***Lactobacillus gasseri* OLL2809 is effective especially on the menstrual pain and dysmenorrhea in endometriosis patients: randomized, double-blind, placebo-controlled study**

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Abstract In this study, we evaluated the efficacy of *Lactobacillus gasseri* OLL2809 on endometriosis by the randomized, double-blind and placebo-controlled clinical study, especially against pain, which is one of the causative factors to decrease the quality of life. Sixty-six patients clinically diagnosed with endometriosis were enrolled in this study, 62 of which have successfully completed the trial. The tablets containing 100 mg of *L. gasseri* OLL2809 (active tablet, $n = 29$) or placebo tablets ($n = 33$) were ingested once a day for 12 weeks. Visual analog scale (VAS) of pain intensity at the menstrual period and verbal rating scale (VRS) of dysmenorrhea were significantly improved by the ingestion of the active tablets as compared with placebo tablets. There was no significant change of blood examination and biochemical examination of blood in the enrolled

patients. Above results show that the tablet containing *L. gasseri* OLL2809 is effective on endometriosis, especially against menstrual pain and dysmenorrhea. Moreover, it was found that the tablet has no adverse effects. Therefore, it was suggested that the tablet containing *L. gasseri* OLL2809 contributes to improve the quality of life in the patients with endometriosis.

Keywords *Lactobacillus gasseri* OLL2809 · Endometriosis · Menstrual pain · Dysmenorrhea

Abbreviations

VAS Visual analog scale
VRS Verbal rating scale
NK Natural killer
IL Interleukin
QOL Quality of life

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Introduction

Endometriosis, which is pathologically defined by the presence of viable endometrial tissue outside the uterine cavity, is one of the most enigmatic and problematic maladies affecting women of reproductive age and a common condition affecting 6–10% of women of reproductive age (Giudice and Kao 2004). Despite being one of the most frequently encountered

gynecological diseases, the pathophysiology of this disease remains controversial.

According to Sampson's classical implantation theory, retrograde menstruation, peritoneal adhesion of endometrial tissue and outgrowth of these endometrial cells, glands and stroma are supposed to be essential elements in the pathogenesis of endometriosis (Sampson 1940). However, retrograde menstruation is a universal phenomenon with viable endometrial cells found in peritoneal fluid of 76–90% of women, and its prevalence is much higher than that of endometriosis (Bartosik et al. 1986). These findings indicate that other factors, such as the amount of retrograde flow or immunological changes, may determine the susceptibility of a woman to endometriosis even though retrograde menstruation is one of the causative factors of endometriosis.

Studies in women with endometriosis have demonstrated that, in addition to changes in its volume, there are functional changes in several immunological components of the peritoneal fluid, for example phagocytic macrophages–monocytes, natural killer (NK) cells, cytotoxic T lymphocytes, B cells or inflammatory mediators such as complements and cytokines (Ho et al. 1997). Especially for NK cells, there are many reports that NK cells play an important role in the pathogenesis of endometriosis (Oosterlynck et al. 1991, 1992; Ho et al. 1995; Quaranta et al. 2006; Kusakabe et al. 2007). For instance, Oosterlynck et al. reported that the decrease in NK activity significantly correlated with the severity of the disease (Oosterlynck et al. 1992). These findings have given rise to the NK cell theory of endometriosis that impaired clearance of ectopic endometrium by NK cells in the peritoneal cavity contributes to the development of the disease.

Sashihara et al. reported that *Lactobacillus gasseri* OLL2809, a probiotic lactic acid bacterium, strongly stimulates interleukin (IL)-12 production by murine splenocytes (Sashihara et al. 2006, 2007, 2008). Therefore, it is expected that its use will lead to ameliorate allergic diseases. Indeed, *L. gasseri* OLL2809 has been shown to reduce allergic symptoms in patients of Japanese-cedar pollinosis with high Japanese-cedar pollen-specific IgE (Gotoh et al. 2009). It has been known that IL-12 induces cytotoxicity of NK cells (Kobayashi et al. 1989; Stern et al. 1990). Therefore, it is expected that *L. gasseri*

OLL2809 may ameliorate endometriosis by activating cytotoxicity of NK cells through the enhanced production of IL-12. Indeed, there are many reports on the role of IL-12 against endometriosis (Mazzeo et al. 1998; Zeyneloglu et al. 1998; Somigliana et al. 1999; Gazvani et al. 2002).

