each individual component disease is mild, the risk of metabolic syndrome is high. Over the past decade, different organizations have proposed various diagnostic criteria. The use of traditional Chinese medicine for the treatment of metabolic syndrome is becoming increasingly common due to its wide availability. Traditional Chinese prescriptions have received much attention as potential sources of novel therapeutic agents due to their multiple beneficial effects and absence of toxic and/or side effects (*12*).

We chose Kangen-karyu extract for the following reasons. Firstly, it has been clinically used as a treatment for cardiovascular diseases (*8,9*). Secondly, Kangen-karyu has received much attention as a source of new therapeutic agents based on pre-clinical animal experiments related to various human diseases (*5-7,13-18*). To add to these findings, we reported experimental evidence supporting its preventive and/or therapeutic potential against metabolic syndrome. The administration of Kangen-karyu significantly improved high-fructose-induced metabolic syndrome such as hyperglycemia, hyperlipidemia, and hypertension through the reductions of triglyceride and cholesterol contents with the regulation of hepatic SREBP-1 and the nuclear factor-kappa B signaling pathway (*6*). The results of our previous study suggest that Kangen-karyu may play a protective role against metabolic syndrome.

In the present case, there was an improvement in metabolic syndrome following the administration of Kangen-karyu extract for 6 months. The levels of serum total cholesterol, LDL-cholesterol, and triglycerides were decreased. The systolic/diastolic blood pressure was decreased compared with non-administration. At that time, the somatic and subjective symptoms had partially disappeared. Herein, we present a therapeutic option of Kangen-karyu based on metabolic parameters.

Although the mechanism underlying the development of the metabolic syndrome is not understood fully, it has been proposed that metabolic syndrome appears as a result of the reciprocal action of several environmental factors. In particular, alcohol consumption is one of the most prevalent habits in the general population (*19*). The beneficial effect of regular, light to moderate alcohol consumption on the development of coronary artery disease can be explained by several factors, including increase HDL-cholesterol and the balance between blood coagulation and fibrinolysis (*20,21*). The harmful effects of heavy alcohol consumption are due to an increase in plasma triacylglycerol and increased blood pressure (*22,23*). Each of these factors is a component of metabolic syndrome. Therefore, it is of interest to evaluate the overall associations of alcohol consumption with the development of metabolic syndrome. In the present case, the levels of serum total cholesterol, LDL-cholesterol, and triglycerides improved following the administration of Kangen-karyu extract. The blood pressure decreased compared with non-administration. In addition, interesting findings were obtained with regard to enzymes related to the hepatobiliary system and myocardial infarction: the levels of AST, ALT, γ-GTP, and CPK decreased compared with non-administration. Although the association of alcohol consumption with metabolic syndrome is complex and controversial, as both protective and detrimental effects have been reported (*19,24*), we report evidence to support the use of Kangen-karyu as an adjunctive therapy for a patient with lifestyle-induced metabolic syndrome. Kangen-karyu exhibits good efficacy in the treatment of lifestyle-induced metabolic syndrome.

Treatment for metabolic syndrome involves the management of a cluster of chronic diseases such as diabetes mellitus, hypertension, dyslipidemia, and obesity. However, traditional Chinese medicine has received much attention as a source of multi-target strategies due to its multiple beneficial effects and absence of toxic and/or side effects. We have been

investigating the multi-target therapeutic effects of Kangen-karyu on patients with metabolic syndrome. The present case provides strong evidence to support the administration of Kangen-karyu extract as a therapeutic agent to prevent the progression of metabolic syndrome.

