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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

Efficient therapeutic effect of *Nigella sativa* aqueous extract and chitosan nanoparticles against experimentally induced *Acanthamoeba* keratitis

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Abstract

Acanthamoeba keratitis (AK) is a devastating, painful corneal infection, which may lead to loss of vision. The development of resistance and failure of the currently used drugs represent a therapeutic predicament. Thus, novel therapies with lethal effects on resistant *Acanthamoeba* are necessary to combat AK. In the present study, the curative effect of *Nigella sativa* aqueous extract (*N. sativa*) and chitosan nanoparticles (*nCs*) and both agents combined were assessed in experimentally induced AK. All inoculated corneas developed varying grades of AK. The study medications were applied on the 5th day postinoculation and were evaluated by clinical examination of the cornea and cultivation of corneal scraps. On the 10th day posttreatment, a 100% cure of AK was obtained with *nCs* (100 μg/ml) in grades 1 and 2 of corneal opacity as well as with *N. sativa* 60 mg/ml-*nCs* 100 μg/ml in grades 1, 2, and 3 of corneal opacity, highlighting a possible synergistic effect. On the 15th day posttreatment, a 100% cure was reached with *N. sativa* aqueous extract (60 mg/ml). Moreover, on the 20th day posttreatment, *N. sativa* (30 mg/ml) provided a cure rate of 87.5%, while *nCs* (50 μg/ml) as well as *N. sativa* 30 mg/ml-*nCs* 50 μg/ml yielded a cure rate of 75%; the lowest percentage of cure (25%) was obtained with chlorhexidine (0.02%), showing a non-significant difference compared to the parasite control. The clinical outcomes were in agreement with the results of corneal scrap cultivation. The results of the present study demonstrate the effectiveness of *N. sativa* aqueous extract and *nCs* (singly or combined) when used against AK, and these agents show potential for the development of new, effective, and safe therapeutic alternatives.

Keywords *Acanthamoeba astronyxis* T7 genotype · Chitosan nanoparticles · Corneal amoebic infection · Nanotherapy · Keratitis · *Nigella sativa*

Introduction

Acanthamoeba species are the causative agents of the sight-threatening corneal infection *Acanthamoeba* keratitis (AK) (Visvesvara 2010). AK occurs in immunocompetent individuals following corneal trauma or, more commonly, as a result of poor hygiene of contact lenses (Visvesvara et al. 2007). AK progresses slowly from the superficial to deep corneal tissue (Lorenzo-Morales et al. 2015). Hence, AK may lead to corneal ulceration, loss of visual acuity and, eventually, blindness (Seal 2003).

So far, no single effective treatment against AK has been described due to the wide range of virulence factors and variable pathogenicity of different strains (Lorenzo-Morales et al. 2015).

A diversity of treatments has been used for AK management (Schuster and Visvesvara 2004). Most have been found to be effective in the treatment of AK but are accompanied by drug toxicity and the development of resistance (Visvesvara et al. 2007). PHMB is effective at a low concentration (0.02%) but is unfortunately more toxic to

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keratocytes than chlorhexidine (Lee et al. 2007). In addition, chlorhexidine is reported to be more effective and less toxic to corneal epithelial cells at minimal concentrations (0.02%) but is still associated with development of resistance (Lorenzo-Morales et al. 2013). Thus, the developed resistance, the variability of species susceptibility to drugs, and the failure to reach the required lethal concentration of anti-*Acanthamoeba* agents in the cornea lead to failure of treatment (O'Day and Head 2000). Additionally, the development of side effects such as cataract, iris atrophy, and peripheral ulcerative keratitis has been attributed to the use of AK chemotherapeutic drugs (Dart et al. 2009). The aforementioned adverse effects enforce the need for effective and safe treatments, and therapeutic plants can provide a different source of drugs with high efficacy and low toxicity (Freitas et al. 2006). Several natural agents have been studied for their effectiveness against *Acanthamoeba*; examples include propolis and garlic, which were found to have a potential anti-*Acanthamoeba* effect (Topalkara et al. 2007; Polat et al. 2008). *N. sativa* is one of the most potential natural sources in traditional medicine (Ali and Blunden 2003). Furthermore, *N. sativa* extracts have been proven to have therapeutic effects on *Giardia lamblia* (Kabbashi 2017), *Cryptosporidium parvum* (El-Refaii 2003), *Blastocystis hominis* (El El Wakil 2007), *Trypanosoma brucei* (Ekanem and Yusuf 2008), *Trichomonas vaginalis* (Aminou et al. 2016), and *Toxoplasma gondii* (Rayan et al. 2011). Ismail et al. (2018) demonstrated that the *N. sativa* aqueous extract (30 mg/ml) had a lethal effect on *Acanthamoeba* in vitro and was comparable to or even superior to chlorhexidine (0.02%). On the other hand, the nanotechnology in microbiology is growing rapidly, as it is expected that nanoparticles will be used in the treatment of various diseases (Allahverdiyev et al. 2011). A study conducted by Aqeel et al. (2015) reported the significant amoebicidal effect of gold nanoparticles conjugated to chlorhexidine on *A. castellanii.* Additionally, silver nanoparticles conjugated to tannic acid were considered as a potential agent against *Acanthamoeba* spp. (Padzik et al. 2018). As stated by Pemberton (1999), insects are an unexploited source of useful compounds in modern medicine. Chitosan (Cs) is a nontoxic biopolymer obtained by deacetylation of chitin (Qi et al. 2004). Many authors extracted the biologically active CS from the housefly larvae (Ding et al. 2006; Jing et al. 2007). *Chitosan* nanoparticles (*nCs*) were used as a drug delivery system for the treatment of *Cryptosporidium*, *Plasmodium falciparum*, and *Leishmania* (Kayser 2001; Föger et al. 2006; Pujals et al. 2008). Moreover, Said et al. (2012) reported the efficiency of *nCs* as anti-*Giardia* agents. Out of the need for a safe and effective treatment of AK, the aim of this study was to evaluate the therapeutic effect of the *N. sativa* aqueous extract and *nCs* on experimentally induced AK.

