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Effect of vanillic acid and exercise training on fatty liver and insulin resistance in rats: Possible role of fibroblast growth factor 21 and autophagy

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ABSTRACT

Background and aims: The prevalence of non-alcoholic fatty liver disease has been alarmingly increased with no lines of effective treatment. Vanillic acid is a naturally occurring polyphenol with promising therapeutic effects. Exercise is well known to be an effective tool against obesity and its consequences. Thus, we aim to study the effect of vanillic acid alone and along with exercise on fatty liver induced by a high-fat diet in a rat model and to investigate possible novel mechanisms involved in their action. *Methods:* In this study, 40 male rats were divided equally into five groups: **control** (standard chow diet), **HFD** (high-fat diet), **HFD+VA** (HFD+ vanillic acid (50 mg/kg/day orally), **HFD+EX** (HFD+ swimming exercise 5 days/week), **HFD+VA+EX** (HFD+ vanillic acid+ swimming exercise) for eight weeks. *Results:* Body mass, liver weight, liver enzymes, cholesterol, and triglycerides were significantly decreased in the combined VA+EX group, with marked improvement in hyperglycemia, hyperinsulinemia, and consequently HOMA-IR index compared to the HFD group. These improvements were also reflected in the pathological view. VA and swimming, either solely or in combination, markedly increased hepatic and circulating fibroblast growth factor 21. Additionally, VA and swimming increased the immunohistochemical expression of the autophagosomal marker LC3 and decreased the expression of P62, which is selectively degraded during autophagy. *Conclusions:* These results suggest the hepatoprotective effect of VA and swimming exercise against fatty liver and the involvement of FGF21 and autophagy in their effect.

KEYWORDS

HFD, vanillic acid, swimming exercise, FGF21, autophagy

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INTRODUCTION

Due to the modern sedentary and food-abundant lifestyle, Non-alcoholic fatty liver disease (NAFLD) is now considered the most frequent liver condition worldwide, ranging from simple hepatic fat accumulation (steatosis) to the more progressive form (non-alcoholic steatohepatitis or NASH), which in turn may progress to fibrosis or even hepatocellular carcinoma and eventually liver failure. Alarmingly, the consequences of the fatty liver may grow into be the leading indication for liver transplantation [1].

NAFLD is closely associated with insulin resistance. The hepatokines and variations in their secretion explain this association. One of these hepatokines is fibroblast growth factor 21 (FGF21), which is a metabolic hormone with various beneficial effects on energy, glucose, and lipid homeostasis. FGF21 is shown to have a glucose-lowering effect as it induces glucose transporter 1 (GLUT1) expression in differentiated adipocytes and consequently increases glucose uptake in an insulin-independent mechanism [2]. Additionally, FGF21 decreases hepatic glucose production, possibly by affecting glucagon metabolism [3]. FGF21-deficient mice show an impairment of glucose homeostasis and weight gain. Moreover, FGF21- knockout (KO) mice fed a ketogenic diet demonstrate marked impairment in ketogenesis and develop hepatic steatosis [4].

Recently, autophagy has become a hot spot for research. It is considered the housekeeper of the cell, being responsible for getting rid of excess or defective organelles. Lipophagy (autophagy of lipid droplets) is shown to be a major pathway of lipid mobilization in hepatocytes [5]. Its inhibition has been linked to the development of fatty liver and insulin resistance. Furthermore, autophagy is found to be involved in the attenuation of liver injury and inflammation [6].

Presently, NAFLD is highly prevalent, with limited therapies, so identifying effective dietary strategies for prevention and treatment of the disease is much focused. Vanillic acid (VA, 4-hydroxy-3-methoxy-benzoic acid) is a phenolic derivative of edible plants and fruits. Also, it is an intermediate in the production of vanillin from ferulic acid. It has several therapeutic properties including antimicrobial activities [7], free-radical scavenging ability [8], antiobesity agent [9] and potential antidiabetic target [10].

Physical inactivity has been associated with increased NAFLD severity [11]. Among obese people, sedentary individuals have increased risks of fatty liver in comparison to weight-matched physically active individuals [12]. These data provide support for the hypothesis that increasing physical activity, through exercise, has beneficial effects on NAFLD. Theoretically, it is also a cheap intervention with both therapeutic and preventive value [13]. So the present work aims to study the effect of VA and exercise on fatty liver diseases and to investigate the possible involvement of FGF21 and autophagy in their mechanism of action.

MATERIALS AND METHODS

Experimental animals

6–8 weeks old, 40 male Sprague-Dawley rats, weighing 150–200 g were included in this study. The housing of animals was in the animal house of Medical Experimental Research Center (MERC), Mansoura University, Egypt. Temperature was maintained at around 23 °C under a 12/12 h light/dark cycle, with free access to water and under complete veterinary care. Before the

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study, we took the ethical approval of our local committee (Mansoura faculty of medicine institutional research board), on March, 15th 2019, code (PhD.19.03.19).

