Int. J. Morphol.,
42(5):1200-1207, 2024.

The Impact of Gestational Alcohol on the Trabecular Bone Structure of the Mandible in 3-Week-Old Sprague Dawley Rats

Impacto del Alcohol Gestacional en la Estructura Ósea Trabecular de la Mandíbula en Ratas Sprague Dawley de 3 Semanas de Edad

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THOBANE, N.; NDOU, R. & PILLAY, D. The impact of gestational alcohol on the trabecular bone structure of the mandible in 3-week-old Sprague Dawley rats. *Int. J. Morphol., 42(5)*:1200-1207, 2024.

SUMMARY: Drinking alcohol while pregnant can cause several diseases, belonging to an umbrella of disorders known as Fetal Alcohol spectrum disorders. Alcohol exposure during pregnancy is linked to reduced birth weights, shorter bones, delayed bone growth, decreased bone resilience, and delayed ossification of the bones. However, the specific effects on the craniofacial bones, particularly the mandible, remain less explored. This study aims to fill this gap by examining the impact of gestational alcohol consumption on mandibular structure. Time-mated pregnant Sprague Dawley dams were divided into an ethanol group (n=6) and a saline control group (n=6). The ethanol group was given 0.015ml/g of 25.2% ethanol, while the control group received 0.9% saline for the first 19 days of gestation. The presence of a vaginal plug indicated the first day of gestation. After birth, the pups were euthanized at three weeks of age, and their mandibles were harvested and analyzed using a 3D-mCT scanner to assess bone ratio, trabecular thickness, number, and spacing. Osteometric measurements for mandibular length and height were also taken. The study found no significant differences in trabecular morphometric parameters between the groups. However, one parameter for mandibular length (Cd-Bi) and two parameters for mandibular height (Cd-Ag and Cr-Ag) were significantly smaller in the ethanol group than in the controls. The study reveals that gestational alcohol consumption can significantly affect the development of the mandible, with potential implications for understanding the mechanisms behind alcohol-induced teratogenicity. The findings highlight the need for further research to explore the intricate interactions between embryological, biological, genetic, and epigenetic factors that contribute to the varied effects of alcohol exposure during pregnancy on different skeletal systems.

KEY WORDS: Gestational alcohol consumption; Mandibular length; Height; Trabecular bone.

INTRODUCTION

The prevalence of alcohol consumption during pregnancy in South Africa is reported to be 3.7% percent (Peltzer & Pengpid, 2019). However, this is excessively higher on the African continent, with an overall prevalence of alcohol use during pregnancy recorded at 22.8% in sub-Saharan Africa (Mulat *et al*., 2022). Alcohol consumption during pregnancy is associated with a series of developmental abnormalities. These include neurodevelopmental impairments (Wilhelm & Guzzetti, 2015), growth retardation (Simpson *et al*., 2005), and craniofacial disorders (Shen *et al*., 2013; Popova *et al*., 2016). These abnormalities can have a long-term impact on individuals' overall health and well-being (Simmons *et al*., 2023).

Alcohol consumption by mothers during pregnancy doubles the risk of bone fractures before the age of eight (Parviainen *et al*., 2020). This could be attributed to the low bone mineral density reported among children and adolescents with confirmed prenatal alcohol exposure (Young *et al*., 2022). Mineral bone density and trabecular structure influence bone strength (Mosekilde *et al*., 2000). In long bones, the trabecular morphometric parameters are affected by exposure to alcohol during gestation. This is evidenced by thinner trabeculae in the tibia (Ndou *et al*., 2023) and fewer trabecular reported in the femur (Pillay & Ndou, 2021). There is a lack of research on the structure of the mandible in postnatal life after gestational alcohol exposure. Therefore, it is difficult to compare mandibular trabecular morphology with previous studies on long bone postnatal development among offspring of mothers who drink alcohol while pregnant.

