

A Pilot Comparative Study of Vocal and Instrumental Acoustic Stimulation on Early Embryonic Brain Morphology in Gallus gallus

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ABSTRACT

Introduction. Early embryonic brain development can respond to environmental factors, including sound. Sound waves create mechanical vibrations that affect cellular activity through mechanotransduction. This study examined the effects of rhythmic vocal and harmonic instrumental acoustic stimulation on early brain development. **Methods.** We randomly assigned 20 fertilized chicken eggs to two groups: rhythmic vocal stimulation (Qur'anic recitation, $n = 10$) and harmonic instrumental stimulation (classical music, $n = 10$). We exposed the eggs to sound for 5 days, from embryonic day 1 to day 5. On day 5, we collected and examined the embryos. We assessed brain development based on embryo viability and the morphology of the primary brain vesicles. We used Fisher's Exact Test to compare developmental outcomes between the two groups. **Results.** Eight embryos (80%) developed in the rhythmic vocal group, while seven embryos (70%) developed in the harmonic instrumental group. The difference was not statistically significant ($p > 0.05$). However, Qualitative microscopic observation noted that primary brain vesicles appeared more clearly delineated in the rhythmic vocal group; however, this observation was descriptive and was not confirmed by quantitative morphometric measurement. **Conclusion.** Although the difference was not statistically significant, rhythmic vocal stimulation raise the hypothesis that rhythmic vocal stimulation may be associated with more distinct brain vesicle morphology. Because this was a pilot study with a small sample size, further studies using larger sample sizes and histological, biochemical, and molecular analyses are needed to better understand the biological effects of acoustic stimulation during embryonic development.

1. Introduction

Early embryonic development represents a critical stage in the life course of an organism. The zygote undergoes a series of coordinated developmental processes through the morula, blastula, and gastrula stages. These early developmental processes establish the basic body plan and cell lineages that will develop into the embryo and later the fetus. The embryonic phase is important because organ formation begins during this period. Disruptions at this stage can cause structural abnormalities and congenital disorders, which may affect an individual's health and quality of life after birth.^{1,2}

Congenital anomalies remain an important global health concern. The World Health Organization (WHO) reports that birth defects contribute to approximately 2.7 million neonatal deaths each year across 193 countries.³ In the United States, approximately 120,000 infants are born with congenital anomalies each year.⁴ Neural Tube Defects

(NTDs) are among the most common congenital malformations and rank second after congenital heart defects.⁵ In China, epidemiological data show an incidence of approximately six NTD cases per 1,000 births. Globally, anencephaly and spina bifida are the most common forms of NTDs and affect an estimated 300,000 newborns each year. In the United States, anencephaly occurs in approximately 1 in 4,859 births, while spina bifida occurs in about 1 in 2,858 births.^{4,6}

Many congenital anomalies develop during the early stages of embryogenesis. Around the third week of development, the neural plate forms and folds to create the neural groove. The neural groove then closes to form the neural tube. The neural tube later develops into the central nervous system, including the brain and spinal cord. This stage is highly sensitive to developmental disturbances. Neural tube defects occur when the neural tube does not close properly between days 21 and 28 of embryonic development.⁷ The central nervous system plays an

important role in receiving sensory input, processing information, and coordinating physiological responses. Therefore, disturbances during its early development can affect neurological function and overall health.⁸

Embryonic development depends on both genetic factors and environmental stimuli. During incubation, embryos respond to external conditions such as temperature, humidity, mechanical vibration, and acoustic exposure. In *Gallus gallus*, embryonic day five marks a period of rapid neuroepithelial proliferation and growth of the primary brain vesicles. At this stage, the developing nervous system remains highly plastic and responsive to environmental stimuli.⁹

Recent studies have suggested that acoustic stimulation may influence biological processes through mechanotransduction pathways. In these pathways, mechanical vibrations generated by sound waves are translated into cellular responses. Such stimulation may modulate cellular metabolism, proliferation, and morphogenetic activity during early development. Patterned acoustic stimuli with distinct rhythmic and harmonic properties may therefore differentially influence embryonic growth and neural development.¹⁰

