Investigating the effect of gold nanoparticles on hydatid cyst protoscolices under low-power green laser irradiation

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ABSTRACT

Objective: Various scolicidal agents are applied for the destruction of protoscolices in cysts media. Undesirable complications of the scolicidal agents limit the techniques to treat the cyst disease. Therefore, new non-toxic scolicidal agents are needed. Upon laser light irradiation, the photothermal gold nanoparticles (AuNPs) convert the absorbed laser light into heat through photothermal effect which kills the surrounding protoscolices by rising the temperature of the cysts media. In this study, we introduced biocompatible AuNPs as a non-toxic scolicidal agent to cure liver hydatid cysts.

Material and Methods: The protoscoleces were collected from the livers of naturally infected sheeps. In each experimental group, 1.5 mL suspensions of hydatid liquid containing protoscolices were added to test tubes. The test tubes were divided into five groups. Control, AuNPs only, Green laser only, High-dose AuNPs + laser and Low-dose AuNPs + laser groups. Two concentrations (0.4 and 0.8 mL) of AuNPs and three laser powers (30, 50, 150 mW) were applied for 30, 60 and 120 minutes to the groups. Then the cysts liquid assessed under a light microscope and determined the viability of protoscoleces.

Results: Protoscolices in high-dose AuNPs group were destructed up to 89.30% deaths under 150 mW laser power for 120 minutes. However, negligible cell deaths were observed in cases where only AuNPs added or only laser irradiated groups. Increasing the dose of AuNPs or laser power or duration of application increased the protoscolosidal death rate.

Conclusion: In the study, we have successfully demonstrated that the AuNPs are an effective therapeutic and scolicidal agent to cure hydatid cyst disease under laser irradiation.

Keywords: Gold, nanoparticle, laser, hydatid cyst

INTRODUCTION

Cystic echinococcosis is a zoonosis disease characterized by the formation of cysts in the internal organs of most humans caused by the larvae of a tape worm called Echinococcus granulosus (EG) (1). In humans, this infection involves cyst evolution in the lungs, liver and other organs (2,3). In some cases, hydatid cysts are fatal. This disease occurs in many countries worldwide and poses major challenges to human health and economy (4).

There is no ideal non-invasive treatment to cure hydatid cyst disease. Current treatment methods for this disease in the liver are surgery, percutaneous aspiration, and drug treatment (mebendazole and albendazole) (5,6). Among these, Puncture-Aspiration-Injection-Reaspiration (PAIR) method, as a percutaneous aspiration treatment, has recently gained much attention as a superior method to treat the hydatid cyst disease, especially gharbi type 1,2 cysts (7). Various scolicidal agents including albendazole, 95% alcohol, hypertonic saline, hydrogen peroxide, silver nitrate, cetrimide and ethyl alcohol are used in PAIR method for the sterilization of the cyst contents (7). However, scolicidal agents lead to many complications and the most important adverse effects of these agents are sclerosis and chemical cholangitis (8-10). The undesirable complications of the scolicidal agents limit the PAIR technique to be a non-invasive method to treat the cyst disease (11). Therefore, new non-invasive scolicidal agents are desperately needed to cure hydatid cysts.

In this study, we introduced biocompatible photothermal gold nanoparticles (AuNPs) as a non-invasive scolicidal agent to treat liver hydatid cysts via PAIR meth-
Cysts in this study. Upon laser light irradiation, the AuNPs absorb the light and convert into heat through photothermal effect which destructs the surrounding hydatid cysts by rising the temperature of the cysts media. We evaluated the scolicidal effect of AuNPs under different laser powers against protoscolices of hydatid cysts in this study.

MATERIAL and METHODS

Ethics approval for this study was granted by the ethical committee. The protoscolices of E. granulosus were collected from the livers of naturally infected sheep and goats. In each experimental group, 1.5 mL suspensions of hydatid liquid containing protoscolices were added to 42 test tubes in total. All the experimental test tubes were randomly assigned to five groups. Each study was repeated five times.

