Original Research Article

Effect of Oral intake of Sodium Benzoate on Serum Cholesterol and Proinflammatory cytokine (Tumor necrosis factor alpha [TNF-α] and Interleukin-6 [IL-6]) levels in the heart tissue of Wistar rats

EFEKEMO, Oghenetekevwe, AKANINWOR, Joyce Oronne, ESSIEN, Eka Bassey

Department of Biochemistry, University of Port Harcourt, PMB 5323, Choba, Port Harcourt, Rivers State, Nigeria.

Email address: efekemo_tekevwe@yahoo.com

AUTHORS CONTRIBUTION

The work was carried out in collaboration between all authors. Author Efekemo Oghenetekevwe as the main author designed, analyzed, interpreted and prepared the manuscript, under the supervision of Essien Eka Bassey and Akaninwor Joyce Oronne. All authors read and approved the final manuscript.

ABSTRACT

The *in vivo* effect of oral administration of varying concentrations (150, 250, 500mg/kg body wt.) of sodium benzoate (a known preservative in the food, cosmetic and

pharmaceutical industry) on serum cholesterol and proinflammatory markers in heart tissue of wistar albino rats were investigated. The oral intake was administered at 24 hour intervals for 7, 14, 21 and 28 days. The groups were labelled; control (group 1), 7days (group 2), 14days (group 3), 21 days (group 4) and 28days (group 5). The rats were fed normal diet *ad libitum* and blood sample for the determination was taken at the end of the duration. For serum cholesterol, the result obtained for sodium benzoate concentrations administered showed significant ($p \leq 0.05$) decrease in cholesterol levels at group 5 for 250mg/kg body wt. and grp 2, 3, 4 and 5 for 500mg/kg body wt of experimental rats. The proinflammatory cytokines TNF-α and IL-6 of heart tissue showed significant decrease at grp 4 and 5 for 250mg/kg body wt and 2, 3, 4 and 5 for 500mg/kg body wt. values were all compared to control. These findings suggest modulation of the inflammatory pathway due to administration of the preservative.

Key words: Sodium benzoate; Cholesterol;Serum; Proinflammatory cytokines.

INTRODUCTION

The investigations of constituents of blood and organ tissue of mammals have continually played a valuable role in the normal functioning assessment of living organisms. Changes from the normal levels have been observed in disease conditions [1]. The effects of various compounds on biochemical parameters of experimental animals have been applied in assessing the safe use of compounds in products consumed. Sodium benzoate (C_6H_5COONa) is widely applicable as a preservative in several products consumed by man[2, 3, 4, 5]. Sodium benzoate metabolizing

occurs in the mitochondria matrix, it is metabolized by conversion to hippurate in two steps: Sodium Benzoate enters the mitochondria and is converted to benzoyl CoA by an ATP-dependent acid, butyrate CoA ligase. Then benzoyl CoA is subsequently converted to hippurate by glycine N-acyltransferase, and then exits the mitochondria. Ingestion of sodium benzoate causes a rise in both serum benzoate and hippurate level [6]. Sodium benzoate is also a component of ucephan, a food and drug administration approved drug used in the treatment of hepatic metabolic defects associated with hyperammonemia such as urea cycle disorder [7, 8]. It has been reported that 2% solution of sodium benzoate in drinking water is safe for lifelong treatment in mice without any noticeable side effects [9]. Recent studies have shown that sodium benzoate is useful in protecting mice from relapsing–remitting experimental allergic encephalomyelitis [10] and that it is also capable of inhibiting the expression of various proinflammatory molecules from activated glial cells [10].Several studies on the short and long term effects of sodium benzoate have reported adverse effects due to both chronic and subchronic intake of sodium benzoate [11, 12]. Some reports suggest the absence of negative consequence of sodium benzoate intake [9, 13].The upper limits of benzoate allowable in foods vary with 0.1% reported for United States of America, while a range of 0.15 to 0.25% had been reported for other countries of the world [14]. For European countries, the limit reported range is from 0.015 to 0.5% [15]. There are thus variations in the acceptable limits of these preservatives in foods. It therefore follows that sodium benzoate could be assimilated widely by consuming a wide range of food products intentionally preserved with it. The present report addressed the effects of oral administration of sodium benzoate on serum cholesterol, and proinflammatory cytokines in heart tissue. The findings in this study indicate that sodium benzoate may be useful in modulating the downstream signaling pathway.

