Original Research Article
IMMUNOHISTOCHEMICAL ANALYSIS OF TUMOR SUPPRESSOR p53 IN p-Dimethylamino benzaldehyde (DMBA) INDUCED HEPATOCARCINOGENESIS IN RATS (Rattus norvegicus L.) MALE WISTAR STRAINS

ABSTRACT

Aims: Hepatocellular carcinoma accounts for approximately 90% of all primary liver malignancies and poses a major global health challenge. The initiation of hepatocellular carcinoma (HCC) is marked by a progressive accumulation of molecular alterations, the origins of which remain largely unclear. This study provides an extensive immunohistochemical analysis of hepatitis, cirrhosis, and HCC using a p-Dimethylamino Benzaldehyde (DMBA)-induced HCC rats model.p-Dimethylamino benzaldehyde (DMBA) is a common environmental polycyclic aromatic hydrocarbon, acting as a mutagen, capable of exerting carcinogenic effects, which can be tested both in cell culture and in vivo animal models.
Study design: This study used an in vivo laboratory test method
Place and Duration of Study: Sample: Department of Pharmacology and Therapy and Department of Histology, Faculty of Medicine, Udayana University, between December 2023to July 2024.
Methodology: This study used an *invivo* laboratory test method. The laboratory test was in the form of a study on experimental animals, Wistar rats induced by *p -Dimethylamino Benzaldehyde* (DMBA) *treatment* . Observation of *Hepatocellular Carcinoma* in rat liver tissue samples with immunohistochemical tests of expression *Tumor Suppressor* p53 in tumor and non-tumor hepatocytes.
Results: The results of the hypothesis test study on tumor diameter and immunohistochemical test on the expression of Tumor Suppressor p53 with ROC analysis showed that the cut off tumor diameter obtained a cutoff value of 0.5 with a sensitivity value of 1.0 and a specificity value of 1.0. The results of the ROC analysis showed that the H-score data of wild-type p53 Immunohistochemistry contained 26 samples with 7 positive HCC results and 19 negative samples.
Conclusion: This study shows that in male Wistar rats p-Dimethylamino benzaldehyde (DMBA) - induced hepatocarcinogenesis can be a viable model for studying Hepatocellular Carcinoma in the future.

Keywords:Hepatocarcinogenesis, DMBA, Tumor Suppressor p53

1. INTRODUCTION

Hepatocellular carcinoma is the fifth most common type of cancer worldwide and is a leading cause of cancer-related deaths globally (1) . Hepatocellular carcinoma constitutes 90% of all primary liver malignancies and poses a major global health challenge (2) . The overall 5-year survival of HCC patients is currently 10–20% (3) . Hepatitis B virus (HBV) infection is a predominant risk factor for hepatocellular carcinoma (HCC), responsible for approximately 60% of cases in Asia and Africa and 20% in Western regions (4) . In Western countries and Japan, hepatitis C virus (HCV) infection has traditionally been the primary cause of HCC (4) . However, the incidence of HCV-related HCC has decreased due to the increasing number of patients achieving sustained virologic responses (SVRs) following the widespread adoption of direct-acting antiviral therapies targeting HCV (4) .

Liver cancer exhibits significant heterogeneity in its pathogenesis, histopathological characteristics, and biological behavior. This variability in etiology contributes to the generally poor prognosis observed in patients with liver cancer (5) . The 2010 fourth edition of the World Health Organization (WHO) Classification of Digestive System Tumors offers a comprehensive overview of the histopathological characteristics of hepatocellular carcinoma (HCC). This encompasses detailed classifications of various "cytological variants," including pleomorphic cell, clear cell, and spindle cell

types. Furthermore, the classification recognizes several "special types" of HCC, such as fibrolamellar carcinoma, sclerotic HCC, undifferentiated carcinoma, lymphoepithelioma-like carcinoma, and sarcomatoid HCC (6) . The classification of HCC relies primarily on morphological characteristics, histopathological diagnosis is considered the gold standard for identifying hepatocellular carcinoma (HCC) and distinguishing it from other potential diagnoses (2) .

