

Meliorative Impact of Daphnetin on Hepato- and Neuro- Toxicity Induced by Acrylamide

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Abstract

Background: Cooking carbohydrate-rich diet produce acrylamide chemical (ACR) that exerts a hepatotoxic and neurotoxic effects. Daphnetin (DAPH), a coumarin derivative, has been reported for its potential therapeutic effects.

Aim of Study: To evaluate the effect of DAPH on hepato- and neurotoxicity of ACR.

Material and Methods: We investigated the effect of DAPH on the hepatic and neural impairment caused by exposure to ACR (40mg/kg) for 15 days in mice. Liver function tests and oxidative stress markers were estimated. Further, the expression level of tumor necrosis factor alpha (TNF- α), hemeoxygenase-1 (HO-1) and caspase-3 activity were assessed in brain and liver tissues.

Results: The administration of DAPH (20 mg/kg) to ACR-intoxicated mice significantly decreased the serum levels of transaminases; with improving the protein and albumin content impairment of liver caused by ACR. The level of malondialdehyde (MDA) significantly decreased in the brain tissue of mice treated with DAPH after the drastically increase of MDA caused by ACR intoxication. DAPH administration down regulate Caspase 3 and TNF- α after their up-regulation in the ACR group while DAPH administration up-regulate HO-1 after the significant down-regulation by ACR.

Conclusion: In conclusion, our results highlight that exposure to DAPH assuaged ACR-induced oxidative stress, inflammation and caspase-3 activation in liver and brain of mice. Thus, DAPH could be effective in reducing hepato- and neuro-toxicity due to its strong antioxidant, anti-inflammatory and antiapoptotic properties.

Key Words: Acrylamide – Daphnetin – Tumor necrosis factor alpha – Hemeoxygenase-1 – Caspase-3.

Introduction

ACRYLAMIDE (ACR) is one of the most common toxins in food. It occurs in food containing high concentrations of hydrocarbons subjected to high temperature [1]. ACR is formed during cooking

with very high temperatures, and this is due to the reaction between the amino acid asparagine and a carbonyl-containing source [2]. High concentration of ACR may be found in food products such as potato chips, fried potatoes, cornflakes or bread. Thus, acrylamide is present in everyday diet of most people [3]. ACR is used widely in the industrial production as synthesis of the polyacrylamide, dye synthesis and cosmetic manufacturing [4].

Toxic effects of acrylamide are mediated by the formation of genotoxic metabolites, oxidative stress, affected propagation of neural signals, ultrastructural and histological defects in central neural system [5]. The discovery that some cooked foods contain acrylamide in 2002 has attracted significant attention to its possible biological effects, and shown the need for further research into neurotoxicity, hepatotoxicity and reproductive harm, but primarily carcinogenicity [6].

At low doses, 50% of acrylamide is metabolized to DNA reactive metabolite, named 'glycidamide' by cytochrome 2E 1 (CYP2E 1)-mediated epoxidation. However, this conversion is saturable and at highdoses only 13% of acrylamide is bio-transformed to glycidamide [7]. Glycidamide can be metabolized by epoxide hydrolase and both acrylamide and glycidamide can undergo conjugation with glutathione (GSH) [8]. The results of different studies support the concept of acrylamide by itself not acting as a genotoxic agent at exposure levels to be expected from food ingestion. Metabolic formation of glycidamide is essential for genotoxic effectiveness [9]. Thus, glycidamide acts like the ultimate genotoxic metabolite of acrylamide and it was found to form DNA adducts and induce micronuclei in different organs of mice [10]. Acrylamide is a well-recognized potent neurotoxin affecting both the central nervous system (CNS) and peripheral nervous system (PNS). The neurotoxic prop-

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erties of acrylamide have been studied because these are the only toxic effects that have been shown both in humans related to occupational exposure and in experimental animal studies [11].

As a compound with high water solubility, ACR could be easily diffused and transferred throughout the body organs including the liver and the kidney. Moreover, the capability of ACR to form adducts with hemoglobin contributes to its accumulation in these organs and subsequent damage [12].