Synthesis of Gold Nanoparticles (AuNPs)

The gold nanoparticles were produced by Turkевич method (13). In typical synthesis, 1 mL of 12.7 mM aqueous chloroauric acid (HAuCl₄) solution was added to 49 mL of deionized water. The mixture was heated till boiling while stirring. After 5 minutes, 0.94 mL of 38.8 M trisodium citrate solution was added to the boiling mixture. Within 2-3 minutes, the color of the mixture turned to red. The mixture was stirred for 15 minutes and cooled to room temperature. The samples were centrifuged and washed several times with distilled water to generate AuNPs.

Collection of Protoscolices

The hydatid fluid was aspirated by a syringe and aseptically transferred in to a flask. This was centrifuged at 2500 rpm for 7 minutes by using centrifuge (Nuve NF 1200R multi-purpose centrifuge, Istanbul, Turkey). The supernatant was discarded and the protoscolices precipitates were washed two times with PBS (pH 7.2) solution. The number of protoscolices per ml was adjusted to 2 × 10⁷ protoscolices in 0.9% NaCl solution with at least 90% viability rate. The viability of the protoscolices was confirmed by their flame cell motility and impermeability to 0.1% eosin solution under a light microscope. The cysts which did not have any protoscolices or sufficient number of live protoscolices were not included in the study.

Experiment Groups

Two concentrations (0.4 and 0.8 mL) of AuNPs and three laser powers (30, 50, 150 mW) were applied for 30, 60 and 120 minutes. 1.5 mL of each hydatid liquid was placed in test tubes and these hydatid liquids were gently mixed. The test tubes were divided into five groups. CNI 532 nm green laser (Changchun New Industries Optoelectronics Technology Co., Ltd. Jilin, China) was used as the laser source.

Groups: There were 42 test tubes in total. Each test was repeated five times. 42 test tubes were used for each repeat.

Group A: (Low-dose AuNPs group) There were 9 tubes in this group. AuNPs were added to test tubes containing hydatid liquid. No laser was applied and no AuNPs was added.

Group B: (Laser group only) There were 9 tubes in this group. AuNPs were added to test tubes containing hydatid liquid. No laser was applied. (AuNPs-30 min, AuNPs-60 min, AuNPs-120 min).

Group C: (control group) There were 12 tubes in this group. This group is the test tubes containing hydatid liquid. No laser was applied and no AuNPs was added.

Group D: (AuNPs group only) There were 3 tubes in this group. AuNPs were added to test tubes containing hydatid liquid. No laser was applied. (AuNPs-30 min, AuNPs-60 min, AuNPs-120 min).

Group G: (Green laser group only) There were 9 tubes in this group. Test tubes containing hydatid liquid were only exposed to laser. AuNPs were not added to the test tubes. These test tubes were exposed to 30 mW (30 mW-30 min, 30 mW-60 min, 30 mW-120 min), 50 mW (50 mW-30 min, 50 mW-60 min, 50 mW-120 min) and 150 mW (150 mW-30 min, 150 mW-60 min, 150 mW-120 min) green lasers.

Group H: (High-dose AuNPs group) There were 9 tubes in this group. A high dose of AuNPs (0.8 mL) was added to the test tubes containing hydatid liquid. Then, these test tubes were exposed to 30 mW (30 mW-30 min, 30 mW-60 min, 30 mW-120 min), 50 mW (50 mW-30 min, 50 mW-60 min, 50 mW-120 min) and 150 mW (150 mW-30 min, 150 mW-60 min, 150 mW-120 min) green lasers.

Processes of Exposed of Groups

Group A: 0.8 mL of AuNPs were added to all the test tubes containing 1.5 mL cyst fluid. Laser was not applied. These tubes were kept for 30, 60 and 120 minutes. At the end of the period, 1.5 mL of 1% Eosin Y was added to each test tube and then the test tubes were kept in the incubator (37°C) for five minutes. After dyeing process completion, equal amounts of cells were taken from each test tube and then assessed under a light microscope.