Experimental design

Rats were divided randomly into 5 groups (8 rats each and for 8 weeks): Control group: fed on a standard chow diet (48.8% as carbohydrate, 21% as protein, 3% as fat, fibers, minerals, and water) [14], the HFD group: fed on a high-fat diet (60.98% of fat: beef tallow, lard, corn oil, 14.47% of protein, 24.55% of carbohydrate) [15], HFD+VA group: fed on a high-fat diet + vanillic acid (50 mg/kg/day) [16], HFD+EX group: fed on a high fat-diet + swimming exercise, HFD+VA+EX: fed on a high-fat diet + vanillic acid (50 mg/kg/day) [16] + swimming exercise. The diet (standard and high-fat diet) was supplied by MERC. Vanillic acid (VA) was obtained from Sigma Chemical Co., USA, CAS number 121-34-6, product number H36001, dissolved in distilled water and administered by oral gavage daily for 8 weeks (1 mL each rat in the morning and before swimming exercise in exercise groups). Rats' body weight was measured weekly until the end of the study to determine weight gain. Food intake was also measured.

Exercise training protocol

Rats were submitted to swimming in a tank filled with 32 ± 1 °C water, measures 60 cm diameter × 80 cm height, 5 days/week for 8 weeks, starting with 5 min/day, which are gradually increased till reaching 30 min per session [17]. The rats were supervised to make sure that they are not touching the bottom or the walls of the tank, one rat at one time to avoid rats drowning each other. The animals were left to swim freely and were gently stimulated to swimming by stirring the water every now and then.

Animal euthanasia and collection of samples

After the eight weeks, overnight fasted rats were sacrificed by an overdose of thiopental anesthesia, blood samples were collected by cardiac puncture. Serum was obtained by centrifugation at 1000 R.P.M for 20 min, frozen, and stored at −20 °C till chemical analysis. Livers were rapidly excised and weighed, and then a fresh liver portion was rapidly dissected for fresh sectioning and Oil Red O (ORO) staining. A very small part of the liver was separated, weighted and kept frozen at −80 for liver FGF21 analysis. For histopathological examination and immunohistochemical analysis of LC3 & P62, the rest of the hepatic tissue was fixed in 10% formalin.

Assay of serum levels of liver enzymes, lipid profile, glucose, insulin, and HOMA index

Aspartate and alanine aminotransferases (AST, ALT) were determined following an enzymatic colorimetric method using a Randox reagent kit (Sigma–Aldrich, USA). Serum triglycerides and total cholesterol concentrations were measured using commercial kits (Spinreact, Spain). Serum glucose concentration was determined by the glucose oxidase method [18] using an enzymatic kit (bioMerieux). Serum insulin level was measured by ELISA insulin kits for rats (Sun-Red biology and technology, Shanghai), homeostatic model of assessment of insulin resistance (HOMA-IR index) = (Fasting glucose (mg/dL) × fasting insulin (mIU/L)) / 405 [19].

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Measurement of serum and tissue levels of FGF21

FGF21 concentration was measured in serum and hepatic tissue after being homogenized in 0.02 M buffer of sodium phosphate, of PH (7.4), and then centrifugation, at 3000 r. p. m at 4 °C, was performed for 20 min. The resulting supernatant was used for determining FGF21 in liver tissue by ELISA kit for rats (Sun-Red biology and technology, Shanghai) following the manufacturer's instructions.

Histopathological assessment

Serial hepatic sections were cut (5 μ) after being placed in 10% neutral buffered formalin, processed by the standard procedure for paraffin embedding and then, these serial sections were stained with hematoxylin and eosin (H&E), evaluated according to Brunt et al. [20] and examined blindly by a pathologist for macro, microvesicular steatosis, ballooning of hepatocytes and inflammatory cells and scored as follow: steatosis <5%, score 0; 5–33%, score 1; >33–66%, score 2; >66% score 3), lobular inflammation (overall assessment of all inflammatory foci, no foci, score = 0; <2 foci per × 200 magnification field, score 1; 2–4 foci per × 200 magnification field, score 2; >4 foci per × 200 magnification field, score 3) and hepatocellular ballooning (none, score 0; few balloon cells, score 1; numerous cells/prominent ballooning, score 2).

Oil red O stain (hepatic lipid)

Oil Red O staining was performed to examine hepatic fat, evaluated blindly by a pathologist. Oil red O positive area was quantified in 5 randomly selected fields per section. The percentage of the Oil Red O positive area was measured using a computerized image J analysis system.

Immunohistochemical analysis of LC3, P62 (autophagy markers)

The tissue sections were deparaffinized, rehydrated, washed, immersed in 3% H_2O_2, and then digested with pepsin for antigen retrieval. After blocking of unspecific binding by serum, the sections were incubated with polyclonal LC3 (cat# A5601, 1:150) and P62 (cat# A7758, 1:75) antibodies, purchased from Abclonal, USA, at 4 °C overnight. Diaminobenzidine/peroxidase substrate was used to produce a brown-colored signal. The section was counterstained, dehydrated, cleared, and cover slipped, evaluated blindly by a pathologist. For the semi-quantitative morphometric analysis, the numbers of LC3 and p62 positive cells were calculated in a blinded fashion and averaged for five fields per slide at a 100X magnification by using image J software.