Daphnetin (DAPH) is one of main extract from *D. marginate* [13]. Structurally, DAPH is a coumarin derivatives with a cell-permeable property [14]. Similar to other coumarin derivatives, DAPH has been reported to have many pharmacological actions, including anti-inflammatory, anti-oxidative and anti-tumor effects [15]. It has been reported that daphnetin ameliorated oxidative stress-induced hepatotoxicity via activating the expression of the cellular antioxidant enzyme, heme oxygenase-1 (HO-1) [16]. Overexpression of HO-1 protects dopaminergic neurons against induced neurotoxicity [17].

Therefore, the current study was designed to explore the potential protective effects of Daphnetin against ACR induced hepatotoxicity and neurotoxicity in mice.

Material and Methods

Chemicals:

Acrylamide and 7,8-dihydroxycoumarin 90% (Daphnetin; DAPH) were purchased from Sigma-Aldrich, Inc. (St. Louis, USA). All other chemicals were of analytical grad.

Animals:

Male Swiss mice weighing between 25 and 30mg bred in animal house of Faculty of Medicine, Ain Shams University were used (during October 2020). The mice were kept under conditions of 12 hour slight/dark cycle at $22\pm 30^{\circ}\text{C}$ with free access to food and water. The study was done in agreement with the Ethical Committee Acts and Guidelines of Ain Shams University, which meet the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care.

Experimental design:

Forty Swiss mice were randomly divided into four groups with ten animals per each group. All treatments started at the same time and lasted for

the specified duration for each chemical. These groups are as follow:

Group I: Normal control group received intraperitoneal (i.p.) normal saline for 15 days. Group II: Mice received ACR (40mg/kg b.w. in physiological saline) i.p for 15 days according to Kopanska et al., [18]. Group III: Mice received DAPH via intraperitoneal injection (20mg/kg) for 15 days diluted in 0.9% saline according to Liu et al., [19]. Group IV: Mice received DAPH via intraperitoneal injection (20mg/kg) along with ACR as in group II for 15 days.

At the end of the experiment, mice were sacrificed by carotid bleeding under ether anesthesia. Blood samples were collected and centrifuged (1,000 xg, 15min, 4°C) to separate the sera.

The livers and brains of all groups were removed, immersed in ice-cold phosphate buffered-saline pH 7.4 (PBS), minced and washed with the same solution. The mince was homogenized [in a ratio of 1gm of wet tissue to 10 times (w/v) 0.05 M ice cold PBS (pH 7.4)] with a Teflon / glass homogenizer. The homogenate was centrifuged (1,500 xg, 15min, 4°C) and the supernatants were collected and stored at 20°C till use.

Biochemical assays:

Liver enzymes including ALT and AST, serum total protein and albumin were measured by Bio diagnostic kit.

Assessment of oxidative stress markers:

Lipid peroxidation was estimated in brain supernatants the amount of thiobarbituric acid reactive substances (TBARS) determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) as described by Ohkawa et al., [20]. Total antioxidant activity (TAA) was determined according to the method of Koracevic et al.,

[21].

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR):

Total RNA was isolated from liver and brain tissue samples using the Thermo Scientific GeneJET RNA Purification Kit (no. K0731), according to the manufacturer's instructions. One microgram of total RNA was then used for cDNA synthesis using the Thermo Scientific Revert Aid TM First Strand cDNA synthesis kit (no. K1621). The relative expression levels of mRNA were measured using the Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) (no. K0221), according to manufacturer's protocol, and the results were computerized using Stratagene (Mx3000PTM) machine.

The used PCR primer pairs were of the following gene sequence: HO-1 (Forward 5'-CAGAAGAGGCTAAGACCGCCT-3'), (Reverse 5'-TCTGGTCTTTGTGTTCCTCTGTCA-3'); TNF- α (Forward 5'-GCCTCTTCTCATTCTGCTTG-3'), (Reverse 5'-CTGATGAGAGGGAGGCCATT-3'); Caspase 3, (Forward 5'-GGC TTG CCA GAA GAT ACC GGT-3'), (Reverse 5'-GCA TAA ATT CTA GCT TGT GCG CGT A-3'); GAPDH (Forward 5'-ACCACAGTCCATGCCATCAC-3'), (Reverse 5'-TCCACCACCTGTTGCTGTA-3').