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References

1. VanWormer JJ, Boucher JL, Sidebottom AC, Sillah A, Knickelbine T. Lifestyle changes and prevention of metabolic syndrome in the Heart of New Ulm Project. Prev Med Rep. 2017; 6:242-245.
2. Garralda-Del-Villar M, Carlos-Chillerón S, Diaz-Gutierrez J, Ruiz-Canela M, Gea A, Martínez-González MA, Bes-Rastrollo M, Ruiz-Estigarribia L, Kales SN, Fernández-Montero A. Healthy lifestyle and incidence of metabolic syndrome in the SUN cohort. Nutrients. 2019; 11:65.
3. Wu H, Tian J, Dai D, Liao J, Wang X, Wei X, Jin D, An X, Lian F, Tong X. Efficacy and safety assessment of traditional Chinese medicine for metabolic syndrome. BMJ Open Diab Res Care. 2020; 8:e001181.
4. Makino T, Wakushima H, Okamoto T, Okukubo Y, Deguchi Y, Kano Y. Pharmacokinetic and pharmacological interactions between ticlopidine hydrochloride and Kangen-karyu – Chinese traditional herbal medicine. Phytother Res. 2003; 17:1021-1024.
5. Yokozawa T, Cho EJ, Sasaki S, Satoh A, Okamoto T, Sei Y. The protective role of Chinese prescription Kangen-karyu extract on diet-induced hypercholesterolemia in rats. Biol Pharm Bull. 2006; 29:760-765.
6. Yokozawa T, Kim HJ, Yamabe N, Okamoto T, Cho EJ. The protective role of Kangen-karyu against fructose-induced metabolic syndrome in a rat model. J Pharm Pharmacol. 2007; 59:1271-1278.
7. Noh JS, Park CH, Kim HY, Zhao Q, Yamabe N, Matsumoto K, Yokozawa T. Chinese prescription Kangen-karyu prevents dyslipidaemia and oxidative stress in mouse model of type 2 diabetes. J Pharm Pharmacol. 2011; 63:111-119.
8. Xu LN, Yin ZZ, Ou YR. The effect of compositus Guan-Xin NO 2 on myocardial ischaemia and hypoxia in experimental animals. Yao Xue Xue Bao. 1979; 14:461-466.
9. Qin F, Huang X. Guanxin II for the management for coronary heart disease. Chin J Integr Med. 2009; 15:472-476.
10. Botev R, Mallié JP, Couchoud C, Schück O, Fauvel JP, Wetzels JFM, Lee N, Santo NGD, Cirillo M. Estimating glomerular filtration rate: Cockcroft-Gault and modification of diet in renal disease formulas compared to renal inulin clearance. Clin J Am Soc Nephrol. 2009; 4:899-906.
11. Agarwal DP. Cardioprotective effects of light-moderate consumption of alcohol: a review of putative mechanisms. Alcohol Alcohol. 2002; 37:409-415.
12. Winslow LC, Kroll DJ. Herbs as medicines. Arch Intern Med. 1998; 158:2192-2199.
13. Takahashi M, Sugaya K, Kubota K. Kangenkaryu prevents the decrease of cholinergic markers following the nucleus basalis magnocellularis lesion. Jpn J Pharmacol. 1992; 60:307-310.
14. Gao M, Ikeda K, Noguchi T, Mori K, Yamori Y. Studies on preventive effect of 'Kangenkaryu', Chinese herbal medicine, on stroke in SHR-SP. J Trad Med. 2001; 18:245-250.
15. Makino T, Wakushima H, Okamoto T, Okukubo Y, Saito K, Kano Y. Effects of Kangen-karyu on coagulation system and platelet aggregation in mice. Biol Pharm Bull. 2002; 25:523-525.
16. Pu F, Kaneko T, Enoki M, Irie K, Okamoto T, Sei Y, Egashira N, Oishi R, Mishima K, Kamimura H, Iwasaki K, Fujiwara M. Ameliorating effects of Kangen-karyu on neuronal damage in rats subjected to repeated cerebral ischemia. J Nat Med. 2010; 64:167-174.
17. Yamabe N, Kim HY, Kang KS, Zhao Q, Matsumoto K, Yokozawa T. Effect of Chinese prescription Kangen-karyu on lipid metabolism in type 2 diabetic *db/db* mice. J Ethnopharmacol. 2010; 129:299-305.
18. Zhao Q, Yokozawa T, Yamabe N, Tsuneyama K, Li X, Matsumoto K. Kangen-karyu improves memory deficit caused by aging through normalization of neuro-plasticity-related signaling system and VEGF system in the brain. J Ethnopharmacol. 2010; 131:377-385.
19. Yoon YS, Oh SW, Baik HW, Park HS, Kim WY. Alcohol consumption and the metabolic syndrome in Korean adults: the 1998 Korean National Health and Nutrition Examination Survey. Am J Clin Nutr. 2004; 80:217-224.
20. Langer RD, Criqui MH, Reed DM. Lipoproteins and blood pressure as biological pathways for effect of moderate alcohol consumption on coronary heart disease. Circulation. 1992; 85:910-915.
21. Krobot K, Hense HW, Cremer P, Eberle E, Keil U. Determinants of plasma fibrinogen: relation to body weight, waist-to-hip ratio, smoking, alcohol, age, and sex. Results from the second MONICA Augsburg survey 1989-1990. Arterioscler Thromb. 1992; 12:780-788.
22. Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A, Zukel WJ. Alcohol and blood lipids. The cooperative lipoprotein phenotyping study. Lancet. 1977; 2:153-155.
23. Marmot MG, Elliott P, Shipley MJ, Dyer AR, Ueshima H, Beevers DG, Stamler R, Kesteloot H, Rose G, Stamler J. Alcohol and blood pressure: the INTERSALT study. BMJ. 1994; 308:1263-1267.
24. Freiberg MS, Cabral HJ, Heeren TC, Vasan RS, Ellison RC. Alcohol consumption and the prevalence of the metabolic syndrome in the U.S.: a cross-sectional analysis of data from the Third National Health and Nutrition Examination Survey. Diabetes Care. 2004; 27:2954-2959.

