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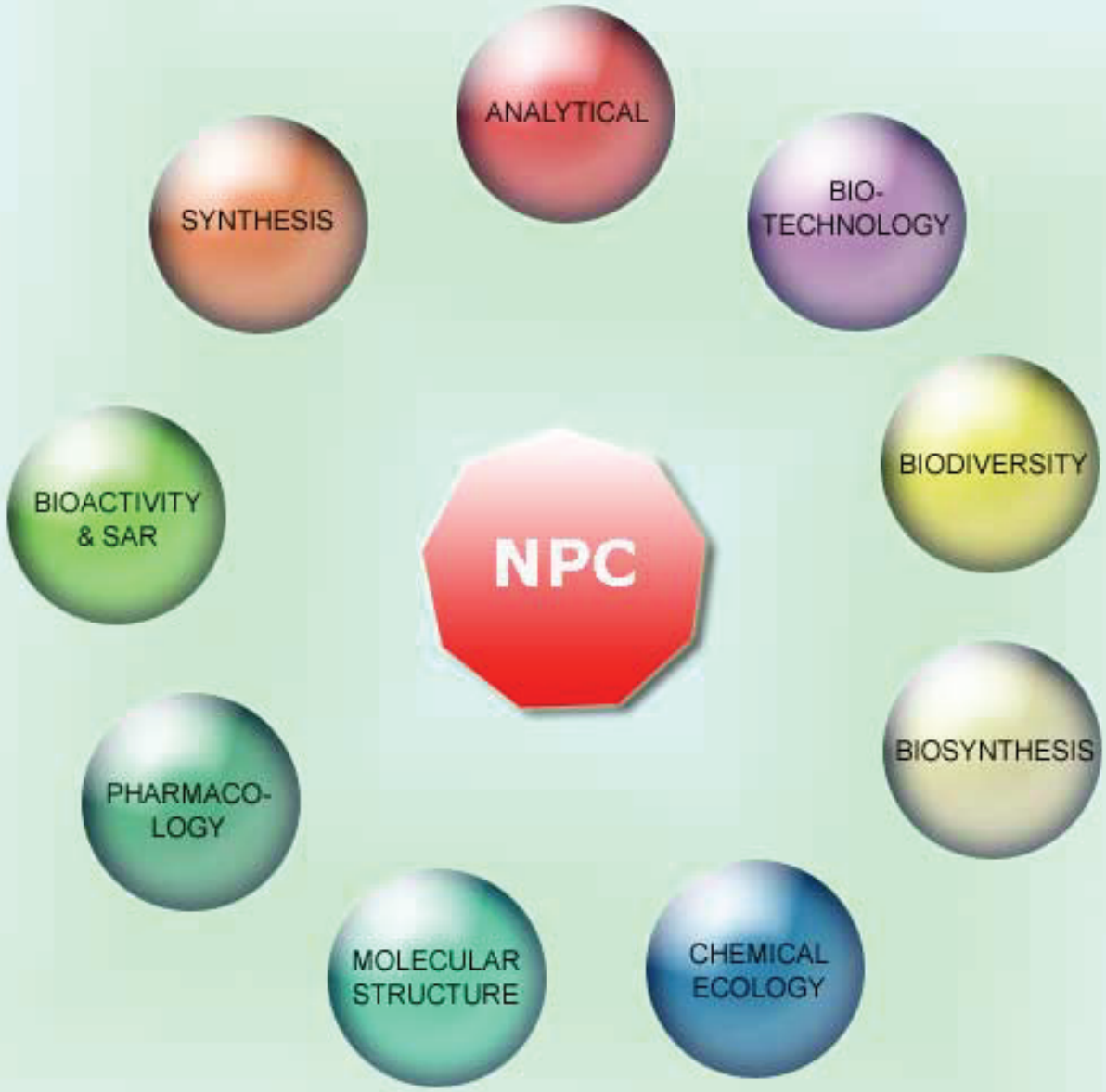

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Polyphenol Compounds Melanin Prevented Hepatic Inflammation in Rats with Experimental Obesity

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Melanin produced by yeast *Nadsoniella nigra* strain X-1 lead to significant reduction of steatosis, lobular inflammation and ballooning degeneration, according to NAFLD activity score (NAS), in liver of rats with monosodium glutamate (MSG) induced obesity. These histological changes were associated with substantial decrease of TNF-α expression in sinusoid cells that prevented NF-kB activation in hepatocytes.

Keywords: Polyphenols, Melanin, Obesity, Non-alcoholic fatty liver disease, Non-alcoholic steatohepatitis, Antioxidants.