Materials and methods

Preparation of the study medications

The *N. sativa* (*Ns*) aqueous extract was prepared according to the method of Hosseinzadeh et al. (2013). *Ns* seeds were obtained from the Medicinal and Aromatic Research Department, Horticulture Research Station, Agriculture Research Center, Alexandria, Egypt, under voucher number N000786. One hundred grams of seeds were grinded, boiled in 1000 ml water for 15 min and filtered through a cloth. The filtrate was evaporated to dryness under reduced pressure. The aqueous extract was preserved at − 20 °C until use. The concentrations of *Ns* were adjusted in distilled water to 30 mg/ml, the minimal lethal concentration ("MLC") for *Acanthamoeba* obtained in a previous study (Ismail et al. 2018), and 60 mg/ml.

Chitosan nanoparticles (*nCs*) were generously provided by Dr. Yousry Mahmoud Gohar, Microbiology Department, Faculty of Science, Alexandria University. Characterization was previously performed by Gaafar et al. (2014), using transmission electron microscope (JEOL 100 CX); they reported that *nCs* were spherical in shape, with an average size of 93.65 nm.

nCs were dissolved in distilled water and adjusted to 50 μg/ml (Said et al. 2012) and 100 μg/ml.

Ns-nCs combinations (30 mg/ml *Ns*-50 μg/ml *nCs* and 60 mg/ml *Ns*-100 μg/ml *nCs*) were prepared.

Chlorhexidine digluconate (20%) (Sigma-Aldrich Corporation) was prepared as 0.02% Ch. (Lim and Kam 2008).

Acanthamoeba isolation, cultivation, molecular identification, and infective inoculum preparation

The isolate used in the present study was obtained from a 38-year-old male contact lens wearer who was presented at the ophthalmic outpatient clinic (RIO) with corneal ulcer refractory to antifungal, antibacterial, and antiviral treatment. Aseptic corneal scraps were performed, samples were cultivated on 1.5% non-nutrient agar (NNA) plates seeded with a heat-killed *Escherichia coli* (*E. coli*) bacterial suspension (Łanocha-Arendarczyk et al. 2018) and incubated in a humidified chamber at 30 °C (Init et al. 2010). Plates were examined daily by inverted microscopy for 7 days, and subcultures were carried out after 2 weeks (Schuster 2002). Trophozoites were collected by pouring 2–3 ml of Page Amoeba Saline (PAS) into the 72-h old culture plates; the agar surface was carefully scraped, and the suspension was collected and washed twice with PAS, followed by the addition of 100 μg/ml gentamicin and centrifugation at 2000 rpm for 5 min. The pellet was resuspended in 1 ml of PAS and mixed well by vortexing; the concentration of the inoculum was adjusted to 1×10^6 amoebae trophozoites/ml by a

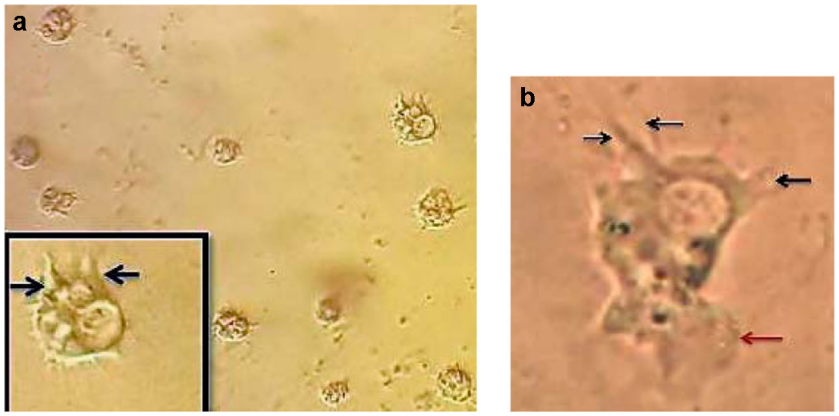

Fig. 1 **a** NNA-*E. coli* culture examined by the inverted microscope (× 200) showing acanthopodia of *Acanthamoeba* trophozoites. **b** *Acanthamoeba* trophozoite by the light microscope (× 1000) (Acanthopodia: black arrows, Lobopodia: red arrows)

hemocytometer "Neubauer Cell Counting Chamber" (Boeckel Co., Germany) (Łanocha-Arendarczyk et al. 2018). Molecular characterization, sequencing, genotyping, and phylogenetic analysis were previously performed by the authors to identify the isolate, which displayed an *Acanthamoeba astronyxis* T7 genotype (Sarhan et al. 2017).

In vitro evaluation of the effect of *nCs* on *A. astronyxis*

An evaluation of the effect of *nCs* on *A. astronyxis* was performed according to (Polat et al. 2008). Briefly, 100 μl of an amoeba suspension in PBS (25×10^4/ml) was inoculated into each well of a 96-well plate, left for 30 min to avoid disturbance of the adherence of amoebae onto the wells' surface. The PBS solution was removed, and 100 μl of 12.5, 25, and 50 μg/ml of *nCs* were added. The plate was sealed and incubated at 30 °C for 24 h. A parasite control and 0.02% Ch. drug control were included. Each experiment was performed in duplicate. After the incubation period, 100 μl from each well was transferred into 100 μl of 0.3% basic methylene blue media, left for 10 min and then counted by the hemocytometer (Polat et al. 2008). The percent of growth reduction = $a - b/a \times 100$, where a is the mean number of stained viable amoebae in control cultures and b is the mean number of stained viable amoebae in cultures treated with *nCs*, was calculated (Palmas et al. 1984).