The fold change in messenger RNA expression was determined in all treated groups compared to controls according to the 2-Ct method, where $CT = CT_{\text{target gene}} - CT_{\text{reference gene}}$ and $CT = CT_{\text{sample 2}} - CT_{\text{sample 1}}$, where sample 1 is the control sample and sample 2 is the experimental sample.

Statistical analysis:

Statistical analysis was performed using SPSS version 21.0 (SPSS, Chicago, IL, USA). Data are presented as mean \pm standard deviation. $p < 0.05$ was considered statistically significant.

Differences in the measured parameters between groups were analyzed by one-way analysis of variance (ANOVA).

Results

As represented in Table (1), exposure to ACR (40mg/kg) induced advanced abnormalities in liver

enzymes and proteins in mice when compared to control group ($p < 0.05$). Administration of DAPH (20mg/kg) after ACR induced liver toxicity improved severe liver function tests abnormalities in mice as compared to ACR-treated group ($p < 0.05$). Exposure to DAPH alone did not change liver enzymes and proteins levels in animals as compared with control.

Administration of ACR (40mg/kg) for 15 days markedly elevated the levels of brain MDA compared with control group ($p < 0.05$). Meanwhile, TAC contents were considerably lower in ACR-treated rats in comparison to the control group ($p < 0.05$). Conversely, administration of DAPH (20mg/kg) inhibited the oxidative damage in the brain tissue. In addition, DAPH significantly ameliorated the level of MDA in comparison to ACR-treated mice ($p < 0.05$). Furthermore, administration of DAPH in ACR-induced toxicity mice, significantly increased TAC contents compared with ACR group ($p < 0.05$) (Table 2).

At the end of the 15 weeks, Caspase 3, heme-oxygenase-1 (HO-1) and tumor necrosis factor alpha (TNF- α) genes expressions were estimated in the brain and liver tissues of all groups. Caspase 3 and TNF- α were up-regulated in the ACR group while HO-1 was down-regulated compared to the naïve group. DAPH administration in treated group resulted in a significant down regulation of Caspase 3 and TNF- α with up-regulation of HO-1 compared to the ACR ($p < 0.05$) (Fig. 1).

Table (1): Effect of DAPH and ACR on liver enzymes, Albumin, and total proteins among all groups.

Groups	ALT (U/L)	AST (U/L)	Total Protein (g/dl)	Albumin (g/dl)
Control	6.54 \pm 0.17 ^b	9.78 \pm 0.27 ^b	6.68 \pm 0.37 ^b	3.80 \pm 0.01 ^b
AC	17.75 \pm 0.44 ^a	18.85 \pm 0.34 ^a	4.26 \pm 0.14 ^a	1.20 \pm 0.01 ^a
DAPH	6.75 \pm 0.14 ^b	9.84 \pm 0.16 ^b	6.56 \pm 0.32 ^b	3.78 \pm 0.04 ^b
DAPH+AC	10.72 \pm 0.21 ^{a,b}	13.82 \pm 0.32 ^{a,b}	4.83 \pm 0.12 ^{a,b}	2.29 \pm 0.06 ^{a,b}

-Data are expressed as mean \pm SD (n=10); different letters indicate a significant difference ($p < 0.05$).

Table (2): Effect of DAPH and ACR on Level of Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC) in brain of all studied groups.

Groups	MDA (nmol/mg protein)	TAC (U/mg protein)
Control	3.52 \pm 0.04 ^b	0.42 \pm 0.02 ^b
AC	5.23 \pm 0.18 ^a	0.23 \pm 0.01 ^a
DAPH	3.74 \pm 0.05 ^{a,b}	0.38 \pm 0.01 ^{a,b}
DAPH+AC	3.68 \pm 0.16 ^b	0.34 \pm 0.01 ^{a,b}

-Data are expressed as mean \pm SD (n=10); different letters indicate a significant difference ($p < 0.05$).