Although craniofacial disorders are common among offspring of mothers who drink alcohol during pregnancy,

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Received: 2024-06-03 Accepted: 2024-06-23

there is a lack of studies on the facial bones, particularly, the mandible. The mandible is a component of the craniofacial complex and may be vulnerable to the effects of alcohol exposure during gestation as demonstrated by micrognathia among children with fetal alcohol spectrum disorders (Shen *et al*., 2013). This is a feature of reduced mandibular dimensions. Furthermore, most existing studies in the scientific literature report on the effects of intrauterine alcohol exposure in rodents during the gestation period and among newborn rats. Fewer studies focus on the postnatal effects of gestational alcohol on bone.

Any amount of alcohol intake during pregnancy is considered detrimental to fetal development as there is no established safe amount of alcohol consumption by pregnant mothers (Armstrong *et al*., 2017). Regarding skeletal effects, other researchers have shown that prenatal exposure to alcohol affects the development of skeletal elements and fetal growth irrespective of the level of maternal consumption (Snow & Kiever, 2007). The effect of prenatal ethanol exposure on the development of the fetal skeleton may contribute to the risk of osteoporosis in the offspring postnatally (Snow & Kiever, 2007).

While it is well known that gestational alcohol exposure alters craniofacial features in the fetus and at birth due to delayed bone growth and development (Shen *et al*., 2013), it remains debatable whether the effects of gestational exposure on craniofacial bones persist in postnatal life. This is particularly important in a binge drinking setting as it has been established that binge drinking is a common problem in South Africa with a prevalence of 35% in women (Johnson *et al*., 2023). Therefore, the purpose of the current study was to determine whether gestational alcohol exposure affects the postnatal development of the mandible in 3-week-old Sprague Dawley rats in a binge maternal drinking simulation. The present study used 3-week-old rats that were exposed to alcohol during the gestation period, unlike other studies that focused on newborn rats (Sampson *et al*., 1996; Snow & Keiver, 2007).

MATERIAL AND METHOD

The research protocol involving animal experimentation was approved by the Animal Ethics Committee, University of Witwatersrand (AESC 2015/27/15C). The study comprised 12 time-mated Sprague Dawley (SD) rats from the Central Animal Services (CAS), University of Witwatersrand. The animals were kept in a controlled environment and provided with standard care.

Group allocation and Micro CT. Time-mated pregnant Sprague Dawley dams (n=12) were randomly assigned to ethanol (n=6) and saline control (n=6) groups. The ethanol group received 0.015ml/g of 25.2% ethanol and the saline control group received 0.9% saline for the first 19 days of gestation. The pups were euthanized at 21 days of age, and 2 pups were collected from each dam, resulting in 12 ethanol and 12 saline control pups (Fig. 1). The pups' mandibles were harvested for osteometric parameters (Figs. 2 & 3) (Table I & Table II) trabeculae morphometry based on the scanning parameters outlined in Table III.

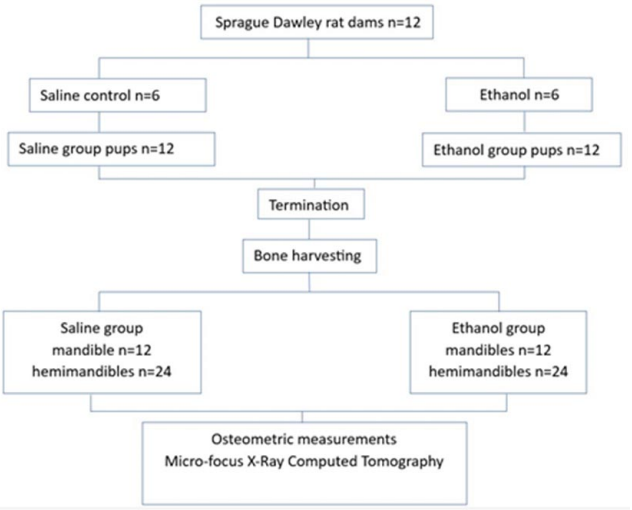

Fig. 1. Study design.

