Identification and quantification of growth factors involved in growth and development of cartilage present in exosomes of lactating mother’s milk of cleft infant as compared to non-cleft infant – a protocol for an analytical observational study

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Abstract

Background: Cleft lip and palate (CLP) have complex etiology and even after successful cleft surgeries in a CLP child, there are chances of relapse as there may be a lack of something at the cellular level. Several growth factors like TGF-β, IGF, and BMP7 are involved in modulating chondrocyte homeostasis and can also induce chondrogenic differentiation in the bone marrow.

Objectives: To identify growth factors involved in the growth and development of cartilage present in exosomes of lactating maternal milk of cleft and non-cleft children.

Methodology: There will be two groups: GROUP-A: The lactating mother of a CLP child. GROUP-B: The lactating mother of a non-cleft child. Mothers with a CLP child and a non-cleft child between the age of 0–6 months will be selected for the study. The need for the study will be explained to the mothers and consent will be taken. A milk sample of 2ml will be collected in a falcon tube and transported in cold storage facilities to the laboratory. Initially, the isolation of exosomes from milk samples will be done, from which total proteins will be isolated by centrifugation process. This will be followed by an analysis of growth factors from the isolated total proteins (from both exosomes) through immunoblotting. Once the analysis is done, the quality and quantity of growth factors responsible for cartilage growth and development will be compared.

Expected results: Isolated maternal exosomes are expected to contain an ample amount of growth factors involved in cartilage growth and development, highlighting its potential for use as a therapy alongside prevalent procedures in CLP.
**Conclusion:** In this study, we expect that maternal exosomes act as a carrier of factors that can have therapeutic significance to the natural compensation of the cartilage to maintain nasal symmetry by naso-alveolar molding.

**Keywords**
Exosome, Growth Factors, cleft lip and palate

This article is included in the Datta Meghe Institute of Higher Education and Research collection.

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Introduction
The most prevalent birth abnormality is cleft lip and palate (CLP), which is linked to several inherited genetic diseases that damage the child’s orofacial region. Many variations and combinations of this syndrome can exist. When compared to African populations, Asian and American populations have a far higher prevalence of CLP. A 2009 study found that India, with a population of over 1.1 billion, produces 24.5 million births annually, with a birth prevalence of clefts of between 27,000 and 33,000 per year.1 The incidence of CLP is higher in males than females. This birth defect creates medical, psychological, and social problems which affect both individuals and their families. CLP has a complex etiology with both genetic and environmental factors that act as key factors for the occurrence of CLP.2 The common risk factors leading to CLP are maternal smoking, alcohol consumption, consumption of certain medications such as Topiramate, Valproic acid, etc during the 1st trimester, nutritional deficiency like folic acid deficiency, vitamin A deficiency, low serum folate level, low vitamin B6 and B12 level, chemical exposure like cosmetics, pesticides, psychological factors such as stress, depression, and consanguineous marriage. The genes responsible for non-syndromic CLP are FGFR2, IRF6, FGF8, BMP4, Wnt, etc.2

The anatomy of CLP highlights abnormal shape and position of the nasal septum and nasal tip cartilage which creates an uneven appearance of the nostril, nasal tip, and nasal dome/contour. This leads to poor esthetics, and difficulty in breathing, speech, and feeding. The deviated nasal cartilage is molded and corrected with the help of various presurgical infant orthopaedics (PSIO) appliances to expand the nasal cartilage pre-surgically. The nasal cartilage is a structure present within the nose that provides form and support to the nasal cavity. This cartilage is made up of hyaline cartilage. Since there is no direct blood supply to the cartilage, it is an avascular connective tissue that obtains its nutrition through diffusion from the environment. The process of diffusion is accelerated by increasing the compressive stresses exerted on the cartilage. Our joints are shielded by this sturdy, pliable tissue, which also serves as a shock absorber.

Because cartilage is made of highly differentiated, specialised cells, maintaining the stability of the matrix elements is its primary purpose. Extracellular matrix (ECM), which makes up the structure and organisation of cartilage, is crucial to the proper operation of cartilage. Its ability to self-repair is limited when it gets injured. The cartilage consists of a large number of chondrocytes that are embedded in the ECM. Several growth factors like TGF-β, IGF, and BMP7 are involved in modulating chondrocyte homeostasis and can also induce chondrogenic differentiation in the bone marrow.3

Rationale
Even after successful cleft palate and lip surgeries in CLP children, there are chances of relapse as there may be a lack something on a cellular level. Direct cell-to-cell interaction and the maintenance of homeostasis depend on intercellular communication. Since the last decade, there has been an increased interest in the role of extracellular vesicles, especially exosomes. Recent studies have illustrated that exosomes act as a potential portal for a cell-free drug delivery system with the native characteristic of the parent cell of origin. Exosomes, called extracellular vesicles, are present in almost all cells, tissue and body fluids. It helps in intercellular signaling and maintaining tissue homeostasis in diseased physiology.4 It consists of 9769 proteins, 2838 miRNAs, 3408 mRNAs & 1116 lipids.