Approximately 1.7 billion people in the world suffer from being overweight, most notably in developed countries. The number of which has more than doubled in children and quadrupled in adolescents in the past 30 years [1-3]. Obesity increase the risk for number of diseases, namely, cardiovascular diseases, type 2 diabetes, dyslipidemia, premature death, non-alcoholic fatty liver disease (NAFLD) as well as different types of cancer [4]. NAFLD is currently being in strict focus of the scientific community because of its increasing prevalence and complicated pathogenesis represented growing challenge in terms of prevention and treatment. The disorder has a prevalence of 15–20% in the general population and 76–90% in the obese [5]. NAFLD is currently a leading cause of chronic liver disease [6,7], which has resulted in significant health concerns such as morbidity, mortality, and liver transplants [8]. High clinical significance and complicated mechanisms of pathogenesis contributes to growing forces of scientist to fulfil the gaps in the understanding of NAFLD. However, currently the exact pathogenetic mechanisms involved in NAFLD, remain incompletely understood. Therapy is manly based on lifestyle modifications to achieve weight loss, health diet, and physical activity [9], on the use of omega-3 fatty acids to reduce hepatic fat accumulation [10-11]; antioxidants [12] and probiotics supplementation [13,14] for liver damage, and to treat the associated metabolic conditions to NAFLD. Despite of this a lot of obese people do not improve their health and the search of nontoxic anti-obesity drugs is still urgent.

Cell culture, animal, and limited human studies suggest that consumption of foods containing certain polyphenols or their corresponding supplements changes lipid and energy metabolism and may facilitate weight loss and prevent weight gain. [15, 16]. Evidence from pre-clinical and some clinical studies indicates that consumption of green and white teas containing catechins, fruits such as blueberries with anthocyanins, foods such as red grapes and wine with resveratrol, and spice like turmeric containing curcumin may provide several health benefits including improving blood glucose and lipid profiles, ameliorating insulin resistance, adiposity and obesity [17, 18]. Current knowledge suggests that the potential complementary effect of these polyphenols may occur through several mechanisms: suppression of fat absorption from the gut, uptake of glucose by skeletal muscles, suppression of anabolic pathways, stimulation of catabolic pathways in adipose tissues, liver and other tissues, inhibition of angiogenesis in adipose tissues, inhibition of differentiation of pre-adipocytes to adipocytes, stimulation of apoptosis of mature adipocytes, and reduction of chronic inflammation associated with adiposity [15].

In the previous study, we have showed the effects of exogenously administered melanin produced by yeast *Nadsoniella nigra* strain X-1 on the obesity parameters of rats and the development of NAFLD/NASH. It was shown significant decrease of mass indexes and fat accumulation in visceral adipose tissue of treated rats that suggests preventive influence of melanin on obesity. Such impact may be one of the cause of the interruption of the NAFLD development confirmed by the histological analysis of liver. It was registered substantial reduction of steatosis, lobular inflammation and ballooning degeneration in liver tissue in 4-month MSG-rats treated with melanin. Melanin reduced the content of IL-1 in rat serum and restored the level of anti-inflammatory cytokines (IL-10, TGF- β) to the control values. [19]. The present study was performed to investigate the anti-inflammatory mechanism of melanin produced by yeast *Nadsoniella nigra* strain X-1 by the expression of tumor necrosis factor α (TNF-α) and nuclear factor kappa of activated B cells (NF-κB) in rat liver with NAFLD/NASH.

In the control group, the structure of the hepatic lobule was normal, hepatic plates were arranged radially to the central vein. Hepatocytes demonstrated central round nuclei with nucleoli and oxyphilic cytoplasm. Sinusoids were arranged in regular pattern radially to the central vein. There were no any features of lobular inflammation or fibrosis.

MSG effects on proinflammatory pathways activation and NAFLD. In the MSG group, the structure of the hepatic lobules was different due to irregular distribution of hepatic plates and disarrangement of sinusoids. Hepatocytes varied in size and shape

