Behavioral States of Isolated Goat Fetuses During Extrauterine Incubation Using Arteriovenous Extracorporeal Membrane Oxygenation

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Abstract

1. OBJECTIVES

To investigate the physiological states of goat fetuses undergoing extrauterine incubation following isolation from their mother, we analyzed fetal electroencephalograms (EEGs) with power spectral analysis.

2. MATERIALS AND METHODS

Ten goat fetuses at 124 ± 3.9 days of gestation (term 150 days) were incubated in an artificial womb connected to an extracorporeal membrane oxygenation (ECMO) apparatus. While these fetuses were in a stabilized condition, their EEGs were recorded using a digital data recorder. Electrocortical activity on the EEGs was classified visually as either a high-voltage period or a low-voltage period. The recorded data were then analyzed using a computer. After analogue-to-digital conversion at a sampling frequency of 300Hz with a resolution of 16bits, power spectral analysis using an auto-regression model was carried out.

3. RESULTS

Both high-voltage and low-voltage periods could be observed on the EEGs of all ten goat fetuses, and these periods appeared to alternate. Power spectral analysis of recorded EEGs in the high-voltage period showed a peak at the 0.5-4Hz frequency range, which corresponds to the delta band. A peak at the 8-13Hz frequency range, which corresponds to the alpha band, was observed in the low-voltage period.

4. CONCLUSION

During extrauterine incubation, goat fetuses showed two different EEG patterns. This suggests that the fetuses show physiological behavioral states that are independent from those of their mother.

Key words : goat fetus, animal experiment, ECMO, behavioral states,
power spectral analysis

Introduction

In the field of maternal fetal medicine, animal experiments such as the chronic preparation mod-

el have been used for many years and provided a substantial amount of knowledge about fetal physiology^{1) 2)}. The chronic preparation model is a well-established method of studying fetal physiology and pathology ; however, its use means that fetuses cannot be directly affected by researchers, and thus can only be affected indirectly through their mother. Furthermore, responses from the fetus that are not influenced by the mother cannot be

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Figure-1 Extrauterine incubation system (from Kozuma *et al*¹¹⁾)

- ① tube occluder
- ② open-top reservoir
- ③ flow detector-controller
- ④ blood pump
- ⑤ membrane oxygenator
- ⑥ closed reservoir
- ⑦ heat exchanger

determined.

There have been several investigations of methods to incubate fetuses outside of the uterus in an artificial womb^{3) 4)}. However, these attempts were abandoned because of the difficulty of maintaining fetuses in a good condition for a sufficient length of time. In 1987, Kuwabara *et al.* developed a new method of incubating goat fetuses in an artificial womb utilizing umbilical arteriovenous extracorporeal membrane oxygenation (A-V ECMO)⁵⁾. By using this system, long-term extrauterine incubation of two goat fetuses for more than three weeks was achieved⁶⁾. This extrauterine incubation system not only allows the fetus to be observed with the naked eye but also enables many different fetal physiological parameters to be easily measured. Furthermore, because fetuses connected to the A-V ECMO system and separated from the placenta are independent of their mother, they can be directly affected, and the fetal state, as well as responses to an introduced effect, can be investigated without maternal influence. Thus, the extrauterine incubation system appears to be another useful experimental model for investigating fetal physiology^{7) 8)}; however, at present, there is little knowledge about the physiology of fetuses during extrauterine incubation.

Fetuses in the late second trimester are known to show specific physiological behavioral states⁹⁾. These states have been extensively investigated

using the chronic preparation model¹⁰⁾. However, there are few studies of fetal behavioral states during extrauterine incubation. Fetuses during extrauterine incubation are known to show a wave of high and low voltages on EEGs¹¹⁾; however, computer analysis of EEGs, which may allow the more precise determination of behavioral states, has never been performed on a fetus during extrauterine incubation. We hypothesize that fetuses in the late second trimester may exhibit a physiological behavioral states even when separated from their mother. We analyzed EEGs from fetuses recorded during extrauterine incubation with power spectral analysis.