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Is amiloride a promising cardiovascular medication to persist in the COVID-19 crisis?

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SUMMARY In the ongoing coronavirus diseases-2019 (COVID-19) crisis that caused immense suffering and deaths, the choice of therapy for the prevention and life-saving conditions must be based on sound scientific evidence. Uncertainty and apprehension are exacerbated in people using angiotensin-converting enzyme (ACE) inhibitors to control their comorbidities such as hypertension and diabetes. These drugs are reported to result in unfavorable outcome as they tend to increase the levels of ACE2 which mediates the entry of SARS-CoV-2. Amiloride, a prototypic inhibitor of epithelial sodium channels (ENaC) can be an ideal candidate for COVID-19 patients, given its ACE reducing and cytosolic pH increasing effects. Moreover, its potassium-sparing and anti-epileptic activities make it a promising alternative or a combinatorial agent.

Keywords ACE2, ACE inhibitors, amiloride, COVID-19, cytosolic pH, ENaC

The world is anxiously watching the escalating spread of a novel pandemic that has already caused immense suffering and deaths (*1*). In this situation, the choice of therapy for coronavirus diseases-2019 (COVID-19) prevention and life-saving purpose must be supported by compelling scientific evidence (*2*). People suffering from co-morbid conditions such as diabetes and hypertension are reportedly at a higher risk of death (*3*). Uncertainty and apprehensions are exacerbated in these people since they rely on certain drugs to control their comorbidities (*4*). If at all angiotensin-converting enzyme (ACE) inhibitors result in unfavorable outcomes (*5,6*), a potassium-sparing diuretic can come to rescue. We hypothesized that amiloride, a prototypic inhibitor of epithelial sodium channels (ENaC) (*7*), can alleviate the expression of ACE2 in human lung epithelial cells.

In our study, amiloride, an epithelial sodium channel (ENaC) inhibitor, was found to reduce the expression of angiotensin-converting enzyme (ACE2) in both human alveolar epithelial and bronchial epithelial cell lines at 24 h as illustrated in Figure 1. Similarly, 'with no lysine kinase' (WNK) inhibitor showed a reduction in both cell lines but it was not significant. WNKs are a novel family of serine-threonine kinase known to regulate ENaC (*8*) and therefore it was inhibited in the study to determine the signaling pathway involved.

Multiple reasons support the use of amiloride in patients with cardiovascular morbidities during the coronavirus diseases-2019 (COVID-19) crisis. Firstly, ACE2 mediates the entry of SARS-CoV-2 (*9*) and therefore it may constitute a pharmacological target to limit cell entry of the virus (*10*). Amiloride induced reduction in ACE2 expression in bronchial and alveolar epithelial cells indicates a beneficial effect of the drug in restricting the entry of SARS-CoV-2. Secondly, a decreased cytosolic pH is believed to be the most important reason behind COVID-19 infection resulting in a higher incidence of the disease in the elderly and smokers. Amiloride can counteract the low cytosolic pH by acting on Na^+/H^+ exchanger (*11*). Thirdly, amiloride is effective in lung tissue (*11*), which is the most frequent site of infection for SARS-CoV-2 indicated by acute respiratory distress syndrome and mortality (*12*). Fourthly, potassium was reported to be significantly lower in severe COVID-19 patients (*13*) and the correction of hypokalemia was found to be challenging because of its continuous renal potassium loss resulting from the degradation of ACE2 (*14*). Amiloride with its potassium-sparing diuretic activity (*15*), helps restore normal serum potassium concentrations in those who develop hypokalemia (*16*). Finally, The Center for Disease Control and Prevention (CDC) suggests that epilepsy, among neurological comorbidities, maybe a predisposing factor for COVID-19 despite lack of evidence (*17*). Fortuitously, the illness associated with abnormal electric discharge (*18*) was found to be alleviated in rodents by anti-seizure and other neurological activities of amiloride (*19*).