Morphological staging of hepatocellular carcinoma (HCC), especially in specimens obtained from resections and transplants, depends on a meticulous assessment of both the macroscopic and histological characteristics of the tumor lesion. This evaluation follows the validated TNM classification, which also includes assessment of resection margins. In addition, tumor grading is usually included in the diagnostic process. However, it should be noted that there is no globally standardized consensus the choice of grading system for hepatocellular carcinoma (HCC) remains uncertain, and the current evidence on the independent prognostic significance of grading in HCC remains inconclusive (2) .

Hepatocellular carcinoma (HCC) comprises several subtypes that collectively represent around 35% of cases. These subtypes, listed in decreasing order of prevalence, include steatohepatic, clear cell, macrotrabecular-massive (MTM), scirrhous, chromophobe, fibrolamellar, as well as neutrophil-rich and lymphocyte-rich subtypes. Except for the clear cell and lymphocyte-rich subtypes, the others share common molecular features (7) .

In certain instances, a conclusive pathological diagnosis may not be achievable through histological evaluation alone, particularly with small biopsy samples. In these situations, immunohistochemical staining proves to be highly beneficial. Immunohistochemistry is commonly employed for molecular pathological diagnosis owing to its broad applicability, ease of execution and interpretation, and relatively low cost (8) .

The development of malignant tumors is preceded by a sequence of molecular biological events (9) . The development of hepatocellular carcinoma (HCC) involves a gradual accumulation of molecular alterations, though the early events remain mostly unclear.

DMBA is a synthetic chemical used to induce breast cancer in preclinical models by activating the cytosolic aryl hydrocarbon receptor (AhR) (10) . In the liver, DMBA is metabolized to DMBA-3,4-diol-1,2-epoxide (DMBA-DE) through a series of reactions involving cytochrome P450 enzymes, particularly CYP1A1 and CYP1B1 (11) .p-Dimethylamino benzaldehyde (DMBA) is a common environmental polycyclic aromatic hydrocarbon, acting as a mutagen, capable of exerting carcinogenic effects, which can be tested both in cell culture and in vivo animal models.

Certain microRNAs (miRNAs) are directly regulated by oncoproteins or tumor suppressor proteins. For instance, the tumor suppressor protein p53 modulates the expression of miR-34 by interacting with p53-responsive elements located upstream of the miR-34 locus (9) . The tumor suppressor p53 is a major tumor suppressor, and loss of p53 tumor suppressor function is often an early precursor to cancer development. The tumor suppressor p53 is the most frequently mutated gene in human cancers, with mutations in tumor suppressor p53 occurring in >50% of all human cancers and in nearly every type of human cancer (12) . Cancer cells gain a selective advantage by maintaining mutant forms of the protein, which radically degrade p53 tumor suppressor function by promoting invasion, metastasis, and chemotherapy resistance (13) .

The tumor suppressor function of p53 in cancer progression is associated with various transcriptional and non-transcriptional activities that precisely control cell proliferation, senescence, DNA repair, and apoptosis. Moreover, increasing evidence suggests that p53 also plays a critical role in metabolic processes in both normal and malignant cells (14) .

This study presents a comprehensive immunohistochemical landscape of tumor suppressor p53 in hepatitis, cirrhosis, and HCC stages using p-Dimethylamino Benzaldehyd (DMBA)-induced Hepatocellular carcinoma mice model.

2. MATERIAL AND METHODS

this study include analytical scales, stainless spoons, ovens, *object glasses* , *cover glasses* , droppers, mouse cages , mouse drinking bottles, husks, Hardware (Asus Vivo Book Flip 14 Laptop with Intel Celeron N4020 processor specifications, 4GB RAM, 64-bit operating system, window 2019), SPSS 26 software , mouse food, gloves, masks, 1 cc and 3 cc syringes, probes, mouse fixation tools, ketamine, data loggers, cameras, microscopes , styrofoam boxes, plastic tissue pots, plastic bottles, scalpels, tweezers, strainers, tissue cassettes, automatic processor machines, vacuum machines, blocking machines, microtome machines, microtome knives, 46 ^{o}C water baths, object glasses, cover slips, special staining racks, 60 ^{o}C ovens.

2.1 METHODOLOGY

The type of research used is *true experimental laboratories in vivo.* The research design applied to observe the process of hepatocarcinogenesis in male rats (Rattus norvegicus L.) Wistar strain induced by p-Dimethylamino benzaldehyde (DMBA).

