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Histopathological and clinicopathological studies on the antioxidant effect of gold nanoparticles in diethylnitrosamine induced hepatocarcinogenesis in rats

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Abstract

This study investigated the antioxidant and therapeutic effect of gold nanoparticles (GNPs) against diethylnitrosamine (DENA)-induced hepatocarcinogenesis. 24 rats were divided into four groups. The first group kept as a negative control group without any treatments. The second group received single intraperitoneal injection of DENA (200 mg/kg) followed by weekly subcutaneous injections of carbon tetrachloride (3 ml/kg) for 10 weeks and kept as a positive control group. The third group was intoxicated by DENA and CCl4 as the second group then treated by i.p. injection of 0.075 mg free GNPs kg^{-1} twice (at day, 0 and 3) then retreated with the same dose after 21 days. The fourth group received the same dose of GNPs as in third group. The results showed that group injected with DENA expressed high levels of lipid peroxidation (MDA) accompanied by significantly decreased levels of antioxidants (reduced glutathione and Catalase) when compared with the control group, on the other hand there was significant decrease in MDA and increase in antioxidants in GNPs treated group. Concomitantly, the improvement of histopathological alterations induced by DENA in livers of GNPS treated groups was observed. In conclusion, these results suggested a positive hepatotherapeutic and antioxidant effect of GNPs by decreasing lipid peroxidation and enhancing the levels of antioxidants in DENA-induced hepatocarcinogenesis by reducing the formation of free radicals.

Key words: antioxidants, hepatocarcinogenesis, diethylnitrosamine, gold nanoparticles.

Introduction

Hepatocellular carcinoma is one of the most common and lethal cancer (Wilson et al., 2012). Diethylnitrosamine (DENA) is a by-product of nitrosation of primary amines in the acidic conditions of stomach. It is further converted into an active ethyl radical metabolite that reacts withDNA, leading to mutations and oncogenesis (Anis et al., 2001). DENA metabolism in the liver of human and rats by cytochrome isoform 2E1 (CYP 2E1) generates reactive oxygen species (ROS) causing oxidative stress (Mandal et al., 2008). It has also been reported that oxidative stress plays a pivotal role during carcinogenesis (Jayakumar et al., 2011).

Nanotechnology is the design, characterization, production and application of structures, devices and systems by controlling the shape and the size at nanometer scale (The Royal Society and The Royal Academy of Engineering, 2004). Biomedical applications of gold nanoparticles (AuNPs) are rapidly increasing due to their attractive properties of relatively low cytotoxicity, a high capacity to target cells, readily functionalized surfaces and a tunable optical absorption peak (Chen et al., 2009). Several biochemical markers have been suggested for biomonitoring the actions of anticancer agents. Estimation of circulatory lipid peroxidation end products [thiobarbituric acid reactive substances (TBARS), non-enzymatic antioxidants (reduced glutathione (GSH), carotene, ascorbic acid and tocopherol), activities of enzymatic antioxidants (superoxide dismutase (SOD), glutathione peroxidase (GPx) and Catalase (CAT))] as biochemical markers is a reliable method for screening the action of

Nowadays, AuNPs are used effectively in laboratory-based in vivo research either as a diagnostic imaging agent or as a therapeutic agent in experimental gene, drug delivery and photothermal therapeutics. Mukherjee et al., 2005 report an intrinsic property of gold nanoparticles (nanogold). They can interact selectively with heparin-binding glycoproteins and inhibit their activity. Gold nanoparticles specifically bound vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)-165 and basic fibroblast growth factor (two endothelial cell mitogens and mediators of angiogenesis), resulting in inhibition of endothelial/fibroblast cell proliferation in vitro and VEGF-induced permeability as well as angiogenesis in vivo. Gold nanoparticles has an effect on the anti oxidant enzymes such as GSH, SOD, Catalase and GPx in diabetic mice, by inhibition of lipid peroxidation and ROS generation during hyperglycemia which showing anti-oxidant effect of AuNPs during hyperglycemia (BarathManiKanth et al., 2010).

chemopreventive agents (Sundaresan and subramanian, 2003).

In the present study, a systematic investigation of the therapeutic and antioxidant effect of GNPs against DENA-induced hepatic carcinoma has been carried out by analyzing the circulatory TBARS, non-enzymatic and enzymatic antioxidants.