Group G: AuNPs were not added to any of the test tubes containing 1.5 mL cyst fluid. A power of 30 mW, 50 mW or 150 mW green laser was applied for 30, 60, 120 minutes. At the end of the period, the test tubes were taken and 1.5 mL of 1% Eosin Y was added to each of the test tubes. After keeping these test tubes in the incubator (37°C) for five minutes, equal amounts of cells were taken from each test tube. These cell samples were assessed under a light microscope.

Group L: A low dose (0.4 mL) of AuNPs was added to all the test tubes having 1.5 mL of cyst fluid. A power of 30 mW, 50 mW or 150 mW green laser was applied for 30, 60, 120 minutes. At the end of the period, 1.5 mL of 1% Eosin Y was added to each test tube and these test tubes were kept in the incubator (37°C) for five minutes. The dyeing process was completed. Equal amounts...
of cells were taken from each test tube and these cell samples were assessed under a light microscope.

**Group H:** A high dose (0.8 mL) of AuNPs was added to all the test tubes containing 1.5 mL of cyst fluid. Laser power of 30 mW, 50 mW or 150 mW was applied for 30, 60 and 120 minutes. At the end of the period, the test tubes were taken and 1.5 mL of 1% Eosin Y was added to each test tube. Then these were kept in the incubator (37°C) for five minutes for dyeing process. Equal amounts of cells were taken from each test tube and the samples were assessed under a light microscope.

**Viability Test**

Eosin exclusion test was used to determine the viability of protoscoleces (14). After exposure to the eosin 0.1% (1 g of eosin powder in 1000 mL distilled water), the alive and dead protoscoleces were analyzed. Alive protoscoleces remain colorless and show characteristic muscular movements and flame cell activity, while dead protoscoleces absorb eosin and have a red color.

**Statistical Analysis**

Mortality rate of protoscoleces (%) = the number of dead protoscoleces/total number of protoscoleces x 100% (15).

All the experiments were performed in quintuplicate. Data collection was performed using Microsoft Excel 2007 (Microsoft, Remond, WA, USA), and statistical analysis was undertaken using Statistical Package for the Social Sciences 16.0 (SPSS Inc., Chicago, IL, USA) with the analysis of variance (ANOVA). Moreover, Tukey’s Honest Significant Difference (HSD) test was used for categorical variables. Continuous variables were reported as the mean ± standard deviation. A value of p< 0.01 indicated significant differences between groups.

**RESULTS**

**Results of Ex-Vivo Microbiological Examinations**

Protoscolex death was only observed in the groups with AuNPs containing protoscoleces in hydatid cyst liquid under laser irradiation (group L and group H) (Table 1). In these groups, the amount of cell deaths depends on the duration of laser irradiation and the laser power. Whenever the duration of the laser irradiation or laser power increases, the amount of cell deaths rises. For instance, protoscoleces in group H were destructed up to 89.30 % deaths under 150 mW laser power for 120 minutes. However, negligible cell deaths were observed in cases where only AuNPs added or only laser irradiated samples which are group G and group A.

In conclusion, a significant increase for the number of protoscoleces deaths in hydatid cyst liquid is observed whenever longer laser irradiation, higher laser power or higher amount of AuNPs is applied.

**Statistical Analysis Results**

Groups were compared according to their AuNPs doses and laser irradiation (low-dose AuNPs with laser, high-dose AuNPs with laser, only laser, and only AuNPs). The highest mean was associated with the high-dose AuNPs group (Group H), then the low-dose AuNP group (Group L) followed by only laser (Group G) and then only AuNP group (Group A) (p< 0.01).

In addition, the groups were also compared according to their processing times (30 min, 60 min, 120 min). The highest mean was associated with the 120 min process groups, followed by the 60 min process groups, and then the 30 min process groups (p< 0.01).

According to the power of the laser applied (30 mW, 50 mW, 150 mW), the groups were compared. The highest mean was associated with the 150 mW group, followed by the 50 mW group, and then 30 mW group.

