Differential Regulation of Eicosanoid and Endocannabinoid Production by Inflammatory Mediators in Human Choriodecidua

M. D. Mitchell*, G. E. Rice, K. Vaswani, D. Kvaskoff*, H. N. Peiris

University of Queensland Centre for Clinical Research, Centre for Clinical Diagnostics, University of Queensland, Royal Brisbane and Women’s Hospital, Queensland, Brisbane, Australia

* Current address: AG Brügger, Heidelberg University Biochemistry Centre (BZH) Im Neuenheimer Feld, Heidelberg, Germany

* murray.mitchell@uq.edu.au

Abstract

An increase in intrauterine prostaglandin production is critical for the onset and progression of labor in women and indeed all mammalian species studied. Endocannabinoids can act as substrates for enzymes of the prostaglandin biosynthetic pathways and can be utilized to generate other related compounds such as prostamides. The end products are indistinguishable by radioimmunoassay. We have separated such compounds by mass spectrometry. We now show that inflammatory stimuli such as LPS and proinflammatory cytokines act differentially on these pathways in human choriodecidua and preferentially create drive through to prostaglandin end products. These findings create doubt about the interpretation of data on prostaglandin biosynthesis in intrauterine tissues from pregnant women especially in the presence of an infection. The possibility is raised that separation of these products might reduce variability in results and lead to potential uses for their measurement in the diagnosis of preterm labor.

Introduction

Preterm delivery (i.e., births occurring <37 weeks gestation) is a major obstetric health problem. In the United States, the preterm birth rate was 11.39% in 2013 [1]. Worldwide, approximately 13 million babies are born prematurely each year [2]. These statistics have remained constant or worsened for decades despite advances in knowledge and medical care. Prematurity is the single most severe complication of pregnancy contributing to poor neonatal outcome. It is strongly associated with low birthweight, increased incidence of perinatal mortality, and greater susceptibility to adult-onset diseases [3]. Parturition is activated by a combination of endocrine and mechanical stimuli from both mother and infant that results in four distinct physiological events: cervical remodeling, uterine contraction, cervical dilatation, and fetal membrane rupture. These events are coordinated by multiple effector pathways, such as NFκB for the activation of inflammation and endocannabinoids and prostaglandin endoperoxidase.
synthase (PGHS)-2 for uterine contractions. Premature birth may occur when these signaling pathways are blocked, mimicked or subverted such that effector pathways are activated irrespective of fetal development. One such pathway that is coming to prominence is the endocannabinoid regulation of labor. Nevertheless, our understanding of how labor is initiated remains poor.

It is, therefore, vital to obtain a better understanding of the basic mechanisms of preterm birth, because even if existing preventive interventions were fully scaled, fewer than 20% of preterm births would be prevented [4]. Classic studies from Mont Liggins provide what is still today the basic outline of the mechanisms of parturition [5]. Unfortunately, these studies in sheep have not been directly transferable to humans without significant anomalies, as the traditional theory of activation of the fetal hypothalamic-pituitary-adrenal axis and the coordinated stimulation of intrauterine prostaglandin production is not fully consistent with what is known about labor onset in women [6]. What is now unequivocal is that the parturient process in all mammals requires increased intrauterine prostaglandin production which is achieved by arachidonic acid metabolism via fatty acid cyclooxygenase (COX). Treatment with prostaglandins induces labor, and inhibition of prostaglandin biosynthesis prevents labor and delivery [7]. This increase in prostaglandin levels with term labor is a cause and not a result of labor [8] and occurs before the onset of labor [9]. We also know that intrauterine-associated infection is a major cause of premature delivery [10]. Indeed, preterm labor may be associated with an exaggerated production of cytokines [11], which stimulate prostaglandin production from intrauterine tissues [10]. Thus, a better understanding of the mechanisms by which cytokines stimulate prostaglandin biosynthesis could lead to the development of novel approaches for preventing and treating preterm labor.