Table 1 Reduction of the number of amoebae in vitro after 24-h incubation with chitosan nanoparticles

	Mean number of amoebae ± SD	Inhibition (%)
Parasite control	119.33 ± 1.52	0
Ch 0.02%	93.3 ± 3.7*	21.7
nCs 50 μg/ml	0.0 ± 0.0**	100
nCs 25 μg/ml	3 ± 1**	97.84
nCs 12.5 μg/ml	5 ± 1.5**	95.8

SD standard of deviation, *nCs* chitosan nanoparticles; *significant difference, **highly significant difference as compared to parasite control

Experimental animals

Sixty-four albino rats were included, with an approximate weight of 200 ± 10 g; they were housed individually and fed with an unrestricted standard diet and water access. Animals were distributed into the following groups (8 animals in each group): group I—*Ns* treated (Ia: *Ns* 30 mg/ml, Ib: *Ns* 60 mg/ml), group II—*nCs* treated (IIa: *nCs* 50 μg/ml, IIb: *nCs* 100 μg/ml), group III—*Ns-nCs* treated (IIIa: *Ns* 30 mg/ml-*nCs* 50 μg/ml, IIIb: *Ns* 60 mg/ml-*nCs* 100 μg/ml), group IV—Ch 0.02% treated, and group V—parasite control group (infected left eye with no treatment). Concerning the drug control for the groups from I to IV, the right eye of each rat served as the drug control (not infected but still treated) and the left eye was the test eye (infected and treated).

Intracorneal inoculation of infective *Acanthamoeba* was performed according to the method of Vural et al. (2007); the rats' corneas were examined prior to inoculation to exclude any abnormalities. The animals were anesthetized, and a half-thickness linear blade incision was made approximately 2 mm from the center of the cornea using a sterile surgical blade no. 15. Next, 1 μl of the prepared inoculum was injected in the left eye by a sterile insulin syringe, and 1 μl of sterile PAS was injected in the right eye. The grading of keratitis was performed according to Herretes et al. (2006) by an experienced ophthalmologist, regarding the area and density of corneal opacity as follows: grading scheme I (area)—grade (0), no opacity; grade (1), opacity less than 1/3 of the corneal surface; grade (2), opacity more than 1/3 and less than 2/3 of the corneal surface; and grade (3), opacity more than 2/3 of the corneal surface; grading scheme II (density)—grade (0), no corneal haze; grade (1), mild corneal haze with iris details clearly visible; grade (2), moderate corneal haze with iris details not clearly visible; grade (3), severe corneal haze with anterior chamber structures not visible.

Table 2 Pretreatment number and percentage of keratitis grade as regards the area and density of corneal opacity

	Ia		Ib		IIa		IIb		IIIa		IIIb		IV		V	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Grading I																
1	5	62.5	1	12.5	6	75	5	62.5	5	62.5	3	37.5	7	87.5	6	75
2	1	12.5	1	12.5	1	12.5	3	37.5	1	12.5	0	0	0	0	2	25
3	2	25	6	75	1	12.5	0	0	2	25	5	62.5	1	12.5	0	0
Grading II																
1	3	37.5	1	12.5	4	50	5	62.5	4	50	3	37.5	7	87.5	6	75
2	5	62.5	3	37.5	2	25	3	37.5	2	25	3	37.5	0	0	2	25
3	0	0	4	50	2	25	0	0	2	25	2	25	1	12.5	0	0

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group

Corneal scrapings were obtained from animals on the 5th day postinoculation after the application of local anesthetic eye drops and were cultured on NNA-*E. coli* plates prior to drug application. The drugs were applied to the rats' eyes starting from the 5th day postinoculation according to Vural et al. (2007), in the form of eye drops administered every 2 h. On days 10, 15, and 20, posttreatment evaluations of the medication were performed. External examination of the corneas was carried out by an experienced ophthalmologist using an operating microscope. The keratitis grade was determined as formerly mentioned. Corneal scrapings were obtained, examined by microscopy and cultured on NNA-*E. coli* plates (Fig. 1).

Statistical analysis

The data were collected, coded, tabulated, and introduced to a personal computer using Statistical Package for Social Science (SPSS 15.0.1 for Windows; SPSS, Inc., Chicago,