MATERIALS AND METHOD

The experimental analysis was carried out in the Department of Biochemistry Research Laboratory, University of Port Harcourt, Choba, Rivers State, Nigeria. The study duration was for a period of one month, twenty eight days being the longest duration. The animals were purchased from the Department of Biochemistry, Animal House. Sodium benzoate was purchased from May & Baker Ltd., England.The reagent for cholesterol determination was purchased from Agape Diagnostics, Switzerland. TNF alpha and IL-6 kits were purchased from Elabscience, Donghu Hi-Tech Development Area, Wuhan, China. while all other reagents were of analytical grade. An approval was given by the Institution ethics committee for the commencement of this study.

Animals

A total of sixty-six (66) wistar albino rats, with an average weight of 140g were obtained from the animal house of the Department of Pharmacology, University of Port Harcourt. They were maintained on normal diet *ad libitum*, grouped into five (5), and housed in stainless steel cages in a well ventilated room under 12h light/dark cycle. The sodium benzoate concentrations were 150mg/kg body wt., 250mg/kg body wt and 500mg/kg body weight. The rats were divided into five groups namely G1 (control group), G2 (7days), G3 (14days), G4 (21days) and G5 (28days). The varying concentrations of sodium benzoate were administered orally in 1ml portions at 24 h intervals for the duration of the experiment (7, 14, 21 and 28 days). At the end of the experimental duration the rats were sacrificed.

Sample collection

The rats were anaesthetized with diethyl ether and dissected for blood collection. Blood samples collected were allowed to coagulate in sample bottles and centrifuged at 2500rpm for 10 mins and stored at $4^{o}C$ and the serum obtained was used to estimate cholesterol. After blood collection, the liver and heart were excised, weighed and rinsed in ice cold normal saline and transferred into ice cold sample containers for determination of the proinflammatory cytokines; interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) assay.

Determination of cholesterol

Principle

Enzymatic colorimetric determination of total cholesterol

$$\text{Cholesterol ester} + H_2O \xrightarrow[\text{Cholesterol esterase}]{} \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{cholesterol oxidase}]{} \text{4-chloesten-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{phenol} + \text{4 - Aminoantipyrine} \xrightarrow[\text{peroxidase}]{} \text{red quinone} + 4H_2O_2$$

Determination of TNF-alpha

This ELISA kit applies to the in vitro quantitative determination of Rat TNF-α concentrations in serum, plasma and other biological fluids. The kit is specific for rat TNF-alpha detection. The ELISA kit uses the sandwich-ELISA principle.

Determination of Interleukin-6

This ELISA kit applies to the in vitro quantitative determination of Rat IL-6 concentrations in serum, plasma and other biological fluids. The kit is specific for rat Interleukin-6 detection. This ELISA kit uses the Sandwich-ELISA principle.

Statistical analysis

All data were subjected to statistical analysis. The values were reported as mean ± standard error of mean (S.E.M), and analysed by one-way analysis of variance (ANOVA). ANOVA was used to test for differences between treatment groups using statistical package for social sciences (SPSS) version 20. The results were considered significant at P-values of less than 0.05, that is, at 95% confidence level ($P<0.05$).

RESULT

The result of the effect of Sodium benzoate on Serum Cholesterol, Interleukin-6 and Tumor necrosis factor – α in heart tissue of wistar rats are shown in fig 1, 2 and 3.

The cholesterol level of experimental rats in group 2, 3, 4 and 5 showed sodium benzoate had no significant difference for 150mg/kg body wt. but significantly ($p\leq0.05$) decrease was observed in group 5 for 250mg/kg body wt. and group 2, 3, 4 and 5 for 500mg/kg body wt. values were all compared to the control.