Various forms of auditory stimulation have been explored in previous research. Exposure to classical music in the context of prenatal development, has been associated with potential benefits for fetal brain maturation and cognitive development.¹¹ Meanwhile, rhythmic vocal recitation, such as the recitation of the Qur'an, has been reported in several studies to produce physiological and psychological effects, including reductions in anxiety, stabilization of blood pressure, and improvements in sleep quality across different age groups.¹² Despite these findings, most studies have focused on postnatal populations, and limited research has examined the potential influence of rhythmic vocal stimulation on early embryonic neurodevelopment.

In recent years, experimental evidence comparing different forms of acoustic stimulation during early embryogenesis remains scarce. In particular, little is known about how distinct acoustic patterns, such as rhythmic vocal recitation and harmonic instrumental music, may influence early brain morphogenesis. Therefore, this pilot experimental study aimed to evaluate the effects of two types of acoustic stimulation, namely rhythmic vocal acoustic stimulation and harmonic instrumental acoustic stimulation, on early brain development in chicken embryos (*Gallus gallus*). Morphological observation of brain vesicles was assessed as indicators of embryonic morphogenesis and metabolic activity on embryonic day five.

2. Methods

A pilot experimental study was conducted to evaluate the effect of patterned acoustic stimulation on early embryonic brain development. The study

protocol was reviewed and approved by the Institutional Ethics Committee of the Faculty of Medicine, UIN Syarif Hidayatullah Jakarta (approval number: B-088/F12/KEPK/TL.00/12/2023). The experiment was conducted at the Laboratory of Medical Biology, Faculty of Medicine, UIN Syarif Hidayatullah Jakarta, from September 2023 to January 2024.

Chicken embryos (*Gallus gallus*) were used as the experimental model. Fertilized eggs were obtained from a local poultry farm in Reni Jaya Pamulang, South Tangerang, Banten. Chicken embryos were selected because their development is well characterized, readily observable, and shares fundamental features with embryogenesis in other vertebrates, including humans. This model also allows direct observation of organogenesis within a relatively short incubation period.¹³ Embryonic developmental stages were assessed according to the Hamburger–Hamilton staging system, a widely used reference for avian embryonic development.¹⁴

Twenty fertilized chicken eggs were randomly allocated to two acoustic stimulation groups, with 10 eggs per group. The first group received rhythmic vocal acoustic stimulation through Qur'anic recitation, while the second received harmonic instrumental acoustic stimulation through classical music. Random allocation was used to minimize potential selection bias.

All fertilized eggs were incubated at 37°C for 120 hours (5 days) under the same laboratory conditions. Each egg was manually turned once every 24 hours to support normal embryonic development and reduce adhesion to the eggshell membranes. Standard incubator humidity conditions were used; however, relative humidity was not quantitatively monitored during this pilot study.

Acoustic stimulation was delivered using the same portable Bluetooth speaker (KTV Bluetooth Speaker) for both groups. The speaker was positioned approximately 10 cm from the eggs inside a closed enclosure to minimize variation in sound exposure. The volume was set at approximately 50% of the maximum output and maintained throughout the experiment. Acoustic stimulation was administered for 60 minutes daily from embryonic day 1 to day 5. Outside the stimulation periods, all eggs were maintained under identical incubation conditions. Sound intensity, sound pressure level (SPL), and frequency spectrum were not quantitatively measured. Accordingly, the stimulation procedure was standardized through the use of the same speaker, a fixed speaker position, an identical volume setting, and equal exposure duration across groups. Future studies should incorporate sound level measurements and frequency spectrum analysis to improve acoustic calibration and experimental standardization.

