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Effect of Aerobic Exercise on Plasma Biomarkers of Pain in Women with Primary Dysmenorrhea: A Controlled Non-Randomized Pilot Trial

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Abstract

Objectives: To (1) evaluate preliminary effects of aerobic exercise on progesterone, prostaglandin metabolites and pro-inflammatory cytokines in women with primary dysmenorrhea, (2) assess the feasibility of methodological procedures and obtain preliminary data (effect size) to estimate the sample size for a future randomized controlled trial.

Methods: Twenty women aged 18-29 years were divided into two groups (high-intensity aerobic-exercise group and no-exercise control group) in a 1:1 ratio. Women assigned to the exercise group performed high-intensity treadmill-based aerobic exercise for three days a week, at 70-85% of maximum heart rate for 30 minutes for 4 weeks. The control group did not receive trial intervention but provided blood for estimation of plasma variables. Blood plasma levels of progesterone, prostaglandin (PG) metabolites, F2 alpha [KDPGF2α]) and PGE2 (KDPGE2), and tumor necrosis factor-alpha (TNF-α) were measured at 4-weeks post-intervention.

Results: There was an increase in progesterone levels (d=0.36) and decreases in KDPGF2α (d=0.35), KDPGE2 (d= 0.47), and TNF-α (d=0.33) from baseline to week 4 in the exercise group compared with the control group. Concerning feasibility outcomes, 90% of the required number of participants were recruited in 3-4 weeks and adherence to the intervention was 97%.

Conclusion: Study findings suggest that aerobic exercise may be effective for primary dysmenorrhea via its influence on progesterone and inflammatory pain mediators. The study methodology appears to be feasible for conducting a full-scale randomized controlled trial.

Keywords: Aerobic exercise; Biomarkers; Pain; Primary dysmenorrhea; Progesterone; Prostaglandins

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Introduction

Primary dysmenorrhea is a common gynaecological problem affecting nearly half of women worldwide [1]. The pathophysiology of primary dysmenorrhea is linked primarily to prostaglandins (PGs) [2-4]. Several studies have reported a close relationship between primary dysmenorrhea and abnormally elevated PG secretion [2-5]. Increased PG production, particularly of the E (PGE2) and F series (PGF2α) has been reported in both humans [6-8] and experimental models [9]. Abnormal PG levels during the menstrual cycle are reported to induce hyper-contractility of the myometrium, leading to ischemia and hypoxia, which are regarded as the primary contributors to the pain in primary dysmenorrhea [10,11].

The PGs are intracellular lipid compounds that are derived enzymatically from the polyunsaturated fatty acid, arachidonic acid [12]. Arachidonic acid is released from the phospholipid molecule by the lysosomal enzyme phospholipase A2 [13]. Lysosomal activity is controlled by several factors, one of which is the level of the hormone progesterone during different phases (follicular and luteal) of the menstrual cycle [3,14]. The decline in progesterone levels during the late luteal phase of the menstrual cycle labilizes the lysosomal activity resulting in greater production of arachidonic acid and consequently larger

production of PGs **Figure 1** [3,14,15]. In summary, the low level of progesterone in the late luteal phase of the menstrual cycle is reported to increase the synthesis of PG, with PGs and progesterone displaying an inverse relationship [3,16].

Pro-inflammatory cytokines are also known to play a role in the pathogenesis of primary dysmenorrhea [17-19]. The human endometrium is highly inflammatory, with the endometrial stromal cells producing large amounts of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) during the mid-to-late luteal phase of the menstrual cycle [20]. Studies have found elevated levels of TNF-α in women with primary dysmenorrhea compared with women without dysmenorrhea [17,18]. TNF-α is reported to stimulate the release of PGs through its effect on the enzyme cyclooxygenase (COX) **Figure 1** [17,19]. There are two isoforms of the COX enzyme (COX-1 and COX-2) that are involved in the generation of PGs from arachidonic acid [21]. Of these two isoforms, COX-2 is the primary enzyme controlling the synthesis of PGs [20]. COX-2 is constitutively expressed in the endometrial stromal cells and is influenced by TNF-α and progesterone levels [20]. Greater expression of TNF-α in the endometrium during the late luteal phase induces higher COX-2 resulting in a greater generation of PGs leading to myometrial hypoxia and pain **Figure 1** [20,22]. In addition, progesterone has an inhibitory effect on cytokine-induced COX-2 expression; decrease in progesterone levels during the late luteal phase reduces the inhibitory effect of COX-2 **Figure 1** [20]. In summary, expression of COX-2 in the endometrial stromal cells is reported to increase significantly in response to increase in levels of TNF-α (i.e. directly proportional) and decrease in progesterone levels (indirectly proportional) [20,22].