2.2 SAMPLE

In this study, specimens were collected from rat that met the inclusion and exclusion criteria with the following provisions:

2.2.1 INCLUSION CRITERIA

a. Male Wistar Rat (Rattus norvegicus L.)
b. Rat weight 100-250 grams
c. Healthy mice aged 2-3 months.

2.2.2 EXCLUSION CRITERIA

a. Rats don't want to eat or drink
b. Ratsdied and liver tissue could not be taken.

2.3 PROCEDURES

This study is a true experimental laboratories in vivo. This research protocol has met the ethical principles of research, the research protocol was reviewed and approved by the Udayana University ethics committee with a letter or Ethical Clearance document Number: B / 259 / UN14.2.9 / PT.01.04 / 2023. This research protocol has received approval for the implementation of research from the Integrated Biomedical Laboratory with a letter or document Number: 1568 / UN14.2.2.VII.6 / LT / 2023.

a. group of mice were induced with DMBA twice a week for 5 weeks at a dose of 25 mg/kg BW. DMBA was administered orally using a probe. DMBA was dissolved in corn oil in a Bio Safety Cabinet for the carcinogenic induction process.
b. Normal rat group and DMBA-induced rat group were given pellets and drink ad libitum during the treatment process. The waiting period for cancer growth was 2 weeks.
c. Final treatment all rats (Rattus novergicus L.) were sacrificed with ketamine. Liver organ was taken for Immunohistochemistry measurement of Tumor Suppressor p53 expression.

3. RESULTS AND DISCUSSION

We employed an established protocol to create a genotoxic hepatocarcinogenesis model, in which 28 male rats were administered 10 doses of DMBA (25 mg/kg body weight) orally, twice a week, over a period of 5 weeks. To investigate the progression of inflammation, cirrhosis, and liver tumors, we performed surgery on rats that died during the DMBA induction process monitored from week 3 to week 10.

Table 1. Results of the Normality Test for Body Weight of Research Rats

Tests of Normality

	Shapiro Wilk		
	Statistics	df	Sig.
Rat Weight Week 1	.900	28	.011
Rat Weight Week 2	.968	28	.532
Rat Weight Week 3	.956	28	.282
Rat Weight Week 4	.983	28	.918
Rat Weight Week 5	.975	28	.712
Rat Weight Week 6	.975	28	.712
Rat Weight Week 7	.975	28	.712
Rat Weight Week 8	.988	28	.982
Rat Weight Week 9	.995	28	1,000

Rat Weight Week 10	.984	28	.939
*. This is a lower bound of the true significance.			
a. Lilliefors Significance Correction			

Table 2. Hepatocarcinogenesis in Rat Model Induced by p-Dimethylaminobenzaldehyde (DMBA)

