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在线全文

激素替代冻胚移植周期生殖道微生态变化与妊娠结局分析*

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【摘要】 目的 分析激素替代冻胚移植周期中不同时间点生殖道微生态变化及代谢差异与妊娠结局的关系。**方法** 选取2022年7月−2023年1月首次行激素替代治疗冻融单囊胚移植患者20例作为研究对象，采集月经周期第2～5天、雌激素替代治疗7 d后、内膜转化日和胚胎移植日的阴道及宫颈分泌物进行16S rRNA测序及非靶代谢组学检测，按照临床妊娠与否进行分组，通过生物信息学方法对测序结果进行分析。**结果** ①阴道及宫颈微生物群α-多样性指数在月经周期第2～5天更高（P<0.01），在口服雌激素替代治疗7 d后、内膜转化日和胚胎移植日差异无统计学意义（P≥0.1）。②妊娠组、非妊娠组阴道及宫颈分泌物在不同时间点存在多种具有显著差异的微生物及代谢物。③不同时间点微生物分析表明妊娠组阴道分泌物*Peptoniphilus*、*Enterocloster*、*Finegoldia*、*Klebsiella*、*Anaerobutyricum*、*Agathobaculum*、*Sporanaerobacter*、*Bilophila*、*Prevotella*和*Anaerococcus*差异有统计学意义（P<0.05）。④不同时间点代谢物分析表明妊娠组阴道分泌物3-Hydroxybenzoic acid、Linatine、（R）-Amphetamine、Hydroxychloroquine和L-Altarate差异有统计学意义（P<0.05）；宫颈分泌物Isocitric acid、Quassin、Citrinin和12（R）-HETE差异有统计学意义（P<0.05）。⑤不同时间点代谢物分析表明非妊娠组阴道分泌物Linatine、Decanoyl-L-carnitine、Aspartame、Sphingosine和Hydroxychloroquine差异有统计学意义（P<0.05）；宫颈分泌物Isocitric acid、Quassin、Citrinin和12（R）-HETE差异有统计学意义（P<0.05）。⑥微生物与代谢组学联合分析表明，某些代谢物与微生物群落具有显著的相关性，尤其是*Klebsiella*。**结论** 激素替代冻胚移植周期中不同时间点存在多种菌属及代谢的显著差异，有可能会成为预测胚胎移植妊娠结局的潜在生物标志物。

【关键词】 生殖道微生物 16S rRNA 代谢组学 冻胚移植周期 激素替代治疗

Analysis of Reproductive Tract Microecological Changes During the Frozen-Thawed Embryo Transfer Cycle and Clinical Pregnancy Outcomes

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【Abstract】 Objective This study aims to analyze the relationship between reproductive tract microecological changes, metabolic differences, and pregnancy outcomes at different time points in the frozen-thawed embryo transfer cycle while patients are undergoing hormone replacement therapy, which will be a breakthrough point for improving outcomes. **Methods** A total of 20 women undergoing frozen-thawed single blastocyst transfer for the first time at the Reproductive Medicine Center of Fujian Maternal and Child Health Hospital between July 2022 and January 2023 were recruited for this study. Their vaginal and cervical secretions were collected for 16S rRNA sequencing and non-targeted metabolomics analysis on days 2-5 of menstruation, day 7 after estrogen replacement therapy started, the day when progesterone was added, and the day of transplantation. The subjects were divided into different groups according to their clinical pregnancy status and the sequencing results were analyzed using bioinformatics methods. **Results** 1) The alpha-diversity index of the vaginal and cervical microbiota was higher on days 2-5 of menstruation (P<0.01), but did not differ significantly on day 7 after oral estrogen replacement therapy started, the day of progesterone administration, and the day of transplantation (P≥0.1). 2) Both the pregnant group and the non-pregnant group showed a variety of microorganisms and metabolites with significant differences in the lower reproductive tract at different time points. 3) Microbial analysis

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at different time points showed that there were significant differences in vaginal flora, including *Peptoniphilus*, *Enterocloster*, *Finegoldia*, *Klebsiella*, *Anaerobutyricum*, *Agathobaculum*, *Sporanaerobacter*, *Bilophila*, *Prevotella*, and *Anaerococcus* in the pregnant group (P<0.05). 4) Metabolite analysis at different time points showed that there were significant differences in 3-hydroxybenzoic acid, linatine, (R)-amphetamine, hydroxychloroquine, and L-altarate in the vaginal secretions of the pregnant group (P<0.05), and that there were significant differences in isocitric acid, quassin, citrinin, and 12(R)-HETE in the cervical secretions (P<0.05). 5) Metabolite analysis at different time points showed that, in the non-pregnant group, there were significant differences in linatine, decanoyl-L-carnitine, aspartame, sphingosine, and hydroxychloroquine in the vaginal secretions (P<0.05), and the isocitric acid, quassin, ctrinin, and 12(R)-HETE in the cervical secretions (P<0.05). 6) Combined microbiome and metabolomics analysis showed that certain metabolites were significantly associated with microbial communities, especially *Klebsiella*. **Conclusions** Significant differences in the microbiota genera and metabolites at different time points were found during the frozen-embryo transfer cycle of hormone replacement therapy, which may be used as potential biomarkers to predict pregnancy outcomes of embryo transfer.