We have shown that anandamide acts as substrate for prostamide production and that stimulation with cytokines mainly induces prostamide output rather than prostaglandin output [12]. Because all presently available antisera to prostaglandins e.g. prostaglandin E2 (PGE2) recognize prostamides, when a substrate is targeted to the site of inflammation, a prostamide may be secreted rather than a prostaglandin (Fig 1). Hence, the overall response to treatment depends on the properties of the prostaglandin or prostamide formed; these substances have widely divergent contractile activities. Furthermore, this induces variation across studies and affects the reproducibility of diagnostic results. In particular, studies of cytokine effects on prostaglandin biosynthesis, which is a critical step in intrauterine infection-induced preterm labor, may have dramatically different results and may fail to reveal key information about specific cytokines. We have now used the gold standard method of mass spectrometry to identify unequivocally products of endocannabinoid and eicosanoid biosynthetic pathways that are formed upon exposure to inflammatory stimuli of human choriodecidua.

Materials and Methods

Patients

Placentae were obtained from women undergoing elective at Caesarean section at term before the onset of labor because of prior Caesarean section or cephalo-pelvic disproportion. Collection was approved by the Human Research Ethics Committees of the Royal Brisbane and Women’s Hospital, and the University of Queensland. Women gave written informed consent for use of placental tissue for research purposes.

Explant Cultures

Five individual placentae from singleton pregnancies of healthy non-smoking mothers were used in the study. In triplicate, chorio-decidual tissue explants were generated and cultured in...
12-well plates as described previously [12, 13]. Briefly, explants were initially placed in DMEM/F12 media supplemented with glutamax (Life Technologies Australia Pty Ltd), 10% FBS (Life Technologies Australia Pty Ltd), and 1% Antibiotic-antimycotic (Life Technologies Australia Pty Ltd) and incubated at 37°C in humid 5% CO2/95% air for 24 h. After a further 24 h incubation with FBS-free media, explants were treated for a further 24 h with 0, 0.1, 1, or 10 μg/mL lipopolysaccharide derived from Escherichia coli (LPS) (Sigma-Aldrich, Australia); 0, 0.1, 1, or 10 ng/mL interleukin 1beta (IL-1β) (BD Biosciences); or 0, 1, 10, or 100 ng/mL tumor necrosis factor alpha (TNF-α) (BD Biosciences). We have previously shown [12, 13] that these timeframes and condition do not alter explant viability.

Lipid Analyses
Lipid metabolites were isolated from cell culture media (50 μL) and tissue (30 mg) by extraction in 1% formic acid in cold methanol (Sigma-Aldrich, Australia; 200 μL, containing 250 fmol internal standards) followed by sonication, shaking, and centrifugation (4,500 x g at 4°C) to precipitate proteins. Solid phase clean-up on reverse-phase cartridges (Phenomenex Inc. NSW Australia), using 10% methanol (Sigma-Aldrich, Australia) as a washing solution, removed most water-soluble interferences (e.g., salts from culture media and proteins). Deuterated internal standards (purchased from Cayman Chemicals-Sapphire Bioscience Pty. Ltd) were used to normalize signal response across samples and to account for any differences in extraction recovery (extraction recovery for all eicosanoids was >85%).

Authentic standards were purchased from Cayman Chemicals-Sapphire Bioscience Pty. Ltd Prostaglandin E2 Ethanolamide (PGE2-EA), Deuterated Prostaglandin E2 Ethanolamide (PGE2-EA-d4), Prostaglandin F2α Ethanolamide (PGF2α-EA), Deuterated Prostaglandin F2α Ethanolamide (PGF2α-EA-d4), Prostaglandin E2 (PGE2), Deuterated Prostaglandin E2 (PGE2-d4), PGF2α (##10007221), Deuterated Prostaglandin F2α (PGF2α-d4), 13,14-dihydro-15-keto PGF2α (PGFM), Deuterated 13,14-dihydro-15-keto PGF2α (PGFM-d4), Arachidonoyl ethanolamide (Anandamide; AEA), Deuterated Arachidonoyl ethanolamide (AEA-d4), 2-Arachidonoyl glycerol (2-AG), Deuterated 2-Arachidonoyl glycerol (2-AG-d8).

Fig 1. Endocannabinoid pathway. Endocannabinoid pathway giving rise to prostaglandins glycerol esters, prostaglandins and prostanoids.

doi:10.1371/journal.pone.0148306.g001
Statistical analyses. Statistical differences were determined using the Wilcoxon signed-rank test.