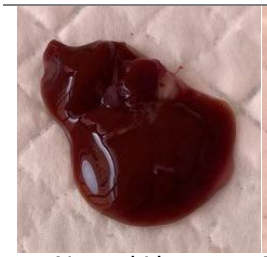
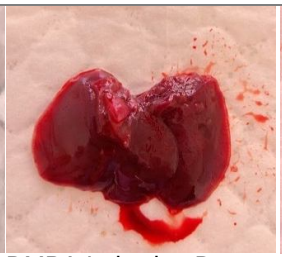
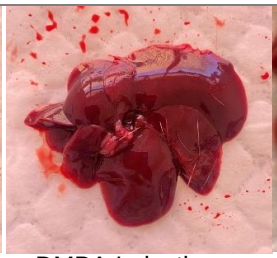
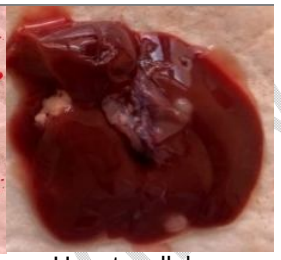
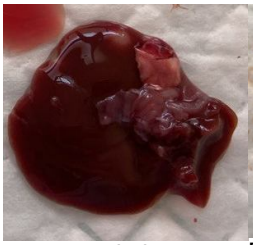
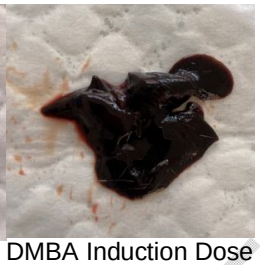
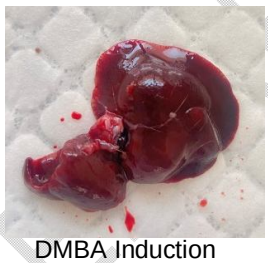
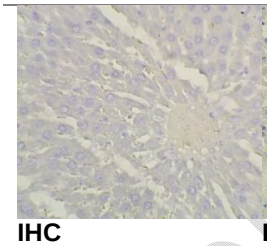
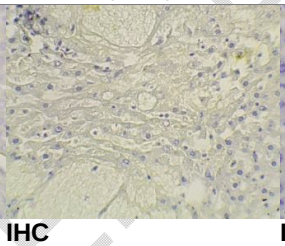
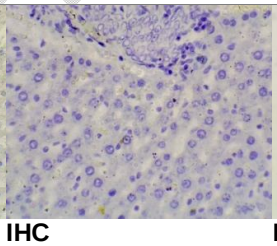
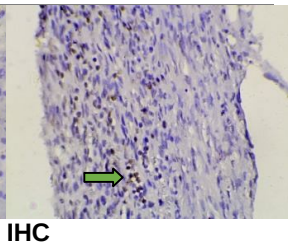
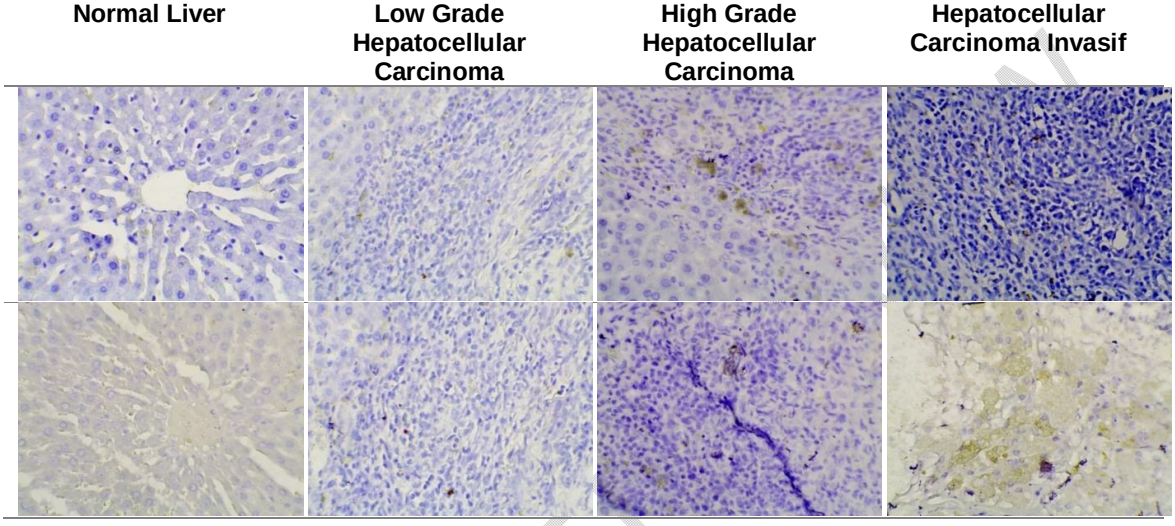
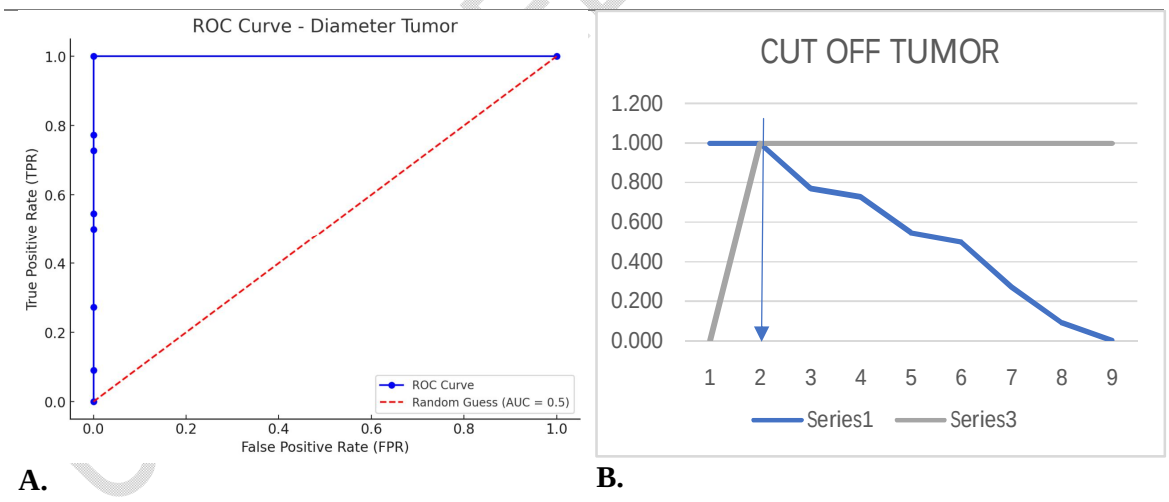
Normal Liver	Liver Hepatitis	Liver Cirrhosis	Hepatocellular Carcinoma
			