Materials and methods

Synthesis and characterization of Gold nanoparticles:-

Gold nanoparticles were prepared by citrate reduction of gold chloride trihydrate by using sodium citrate tribasic dihydrate (all chemicals purchased from Sigma Chem. Comp. USA) according to the method of Frens, 1973.

The prepared nanoparticles were characterized by using UV-Visible absorption spectroscopy, TEM and Zetasizer nano according to the methods described previously (Kalishwaralal et al., 2009) to determine GNPs size and shape.

Experimental design:-

Animals and their treatments:-

Twenty four male Wister albino rats, 70-100 g were obtained from Holding Company for Biological Products and Vaccines (VACSERA) - Helwan. All animals were housed in plastic cages (5 rats/ cage) in a well-ventilated environment and received a daily illumination of 16 hours of light. They were fed on dry commercial standard pellets and gain access to tap water ad- libitum throughout the experimental period. They were acclimatized to the environment for 2 weeks prior to experiment to ensure their healthy state.

Animals were randomly divided into four groups (n = 6 in each group). Animals in group I (untreated control negative) received neither DENA nor GNPs. Group II animals received single intraperitoneal injection of DENA (200 mg/kg) followed by weekly subcutaneous injections of carbon tetrachloride (3 ml/kg) for 12 weeks. Group III animals received single intraperitoneal injection of DENA (200 mg/kg) followed by weekly subcutaneous injections of carbon tetrachloride (3 ml/kg) for 12 weeks as in group II, then the animals were treated by i.p. injection of 0.075 mg free gold nanoparticles kg^{-1} twice (at day, 0 and 3) then retreated with the same dose after 21 days. Group IV animals received GNPs alone as in group III.

Histopathological examination:-

All rats in different experimental groups were submitted to post mortem examination at euthenization time. On completion of the experimental period (21 weeks), animals were euthenized by cervical dislocation. Liver tissue specimens were taken from all rats and preserved in 10% neutral buffer formalin (PH 7.0). Tissue processing was done to obtained paraffin section and stained by Hematoxylin and eosin (H&E) (Bancroft and Gamble, 2013).

Determination of oxidative stress:-

Homogenization of liver tissue was done in 5-10 ml cold buffer (50 mM potassium phosphate, pH7.5) per gram tissue for MDA Assay (Ohkawa et al., 1979). While for CAT assay, tissue homogenization was carried out in 5 -10 ml cold buffer (i.e., 50 mM potassium phosphate, PH 7.5.; 1mM EDTA and 1ml /l Triton X-100) per gram liver tissue using tissue homogenizer (Aebi, 1984). On the other hand for GSH assay, tissue homogenization was done in 5 -10 ml cold buffer (i.e., 50 mM potassium phosphate, PH 7.5. and 1mM EDTA) per gram hepatic tissue using tissue homogenizer (Beutler et al., 1963). All tissue homogenates were then centrifuged at 4000 rpm for 15 minutes at 4 °c and the supernatants were aspirated for MDA, catalase and GSH assays.

Statistical analysis:-

All the previous data were expressed as mean ± SD for six rats in each group. Variables were statistically analyzed by one way analysis of variance (ANOVA) test was used to compare means of more than two groups. When differences were significant, Least Significant Difference (LSD) test was performed to find the individual differences between groups. The significance level was set as P value ≤ 0.05 significant. Statistical analysis was performed using SPSS version 16 (Snedecor and Cochran, 1982).

Results

Characterization of GNPs:-

Spectrophotometer results showed peak absorption (0.9702) at wavelength 530nm. The morphology and size of the biologically synthesized gold nanoparticles was determined using Transmission electron microscopy (TEM). The images showed that GNPs exhibited spherical shape with different sizes ranging from 13 to 50nm in diameter. For further

identification of particles average size, Zetasizer nano was used which showed peak size (21.03%) at 17.5nm diameter.