Materials and Methods

1. Animals

We used 10 goat fetuses in this study. The fetal age at the time of the experiment was 124 ± 3.9 days (mean $\pm$ SD) of gestation (term 150 days), and the estimated body weight at the start of extrauterine incubation was $1770 \text{g} \pm 261 \text{g}$.

2. Extrauterine Incubation System

The surgical procedure to introduce a goat fetus into an extrauterine incubation system have been described in detail previously¹²⁾. The system can be summarized as follows. The goat fetus is incubated in artificial amniotic fluid and an artificial placental system that consists of a membrane oxygenator (Mera SILOX-S 0.5 : Senko Medical Instrument Manufacturing Co., Japan) is used for A-V ECMO. The fetal blood flow is drained to an arterial open-top reservoir by fetal cardiac output and then transferred to the membrane oxygenator using a roller pump equipped with a controller that regulates its rotation rate in accordance with the volume of blood in the open-top reservoir. After complete oxygenation, the fetal blood is returned to the fetal umbilical vein via closed reservoirs for attenuation of rotation of the roller pump.

During extrauterine incubation in this study, a heparinized solution was infused continuously into the extracorporeal circuit, and its infusion rate was adjusted to 80-100 IU/kg/hr. Glucose and calcium gluconate were administered to the fetus at rates of 5-8 mg/kg/min and 40 mg/kg/day, respectively,

Figure-2 Physiological parameters of a goat fetus during extrauterine incubation

ECG : electrocardiogram
BP : blood pressure
EEG : electroencephalogram

LV : low-voltage period in EEG
HV : high-voltage period in EEG

and pancronium bromide was continuously administered at 0.02mg/kg/hr to suppress fetal gross movement. Fetal blood pressure was measured through a catheter inserted into the umbilical artery. To measure ECGs, 3 electrodes were fixed to the fetal anterior chest wall. To measure EEGs, 2 pairs of spiral electrodes (Intermedical Co., Japan) were placed on the bilateral sides of the scalp skin. The protocol of this experiment was approved by the Juntendo university school of medicine committee concerned with animal experiments.

3. Experimental Protocol

After a minimal recovery period of 24 hours had passed from the start of the extrauterine incubation, we started recording fetal physiological parameters for 120 minutes to 180 minutes in a dark and quiet environment. The measured signals were recorded on a multichannel recorder and stored on a PCM data recorder (PC208Ax, Sony Co., Japan). All analog tape records of the EEG signals were filtered at 0.5-50Hz (NF Electronic Instruments, FV-664, 24dB/OCT) and then digitized at a frequency of 300Hz with a resolution of 16 bits. All digitized data were analyzed using a computer (Compaq Alpha Server 4100 5/ 466, Compaq, U.S.A.).

Electrocortical activity on the EEGs was classified visually as either a high-voltage period or a low-voltage period. For each subject, we selected 3 parts of the data record, each of which included at least one series of two complete cycles of high-voltage and low-voltage periods on the EEG. Pow-

Figure-3 Three dimensional display of frequency variation of EEG by spectral analysis employing auto regression model

X axis : time (second)
Y axis : frequency variation (Hz)
Z axis : power (%)

←——→ Solid line : high-voltage period (HV)
←-----→ Broken line : low-voltage period (LV)

er spectral analysis using an auto-regression model (AR-model) was carried out for each part. The power spectrum of each EEG for 5.12 seconds was calculated, which was repeated 5 times, and then their ensemble average was obtained for each frequency domain. The order of the AR model was determined to be 45 by the method of final prediction error.

After the power spectral analysis, peak power at the ranges of 0.5-4Hz, 8-13Hz and 13-35Hz was defined as a delta-wave signal, an alpha-wave signal, and a beta-wave signal, respectively.

4. Statistical Analysis

The correlation between the delta and alpha components of EEGs was analyzed. The data were expressed as mean $\pm$ SD. We used SAS software for the statistical analysis, and a significant difference was defined as $p < 0.05$.