Normal Liver	DMBA Induction Dose 5	DMBA Induction Dose 9	Hepatocellular Carcinoma
			
Normal Liver	DMBA Induction Dose 7 (P2.4)	DMBA Induction Dose 10 (P2.3)	Hepatocellular Carcinoma
			
IHC p53 *wild type*	**IHC p53 *wild type***	**IHC p53 *wild type***	**IHC p53 *wild type***
	Inflammatory infiltration	Over expression of TGF-α (Proliferation)	P53 signaling (DNA damage)
	swelling of hepatocytes in the portal area	IGF-II Overexpression (Survival)	TGF-β signaling (loss growth inhibition)
	Cell inflammation	Fibrous tissue hyperplasia	WHT signaling (proliferation differentiation survival)
		Pseudolobular in the liver	MAPK signaling (HGF overexpression)

Table 3. Immunohistochemical Classification of Hepatocellular Tumor Suppressor p53 p-Dimethylamino benzaldehyde (DMBA) Induced Carcinoma in a Rat Model

Normal Liver	Low Grade Hepatocellular Carcinoma	High Grade Hepatocellular Carcinoma	Hepatocellular Carcinoma Invasif





A. **B.**

Fig 1. (A) ROC Curve Tumor Diameter ; (B) Tumor Diameter Cut Point

Hepatocellular carcinoma (HCC) is among the most aggressive malignancies, characterized by a poor prognosis despite considerable advancements in surgical and local-regional therapies over the past two decades (15) . Hepatocellular carcinoma (HCC) is a condition primarily resulting from inflammation, with 90% of cases arising from chronic liver inflammation and the subsequent development of cirrhosis. The liver possesses the capacity for self-repair following acute injury (16) . Cancer cells exhibit substantial heterogeneity, and many cells undergo apoptosis as the disease advances. Upon reaching a critical stage, a majority of cancer cells activate telomerase to repair and preserve their telomeres, thus enabling continued cell division. The precise mechanisms triggering the

reactivation of telomerase remain unclear; however, It seems to involve the activation of specific oncogenes like RAS or MYC, in conjunction with the loss of function of certain tumor suppressor proteins, including p53 and pRB. Telomerase activity is reactivated in approximately 90% of cancer cases. Other cancers appear to take up or originate from stem cells. Stem cell cancers retain a degree of telomerase activity, This characteristic is typical of somatic stem cells. Telomerase is specifically activated in cancer cells and potentially in stem cell-related cancers as well, making it a promising target for therapy (17).

The onset of hepatocellular carcinoma (HCC) is characterized by a gradual accumulation of molecular changes, whose origins are still largely unknown. This study presents a comprehensive immunohistochemical analysis of hepatitis, cirrhosis, and HCC using a p-Dimethylamino Benzaldehyde (DMBA)-induced HCC mouse model. The researchers observed early genomic instability and disrupted cancer-related signaling pathways during liver hepatitis. Despite significant pathological differences, the mutational signatures and expression profiles between the hepatitis and cirrhosis stages are remarkably similar. Cirrhosis is a severe liver condition marked by the replacement of healthy liver tissue with fibrous scar tissue, resulting in irreversible damage to the liver. The accumulation of scar tissue impairs the liver's functionality by obstructing blood flow and reducing its capacity to process nutrients, hormones, medications, and endogenous toxins. These substances can reduce the production of proteins and other substances made by the liver, ultimately making the liver unable to function properly. Characterization of the progressive molecular changes during hepatocarcinogenesis, where there is intense competition between tumor-suppressive and oncogenic forces in cirrhosis.