【Key words】 Reproductive tract flora　16S rRNA　Metabolomic　Frozen-thawed embryo transfer cycle　Hormone replacement therapy

不孕症是常见的生殖障碍性疾病之一, 全世界育龄期夫妇不孕症的发生率约为8% ~ 12%[1]。生殖道菌群作为人体最重要的微生物群落之一, 在维持女性生殖健康中扮演重要角色[2-3]。既往研究认为女性雌激素水平的变化与阴道微生物群组成有关[4-5]。激素替代治疗(hormone replacement therapy, HRT)冻融胚胎移植术(frozen-thawed embryo transfer, FET)是辅助生殖技术的重要方案之一, 本研究分析雌、孕激素的应用对于阴道、宫颈微生物群落和代谢的影响及其与助孕结局的关系, 为发现改善FET助孕结局的潜在生物标志物提供依据。

1　资料与方法

1.1　样本来源

随机选取2022年7月–2023年1月就诊于福建省妇幼保健院生殖医学中心, 首次行FET采用HRT内膜准备且移植1枚优质囊胚的患者作为研究对象。所有研究对象均为已行体外受精(*in vitro* fertilization, IVF)或卵胞浆内单精子显微注射(intracytoplasmic sperm injection, ICSI), 但因各种原因未行新鲜胚胎移植的患者。本研究已通过福建省妇幼保健院伦理审查委员会伦理审批(批准号2022KYLLR03029), 并在中国临床试验注册中心注册(注册号ChiCTR2200058698)。所有研究对象均已签署知情同意书。

纳入标准: ①年龄20 ~ 40岁; ②体质量指数(body mass index, BMI) 18.5 ~ 23.9 kg/m^2; ③采集标本72 h内无同房、阴道用药或冲洗。

排除标准: ①既往肿瘤病史; ②曾诊断为生殖道畸形、宫腔粘连、瘢痕憩室等; ③近3个月内使用过可能影响细菌存活状态的药物, 如抗生素、糖皮质激素或免疫抑制剂等; ④有结核感染病史。

采样时间: 月经周期第2 ~ 5天、口服雌激素替代治疗7 d后、内膜转化日和胚胎移植日。

1.2　内膜准备方案

月经周期第2 ~ 5天开始口服雌激素3~9 mg/d (补佳乐, 拜耳, 德国), 根据患者既往子宫内膜条件决定起始剂量, 用药7 d后经阴道超声评估子宫内膜条件并根据内膜厚度调整用药。当子宫内膜厚度≥8 mm时, 每日肌注黄体酮40 mg(黄体酮注射液, 浙江仙琚, 中国), 并口服地屈孕酮10 mg bid(达芙通, 雅培, 尼德兰)。以开始加用孕酮日为第0天, 在第5天解冻并移植D5或D6囊胚。在受精后第5天和第6天, 综合考虑囊胚扩张状态、内细胞团和滋养层细胞情况, 根据Gardner评分系统进行囊胚质量分级。Gardner评分3BB及以上的囊胚为优质囊胚。移植后按原剂量继续使用雌激素, 并口服地屈孕酮10 mg bid(达芙通), 阴道用黄体酮阴道缓释凝胶90 mg qd(雪诺同, 默克, 英国)直至移植后14 d行血β-人绒毛膜促性腺激素(human chorionic gonadotropin, β-hCG)检测。β-hCG阳性后2周经阴道超声检查发现宫腔内至少一个妊娠囊确认为临床妊娠。

1.3　样本采集

在HRT冻胚移植周期中特定时间点使用一次性取样拭子采集阴道后穹窿部和宫颈管分泌物标本。所有样本均在实施妇科检查和任何阴道操作前采集。使用一次性无菌拭子蘸取阴道后穹隆部分泌物, 并在擦拭宫颈表面后另取一次性无菌拭子置入宫颈管内约1 ~ 2 cm, 旋转并放置至少20 s, 取出拭子时避免拭子头部与其他部位接触污染, 折下拭子头部置于2 mL无菌冻存管内, 转入–80 ℃冰箱冻存直至检测。

1.4 16S rRNA 测序及数据处理

以16S rRNA基因V3-V4区为引物，采用聚合酶链反应(polymerase chain reaction, PCR)扩增基因组DNA。利用1%琼脂糖凝胶电泳检测抽提的基因组DNA。参照电泳初步定量结果，将PCR产物用QuantiFluor™ -ST蓝色荧光定量系统(Promega公司)进行检测定量，将PCR产物进行混合、纯化、文库构建，用Pacbio测序系统进行测序。对原始读长数据进行过滤、拼接，得到有效片段。按照97%相似性对非重复序列进行操作分类单元(operational taxonomic units, OTU)聚类，对OTU序列进行物种注释后以进行群落结构及物种差异分析等。

1.5 非靶代谢组学分析及数据处理

确保样品的收集和处理符合实验要求以防止代谢物降解和污染。对收集的样品进行预处理，去除杂质和干扰，提取代谢物。利用Thermo Ultimate 3000(Thermo Fisher Scientific, 美国)超高效液相系统对提取的样品进行分离纯化和用Thermo Q Exactive质谱检测器进行质谱分析，将代谢物转化为离子，实现基于质荷比(m/z)的定性和定量分析。利用峰检测、峰过滤、峰对齐、聚类分析和差异分析等对质谱数据进行后处理和分析。