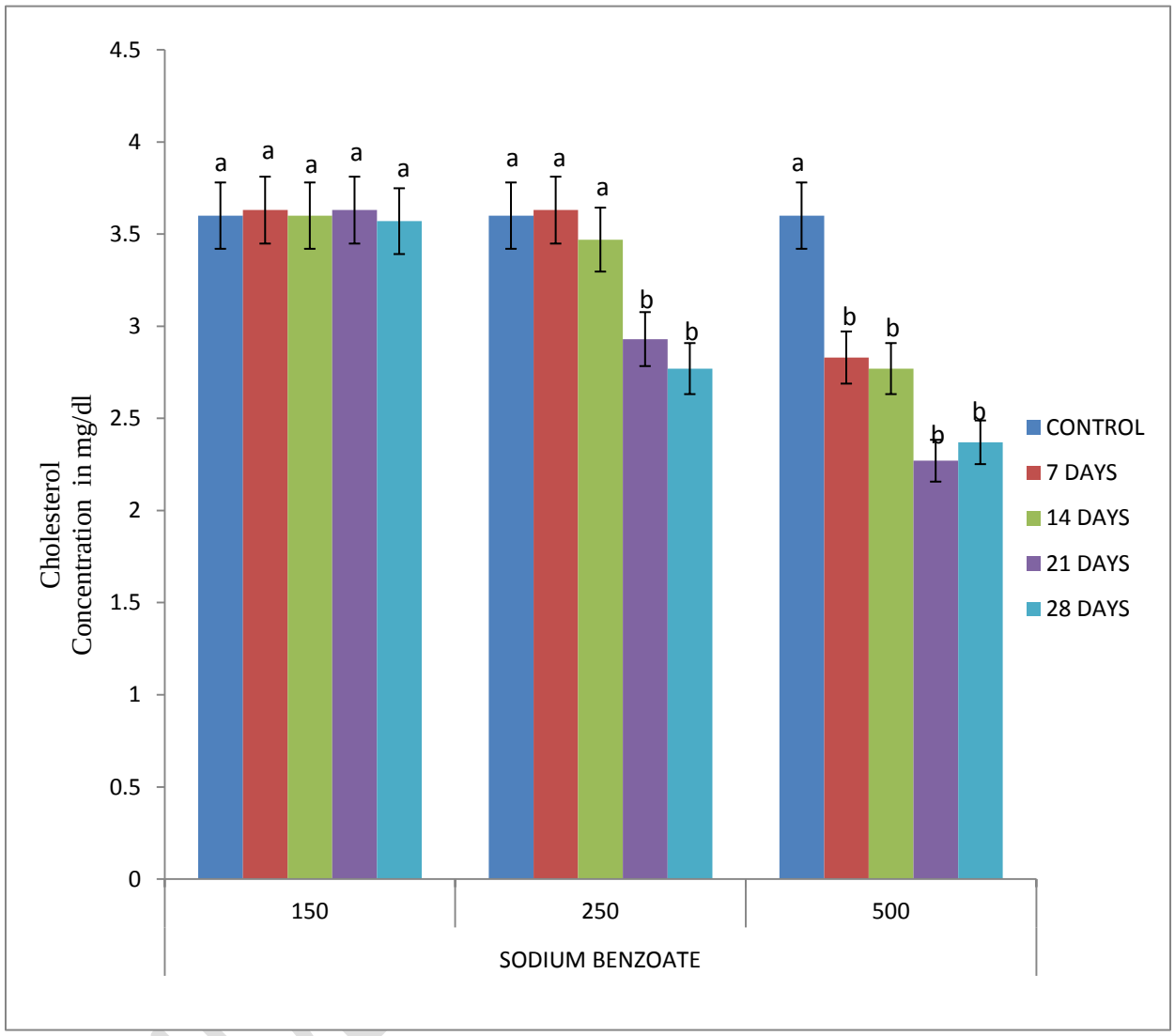

Fig 1: Effects of varying concentrations of sodium benzoate on cholesterol levels in serum.

Values are means ± Standard Error Mean (SEM). Values with different superscript are statistically significant at ($p \leq 0.05$). Superscript (a,b) compares 7 Days, 14 Days, 21 Days and 28 Days to control.

For the proinflammatory cytokines of experimental animals in group 2, 3, 4 and 5, tumor necrosis factor-α and interleukin-6 showed significant decrease in the heart tissue at group 4 and

5 of 250mg/kg and group 2, 3, 4 and 5 of 500mg/kg body wt. Values were all compared to the control.