Results

The EEG activities in all 10 goat fetuses during extrauterine incubation were classified into two visually discernable types : a high-voltage period and a low-voltage period that appeared alternately (Figure-2). The blood pressure level was higher in the low-voltage period than in the high-voltage

Figure-4 Two dimensional display of frequency variation of EEG signals in a high-voltage period (Figure-4-a) and low-voltage period (4-b) Each line represents a frequency variation in each spectral analysis

X axis : frequency variation (Hz)

Y axis : power (%)

————— Solid line : high-voltage period (HV)

- - - - - Broken line : low-voltage period (LV)

period.

Power spectral analysis of global time variation in the EEGs showed that a power peak was located in the 0.5-4Hz frequency range both in the high-voltage and low-voltage periods (Figure-3).

Power spectral analysis of the EEGs in the high-voltage period revealed that there was a single peak at the 0.5-4Hz frequency range, which corresponds to the delta band. There was also a power peak at the same range in the low-voltage period. The peak power in the high-voltage period was about 3 times greater than that in the low-voltage period. On the other hand, in the low-voltage period, there was another power peak at the 8-13Hz frequency range, which corresponds to the alpha band (Figure-4-a, 4-b). Changes in the power at the delta and alpha bands in the global time variation of EEG signals showed a biphasic pattern. In other words, in the high-voltage period, power at the delta band showed a high phase and that at the alpha band showed a low phase. In the low-voltage period, the opposite occurred (Figure-5).

Discussion

In 1982, Nijhuis¹³⁾ further developed the theory regarding the behavioral states of newborn infants, which had originally been established by Precht⁹⁾, and classified fetal behavioral states into 4 categories. Since then, many studies on fetal behavioral states have been conducted^{14)~17)}. Because methods of studying human fetuses should be noninvasive both to the mother and the fetus, observations of fetal behavioral states have been carried out in cases of premature neonates who were born at various weeks of gestation. However, even at the same fetal age, the behavioral states of premature neonates is expected to be significantly different from that of immature fetuses in utero, because the extrauterine environment should have various effects on premature neonates. Recently, progress in ultrasonography has enabled more precise observation of human fetuses in utero. Subsequent studies of fetal behavioral states have progressed remarkably through observation of fetal eye movements or the diameter of the pupil^{18)~21)}. However, it has remained impossible to obtain substantial information without invasive methods. In studies of fetal behavioral states, it is important to measure and continuously assess changes in fetal physiological parameters, such as EEGs, eye movements, muscle tone, blood pressure, heart rate and so on. Many investigators have used chronically pre-

pared animal subjects^{1) 22)}. Experiments using chronically prepared animal subjects have indicated that a fetus close to term shows REM and NREM behavioral states²³⁾. However, a fetus in utero is not independent of maternal influences such as hormones and catecholamines. Accordingly, it has not been clear that a fetus exhibits its own behavioral states. In our experiment, goat fetuses were maintained on fetal circulation and incubated so as to isolate them from maternal influences using our system. Transparency and good accessibility to the fetus are very useful for simultaneously observing both the behavior of a goat fetus and physiological parameters such as fetal heart rate, fetal blood pressure, fetal EEGs and so on. Furthermore, the fetal goat's independence from maternal influences makes it possible to assess the functional development of the fetus itself. By using this system, Unno *et al.* incubated 2 fetal goats for more than 500 hours⁶⁾. After 500 hours of incubation, the development of fetal various organs could be demonstrated. This shows that this system is suitable for investigating fetal physiology. Kozuma *et al.*¹¹⁾ investigated fetal breathing movements using this extrauterine fetal incubation system, and concluded that the fetal breathing movements occurred almost exclusively during periods of low-voltage electrocortical activity and were episodic suggesting that intermittent breathing movements are intrinsic to fetuses, independent of placental-derived factors. This also showed that this extrauterine incubation system is an effective experimental model for investigating fetal physiology. In this study, we investigated fetal electrocortical activity by analyzing biological signals under quiet conditions in order to assess fetal behavioral states. EEG signals could be clearly obtained from all 10 fetuses and then classified visually into high-voltage (HV) and low-voltage (LV) periods. These findings confirmed previous observations of chronically prepared animal fetuses that EEG activity of fetuses can be classified into 2 states.