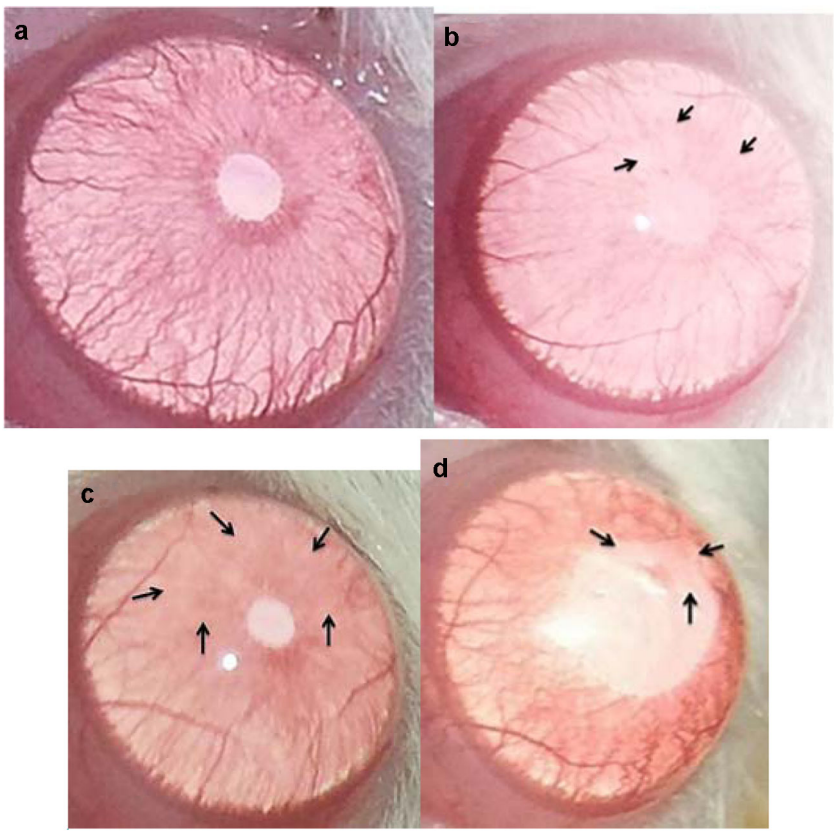

Fig. 2 **a** Normal clear corneal appearance with clearly visible iris vessels. **b** Corneal opacity of grade 1 in area (< 1/3 of corneal surface) and grade 1 in density (mild corneal haze). **c** Corneal opacity of grade 1 in area (< 1/3 of corneal surface) and grade 2 in density (moderate corneal haze, iris vessels are not clearly visible). **d** Corneal opacity of grade 1 in area (< 1/3 of corneal surface) and severe corneal opacity arrowed, where iris vessels are not visible (grade 3 in density)

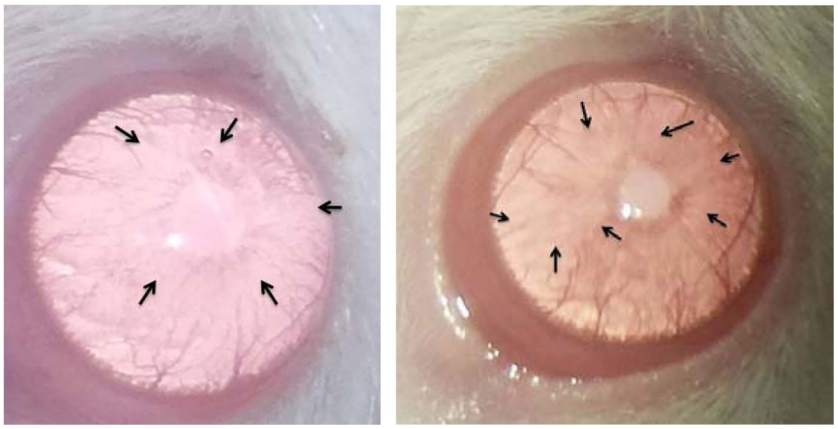

Fig. 3 Showing corneal opacity affecting 1/3–2/3 of corneal surface (grade 2 in area) and moderate corneal haze, iris vessels are not clearly visible (grade 2 in density)

IL, 2001). Descriptive statistics: Median and interquartile range (IQR) was used for non-parametric numerical data, and number and percentage for non-numerical data. Analytical statistics: Kruskal-Wallis test was used to assess the statistical significance of the difference between more than two study group ordinal variables. Post hoc test was used for comparisons of all possible pairs. Significance level was set at 0.05 while the highly significance level was set at 0.001.

Results

Reduction in the number of amoebae treated by *nCs* in vitro

A 100% reduction in number of viable amoebae was shown with the *nCs* concentration of 50 μg/ml after 24 h of incubation that showed a highly significant difference compared to parasite control (Table 1).

Postinoculation keratitis grading

All left corneas of *Acanthamoeba*-inoculated rats developed varying degrees of AK (Table 2 and Figs. 2, 3, 4, and 5).

Effects of *Ns*, *nCs*, and both treatments combined on AK

With *Ns* (30 mg/ml), 75% of rats were cured on the 10th day, reaching 87.5% on the 15th day (Tables 3, 4, 5, and 6); these results were significantly different from those in group V along the studied intervals and from those in group IV on the 15th and 20th day (Tables 7, 8, and 9). With *Ns* (60 mg/ml), 87.5% of rats were cured by the 10th day, reaching 100% cure on the 15th day (Tables 3, 4, 5, and 6); these results differed significantly from those in groups IV and V along the studied intervals (Tables 7, 8, and 9).

With *nCs* (50 μg/ml), 12.5% of rats were cured on the 10th day, rising to 75% on the 20th day (Tables 3, 4, 5, and 6). A significant difference was shown between group IIa and both groups IV and V as regards the area of opacity on the 20th day (Tables 7, 8, and 9). On the other hand, *nCs* (100 μg/ml) yielded a 100% cure rate on the 10th day (Tables 3, 4, 5, and 6), and the difference between group IIb and both groups IV and V was significant along the studied intervals (Tables 7, 8, and 9).

For group IIIa (*Ns* 30 mg/ml + *nCs* 50 μg/ml), 75% of rats were cured on the 10th day (Tables 3, 4, 5, and 6), and these results differed significantly from those of group V on the 10th day and from those of both groups IV and V on the 15th day

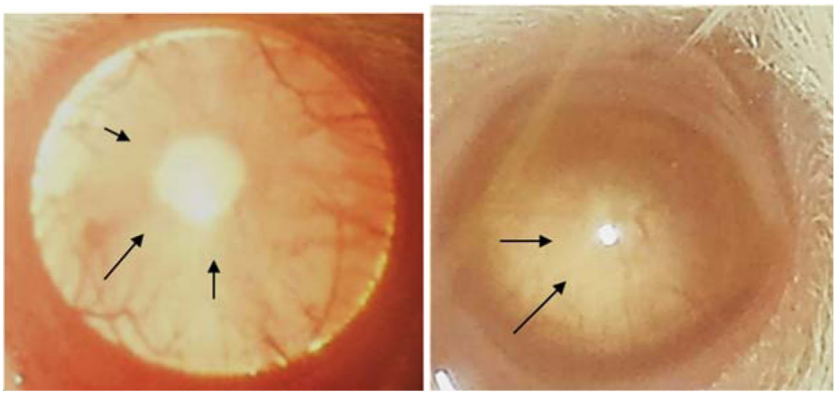

Fig. 4 Showing diffuse corneal opacity (grade 3 in area) and moderate corneal haze, iris vessels are not clearly visible (grade 2 in density)