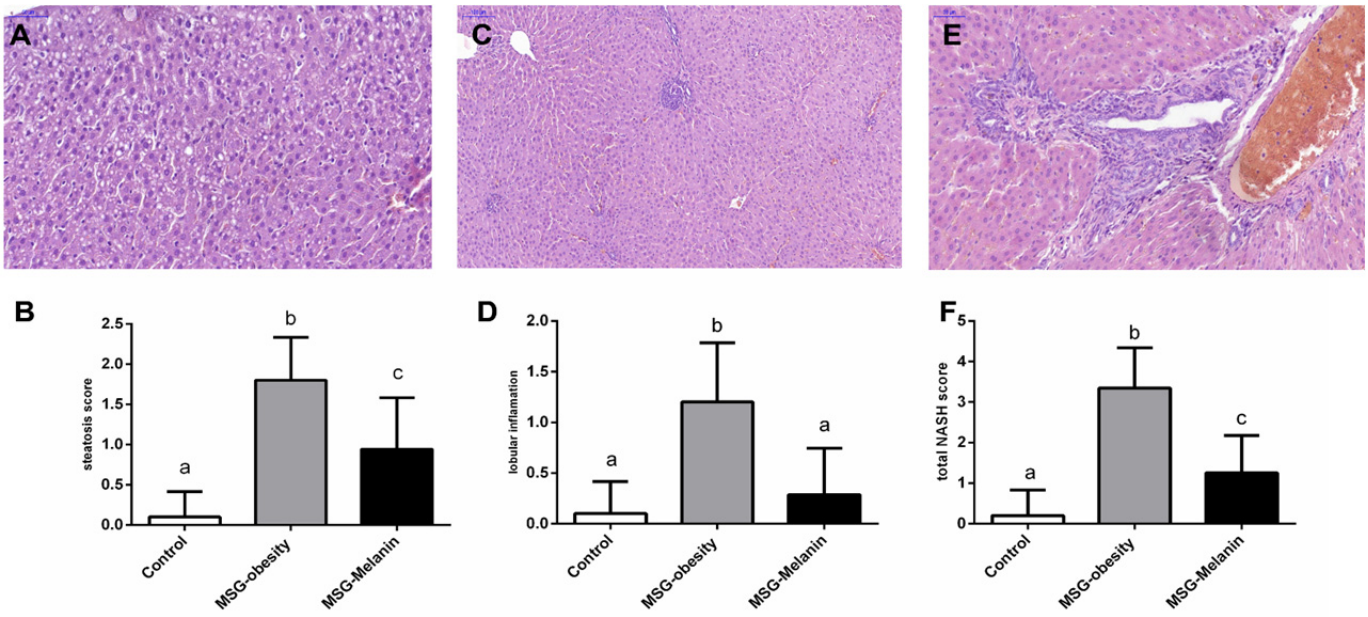

Figure 1: Histopathological changes in the liver of MSG animals, and attenuating effect of Melanin on NAFLD and NAS development. A, C, E – demonstrate steatosis, lobular inflammation and portal inflammation with fibrosis in MSG obese rats respectively. B – effect melanin on steatosis score, D – effect of Melanin on lobular inflammation score, F – effect of melanin on total NASH score. [a, b, c] Values at the same row with different superscript letters show significant differences at $p < 0.05$.

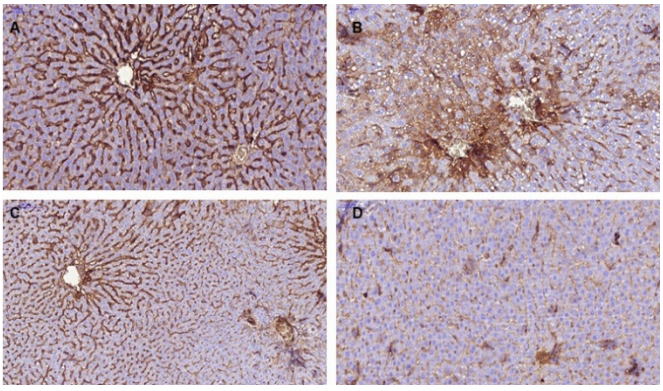

Figure 2: Number of CD68 positive cells (A and C) and TNF-α expression (B and D) in the liver of experimental rats. A – numerous CD68 cells in MSG group, B – high TNF-α expression in cells of sinusoids and hepatocytes of the liver in MSG group, C – CD68 cells in MSG + Melanin group, D – mild expression of TNF-α in the liver of MSG + Melanin group.