Aerobic exercise has been shown to alleviate the pain of primary dysmenorrhea in several observational [23-25], randomized [26,27], and non-randomized case series studies [28,29]. A recent systematic review recommended physical activity as an effective

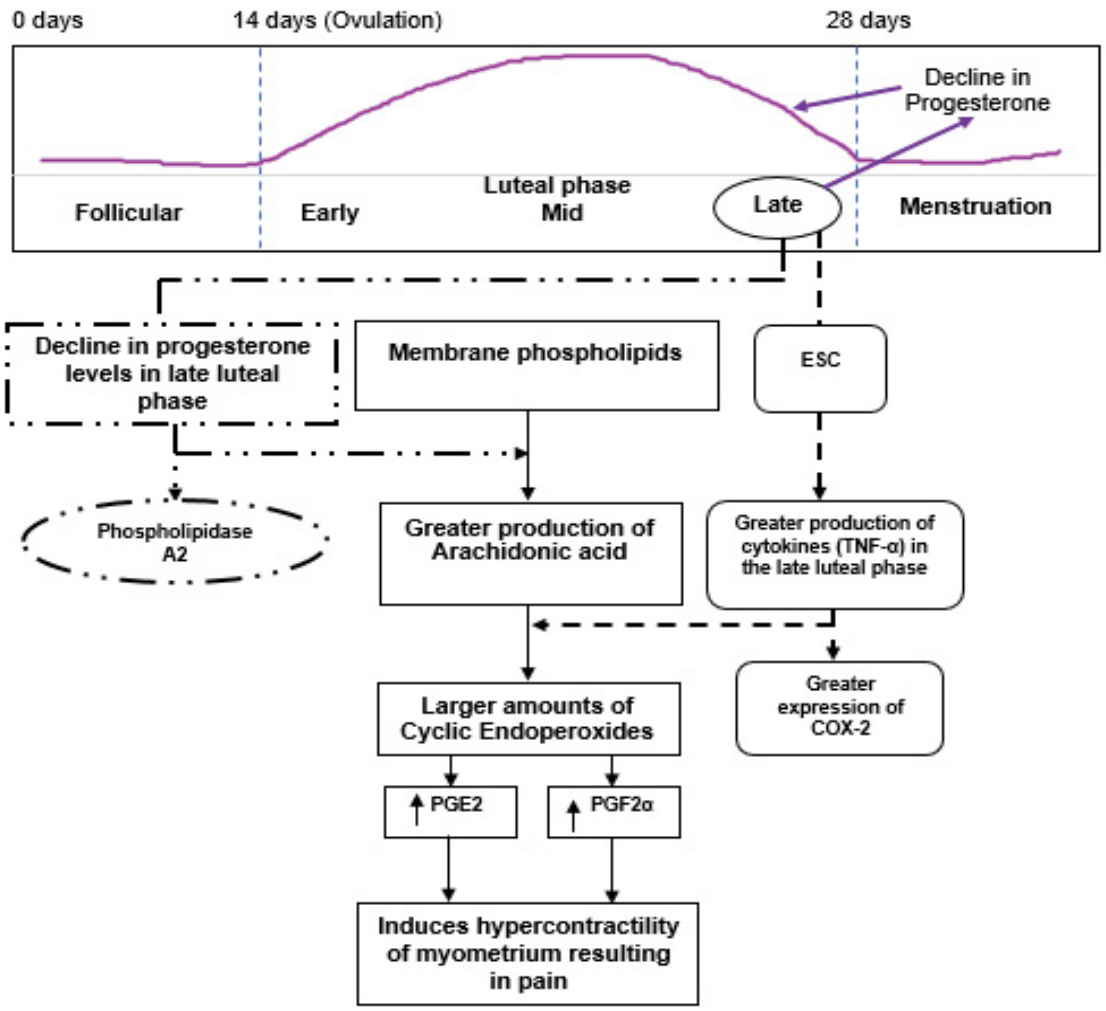

Figure 1 Biosynthesis of prostaglandins in primary dysmenorrhea. *Purple line:* Progesterone levels in different phases of the menstrual cycle. *Purple arrow:* Progesterone levels in the late luteal phase of the menstrual cycle. *Long dash with double dots:* Hormone (progesterone) mediated pain mechanism- the influence of low progesterone levels during the late luteal phase on the lysosomal enzyme phospholipase A2 resulting in greater production of arachidonic acid and prostaglandins. Bold dashes: Inflammatory cytokine (TNF-α)-mediated pain mechanism-the influence of pro-inflammatory cytokines (TNF-α) on COX-2 (the enzyme controlling the synthesis of PGs). *Note:* COX-2=Cycloooxygenase-2; ESC=Endometrial Stromal Cells; PGE2=Prostaglandin E2; PGF2α=Prostaglandin F2-alpha; TNF-α=Tumor Necrosis Factor-alpha.

treatment for primary dysmenorrhea [30]. However, no study has yet identified the physiological mechanisms underlying the beneficial effects of aerobic exercise-induced pain relief in primary dysmenorrhea. Our preliminary studies [14,31] identified high-intensity aerobic exercise as effective for women with primary dysmenorrhea. Our previous study to evaluate the feasibility, safety and preliminary effectiveness of the aerobic exercise intervention on primary dysmenorrhea found large beneficial effects for a 4-week supervised treadmill-based aerobic exercise intervention on primary dysmenorrhea-associated pain quality and intensity [31]. Our randomized controlled trial showed that four weeks of supervised treadmill-based aerobic exercise supplemented with a further 24 weeks of unsupervised aerobic exercise was effective for decreasing pain quality, intensity, and interference and improving quality of life and physical