Shinozuka *et al.*²⁴⁾ evaluated and mathematically analyzed electrocortical activity in sheep fetuses. For the differentiation of high-voltage and low-voltage states, they calculated the mean voltage after obtaining full wave rectification of all EEG signals, and voltages above the upper 95% confidence limit

Figure-5 Change in the power after filtering at the delta band and alpha band

X axis : time (second)

Y axis : power (%)

———> Solid line : high-voltage period (HV)

- - - - -> Broken line : low-voltage period (LV)

====> Double solid line : delta band (0.5-4.0Hz)

- - - - -> Double broken line : alpha band (8.0-13Hz)

of the mean voltage were classified as HV, while those below the lower 5% confidence limit of the mean voltage were considered LV. By using this method, their observations for the time spent at both HV and LV states were almost the same as those made by Dawes *et al*²⁵⁾ in 1976 using HV and LV classification carried out with the naked eye. In our experiment, fetuses that were undergoing extrauterine incubation, separated from the placenta but connected to an artificial placenta, showed low-voltage and high-voltage EEG periods.

Moreover, it was evident from spectral analysis of the EEGs that delta wave signals appeared predominantly in the LV period, at which point the fetuses were in a state of REM sleep or were awake. In the HV period, alpha wave signals appeared showing that the fetuses were in a state of NREM sleep. In our experimental system, each goat fetus was completely isolated from its mother and was free from maternal hormones and catecholamines. It was shown that immature fetal goats at around 125 days of gestation demonstrated REM and NREM behavior, but the mechanism by which fetal behavior develops remains unclear.

In this study, pancronium bromide was continuously administered at a rate of 0.03 mg/kg/hr so as to avoid the influence of electromyograms on EEGs and to obtain stabilized data. We confirmed that EEGs and ECGs were unaffected by the administration of this drug. However, the use of a muscle relaxant made it impossible to obtain data about fetal movements. Further study concerning EEG activity and fetal movements without the administration of a muscle relaxant should be performed.

In conclusion, goat fetuses undergoing extrauterine incubation showed two different EEG patterns. This may suggest that, during extrauterine incubation, goat fetuses show physiological behavioral states independent from those of their mother.

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A-VECMO (膜型人工肺による動-静脈式体外酸素化法) による子宮外保育中という
母体から独立した状況下でのヤギ胎仔の behavioral state について

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抄 録

【目的】母体からの影響を除いた状況下での胎児の behavioral states を評価することを目的として、われわれが開発したヤギ胎仔子宮外保育装置を用いて動物実験を行った。

【方法】妊娠118日～126日(満期:150日)のヤギ胎仔10頭を用いて子宮外保育実験を行った。子宮外保育実験は、われわれが独自に開発したA-VECMO(膜型人工肺による動-静脈式体外酸素化法)を用いた子宮外保育実験装置を用いて行った。子宮外保育開始後、胎仔の状態が安定したところで、血圧、心電図(ECG)、脳波(EEG)などの生理学的パラメータをデジタルデータレコーダに記録した。記録したデータはオフラインでサンプリング周波数300Hz、解像度16ビットでAD変換の後、コンピュータ解析を行った。それぞれのデータはEEGにおいて高振幅(high-voltage)と低振幅(low-voltage)の繰り返しが少なくとも1回は含まれる部分を3ヵ所ランダムに選び、自己相関モデルによるパワースペクトル解析を実施した。

【結果】10頭すべての胎仔において、子宮外保育実験中のEEGは高振幅期と低振幅期の2つに分類され、それぞれが相互に繰り返し出現した。パワースペクトル解析の結果、高振幅期脳波は0.5～4Hz、つまりデルタ波帯域に一致するピークを有する脳波であり、低振幅期脳波は、これに加えて8～13Hz、つまりアルファ波帯域にもピークを有する脳波であることが明らかとなった。

【結論】A-VECMO(膜型人工肺による動-静脈式体外酸素化法)を用いた子宮外保育実験中においても、妊娠125日前後のヤギ胎仔はnon-REM期およびREM期あるいは覚醒期に匹敵する2つの異なる behavioral states を示すことから、未熟ヤギ胎仔において自律的な behavioral states の発達が認められることが判明した。

キーワード: 羊胎仔, 動物実験, ECMO, behavioral states, スペクトル解析