1.6 统计学方法

临床资料分析：采用SPSS 23.0版本进行统计。呈正态分布定量变量表示为$\bar{x}\pm\sigma$，并采用两独立样本t检验；非正态分布定量变量表示为中位数(P_{25}, P_{75})，并采用Wilcoxon秩和检验。16S rRNA测序结果采用Kruskal-Wallis H秩和检验。$P<0.05$为差异有统计学意义。

代谢组学结果分析：采用R XCMS软件包分别对样本数据进行主成分分析、偏最小二乘判别分析、正交偏最小二乘判别分析降维分析。据统计检验计算P值、OPLS-DA 降维方法计算变量投影重要度(variable importance in projection, VIP)、fold change 计算组间差异倍数。FDR< 0.05和(或)VIP 值 > 1时差异具有统计学意义。

联合分析采用皮尔森相关性分析及冗余分析。选取关联系数较大且 $P<0.05$ 的变量，绘制冗余分析图。两个变量之间如果为锐角，代表正相关，即存在协同作用；如果为钝角，代表负相关，即存在拮抗作用。

2 结果

2.1 受试者临床资料分析

见图1。本研究共招募受试者142例，在排除不合格标本及未完成全部采样受试者后，20名受试者最终被纳入分析。按照临床妊娠与否分组，非妊娠组与妊娠组相比，年龄、BMI、基础性激素水平及内膜转化日性激素水平差异无统计学意义(表1)。

2.2 整体菌群结构特征

整体菌群结构分析表明，门水平上丰度排名前10位的分别是*Firmicutes*、*Actinobacteria*、*Proteobacteria*、*Tenericutes*、*Bacteroidota*、*unclassified_d__Bacteria*、*Fusobacteria*、*Acidobacteria*、*Verrucomicrobia*及*Chloroflexi*。其中属水平上以*Firmicutes*中乳酸杆菌属在所有样本中丰度排名第一(图2)。

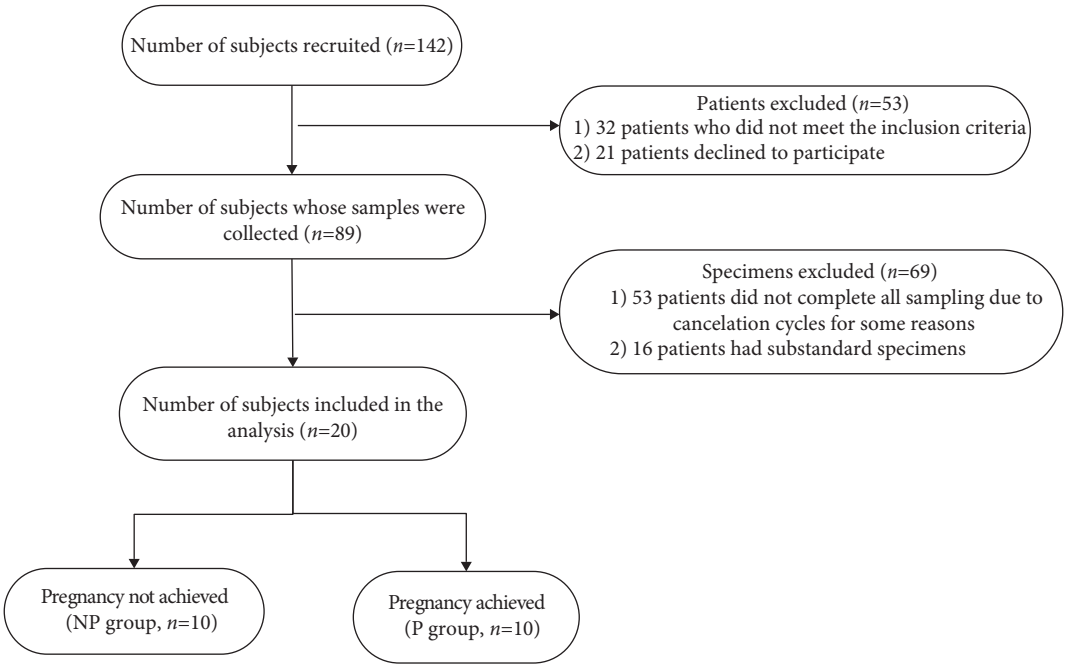

图 1 项目招募流程图

Fig 1 Flowchart showing the subject recruitment process

表 1　受试者基本信息

Table 1　Basic information about the subjects

Index	Pregnancy not achieved group (n=10)	Pregnancy achieved group (n=10)	P
Age/yr.	28.60±3.69	30.10±4.36	0.42
BMI/(kg/m^2)	20.57±2.12	20.97±2.41	0.70
Basal FSH/(mIU/mL)	6.08±2.23	5.43±1.53	0.46
Basal LH/(mIU/mL)	3.77±1.76	3.84±1.44	0.92
Basal E_2/(pg/mL)	28.60±16.80	32.20±11.97	0.59
Basal P/(pg/mL)*	0.29 (0.20, 0.36)	0.26 (0.18, 0.36)	0.57
LH on the day of progestrone administration/(mIU/mL)	10.22±4.62	11.06±4.00	0.67
E_2 on the day of progestrone administration/(pg/mL)*	147.00 (124.25, 189.25)	111.50 (87.75, 137.50)	0.05
P on the day of progestrone administration/(pg/mL)*	0.20 (0.13, 0.31)	0.15 (0.10, 0.19)	0.29

BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; E_2: estrogen; P: progesterone. * Median (P_{25}, P_{75}).