On the other hand, endometriosis is associated with pain and decreases the quality of life (QOL). Therefore, it is important to maintain the QOL even in the menstrual period. To relieve the pain in the menstrual period, non-steroidal anti-inflammatory drugs (NSAIDs) have been used in general. However, NSAIDs have been known to induce gastrointestinal disorders, such as erosive lesions or ulcers, for their main pharmacological property inhibiting cyclooxygenase which generates prostaglandins from arachidonic acid to protect mucosal functions. Moreover, hormonal side effects were observed for the therapy of endometriosis using such as danazol and gonadotropin-releasing hormone agonist (Olive and Pritts 2001; Crosignani et al. 2006). *Lactobacilli* have been generally regarded as safe (Mäyrä-Mäki and Bigret 1993; Naidu et al. 1999). Therefore, if *L. gasseri* OLL2809 was effective against endometriosis, it may become very useful functional material to improve the QOL and have a therapeutic effect for the patients with endometriosis.

Then, in this study we evaluated the efficacy of *L. gasseri* OLL2809 especially on the pain, which is one of the causative factors to decrease QOL in endometriosis patients, by the double blind placebo-controlled study in patients with endometriosis.

Materials and methods

Subjects

Patients visited the Department of Gynecology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine in Shanghai from September to November in 2008, and who were diagnosed with endometriosis and fulfilled the following inclusion criteria were enrolled. Inclusion criteria were: (1) patients whose age are between 18 and 45 years; (2) patients who have regular menstrual cycles (28 ± 3 days). Exclusion criteria were: (1) Patients who had received hormone therapy in the past 3 months; (2) Patients with severe primary cardiac,

hepatic, renal or hematopoietic diseases; (3) Those suffering from malignant tumors or suspected malignant tumor; (4) CA-125 $\geq$ 100 IU/ml; (5) Patients with endometriosis around simple incision in the abdominal wall; (6) Patients who may suffer from diarrhea after having dairy products; (7) Patients receiving treatment for current allergic diseases; (8) Patients who often take drugs which may affect the test results (such as medicines treating constipation or digestive medicine etc.); (9) Patients who take health foods for more than 4 days per week which may affect the test results (e.g. lactobacilli products such as yogurt or beverages).

Clinical diagnosis with endometriosis was followed by “The clinical diagnosis criteria of endometriosis described in the 6th edition of *Obstetrics and Gynecology* published by People’s Medical Publishing House in 2003” and was made when one of the following symptoms (from i to iv) and one of following two physical signs (vi to vii) were observed. Symptoms: (i) Secondary dysmenorrhea aggravating progressively; (ii) Lower abdominal pelvic pain during non-menstrual period; (iii) Coital discomfort or algopareunia; (iv) Periodicrectal irritation symptoms: diarrhea, constipation or straining feeling in anus; (v) Infertility. Physical signs: (vi) Tender nodules can be observed in sites of rectouterine pouch, uterosacral ligament of the lower segment of uterine posterior wall; (vii) Cystic, solid and immovable mass connected with uterus can be palpable in adnexa area.

Study protocol

This study was a randomized, double-blind, placebo-controlled clinical trial performed at a single institution in the Department of Gynecology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine in Shanghai, China, from September 2008 to February 2009.

Sixty-six patients were clinically diagnosed with endometriosis and sequentially enrolled in this study. The subjects were randomly divided into two groups, Group 1 (Placebo group) and Group 2 (Active group) by a controller who was not directly involved in the study. A group allocation number was given each subject. To prevent leakage of the information, this number was closely guarded both by the controller and the member of ethic committee until accessing the key after completion of the study.