Mandibular osteometry. The measurements of linear mandibular length and linear mandibular height were taken using a digital vernier caliper (to the nearest 0.05mm), according to the method of Maki *et al*. (2002). Each measurement was taken 3 times, and the average (mean) was determined to represent the value of each osteometric parameter (Figs. 2 & 3) (Tables I & II).

Parts of the mandible studied. The trabecular and cortical bones of the mandible were evaluated using V-G studios. The assessment focused on the trabecular bone located between the bodies of molars 1 and 2 (Fig. 4a). The cortical thickness and cross-sectional area of the bone were measured on the same slice, between molar 1 and 2, specifically along the labial surfaces of the bone (Fig. 4b).

Trabecular parameters to be studied. Following reconstruction, V-G studio was used for data processing. The mandible's trabecular morphometric characteristics were investigated (Table II). The ratio of bone volume to total volume (BV/TV), trabecular thickness (TbTh), trabecular number (TbN), and trabecular spacing (TbSp) were measured as shown in (Table II). A cross-sectional slice from each mandible was saved for further analysis on V-G studio. From these slices, the cross-sectional area of the mandible was taken.

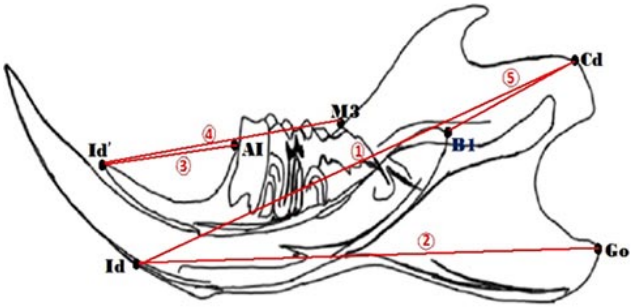

Fig. 2. Reference points for measurements of rat linear mandibular length. Bi indicates the alveolar base of the lower incisor; ¿Cd-Id, indicates the mandibular length; ¡Go-Id, indicates the mandibular body length; ¬Al-Id', indicates the length of the lingual side of the alveolar bone; √Id'-M3, indicates the length of the lower dental arch; and ƒCd-Bi, indicates the distance from the condylar head to the alveolar base of the lower incisor. Adapted from Makai *et al.* (2002).

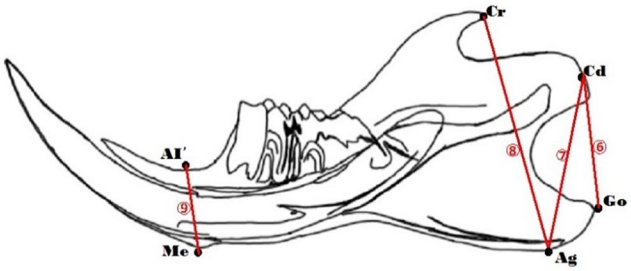

Fig. 3. Reference points for measurements of rat linear mandibular height. ≈Cd-Go indicates mandibular ramus height; ΔCd-Ag, indicates the height of the condylar head; «Cr-Ag, indicates the coronoid process height; and »AI'-Me, indicates the height of the central portion of the alveolar bone. Adapted from Makai *et al.* (2002).

Table I. Measurements of the length of the rat mandible (Maki *et al.*, 2002)

Measurement	Reference points	Explanation	Anatomical landmark
Mandibular length	Cd-Id	The distance from the posterior point of the condyle (Cd) to the infradentale (Id) on the labial side was measured from point Cd to Id.	Cd-Condyle Id-Infradentale on the labial side of the rat mandible.
Mandibular body length	Go-Id	The distance between the gonion (Go) and the infradentale (Id) on the labial side was measured from point Go to Id.	Go-Gonion is the point or apex on the angle of the mandible. Id-is the Infradentale on the labial side of the rat mandible.
Length of the lingual side of the alveolar bone	AI-Id'	The distance between the highest point of the mesial alveolar (Al) at the lower margin of the first molar and the infradentale (Id') on the lingual side was measured from point Al to Id'.	Al-mesial alveolar at lower margin of the first molar. Id'-Infradentale on the lingual side of the rat mandible.
Length of the lower dental arch	Id'-M3	The distance between the infradentale (Id') on the lingual side and the highest point of the central cusp of the lower third molar (M3) were measured from point Id' to M3.	Id'-Infradentale on the lingual side of the rat mandible. M3-the highest point of the central cusp, of the lower 3rd molar.
Distance from the condylar head to the alveolar base of the lower incisor.	Cd-Bi	The distance from the condyle (Cd) to the alveolar base of the lower incisor (Bi) was measured from point Cd to Bi.	Cd-Condyle Bi–base of the lower incisor.