Statistical analysis

Obtained data were represented as Mean ± SD or medians. Comparison for parametric data was done by the analysis of variance (ANOVA) followed by Tukey's post hoc analysis. $P < 0.05$ was considered significant. Non-parametric data, Mann Whitney and Kruskal – Wallis tests were used.

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RESULTS

Effect of vanillic acid and exercise training on body mass, liver weight, liver index, serum liver enzymes, cholesterol, and triglycerides

As shown in Table 1, the high-fat diet resulted in significant increases in the total body mass by (42%), liver weight (69%), liver index, liver enzymes (AST, ALT) (221%, 157%) respectively, cholesterol (171%), and triglycerides (124%) compared to the control group.

On administration of VA, or exercise training or combined VA and exercise training, body masses and liver weights significantly decreased as compared to the HFD group, while food intake remained unchanged, with no significant difference between HFD+VA and HFD+EX group, and with the most noticeable decrease among combined HFD+VA+EX group. Weekly changes in body weights among different studied groups are demonstrated in Fig. 1.

As regard liver enzymes, exercise-trained rats showed more improvement by (−46%, −41%) than the vanillic acid administered rats which showed (−18%, −27%) percent of change as compared to the HFD group.

On the contrary, in comparison to the HFD group, the vanillic acid effect on lipid profile (cholesterol, triglycerides) was more noticeable (−39%, −50%) than the exercise group (−35%, −38%) respectively. The combined approach of vanillic acid and exercise resulted in a more pronounced decrease in these parameters, liver enzymes by (−66%, −60%), lipid profile by (−60%, −53%) than each of them on its own, reaching values near the control group.

Effect of vanillic acid and exercise training on insulin resistance

This study also revealed that the high-fat diet for eight weeks in rats produced a significant elevation of the serum levels of glucose (96%), insulin (58%), and the HOMA-IR index (214%) compared to the Control group. The oral vanillic acid and the swimming exercise produced a

Table 1. comparison of body mass, liver weight, liver index, food intake, serum liver enzymes, cholesterol and triglycerides among different studied groups

$N=8$	Control	HFD	HFD+VA	HFD+EX	HFD+VA+EX
Body mass (gm)	220.0 ± 11.45	311.37 ± 33.42*	245.0 ± 27.09$^{\#}$	246.5 ± 44.42$^{\#}$	237.50 ± 8.86$^{\#}$
Liver weight (gm)	6.95 ± 0.37	11.78 ± 0.98*	9.11 ± 0.25$^{*\#}$	9.51 ± 0.60$^{*\#}$	7.26 ± 0.67$^{\#\$@}$
Liver index (%)	3.13 ± 0.23	3.75 ± 0.17*	3.71 ± 0.39*	3.68 ± 0.33*	3.46 ± 0.13$^{*\#}$
Food intake (gm/week)	105.88 ± 3.48	136.38 ± 4.07*	134.63 ± 3.42*	132.88 ± 2.75*	129.88 ± 1.73*
AST (U/L)	31.25 ± 3.73	100.38 ± 9.55*	82.25 ± 3.73$^{*\#}$	54.50 ± 6.91$^{*\#\$}$	34.63 ± 5.07$^{\#\$@}$
ALT (U/L)	35.88 ± 4.22	92.38 ± 7.59*	67.38 ± 3.78$^{*\#}$	54.50 ± 2.98$^{*\#\$}$	36.88 ± 4.12$^{\#\$@}$
Cholesterol (mg/dL)	49.75 ± 8.89	134.88 ± 15.97*	81.63 ± 4.37$^{*\#}$	87.0 ± 7.48$^{*\#}$	54.25 ± 8.01$^{\#\$@}$
TG (mg/dL)	47.25 ± 5.28	106.0 ± 7.76*	52.63 ± 7.23$^{\#}$	65.25 ± 4.86$^{*\#\$}$	49.37 ± 4.68$^{\#@}$

One Way ANOVA test was used, all parameters described as mean ± Standard deviation, within group significance detected by post HOC Tukey test, $P < 0.05$ was considered significant. *significant difference with control group, # significant difference with HFD group, $ significant difference with HFD+VA group, @ significant difference with HFD+EX group, $n = 8$ rats per group.

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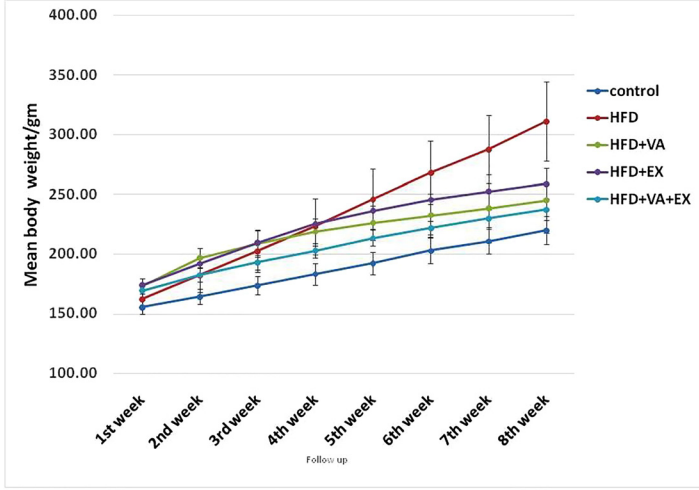