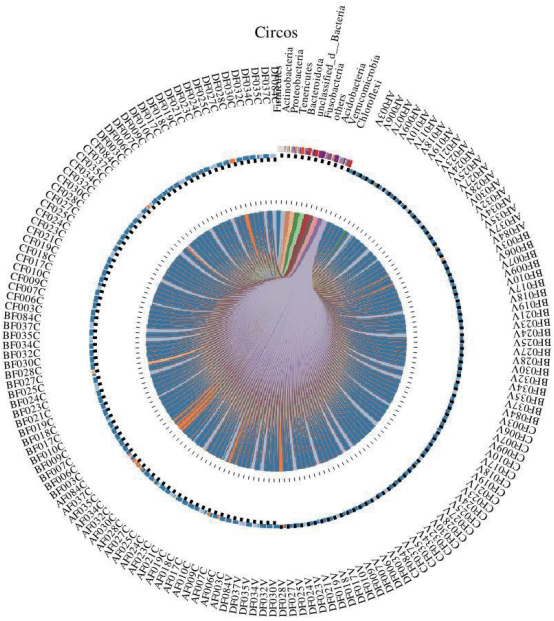

图 2 门水平基因组circos图

Fig 2 Genome circos map at the phylum level

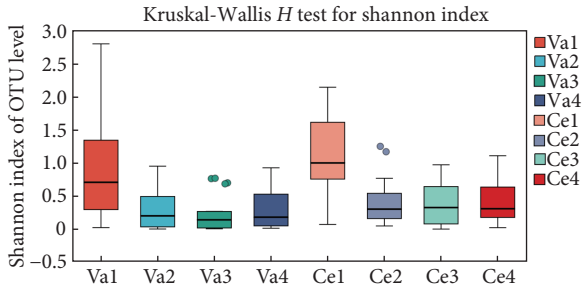

图 3 不同时间点阴道及宫颈微生物Shannon指数比较箱形图

Fig 3 Boxplot of the Shannon index of vaginal and cervical microorganisms at different time points

Va1: vaginal secretions on days 2-5 of menstruation; Va2: vaginal secretions on day 7 after oral estrogen replacement therapy started; Va3: vaginal secretions on the day progesterone was added; Va4: vaginal secretions on the day of transplantation; Ce1: cervical secretions on days 2-5 of menstruation; Ce2: cervical secretions on day 7 after oral estrogen replacement therapy started; Ce3: cervical secretions on the day progesterone was added; Ce4: cervical secretions on the day of transplantation.

2.3　多样性分析

利用Shannon指数分析评估不同时间点样本α-多样性发现, 与其他采样时间点相比, 月经周期第2～5天阴道和宫颈分泌物中具有更高微生物群多样性指数(P< 0.01), 而在口服雌激素替代治疗7 d后、内膜转化日和胚胎移植日, 阴道及宫颈微生物群多样性指数无明显差异(P≥0.1)(图3)。对不同时间点样本进行主成分分析显示, β-多样性指数无明显差异(P> 0.05)(图4)。

2.4　不同时间点阴道及宫颈微生物群分布特征

本研究采用Kruskal-Wallis秩和检验评估妊娠组和非妊娠组不同时间点样本之间属水平上微生物群差异。结果显示, 妊娠组阴道分泌物中*Peptoniphilus*、*Enterocloster*、*Finegoldia*、*Klebsiella*、*Anaerobutyricum*、*Agathobaculum*、*Sporanaerobacter*、*Bilophila*、*Prevotella*和*Anaerococcus*差

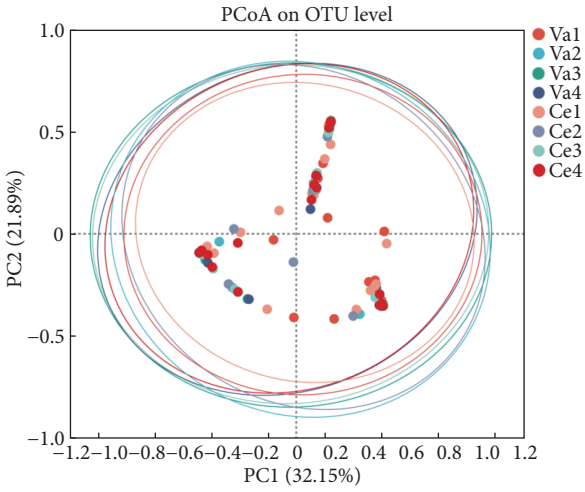

图 4 不同时间点阴道及宫颈微生物主成分分析

Fig 4 Principal Component Analysis of vaginal and cervical microorganisms at different time points

OTU: operational taxonomic units. The other abbreviations are given in the note to Fig 3.

