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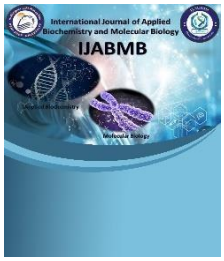

Detection of AMPK Gene Expression in Mice and Its Relationship with Clopidogrel Effect, With the Study of Some Vital Variables

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Abstract

Background:
Plavix comprises clopidogrel and is classified as an antiplatelet medication. Platelets are diminutive entities in the bloodstream that aggregate during hemostasis. By inhibiting this aggregation, antiplatelet pharmaceuticals diminish the likelihood of thrombus formation (a process known as thrombosis). Adenosine monophosphate-activated protein kinase (AMPK) is a well-established mechanism for the treatment of metabolic dysfunction-associated steatotic liver disease (MASLD).

Objective:
We hypothesised that clopidogrel could activate the AMPK pathway.

Methods: Thirty male mice (Mus musculus) were collected. No statistically significant alterations in animal weights were evident across all groups before and after the trial; however, liver weight diminished with clopidogrel, as shown by Quantitative Real-Time PCR (qRT-PCR).

Results:
The expression of the AMPK gene was markedly elevated in mice administered clopidogrel compared to the control group. Micronuclei primarily originate from genetic damage to live cells. The micronucleus (MN), a visible nuclear structure within cells, forms when chromosomes fracture or segregate during cellular division. The assessment was executed in accordance with the Schmid (1975) technique. This study sought to investigate if Indian Plavix increases or decreases MN levels in mice. PCEs *79 ± 4.109, MNiPCEs *11.4 ± 2.458, NCE *21.4 ± 3.727, and MNiRBCs *5.2 ± 1.666 are significantly higher than those in the control group.

Conclusion:
We propose that clopidogrel may improve fatty liver in mice via augmenting the activation of the AMPK pathway. The findings demonstrated that Indian Plavix elevates MN levels in mice.

Keywords: AMPK, clopidogrel, micronucleus assay, *MUS* MUSCULUS, qRT-PCR.

Introduction

Clopidogrel, an antiplatelet drug, reduces instances of ischemia by inhibiting platelet aggregation triggered by adenosine diphosphate (ADP) (1, 2).. It acts as an inhibitor of platelet function in vivo, specifically targeting the binding of ADP to its platelet receptor and subsequent stimulation of the GPIIb/IIIa complex. Clopidogrel's mechanism involves permanently affecting the platelet ADP receptor, leading to prolonged effects on platelets exposed to it. Through bioconversion, it generates an irreversible, non-competitive inhibitor of the P2Y12 receptor, which remains attached to the platelet surface for the receptor's lifespan (7-10 days) (3). In the United States of America and Canada, clopidogrel continues to be the antiplatelet medication that is prescribed the most frequently(4,5). As a matter of fact, according to a study that examined 64,600 patients who underwent percutaneous coronary intervention (PCI) between the years 2012 and 2014, the percentage of individuals who were given clopidogrel, prasugrel, and ticagrelor was respectively 72%, 20%, and 8% (3). The combination of clopidogrel and aspirin is a well-established and widely used DAPT for the purpose of reducing the risk of ischemic heart disease in patients who have ischemic heart disease (6). In the treatment of MASLD, one of the most well-established mechanisms is the stimulation of adenosine monophosphate (AMP)-activated protein kinase (AMPK). Clopidogrel has the potential to activate the CaMKKβ/AMPK/Nrf2 pathway, which can successfully treat atherosclerosis. This is achieved by decreasing the amount of cells that express LRP1 (low-density lipoprotein receptor-related protein 1) and α-SMA (α-smooth muscle actin) in plaques derived from ApoE−/− mice that are fed a high-fat diet(7). One promising possibility for this type of biomarker is the MN test, which has the potential to detect chromosomal abnormalities or mitotic spindle malfunction caused by a neurogenic pathway (8). The question of whether a higher frequency of MN in particular tissues, like oral epithelia, would suggest an increased risk of future cancer development or may be answered by removing the cells from the affected tissues remains unanswered. point to other malignancies in different parts of the body (9). Evaluation of MN due to genotoxic chemical activity has been emphasised by numerous

cytogeneticists. It is stated that the United States Food and Drug Administration has given its approval to this Medication Guide. Clopidogrel has not been found to have any genotoxic activity in several in vitro and in vivo genotoxicity tests (10). The purpose of this research is to assess the prevalence of cytogenic damage-induced micronuclei (MN) in Clopidogrel-treated patients.