Subjects received two active tablets, containing *L. gasseri* OLL2809, or placebo tablets once a day for 12 weeks (3 menstrual periods). Tablets with (active tablet) or without (placebo tablet) *L. gasseri* OLL2809 were produced by Meiji Dairies Corporation (Tokyo, Japan). One tablet (250 mg) contained freeze-dried 50 mg of heat-killed (75 °C for 60 min) *L. gasseri* OLL2809. Placebo tablet contained dextrin instead of freeze-dried *L. gasseri* OLL2809 and was identical in color and taste to the active tablet.

Subjects were asked to visit the hospital every 1 month a total of 3 times (until 3 month later) after starting ingestion of the tablets and to record the symptoms of endometriosis using questionnaires as well as to receive clinical examinations by physicians in charge.

Withdrawal and rejection criteria from the study were as follows. Withdrawal criteria: (1) Subjects withdrawal at their own will; (2) Loss to follow up; (3) Subjects with serious complications or severe adverse events; (4) Unblinded cases being urgently uncovered. Rejection criteria: (1) Subjects who did not take medicine in accordance with prescription (within 80 to 120% of the prescribed range); (2) Subjects of whom the efficacy could not be determined or of whom the determination on efficacy or safety could be largely affected by insufficient data etc.

Informed consent was obtained from all enrolled subjects, and the study was carried out in accordance with the Declaration of Helsinki. In addition, this study received the approval of the Ethics Committee of the Division of Research and Development of Meiji Dairies Corporation.

Evaluation items

The primary efficacy endpoints were patient response to the treatment for pain at the menstrual period and dysmenorrheic score associated with endometriosis, as evaluated visual analog scale (VAS) of pain intensity and verbal rating scale (VRS) of dysmenorrhea, respectively. Secondary efficacy endpoints were the pain in non-menstrual period and pain score in non-menstrual period, as evaluated VAS of pain intensity and VRS of non-menstrual pelvic pain, respectively.

1. VAS of pain intensity in the menstrual and non-menstrual period

The VAS of pain intensity in the menstrual period and non-menstrual period was a subjective assessment of the pain on a scale of 0 (no pain) to 10 (most severe pain). It was recorded on a 10-cm ruler in the diary at each follow-up visit and reflected the severity of this symptom as perceived by the subject in the preceding month.

2. VRS of dysmenorrhea and non-menstrual pelvic pain

VRS of dysmenorrhea (dysmenorrheic score) was evaluated using a modified version of Andersch and Milsom's multidimensional VRS (Andersch and Milsom 1982), which defines pain according to the limitation of ability to work (unaffected = 0; rarely affected = 1; moderately affected = 2; clearly inhibited = 3) and need for analgesics (no = 0; once a day = 1; twice a day = 2; three or over a day = 3), and simple sum of these scores were used as a score of dysmenorrheic score (maximum score is 6).

3. VRS of non-menstrual pelvic pain was evaluated using a modified version of Andersch and Milsom's multidimensional VRS (Andersch and Milsom 1982), which defines pain according to the limitation of ability to work (unaffected = 0; rarely affected = 1; moderately affected = 2; clearly inhibited = 3) and need for analgesics (no = 0; one day in non-menstrual days = 1; 2–7 days in non-menstrual days = 2; 8 days or over in non-menstrual days = 3), and simple sum of these scores were used as the VRS of non-menstrual pelvic pain (maximum score is 6).

These evaluations mentioned above were performed before and 1, 2 and 3 months after tablets ingestion.

4. Serum levels of CA-125

Serum levels of CA-125 were measured as a valuable marker of endometriosis before and 3 months after tablet ingestion.