异有统计学意义($P<0.05$)(图5A);宫颈分泌物中*Peptoniphilus*、*Anaerococcus*、*Bradyrhizobium*、*Bilophila*、*Peptostreptococcus*、*Sphingomonas*、*Prevotella*和*Cutibacterium*存在一定差异,但无统计学意义(图5B)。

非妊娠组阴道分泌物中*Anaerobutyricum*、*Klebsiella*和*Enterocloster*存在一定差异,但无统计学意义(图5C);宫颈分泌物中*Bilophila*、*Sphingomonas*和*Bradyrhizobium*、*Anaerococcus*存在一定差异,但无统计学意义(图5D)。

2.5 不同时间点阴道及宫颈代谢差异分析

不同时间点阴道分泌物差异代谢物分析表明,妊娠组3-Hydroxybenzoic acid、Linatine、(R)-Amphetamine、Hydroxychloroquine和L-Altarate差异有统计学意义(图6A);非妊娠组Linatine、Decanoyl-L-carnitine、Aspartame、Sphingosine和Hydroxychloroquine差异有统计学意义(图6B)。差异代谢物功能通路富集分析表明,妊娠组与非妊娠组差异代谢物均与突触囊泡循环、丙氨酸、天冬氨酸和谷氨酸代谢、癌症的中心碳代谢、蛋白质消化吸收、谷胱甘肽代谢等代谢通路有关(图6C、6D)。

不同时间点宫颈分泌物差异代谢物分析表明,妊娠组、非妊娠组Isocitric acid、Quassin、Citrinin和12(R)-HETE差异均有统计学意义(图7A、7B)。差异代谢物功能通路富集分析表明,妊娠组差异代谢物主要与谷胱甘肽代谢、丙氨酸、天冬氨酸和谷氨酸代谢、花生四烯酸代谢、精氨酸生物合成等代谢通路有关(图7C)。非妊娠组差异代谢物主要与柠檬酸循环、癌症的中心碳代谢、胰高血糖素信号通路、丙氨酸、天冬氨酸、谷氨酸代谢等代谢通路有关(图7D)。

2.6 微生物组学与代谢组学联合分析

本研究采用冗余分析进一步对不同时间点阴道及宫颈群落丰度与代谢物丰度进行相关性分析表明,某些代谢物与微生物群落具有显著的相关性,尤其是*Klebsiella*(图8)。