Histopathological findings of liver tissue:-

Group of rats kept as control (non treated group):

There was no histopathological alteration and the normal histological structures of central vein and surrounding hepatocytes were recorded in **Fig.1.**

Group of rats receiving DENA (control positive group):

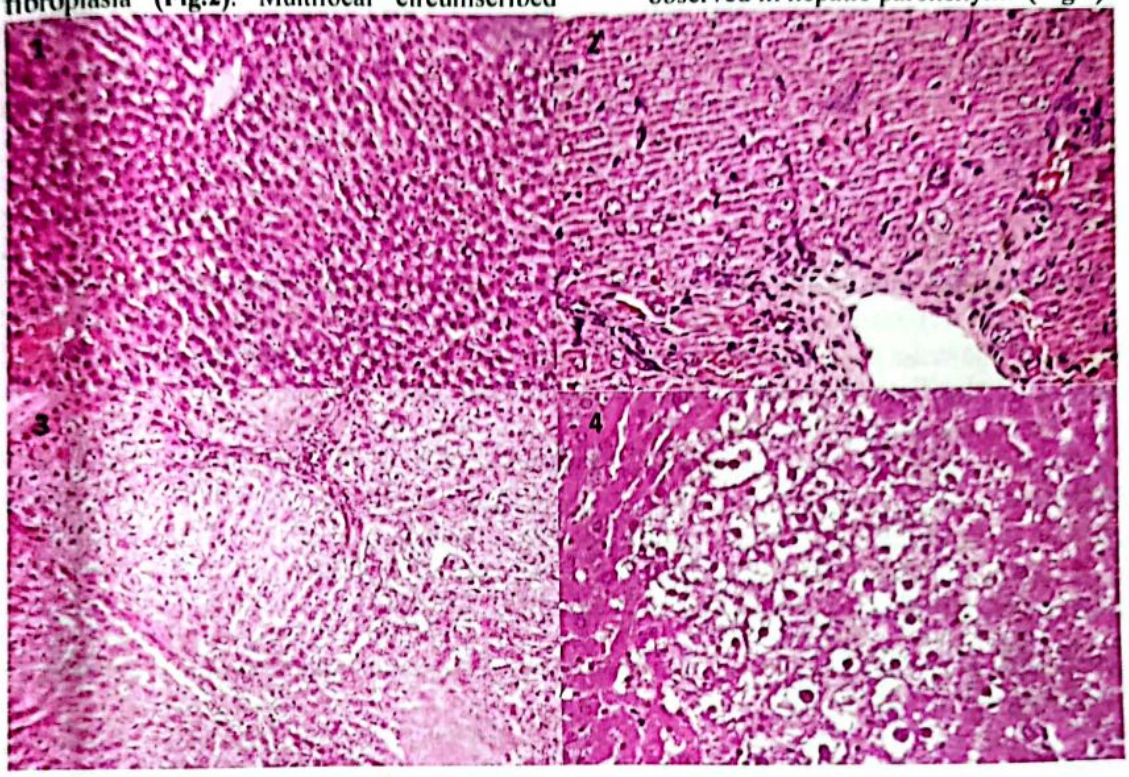
There was diffuse Kupffer cells proliferation in between hepatocytes with marked portal fibroplasia **(Fig.2)**. Multifocal circumscribed nodules of vacuolated, degenerated and cytomegalic hepatocytes were recorded and surrounded by compressed cells **(Fig.3)**. Clear focus of hepatocellular alteration was also recorded **(Fig.4).**