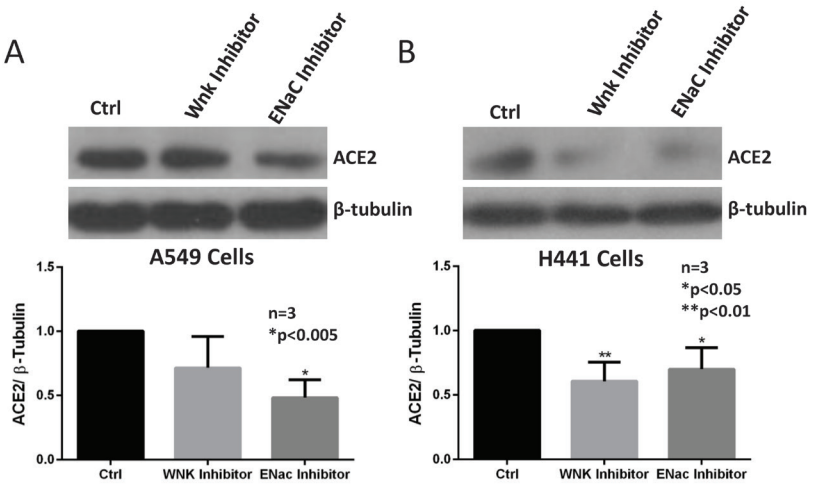

Figure 1. Amiloride suppresses ACE2 expression in A549 and H441 cells. Immunoblots and densitometric analyses of ACE2 expression on WNK and ENaC inhibitors at 24 h in **(A)** A549 and **(B)** H441 cell lines.

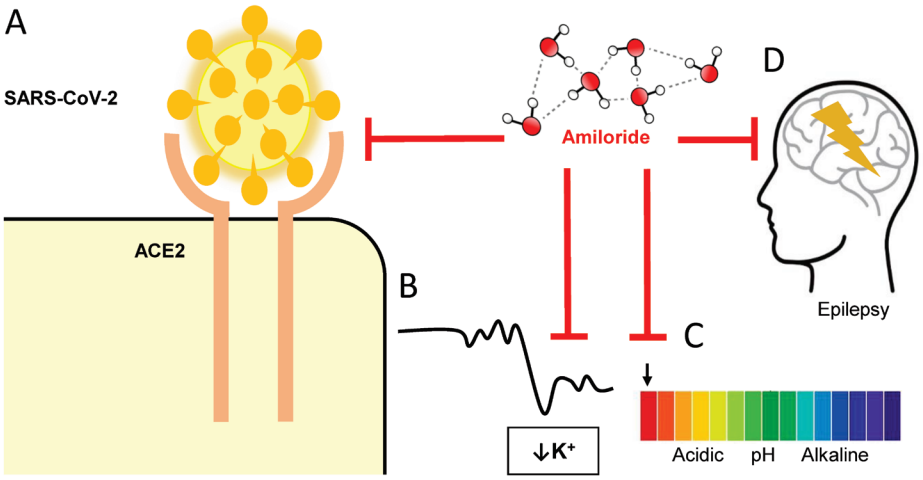

Figure 2. Diagrammatic illustration of amiloride effects. It inhibits **(A)** ACE2 expression; **(B)** Hypokalemia; **(C)** Acidic pH in the cytosol and **(D)** Seizures.

Regardless of the salt-sensitive status, large meta-analyses have reported the superiority of low-dose diuretics against other alternatives in hypertension (*20*). In the ongoing uncertainty over the safety of ACE inhibitors during the COVID-19 crisis, amiloride usage as an alternative or a combinatorial agent in the aforesaid co-morbidities can not only ameliorate the cardiovascular events but can also help in COVID-19 as pictorially illustrated in Figure 2.

Materials and Methods

Cell lines and reagents: The human A549 adenocarcinoma (ATCC® CCL-185™) and Human bronchial (H441) epithelial (ATCC® HTB-174™) cell lines obtained from ATCC (Manassas, VA) were cultured in DMEM high glucose and RPMI 1640 media (Hyclone, Logan, UT), respectively supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator at 37°C and 5% CO_2. Cells were routinely passaged when they reached 80-90% confluency. Compound inhibitors were obtained from Selleckchem, Houston, TX for WNK (WNK 463, Cat#S8358) and epithelial sodium channel (Amiloride, Cat#S1811) to be used in the concentrations of 1 µM and 10 µM, respectively.