Results and Discussion

Results

Data were collected using positive/negative periods, which enabled the measurement of both prostaglandins (negative ions) and endocannabinoids and prostamides (positive ions) concomitantly (Fig 2). This approach is flexible for including additional lipid metabolites of interest in the future. A gradient elution under reverse-phase (C18) high-performance liquid chromatography conditions led to separation of individual peaks in under 7.5 min, which were then quantified using a matrix-matched standard curve (20–5,000 pmol/L). Mobile phase conditions were obtained from the literature [14, 15]. High specificity (i.e., unique mass pairs) and sensitivity (< 8 pg/mL for prostaglandins and < 2 pg/mL for prostamides; see lower limit of quantification (LLOQ) in Table 1) enabled us to measure physiologically relevant levels of lipids in choriodecidual explants (Fig 3). Analysis of each lipid type is achieved by monitoring characteristic mass fragment pairs for each molecule at their distinct retention times (Rt; scheduled multiple reaction monitoring (MRM) transitions in Table 1). The concentrations of eicosanoids generated by choriodecidual explants (in explant culture media) measured by LC-MS/MS data for explants treated with LPS, IL-1β and TNF-α are shown in Figs 4–6. As would be predicted all three treatments stimulated the production of PGF2α, PGE2 and PGFM, with
minor variations (Figs 4–6A). However, none of the inflammatory stimuli caused a significant change in the productions of prostamides or endocannabinoids (Figs 4–6B and 6C). Concentrations of prostaglandins, prostamides and endocannabinoids were well above the limit of

| Endogenous lipids: | Rt (min) | Period 1 (Positive) | Period 2 (Negative) | Period 3 (Positive) | LLOQ (pg/mL) |
|--------------------|---------|---------------------|---------------------|---------------------|--------------|
| PGF2α-EA          | 3.12    | Q1 380.4 62.0       | Q1 353.2 309.0     | Q1 348.4 62.0     | 45 2         |
| PGE2-EA           | 3.14    | Q1 382.4 62.0       | Q1 353.2 113.0     | Q1 348.4 62.0     | 45 2         |
| PGF2α             | 3.63    | Q1 353.2 309.0     | Q1 353.2 113.0     | Q1 348.4 62.0     | -28 8        |
| PGE2              | 3.71    | Q1 351.1 271.1     | Q1 351.1 271.1     | Q1 348.4 62.0     | -24 8        |
| PGFM              | 4.00    | Q1 353.2 217.1     | Q1 353.2 217.1     | Q1 348.4 62.0     | -36 8        |
| AEA               | 5.11    | Q1 353.2 217.1     | Q1 353.2 217.1     | Q1 348.4 62.0     | 25 1         |
| 2-AG              | 5.36    | Q1 379.1 287.2     | Q1 379.1 287.2     | Q1 379.1 287.2    | 21 10        |

| Internal standards: | Rt (min) | Period 1 (Positive) | Period 2 (Negative) | Period 3 (Positive) | LLOQ (pg/mL) |
|---------------------|---------|---------------------|---------------------|---------------------|--------------|
| PGF2α-EA-d4        | 3.11    | Q1 384.4 62.0       | Q1 357.2 313.0     | Q1 352.4 66.0     | 45 2         |
| PGE2-EA-d4         | 3.13    | Q1 382.4 62.0       | Q1 355.1 319.0     | Q1 357.2 186.9    | 45 2         |
| PGF2α-d4           | 3.63    | Q1 357.2 186.9     | Q1 357.2 186.9     | Q1 357.2 186.9    | -28 8        |
| PGE2-d4            | 3.70    | Q1 355.1 319.0     | Q1 355.1 319.0     | Q1 357.2 186.9    | -24 8        |
| PGFM-d4            | 3.99    | Q1 357.2 186.9     | Q1 357.2 186.9     | Q1 357.2 186.9    | -36 8        |
| AEA-d4             | 5.11    | Q1 352.4 66.0     | Q1 352.4 66.0     | Q1 352.4 66.0     | 25 1         |
| 2-AG-d8            | 5.36    | Q1 387.2 295.0     | Q1 387.2 295.0     | Q1 387.2 295.0    | 21 10        |

Table 1. Optimized scheduled MRM pairs and parameters for eicosanoids.
detection of the assay (as indicated by the dashed line in Figs 4–6). The mean ratio of PGE$_2$ and PGF$_{2\alpha}$ to ethanolamide metabolites produced by term choriodecidua after treatment with LPS, IL-1$\beta$, or TNF-$\alpha$ is given in Fig 7. Significant changes in the ratios were noted with all the treatments. These data suggest a higher proportion of PGE$_2$ and PGF$_{2\alpha}$ are produced following treatment with LPS, IL-1$\beta$, or TNF-$\alpha$.