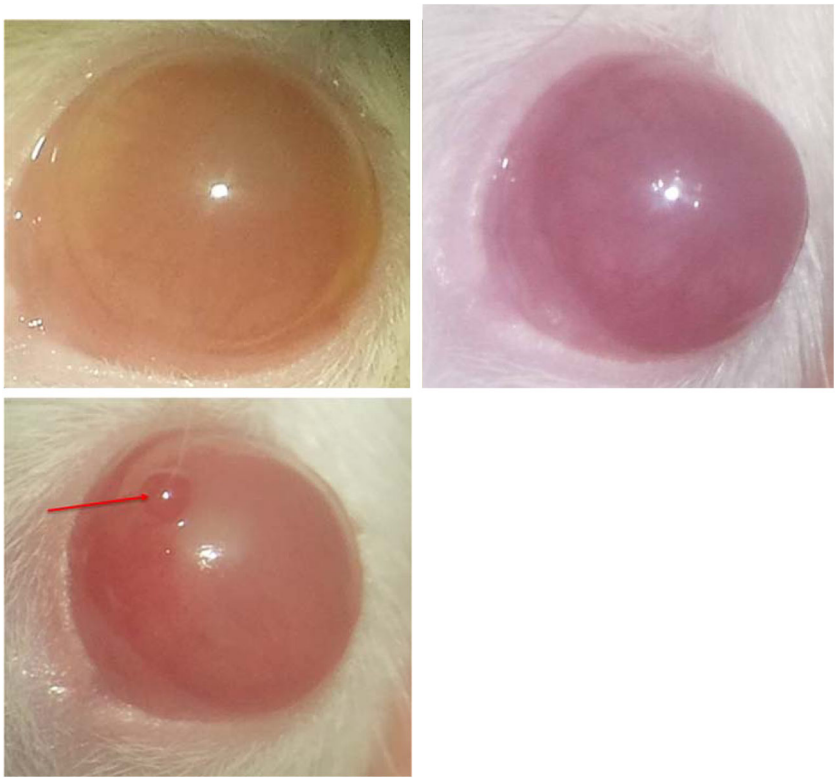

Fig. 5 Showing diffuse corneal opacity grade 3 in area and grade 3 in density (severe corneal opacification, iris vessels are not visible), with descematocele, red arrow

regarding the area (Tables 7, 8, and 9). Concerning group IIIb (*Ns* 60 mg/ml + *nCs* 100 μg/ml), 100% cure was reached from the 10th day (Tables 3, 4, 5, and 6), with a significant difference compared to that of group IV and group V along studied intervals (Tables 7, 8, and 9).

Concerning Ch (0.002%), only 25% were cured from the 10th day (Tables 3, 4, 5, and 6), with no significant difference compared to group V along all the studied intervals (Tables 7, 8, and 9).

Spontaneous cure of 25% of rats was observed in group V (parasite control, Tables 5 and 6). The results of cultivation were consistent with those of clinical evaluation (Fig. 6).

All drug-control eyes showed normal, clear corneas along the study course.

Table 3 The keratitis grading according to area and density of corneal opacity on day 10 posttreatment

	Ia		Ib		IIa		IIb		IIIa		IIIb		IV		V	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Grading I																
0	6	75	7	87.5	1	12.5	8	100	6	75	8	100	1	12.5	0	0
1	0	0	0	0	5	62.5	0	0	0	0	0	0	6	75	6	75
2	0	0	0	0	1	12.5	0	0	0	0	0	0	0	0	2	25
3	2	25	1	12.5	1	12.5	0	0	2	25	0	0	1	12.5	0	0
Grading II																
0	6	75	7	87.5	1	12.5	8	100	6	75	8	100	1	12.5	0	0
1	1	12.5	0	0	3	37.5	0	0	0	0	0	0	6	75	6	75
2	1	12.5	1	12.5	2	25	0	0	0	0	0	0	0	0	2	25
3	0	0	0	0	2	25	0	0	2	25	0	0	1	12.5	0	0

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group

Table 4 The keratitis grading as regards area and density of corneal opacity on day 15 posttreatment

	Ia		Ib		IIa		IIb		IIIa		IIIb		IV		V	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Grading I																
0	7	87.5	8	100	1	12.5	8	100	6	75	8	100	1	12.5	0	0
1	1	12.5	0	0	5	62.5	0	0	0	0	0	0	6	75	6	75
2	0	0	0	0	1	12.5	0	0	0	0	0	0	0	0	2	25
3	0	0	0	0	1	12.5	0	0	2	25	0	0	1	12.5	0	0
Grading II																
0	7	87.5	8	100	1	12.5	8	100	6	75	8	100	1	12.5	0	0
1	1	12.5	0	0	3	37.5	0	0	0	0	0	0	6	75	6	75
2	0	0	0	0	2	25	0	0	0	0	0	0	0	0	2	25
3	0	0	0	0	2	25	0	0	2	25	0	0	1	12.5	0	0