Fig. 1. weekly changes in body weight among different studied groups ($n = 8$ per group)

significant reduction in the previously mentioned parameters compared to the HFD group, exercise-trained rats showed more improvement (−20%, −20% and −37%) in glucose, insulin, and HOMA-IR index respectively, than the vanillic acid administered rats which revealed (−13%, −16% and −28%) percent of change, while the combined approach showed the most improvement by (−49%, −34% and −66%) respectively in comparison to the HFD group, as shown in Table 2.

Effect of vanillic acid and exercise training on circulating and hepatic levels of FGF21

As compared to the HFD group, serum and hepatic levels of FGF21 increased by (37% and 33% respectively) with vanillic acid intervention, the exercise-trained group revealed 73% and 46% increases of serum and hepatic levels of FGF21. Lastly, the combined vanillic acid and exercise group revealed more increase in the serum and hepatic levels by 109% and 69% (Fig. 2).

Effect of vanillic acid and exercise training on the histopathological view

As for the pathological view Fig. 3, vanillic acid, exercise training, and their combination resulted in marked improvement with a significant decrease in the fatty infiltration of the

Table 2. comparison of serum glucose, insulin and HOMA IR index among different studied groups

	Control	HFD	HFD+VA	HFD+EX	HFD+VA+EX
Glucose (mg/dL)	77.13 ± 7.34	151.38 ± 8.41*	131.38 ± 3.99$^{*\#}$	121.56 ± 4.25$^{*\#\$}$	77.5 ± 7.56$^{\#\$@}$
Insulin (mIU/L)	9.29 ± 0.72	14.70 ± 0.33*	12.30 ± 0.35$^{*\#}$	11.81 ± 0.38$^{*\#}$	9.74 ± 0.69$^{\#\$@}$
HOMA IR	1.75 ± 0.23	5.49 ± 0.39*	3.97 ± 0.17$^{*\#}$	3.47 ± 0.12$^{*\#\$}$	1.85 ± 0.26$^{\#\$@}$

One Way ANOVA test was used, all parameters described as mean ± Standard deviation, within group significance detected by post HOC Tukey test, $P < 0.05$ was considered significant. *significant difference with control group, # significant difference with HFD group, $ significant difference with HFD+VA group, @ significant difference with HFD+EX group, $n = 8$ rats per group.

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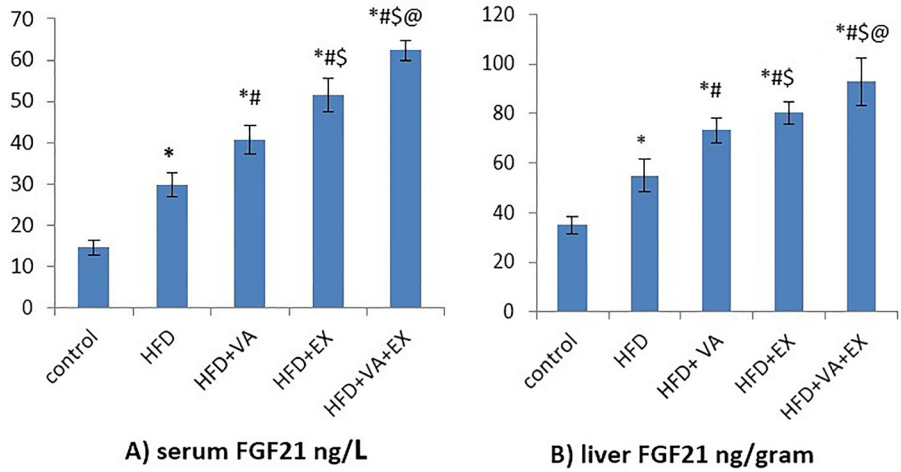

Fig. 2. Levels of circulating (A) and hepatic FGF21 (B) in different studied groups. *significant difference with control group, # significant difference with HFD group, $ significant difference with HFD+VA group, @ significant difference with HFD+EX group. The data are presented as the mean ± SD, $n = 8$ rats per group. $P < 0.05$ considered significant