Western blotting: The cell lysates were prepared using 1X RIPA lysis buffer (Millipore, Temecula, CA) supplemented with protease and phosphatase inhibitor tablets (Roche Applied Science, Indianapolis, IN). Protein concentration was measured by the DC protein assay (Bio-Rad Laboratories, Hercules, CA) and approximately 40-50 µg of cell lysates in Laemmli buffer were used. Densitometry was performed using NIH ImageJ software. Primary antibodies against ACE2 (Cat# MABN59) and β-tubulin (Cat#2118S)

were purchased from Millipore Sigma (Burlington, MA) and Cell Signaling Technology (Danvers, MA), respectively.

Statistical analysis: All the data are presented as mean ± SEM. The 'n' value for each figure implies the number of samples in each group. All the data were analyzed by parametric testing using the Student's unpaired *t*-test or one-way ANOVA, followed by the posthoc test using the GraphPad Prism 6.01 software. Data with $p < 0.05$ were considered significant.

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References

1. Morens DM, Daszak P, Markel H, Taubenberger JK. Pandemic COVID-19 Joins History's Pandemic Legion. mBio. 2020; 11:e00812-20
2. Trifirò G, Crisafulli S, Andò G, Racagni G, Drago F. Should patients receiving ACE inhibitors or angiotensin receptor blockers be switched to other antihypertensive drugs to prevent or improve prognosis of novel coronavirus disease 2019 (COVID-19)? Drug Saf. 2020; 43:507-509.
3. Khulood D, Adil MS, Sultana R, Nimra. Convalescent plasma appears efficacious and safe in COVID-19. Ther Adv Infect Dis. 2020; 7:2049936120957931.
4. Khashkhusha TR, Chan JSK, Harky A. ACE inhibitors and COVID-19: We don't know yet. J Card Surg. 2020; 35:1172-1173.
5. Tadic M, Cuspidi C, Sala C. COVID-19 and diabetes: Is there enough evidence? J Clin Hypertens (Greenwich). 2020; 22:943-948.
6. Kai H, Kai M. Interactions of coronaviruses with ACE2, angiotensin II, and RAS inhibitors-lessons from available evidence and insights into COVID-19. Hypertens Res. 2020; 43:648-654.
7. Kleyman TR, Sheng S, Kosari F, Kieber-Emmons T. Mechanism of action of amiloride: a molecular prospective. Semin Nephrol. 1999; 19:524-532.
8. Huang CL, Kuo E. Mechanisms of disease: WNK-ing at the mechanism of salt-sensitive hypertension. Nat Clin Pract Nephrol. 2007; 3:623-630.
9. Wang Q, Zhang Y, Wu L, Niu S, Song C, Zhang Z, Lu G, Qiao C, Hu Y, Yuen KY, Wang Q, Zhou H, Yan J, Qi J. Structural and functional basis of SARS-CoV-2 entry by using human ACE2. Cell. 2020; 181:894-904.e899.
10. McKee DL, Sternberg A, Stange U, Laufer S, Naujokat C. Candidate drugs against SARS-CoV-2 and COVID-19. Pharmacol Res. 2020; 157:104859.
11. Cure E, Cumhur Cure M. Comment on "Organ-protective effect of angiotensin-converting enzyme 2 and its effect on the prognosis of COVID-19". J Med Virol. 2020.
12. Jain A. COVID-19 and lung pathology. Indian J Pathol Microbiol. 2020; 63:171-172.
13. Lippi G, South AM, Henry BM. Electrolyte imbalances in patients with severe coronavirus disease 2019 (COVID-19). Ann Clin Biochem. 2020; 57:262-265.
14. Chen D, Li X, Song Q, Hu C, Su F, Dai J, Ye Y, Huang J, Zhang X. Assessment of hypokalemia and clinical characteristics in patients with coronavirus disease 2019 in Wenzhou, China. JAMA Netw Open. 2020; 3:e2011122.
15. Bull MB, Laragh JH. Amiloride. A potassium-sparing natriuretic agent. Circulation. 1968; 37:45-53.
16. Maronde RF, Milgrom M, Vlachakis ND, Chan L. Response of thiazide-induced hypokalemia to amiloride. JAMA. 1983; 249:237-241.
17. Kuroda N. Epilepsy and COVID-19: Associations and important considerations. Epilepsy Behav. 2020; 108:107122.
18. Mir S Adil, Azizullah G, M.d Amer K, M Nematullah K, M Aamer K, Ihtisham S. Metronidazole induced seizures. World Journal of Pharmaceutical Sciences. 2014; 2:108-111.
19. Ali A, Ahmad FJ, Pillai KK, Vohora D. Evidence of the antiepileptic potential of amiloride with neuropharmacological benefits in rodent models of epilepsy and behavior. Epilepsy Behav. 2004; 5:322-328.
20. Huot SJ, Aronson PS. Na(+)-H+ exchanger and its role in essential hypertension and diabetes mellitus. Diabetes Care. 1991; 14:521-535.