3 讨论

生殖道微生物群不仅影响生殖健康,也有可能是不孕症的病因之一。随着辅助生殖技术应用的蓬勃发展,

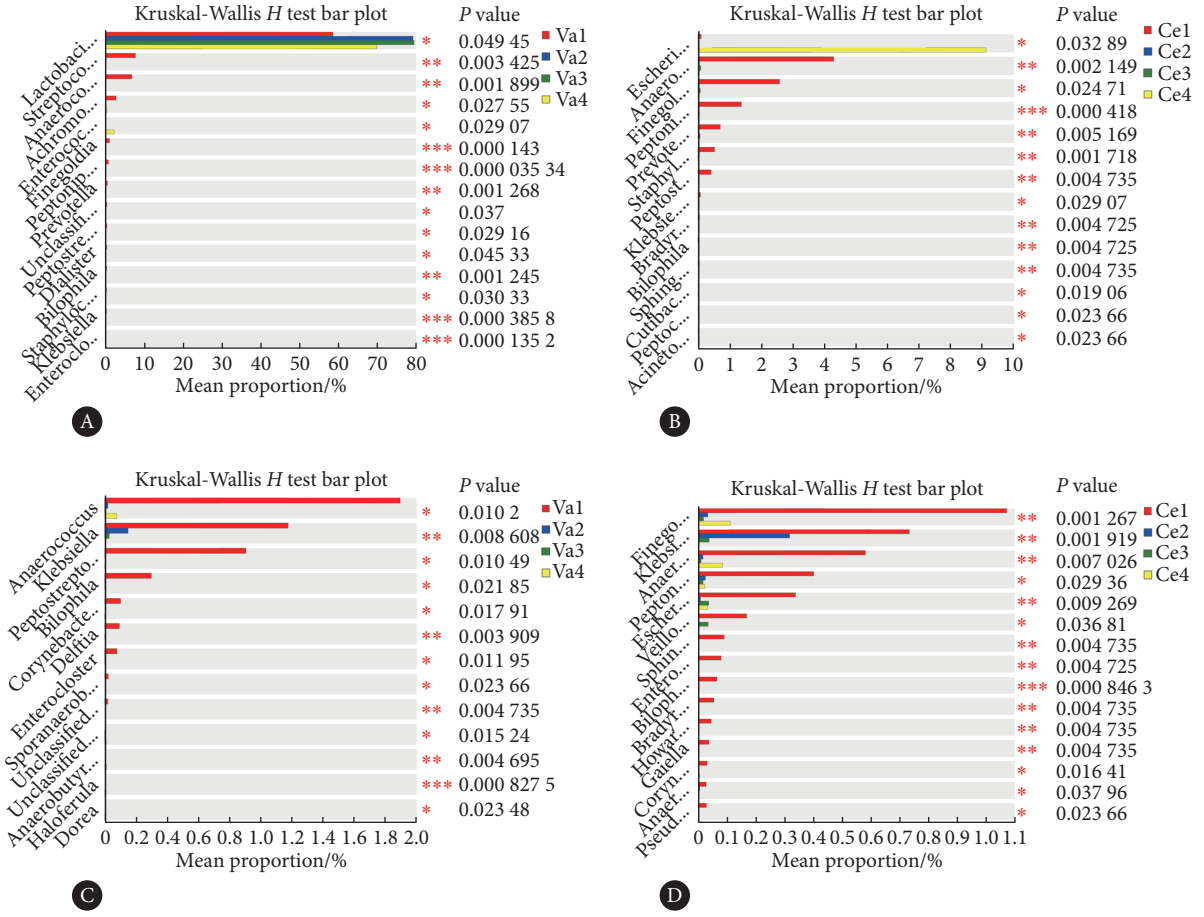

图 5 妊娠组(A、B)与非妊娠组(C、D)属水平上不同时间点阴道(A、C)及宫颈微生物群(B、D)的比较

Fig 5 Comparison of vaginal (A and C) and cervical microbiota (B and D) at different time points at the genus level in the pregnant group (A and B) and the non-pregnant group (C and D)

The abbreviations are given in the note to Fig 3.

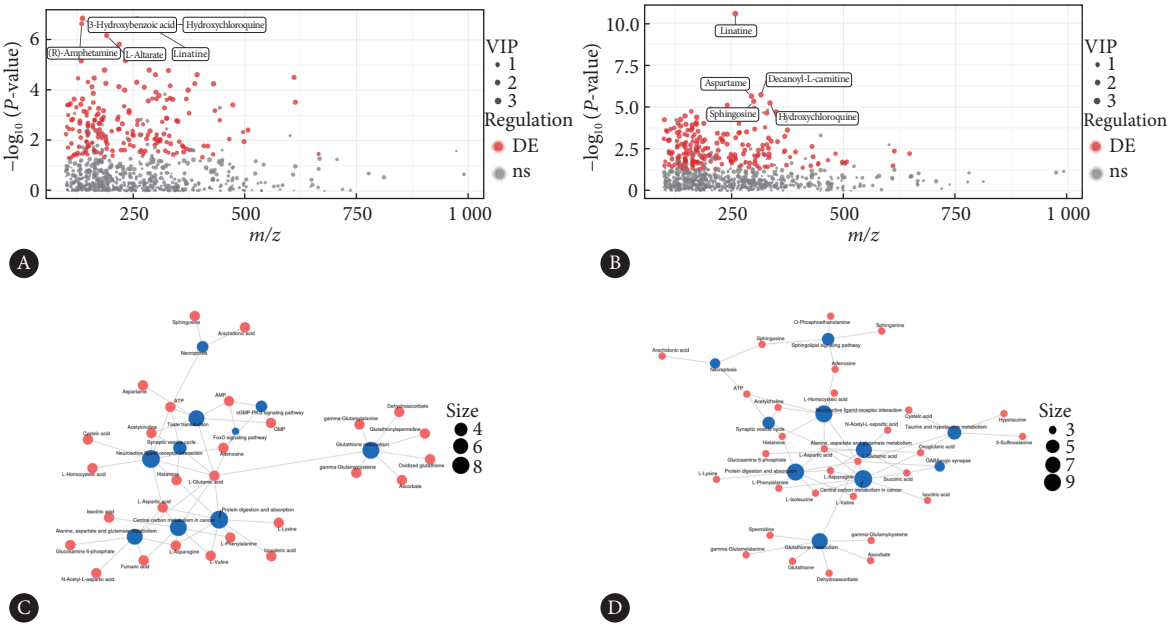

图 6 不同时间点阴道分泌物代谢差异分析

Fig 6 Analysis of the differences in the metabolism of vaginal secretions at different time points

A, Scatter plot of the charge ratio and the *P*-value of the differential metabolites of vaginal secretions at different time points in the pregnant group. B, Scatter plot of the charge ratio and the *P*-value of the differential metabolites of vaginal secretions at different time points in the non-pregnant group. C, Enriched metabolic pathway of the differential metabolites of vaginal secretions at different time points in the pregnant group. D, Enriched metabolic pathway of the differential metabolites of vaginal secretions at different time points in the non-pregnant group. VIP refers to variable importance in projection, a measure used to assess the correlation between a metabolite and the classification of a sample. The higher the VIP value, the greater the contribution of the metabolite to the sample classification. DE represents differentially expressed metabolites, and ns represents metabolites detected but not filtered by filtering parameters. Supplementary description for C and D, Blue dots represent the metabolic pathways and red dots represent metabolites. The size of the dot increases as the number of connected points increases.