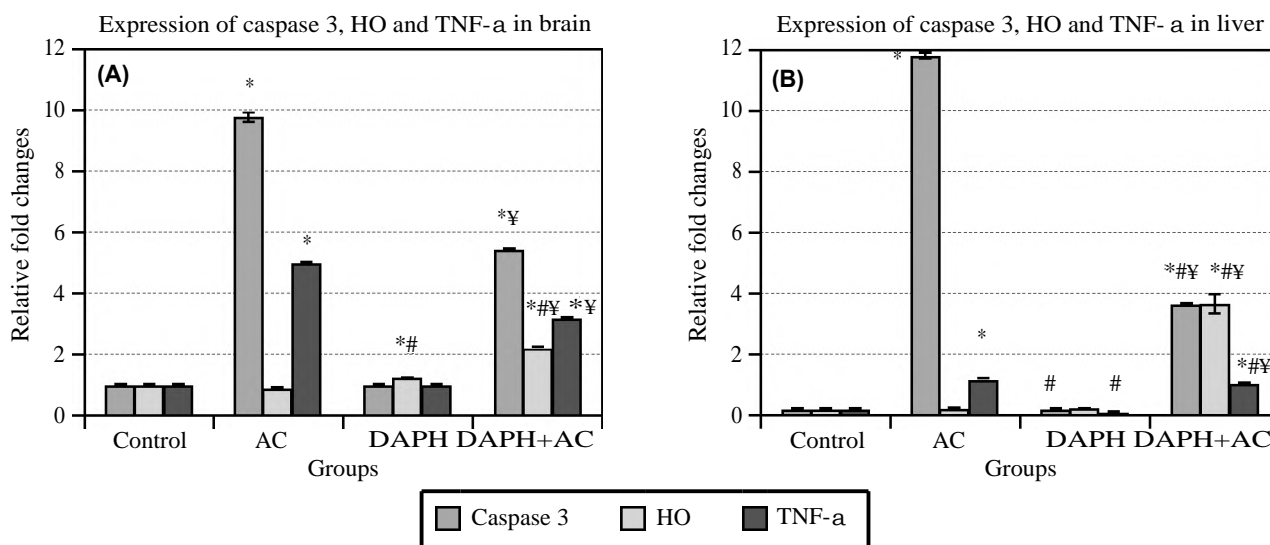


Fig. (1): Expression of Caspase 3, hemeoxygenase-1 and TNF alpha genes expression among different groups in (A) Liver tissue, (B) Brain tissue.

* Significant when compared to control group. # Significant when compared to AC group. ¥ Significant when compared to DAP group.

Discussion

Acrylamide (ACR) is a well-known environmental pollutant, which exerts a variety of systemic toxic effects on human beings following either occupational or dietary exposure [22]. More importantly, the well-being of the public is being adversely affected by the intake of dietary ACR that is formed during heating processes of tobacco and carbohydrate-rich foods at high temperatures [22]. So far, in addition to its high-profile nephrotoxicity, reproductive toxicity, and carcinogenicity [23], other toxic effects of ACR, for example, hepatotoxicity [24] and neurotoxicity [25], have had more attention paid to them.

Attention is focused on natural antioxidants, of which, coumarins have been considered to play an important role as dietary antioxidants for prevention of oxidative damage in living system [26]. Therefore, the current study was designed to explore the potential protective effects of Daphnetin (DAPH) against ACR induced hepatotoxicity and neurotoxicity in mice.

Administration of ACR induced a significant liver damage which was manifested by inflammatory cell infiltration and hepatocellular necrosis [12]. Aminotransferases such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are used in the specific liver damage diagnosis. ACR was reported to increase serum level of these enzymes in rodents and also produced significant changes in serum proteins [24]. The current results showed that liver function markers, including ALT and AST activity were significantly

higher in the ACR group than in control group which agreed with [27,28].