functioning in women with primary dysmenorrhea, relative to usual care [32]. The critical next step in our research program is to identify the physiological mechanisms underlying the beneficial effects of aerobic exercise-induced pain relief in primary dysmenorrhea. The proposed study will address this important knowledge gap by investigating the mechanisms underlying the beneficial effects of aerobic exercise on pain associated with primary dysmenorrhea. To address this knowledge gap, we plan to conduct a prospective randomized controlled trial to investigate the physiological mechanisms underlying aerobic exercise-induced analgesia in women with primary dysmenorrhea. Prior to undertaking a large-scale definitive trial, a pilot study was carried out as an essential precursor.

The objectives of this pilot study are to (1) evaluate the preliminary effects of aerobic exercise on progesterone, prostaglandin metabolites and pro-inflammatory cytokines in women with primary dysmenorrhea and (2) assess the feasibility of the protocol and obtain preliminary data (effect size) to estimate the sample size for a future randomized controlled trial to investigate the physiological mechanisms underlying the beneficial effects of aerobic exercise on the pain associated with primary dysmenorrhea.

Methods

Design

This was a controlled non-randomized trial conducted at the Hong Kong Polytechnic University (PolyU) between April and December 2018. Sample sizes between 10 and 40 per group are reported to be sufficient for trials conducted to obtain preliminary results that could be used to evaluate the feasibility and estimate effect sizes for future full-scale randomized controlled trials [33,34]. Based on the available resources, a sample size of 20 participants (10 per group) was planned.

Ethics approval and consent to participate

All study procedures were approved by the Human Subjects Ethics Sub-committee of PolyU (Ref: HSEARS20180426001) and written informed consent was obtained from each participant prior to data collection.