Materials and Methods

Study design: The main study used 30 male Swiss albino mice ranging in age from 12 to 20 weeks and weighing 30-37 g. Food and water were readily available to the mice. Between fourteen o'clock and fifteen o'clock each day, dosages were administered. A record of the animals' overall health was kept throughout the experiment. Mice were orally dosed with Plavix 75 mg once day, a negative control, and a positive control consisting of 0.001 mg of retinoic acid. After fourteen days, the experiment was over. Every day, we tracked the weight of each mouse.

Measuring gene expression: The samples' mRNA was isolated using real-time polymerase chain reaction (PCR). RNA extraction kit Transzol Up plus (RNA Kit) from TRANS was used to measure gene expression. Table 1 lists gene primer sequences.

Table 1. Primer Sequences of the studied genes.

Gene name	Primer Sequences	Product size(bp)
AMPK	AMPK F 5′GTACCAGGTCATCAGTACAC3′ AMPK R 5′ACTACTCCAGGTACATCAGA 3′	117(11)
GADPH(Housekeeping gene)	GADPH F5′ACCACAGTCCATGCCATCAC 3′ GADPH R5′ TCCACCACCCTGTTGCTGTA 3′	472(11)

Quantitative Real-Time PCR (qRT- PCR) Run : Quantified mature RNA expression fold changes were estimated using the relative cycle threshold (2-ΔΔCt) technique. Relative gene expression ratio between control and test groups. Housekeeping reference genes were utilised to measure gene expression using double delta Ct (threshold cycle) analysis. The calculations were: Each sample's CT was computed using real-time cycler software. All samples were duplicated and mean values determined. Patients and controls' target gene Ct values were recorded. To determine the difference between the CT values (ΔCt) for each target gene and the housekeeping gene, subtract the specified normalisation factor from the Ct value of each gene of interest.Gene expression was measured and estimated using these equations:

1- ΔCT = CT (gene)−CT (housekeeping gene)
2- ΔΔCT = ΔCT Treated− ΔCT Control
3- CT gene = threshold value for each gene.
4- CT Housekeeping gene = threshold value for the reference gene

Micronucleus assay followed the procedures outlined by Schmid (1975) (13). Cervical dislocation was the method of death for the animals. Having obtained one and a half ml of PBS by use of a syringe with a capacity of five millilitres, the cells were given 10 minutes to rest in the incubator before the centrifuge tube was used again. After that, it was taken out of the incubator and spun in a centrifuge at 800 rpm for five minutes. Using a Pasteur pipette, the liquid above the sediment was removed, retaining half a millilitre of the supernatant. The cells were subsequently removed with a pipette and deposited into a drop of foetal calf serum, produced in accordance with the technique, on a clean glass slide. The slides were then left to air dry until the following day. The sample was further dried by submerging the slides in ethyl alcohol for three minutes after drying. After 7 minutes with May-Gruenwald stain and 3 minutes with Giemsa, the slides were prepared for analysis. Scanning 500 cells per glass slide allowed us to record the immature red blood cells harbouring micronuclei. After rinsing with Sorenson buffer and letting them dry, we analysed the slides.

Statistical Analysis: The data analysis was conducted using SPSS V20, a statistical programme. Data were presented as the mean plus or minus the standard error (SE). We used an independent samples t-test to look for differences in the means. (10) Distinctions were deemed significant if their probability values were equal to or lower than 0.05.

Results and Discussion

Body weight results: The results showed no significant differences in the

weights of the studied animals before and after the experiment the value ($p < 0.05$), but the results showed a significant increase in liver weight with clopidogrel (Figure 1)**.** Enhanced phosphorylation of the AMPK pathway is the mechanism by which clopidogrel inhibits the production of fatty acids by hepatocytes in vitro.