**Discussion**

Our results are consistent with earlier work [12] and our hypothesis. We have now shown, with specific identification of eicosanoid products, that there is differential regulation of
prostaglandin and prostamide production highlighted by a massive stimulation of prostaglandin E2, F2α and FM production by IL-1β with no response in prostamide production (Fig 5). This is consistent with and provides further evidence for our hypothesis and is identical to the data in [12]. The significant enhanced differential response of prostamide production to IL-1β in the earlier paper by Glass et al [12] was only when added anandamide substrate was added to mimic a site of inflammation at which cells had been recruited that provided such substrate (as described below). Unfortunately, we have not replicated that part of the earlier study but aim to do so in the near future.

We provide unequivocal evidence that there is differential regulation of prostaglandin and prostamide biosynthesis in human choriodecidua in response to inflammatory stimuli. Each of the inflammatory mediators tested consistently provoked a preferential drive through the prostaglandin biosynthetic pathways. This is consistent with findings in amnion and an amnion...
derived cell line using crude chromatographic separation techniques [12]. It is interesting and valuable information since differences have been discerned in responses of fetal versus maternal tissues in cannabinoid stimulation of prostaglandin biosynthetic pathways [13]. Our data demonstrate that both amnion and choriodecidua respond similarly with this differential drive through the prostaglandin biosynthetic pathways. Moreover, and importantly, this has been shown using the “gold standard” of measurement by mass spectrometric means. How might this relate to the situation in vivo? These findings create doubt about the interpretation of data on prostaglandin biosynthesis in intrauterine tissues from pregnant women especially in the presence of an infection. The possibility is raised that separation of these products might lead to potential uses for their measurement in the diagnosis of preterm labor. The major identifiable cause of preterm labor is intrauterine infection [10], which may induce the secretion of cross-reacting prostamides, as anandamide release has been observed in response to hemorrhagic shock [16], LPS treatment of macrophages [17], and LPS challenge of human peripheral lymphocytes [18]. Likewise, COX-2 is induced by several inflammatory stimuli, such as IL-1β and LPS. These findings suggest that at the site of inflammation or infection, increases in both anandamide and COX-2 may synergistically induce secretion of PGE₂-ethanolamide. Increased intrauterine prostaglandin production is considered a critical step in the process of human parturition. Hence it is vital to ascertain the results of experiments similar to those presented here, but determining the influence of additional amounts of anandamide and 2-AG on the rates of production and ratio of all end products.

Endocannabinoid signaling is tightly regulated throughout normal pregnancy. Plasma anandamide concentrations, as measured by MS, are markedly elevated in association with term labor [19, 20]. Interestingly, plasma anandamide concentrations measured in the first trimester of women presenting with signs of potential miscarriage are 3-fold higher in those who subsequently miscarry compared with those who progress to term [21]. This suggests that high plasma anandamide concentrations may lead to uterine activation and labor onset. Increased anandamide availability could be due to upregulated synthesis by N-acyltransferase and phospholipase D or reduced hydrolysis by fatty acid amide hydrolase (FAAH). In fact, FAAH protein and mRNA expression as well as activity in peripheral lymphocytes is reduced in women who miscarry spontaneously or fail to maintain pregnancy post-in vitro fertilization in the first trimester compared with gestational age-matched women undergoing voluntary pregnancy termination [22, 23].

Overall, however, the potential of prostaglandins, prostamides, and endocannabinoids to serve as clinically useful diagnostic and predictive factors for preterm labor may have been overlooked due to erroneous interpretation of immunoassay-related data. Examination of the similar molecular structures of key substances [24] demonstrates why immunoassays may have difficulty in distinguishing among these moieties.