Participants

Twenty women were assigned to one of two groups: (1) a high-intensity aerobic-exercise group (n= 10) and (2) a no-exercise control group (n=10). Women who were able to report to PolyU for exercise three times a week entered the exercise group. Women who were unable to come to PolyU for exercise entered the no-exercise control group. Study inclusion and exclusion criteria are consistent with the guidelines from the Society of Obstetrics and Gynaecologists of Canada (SOGC) for the diagnosis of primary dysmenorrhea [35]. Criteria for inclusion were: (1) 18-29 years of age; (2) non-pregnant; (3) having regular menstrual cycles with cycle lengths between 24 and 30 days (average, 28 days); (4) experiencing average menstrual pain (i.e., pain intensity when they experience menstrual pain) equal to or greater than 5 on a 0-10 Numerical Rating Scale (NRS); and (5) having not performed vigorous/high-intensity aerobic exercise in the past 6 months. Women were excluded if: (1) they used oral contraceptive pills, hormonal therapy, or intrauterine devices; (2) reported having no pain relief with over-the-counter analgesics; or (3) they were currently participating in a formal exercise programme.

Recruitment and study procedure

Participants were recruited by posting study flyers around the university campus and waiting rooms of the University Health Service (UHS) at PolyU. The advertisement flyers contained simple inclusion information as well as the contact details of the principal investigator. Interested women who contacted the principal investigator via phone or email were scheduled for a face-to-face visit to discuss the study further. On this first day of contact, study procedures were explained and potential participants were presented with an information sheet and consent form. Women who provided written informed consent were then screened for eligibility. On completion of the screening questionnaire, eligible women completed the short-form of the International Physical Activity Questionnaire to determine baseline physical activity levels. To assess the risk of exercising, each participant was screened using the Physical Activity Readiness Questionnaire (PAR-Q) before starting the intervention. PAR-Q is recommended as a minimum standard for screening participants before beginning an exercise programme [36]. If participants answered 'yes' to any one of the questions in the PAR-Q, they were then requested to have a health care provider approve participation in an exercise programme prior to participation. All study participants were asked to refrain from taking analgesics or anti-inflammatory medications 12 hours prior to study participation. Participants were then requested to notify the investigator at the start of their next menstrual period for blood collection and intervention. Blood collection for evaluation of mediators was performed by registered nurses from UHS at PolyU.

Intervention

The exercise intervention for women in the high-intensity (aerobic) exercise group lasted for four weeks and began on the first day of the menstrual period. Exercise sessions were supervised by a physiotherapist (with seven years of specialist experience in the area of pain and women's health) on a one-to-one basis. Women performed high-intensity treadmill-based

aerobic exercise for three days a week, at a perceived exertion of 11.0 (Borg scale) for the first five minutes (warm-up period), followed by aerobic exercise at 70-85% of maximum heart rate (MHR) (16.0-18.0 Borg scale) for 30 minutes. At the end of the exercise session, women completed a 5-minute cool-down (11.0 Borg scale). MHR was calculated using a formula developed for estimating the peak heart rate for healthy women [37].

Women in the no-exercise control group did not receive trial intervention but completed the study measures and provided blood for the estimation of plasma variables. The control group participants received weekly phone calls lasting about 5-8 minutes, during which primary dysmenorrhea topics were discussed to control for the effects of attention. Women in the no-exercise group were also asked not to change their physical activity levels during the study.

Outcome measures

Pain intensity: Average pain intensity in the last 24 hours was measured using the 0-10 NRS [38] with 0 representing 'no pain' and 10 representing 'worst imaginable pain'. The NRS has been recommended by the Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials (IMMPACT) consensus group as the measure with the most strengths and fewest weaknesses for assessing pain intensity in studies evaluating effects of interventions on pain [39].

Plasma mediators: Levels of progesterone, PGF2α metabolite (13,14-dihydro-15-keto-prostaglandin F2 alpha [KDPGF2α]), and PGE2 metabolite (13,14-dihydro-15-keto-prostaglandin E2 [KDPGE2]) in plasma were measured using Enzyme-Linked Immunosorbent Assay (ELISA) kits (Cayman, USA). Plasma levels of TNF-α were measured using the Milliplex Human Cytokine Magnetic Bead Panel kit (Millipore, USA). The concentrations of PGF2α and PGE2 are very low in the plasma because they are metabolized quickly to their respective metabolites during their initial passage through the pulmonary circulation [40-42], and that measurement of PGF2α and PGE2 does not reflect ongoing changes [40]. Because the metabolites of PGF2α and PGE2 have a longer half-life in peripheral circulation than the compounds, their metabolites have been used as an analytical marker of PGF2α and PGE2 [43]. To measure plasma mediators, approximately 8-10 millilitres of peripheral venous blood samples were collected from all participants on the first day of their menstruation.