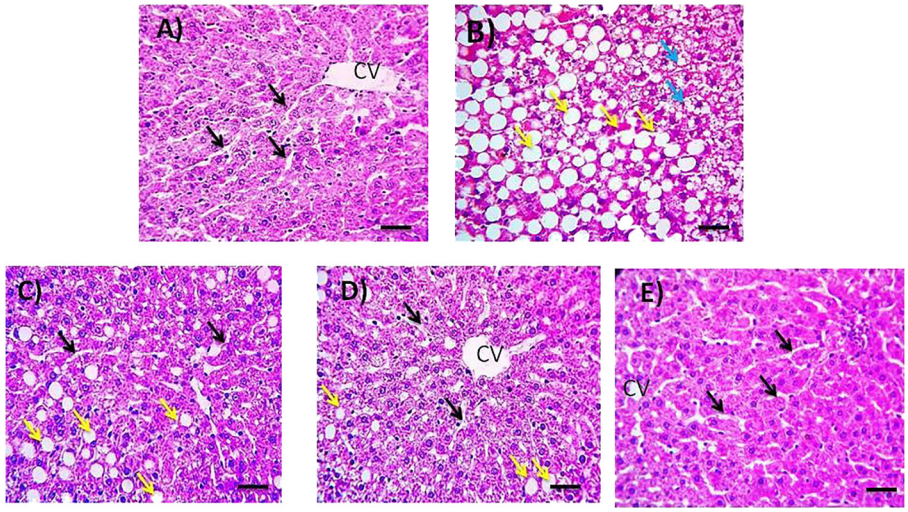

Fig. 3. Representative microscopic pictures of H&E stained hepatic sections showing normal arrangement of hepatic cords around central vein (CV) with normal portal areas and sinusoids (black arrows) in control group **(A)**. Hepatic sections from HFD group **(B)** showed diffuse micro- (blue arrows) to macrovesicular (yellow arrows) hepatic steatosis which is attenuated by vanillic acid treatment **(C)**, few fat vacuoles appear in hepatic cords from exercised group **(D)** and almost disappeared in combined vanillic acid and exercise group **(E)**. Magnification X400, bar 50 µM

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Table 3. comparison of NAFLD activity score (NAS) among different studied groups

	Control	HFD	HFD+VA	HFD+EX	HFD+VA+EX
NAS	0.0 ± 0.0	6.12 ± 0.64*	4.75 ± 0.71$^{*\#}$	3.63 ± 0.52$^{*\#\$}$	2.25 ± 0.71$^{*\#\$@}$

One Way ANOVA test was used, all parameters described as mean ± Standard deviation, within group significance detected by post HOC Tukey test, $P < 0.05$ was considered significant. *significant difference with control group, # significant difference with HFD group, $ significant difference with HFD+VA group, @ significant difference with HFD+EX group, $n = 8$ rats per group.

hepatocytes, inflammatory cells, and ballooning, reflected on NAS as shown in Table 3 and also a significant decrease in the percentage of ORO positive area as compared to the HFD group with the most improvement in the combined group (Fig. 4).

Effect of vanillic acid and exercise training on immunohistochemical expression of autophagic markers (LC3, P62)

HFD group showed decreased immunohistochemical expression of LC3 positive cells which then is robustly increased by vanillic acid and exercise training intervention (Fig. 5), also the number of P62 positive cells showed a marked increase in HFD group due to lack of consumption, but with the intervention groups P62 positive cells starts to decrease again with the greatest decrease among combined vanillic acid and exercise group reaching near-normal levels (Fig. 6).

DISCUSSION

This study is considered as a trial on the path of finding an effective defense against NAFLD, whose prevalence has been alarmingly increasing worldwide because of obesity and sedentary lifestyle. We tried a naturally occurring polyphenol (vanillic acid) alone and combined it also with exercise training (swimming) in a rat model, fed a high-fat diet for 8 weeks. Our study (up to our knowledge) is the first study to try the combined approach of vanillic acid and exercise training against NAFLD in a rat model, fed a high-fat diet. The use of HFD resulted in preclinical models which mimic metabolic and histological aspects of NAFLD in humans, even though being time-consuming [21]. In agreement with Andrade et al. [15], HFD for eight weeks resulted in increasing body mass, liver weight, liver enzymes, and lipid profile, also histopathologically resulted in marked hepatic fatty infiltration. Along with fatty liver changes, there was also evidence of developing insulin resistance (hyperglycemia, hyperinsulinemia, and increased HOMA-IR index). The relationship between steatosis and insulin resistance is more of a vicious cycle, in which systemic insulin resistance leads to hepatic steatosis (increased lipolysis and Free fatty acids flux to the liver), and hepatic steatosis then leads to an exacerbation of hepatic insulin resistance [22].

Vanillic acid is a phenolic acid with antimicrobial, antioxidant, and anti-cancerous properties [23]. Jung and his colleagues [9] concluded that vanillic acid through activation of the AMP-activated protein kinase (AMPK) pathway and other thermogenic factors, is considered as a promising anti-obesity agent. In agreement with these results, VA indeed resulted in