After 120 hours of incubation, the embryos were carefully extracted for examination. Each eggshell

was gently cracked by tapping it against a sharp edge while maintaining its original orientation. The egg contents were then transferred into a Petri dish containing 0.9% sodium chloride (NaCl) solution. The embryos were carefully separated from the surrounding albumen and examined under a stereomicroscope.¹⁵

Morphological assessment focused on the development of brain vesicles. Specimens were photographed using a digital camera for subsequent analysis. The primary outcome was brain vesicle development on embryonic day 5, assessed as an indicator of embryonic brain morphogenesis.

Embryonic development was evaluated on day 5 by stereomicroscopic examination. Based on morphological criteria, embryos were classified into two categories: (1) developing embryos, defined as clearly identifiable embryos with visible primary brain vesicles (prosencephalon, mesencephalon, and rhombencephalon) exhibiting normal morphology appropriate for the developmental stage; and (2) non-developing embryos, defined by the absence of an identifiable embryo, severe underdevelopment, or marked degeneration, including indistinct or collapsed brain vesicles, tissue shrinkage, or failure to form recognizable embryonic structures. To minimize observer bias, specimens were coded before morphological assessment, and the observer was blinded to group allocation during classification.

Categorical developmental outcomes were summarized as frequencies and percentages. Fisher's exact test was used to compare the proportions of

developing and non-developing embryos between the rhythmic vocal and harmonic instrumental groups. The effect size was expressed as the risk difference between groups. Statistical significance was set at $p < 0.05$.

3. Results

Preliminary Study and Optimization of Embryo Incubation

A preliminary experiment was conducted in September 2023 to optimize the incubation procedure for obtaining chicken embryos at specific developmental stages. Initially, twenty fertilized chicken eggs were incubated at a constant temperature of 37°C and allocated into two experimental groups: (1) harmonic instrumental acoustic stimulation (classical music) and (2) rhythmic vocal acoustic stimulation (Qur'anic recitation). During the initial trial, embryos were not consistently identified after incubation. Therefore, the incubation protocol was refined through methodological optimization. Following these improvements, embryos were successfully obtained after continuous incubation at 37°C for approximately 120 hours. Fertilized eggs containing developing embryos exhibited several macroscopic characteristics, including: the appearance of dark spots and reddish vascular streaks visible through the eggshell; and the presence of a developing air cavity, indicating embryonic growth and yolk utilization (Figure 2).