Feasibility: Study feasibility outcomes included: (1) recruitment rate, (2) adherence to the exercise intervention, (3) adherence to the blood collection procedures, and (4) safety of the intervention. Eighty percent adherence was deemed to be acceptable *a priori*.

Statistical analysis: Descriptive statistics such as means and standard deviations (SD) were calculated. Based on the guidelines for pilot/feasibility studies, p-values were not calculated [44]. Group means were used to replace missing data. The effect size

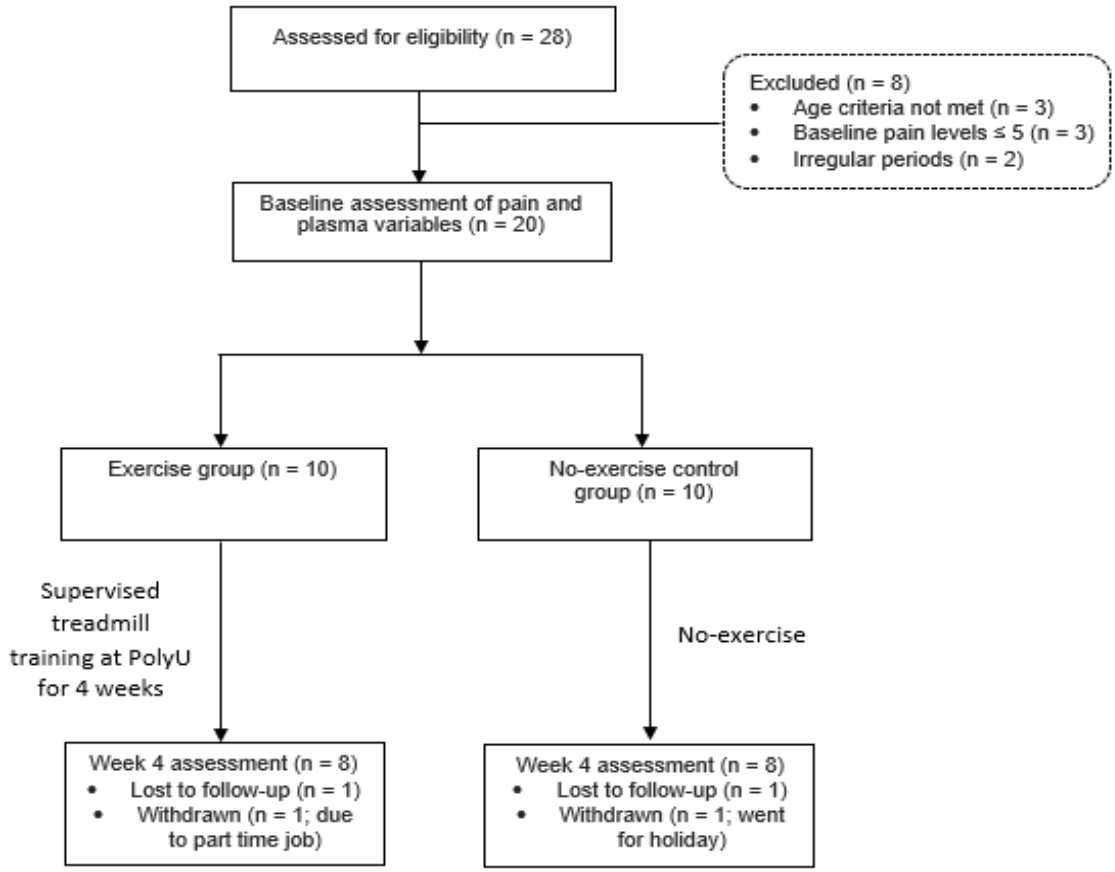

Figure 2 Flow diagram of participants through the study.