5. Blood examination and biochemical examination of blood

Blood examination (red blood cell count, white blood cell count, platelet count and hemoglobin) and biochemical examination of blood (blood sugar, total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total bilirubin, γ -transpeptidase, alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, uric acid, total cholesterol, triglyceride

and high density lipoprotein (HDL) were performed before and after tablets ingestion.

Statistical analysis

A blind data review was performed before decoding, and decision concerning the handling of drop-outs was made on the basis of blinded results. The assessment of efficacy was based on all subjects who completed the study.

All results are expressed as mean $\pm$ SE. Statistical difference between two groups was analyzed by Student's *t*-test or Mann–Whitney's *U*-test. A probability of < 0.05 was considered significant.

Results

Among the 66 subjects enrolled (placebo: 34, active 32), 4 subjects were withdrawn from the study citing personal reasons, and the remained 62 subjects successfully completed the trial. There was no subject who met the rejection criteria. Finally 33 subjects in the Group 1 (placebo) and 29 subjects in the Group 2 (active) were subjected for analysis. Clinical features of the subjects divided into two groups are shown in Table 1. No significant difference was observed between the two groups.

Change in VAS of pain intensity at menstrual period

The time-course changes in VAS of pain intensity are shown in Fig. 1. The scores were 7.73 ± 0.26 and 7.51 ± 0.22 in the placebo and active groups, respectively, before tablet ingestion. No significant difference in the scores was observed between two groups. Significant differences were observed between placebo and active tablet ingested groups at 2 ($P < 0.05$) and 3 ($P < 0.01$) months after tablet ingestion, showing that active tablet ingestion was effective on the pain in menstrual period.

After 3 month of ingestion, the decrease in VAS of pain intensity in the active group (-3.28 ± 0.36) was significantly greater than that in the placebo group (-2.00 ± 0.29) ($P < 0.01$, Fig. 2).

Table 1 Clinical features of analyzed subjects

	Group 1 (Placebo)	Group 2 (Active)	Statistical analysis
No. of subjects	33	29	NS
Age (years old)	32.5 ± 5.6	31.6 ± 4.9	NS
Married/unmarried	31/2	29/0	NS
Menarche (years old)	12.9 ± 1.2	12.9 ± 1.3	NS
Menstrual period (day)	5.3 ± 0.9	5.2 ± 0.7	NS
Menstrual cycle (day)	29.0 ± 1.3	28.9 ± 1.7	NS
Menstrual pain (+/–)	33/0	29/0	NS
Chronic pelvic pain (+/–)	30/3	27/2	NS
Coital pain (+/–)	27/6	25/4	NS

NS not significant

Fig. 1 Time-course changes in VAS of pain intensity at menstrual period. The ingestion of tablets containing *Lactobacillus gasseri* OLL2809 (Active, $n = 29$) showed a significant pain relief in the menstrual period as compared with the ingestion of placebo tablets (Placebo, $n = 33$). *, **: $P < 0.05$, 0.01, respectively, versus placebo tablet

Fig. 2 Change in VAS (visual analog scale) of pain intensity at menstrual period. The ingestion of tablets containing *Lactobacillus gasseri* OLL2809 (Active) showed a significant decrease in VAS of pain intensity in the menstrual period as compared with the ingestion of placebo tablets (Placebo). **: $P < 0.01$ versus placebo tablet

Change in VRS of dysmenorrhea

The time-course changes in VRS of dysmenorrhea are shown in Fig. 3. The scores were 3.45 ± 0.20 and 3.14 ± 0.17 in the placebo and active groups, respectively, before tablet ingestion. No significant difference in the scores was observed between both groups. After tablet ingestion, a significant difference was observed between both groups at 3 months ($P < 0.05$).

The active group showed a tendency to decrease VRS of dysmenorrhea after 3 month of ingestion more effectively than the placebo tablet ingested group (-1.44 ± 0.17 vs -1.03 ± 0.16) ($P = 0.0596$, Fig. 4).