Conclusions

Our results cannot be applied to all physiological and pathophysiological situations but may certainly completely alter our thinking in others. The key factors will be the availability of substrate for the action of key enzymes of arachidonic acid metabolism—particularly recruitment of endocannabinoids via infiltrating cells as we mention earlier. Under these conditions and dependent upon the enzymes present then major products might be prostaglandins or prostamides or indeed other related substances e.g. from the lipoxygenase pathway. In a situation in which prostaglandins were being assessed for utility as diagnostics for e.g. preterm labor then the additional prostamides would register in assays but have little bioactivity on the myometrium and thus provide variability in results that would make the difference between clinical
utility and not. This is especially true for pregnancy and labor and high risk pregnancies since the use of prostaglandins and inhibitors is widespread in clinical practice and it has always been a source of frustration that to date measurements were not useful diagnostically or prognostically. In future the measurement of products of the different pathways must be assessed using mass spectrometry as this is not compromised by antibody specificity or lack thereof. This may at least provide confirmation of identity and validation of results and although this is the “gold standard” and should be in regular use, that is not always possible and it should at least be done in a comparison and validation experiment initially. It remains possible that many of our ideas about prostaglandins and their roles physiological and pathophysiological mechanisms may be radically altered by this knowledge.

Acknowledgments
Thank you to Dr Sarah Reed for assistance with mass spectrometry analyses.

Author Contributions
Conceived and designed the experiments: MDM. Performed the experiments: DK KV HNP. Analyzed the data: DK MDM. Contributed reagents/materials/analysis tools: MDM GR. Wrote the paper: MDM GR DK.

References
1. Martin JA. Department of Health and Human Services Centers for Disease Control and Prevention National Center for Health Statistics NCHS Data Brief 2014;(December).
2. Beck S, Wojdyla D, Say L, Betran AP, Merialdi M, Requejo JH, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. Bull World Health Organ. 2010; 88(1):31–8. Epub 2010/04/30. doi: 10.2471/BLT.08.062554 PMID: 20428351; PubMed Central PMCID: PMC2802437.
3. Li Z, Zeki R, Hilder L, Sullivan EA. Australia’s mothers and babies 2010. Canberra: AIHW National Perinatal Epidemiology and Statistics Unit; 2012.
4. Simmons LE, Rubens CE, Darmstadt GL, Gravett MG. Preventing preterm birth and neonatal mortality: exploring the epidemiology, causes, and interventions. Semin Perinatol. 2010; 34(6):408–15. Epub 2010/11/26. doi: 10.1053/j.semperi.2010.09.005 PMID: 21094415.
5. Liggins GC, Fairclough RJ, Grieves SA, Kendall JZ, Knox BS. The mechanism of initiation of parturition in the ewe. Recent Prog Horm Res. 1973; 29:111–59. Epub 1973/01/01. PMID: 4356273.
6. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. Endocr Rev. 2000; 21(5):514–50. Epub 2000/10/21. doi: 10.1210/edrv.21.5.0407 PMID: 11041447.
7. Turnbull AC, Lucas A, Mitchell MD. Prostaglandins in the Perinatal-Period. Rev Perinat Med. 1981; 4:273–97. PMID: WOS:A1981QX86000007.
8. Romero R, Gonzalez R, Baumann P, Behnke E, Rittenhouse L, Barberio D, et al. Topographic differences in amniotic fluid concentrations of prostanoids in women in spontaneous labor at term. Prostaglandins Leukot Essent Fatty Acids. 1994; 50(2):97–104. Epub 1994/02/02. PMID: 8171074.
9. Romero R, Munoz H, Gomez R, Parra M, Polanco M, Valverde V, et al. Increase in prostaglandin bioavailability precedes the onset of human parturition. Prostaglandins Leukot Essent Fatty Acids. 1996; 54(3):187–91. Epub 1996/03/01. PMID: 8860106.
10. Romero R, Munoz H, Gomez R, Galasso M, Sherer DM, Cotton D, et al. Does Infection Cause Premature Labor and Delivery. Semin Reprod Endocrinol. 1994; 12(4):227–39. doi: 10.1055/s-2007-1016404 PMID: WOS:A1994PY22800005.
11. Keelan JA, Wong PM, Bird PS, Mitchell MD. Innate inflammatory responses of human decidual cells to periodontopathic bacteria. Am J Obstet Gynecol. 2010; 202(5):471 e1-11. Epub 2010/05/11. doi: S0002-9378(10)00244-9 [pii] doi: 10.1016/j.ajog.2010.02.031 PMID: 20452492.
12. Glass M, Hong JW, Sato TA, Mitchell MD. Misidentification of prostamides as prostaglandins. J Lipid Res. 2005; 46(7):1364–8. doi: 10.1194/jlr.C500006-JLR200 PMID: WOS:000229741700002.
13. Mitchell MD, Sato TA, Wang A, Keelan JA, Ponnampalam AP, Glass M. Cannabinoids stimulate prostaglandin production by human gestational tissues through a tissue- and CB1-receptor-specific
mechanism. American journal of physiology Endocrinology and metabolism. 2008; 294(2):E352–6. doi: 10.1152/ajpendo.00495.2007 PMID: 18042663.