Table II. Measurements of the height of the rat mandible (Maki *et al.*, 2002).

Measurement	Reference points	Explanation	Anatomical landmark
Mandibular ramus height	Cd-Go	Distance between the posterior point of the condyle (Cd) and gonion (Co) was measured from point Cd to Co.	Cd-Condyle. Go-Gonion.
Height of condylar head	Cd-Ag	Distance between posterior point of condyle (Cd) and ante-gonion (Ag) was measured from point Cd to Ag.	Cd-Condyle Ag-Ante-gonion.
Coronoid process height	Cr-Ag	Distance between posterior point of coronoid process (Cr) and ante-gonion (Ag) was measured from point Cr to Ag.	Cr-Coronoid process. Ag-Ante-gonion.
Height of the central portion of the alveolar bone	AI'-Me	Distance between deepest point of outer margin of bone (Al') that connects menton (Me) and ante-gonion was measured from point Al' to Me.	Al'-Deepest point of the outer margin of bone. Me-Menton, which is the lowest most inferior point of mandibular symphysis.

Table III. Scanning parameters.

Parameter	Value
X-ray voltage	70kv
X-ray	400µa
Filter	1mm aluminium
Scanning resolution	15µm
Tomographic rotation	360°
Rotation step	1°
Frame averaging	4
Scan duration	8 minutes

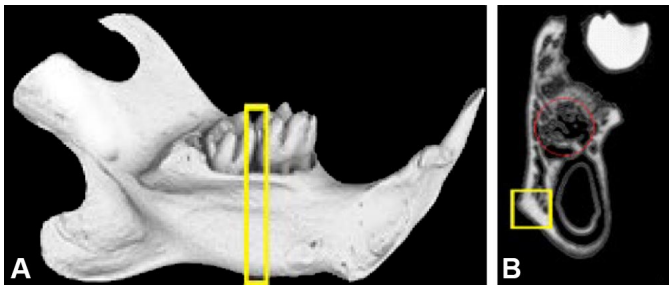

Fig. 4. Region of interest (ROI). a) Shows the selected ROI found between molar 1(M1) and molar 2(M2); b) Shows the trabecular bone found between molar 1(M1) and molar 2(M2) as well as the cortical bone found on the labial side, the manually drawn ROI is represented with the circle and square shapes. Courtesy of N Thobane.

Table IV. Trabecular parameters.

Variable	Description	unit
BV/TV	The volume of the material (bone) to the total volume.	mm^3
Trabecular number (TbN)	Average number of trabeculae per unit length	1/mm
Trabecular thickness (TbTh)	Mean thickness of trabeculae	mm
Trabecular separation (TbSp)	Mean distance between trabeculae	mm

RESULTS

Osteometry of the mandible

Length measurement. There were no significant differences between the ethanol and saline control groups for the following length measurements Cd-Id, which indicates the mandibular length, Go-Id, which indicates mandibular body length, AI-Id, which means the lingual side of the alveolar bone length or M3-Id which indicates the length of the lower dental arch. Conversely, the Cd-Bi, which indicates the length of the lower incisor, was significantly higher in the ethanol group than in the saline control (p = 0,038) (Table V).

Table V. Linear measurements of the SD rat mandibular length.