These exosomes can act as a key player in treating a cleft patient. It acts as the drug delivery tool as its characteristics are derived from the parent cell. Almost all cells, blood, tissue, saliva, tear, breast milk, urine, and the gastrointestinal tract (GIT) secrete exosomes. Exosomes have the potential to cross the blood-brain barrier.4 Its lipid-bilayer-coated with extracellular vesicles protect against immune cells and enzymes. It can promote cellular growth and the regeneration of new blood vessels.3

Recently many studies have been done which state that exosome contains many factors:

Mengna Duan et al. in 2020 studied the effects of exosomes derived from epidermal stem cells on the rate of wound healing in rat skin. When epidermal stem cells were combined with exosomes, scar formation was found to be reduced and wound healing increased.5 Kan Yin et al. in 2019 found that exosomes derived from mesenchymal cells have many growth factors like TGF-β1, VEGF, HGF, cytokines, and proteins.6

Extracellular vesicles that are secreted are known as exosomes, and they are vital components of cells that also contain growth factors. Exosomes contain signalling growth factors that are responsible for the growth and development of cartilage, and this has therapeutic potential in the management of CLP.

A variety of molecules, including proteins, enzymes, growth hormones, genes, DNA, and RNA, are found in exosomes. Exosomes are believed to include growth factors that are crucial for the growth and development of cartilage, thus
it was expected to evaluate their quantity and quality. It was necessary to determine the elements influencing cartilage development in a patient with CLP since the exosome can express the growth of cartilage in a newborn with CLP.

The following study is one of a kind. It was thereby thought to assess the quality and quantity of growth factors in the mother of a child with CLP and a child with non-cleft and to evaluate the mother’s growth factors, which are genetically passed on to the child and produce a particular type of cartilage with a particular set of properties.

This literature highlights the current conventional treatment regime for CLP management which is not addressing the cellular aspect and especially the cellular signaling for cartilage molding. This is a rate-limiting factor towards CLP management, which may be overcome by providing cartilage growth and development-triggering growth factors.

The following study was thereby designed with a hypothesis to assess the quality and quantity of growth factors in lactating mothers of cleft children and non-cleft children.

Objectives
1. To identify growth factors involved in the growth and development of cartilage present in exosomes of lactating maternal milk of cleft and non-cleft children.
2. To quantify the identified growth factors in the exosomes of lactating maternal milk of cleft and non-cleft children.
3. To compare the expression of the growth factors between the isolated exosomes and the source of the exosomes (milk) in a cleft and non-cleft child.

Methods
Ethical considerations
Ethical approval for the study was obtained from the institutional ethics committee of Datta Meghe Institute of Higher Education and Research (Deemed to be University) (ref. no: DMIHER (DU)/IEC/2023/571) Date of approval: 06-02-2023. A written participant information sheet will be given regarding the details of the study, and it will be explained to participants and their parents before enrolment to the study. Their involvement benefits and harm will be explained to the participants. Written informed consent from the participants will be obtained before involving them in study.

Trial design
This will be a parallel group, analytical observational study.

Study setting
The following study will be conducted in the Department of Orthodontics and Dentofacial Orthopedics at Sharad Pawar Dental College in collaboration with Central Research Laboratory (Center of Translation Sciences), Sawangi, Wardha.

Eligibility criteria
Inclusion criteria
- Lactating mothers of a child aged 0–6 months.
- Age of mother <35 years.
- No systemic conditions such as diabetes, hypertension, etc.
- Mother with no developmental and congenital disease.

Exclusion criteria
- Lactating Mother of child age > 6 months.
- Age of mother >35 years (due to hormonal change).
• Mother with systemic disease such as diabetes, hypertension.

• Mother with developmental and congenital disease.

**Intervention**

Lactating mothers reporting in the Department of Orthodontics and Dentofacial Orthopedics and Department of Gynecology & Obstetrics fulfilling the inclusion criteria will be included in the study.

The study will include a total sample size of 30. There will be two groups with 15 patients in each group:

GROUP-A : The lactating mother of the CLP child.

GROUP-B : The lactating mother of a non-cleft child

The patient will be selected from the smile train outpatient department (OPD) of the Orthodontic Department, while the non-cleft patient will be selected from the Department of Gynaecology. Mothers with a CLP child and a non-cleft child between the age of 0–6 months will be selected for the study. The purpose of the study will be explained to mothers, and those who are willing to participate will be given the consent form in a language they can understand, after which their signature will be obtained. The milk sample from the mother with a CLP child will be collected in the breastfeeding room of the smile train unit in the Department of Orthodontic and Dentofacial Orthopaedic, Sharad Pawar Dental College, and from the mother with the non-cleft child it will be collected from the Department of Gynecology. The milk sample of 2 ml will be collected in a falcon tube and transported with cold storage facilities to a laboratory in R&D house (DMIHER) for evaluation. It will be stored at a temperature of -80°C until the experiment is performed. Initially, the isolation of exosomes from the milk sample will be done from which total proteins will be isolated under the centrifugation process. This will be followed by an analysis of growth factors from the isolated total proteins (from both exosomes) through immunoblotting. Once the analysis is done, the quality and quantity of growth factors responsible for cartilage growth and development will be compared for both groups.