Therefore, liver size is directly affected, but body weight is less affected. Clopidogrel liver weights (8.44 value) increased significantly compared to the controls **(**3.99 value). Similar outcomes have been presented by Tai *et al* 2024 (14).

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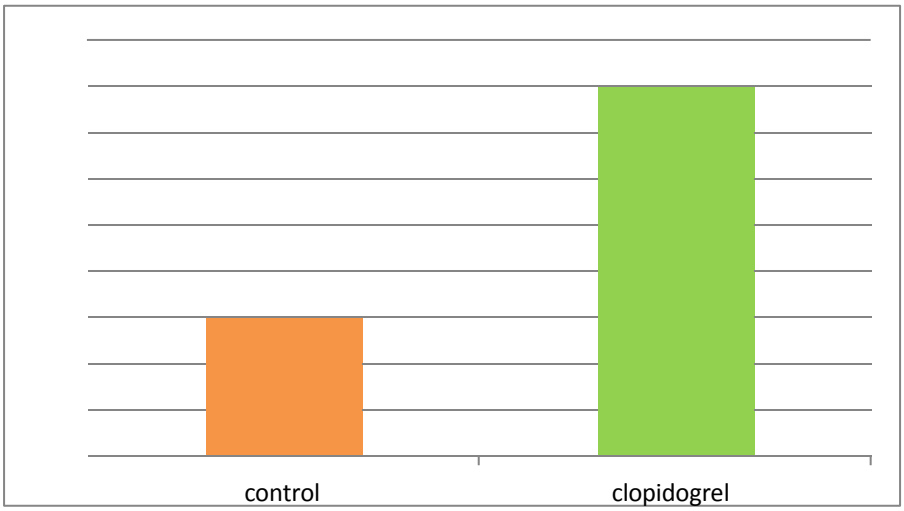

Figure 1: The liver weight compared to the Control and Clopidogrel groups is statistically significant (*$p < 0.05$)

AMPK gene expression :

The results revealed that AMPK gene expression was significantly higher in mice that were treated with Clopidogrel than in control mice ($p = 0.005$), as shown in Figures 2, 3, and Table 2.

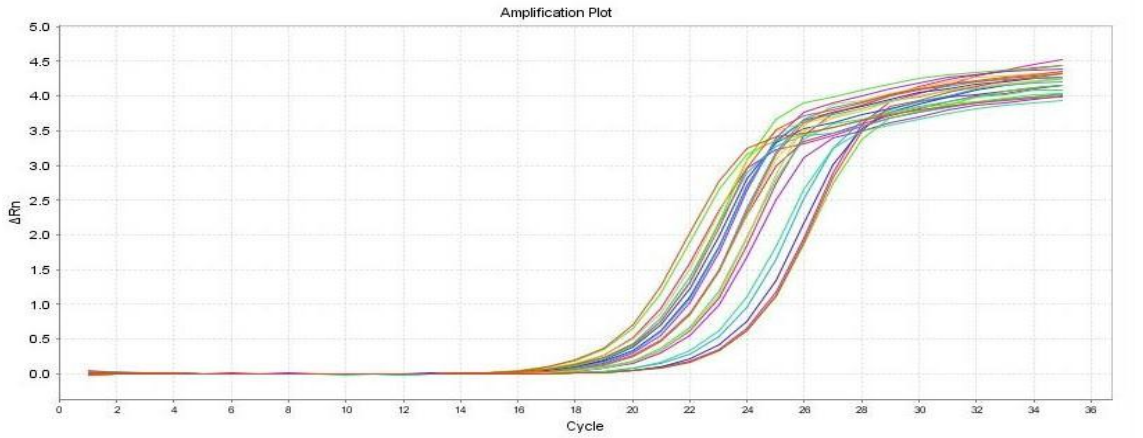

Figure 2. RT-PCR results of related mRNA expression tested by threshold cycle (Ct)