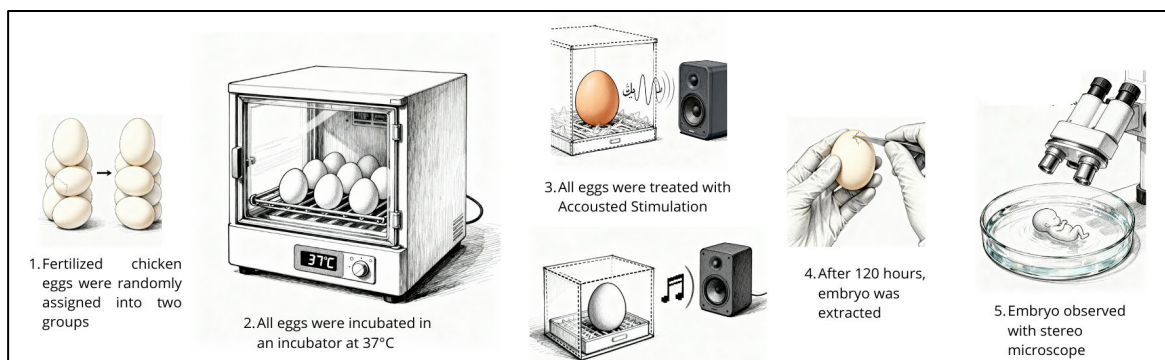


Figure 1. The procedure of this research

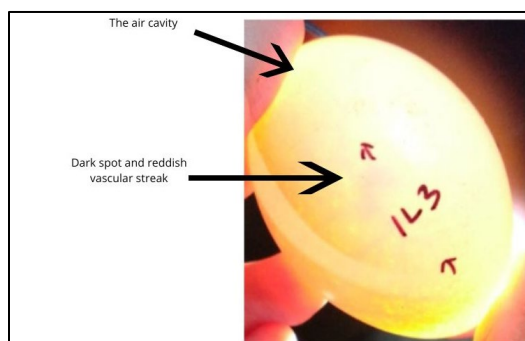


Figure 2. Fertilized eggs characteristic

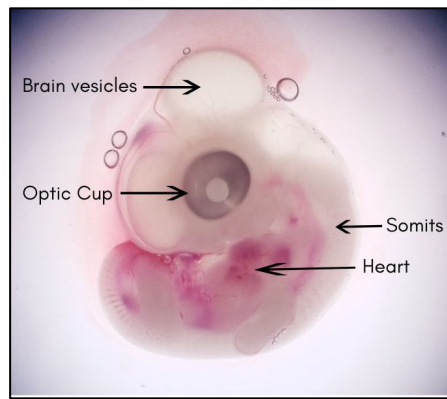


Figure 3. Developing chicken embryo after 120 hours of incubation

Table 1. Statistical comparison of embryonic development between acoustic stimulation groups

Variable	Rhythmic Vocal	Harmonic Instrumental	p-value
Developing Brain Vesicles of Embryos	8	7	
Non- developing Brain Vesicles of Embryos	2	3	p = 0.59

Embryo Morphology During Incubation

Embryos obtained after 120 hours of incubation exhibited several recognizable embryonic structures (Figure 3), including: primary brain vesicles, developing heart, somites, and optic cup. These findings confirmed successful embryonic development to the expected developmental stage.

Embryonic Development Following Acoustic Stimulation

Acoustic stimulation was applied daily from embryonic day 1 until day 5. Twenty fertilized eggs were randomly assigned into two groups (n = 10 each) rhythmic vocal acoustic stimulation and harmonic instrumental acoustic stimulation. Embryonic development was evaluated on day 5 based on the presence of identifiable brain vesicles.

As shown in Table 1, eight embryos (80%) developed successfully in the rhythmic vocal acoustic stimulation group, whereas seven embryos (70%) developed in the harmonic instrumental acoustic stimulation group. Because embryonic development was recorded as a categorical outcome and the sample size was relatively small, Fisher's Exact Test was used to compare the proportions between groups. The statistical analysis demonstrated no significant difference in embryonic development between the two acoustic stimulation groups (p = 0.59).

Microscopic examination revealed qualitative differences between the two treatment groups. Embryos exposed to rhythmic vocal acoustic stimulation generally exhibited more clearly defined primary brain vesicles than embryos exposed to harmonic instrumental acoustic stimulation. The researchers observed three primary brain vesicles: the prosencephalon, mesencephalon, and rhombencephalon. However, they assessed these structures qualitatively without quantitative morphometric measurements.

4. Discussion

In this pilot study, we compared the effects of rhythmic vocal and harmonic instrumental acoustic stimulation on early embryonic development in *Gallus gallus*. Eight of 10 embryos (80%) in the rhythmic vocal group showed identifiable embryonic development, compared with 7 of 10 embryos (70%) in the harmonic instrumental group. This difference was not statistically significant. However, qualitative observation showed that the primary brain vesicles in the rhythmic vocal group tended to be larger and more clearly defined. These findings should be considered preliminary because of the small sample size and the qualitative nature of the morphological assessment.

Previous studies have shown that acoustic stimulation can influence developmental and physiological responses in chicken embryos, although the effects vary according to the type, intensity, timing, and duration of exposure. Kesar (2014) reported that chronic exposure to high-intensity noise at 110 dB from embryonic day 10 until hatching adversely affected body weight, brain weight, and brain size in chick embryos.¹⁶ In another study, prenatal exposure to music or noise at 90 dB from embryonic day 12 until hatching altered physiological stress-related responses in developing chicken embryos.¹⁷ These findings indicate that developing chicken embryos can respond to acoustic stimulation. However, the protocols used in these studies differed substantially from the present study, particularly in terms of developmental stage, sound intensity, duration of exposure, and outcome measures. Therefore, direct comparison should be made with caution.