Measurements	Treatment Groups	Mean	Standard deviation	P-value
Cd-Id	Ethanol	17,64	0,75	0,11
	Saline	17,29	0,48	
Go-Id	Ethanol	16,55	0,78	0,35
	Saline	16,35	0,43	
Al-Id'	Ethanol	6,051	0,26	0,67
	Saline	6,09	0,31	
M3-Id'	Ethanol	11,95	0,48	
	Saline	11,83	0,55	0,5
Cd-Bi	Ethanol	5,62	0,56	0,038
	Saline	5,30	0,27	
	Saline	3,29	0,17	

Height measurement. In Table VI, the Cd-Go, which indicates the mandibular ramus height, was similar in both groups. Conversely, the Cd-Ag, which means the height of the condylar process, was significantly larger in the ethanol group than in the saline control group. Also, the Cr-Ag, which indicates the height of the coronoid process, was significantly larger in the Ethanol group than the saline control group. AI'-Me', which means the height of the central portion of the alveolar bone showed no significant differences between the ethanol group and the saline control.

Table VI. Linear measurements of the SD rat mandibular height.

Measurements	Treatment Groups	Mean	SD	P-value
Cd-Go	Ethanol	4,56	0,18	
	Saline	4,47	0,22	0,201
Cd-Ag	Ethanol	6,89	0,33	
	Saline	6,58	0,28	0,006
Cr-Ag	Ethanol	8,59	0,41	
	Saline	8,10	0,28	≤0,001
Al'-Me	Ethanol	3,38	0,18	
	Saline	3,29	0,17	0,113

Trabecular morphometry and cortical area

Mandible bone-to-total volume ratio (BV/TV): The bone-to-total volume ratio (BV/TV) was marginally lower in the ethanol group (mean= 30.14% ± 0,069) than in the saline controls (mean= 32, 37% ± 0,089). However, no significance was detected between the groups (p=0.411) (Figs. 5A & 6A-B).