Immunoblotting is a technique in which we use host antibodies to identify a target protein via antigen-antibody reaction as it identifies the target protein among the number of unrelated proteins. Proteins are separated by electrophoresis and transferred onto the nitrocellulose membrane. This technique uses three elements which are:

1. Separation by size
2. Transfer to the solid support
3. Marking of a target protein using a primary and secondary antibody to visualize

**Outcomes**

*Primary outcome*

The proposed study is intended to highlight the therapeutic potential of maternal exosomes which can be genetically identified for their quality and quantity. Results will be highlighting the growth factors in infants with CLP. Likewise, further modalities for the management of the nasal cartilage can be done. In CLP subjects, the availability of maternal growth factors is limiting owing to its deficiency.

*Secondary outcome*

Because of this, isolated maternal exosomes are expected to contain ample growth factors involved in cartilage growth and development, highlighting its potential for use as a therapy alongside prevalent procedures in CLP.

**Sample size**

The sample size was calculated by using Daniel’s formula for sample size:

\[
 n = \frac{Z_{\alpha/2}^2 \times P(1-P)}{d^2}
\]
Where,

\[ Z_{0.02} = 1.96 \] is the level of significance at 5% i.e. 95% confidence interval = 1.96

\[ P = \text{Prevalence of cleft lip and palate} = 1\% = 0.01 \]

\[ d = \text{Desired error of margin} = 6\% = 0.06 \]

\[ n = \frac{1.96^2 \times 0.01 \times (1 - 0.01)}{0.06^2} = 10.56 \]

\( n \) is the population size

Total sample size is 30

There is a total two groups which means 15 patients in each group.

**Statistical method**

All the demographic and outcome data will be presented using descriptive statistics for continuous variables can be categorized using mean, standard deviation, and median for discrete variables, and frequency and proportion for continuous variables.

The outcome variable will be tested for normality using the Kolmogorov-Smirnov test for continuous data. Results will be analysed using SPSS version 27.0.

Growth factors will be categorized according to the range that will be distributed for analysing the data into the normal range and not in the normal range. The chi-squared test will be used for finding the result of the association of growth factors with milk from the mother of cleft and non-cleft infants.

An odds ratio will be used to find the risk involved multiple times.

An independent t-test will be used to find the results of the two groups for outcome variables if data comes under the normal distribution, or a non-parametric test will be used to find the significant difference if data doesn’t come under the normal distribution.

**Dissemination**

In this study, we expect that maternal exosomes act as carriers of factors that can have therapeutic significance to the natural compensation of the cartilage to maintain nasal symmetry by naso-alveolar molding. Because of growth factors, which in the first 2–3 months help/enhance cartilage development and growth. However, due to a lack of these growth factors in children, this period declines to 1 month, which can result in the failure of many mechanical appliances like stunts in the 3- to 4-month range getting no results due to a lack of certain factors.

Identifying the factors like growth factors, estrogen or hormones can enhance the growth and development of cartilage and, by highlighting their therapeutic significance in CLP management as isolated maternal exosomes are expected to contain ample amount of growth factors involved in cartilage growth and development, highlighting its potential for use as a therapy alongside prevalent procedures in CLP.

**Study status**

Not started yet.

**Discussion**

Considering the complexity of naso-alveolar cartilage molding, the management of CLP is a great challenge and time-factor is very crucial. According to the literature, the initial 2–3 months of a child’s life is a crucial time for cartilage molding but children with CLP are deprived of important nutrients present in their mother’s milk. Certain developed countries provide fortified milk to CLP children which provides important nutrients necessary for the growth and development of cartilage, but developing countries like India lack that as 90% of the population there provide animal (cow/buffalo/goat) derived milk which has fewer nutrients compared to mother’s milk or fortified milk.
The importance of the transforming growth factor-β family and the potential for treating osteoarthritis with stem cell-derived exosomes were concluded by Kwang Ho Yoo et al. in their study published in 2022. TGF-β plays an important role in many cellular processes, including cellular proliferation, and the role of exosomes in chondroprotection has also been established. In 2015, Qiongqiong Yu et al. investigated the role of the BMP7 gene in nonsyndromic orofacial clefts. Bmp7 plays an important role in the development of the palate and other orofacial structures. Bmp7 promotes cartilage matrix synthesis and inhibits catabolic cytokine activity. In 2022, Nathlic G Thielen et al. evaluated the transcription factor responsible for the TGF-β-driven hypertrophy-like phenotype in human osteoarthritic chondrocytes. TGF-β induces the expression of a critical hypertrophy factor in chondrocytes. In 2021, Mengmeng Duaner et al. studied the effect of TGF-β on cartilage development and disease. TGF-β2 controls all aspects of endochondral ossification and upholds homeostasis.

**Conclusion**

In this study, we expect that maternal exosomes act as carriers of factors that can have therapeutic significance to the natural compensation of the cartilage in order to maintain the nasal symmetry by naso-alveolar moulding.

**Data availability**

No data are associated with this article.

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**References**


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