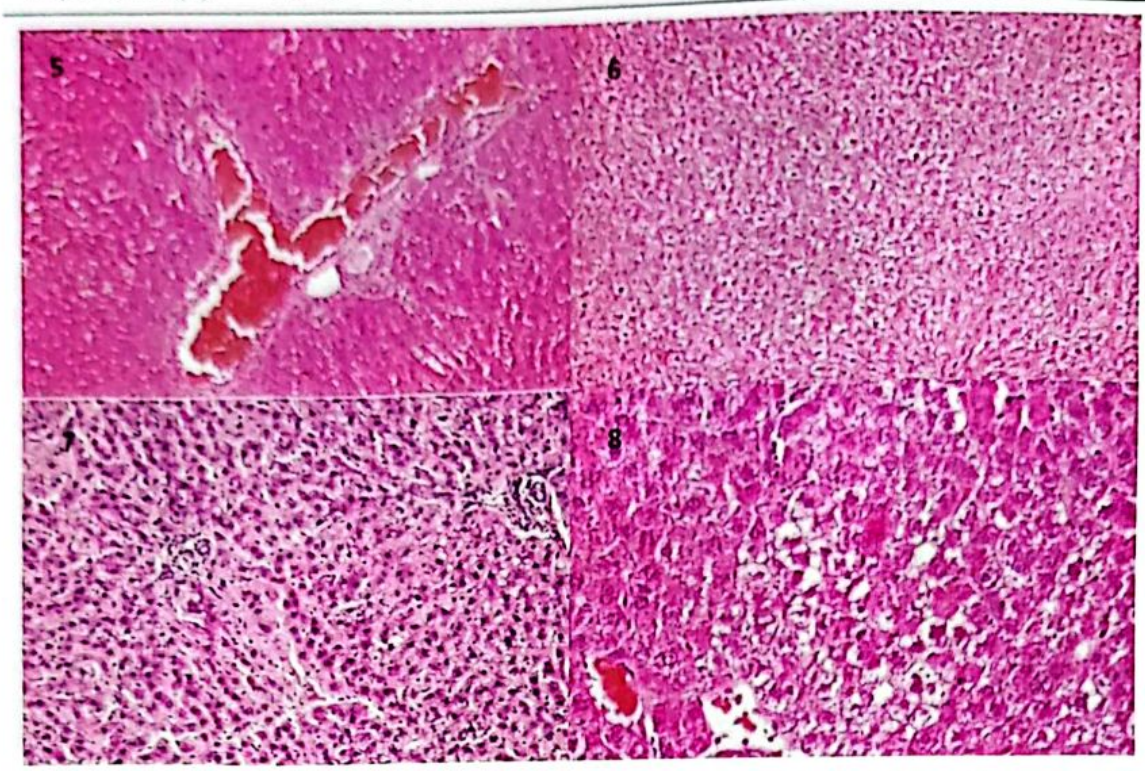
Group of control rats treated with GNPs:

Severe dilatation and congestion were observed in portal vein **(Fig.5)** associated with vacuolar degeneration in some of hepatocytes **(Fig.6).**

Group of rats receiving DENA and treated with GNPs:

This group showed few inflammatory cells infiltration in portal areas **(Fig.7).** Focal area of dysplastic and degenerated hepatocytes was observed in hepatic parenchyma **(Fig.8).**





Finding of antioxidant analysis:-

The results in **table (1)** revealed that DENA intoxicated group showed a significant increase in MDA level and significant decrease in GSH and CAT levels in liver homogenate as compared to control negative group. Treatment of rats by GNPs significantly diminished the elevated levels of MDA and increased levels of GSH and CAT.

Table (1): The effect of GNPs treatment on MDA, GSH and CAT levels in liver homogenate at the end of the experiment

GP	MDA	GSH	CAT
1	262.33±2.33 b	45.5±1.78 b	1.47±0.18 b
2	295.33±4.7 a	20.03±1.33 a	0.57±0.03 a
3	282.33±6.84 ab	36.37±5.32 ab	1.43±0.07 ab

All data presented as mean value (n=3) ± Standard error. Values bearing different superscripts (a, b) are significant at $P < 0.05$.

Discussion

Nanotechnology is undergoing explosive expansions in many areas serving mankind, due to which even poorer developing countries have also decided that this new technology could represent a considered investment in future economic and social well-being that they cannot ignore. The gold nanoparticles are known for their tremendous applications in the field of therapeutics and diagnosis (BarathManiKanth et al., 2010).

In this study, the results obtained in the synthesis and characterization of gold nanoparticles is strongly supported by previously published reports on synthesis and characterization of gold nanoparticles using the same reduction method (Sheikpranbabu et al., 2009).