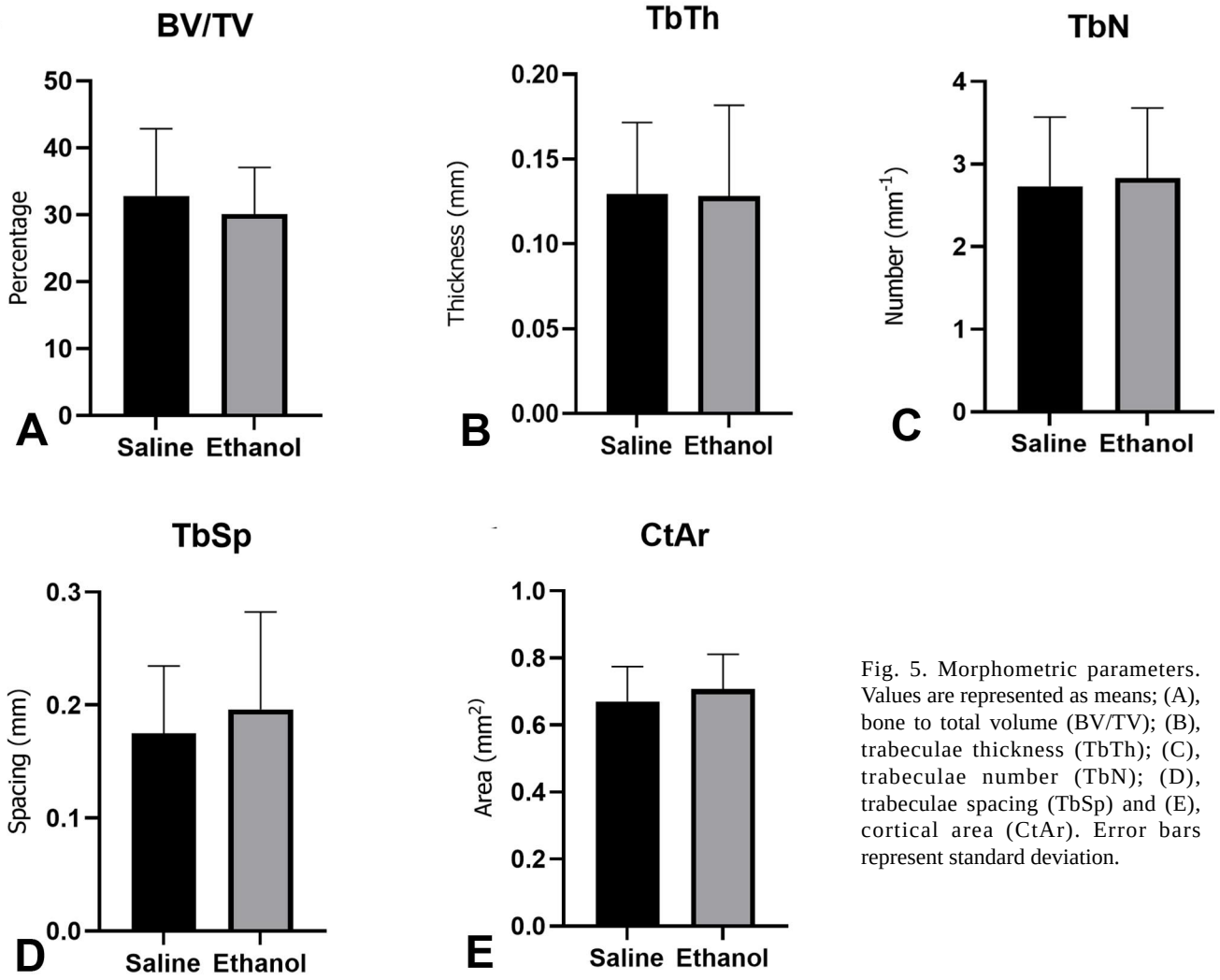

Fig. 5. Morphometric parameters. Values are represented as means; (A), bone to total volume (BV/TV); (B), trabeculae thickness (TbTh); (C), trabeculae number (TbN); (D), trabeculae spacing (TbSp) and (E), cortical area (CtAr). Error bars represent standard deviation.

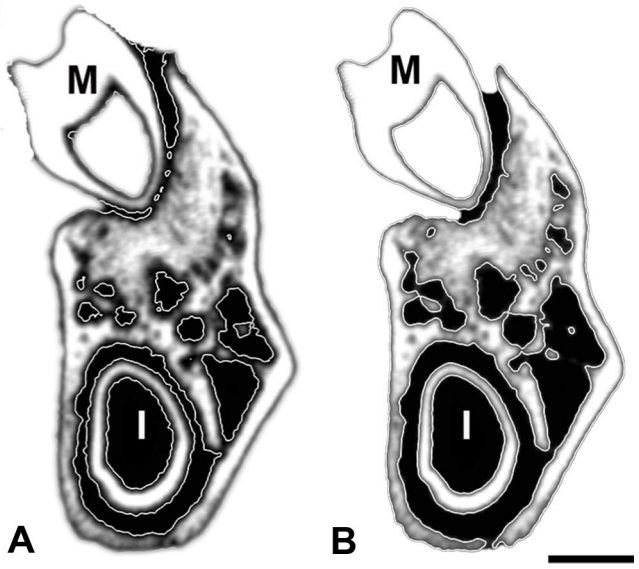

Fig. 6. Trabecular morphology. Representative slices of (A), saline control mandible showing trabeculae and spacing, and (B), the ethanol group showing similar trabeculae and spacing as control. The scale bar represents 1mm. M-Molar; I-Incisor

Mandible trabecular thickness (TbTh): The Trabecular thickness (TbTh) was similar between the ethanol group (mean= 0,128 ± 0,053) and the saline control (mean= 0,129 ± 0,986) (p= 0,942) (Figs 5B & 6 A-B).

Mandible trabecular number (TbN): Concerning the trabecular number (TbN) a similar pattern was observed between the ethanol (mean= 2,830 ± 0,845) and the saline groups (mean= 2,731 ± 0,836) (p= 0,725) (Figs. 5C & 6A-B).

Mandible trabecular spacing (TbSp): Concerning, trabecular spacing (TbSp), marginally wider trabecular were observed in the ethanol group (mean= 0,196± 0,086) than in the saline control (mean= 0,175 ± 0,059). However, no significance was detected (p= 0,896 (Figs. 5D & 6 A-B).