When the AuNP dose, process time and laser power were taken into account, the highest average protoscoleces deaths in hydatid cyst liquid was associated with the group H having high-dose AuNPs which exposed to 150 mW laser power for 120 minutes irradiation (p< 0.01) as shown in Figure 1. In contrast, the lowest average protoscoleces deaths was associated with the group G or group A which are having AuNPs without any laser exposure or only laser irradiation without any AuNPs addition, respectively (p< 0.01) (Figure 2).

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<th>Table 1. The death rates of protoscoleces in group L and group H</th>
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DISCUSSION

Hydatid cyst disease is an important and common public health problem worldwide. In the endemic areas of countries and regions such as Peru, Argentina, East Africa, Middle Asia and China, the disease affects 50 out of 100,000 people each year while its prevalence is between 5% and 10% (1,2).

The present optional therapies for hydatid cyst disease are problematic in many ways. Surgical methods are all invasive and come with the risk of recurrence and the rupture and spillage of the contents (14). Where chemotherapy is concerned, albendazole and mebendazole are the preferred drugs. However, their concentrations in the hydatid cyst are not sufficient to cure the disease because the cyst’s wall is too thick (16). Clinical studies have shown that the medication used for treatment could not always kill the protoscolices (17). Percutaneous aspiration, injection and reaspiration (PAIR) is a percutaneous technique which is a significant and versatile alternative for the conventional methods, especially in the treatment of early-stage cysts (18).

There is a desperate need for a scolicidal agent to be used in PAIR method to effectively cure hydatid cyst protoscolices.
According to the literature, a large number of scolicidal agents are used in PAIR, such as; Albendazole, 95% alcohol, hypertonic saline, hydrogen peroxide, silver nitrate, cetrimide and ethyl alcohol. However, many of these agents have adverse effects, therefore, limit their usage in the treatment of hydatid cyst disease (19). Formalin was the first scolicidal agent used, however, it is not used today due to its toxic effect (20). Radiologists prefer using another scolicidal agent, ethyl alcohol which is inflammable and volatile (21). This characteristic of ethyl alcohol has led to the restriction of its use in surgery. Also, ethyl alcohol can damage the bile ducts epithelium, leading to sclerosing cholangitis (22). That is why, it is not used to treat hydatid cysts which have bile duct communication. In addition, due to low scolicidal efficiency and the resultant complications, the usage of hydrogen peroxide as a scolicidal agent in the treatment of hydatid disease is very limited (23). A disinfectant chemical, povidone-iodine (PVP-I), is also used as a scolicidal agent. The complications of PVP-I are PVP (polyvinylpyrrolidone) storage disease, renal shutdown and sclerosing serositis (23). Nowadays, most effective scolicidal agent frequently used is hypertonic saline. Hypertonic saline is nearly 100% effective on protoscolices (24); however, it could lead to hyponatremia, neurological side effects and intracranial bleeding (25). Furthermore, the usage of hypertonic saline should be avoided in treatments of cysts which open to the bile duct due to very high possibility to have chronic sclerosing cholangitis disease (20). Another effective scolicidal agent is cetrimide-chlorhexidine (Savlon) and it has scolicidal effect even a minimal usage of 0.1%. However, like hypertonic saline, cetrimide-chlorhexidine should also not be used to treat cysts associated with the bile duct (26). Although effective cure rate of scolicidal agents are present for the treatment of hydatid cyst protoscolices, these agents possess very harmful adverse effects including chronic sclerosing cholangitis (12,14). Even if the surgeons are not willing to use these toxic agents, the usage of these agents is unfortunately a common practice in clinic due to deficiency of ideal non-toxic scolicidal agent (27). Therefore, there is an extreme need for a non-invasive agent which has a significant scolicidal effect to cure the hydatid cyst protoscolices without any harmful effects and complications.