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Stabilizing mast cells by commonly used drugs: a novel therapeutic target to relieve post-COVID syndrome?

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SUMMARY Regardless of the severity of coronavirus disease 2019 (COVID-19), a high proportion of patients struggle with persistent respiratory or systemic symptoms after recovery. This is called "post-COVID syndrome", for which pulmonary fibrosis is one of the pathogenesis. Besides T-lymphocytes and macrophages, mast cells also contribute to the development of cytokine storm and thus stimulate the activity of fibroblasts. Additionally, by the exocytotic release of fibroblast-activating factors, mast cells directly facilitate the progression of pulmonary fibrosis. In our previous basic studies, anti-allergic drugs (olopatadine, ketotifen), antibiotics (clarithromycin) and corticosteroids (hydrocortisone, dexamethasone) inhibited the process of exocytosis and showed their potency as highly effective mast cell stabilizers. Given such pharmacological properties of these commonly used drugs, they may be useful in the treatment of post-COVID-19 pulmonary fibrosis and in relieving the symptoms of post-COVID syndrome.

Keywords COVID-19, post-COVID syndrome, pulmonary fibrosis, mast cell

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In most cases, patients are asymptomatic or only present with mild to moderate symptoms, including fever, dry cough and shortness of breath. Nevertheless, some patients develop severe pneumonia or acute respiratory distress syndrome (ARDS), sometimes complicated by multiple organ dysfunction due to systemic thrombotic microangiopathy (*1*). Recently, regardless of the severity of the disease, a high proportion of patients with COVID-19 are reported to struggle with persistent respiratory or systemic symptoms after recovery (*2,3*). This so-called "post-COVID syndrome" includes dyspnea, chest pain, generalized fatigue and joint pain, for which pulmonary fibrosis is one of the pathogenesis (*4,5*).

Pulmonary fibrosis is the interstitial pulmonary disease characterized by the proliferation of fibroblasts, the excessive deposition of collagen and extracellular matrix, and the destruction of normal pulmonary architecture (*6*). For the development of idiopathic pulmonary fibrosis, several growth factors, such as transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), are generally considered to be responsible (*7,8*), since these growth factors promote the profibrogenic process of the lung after injury. For the development of "post-COVID-19" pulmonary fibrosis, cytokine storm, which is characterized by the over-activation of leukocytes and the uncontrolled secretion of pro-inflammatory cytokines, is additionally thought to be responsible (*9,10*). These cytokines impair the recovery process from the lung injury and stimulate the activity of fibroblasts to produce collagen, and thus promote the progression of pulmonary fibrosis (*11*).

We have demonstrated in several animal studies that the overexpression of Kv1.3-channels in T-lymphocytes and macrophages is strongly associated with their over-activation and the progression of renal fibrosis (*12,13*). In these studies, margatoxin, a selective Kv1.3-channel inhibitor, actually suppressed the activity of the leukocytes and slowed the progression of renal fibrosis. On the other hand, in a series of patch-clamp studies, we have revealed the suppressive properties of nonsteroidal anti-inflammatory drugs (NSAIDs), anti-hypertensive drugs, anti-cholesterol drugs and anti-allergic drugs on lymphocyte Kv1.3-channels (*14-16*). Taking into account such pharmacological properties of these drugs, they may also be useful in the treatment of post-COVID-19 pulmonary fibrosis, in addition to their usefulness in suppressing cytokine storm (*9*).

Besides T-lymphocytes and macrophages, recent studies additionally indicate a large contribution of mast

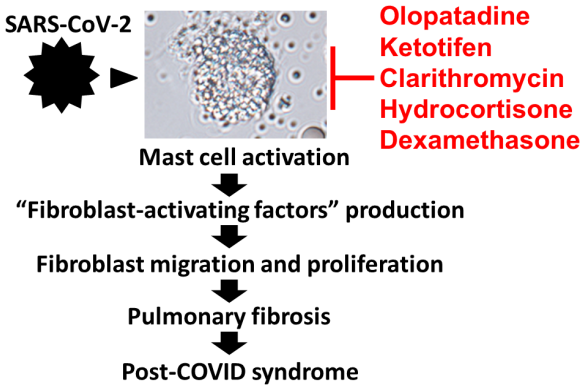

Figure 1. Mast cells in the development of pulmonary fibrosis and as the target of anti-allergic drugs, antibiotics and corticosteroids. Once mast cells are activated by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), they produce fibroblast-activating factors. These factors promote the migration and proliferation of fibroblasts and facilitate the progression of pulmonary fibrosis, causing post-COVID syndrome. Anti-allergic drugs (olopatadine, ketotifen), antibiotics (clarithromycin) and corticosteroids (hydrocortisone, dexamethasone) that stabilize mast cells suppress the progression of pulmonary fibrosis and relieve the symptoms of post-COVID syndrome.