(Cohen's *d*) of the difference in post-intervention means between groups was calculated. As this pilot study was designed to evaluate the feasibility of methodological procedures and obtain data (variance) to estimate the sample size for a future randomized controlled trial, a sensitivity analysis was not planned.

Results

Participant characteristics

The flow diagram of participants through the study is presented in **Figure 2**. Twenty-eight women volunteered to be in the study; 20 of these were deemed eligible. These 20 women were distributed equally between the two groups (high-intensity exercise and no-exercise control groups). Most participants were from Hong Kong (18/20). The study ended in December 2018 after week-4 measurements were completed. One woman from the control group and one from the exercise group withdrew from the study after completing baseline assessment reasons for withdrawal are summarized in **(Figure 2)**. One woman from the control group and one from the exercise group was lost to follow-up at week 4 assessment. For technical reasons, progesterone data could not be obtained from the plasma of one participant in the control group and one participant from the exercise group. The baseline characteristics of the study participants are reported in **Table 1**.

Effect of intervention on pain intensity and plasma mediators

Descriptive statistics for pain and plasma mediators are summarized in **Table 2**. There was a small to medium increase in progesterone levels (*d*=0.36) from baseline to week 4 in the exercise group but not in the control group. Mean KDPGF2α, KDPGE2, TNF-α levels, and pain intensity decreased from baseline to week 4 in the exercise group but not in the control group. The effect size ranged from small to medium for prostaglandin metabolites (KDPGF2α [*d*=0.36], KDPGE2 [*d*=0.47]), TNF-α (*d*=0.33), and pain intensity (*d*=0.5).

Feasibility outcomes

Eighteen out of 20 participants (90%) were recruited in 3-4 weeks. The remaining two participants were recruited over a period of three months. With regard to the feasibility of blood collection,

Table 1 Participant demographic characteristics at baseline.

Characteristics	Exercise group (n=10)	No-exercise control group (n=10)
Age (yrs), mean (SD)	24.1 (4.4)	24 (3.4)
Physical activity levels	Moderate	Moderate
Mean age at menarche in years, mean (SD)	11.3 (0.6)	12.5 (0.6)
Menstrual flow in days, mean (SD)	5.8 (0.91)	5.2 (1.3)
Pain on 0-10 NRS, mean (SD)		
Cycle 1	6.0 (0.95)	5.7 (0.7)
Cycle 2	6.6 (1.3)	5.9 (1.0)
Ethnicity, n (%)		
Chinese	8 (80)	10 (100)
Indian	1 (10)	
Indonesian	1 (10)	
Marital status, n (%)		
Single	9 (90)	10 (100)
Married	1 (10)	
Employment status, n (%)		
Yes, n (%)	2 (20)	10 (100)
No, student, n (%)	8 (80)	
Pregnancies, n (%)		
0	9 (90)	9 (90)
1	1 (10)	1 (10)
Note: NRS=Numeric Rating Scale; SD=Standard Deviation.		

Table 2 Means (SD) and effect sizes (Cohen's *d*) for plasma variables.

Outcome measure	Exercise group (pg/ml) mean (SD)		No-exercise control group (pg/ml) mean (SD)	
	Pre	Post	Pre	Post
Progesterone (pg/ml)	3686.1 (2203.5)	4057.3 (2982.6)	4770.0 (2324.7)	3227.4 (1266.0)
TNF-α	5.4 (2.7)	5.1 (1.9)	4.3 (2.6)	4.9 (2.8)
KDPGF2- α (pg/ml)	55.8 (44.1)	42.8 (20.9)	33.0 (11.0)	37.2 (7.7)
KDPGE2 (pg/ml)	64.0 (23.0)	55.8 (17.5)	50.6 (18.3)	66.8 (27.8)
Pain intensity (0-10 NRS)	5.9 (2.4)	4.9 (2.2)	4.1 (2.1)	3.8 (2.0)
Note: KDPGE2=15-keto-13,14-dihydro-Prostaglandin E2; KDPGF2-α=13,14-dihydro-15-keto Prostaglandin F2-alpha; NRS=Numeric Rating Scale; TNF-α=Tumor Necrosis Factor-alpha.				

women who started their period on Saturday afternoon or Sunday were unable to give blood because the UHS conducted blood collection only on weekdays and until 12 noon on Saturdays. Treatment adherence met the *a priori* level indicating that adherence was acceptable. Nine of the 10 participants in the exercise group had 108 exercise sessions prescribed over the study period. The percentage of adherence to exercise sessions was 97%. The intervention was found to be safe and no adverse events were reported.