Mandible cortical area (CtAr): Similar observations were shown concerning the cortical area in the ethanol (mean= 0,707 ± 0,103) and saline groups (mean= 0,67± 0,104) (Figs. 5E & 6A-B). No significance was detected (p= 0,292).

DISCUSSION

The purpose of the current study was to determine whether gestational alcohol exposure affects the postnatal development of the mandible in 3-week-old Sprague Dawley rats. It is well known that gestational alcohol exposure alters craniofacial features due to delayed bone growth and development (Shen *et al*., 2013). However, it is debatable whether the effects of gestational exposure on bone are permanent or that catch-up development may occur in postnatal life. Therefore, the present study used 3-week-old rats that were exposed to alcohol during the gestation period, unlike other studies that focused on newborn rats (Sampson *et al*., 1996; Snow & Keiver, 2007).

The study used a model of binge drinking. Binge drinking is defined as having 4-5 glasses of alcohol or taking 20 ml of 40% whisky diluted in 200 mL per glass 4-5 times in quick succession (Sokol *et al*., 2003) . This is an appropriate model in the South African context, as it mimics the large number of women who secretly drink to avoid detection by spouses and other family members or members of society (Conover & Jones, 2012; Watt *et al*., 2014). Women may be unaware of their pregnancy in the first 4 weeks, and this may expose the embryo to alcohol.

This study found that one parameter for mandibular length (Cd-Bi) and two parameters used as a proxy for mandibular height (Cd-Ag and Cr-Ag) were significantly smaller in the ethanol group than in the controls. Since fetal alcohol syndrome affects the craniofacial morphology, this result is consistent with that phenomenon (Shen *et al*., 2013). It was expected for more of these mandibular length and height variables to show group differences as previous studies have reported long bones with significantly shorter bones in the ethanol group (Pillay & Ndou, 2021; Ndou *et al*., 2023). The dosage of alcohol in our study which was lower compared to previous studies may have played a role in the findings of the study. Also, in the present study, alcohol was given once daily through oral gavage to mimic binge drinking unlike providing ethanol in drinking water. This binge drinking model may enable alcohol clearance from the bloodstream, providing many hours of low blood alcohol levels than if ethanol was provided in drinking water.

Also, it is important to highlight the unique embryological origin and developmental processes of the mandible compared to long bones. The mandible, which forms the lower jaw, develops through endochondral ossification and intramembranous ossification, a process where bone tissue is formed directly within a membranous connective tissue, without the presence of a cartilage precursor (Amano *et al*., 2010). Endochondral ossification is when a cartilage model is first formed and subsequently replaced by bone tissue (Lee & Leichter, 1980). Long bones are known only to undergo endochondral ossification) (Amano *et al*., 2010). Distinction in the mode of bone development may partly explain why the mandible is less affected by gestational alcohol exposure. The timing and specific cellular processes involved in mandible development may render it less vulnerable to the detrimental effects of alcohol. Therefore, the findings of this study raise a new question as to whether gestational alcohol affects intramembranous ossification differently from endochondral ossification that occurs in long bones regarding bone size. This remains an area that requires further research.

Another crucial factor to consider is the differential susceptibility in different tissues and cell types to alcohol-induced damage. Ethanol readily crosses the placenta, exposing the developing fetus to its toxic effects (Salihu *et al*., 2011). Various mechanisms have been proposed to explain the teratogenic effects of alcohol, including oxidative stress, disruption of cell signaling pathways, and interference with cellular migration and differentiation (Chung *et al*., 2021). Long bones undergo active cell proliferation, differentiation, and cartilage-to-bone transformation during gestation and early postnatal life (Roselló-Díez & Joyner, 2015), making them more susceptible to alcohol-induced interference in these processes. In contrast, the mandible completes most of its development postnatally and experiences limited prenatal growth (Lui *et al*., 2011), potentially making it less vulnerable to the disruptive effects of alcohol exposure. In our study the alcohol was stopped at 19 days of gestation and the alcohol was then withdrawn

from the study groups. It is possible that withdrawing the insult to the offspring could have mitigated the effects seen in the mandible when exposed to alcohol.