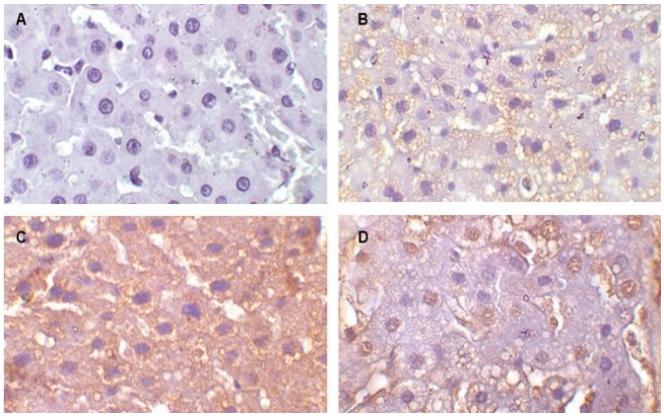

Figure 3: NF-kB expression in healthy (A) and experimental rats with MSG-induced obesity (B-D). Steatosis development was associates with mild to strong cytoplasmic expression of NF-kB. In addition, in about 40% of hepatocytes the nuclear expression of NF-kB was found, that was accompanied with hepatic plates disarrangement and sinusoidal cells immunopositivity. Immunohistochemistry with using monoclonal antibodies against NF-kB, ×400.

due to diffuse and numerous fat vacuoles of varying size (steatosis score was 1.8±0.17). Number of hepatic cells contained large fat vacuoles (Figure 1). Most of fat-rich hepatocytes were located at the periphery of hepatic lobules. In some animals, hydropic degeneration and inflammatory cells infiltration were observed (lobular inflammation score =1.20±0.17). The total NASH score reached 3.33±0.36 points. In addition to inflammation and ballooning degeneration the mild perivenular and perisinusoidal fibrosis associated with portal inflammation and fibrosis typical for NASH were found in 3 rats (33.3%). These changes were accompanied with increased number of macrophages in the liver.

Numerous CD68 positive cells covered almost the whole surface of sinusoidal capillaries that allowed to refer them to Kupffer cells. In addition, numerous CD68 positive cells were identified in periportal infiltrates. The very similar was the distribution of TNF-α positive cells. As macrophages and in particular Kupffer cells are considered demonstrate the role of TNF-α in NASH development. In addition, the moderate immunopositive reaction to TNF-α was found in some to be the main source of TNF-α production, our findings could hepatocytes in rats with NASH (Figure 2). High TNA-α expression correlated with NF-kB immunopositivity ($r=0.782$, $P < 0.001$). We found diffuse cytoplasmic reaction to NF-kB in hepatocytes and sinusoid cells. In addition, severe steatosis and cell injury were accompanied with nuclear expression of NF-kB in hepatocytes (Figure 3). Thus, neonatal MGS-induced obesity is associated with proinflammatory pathways activation in liver. The activation of NF-κB signaling pathway may cause NAFLD due to TNF-α overexpression in Kupffer cells.

Melanin effects on NAFLD development in MSG-obese rats. The administration of melanin provided ameliorating effect on liver structure significantly decreasing the degree of steatosis and preventing injury of hepatocytes. We established substantial attenuation of lobular inflammation in MSG rats treated with melanin ($p < 0.001$). The degree of steatosis in the conditions of

melanin administration was lower by 59.4% ($p < 0.01$) as compared to MSG-group. It was also found significantly lower total NASH score ($p < 0.001$).

These histological changes were associated with substantial decrease of TNF-α expression in sinusoid cells that prevented NF-kB activation in hepatocytes (table 1). Taking all together, these data confirmed the therapeutic impact of melanin on the MSG-induced NAFLD development.

Table 1: Impact of melanin on NF-kB and TNF-α expression in the liver.

	MSG	MSG + Melanin	P-value
TNF-α score	7.2±0.4	1.2±0.1	< 0,001
NF-kB score	3.5±0.3	1.6±0.1	< 0,001

NAFLD, which is a multi-factorial disorder associated with a variety of genetic and environmental contributory factors, is considered to be the most common cause of liver disease [20]. Whether the initial '2-hit hypothesis' or '3-hit hypothesis' is applied in determining the etiology, insulin resistance, oxidative stress and inflammatory cascades are believed to serve integral roles in the pathogenesis and progression [21]. The presence of steatosis induced by the over accumulation of free fatty acids and cholesterol is closely associated with chronic hepatic [19, 22], which is partly mediated by the activation of the inhibitor of the NF-κB kinase subunit β/NF-κB signaling pathway. In the present study, the results indicated that neonatal MGS-induced obesity is associated with proinflammatory pathways activation in liver. Histological analysis of liver micropreparations confirmed the development of NAFLD in rats. It was registered evidence of steatosis, lobular inflammation and ballooning degeneration in liver tissue in 4-month rats treated with MSG neonatally. We indicate the activation of NF-κB signaling pathway which cause NAFLD due to TNF-α overexpression in liver Kupffer cells of 4-month MSG-rats. TNF-α is a major proinflammatory cytokine and plays an important role in the development of NAFLD. Thus, activated Kupffer cells can increase the production of TNF-α, which may be responsible for NAFLD. Current studies have exhibited that TNF-α inhibition could decrease the level of hepatic fatty storage in mice on high fat diet [23]. In this study, the effects of TNF-α were evaluated by immunohistochemistry in the damaged liver. The administration of melanin significantly decreased the degree of steatosis and prevented injury of hepatocytes. These histological changes were associated with substantial decrease of NF-kB activation in hepatocytes. In conclusion melanin treatment up-regulated the expression of TNF-α, compared with the control group, and down-regulated the expression of TNF-α, compared with the MSG-NAFLD group.