Table 2. AMPK gene expression is higher in mice that were treated with Clopidogrel and controls.

mRNA	Mean ΔCT± ES for control	Mean ΔCT± ES for Patients	p-value	ΔΔCt	Folding expression
AMPK gene mRNA	20.81±0.38	25.36±0.81*	0.05	-1.03	2.088

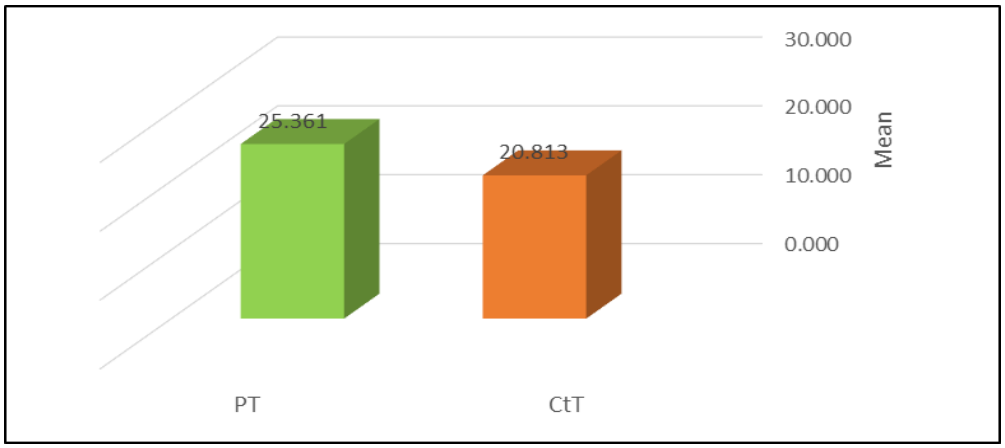

Figure 3. AMPK gene expression in mice treated with clopidogrel.

The CaMKKβ/AMPK/Nrf2 pathway may be activated by clopidogrel (15), and clopidogrel may be useful in treating atherosclerosis by lowering the amount of cells positive for LRP1 and α-SMA in plaques from ApoE −/− mice that are fed a high-fat diet (15). In the assessment of clopidogrel-induced hepatic steatosis using oil red O staining and triglyceride kit assay, researchers observed a decrease in blood aminotransferases, liver weight, and the liver-to-body weight ratio. Clopidogrel enhanced AMPK and ACC phosphorylation and downregulated numerous important lipogenic, profibrotic, and proinflammatory genes. These genes include Acaca/Acacb, Fasn, Scd1, Elovl6, Mo gat1, Pparg, Cd36, and Fabp4. However, AMPK inhibitor compound C mitigated clopidogrel-induced enhanced AMPK and ACC phosphorylation in primary hepatocytes from clopidogrel-treated animals and decreased intracellular lipid accumulation. Clopidogrel has shown promise in preventing and reversing hepatic steatosis in mice that is produced by a high-fat diet, which raises the possibility that it could be repurposed to combat fatty liver in patients (7).

The results of the Micronucleus assay: A higher frequency of micronuclei in bone marrow cells was seen as a consequence of taking clopidogrel, as shown in Table 3, and cellular genetic damage in MnPCE, polychromatic erythrocytes, and normochromic erythrocytes is illustrated in Figure 4.