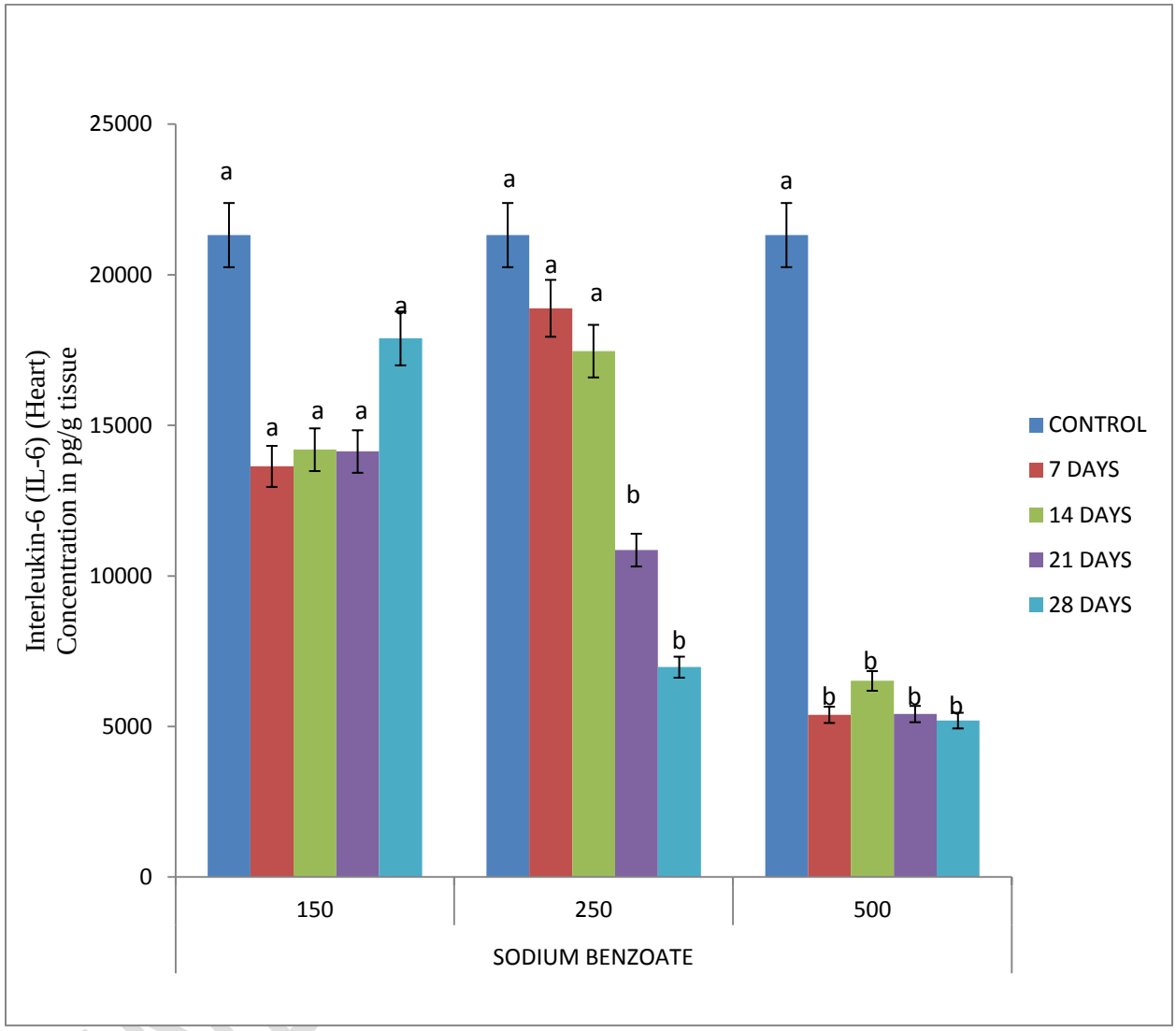

Fig 2: Effects of varying concentrations of sodium benzoate on interleukin-6 (IL-6) levels in heart tissue.

Values are means ± Standard Error Mean (SEM). Values with different superscript are statistically significant at ($p \leq 0.05$). Superscript (a,b) compares 7 Days, 14 Days, 21 Days and 28 Days to control.