Discussion

This study was designed to (1) evaluate preliminary effects of aerobic exercise on progesterone, prostaglandin metabolites and pro-inflammatory cytokines in women with primary dysmenorrhea and (2) assess the feasibility of methodological procedures and obtain preliminary data (effect size) to estimate the sample size for a future randomized controlled trial.

The findings indicate a trend towards increases in progesterone levels and decreases in KDPGF2α, KDPGE2, and pain intensity in the exercise group compared with the control group. Given that the synthesis of PGs is influenced by the progesterone level in the late luteal phase of the menstrual cycle (progesterone and PGs displaying an inverse relationship), the findings herein suggest that high-intensity aerobic exercise-induced analgesia in primary dysmenorrhea might occur via hormone (progesterone)-mediated mechanisms. However, it is critical to emphasize that this pilot study is underpowered to evaluate changes in effectiveness between groups. The small sample size (n=20) explains the differences baseline values in outcomes between groups and subsequent changes over time. Given the limitations associated with the small sample size, the study requires replication before any strong conclusions can be made. This justifies the need to conduct an adequately powered randomized controlled trial.

Our study demonstrated a small to medium effect size difference in plasma TNF-α between the exercise and no-exercise control groups. Considering the role of TNF-α in the pathogenesis of primary dysmenorrhea, post-intervention decreases in TNF-α levels indicate that high-intensity aerobic exercise-induced analgesia might occur via cytokine (pro-inflammatory)-mediated mechanisms. The findings of a small to medium effect size difference in plasma progesterone and PGs and a small effect size difference in TNF-α levels suggests that progesterone and prostaglandins might be viable mediators compared with TNF-α. Notwithstanding, the study findings suggest that these three mediators are worth evaluating in a full clinical trial. The current study findings were also consistent with previous research showing the beneficial effects of exercise on dysmenorrhea [26,27,31]. In addition, the findings support the feasibility of the study procedures for conducting a fully powered randomized controlled trial with regard to recruitment, adherence, and safety of the intervention.

Women were scheduled to undergo blood collection at the UHS at PolyU on the first day of their menstrual period. The UHS admitted participants for blood collection on all weekdays (9 am to 5 pm) and Saturdays (from 10am-12 noon). Women who started their period on Saturday afternoons or Sundays were unable to give blood and had to wait until their next period for providing blood for estimation of study outcomes. Women in the exercise group were willing to wait until their next period and continued exercise for four more weeks. However, women in the no-exercise control group were unwilling to wait until their next period. Therefore, some women in the control group gave blood on the following Monday which was either the second or the third day of menstruation. Given that the progesterone levels begin to decline in the follicular phase, the decrease in mean progesterone levels in the no-exercise control group could potentially be due to the collection of blood samples on the second or third day of menstruation. To avoid issues relating to blood sample collection in any future randomized controlled trial, it would be important to employ a registered nurse to collect blood on weekends.

The current study demonstrated a very high-treatment adherence (97%) to the exercise training sessions. The adherence rate was higher than the 80% cut-off established *a priori* for determining that the adherence was acceptable. Eighteen out of 20 participants (90%) were recruited within 3-4 weeks, but it took a full three months to recruit the remaining two participants. The delay in recruitment was due mostly to the start of summer holidays at the university, although some of it might have been due to the possibility that the women who were interested in participating in the trial enrolled early. Given the limitations to recruiting students from the university, it would be a good idea to include women from the general public and to begin recruitment prior to or following summer holidays/ semester break at the University for any future trial.