The present study was conducted to study the antioxidant and therapeutic effect of GNPs against DENA induced hepatocellular carcinogenicity in rats. It has been reported that DENA is metabolized to alkylating reactants, which could interact with DNA molecule and initiate carcinogenesis (Al.Rejaie et al., 2009). Although many risk factors have been reported, lipid peroxidation plays an important role in hepatic carcinogenesis. Further, enhanced lipid peroxidation associated with depletion of antioxidants is a characteristic finding in a variety of malignancies. Promotion by using CCL4 has been implicated in several models of multistage hepatocarcinogenesis. Liver injuries induced by DENA and CCL4 are the best characterized system of the xenobiotics-induced

hepatotoxicity and is a commonly used model forscreeningtheantihepatotoxic, hepatoprotective and hepatotherapeutic activity of drugs (Sundaresan and subramanian, 2003). In the current study, the histopathological lesions observed in DENA intoxicated group relatedto the hepatotoxic and hepatocarcinogenic effect of DENA and CCL4. Our results were agreed with many authors who found hepatocellular degenerations, necrosis and inflammations in DENA injected rats (Ha et al., 2001 and Bansal et al., 2005). In the present study, there were multiple altered hepatocellular foci with different sizes in liver of rats in group II, which were believed to be due to DENA injection. In rats, DENA is a potent hepatocarcinogen influencing the initiation stage of carcinogenesis during a period of enhanced cell proliferation accompanied by hepatocellular necrosis and induces DNA carcinogen adducts, DNA-strand breaks and in turn hepatocellular adenoma and carcinomas without cirrhosis through the development of putative preneoplastic focal lesions (Bansal et al., 2005). Al-Rejaie et al., 2009 showed that DENA produced a massive degenerative change and increased the evidence of pre-neoplastic lesions in liver tissues. In the current study, liver sections from rats treated with DENA and GNPs (group III) showed regression in inflammatory reaction, sizes and numbers of altered hepatocellular foci, this reduction related to anti-inflammatory and antiangiogenic effect of GNPs. Gold nanoparticles have received great attention as anti-inflammatory agents through their ability to inhibit expression of NF-kappa B and subsequent inflammatory reactions (Norton, 2008).The inhibitory activity of gold nanoparticles against VPF/VEGF165 induced proliferation of endothelial cells provides clear evidence over their therapeutic potential in the treatment of diseases like chronic inflammations, pathological neo-vascularization, rheumatoid arthritis, and neoplastic disorders (Mukherjee et al., 2005). Thus, nanogold has antiangiogenic effects; it may be beneficial for the treatment of arthritis. Moreover, nanogold inhibits VEGF-induced permeability in models of ear tumor and ovarian tumor in mice (Bhattacharya et al., 2004).

In our study, DENA injected rats showed significant increase in the levels of TBARS (MDA) which could be ascribed to the excessive generation of free radicals due to the effect of both DENA and CCL4. It is known that DENA metabolism in the liver of rats by cytochrome isoform 2E1 (CYP 2E1) generates reactive oxygen species (ROS) causing oxidative stress (Mandal et al., 2008). Also CCL4 metabolism in the liver of rats by cytochrome P_{450} generates ROS (Poli, 1993). Excessive generation of ROS can cause oxidative damage, and it has also been reported that oxidative stress plays a pivotal role during DENA induced hepatotoxicity and carcinogenicity (Jayakumar et al., 2011). DENA was found to cause elevation of lipid peroxidation levels and decreased levels of both enzymatic and non enzymatic antioxidants in the liver tissues (Pradeep et al., 2007). Also Szatrowski and Nathan, 1991 have suggested that tumor cells produce substantial amount of hydrogen peroxide and reactive oxygen metabolites that are released into the circulation. Therefore, the elevation of MDA concentration in this group indicates increased lipid peroxidation and occurrence of oxidative stress.

In the current study, there were significant depletion in GSH and CAT level in DENA injected group. These results in agreement with **Granado et al., 2009,** who attributed the depletion in GSH and CAT levels in rats administered with DENA due to decreased expression of antioxidant enzyme during hepatocellular damage. Treatment of DENA intoxicated rats by GNPs in group (III) have antioxidant effects manifested by decreased MDA levels and increased GGT and CAT levels, which believed to be due to GNPs injections. Sahin and Turkmen, 2005 showed that there were significant increase in the levels of GSH and CAT in the diabetic treated mice with AuNPs in comparison to diabetic control which believed to be due to the significant decrease in lipid peroxidation and ROS generation in GNPs treated mice suggesting that AuNPs prevents disruption of organs by protecting lipids from peroxidation by ROS. The ability of gold nanoparticles in inhibiting the lipid from peroxidation thereby preventing the ROS generation has restored the imbalances in the antioxidants and liver enzymes responsible for the cell dysfunction and destruction. Our result suggesting gold nanoparticles' potential as antioxidant is shored up with previous reports

delivering the effects of gold nanoparticles as an antioxidant (Yakimovich et al., 2008). Thus these findings over the ability of AuNPs in the elimination of ROS induced by DENA, thereby restoring the balanced level of anti-oxidant defense system affirms the therapeutic application of gold nanoparticles as a promising antioxidant.