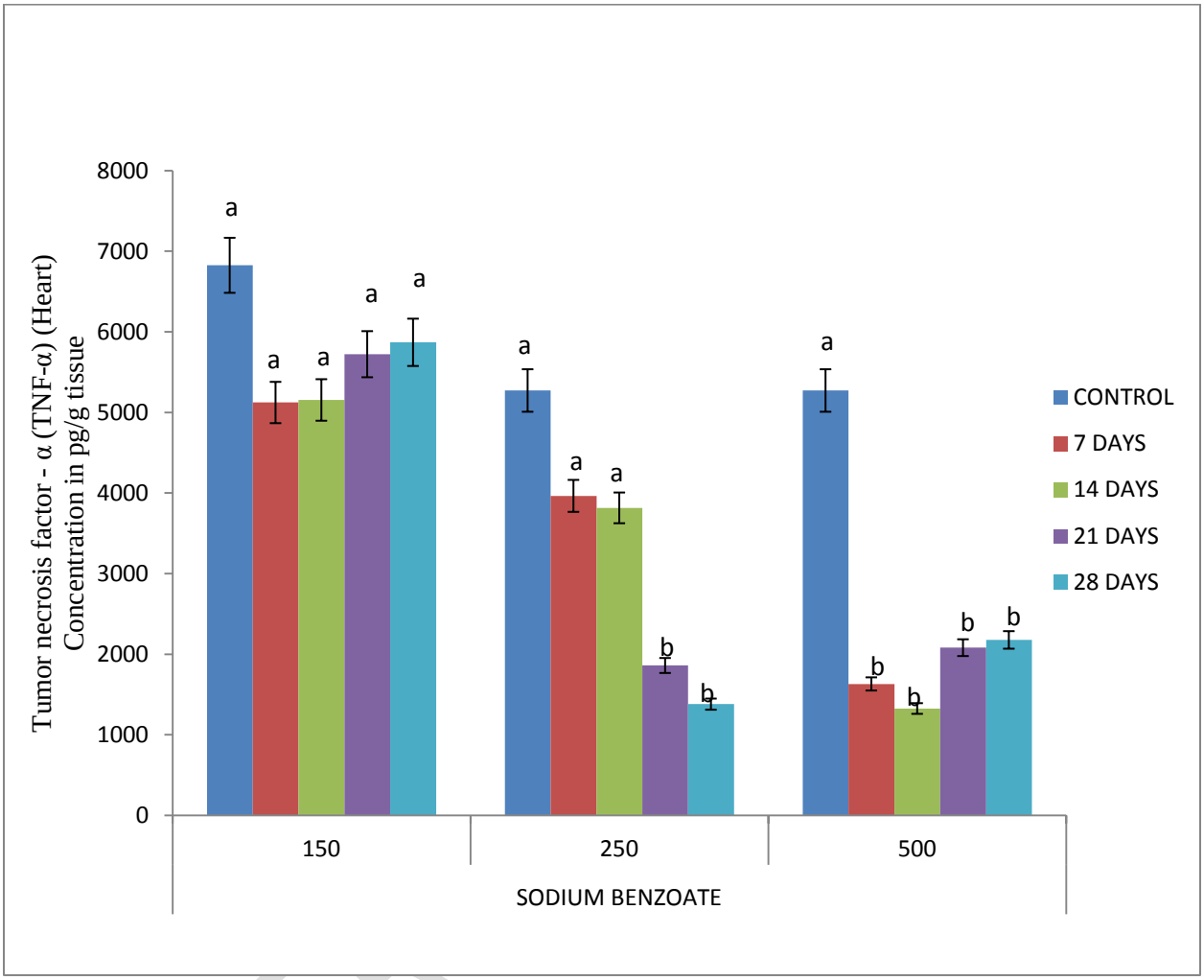

Fig 3: Effects of varying concentrations of sodium benzoate on tumor necrosis factor (TNF) levels in heart tissue.

Values are means ± Standard Error Mean (SEM). Values with different superscript are statistically significant at ($p \leq 0.05$). Superscript (a,b) compares 7 Days, 14 Days, 21 Days and 28 Days to control.

DISCUSSION

The total body content of cholesterol depends on the balance between the amount of cholesterol formed in the body plus that absorbed from diet. Intestinal cholesterol absorption represents another major route for the entry of cholesterol into the body, and, thus, this source can influence the plasma LDL-cholesterol concentration. The cholesterol pool in the intestine comes from dietary cholesterol and the majority from biliary excretion [16]. The deviation from normal values of cholesterol, may be an indication of a change in the cholesterol biosynthesis pathway [17]. This study revealed that cholesterol showed a significant ($p \leq 0.05$) decrease in levels, indicating an effect on lipid mobilization, storage processes, membrane structure and function. Alterations in the concentration of cholesterol can give useful information on the lipid metabolism as well as predisposition of the animals to atherosclerosis and its associated coronary heart diseases [18]. From this study it is seen that sodium benzoate suppressed the mevalonate pathway thereby lowering cholesterol synthesis leading to the depletion of intermediates in the cholesterol biosynthetic pathway as well as lowering cytokine expression. Sodium benzoate is first metabolized by conversion to benzoyl CoA by butyrate CoA ligase, then benzoyl CoA conjugates with glycine-N- acyltransferase to form hippurate. The benzoyl CoA formed inhibits the rate limiting enzyme (3-hydroxy-3-methyglutaryl CoA reductase) leading to the depletion of intermediates in the cholesterol biosynthetic pathway [19]. An earlier study, demonstrated that sodium benzoate is capable of reducing the level of cholesterol in vivo in mice at a level comparable to pravastatin [10], suggesting that the preservative attenuates the cholesterol biosynthesis pathway. This result is similar to that of the present study. Sodium benzoate is seen to behave in a similar way with the statin drug family in their cholesterol lowering effect by inhibiting HMG-CoA reductase as well as specific prenylated proteins. Intermediates of the