cells to the pathogenesis of cytokine storm triggered by SARS-CoV-2 (*17,18*). Once activated by the virus, mast cells that reside in the respiratory mucous membrane produce pro-inflammatory cytokines, such as interleukin (IL)-1, IL-4, IL-5, IL-6 and tumor necrosis factor (TNF)-α, in addition to their exocytotic release of chemokines (*19*). Additionally, several studies revealed that mast cells directly facilitate the progression of pulmonary fibrosis by the exocytotic release of fibroblast-activating factors (*20,21*). In these studies, the factors promoted the migration and proliferation of fibroblasts and stimulated their activity to produce collagen (Figure 1).

Previous studies revealed that mast cells are also responsible for the development and progression of organ fibrosis, such as liver cirrhosis, systemic sclerosis and renal fibrosis (*22-24*). Therefore, based on these findings, these studies suggested the pharmacological efficacy of suppressing the mast cell activity in the treatment or protection against organ fibrosis. The pharmacological approaches that were taken were either directly stabilizing mast cells or indirectly inhibiting the chemokines released from mast cells. In our previous animal study, tranilast, one of the potent mast cell stabilizers, actually ameliorated the progression of peritoneal fibrosis complicated with chronic renal failure (*25*). On the other hand, in a series of patch-clamp studies, by monitoring the changes in the whole-cell membrane capacitance in mast cells, we provided *in vitro* evidence that anti-allergic drugs (olopatadine, ketotifen), antibiotics (clarithromycin) and corticosteroids (hydrocortisone, dexamethasone) strongly inhibit the process of exocytosis (*26-29*). Morphologically, these drugs actually suppressed the degranulation from mast cells, showing their potency as highly effective mast cell stabilizers. Of note, prazosin, an α_1-adrenergic receptor blocker, synergistically enhanced the mast cell-stabilizing property of adrenaline (*30*). Given such pharmacological properties of these commonly used drugs, they may also be useful in the treatment of post-COVID-19 pulmonary fibrosis and in relieving the symptoms of post-COVID syndrome (Figure 1).

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References

1. Leisman DE, Deutschman CS, Legrand M. Facing COVID-19 in the ICU: vascular dysfunction, thrombosis, and dysregulated inflammation. Intensive Care Med. 2020; 46:1105-1108.
2. Carfi A, Bernabei R, Landi F, Gemelli Against C-P-ACSG. Persistent symptoms in patients after acute COVID-19. JAMA. 2020; 324:603-605.
3. Davido B, Seang S, Tubiana R, de Truchis P. Post-COVID-19 chronic symptoms: a postinfectious entity? Clin Microbiol Infect. 2020.
4. Vasarmidi E, Tsitoura E, Spandidos DA, Tzanakis N, Antoniou KM. Pulmonary fibrosis in the aftermath of the COVID-19 era (Review). Exp Ther Med. 2020; 20:2557-2560.
5. Tale S, Ghosh S, Meitei SP, Kolli M, Garbhapu AK, Pudi S. Post COVID-19 pneumonia pulmonary fibrosis. QJM. 2020.
6. Vitiello A, Pelliccia C, Ferrara F. COVID-19 patients with pulmonary fibrotic tissue: Clinical pharmacological rational of antifibrotic therapy. SN Compr Clin Med. 2020; 2:1709-1712.
7. Allen JT, Spiteri MA. Growth factors in idiopathic pulmonary fibrosis: relative roles. Respir Res. 2002; 3:13.
8. Ojo AS, Balogun SA, Williams OT, Ojo OS. Pulmonary fibrosis in COVID-19 survivors: Predictive factors and risk reduction strategies. Pulm Med. 2020; 2020:6175964.
9. Kazama I. Targeting lymphocyte Kv1.3-channels to suppress cytokine storm in severe COVID-19: Can it be a novel therapeutic strategy? Drug Discov Ther. 2020; 14:143-144.
10. Garcia-Revilla J, Deierborg T, Venero JL, Boza-Serrano A. Hyperinflammation and fibrosis in severe COVID-19 patients: Galectin-3, a target molecule to consider. Front Immunol. 2020; 11:2069.
11. Kaur G, Lungarella G, Rahman I. SARS-CoV-2 COVID-19 susceptibility and lung inflammatory storm by smoking and vaping. J Inflamm (Lond). 2020; 17:21.
12. Kazama I. Physiological significance of delayed rectifier K^+ channels (Kv1.3) expressed in T lymphocytes and