14. Dumlao DS, Buczynski MW, Norris PC, Harkewicz R, Dennis EA. High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylthanolamines. Biochimica et biophysica acta. 2011; 1811 (11):724–36. Epub 2011/06/22. doi: 10.1016/j.bbapal.2011.06.005 PMID: 21689782; PubMed Central PMCID: PMCPM3205334.

15. Sergi M, Battista N, Montesano C, Curini R, Maccarrone M, Compagnone D. Determination of the two major endocannabinoids in human plasma by mu-SPE followed by HPLC-MS/MS. Analytical and bioanalytical chemistry. 2013; 405(2–3):785–93. Epub 2012/08/01. doi:10.1007/s00216-012-6273-3 PMID: 22847477.

16. Wagner JA, Varga K, Ellis EF, Rzigalinski BA, Martin BR, Kunos G. Activation of peripheral CB1 cannabinoid receptors in haemorrhagic shock. Nature. 1997; 390(6659):518–21. Epub 1997/12/11. doi:10.1038/37371 PMID: 9394002.

17. Liu J, Batkai S, Pacher P, Harvey-White J, Wagner JA, Cravatt BF, et al. Lipopolysaccharide induces anandamide synthesis in macrophages via CD14/MAPK/phosphoinositide 3-kinase/NF-kappaB independently of platelet-activating factor. The Journal of biological chemistry. 2003; 278(45):45034–9. Epub 2003/09/02. doi: 10.1074/jbc.M306062200 PMID: 12949078.

18. Maccarrone M, De Petrocellis L, Bari M, Fezza F, Salvati S, Di Marzo V, et al. Lipopolysaccharide downregulates fatty acid amidase hydrolase expression and increases anandamide levels in human peripheral lymphocytes. Archives of biochemistry and biophysics. 2001; 393(2):321–8. Epub 2001/09/15. doi: 10.1006/abbi.2001.2500 PMID: 11556820.

19. Habayeb OM, Taylor AH, Evans MD, Cooke MS, Taylor DJ, Bell SC, et al. Plasma levels of the endocannabinoid anandamide in women—a potential role in pregnancy maintenance and labor? The Journal of clinical endocrinology and metabolism. 2004; 89(11):5482–7. Epub 2004/11/09. doi: 10.1210/jc.2004-0681 PMID: 15531501.

20. Lam PM, Marczyn TH, El-Talatini M, Finney M, Nallendran V, Taylor AH, et al. Ultra performance liquid chromatography tandem mass spectrometry method for the measurement of anandamide in human plasma. Analytical biochemistry. 2008; 380(2):195–201. Epub 2008/06/17. doi: 10.1016/j.ab.2008.05.033 PMID: 18555789.

21. Habayeb OM, Taylor AH, Finney M, Evans MD, Konje JC. Plasma anandamide concentration and pregnancy outcome in women with threatened miscarriage. JAMA: the journal of the American Medical Association. 2008; 299(10):1135–6. Epub 2008/03/13. doi: 10.1001/jama.299.10.1135 PMID: 18334688.

22. Maccarrone M, Bisogno T, Valensise H, Lazzarin N, Fezza F, Manna C, et al. Low fatty acid amidase hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. Molecular human reproduction. 2002; 8(2):188–95. Epub 2002/01/31. PMID: 11818522.

23. Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C, Finazzi-Agro A. Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. Lancet. 2000; 355(9212):1326–9. Epub 2000/04/25. doi: 10.1016/s0140-6736(00)02115-2 PMID: 10776746.

24. Chan HW, McKirdy NC, Peiris HN, Rice GE, Mitchell MD. The role of endocannabinoids in pregnancy. Reproduction. 2013; 146(3):R101–9. doi: 10.1530/REP-12-0508 PMID: 23744614.