cholesterol biosynthesis pathway are key regulators of isoprenylation of small G proteins like $p21^{ras}$ and $p21^{rac}$[20]. Isoprenoids (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) are important attachments for the post-translational modification of a multitude of proteins involved in intracellular signal transduction pathways, including small GTP-binding proteins, which play crucial roles in the regulation of cell growth and differentiation, gene expression, cytoskeletal assembly and cell motility, protein and lipid trafficking, nuclear transport, and host defense [21, 22]. Whereas geranylgeranylation is required for activation of most of the small GTP-binding proteins (e.g. Rho, Rac, Rab, Rap), only few are farnesylated (e.g. Ras) [21]. Prenylation of protein (the GTP-bound protein family eg. Ras) by farnesyl pyrophosphate and geranylgeranyl pyrophosphate as substrates activates several downstream signaling pathway that lead to activation of neutral factor kappa b that plays a role in expression of proinflammatory molecules [20]. The Ras proto-oncogene proteins, a family of GTP-binding proteins, function by binding to the cytoplasmic surface of the plasma membrane. This membrane localization of $p21^{ras}$ involves prenylation of cysteine in a CAAX motif present at the C terminus, proteolytic removal of AAX tripeptide, and then carboxymethylation of the C-terminal cysteine [23]. The activation of $p21^{ras}$ by receptor tyrosine kinase occurs through conversion of the GDP-bound inactive form to the GTP-bound active form by Sos and Grb2 and then transduction of signal to downstream effector molecules [24]. The GTP-bound form is converted to the inactive form by the intrinsic GTPase activity, which is accelerated by GTPase-activating proteins [20]. Sodium benzoate (NaB) preferentially attenuates farnesylation of $p21^{ras}$ and thereby inhibits the signal transmission to the downstream signaling molecules [25, 26]. One such downstream candidate is Raf-1 (serine-threonine kinase). The $p21^{ras}$ interacts directly with Raf-1 and is believed to function by positioning Raf-1 at the plasma membrane in the vicinity of its activator, and

tyrosine phosphorylation of Raf-1 seems to be essential for $p21^{ras}$- induced activation of Raf-1 [25, 26]. Raf-1, in turn, phosphorylates and activates MEKs and ERKs (members of the MAPK cascade). Therefore, the observed inhibition of cytokine expression may be due to inhibition of NF-κB activation by NaB due to decrease and/or lack of signal transmission from receptor tyrosine kinase to Raf/MAPK cascade via $p21^{ras}$. Proinflammatory molecules have been implicated in the pathogenesis of cardiovascular diseases [27][42]. Transcription factors such as NF-κB, C/EBPβ, AP-1, STAT, IRF-1, etc., play a role in the expression of various proinflammatory molecules, activation of NF-κB seems essential for the transcription of most of the proinflammatory molecules [28, 29, 30, 31, 32, 33]. Therefore, for a drug to exhibit an anti-inflammatory effect, it is almost mandatory to attenuate the activation of NF-κB. Importantly, inflammation was shown to be a prominent hallmark of ventricular hypertrophy [34, 35]. Interstitial inflammatory cell infiltration involving macrophages, T-lymphocytes, fibrosis, high expression levels of cytokines such as interleukins (IL)-6, IL-1β, IL-1RA, and tumor necrosis factor-alpha (TNF-α), and activation of inflammatory signaling pathways such as nuclear factor kappa B (NF-κB) are all characteristic hallmarks of a pathologically hypertrophied heart [36, 37]. The pathogenic role inflammation plays is not clearly understood; however, it most probably exacerbates the disease condition. For example, IL-6 was shown to directly induce hypertrophy both *in vitro* and *in vivo*[38, 39]. Furthermore, macrophage microRNA-155, induced by pro-inflammatory stimuli, including lipopolysaccharide (LPS), TNF-α, and interferon-gamma (INF-γ), promotes cardiac hypertrophy and failure [27]. Additionally, targeting inflammatory cell receptors and mediators was shown to modify the disease process and might preserve cardiac function [40, 41].

CONCLUSION

The experimental findings at these concentrations of sodium benzoate, reflects its effect on cholesterol, and proinflammatory cytokines; suggesting modulation of the inflammatory pathway due to its administration.This highlights a novel anti-inflammatory role via modulation of the mevalonate pathway and $p21^{ras}$.