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group

Discussion

The AK incidence is rising worldwide, especially in contact lens wearers (Khan 2006); AK is usually treated by chlorhexidine, but failure of treatment and keratocyte toxicity have been reported (Lorenzo-Morales et al. 2013); therefore, the development of novel therapeutic agents is required (Visvesvara et al. 2007). Natural substances can be an alternative treatment, with high efficiency and low cytotoxicity (Freitas et al. 2006). Accordingly, we evaluated the therapeutic effect of *Ns* aqueous extract, n*Cs*, and the combination of both agents on experimentally induced AK. All A*canthamoeba*-inoculated left corneas developed AK, though with variable grades and variable association between the different grades, in agreement with Polat and Vural (2012); these discrepancies were mostly attributed to the difference in virulence in each rat, depending on its general and local immunological state (Polat et al. 2014). In the present study, *Ns* aqueous extract (30 mg/ml) gave a total cure of grades 1 and 2 in the area and density of corneal opacity on the 10th day posttreatment in 75% of rats; however, the remaining 2 rats (25%), presented with a pretreatment grade 3 in area and grade 2 in density; one showed total cure by the 15th day, while the other showed improvement to grade 1 in area and density on the 15th day, with no further improvement until the end of the study. The variations in response to treatment are in accordance with Lorenzo-Morales et al. (2015), who stated that the cure is partially dependent on the host response and the extent of the disease. In an in vitro study conducted by Ismail et al. (2018), *Ns* aqueous extract (30 mg/ml) gave 100% growth inhibition of *Acanthamoeba* after 24-h incubation, which was not obtained

Table 5 The keratitis grading as regards area and density of corneal opacity on day 20 posttreatment

	Ia		Ib		IIa		IIb		IIIa		IIIb		IV		V	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Grading I																
0	7	87.5	8	100	6	75	8	100	6	75	8	100	2	25	2	25
1	1	12.5	0	0	1	12.5	0	0	0	0	0	0	5	62.5	4	50
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	25
3	0	0	0	0	1	12.5	0	0	2	25	0	0	1	12.5	0	0
Grading II																
0	7	87.5	8	100	6	75	8	100	6	75	8	100	2	25	2	25
1	1	12.5	0	0	0	0	0	0	0	0	0	0	5	62.5	4	50
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	25
3	0	0	0	0	2	25	0	0	2	25	0	0	1	12.5	0	0

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group

Table 6 The overall progression of corneal opacity along different time intervals

	Pretreatment				Posttreatment											
	0 days				10 days				15 days				20 days			
	No opacity		Opacity		No opacity		Opacity		No opacity		Opacity		No opacity		Opacity	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
V	0	0	8	100	0	0	8	100	0	0	8	100	2	25	6	75
Ia	0	0	8	100	6	75	2	25	7	87.5	1	12.5	7	87.5	1	12.5
Ib	0	0	8	100	7	87.5	1	12.5	8	100	0	0	8	100	0	0
IIa	0	0	8	100	1	12.5	7	87.5	1	12.5	7	87.5	6	75	2	25
IIb	0	0	8	100	8	100	0	0	8	100	0	0	8	100	0	0
IIIa	0	0	8	100	6	75	2	25	6	75.0	2	25.0	6	75.0	2	25
IIIb	0	0	8	100	8	100	0	0	8	100	0	0	8	100	0	0
IV	0	0	8	100	1	12.5	7	87.5	1	12.5	7	87.5	2	25	6	75

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group

in our study using the same concentration. This discrepancy highlights the difference in response to therapy between in vitro and in vivo studies, in which the deep encystation in corneal tissues increased the resistance to therapy (Polat et al. 2008). The results obtained with *Ns* aqueous extract (30 mg/ml) differ significantly from those obtained with Ch 0.02% and the parasite control throughout the study. In the current study, the Ch 0.02%-treated group showed cure of 2/8 rats (25%); both rats exhibited grade 1 in area and density, and this poor response to Ch 0.02% is mostly due to its binding to tissues, which hinders optimal therapeutic concentrations (Pérez-Santonja et al. 2003). In contrast with our results, the findings of Polat and Vural (2012) showed a 75% cure rate using Ch 0.02% in a rat model; however, these results were obtained by combining Ch 0.02% with Neosporin. Interestingly, in the current study, 25% of the parasite control group showed spontaneous cure on the 20th day, possibly due to higher level of IgA in tears, as explained by Said et al. (2004).

Ns (60 mg/ml) yielded a prompt cure of 87.5% of rats on the 10th day, reaching up to 100% cure on the 15th day, despite the finding that 6/8 rats showed pretreatment grade 3 in area and 4/8 rats displayed grade 3 in density; these results significantly differed from those of the parasite control and Ch 0.02% along the studied intervals.

Table 7 Keratitis grading differences between the treated groups and control groups (parasite and *Ch* 0.02%) on day 10 posttreatment

	Grading I		Parasite control		*Ch* 0.02%		Grading II		Parasite control		*Ch* 0.02%	
	Median	IQR	Post hoc test		Post hoc test		Median	IQR	Post hoc test		Post hoc test	
V	1	1–1.5	*p* value	Sig.	0.628	NS	1	1–1.5	*p* value	Sig.	0.629	NS
Ia	0	0–1.5	0.021	S	0.069	NS	0	0–0.5	0.01	S	0.037	S
Ib	0	0–0	0.003	S	0.014	S	0	0–0	0.003	S	0.013	S
IIa	1	1–1.5	0.739	NS	0.880	NS	1.5	1–2.5	0.922	NS	0.561	NS
IIb	0	0–0	< 0.001	HS	0.002	S	0	0–0	< 0.001	HS	0.002	S
IIIa	0	0–1.5	0.021	S	0.069	NS	0	0–1.5	0.025	S	0.08	NS
IIIb	0	0–0	< 0.001	HS	0.002	S	0	0–0	< 0.001	HS	0.002	S
IV	1	1–1	0.628	NS			1	1–1	0.629	NS		
Kruskal-Wallis test	*p* value < 0.001	sig. HS					*p* value < 0.001	sig. HS				

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group. *IQR* interquartile range, *S* significant, *p value* probability value, *NS* non-significant, *HS* highly significant. Comparisons of median and IQR were done by Kruskal-Wallis test and then adjusted by post hoc test