Furthermore, this study exhibited a significant decrement in the total protein and albumin after ACR administration, which could be due to nutrition insufficiency, failure of protein synthesis or excess of excretion. Declining of total protein level might be due to the hepatic inflammation because of ACR exposure, which disarranged the protein synthesis [29]. The present results agreed with Hamdy et al., [30], who demonstrated that ACR induced damage in liver tissues. These data suggested the potential hepatotoxicity of ACR on humans. Therefore, extensive research has been conducted to reduce the hepatic impairment induced by ACR [12,30]. In the present study, administration of DAPH ameliorated the hepatotoxic effect of ACR presented in significant decreasing of liver enzymes (ALT, AST) and significant increasing of total proteins and albumin when compared to control group.

The ACR causes important risks to the human health due to its genotoxic characteristics. ACR leads to free radicals' generation by disturbing the antioxidant balance and thus causing oxidative stress [31]. Several in vitro studies on liver and brain tissues showed that ACR enhanced lipid peroxidation [32]. Considerable experimental data from animal studies have shown that ACR potentiates the oxidative stress indices including malondialdehyde (MDA) in different organs such as brain, testes and liver [33]. It has been shown that the over generation of free radicals is linked to functional alterations in the brain tissue [34].

In agreement with these studies, we showed that ACR elevates MDA and decreases the total antioxidant capacity (TAC) levels in brain tissue when compared to control group. Also, in a study conducted with gestation model, Erdemli et al., [35] reported a significant increase in MDA and decrease in TAC levels of fetal brain tissues in ACR-induced neurotoxicity rats' model.

This finding is consistent with Al-Salmi [36], who reported that there was a significant increment in serum MDA levels in ACR group when compared with the control group. Moreover, Ven Katasubbaiah et al., [37] reported that ACR induced oxidative stress.

DAPH shows protective potential against oxidative stress-driven hepatotoxicity [38]. The current results also showed that co-administration of DAPH with ACR combating this elevation of MDA and TAC in brain tissues. Available data by our research team have previously suggested that DAPH (a coumarin derivative) suppressed the production of oxidative stress indices such as MDA and restored near control levels of TAC in different oxidative stress condition [16].

Oxidative stress and apoptosis pathways are important mechanisms behind ACR-induced hepatotoxicity and neurotoxicity [39]. The current study showed a significant overexpression of liver tissue caspase-3 in ACR-intoxicated mice when compared to control. ACR induced caspase-3 activation in hepatocytes, the final mediator of apoptosis signaling. It is assumed that the oxidative stress damage to mitochondrial and lysosomal membranes are consequences of the increased ROS formation, which leads to mitochondrial permeability transition (MPT)-mediated caspase-3 activation and cell death [40]. Caspase 3 is pivotal in liver damage etiology, apoptosis induction and processing [41].

Furthermore, studies have shown that ACR could initiate the apoptotic pathway via mitochondrial collapse in human astrocytoma cells [42]. Additionally, an increase in MDA and caspase-3 levels in the sciatic nerve was considered as important mechanisms behind ACR-induced peripheral nervous system toxicity [43]. In our study, ACR-induced group indicated an increase in caspase-3 gene expression compared with control group.

Daphnetin, a coumarin derivative, has been reported to have multiple pharmacological actions, including the potential to treat certain neurodegenerative diseases [44]. The current results showed a noticeable decrease in liver and brain caspase-3

gene expression among DAPH treated group when compared to ACR-induced group. In agreement with our data, DAPH exerted neuroprotection against H₂O₂-induced apoptosis in rat [44]. DAPH-induced apoptosis via a caspase-dependent pathway, in particular the mitochondrial pathway [45].

Natural antioxidants with neuroprotective potential are being considered as a promising approach to prevent or slow the effects of neurological illness, due to their low toxicity and absence of clear side effects [46]. Daphnetin, a natural antioxidant, may have the potential to treat certain neurodegenerative diseases [44].

Heme oxygenase (HO)-1, the rate-limiting enzyme in heme catabolism, can be induced in response to various oxidative stimuli, and its induction is thought to be critical in the cellular defense against oxidative tissue injuries [47]. Overexpression of HO-1 would confer some cellular protection in a variety of pathophysiological conditions [49]. Moreover, the current study showed a significant decrease in liver and brain heme oxygenase gene expression after ACR intoxication which was significantly improved after administration of DAPH.