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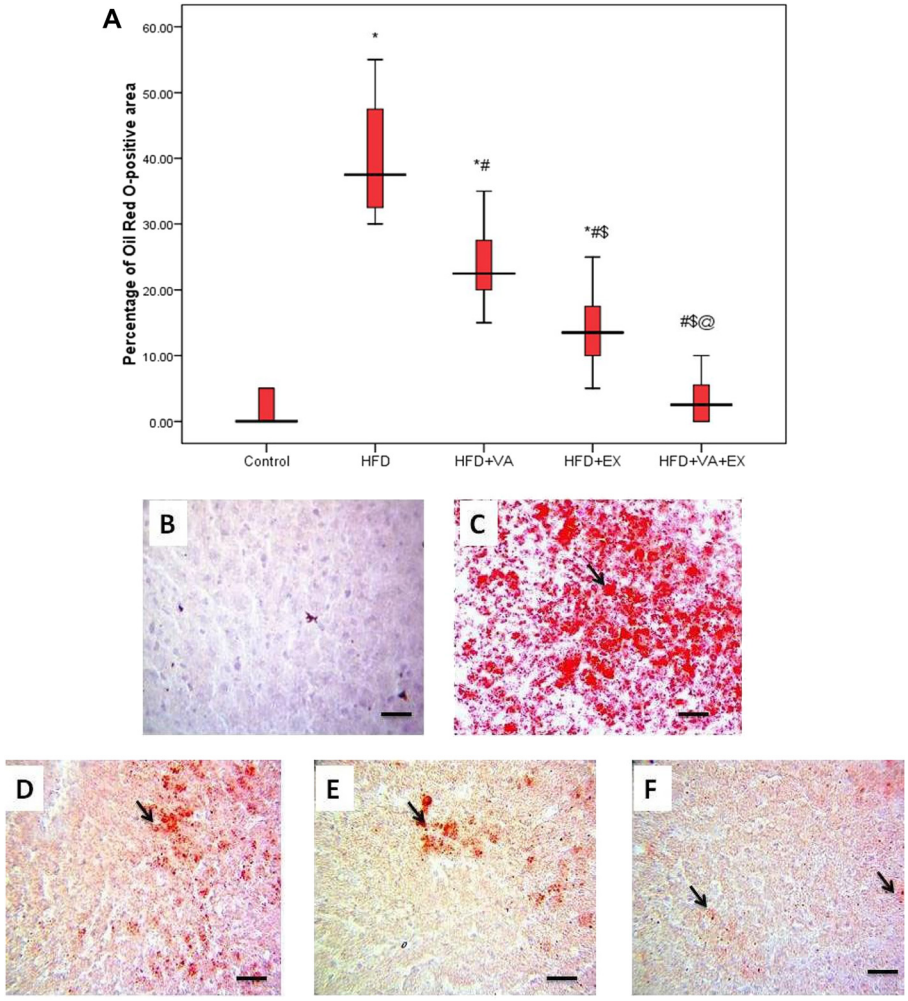

Fig. 4. **A)** Box and whisker plot showing quantification of the percentage of Oil Red O positive area, horizontal lines represent medians and whiskers represent minimum and maximum values. Within group significance detected by Mann Whitney U test.
*significant difference with control group, # significant difference with HFD group, $ significant difference with HFD+VA group, @ significant difference with HFD+EX group. $n = 8$ rats per group. Microscopic pictures of oil red O stained fresh frozen hepatic sections showing almost no fat deposition in control group **(B)**. Hepatic sections from HFD group **(C)** showed orange-red stained diffuse fat deposition (black arrows) which is moderately attenuated by vanillic acid treatment **(D)**, markedly attenuated by exercise **(E)**. The hepatic sections of the combined vanillic acid and exercise group **(F)** showed very few orange-red stained fat droplets and near normal hepatocytes. Magnification X400, bar 50 μM

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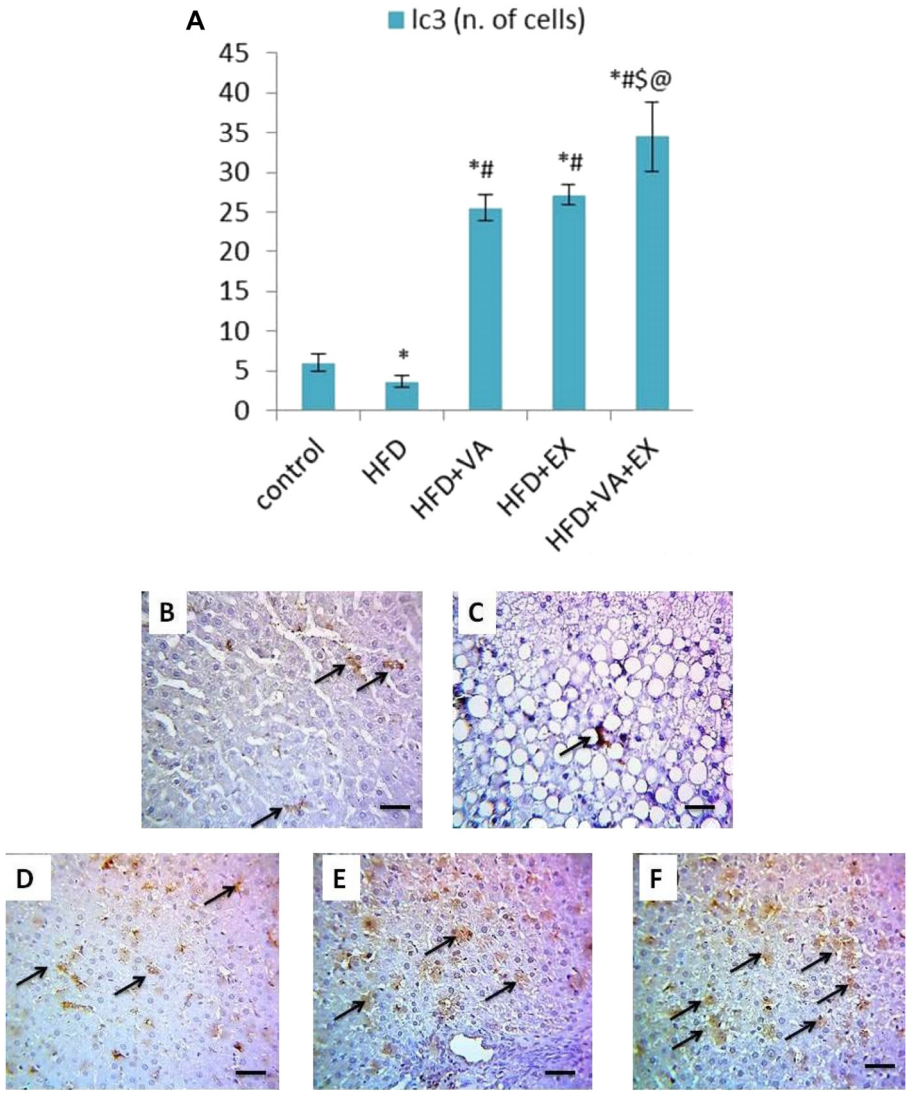