Previous studies on long bone have shown that prenatal alcohol exposure affects trabecular morphometry (Pillay & Ndou, 2021; Ndou *et al*., 2023). However, none of the trabecular morphometric parameters in the mandible exhibited significant differences between the groups when the Mann-Whitney test was conducted. However, BV/TV had a lower value in the ethanol group than the controls although not significantly different using the Mann-Whitney test. Additionally, the preservation of trabecular morphometry in the mandible despite gestational alcohol exposure may be attributable to functional loading, the differences in embryological development, and the timing and intensity of alcohol exposure. Nonetheless, further research is needed to understand the underlying mechanisms and confirm the observed phenomenon.

Additionally, the differential impact of gestational alcohol exposure on long bones and the mandible may be influenced by genetic and epigenetic factors which this study did not explore. Genetic variations might modulate teratogenicity, potentially affecting the extent of skeletal anomalies observed. Epigenetic mechanisms, including DNA methylation and histone modifications, can also regulate gene expression patterns and might contribute to tissue-specific vulnerability to alcohol exposure. Differences in the epigenetic regulation of genes involved in mandible development compared to long bone development could potentially account for the disparate effects observed.

CONCLUSION

The differential effects of gestation alcohol exposure on long bones and the mandible likely result from a complex interplay of embryological, cellular, genetic, and epigenetic factors. Exploring these factors further through research may provide valuable insights into the mechanisms of alcohol-induced teratogenicity and shed light on the specific vulnerabilities and resilience of different skeletal structures to prenatal alcohol exposure.

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RESUMEN: Beber alcohol durante el embarazo puede causar varias enfermedades, pertenecientes a un conjunto de trastornos conocidos como trastornos del espectro alcohólico fetal. La exposición al alcohol durante el embarazo está relacionada con un peso reducido al nacer, huesos más cortos, retraso en el crecimiento óseo, disminución de la resistencia ósea y retraso en la osificación de los huesos. Sin embargo, los efectos específicos sobre los huesos craneofaciales, particularmente la mandíbula, siguen siendo menos analizados. Este estudio tiene como objetivo llenar este vacío examinando el impacto del consumo de alcohol gestacional en la estructura mandibular. Las madres preñadas Sprague Dawley apareadas en el tiempo, se dividieron en un grupo de etanol (n=6) y un grupo control de solución salina (n=6). El grupo de etanol recibió 0,015 ml/g de etanol al 25,2 %, mientras que el grupo control recibió solución salina al 0,9 % durante los primeros 19 días de gestación. La presencia de un tapón vaginal indicó el primer día de gestación. Después del nacimiento, las crías fueron sacrificadas a las tres semanas de edad, y sus mandíbulas fueron extraídas y analizadas utilizando un escáner 3D-mCT para evaluar la proporción ósea, el grosor trabecular, el número y el espaciado. También se tomaron medidas osteométricas de longitud y altura mandibular. El estudio no encontró diferencias significativas en los parámetros morfométricos trabeculares entre los grupos. Sin embargo, un parámetro para la longitud mandibular (Cd-Bi) y dos parámetros para la altura mandibular (Cd-Ag y Cr-Ag) fueron significativamente menores en el grupo de etanol que en los controles. El estudio revela que el consumo de alcohol gestacional puede afectar significativamente el desarrollo de la mandíbula, con implicaciones potenciales para comprender los mecanismos detrás de la teratogenicidad inducida por el alcohol. Los hallazgos resaltan la necesidad de realizar más investigaciones para explorar las intrincadas interacciones entre factores embriológicos, biológicos, genéticos y epigenéticos que contribuyen a los diversos efectos de la exposición al alcohol durante el embarazo en diferentes sistemas esqueléticos.

PALABRAS CLAVE: Consumo de alcohol gestacional; Longitud mandibular; Altura; Hueso trabecular.

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