Conclusion List of figures

From the present study, we concluded that 13nm sized gold nanoparticles have antioxidant effect against DENA-induced hepatocellular injury which diminished the elevated MDA levels and increased GST and CAT levels in liver tissues. Also GNPs improved the histopathological finding induced by DENA intoxication so, GNPs have hepatotherapeutic effects against DENA-induced hepatocellular injury.

Fig. 1	Liver of rat in group (I) showing normal histological structures of central vein and surrounding hepatocytes.
Fig. 2	Liver of rat in group (II) showing anaplastic karyomegalic nuclei with prominent nucleolus in most of hepatocytes with diffuse Kupffer cells proliferation in between hepatocytes and portal fibroplasia.
Fig. 3	Liver of rat in group (II) showing Multifocal circumscribed nodules of vacuolated and cytomegalic hepatocytes with defined adjacent compressed cells.
Fig. 4	Liver of rat in group (II) showing large clear focus of hepatocellular alteration.
Fig. 5	Liver of rat in group (III) showing severe congestion and dilatation of portal vein with surrounding degenerated hepatocytes.
Fig. 6	Liver of rat in group (III) showing vacuolar degeneration of hepatocytes.
Fig. 7	Liver of rat in group (IV) showing few inflammatory cells infiltration in portal areas.
Fig. 8	Liver of rat in group (IV) showing Focal area of dysplastic and degenerated hepatocytes in hepatic parenchyma.

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الملخص العربى

دراسات هستوباثولوجيه و كلينيكوباثولوجيه علي التأثير المضاد للاكسدة

لجزيئات الذهب المتناهية الصغر لعلاج الاورام الكبدية التى يسببها ثنائى اثيل النيتروسامين في الفئران

ايمان ابراهيم حسنين: رضا محمد سيد: طاهر احمد صلاح الدين: عادل محمد بكير

تم تصميم هذه الدراسة لمعرفة التأثيرالعلاجى لجزيئات الذهب المتناهية الصغر المضادة للاكسدة ضد الاورام الكبدية التى يسببها ثنائى اثيل النيتروسامين . وقد أجريت التجربة على 24 ذكر من فئران الالبينو ويستر (متوسط الوزن 120-70). تم تقسيم الحيوانات إلى اربع مجموعات وفقا لأساليب العلاج المختلفة. تم اعتبارالمجموعة الاولى مجموعة ضابطة للمواد المستخدمة بينما المجموعة الثانية تم حقنها بمادة ثنائى اثيل النيتروسامين ورابع كلوريد الكربون والمجموعة الثالثة تم حقنها بمادة ثنائى اثيل النيتروسامين ورابع كلوريد الكربون ثم علاجها بجزيئات الذهب المتناهية الصغر. والمجموعة الرابعة تم علاجها بجزيئات الذهب فقط. أظهرت النتائج أن المجموعة التي حقنت بثنائى اثيل النيتروسامين شاهدت مستويات عالية من بيروكسيد الدهون (MDA) يرافقه انخفاض كبير فى مستويات المواد المضادة للاكسدة (الجلوتاثيون المختزل والكاتلاز) بالمقارنة مع المجموعة الضابطة، من ناحية أخرى كان هناك انخفاض كبير في MDA وزيادة في المواد المضادة للاكسدة في المجموعة التى تم علاجها بجزيئات الذهب. كما لوحظ تحسن فى التغيرات الباثولوجية الناجمة عن ثنائى اثيل النيتروسامين في المجموعة التى تم علاجها بجزيئات الذهب. و قد أوضحت هذه النتائج التأثيرالعلاجى الإيجابي والمضاد للأكسدة لجزيئات الذهب ضد اورام الكبد التي يسببها ثنائى اثيل النيتروسامين من خلال خفض الدهون المؤكسدة وتعزيز مستويات المواد المضادة للاكسدة وذلك عن طريق الحد من تكوين الشوارد الحرة.