Fig. 5. **A)** quantification of the number of LC3 positive cells among different groups. The data are expressed as means ± SE. *significant difference with control group, # significant difference with HFD group, $ significant difference with HFD+VA group, @ significant difference with HFD+EX group, $n = 8$ rats per group. Microscopic pictures of immunostained hepatic sections against LC3 showing significantly reduced numbers of positively stained cells with LC3 (black arrows) in HFD group **(C)** when compared with the control group **(B)**. Numbers of positively stained cells with LC3 slightly increased in hepatic sections from vanillic acid treatment **(D)**, markedly increased by exercise **(E)** and lastly, the hepatic sections of the combined vanillic acid and exercise group **(F)** showed the most increase in the number of LC3B positive cells. Magnification X: 400 bar 50 μM

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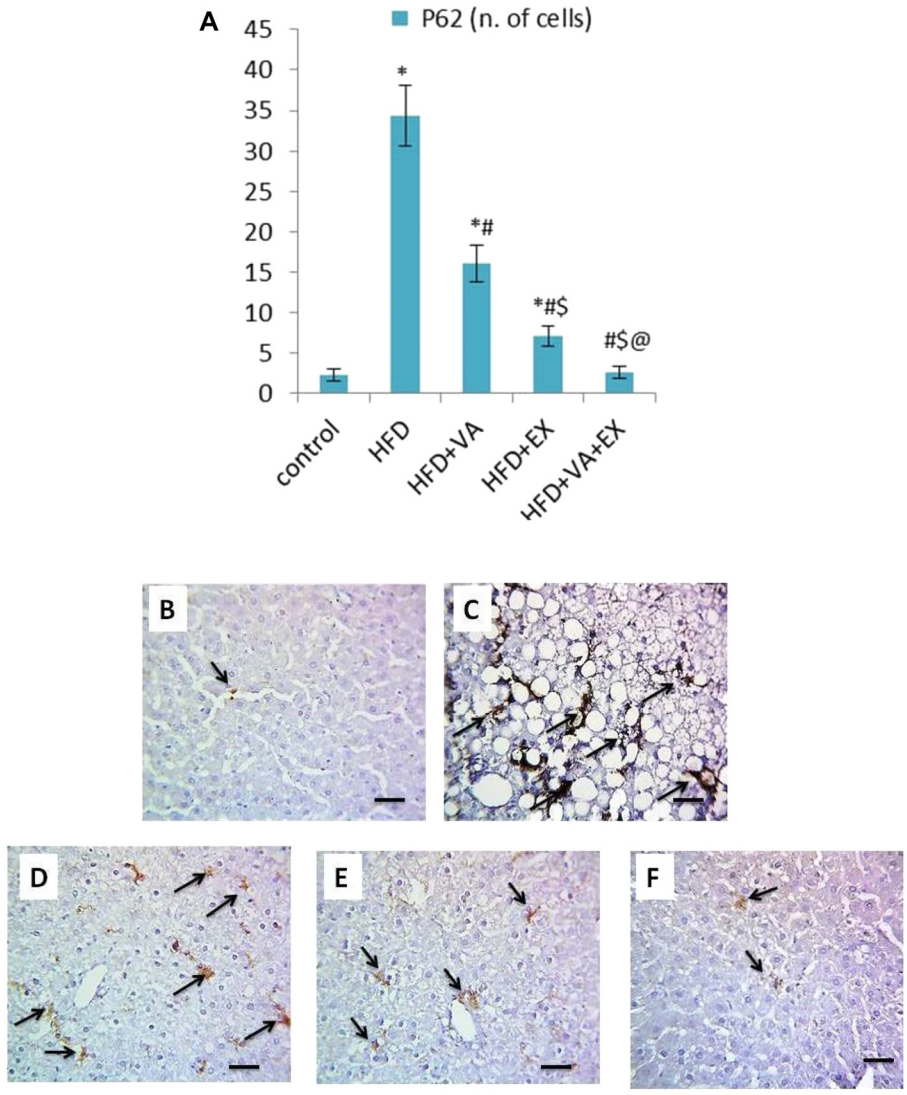