Fig. 3 Time-course changes in VRS of dysmenorrhea at menstrual period. The ingestion of tablets containing *Lactobacillus gasseri* OLL2809 (Active, $n = 29$) showed a significant decrease in VRS of dysmenorrhea as compared with the ingestion of placebo tablets (Placebo, $n = 33$). *: $P < 0.05$ versus placebo tablet

Fig. 4 Change in VRS of dysmenorrhea at menstrual period. The ingestion of tablets containing *Lactobacillus gasseri* OLL2809 (Active) showed a tendency to decrease in VRS at the menstrual period as compared with the ingestion of placebo tablets (Placebo)

Change in VAS of pain intensity at non-menstrual period

The time-course changes in VAS of pain intensity in non-menstrual period are shown in Fig. 5. The scores were 2.18 ± 0.29 , and 1.93 ± 0.29 in the placebo and active groups, respectively, before tablet ingestion; these were much lower than those at menstrual period. The scores decreased with the ingestion periods in both groups. However, there were no significant differences in the scores between the two groups.

Change in VRS of non-menstrual pelvic pain at non-menstrual period

The time-course changes in VRS of pelvic pain are shown in Fig. 6. The scores were 1.27 ± 0.16 , and

Fig. 5 Time-course changes in VAS of pain intensity at non-menstrual period. The ingestion of tablets containing *Lactobacillus gasseri* OLL2809 (Active, $n = 29$) showed no significant pain relief in the non-menstrual period as compared with the ingestion of placebo tablets (Placebo, $n = 33$)

Fig. 6 Time-course changes in VRS of pelvic pain at non-menstrual period. The ingestion of tablets containing *Lactobacillus gasseri* OLL2809 (Active, $n = 29$) showed no significant decrease in VRS as compared with the ingestion of placebo tablets (Placebo, $n = 33$)

1.17 ± 0.12 in the placebo and active groups, respectively, before tablet ingestion. The scores decreased with the ingestion periods in both two groups. However, there were no significant differences in the scores between the two groups as well as the VAS of pain intensity at non-menstrual period.

Change of serum level of CA-125

The serum levels of CA-125 before tablet ingestion were 32.7 ± 6.7 and 20.4 ± 4.7 IU/ml in placebo ($n = 31$) and active ($n = 29$) groups, respectively, being no significant difference between both groups. After the tablets ingestion, the serum levels of CA-125 changed to 32.2 ± 8.3 and 19.1 ± 3.3 IU/ml in placebo and active groups, respectively, being no significant difference between both groups.

Blood examination and biochemical examination of blood

Red blood cell count, white blood cell count, platelet count and hemoglobin did not change significantly after the tablets ingestion in both placebo tablet ingested group and active tablet ingested group. Blood sugar, total protein, ALT, AST, LDH, total bilirubin, γ -transpeptidase, ALP, BUN, creatinine, uric acid, total cholesterol, triglyceride and HDL also did not show significant change after the tablets ingestion in both placebo and active groups.

Discussion

The present double-blind, placebo-controlled clinical trial revealed for the first time that oral *L. gasseri* OLL2809 effectively reduced the pain at the menstrual period and dysmenorrhea associated with endometriosis as compared with placebo, suggesting that *L. gasseri* OLL2809 is useful for improving QOL in patients with endometriosis, because the pain in the menstrual period and dysmenorrhea are causative factors to decrease QOL. Moreover, our results support common clinical practice of treating dysmenorrhea resulting from endometriosis with *L. gasseri* OLL2809, because no adverse effects were observed in the present study.