The absence of a statistically significant difference in the present study may be related to the small sample size and the relatively small difference in developmental outcomes between groups. The

difference was only one developing embryo (80% versus 70%). In addition, the acoustic exposure in this study was not quantitatively calibrated. Sound intensity, sound pressure level, and frequency spectrum were not measured. Therefore, the actual acoustic exposure received by the embryos cannot be precisely determined. These factors may have contributed to the variability in embryonic responses and may have limited the ability to detect a statistical difference. Previous experimental studies have emphasized the importance of standardized sound pressure levels and acoustic exposure conditions when investigating prenatal auditory stimulation in avian embryos.¹⁷

Despite the lack of statistical significance, the qualitative morphological findings provide an initial observation that may be relevant for further investigation. The primary brain vesicles are important early structures in vertebrate brain development, and their morphology changes rapidly during early embryogenesis. The clearer and more distinctly defined brain vesicles observed in the rhythmic vocal group should therefore be regarded as a descriptive, hypothesis-generating observation rather than evidence of a difference in the rate or pattern of brain morphogenesis. However, this interpretation remains descriptive because brain vesicle size was not quantitatively compared between groups in the present analysis.

The findings also suggest that the characteristics of acoustic stimulation may be important. Previous studies have demonstrated that different forms of acoustic stimulation can produce different biological responses in chicken embryos, even when the sound pressure level is comparable. Sanyal et al. (2013) reported differential effects of patterned music and unpatterned noise at the same high sound pressure level on neuronal morphology and volume in several brain regions of developing chicks.¹⁸ Similarly, more recent work has shown that music and noise exposure at the same sound pressure level can produce physiological responses in developing chicken embryos.¹⁷ However, the developmental stages, acoustic intensities, exposure durations, and outcome measures in these studies differed from those used in the present study. Therefore, it remains unclear whether the morphological tendency observed in the present study was related to the rhythmic structure of the vocal stimulation, the harmonic characteristics of the instrumental music, or other acoustic properties. Further studies using standardized acoustic measurements are needed to distinguish these possibilities.

This study has several limitations. First, the sample size was small, with only 10 eggs in each group, which limited the statistical power to detect differences between groups. Second, the researchers assessed brain development mainly through qualitative morphological observations. Third, they did not quantitatively measure sound intensity,

sound pressure level, or frequency spectrum. Fourth, the study did not include histological, biochemical, or molecular analyses. Finally, the study did not include a non-exposed control group. These limitations make it difficult to determine whether the observed findings resulted from acoustic stimulation itself or from differences between the two types of acoustic stimuli.

As a pilot study, these findings provide initial observations for future research. Future studies should use more eggs, include a non-exposed control group, and measure acoustic exposure more consistently. Researchers should also perform quantitative measurements of the primary brain vesicles. Histological and molecular analyses could help determine whether the observed morphological differences relate to changes in neural tissue development. These methods may provide stronger evidence of the biological effects of acoustic stimulation during early embryonic development.

5. Conclusion

Acoustic stimulation did not significantly affect the proportion of developing embryos between the two groups. However, embryos exposed to rhythmic vocal acoustic stimulation showed more clearly developed brain vesicles based on qualitative observations. Further studies with larger sample sizes and histological and biochemical analyses are needed to better understand the potential effects of acoustic stimulation on early embryonic neurodevelopment.

6. Author Contribution

NNF conceived the research idea, designed and supervised the study, conducted the experiments, analyzed and interpreted the data, and wrote the manuscript. SAWRZ and ANU assisted with the laboratory experiments, data collection, and other experimental procedures. All authors reviewed and approved the final manuscript and agreed to its publication.

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