Evaluation of the role of HO-1 in the observed current hepatoprotective effects of Daphnetin revealed that co-administration of it along with ACR induced HO-1 mRNA expression, resulting in increased HO-1 enzyme activity. This may in turn lead to increased endogenous CO concentration, which is important in antioxidant defense and detoxification of reactive intermediates as reported previously [49]. Previous data demonstrate that Daphnetin has an anti-inflammatory and neuroprotective role during the pathogenesis of experimental autoimmune encephalomyelitis (EAE), which is partially at least, dependent on its regulation of HO-1 [50]. Pro-inflammatory cytokines, such as, TNF- α , have recently been shown to play major roles in pathogenesis of liver disease, and their serum levels have been shown to be enhanced in animal models of alcohol-induced liver damage [51]. In the current study TNF- α gene expression level was upregulated in ACR intoxicated group, which was lately downregulated after DAPH supplementation. After exposure to ACR, hepatic pro-inflammatory cytokines expression including tumor necrosis factor- α was increased suggesting leukocytes infiltration in the liver [52].

In conclusion, the present study showed the ability of daphnetin (DAPH) to ameliorate the hepatotoxicity and neurotoxicity of acrylamide, as

the oral administration of DAPH improved and remarkably decreased the levels of serum AST and ALT, increased the activities of total antioxidant capacity (TAA) and reduced (MDA) in mice. Further, our results highlighted that DAPH exposure assuaged ACR-induced inflammation and caspase-3 activation in the liver and brain of mice. Thus, DAPH could be effective in reducing hepato- and neuro-toxicity due to its strong antioxidant, anti-inflammatory and antiapoptotic properties.

Declarations:

Ethics approval and consent to participate:

All procedures for the care and use of laboratory animals were accepted by institutional animal ethics committee for Ain Shams University, animal's procedures were approved in compliance with institutional standards for human care and use of laboratory animals (WHO 1985).

Consent for publication:

Not applicable.

Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

All authors contributed equally to this work.

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التأثير التحسني للدافنتين على السمية الكبدية والعصبية الناجمة عن مادة الأكريلاميد

الخلفية: إن طهي النظام الغذائي الغني بالكربوهيدرات ينتج مادة الأكريلاميد الكيميائية التي لها تأثيرات سامة للكبد والأعصاب. وجد أن الدافنتين، وهو مشتق من الكومارين، له تأثيرات علاجية محتملة.

الهدف من الدراسة: تقييم تأثير الدافنتين على الكبد والسمية العصبية للأكريلاميد.

المواد والطرق: تم دراسة تأثير الدافنتين على القصور الكبدى العصبى الناجم عن التعرض للأكريلاميد (٤٠ مجم / كجم) لمدة ١٥ يوماً فى الفئران. تم تقدير اختبارات وظائف الكبد وعلامات الإجهاد التأكسدى. علاوة على ذلك، تم تقييم مستوى التعبير عن عامل نخر الورم ألفا (TNF- α) ونشاط الهيمأوكسجيناز-١ (HO-1) ونشاط Caspase 3 فى أنسجة المخ والكبد.

النتائج: أدى إعطاء الدافنتين (٢٠ مجم / كجم) إلى الفئران المصابة بالأكريلاميد إلى إنخفاض كبير فى مستويات الأنزيمات الناقلة لمجموعة الأمين مع تحسين البروتينات. إنخفض مستوى malondialdehyde (MDA) بشكل ملحوظ فى أنسجة المخ لدى الفئران التى عولجت بالدافنتين بعد الزيادة الكبيرة فى MDA الناتجة عن تسمم الأكريلاميد. الدافنتين أدى إلى إنخفاض مستوى التعبير الجينى ل Caspase 3 و TNF- α وكذلك أدى إلى ارتفاع مستوى التعبير الجينى HO-1 مقارنة بالمجموعة المعالجة بالأكريلاميد.

الخلاصة: التعرض للدافنتين خفف من الإجهاد التأكسدى والالتهاب الناجم عن الأكريلاميد. وبالتالي، يمكن أن يكون الدافنتين فعالاً فى الحد من سمية الكبد والأعصاب نظراً لخصائصه القوية المضادة للأكسدة والمضادة للالتهابات.