COMPETING INTERESTS

Authors have declared no competing interest exist.

ACKNOWLEDGEMENT

I want to acknowledge the contribution of Dr. D. E. Peters and Mr. B. Aleme to the success of this research work.

Consent : NA

Ethical :

As per international standard informed written ethical approval has been collected and preserved by the author(s).

REFERENCES

1. Cheesbrough M. Medical laboratory manual for tropical countries. 2nd edition. Tropical Health Technology and Butterworth Scientific Limited. 1991;1:494-526.
2. Baldwin EA, Nisperos-Carriedo MO, Baker RA. Use of edible coatings to preserve quality of lightly (and slightly) processed products. Critical Review on Food Science and Nutrition. 1995;35(6):509-524.

3. Ishida H. Levels of preservatives in toothpastes and possibility of their intake during brushing of teeth. Shokuhin Eiseigaku Zasshi. Journal of Food Hygiene Society of Japan. 1996;37:234-239.
4. Villanueva MBG, Jonai H, Kanno S, Takeuchi Y. Dietary sources and background levels of hippuric acid in urine: Comparison of Philippine and Japanese levels. Industrial Health. 1994;32:239-249.
5. Nair B. Final report on the safety assessment of Benzyl Alcohol, Benzoic Acid, and Sodium Benzoate. International Journal of Toxicology. 2001;20(3):23-50.
6. Kubota K, Ishizaki T. Dose-dependent pharmacokinetics of benzoic acid following oral administration of sodium benzoate to humans. European Journal of Clinical Pharmacology. 1991;41:363-368.
7. Leonard JV, Morris AA. Urea cycle disorders. Seminar Neonatology. 2002;7:27–35.
8. Scaglia F, Carter S, O'Brien WE, Lee B. Effect of alternative pathway therapy on branched chain amino acid metabolism in urea cycle disorder patients. Molecular and Genetic Metabolism. 2004;81(1):S79–S85.
9. Toth B. Lack of tumorigenicity of sodium benzoate in mice. Fundamental of Applied Toxicology. 1984;4:494-496.
10. Brahmachari S, Jana A, Pahan K. Sodium benzoate, a metabolite of cinnamon and a food additive, reduces microglial and astroglial inflammatory responses. Journal of Immunology. 2009;183(9):5917–27.
11. Fujitani T. Short-term effect of sodium benzoate in F344 rats and B6C3F1 mice. Toxicology Letters. 1993;69:171-179.

12. Vogt T, Landthaler M, Stolz W. Sodium benzoate-induced acute leukocytoclastic vasculitis with unusual clinical appearance. Archives of Dermatology. 1999;135:726-727.
13. Sodemoto Y. Enomoto M. Report of carcinogenesis bioassay of Sodium benzoate in rats: absence of carcinogenicity of sodium benzoate in rats. Journal of Environmental Pathology, Toxicology and Oncology. 1980;4:87-95.
14. Chipley JR. Sodium benzoate and benzoic acid. In: Branen AL, Davidson PM, eds. Antimicrobials in foods. New York, M. Decker. 1983;11-35.
15. European Commission. European Union Directive 95/2/CE from 20.02.1995 on food additives, colourants and sweeteners. European Commission; 1995
16. Kwiterovich PO. "The metabolic pathways of high-density lipoprotein, low-density lipoprotein, and triglycerides: a current review". The American Journal of Cardiology. 2000;86(12A):5L–10L.
17. Shekhar M., Pramod RS, Vankatesh KV. Control of cholesterol homeostasis by entero-hepatic bile transport – the role of feedback mechanisms. Royal society of chemistry. 2014;4:58964-58975.
18. Yakubu, M. T., Akanji, M. A. and Oladiji, A. T. (2008). Alterations in serum lipid profile of male rats by oral administration of aqueous extract of Fadogia agrestis stem. Research Journal of Medicinal Plants, 2:66–73.
19. Saurabh k, kalipada P. Sodium benzoate, a metabolite of cinnamon and a food additive , upregulates neuroprotective Parkinson disease protein DJ-1 in astrocytes and neurons. Journal of Immunology and Pharmacology. 2012;7(2):424–435.
20. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. Nature.1993;366:643–654.