Experimental

This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the general ethical principles of animal experiments, approved by the First National Congress on Bioethics Ukraine (September 2001). The rats were kept in collective cages in controlled conditions of temperature (22±3° C), light (12 h light/ dark cycle) and relative humidity (60±5%). The animals were fed laboratory chow (PurinaW) and tap water ad libitum.

There were 30 newborn Wistar male rats, divided into 3 groups: intact (n=10), MSG (n=10) and MSG + melanin treated (n=10). Newborns rats of intact group were administered with saline subcutaneously (s.c.) in the volume of 8 mL/g at 2nd, 4th, 6th, 8th and 10th postnatal days. Newborns rats of MSG-group and MSG + melanin group received a solution of MSG (4.0 mg/g of body weight) s.c. at 2nd, 4th, 6th, 8th and 10th days after birth [24-26]. Within 4 months after birth, rats had a normal diet. MSG + melanin group received aqueous solution of melanin in dose 1 mg/kg at volume 2.5 mL/kg per os (p.o.). Melanin was obtained from yeast-like fungi *Nadsoniella nigra* X1 strain from Ukrainian Antarctic station [27]. Melanin administration was started at the age of 4 weeks just after wean and continued for 3 months intermittently alternating two- week course of introduction with two-week course of break in dose 1 mg/kg dissolved in water (0.25 mL/100g) [19]. MSG-group respectively received 2.5 mL/kg of water (p.o.).

The impact of MSG on NAFLD development was assess by histological evaluation of the liver. After excision it was fixed in 10% formalin, embedded in paraffin and cut into 4 μm sections. The tissue sections were stained with hematoxylin and eosin. After that the histopathological changes in liver were observed and scored. Slides were evaluated histologically by 2 independent pathologists. NAFLD was interpreted according to widely recognized histopathological criteria: steatosis, lobular inflammation and ballooning degeneration of hepatocytes. The histological changes were assessed using NAS score:

- Steatosis was evaluated as 0 when <5% of cells demonstrated ectopic fat accumulation; 1 - if 5-33%; 2 when 34-66% and 3 if > 66% of cells were with fat droplets,
- Lobular inflammation was counted as following: 0 - none; 1 < 2 foci/20xfield; 2 if 2-4 foci/20x field; 3 when > 4 foci/20x field)
- Ballooning degeneration of hepatocytes (0 - none; 1 - few; 2 - many)

As low-grade inflammation is one of the leading mechanisms of liver lesion in obesity, the proinflammatory activation of liver cells was analyzed by immunohistochemical assessment of CD68 cells, NF-kB and TNF-α expression. CD68 cells number, NF-κB subunits (p50 and p65) and TNF-α expression was detected in formalin-fixed paraffin-embedded tissue sections according to standard immunohistochemistry techniques. The percent of NF-κB positive cells was semi-quantitatively scored on the basis of the percentage of positive cells as 0%=negative; 1–25% = 1+; 26–50% = 2+; and >50% = 3+. The intensity of NF-κB and TNF-α expression was scored as weak (1+), moderate (2+) and strong (3+). The immunohistochemistry index of NF-κB and TNF-α expression of each section was calculated as intensity multiplied by frequency and categorized as low (< 6) or high (> 6).

Statistical analysis performed by using SPSS-20 software. All data in this study were expressed as means ± standard deviation (M±SD) or %. Data distribution was analyzed using the Kolmogorov-Smirnov normality test. Continuous variables with parametric distribution were analyzed using Analysis of Variance (ANOVA) and if the results were significant, a post-hoc Turkeys test was performed. For data with non-parametric distribution Kruskall-Wallis and post-hoc Dunn's test were conducted for multiple comparisons. The difference between groups was defined to be statistically significant when a p-value was less than 0.05.

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