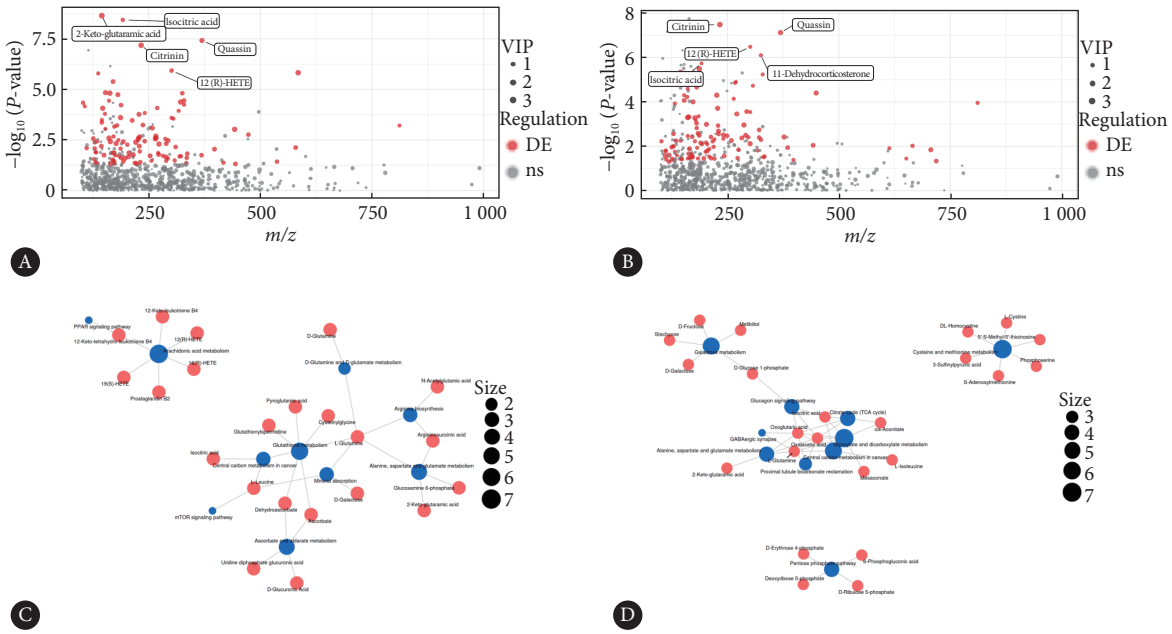

图 7 不同时间点宫颈分泌物代谢差异分析

Fig 7 Analysis of the differences in the metabolism of cervical secretions at different time points

A, Scatter plot of the charge ratio and the *P*-value of the differential metabolites of cervical secretions at different time points in the pregnant group. B, Scatter plot of the charge ratio and the *P*-value of the differential metabolites of cervical secretions at different time points in the non-pregnant group. C, Enriched metabolic pathway of the differential metabolites of cervical secretions at different time points in the pregnant group. D, Enriched metabolic pathway of the differential metabolites of cervical secretions at different time points in the non-pregnant group. The abbreviations are given in the note to Fig 6. Supplementary description for C and D, Blue dots represent the metabolic pathways and red dots represent metabolites. The size of the dot increases as the number of connected points increases.