their pathological significance in chronic kidney disease. J Physiol Sci. 2015; 65:25-35.
13. Abe N, Toyama H, Saito K, Ejima Y, Yamauchi M, Mushiake H, Kazama I. Delayed rectifier K^+-channel is a novel therapeutic target for interstitial renal fibrosis in rats with unilateral ureteral obstruction. Biomed Res Int. 2019; 2019:7567638.
14. Kazama I, Tamada T, Tachi M. Usefulness of targeting lymphocyte Kv1.3-channels in the treatment of respiratory diseases. Inflamm Res. 2015; 64:753-765.
15. Baba A, Tachi M, Maruyama Y, Kazama I. Suppressive effects of diltiazem and verapamil on delayed rectifier K^+-channel currents in murine thymocytes. Pharmacol Rep. 2015; 67:959-964.
16. Saito K, Abe N, Toyama H, Ejima Y, Yamauchi M, Mushiake H, Kazama I. Second-generation histamine H1 receptor antagonists suppress delayed rectifier K^+-channel currents in murine thymocytes. Biomed Res Int. 2019; 2019:6261951.
17. Kritas SK, Ronconi G, Caraffa A, Gallenga CE, Ross R, Conti P. Mast cells contribute to coronavirus-induced inflammation: new anti-inflammatory strategy. J Biol Regul Homeost Agents. 2020; 34:9-14.
18. Afrin LB, Weinstock LB, Molderings GJ. Covid-19 hyperinflammation and post-Covid-19 illness may be rooted in mast cell activation syndrome. Int J Infect Dis. 2020; 100:327-332.
19. Graham AC, Temple RM, Obar JJ. Mast cells and influenza a virus: association with allergic responses and beyond. Front Immunol. 2015; 6:238.
20. Bagher M, Larsson-Callerfelt AK, Rosmark O, Hallgren O, Bjermer L, Westergren-Thorsson G. Mast cells and mast cell tryptase enhance migration of human lung fibroblasts through protease-activated receptor 2. Cell Commun Signal. 2018; 16:59.
21. Gruber BL. Mast cells in the pathogenesis of fibrosis. Curr Rheumatol Rep. 2003; 5:147-153.
22. Gruber BL. Mast cells: accessory cells which potentiate fibrosis. Int Rev Immunol. 1995; 12:259-279.
23. Holdsworth SR, Summers SA. Role of mast cells in progressive renal diseases. J Am Soc Nephrol. 2008; 19:2254-2261.
24. Blank U, Essig M, Scandiuzzi L, Benhamou M, Kanamaru Y. Mast cells and inflammatory kidney disease. Immunol Rev. 2007; 217:79-95.
25. Kazama I, Baba A, Endo Y, Toyama H, Ejima Y, Matsubara M, Tachi M. Mast cell involvement in the progression of peritoneal fibrosis in rats with chronic renal failure. Nephrology (Carlton). 2015; 20:609-616.
26. Baba A, Tachi M, Maruyama Y, Kazama I. Olopatadine inhibits exocytosis in rat peritoneal mast cells by counteracting membrane surface deformation. Cell Physiol Biochem. 2015; 35:386-396.
27. Baba A, Tachi M, Ejima Y, Endo Y, Toyama H, Matsubara M, Saito K, Yamauchi M, Miura C, Kazama I. Anti-allergic drugs tranilast and ketotifen dose-dependently exert mast cell-stabilizing properties. Cell Physiol Biochem. 2016; 38:15-27.
28. Mori T, Abe N, Saito K, Toyama H, Endo Y, Ejima Y, Yamauchi M, Goto M, Mushiake H, Kazama I. Hydrocortisone and dexamethasone dose-dependently stabilize mast cells derived from rat peritoneum. Pharmacol Rep. 2016; 68:1358-1365.
29. Kazama I, Saito K, Baba A, Mori T, Abe N, Endo Y, Toyama H, Ejima Y, Matsubara M, Yamauchi M. Clarithromycin dose-dependently stabilizes rat peritoneal mast cells. Chemotherapy. 2016; 61:295-303.
30. Abe N, Toyama H, Ejima Y, Saito K, Tamada T, Yamauchi M, Kazama I. Alpha 1-adrenergic receptor blockade by prazosin synergistically stabilizes rat peritoneal mast cells. Biomed Res Int. 2020; 2020:3214186.

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DD&T
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