The p53 tumor suppressor signaling pathway has been identified as a critical pathway in hepatocarcinogenesis through integrative analysis. Additionally, dynamic immune responses during hepatocarcinogenesis, including sustained reductions in monocyte levels, have been observed, suggest immunological intervention strategies in addition to chemoprevention for liver cancer.

Cancer cell heterogeneity arises during proliferation and mutation. Tumor-associated endothelial cells (TAECs), fibroblasts, and inflammatory cells each exhibit distinct gene expression profiles, characterized by unique cell surface molecules and secretory patterns. Throughout this process, numerous cancer cells undergo apoptosis, but the surviving cells often become more aggressive and exhibit metastatic characteristics. Somatic mutations in cancer cells occur randomly, leading to genetic variability among cancer cells in different regions of the tumor. Sometimes stem cell populations appear, the origin of which is still unknown. Many major cancer markers are the result of cancer-stromal interactions (17). In the normal control group, tumor diameter growth was found in this study. This phenomenon can explain that Small hepatocellular carcinomas can form epigenetically without DMBA induction.

The analysis of tumor diameter data was continued with ROC analysis in SPSS to determine the tumor cut off. The ROC analysis results indicated that the tumor diameter dataset included 35 samples, comprising 22 positive samples, 10 negative samples, and 3 dropout samples. The analysis results demonstrated that the test result variable (tumor diameter) had an AUC value of 1,000. This shows that the model has perfect ability to distinguish between positive and negative classes.

The following is the ROC curve generated based on tumor diameter data. This curve shows that the classification model with an AUC (Area Under the Curve) value of 1,000 has perfect performance in distinguishing between positive and negative classes. This means that the model does not make errors in classification. The tumor diameter analysis identified a cutoff value of 0.5, with a sensitivity of 1.0 and a specificity of 1.0, indicating optimal discrimination between positive and negative cases. The results of the ROC analysis of the tumor diameter are used as a consideration in assessing the diagnostic ability of biomarkers of Small Hepatocellular carcinoma in this study.

In this study, the oncogene studied was p53 in wild type form. This oncogene analysis was conducted to understand p53 expression and its relationship to the development of hepatocellular carcinoma (HCC). The results of the Immunohistochemistry test in this study showed the accumulation of *Tumor Suppressor* p53 in the cytoplasm. The results of the data from the Immunohistochemistry *of Tumor Suppressor* p53 were entered into the Image J application to count active p53 cells (brown) processed with *cellcounter* . Determination of p53 *Tumor Suppressor* immunostaining score is done by interpreting immunoreactivity based on *Histochemical scoring* (H-score) *assessment.*

Albino rats (Rattus norvegicus L.), better known as laboratory rats, are used as a research model in biomedicine. Rats can represent mammalian biological systems, making them very suitable for preclinical research. The reproductive period of rats can be determined by observing their various life stages and behaviors. The liver of Wistar rats has a structure and function similar to the liver of

other mammals, used in biomedical research to study liver function, metabolism, and the effects of various compounds or drugs.

4. CONCLUSION

This study shows that in male Wistar rats p-Dimethylamino benzaldehyde (DMBA) -induced hepatocarcinogenesis can be a viable model for studying *HepatocellularCarcinoma* in the future. *Hepatocellular morpho-molecular correlationCarcinoma* and identification of various histopathological variants *HepatocellularCarcinoma* , based primarily on its molecular features, we can now not only gain a deeper understanding of the pathogenesis of hepatocellular carcinoma but also use this knowledge to predict patient outcomes and advance the development of targeted therapies.

CONSENT

It is not applicable.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

This study is a true experimental laboratories in vivo. This research protocol has met the ethical principles of research, the research protocol was reviewed and approved by the Udayana University ethics committee with a letter or Ethical Clearance document Number: B / 259 / UN14.2.9 / PT.01.04 / 2023. This research protocol has received approval for the implementation of research from the Integrated Biomedical Laboratory with a letter or document Number: 1568 / UN14.2.2.VII.6 / LT / 2023.

Disclaimer (Artificial intelligence)
Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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