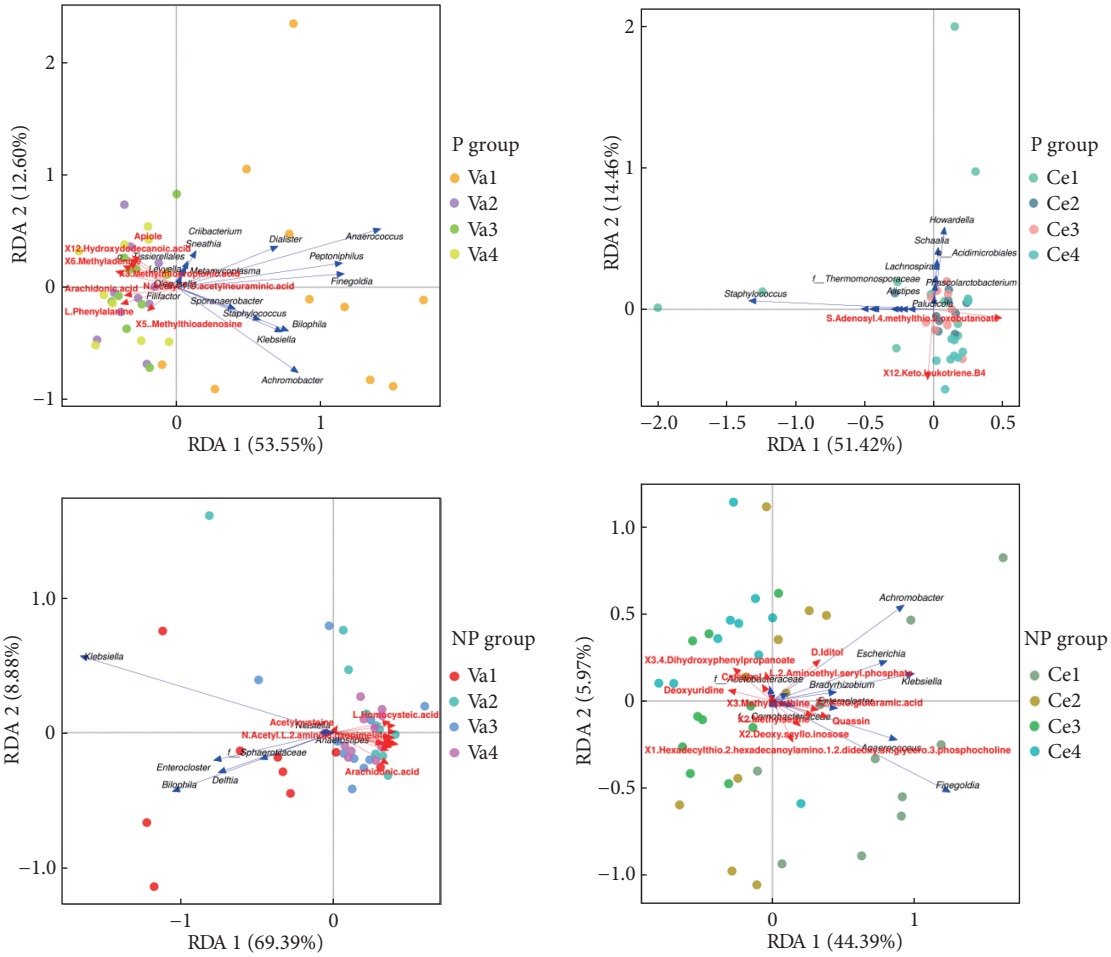

图 8 不同时间点多组学冗余分析图

Fig 8 Multiomics redundancy analysis diagramin at different time points

P group: the group of pregnancy achieved; NP group: the group of pregnancy not achieved. The other abbreviations are given in the note to Fig 3.

关注生殖道微生物对助孕结局的影响是目前的热点。已有研究证实,在控制性超促排卵过程中和外源性应用孕激素黄体支持后,阴道、宫腔微生物的不稳定性变化增加,这可能是影响新鲜周期胚胎移植成功率的原因之一[6]。然而,关于冻胚移植HRT内膜准备过程中生殖道微生物群变化与妊娠结局关系的研究甚少,或仅局限于研究月经周期中某个时间点,因此HRT内膜准备过程中不同时间点生殖道微生物群变化是否与FET妊娠结局相关有待进一步研究。

本研究采用16S rRNA检测对下生殖道分泌物标本进行微生物群落分析,结果证实乳酸杆菌属丰度排名第一,并在月经周期内高度稳定,这与MUHLEISEN等研究结果一致[4-5, 7]。值得注意的是,SOUZA等[8]研究发现尽管乳酸杆菌是生殖系统微生物群中的优势属,但在激素替代治疗胚胎移植周期中乳酸杆菌的丰度对妊娠结局并无显著影响。GAJER等[9]认为月经周期中一些群落在短时间内发生显著变化,而另一些群落则相对稳定,但并没有显著影响群落功能。本研究对比不同时间点阴道及宫颈微生物α-多样性发现,月经期微生物群α-多样性更高,而运用外源性激素替代治疗后阴道及宫颈微生物变化具有相对稳定性。这可能是由于月经期阴道pH值明显高于其他时间点,且雌激素水平在整个月经周期中达到最低,阴道黏膜上皮细胞内糖原含量降低,不利于乳酸杆菌的生存而为其他细菌的繁殖提供了机会,故具有更高的多样性指数。

本研究发现多种菌属在妊娠组与非妊娠组不同时间点阴道及宫颈分泌物中存在差异,尤其值得注意的是妊娠组不同时间点阴道分泌物中*Peptoniphilus*、*Klebsiella*、*Anaerococcus*、*Prevotella*等菌属存在显著差异。*Peptoniphilus*是一种存在于皮肤、阴道和肠道的革兰阳性厌氧球菌[10-12]。多项研究发现,*Peptoniphilus*与未足月胎膜早破、早产、宫颈功能不全以及临床和组织学羊膜绒毛膜炎等有关[13-16]。*Anaerococcus*也是一种革兰阳性厌氧球菌,与人体皮肤和软组织感染、尿路感染等疾病有关,

阴道厌氧球菌属的相对丰度与人乳头瘤病毒感染具有相关性, 阴道厌氧球菌比例增加可能与宫颈上皮内瘤变严重程度有关[17-19]。*Klebsiella*为革兰阴性杆菌, CAREY等[20]对孕期及分娩期孕产妇阴道菌群的研究发现, 阴道中大量增加的肺炎克雷伯菌是早产的独立危险因素。*Prevotella*是内源性阴道菌群的成员之一, 阴道中乳酸杆菌被包括*Prevotella*在内的混合菌群所取代而导致细菌性阴道病。细菌性阴道病与胎膜早破、绒毛膜羊膜炎、流产等不良妊娠结局有关[21]。多种菌属在FET周期不同时间点的显著变异与不同妊娠结局相关, 可能作为预测胚胎移植妊娠成功率的潜在生物标志物, 但多个时间点菌群波动可由某些非特征因素(如经血)驱动, 微生物群研究中的个体内变异性是不可忽视的因素, 而且目前缺乏关于其与妊娠结局和相关妊娠并发症之间相互作用或干预作用的直接研究, 未能得出明确的结论。

此外, 本研究联合非靶向代谢组学分析发现妊娠组与非妊娠组不同时间点阴道及宫颈分泌物均存在一定的代谢差异, 主要与某些氨基酸代谢通路有关, 且某些代谢物与微生物群落具有显著的相关性。但由于本研究为探索性研究, 样本量较局限, 且非靶向代谢组学无偏向性,筛选出的代谢物较为广泛, 没有公认的一组特异性差异代谢产物, 使得相关临床意义受到限制, 进一步提高对阴道和宫颈微生物及其代谢特点的认识, 对改善不孕症患者菌群改变的临床管理具有重要临床指导意义。

综上所述, 在激素替代FET周期中不同时间点存在多种菌属及代谢的显著差异, 有可能会成为预测胚胎移植妊娠成功的潜在生物标志物, 这还需更大规模、更全面的研究来验证。

*　　*　　*

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