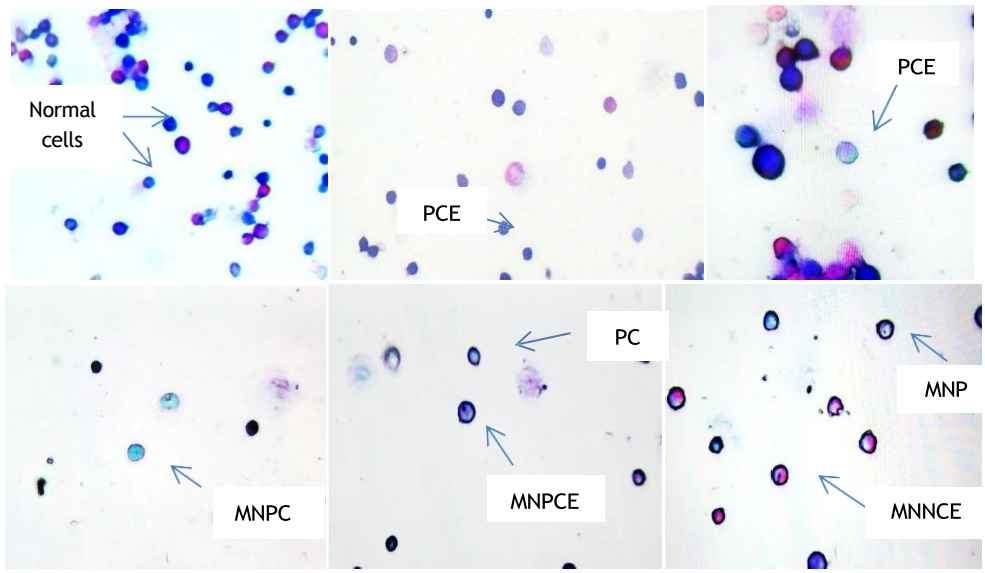

Figure 4. Cellular genetic damage in mice. MnPCE, PCE, and NCE by clopidogrel drug.

Table 3. The average discrepancies in the occurrence of micronuclei inside the bone marrow

Groups	PCEs	MNiPCEs	NCE	MNi NCE	P vlue
	Mean ± std. D				
control	1.800 ± 0.788	0.400 ± 0.516	1.800 ± 1.229	1.000 ± 0.942	0.05
clopidogrel 75mg	*79.000 ± 4.109	*11.400 ± 2.458	*21.400 ± 3.747	*5.200 ± 1.686	0.05
Retinoic 0.001 acid mg	*38.400 ± 1.264	2.677 ± ^2.080	*5.200 ± 3.155	*3.500 ± 1.080	0.05

Numerous in vitro and in vivo genotoxicity investigations have shown that clopidogrel does not have any genotoxic effects. The teratogenic effects of clopidogrel were not observed in rats or rabbits, and it did not affect the fertility of either sexe in rats. When nursing rats were given clopidogrel, it somewhat slowed down the growth of the offspring. Radiolabeled clopidogrel pharmacokinetic investigations have demonstrated that either the parent molecule or its metabolites are eliminated in breast milk. So, it's not out of the question that there could be an indirect influence (poor palatability) or a direct effect (slight toxicity). (9).

According to the research carried out by Bayar et al. in 2021, in AML-12 hepatocytes (p-value <0.01) and 3T3-L1 adipocytes (p= value <0.001), DNA fragmentation was seen to be elevated after treatment with 7.5 μM, Forty μM, and seventy-five μM clopidogrel compared to untreated control groups. Clopidogrel therapy induced a greater extent of DNA damage in 3T3-L1 adipocytes, although no such effect was observed in AML-12 hepatocytes. Additionally, the levels of DNA damage in adipocytes and hepatocytes were seen to rise in a dose-dependent manner for 7.5 and 40 μM clopidogrel, but to fall in reaction to 75 μM.Adipocytes are more susceptible to DNA damage from clopidogrel than hepatocytes. Additional research into the molecular basis of clopidogrel genotoxicity is necessary, particularly in adipose tissue. (16).

Conclusions

It has been suggested that clopidogrel could be repurposed to combat fatty liver in people, based on our conclusion that the drug may reduce fatty liver weight in mice. Mice given clopidogrel had considerably more AMPK gene expression compared to controls. We propose that clopidogrel may improve fatty liver in mice via increasing AMPK pathway phosphorylation. According to the findings, Indian Plavix elevates MN in a mouse model.