Table 8 Keratitis grading differences between the treated groups and control groups (parasite and *Ch* 0.02%) on day 15 posttreatment

	Grading I		Parasite control		*Ch* 0.02%		Grading II		Parasite control		*Ch* 0.02%	
	Median	IQR	Post hoc test		Post hoc test		Median	IQR	Post hoc test		Post hoc test	
V	1	1–1.5	*p* value	Sig.	0.582	NS	1	1–1.5	*p* value	Sig.	0.509	NS
Ia	0	0–0	0.001	S	0.004	S	0	0–0	0.001	S	0.006	S
Ib	0	0–0	< 0.001	HS	0.001	S	0	0–0	< 0.001	HS	0.001	S
IIa	1	1–1.5	0.699	NS	0.871	NS	1.5	1–2.5	0.982	NS	0.595	NS
IIb	0	0–0	< 0.001	HS	0.001	S	0	0–0	< 0.001	HS	0.001	S
IIIa	0	0–1.5	0.011	S	0.047	S	0	0–1.5	0.016	S	0.058	NS
IIIb	0	0–0	< 0.001	HS	0.001	S	0	0–0	< 0.001	HS	0.001	S
IV	1	1–1	0.582	NS			1	1–1	0.611	NS		
Kruskal-Wallis test	*p* value < 0.001	sig. HS					*p* value < 0.001	sig. HS				

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group. *IQR* interquartile range, *S* significant, *p value* probability value, *NS* non-significant, *HS* highly significant. Comparisons of median and IQR were done by Kruskal-Wallis test and then adjusted by post hoc test

The *Ns* curative effect on AK can be attributed to its antioxidant activity (Abdelmeguid et al. 2010). Additionally, phenolic, alkaloid, and saponin constituents of *Ns* were found to enhance IgA secretion in tears leading to opsonization and phagocytosis of *Acanthamoeba* (Said et al. 2004). Furthermore, *N. sativa's* TQ is known to inhibit bacterial biofilm (Chaieb et al. 2011) and thus may hinder *Acanthamoeba's* binding to the cornea by inhibiting biofilm formation (Khan 2006).

On the other hand, *nCs* (50 μg/ml) provided a 12.5% cure rate on day 10, which reached 75% on day 20; all rats showed grades 1 and 2 in area and density, and we noticed no improvement in the remaining 25% that were presented with grade 3 in area or density. The *nCs* (50 μg/ml) results differed significantly from the Ch 0.02% and parasite control results on the 20th day regarding the area of opacity. A higher concentration of *nCs* (100 μg/ml) yielded 100% cure on day 10; still, all the rats showed pretreatment grades 1 and 2 in the area and density of the corneal opacity; a significant difference was shown compared to the Ch 0.02% and parasite control along the studied intervals. The aforementioned results of *nCs* highlight their curative effect on mild and moderate lesions, though a faster cure was obtained with *nCs* (100 μg/ml); the demonstrated curative effect of *nCs* on AK can be explained by their ability to inhibit bacterial and fungal biofilms (Martinez et al. 2010; Chávez de Paz et al. 2011; Holban et al. 2014). Thus, we expect that *nCs* may display the same

Table 9 Keratitis grading differences between treated groups and control groups (parasite and *Ch* 0.02%) on day 20 posttreatment

	Grading I		Parasite control		*Ch* 0.02%		Grading II		Parasite control		*Ch* 0.02%	
	Median	IQR	Post hoc test		Post hoc test		Median	IQR	Post hoc test		Post hoc test	
V	1	0.5–1.5	*p* value	Sig.	0.952	NS	1	0.5–1.5	*p* value	Sig.	0.965	NS
Ia	0	0–0	0.006	S	0.006	S	0	0–0	0.007	S	0.008	S
Ib	0	0–0	0.001	S	0.001	S	0	0–0	0.002	S	0.002	S
IIa	0	0–0.5	0.038	S	0.044	S	0	0–1.5	0.064	NS	0.07	NS
IIb	0	0–0	0.001	S	0.001	S	0	0–0	0.002	S	0.002	S
IIIa	0	0–1.5	0.056	NS	0.065	NS	0	0–1.5	0.064	NS	0.07	NS
IIIb	0	0–0	0.001	S	0.001	S	0	0–0	0.002	S	0.002	S
IV	1	0.5–1	0.952	NS			1	0.5–1	0.965	NS		
Kruskal-Wallis test	*p* value 0.001	sig. S					*p* value 0.001	sig. S				

Group Ia: *Ns* 30 mg/ml, group Ib: *Ns* 60 mg/ml, group IIa: *nCs* 50 μg/ml, group IIb: *nCs* 100 μg/ml, group IIIa: *Ns* 30 mg/ml-nCs 50 μg/ml, group IIIb: *Ns* 60 mg/ml-nCs 100 μg/ml, group IV: *Ch* 0.02% treated, and group V: parasite control group. *IQR* interquartile range, *S* significant, *p value* probability value, *NS* non-significant. Comparisons of median and IQR were done by Kruskal-Wallis test and then adjusted by post hoc test