Oosterlynck et al. reported that a defect in NK cell activity in women with endometriosis resulted in a decreased cytotoxicity to autologous endometrium (Oosterlynck et al. 1991). Ho et al. reported that in the peritoneal fluid of women with endometriosis, NK cytotoxicity was significantly lower than that in women without endometriosis (Ho et al. 1995). Moreover, NK cell activity is decreased in the peripheral blood as well as in the peritoneal fluid of women with endometriosis, and this decrease was significantly related to an increase in disease stage (Oosterlynck et al. 1992). Kusakabe et al. reported that danazol has an effect to induce recruitment of NK cells in mouse uteri (Kusakabe et al. 2007). Quaranta et al. found that NK cell activity was significantly down-regulated in endometriosis patients (Quaranta et al. 2006). These findings have given rise to the NK cell theory of endometriosis, which presupposes that endometriotic cells are natural NK target cells, and that impaired clearance of ectopic endometrium by NK cells in the peritoneal cavity contributes to the development of the disease. However, it can still be argued that alterations in NK cell activity associated with endometriosis can be an effect of the disease. Hill et al. reported that aberrant NK cell activity may be the result of an imbalance in the immune response to a chronic antigenic stimulus, such as ectopic endometrium, or to a previously initiated autoimmune event unrelated to endometriosis (Hill 1992). Moreover, Kikuchi et al. suggested that changes in the differentiation of NK cells were the consequence of the presence of endometriotic implants (Kikuchi et al. 1993).

IL-12 has been known to induce proliferation and cytotoxicity of NK cells (Kobayashi et al. 1989; Stern et al. 1990). Gazvani et al. 2002. reported that IL-12 significantly inhibited the survival of endometrial cells from women with and without endometriosis. Moreover, Mazzeo et al. showed that NK cells pretreated with IL-12 exhibited an enhanced cytotoxic response toward endometrial targets (Mazzeo et al. 1998). Somigliana et al. reported that intraperitoneal injection of IL-12 was able to reduce total weight and total surface area of endometriotic lesions in mouse model (Somigliana et al. 1999). *L. gasseri* OLL2809 has been reported to strongly stimulate IL-12 production by murine splenocytes (Sashihara et al. 2006, 2007, 2008). Moreover, it has been known that IL-12 activates the NK cell cytotoxicity. There are many reports on the useful role of IL-12 against endometriosis as mentioned above. In the present study, *L. gasseri* OLL2809 showed a significant improvement of VAS of pain intensity during the menstrual period and VRS of dysmenorrhea. Therefore, *L. gasseri* OLL2809 might play on the pain relief through the enhanced generation of IL-12. Further studies would be needed to clarify the mechanism of *L. gasseri* OLL2809.

Although both VAS of pain intensity and VRS of pelvic pain during non-menstrual period decreased with the ingestion period, there was no significant effect of *L. gasseri* OLL2809. Regarding the VAS of pain intensity during menstrual period, the scores were higher than 7 before tablet ingestion while they were relatively low (around 2) during non-menstrual period. This observation was similar with the VRS of non-menstrual pelvic pain. Therefore, the reasons for that the clinical effect of *L. gasseri* OLL2809 was not observed during non-menstrual period were assumed to be because the basal values before the tablets ingestion were low and, in addition to that the pain intensity could be largely affected by psychological factors.

There is a positive correlation between serum and peritoneal values of CA-125 in women with and without endometriosis, and their levels are higher in peritoneal fluid (Amaral et al. 2006). Advanced endometriosis is related to higher levels of CA-125 in both serum and peritoneal fluid (Amaral et al. 2006). In this study, no significant decrease in the serum levels of CA-125 was observed although significant pain relief was observed by the treatment

with *L. gasseri* OLL2809. Maiorana et al. reported that no correlation was found between serum levels of CA-125 and pelvic pain in patients with endometriosis (Maiorana et al. 2007). Therefore, significant pain relief might not be affected by the serum CA-125 levels. Indeed, no significant correlations were observed between the levels and VAS of pain intensity or VRS of dysmenorrhea during the menstrual period (data not shown).

In conclusion, the tablet containing *L. gasseri* OLL2809 was effective on endometriosis, which was indicated by the improvement of menstrual pain and dysmenorrhea and found that this tablet containing *L. gasseri* OLL2809 had no adverse effects. These findings support the common clinical practice of the strain to improve QOL in patients with endometriosis.

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