21. Laufs U, Liao JK. Direct vascular effects of HMG-CoA reductase inhibitors. Trends in Cardiovascular Medicine. 2000;10:143–148.
22. Takai Y, Sasaki T, Matozaki T.Small GTP-binding proteins. Physiological Review. 2001;81:153–208.
23. Hancock JF, Cadwallader K, Marshall CJ. Methylation and proteolysis are essential for efficient membrane binding of prenylated p21K-ras (B). EMBO Journal. 1991;10:641–646.
24. Kikuchi A, Williams LT. The post-translational modification of ras p21 is important for raf-1 activation. Journal of Biological Chemistry. 1994;269:20054–20059.
25. Garnovskaya MN, Van Biesen T, Hawe B, Casanas-Ramos S, Lefkowitz RJ, Raymond JR. Ras-dependent activation of fibroblast mitogen-activated protein kinase by 5-HT1A receptor via a G protein beta gamma-subunit-initiated pathway. Biochemistry. 1996;35:13716–13722.
26. Qiu RG, Chen J, Kirn D, McCormick F, Symons M. An essential role for Rac in Ras transformation. Nature. 1995;374:457–459.
27. Heymans S, Corsten MF, Verhesen W. Macrophage microRNA-155 promotes cardiac hypertrophy and failure. Circulation. 2013;128(13):1420–32.
28. Jana A, Pahan K. Oxidative stress kills human primary oligodendrocytes via neutral sphingomyelinase: implications for multiple sclerosis. Journal of Neuroimmune Pharmacology. 2007;2:184–193.
29. Jana A, Pahan K. Fibrillar amyloid-beta-activated human astroglia kill primary human neurons via neutral sphingomyelinase: implications for Alzheimer's disease. Journal of Neuroscience. 2010;30:12676–12689.

30. Kleinert H, Wallerath T, Fritz G, Ihrig-Biedert I, Rodriguez-Pascual F, Geller DA, Forstermann U. Cytokine induction of NO synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-κB-signaling pathways. Britain Journal of Pharmacology. 1998;125:193–201.

31. Liu X, Jana M, Dasgupta S, Koka S, He J, Wood C, Pahan K. (Human immunodeficiency virus type 1 (HIV-1) Tat induces nitric-oxide synthase in human astroglia. Journal of Biological Chemistry. 2002;277:39312–39319.

32. Pahan K, Jana M, Liu X, Taylor BS, Wood C, Fischer SM. Gemfibrozil, a lipid-lowering drug, inhibits the induction of nitric-oxide synthase in human astrocytes. Journal of Biological Chemistry. 2002;277:45984–45991.

33. Pahan K, Liu X, McKinney MJ, Wood C, Sheikh FG, Raymond JR. Expression of a dominant-negative mutant of p21ras inhibits induction of nitric oxide synthase and activation of nuclear factor-kappaB in primary astrocytes. Journal of Neurochemistry. 2000;74:2288–2295.

34. Yang J.Cardiac-specific overexpression of farnesyl pyrophosphate synthase induces cardiac hypertrophy and dysfunction in mice. Cardiovascular Research. 2013;97:490-499.

35. Yang J.Inhibition of farnesyl pyrophosphate synthase attenuates angiotensin II-induced cardiac hypertrophy and fibrosis in vivo. International Journal of Biochemistry and Cell Biology. 2013;45:657–666.

36. Kuusisto J, Kärjä V, Sipola P. Low-grade inflammation and the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy. Heart. 2012;98(13):1007–13.

37. Erten Y, Tulmac M, Derici U, Pasaoglu H, Reis KA, Bali MN, Sindel S. An association between inflammatory state and left ventricular hypertrophy in hemodialysis patients. Renal failure. 2005;27(5), 581-589.
38. Hilfiker-Kleiner D, Shukla P, Klein G, Continuous glycoprotein-130-mediated signal transducer and activator of transcription-3 activation promotes inflammation, left ventricular rupture, and adverse outcome in subacute myocardial infarction. Circulation. 2010;122(2):145–55.
39. Hirota H, Yoshida K, Kishimoto T, Taga T. Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice. Proceedings of the National Academy of Science, USA. 1995;92(11):4862–66.
40. Velten M, Duerr GD, Pessies T. Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice. Cardiovascular Research. 2012;96(3):422–32.
41. Heymans S, Hirsch E, Anker SD.Inflammation as a therapeutic target in heart failure? A scientific statement from the Translational Research Committee of the Heart Failure Association of the European Society of Cardiology. European Journal of Heart Failure. 2009;11(2):119–29.
42. Brahmachari, S., Jana, A., & Pahan, K. (2009). Sodium benzoate, a metabolite of cinnamon and a food additive, reduces microglial and astroglial inflammatory responses. *The Journal of Immunology*, *183*(9), 5917-5927.

363

364

365