Fig. 6. **A)** quantification of the number of P62 positive cells among different groups. The data are expressed as means ± SE, *significant difference with control group, # significant difference with HFD group, $ significant difference with HFD+VA group, @ significant difference with HFD+EX group, $n = 8$ rats per group. Microscopic pictures of immunostained hepatic sections against P62 showing significantly increased positive brown p62 protein expression (black arrows) in HFD group **(C)** when compared with control group **(B)**. Numbers of positively stained cells with p62 slightly decreased in hepatic sections from vanillic acid group **(D)**, markedly decreased by exercise **(E)**. In the hepatic sections of the combined vanillic acid and exercise group **(F)**, very few positively stained cells with p62 are seen. Magnification X: 400 bar 50 μM

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significant decreases in the body mass and liver weight, serum cholesterol, and triglycerides of rats fed HFD for eight weeks. As for the HFD+EX group, there was a significant decrease in their body mass and liver weight and also in their lipid profile when compared to the HFD group. In adipose tissue, exercise training, through modulation of adipokine secretion, lowers lipids and inflammatory markers and regulates browning and thermogenesis [24, 25]. It's a low-cost natural multi pill for prevention and treatment of obesity-related illnesses which affect the functions of multiple organs, including adipose tissue, skeletal muscle, heart, brain, and others [26].

Our results elucidated that rats fed HFD, administered VA on daily basis, and exposed to swimming exercise five days/week, act synergistically not only as antiobesity, but also they almost normalized glucose, insulin, liver enzymes levels in rats fed HFD. Also, the combination of vanillic acid and exercise training has proven to have a more pronounced hepatoprotective effect (than each of them on its own) against high-fat diet induced NAFLD in rats, as evidenced by the marked improvement in the pathological view shown by the robust decrease in lipid accumulation (by the ordinary H&E stain and also by oil red o stain).

Since the hepatokine FGF21 has recently emerged as a shining armor against NAFLD and insulin resistance, it also raised a debate about its paradoxical increase in obesity and NAFLD [27], whether this increase is a compensatory protective release or a state of resistance has occurred. We measured its levels in serum and liver tissue, we found that its level increases in harmony with the improvement that has occurred using vanillic acid and exercise training, suggesting it's more of a protective compensatory increase. As we said it is debatable, as some studies agreed with our results [28] that dietary betaine supplementation, increases FgF21 levels, improves glucose homeostasis and reduces hepatic steatosis in HFD-fed mice. The results of the present work were also in agreement with those of Sun et al. [29], who demonstrated that berberine attenuates hepatic steatosis through increasing FGF21 in mice. On the contrary, long-term curcumin administration to rats fed HFD resulted in attenuating FGF21 production but increases the expression of its receptors [30], these conflicting results might be explained by the duration of the model.

The present work also investigated the involvement of autophagy in the mechanism of action of vanillic acid and exercise training, since the impairment of autophagy has been proven to contribute to the pathogenesis of NAFLD [31], as the selective autophagy pathways; lipophagy (for excess lipids) and mitophagy (for dysfunctional mitochondria) play an essential role in energy homeostasis and lipid metabolism, defective autophagy causes cells to accumulate aberrant mitochondria and surplus lipids, leading to bioenergetics failure and impaired lipid homeostasis [32]. We studied the immunohistochemical expression of two important autophagy markers; LC3 (formed during the process of autophagosome formation) and P62 (which is selectively consumed with the progression of the autophagy process). In our study, HFD resulted in attenuation of the number of LC3 positive cells and on the other hand, increased the number of P62 positive cells in agreement with Ohashi et al. [33], as the increased fat content changes the composition of lipid in the membrane and decreases the fusion between autophagosome and lysosome, which may partly explain why increased fat content impairs autophagy and consequently aggravates lipid accumulation [34]. The usage of vanillic acid along with exercise training has a positive impact on the autophagy process as evidenced by the increased number of cells stained positive for LC3 and decreased P62 positive cells. Whether this improvement is a direct effect of vanillic acid and exercise training or an indirect effect of the decrease of the

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hepatic fat content, is unknown and needs further studies. Also, further studies on obesity models of different durations are needed to investigate the levels of FGF21, and inspection of the state of its receptors needed to be included in our perspectives. Moreover, the linkage between vanillic acid and the induction of autophagy needs further studies on the mechanistic pathways.

CONCLUSIONS

Our data suggest that the combined strategy of vanillic acid and exercise training, through induction of FGF21 and autophagy, could be a useful tool in combating fatty liver and insulin resistance associated with obesity. This study might open new insight into the linkage between vanillic acid (a naturally occurring polyphenol), exercise (easy, cheap, and non-invasive tool) and the induction of FGF21 and autophagy which could be applied widely in different therapeutic fields.

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