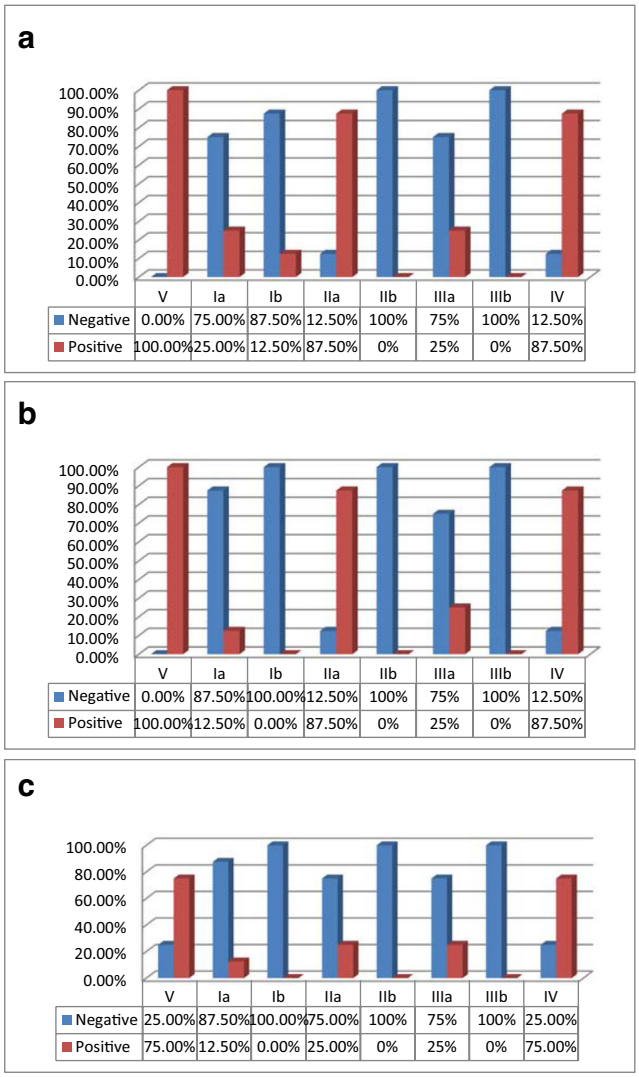

Fig. 6 The percentage of negative and positive samples by culture **a** on day 10 posttreatment, **b** on day 15 posttreatment, and **c** on day 20 posttreatment

inhibitory effect on *Acanthamoeba*. Additionally, the mucoadhesive character of *nCs* (Lim et al. 2016), as well as the high surface-to-volume ratio that enables them to cross the biological membranes (Majumdar et al. 2008) and increase their interaction with microbial cell membrane causing its rupture (Xing et al. 2009), may explain the AK curative effect of *nCs* in the current study.

Concerning the antiprotozoal effects of *nCs*, potent effects were reported when combined with other medications. *nCs*-antimoniate produced in vitro inhibition of *Leishmania infantum* (Pujals et al. 2008). Moreover, Barrera et al. (2010) reported that oral treatment of experimental visceral larva migrans by *nCs*-albendazole reduced the number of larvae. Additionally, *nCs*-curcumin resulted in 100% cure of *Plasmodium yoelii*-infected mice (Akhtar et al. 2012).

In the current work, combined *Ns* 30 mg/ml + *nCs* 50 μg/ml did not cure 25% of rats (group IIIa) presenting with grade 3, and these results differ significantly from those of the parasite control group on days 10 and 15. In contrast to the results of combined low concentrations, combined higher concentrations (*Ns* 60 mg/ml + *nCs* 100 μg/ml) yielded 100% cure of group IIIb on the 10th day, despite the finding that 3 rats exhibited grade 3 in area and grade 2 in density, and 2 had grade 3 in area and density; the curative effect was faster than that exhibited by *Ns* (60 mg/ml) alone, indicating a potential synergistic effect that differed significantly from the Ch 0.02% and parasite control results. A limited number of studies have reported the effects of nanoparticles on *Acanthamoeba* spp.; Anwar et al. (2019) demonstrated 100% inhibition of *A. castellanii* and *N. fowleri* viability by hesperidin-silver nanoparticles at a concentration of 50 μg/ml, in agreement with our in vitro results using *nCs* (50 μg/ml). Additionally, tannic acid-modified silver nanoparticles showed a significant antiamoebic effect on clinical strains of *Acanthamoeba* spp. (Padzik et al. 2018). Furthermore, pretreatment of *A. castellanii* amoebae with cinnamic acid-gold nanoparticles inhibited amoebae cell cytotoxicity (Anwar et al. 2018). In contrast to our results, the findings of Mahboob et al. (2018) showed that periglaucine A-poly (dl-lactide-co-glycolide) nanoparticles inhibit *A. triangularis* trophozoites by 74.9%, with 59.9% inhibition at concentrations of 100 μg/ml and 50 μg/ml.

Concerning the safety of the administered agents, all drug control corneas (the right eye of rats in each group) showed a normal clear corneal architecture throughout the study, indicating the safety of *N. sativa*, *nCs*, and the *N. sativa-nCs* combination and agreeing with previous reports on the tissue safety of *N. sativa* (Ali and Blunden 2003) and *nCs* (Rampino et al. 2013; Gaafar et al. 2014; Piras et al. 2014).

To the best of our knowledge, this is the first report on the effect of *nCs*, *N. sativa* and both agents combined in the treatment of experimentally induced AK. Based on the results demonstrated in our study, it could be assumed that chitosan nanoparticles, *N. sativa*, and the combination of both can successfully cure experimentally induced AK. Further studies are indeed required to determine the effects of these agents on *Acanthamoeba* cyst and trophozoite stages of different pathogenic *Acanthamoeba* species, as well as to explore their underlying mechanisms of antiamoebic actions. A greater number of experimental animals are recommended in future studies to increase the chances of evaluating a wider scale of severe AK.

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Compliance with ethical standards

Ethical consideration The study was approved by the Ethical Committee of Scientific Research, Faculty of Medicine, Ain Shams University, under authorization number FMASU-FWA00006444, and complies with the regulations of the Egyptian ministry of higher education and Helsinki declaration, 1964. The animal experiment was carried out according to the ILARC (Institute of Laboratory Animal Resources Commission) guidelines and principles for use of laboratory animals. Informed consent for patient was approved by the Institutional Review Board.

Conflict of interest The authors declare that there is no conflict of interest.

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