References

1. Bates E. R., Lau W. C., Angiolillo D. J. Clopidogrel-drug interactions. Journal of the American College of Cardiology. 2011;57(11):1251–1263.
2. Savi P., Pereillo J. M., Uzabiaga M. F., et al. Identification and biological activity of the active metabolite of clopidogrel. Thrombosis and Haemostasis. 2000;84(5):891–896.
3. Siller-Matula J. M., Trenk D., Schrör K., et al. Response variability to $P2Y_{12}$ receptor inhibitors. JACC: Cardiovascular Interventions. 2013;6(11):1111–1128.
4. Gandhi S, Zile B, Tan MK, Saranu J, Bucci C, Yan AT, Robertson P, Quantz MA, Letovsky E, Tanguay J-F, Dery J-P, Fitchett D, Madan M, Cantor WJ, Heffernan M, Natarajan MK, Wong GC, Welsh RC, Goodman SG. Increased uptake of guideline-recommended oral antiplatelet therapy: insights from the Canadian Acute Coronary Syndrome Reflective. Can J Cardiol. 2014;30:1725–1731.
5. Karve AM, Seth M, Sharma M, LaLonde T, Dixon S, Wohns D, Gurm HS. Contemporary use of ticagrelor in interventional practice (from Blue Cross Blue Shield of Michigan Cardiovascular Consortium). Am J Cardiol. 2015;115:1502–1506.
6. Price MJ, Murray SS, Angiolillo DJ, Lillie E, Smith EN, Tisch RL, Schork NJ, Teirstein PS, Topol EJ. Influence of genetic polymorphisms on the effect of high- and standard-dose clopidogrel after percutaneous coronary intervention: The GIFT (Genotype Information and Functional Testing) Study. J Am Coll Cardiol. 2012; 59:1928–1937.
7. Ting Tai , Yuan-Yuan Shao ,Yu-Qi Zheng, Li-Ping Jiang ,Hao-Ru Han , Na Yin , Hao-Dong Li , Jin-Zi Ji , Qiong-Yu Mi , Li Yang ,Lei Feng ,Fu-Yang Duan ,Hong-Guang Xie . Clopidogrel ameliorates high-fat diet-induced hepatic steatosis in mice through activation of the AMPK signaling pathway and beyond. Front Pharmacol. 2024 Oct 23;15:1496639. doi: 10.3389/fphar.2024.1496639.
8. Bina Kashyap, Padala Sridhar Reddy. Micronuclei assay of exfoliated oral buccal cells: Means to assess the nuclear abnormalities in different diseases. Journal of Cancer Research and Therapeutics - April-June 2012 - Volume 8 - Issue 2.
9. Rosin MP, Saad el Din Zaki S, Ward AJ, Anwar WA. Involvement of inflammatory reactions and elevated cell

proliferation in the development of bladder cancer in schistosomiasis patients. Mutat Res. 1994;305:283- 92.

10. This Medication Guide has been approved by the U.S. Food and Drug Administration. Revised September 2016.
11. Heqi Bu, Dianlei Liu, Guolin Zhang, Li Chen, Zhangfa Song.(2020)AMPK/mTOR/ULK1 Axis-Mediated Pathway Participates in Apoptosis and Autophagy Induction by Oridonin in Colon Cancer DLD-1 Cells. OncoTargets and Therapy , Volume 13 Pages 8533—8545
12. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.), 25(4), 402–408. Schmid,W.(1975).
13. The micronucleus test. Mutat.Res.,Vol31.pp.9-15.
14. Yang H., Zhao P., Tian S. (2016). Clopidogrel protects endothelium by hindering tnfα-induced VCAM-1 expression through CaMKKβ/AMPK/Nrf2 pathway. J. Diabetes Res. 2016, 9128050.
15. Chen J., Pi S., Yu C., Shi H., Liu Y., Guo X., et al. (2020). sLRP1 (soluble low-density lipoprotein receptor-related protein 1): a novel biomarker for P2Y12 (P2Y purinoceptor 12) receptor expression in atherosclerotic plaques. Arteriosclerosis, Thrombosis, Vasc. Biol. 40 (6), e166–e179.
16. Bayar , Elif , Cevik Mehtap , Caker Selen , Cagatay Penbe and Susleyici Belgin (2021). DNA Damage in AML-12 Hepatocytes and 3T3-L1 Adipocytes Treated with Clopidogrel. Current Drug Safety. Vol 16, Issue 3, 2021.