Non-adherence to unsupervised home exercise is reported to have detrimental effects on clinical outcomes [45]. Evidence suggests that non-adherence to unsupervised home exercises in people with musculoskeletal conditions ranges between 30% and 50%, making it a potentially significant problem that places an additional burden on health care providers [45]. Because we intend for the future randomized controlled trial to include 6 months of unsupervised home exercise, we plan to utilize the following strategies to increase adherence during this period: (1) educational approaches and self-monitoring of exercise performance (fitness trackers) to improve self-efficacy ('defined as *an individual's belief in their own capability to achieve a task*') [45]; (2) use of online electronic diaries instead of paper diaries to report exercise activity (participants who used electronic diaries are reported to be more adherent than those using paper diaries) [46]; and (3) use of weekly telephone calls to remind participants to do the exercises.

It is known that studies that place high demands on participants, including frequent appointments, significant travel and travel costs, and long duration may deter participants from continuing in the study [47]. In the randomized controlled trial being planned, the exercise intervention will be for 7 months beginning with a one-month in-person supervised training programme at PolyU. For the subsequent 6 months of unsupervised home exercises, the exercise group participants from the community will be provided with a subscription for 6 months at the gym that is most convenient to them to minimize travel time and travel costs. In the future randomized controlled trial, post-

treatment assessments of outcomes (including blood collection) will be completed at least four times during the study period. To minimize travel time and costs, we plan to arrange for blood collection either at participants' residence or PolyU, depending on their convenience.

The current study is the first to investigate the mechanisms underlying the beneficial effects of aerobic exercise on primary dysmenorrhea. The primary limitation of the current study was the small sample size and lack of power. However, it is acknowledged that this pilot study was designed to test the methods (proposed for use in the main randomized controlled trial) and not the effectiveness of the intervention. Based on the findings from this study, a prospective, fully powered randomized controlled trial to determine the extent to which exercise-induced analgesia in primary dysmenorrhea operates via its effects on progesterone, prostaglandin, and pro-inflammatory cytokines levels appears feasible.

Conclusion

Changes in pain intensity, progesterone, and KDPGF2α, KDPGE2 in the exercise group compared with the no-exercise control group suggest that high-intensity aerobic exercise-induced analgesia might occur via hormone (progesterone)-mediated mechanisms. High-intensity aerobic exercise-induced decreases in TNF-α suggest a minor influence of exercise on cytokine-mediated mechanism. Future clinical trials can utilize these mediators as outcomes to investigate aerobic exercise-induced analgesia in primary dysmenorrhea. With respect to pain, women in the aerobic-exercise group reported greater improvements in pain intensity compared with the no-exercise group, consistent with previous research in this area. The results also demonstrated that it is feasible to conduct a fully powered randomized controlled trial with regard to recruitment, adherence, and safety of the intervention.

The current study has also given us an insight into the issues that would arise if recruitment and blood collection is restricted to the UHS at PolyU. To overcome the problems of blood collection on weekends for women who have their period on weekends, we plan to recruit a nurse to collect blood on weekends. With respect to recruitment, we plan to recruit participants from the community as well as from PolyU. Using the findings from this study, a future, large-scale randomized controlled trial will aim to evaluate definitively the extent to which changes in progesterone, prostaglandins, and pro-inflammatory cytokines mediate the beneficial effects of aerobic exercise-induced analgesia in primary dysmenorrhea. A greater understanding of the mechanisms underlying aerobic exercise-induced analgesia in primary dysmenorrhea pain would help identify mediator outcomes in future pain interventions in this population.

Declarations

Ethics approval and consent to participate

Ethics approval for the study was obtained from the institutional review board of the Hong Kong Polytechnic University (Ref: HSEARS20180426001). Written informed consent was obtained from all study participants. Participants were free to withdraw from the study at any time. Personal and health information and raw data collected in the study are stored securely on the password-protected personal computer of the primary investigator.

Consent for publication

Written informed consent to publish study data was obtained from all participants.

Availability of data

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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Author's contributions

PK contributed towards design and conduct of the study; study supervision, collection of data, supervision of intervention; and drafting and revision of the manuscript. KKC and SCCW contributed to ELISA, statistical analysis and interpretation of data. CSC, LKY, LYX, NSK, SHY contributed towards data collection and supervision of intervention. All authors read and approved the final manuscript.

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