Anderson and Loveless have reported a successful destruction of protoscolices, namely, E. granulosus protoscolices, by varying temperature in cyst media (28). In their study, different degrees of temperature were applied to protoscolices in cyst fluid from the lungs and livers of infected sheep. The durations of the protoscolices deaths were 16 days at 20°C, 8 days at 30°C, 4 days at 40°C, and 2 hours at 50°C. According to this study, high temperatures above 40°C effectively kill the protoscolices which is also reported in various studies in literature (29,30). As a result, a scolicidal agent which releases heat to the surroundings meaning increase the temperature of the protoscolices media above 40°C could be used as a new scolicidal agent to cure the protoscolices in cyst fluid.

In this study, non-toxic AuNPs was introduced and successfully applied as a new scolicidal agent to cure the hydatid cysts protoscolices in the liver. Under laser irradiation, the AuNPs is a heat generator which increase the temperature of the surroundings, protoscolices in this case, and eventually kill the protoscolices. Under different laser powers and irradiation durations, we evaluated the scolicidal effect of AuNPs against protoscolices of hydatid cysts on ex-vivo model.

Gold nanoparticles are considered as harmless, stable and biocompatible materials which are frequently used in medical research (15). Various reports in literature have shown that AuNPs do not possess ancytotoxic and genotoxic effects, and any known systematic or local side effects (31,32). Besides the inertness and non-toxic character, AuNPs have a unique property which is called “photothermal effect”. Spherical AuNPs can transform the absorbed green laser light energy into heat energy through photothermal effect (33,34). The heat will dissipate into the surrounding media and this localized heating by using AuNPs can cause thermal cellular destruction (35,36). We benefit this destruction process in the treatment of protoscolices in liver hydatid cysts and kill this protoscolices via the temperature increase after localized heating by AuNPs. That is to say, this study facilitated the application of laser to AuNPs in order to rise the temperature of the cyst fluid which eventually cause the deaths of all the protoscolices.

In the present study, besides a control group (Group C), we used 4 different groups such as; only AuNPs added samples (Group A), only laser irradiation applied samples (Group G), low-dose AuNPs added and laser irradiated groups (Group L), high-dose AuNPs added and laser irradiated groups (Group H). We applied three different laser powers of 30 mW, 50 mW, and 150 mW for different durations (30 min, 60 min, 120 min). Due to the usage of low-power and harmless laser powers, we irradiated the cyst liquid containing protoscolices for longer periods in order to result a successful mortality. The experimental results show that scolicidal activity of AuNPs was increased by raising the laser power with higher concentrations of AuNP dose and extending the duration of irradiation process. These result a rise in temperature of the hydatid cysts media which kills protoscolices in significant incidences of mortality. However, when only irradiation of laser or only inclusion of AuNPs to hydatid cysts liquid, there was only negligible deaths of protoscolices which is within the range of acceptable deviation.

**CONCLUSION**

We have successfully demonstrated the usage of AuNPs as a therapeutic and scolicidal agent to cure hydatid cyst disease under laser irradiation. The main advantages of AuNPs compared...
with the other scolicidal agents are non-invasive and biocompatible scolicidal agent. Our results show that higher laser powers and AuNPs amounts with longer irradiation durations result effective destruction of protoscolices in liver hydatid cysts due to a sharp increase in temperature. AuNPs possess a scolicidal effect and could use in PAIR applications. Hence, this approach could produce a better treatment for various hydatid cyst diseases, even in the treatment of cysts associated with the bile duct.

**Ethics Committee Approval:** Ethics approval for this study was granted by the ethical committee.

**Informed Consent:** Written informed consent was obtained from patients who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - B.Ç.; Design - B.Ç., FA.; Supervision - FA., S.Y.; Resource - B.Ç., M.E.D.; Materials - B.Ç.; Data Collection and/or Processing - FA., B.Ç.; Analysis and/or Interpretation - B.Ç., M.E.D.; Literature Search - S.Y., B.Ç.; Writing Manuscript - B.Ç., FA.; Critical Reviews - FA.

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Düşük güçte lazer uygulannmış altın nanoparçacıklarının hidatik kist protoskoleksleri üzerine etkisinin araştırılması

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ÖZET


Fototermal altın nanopartiküller (AuNPs) kist sıvısı içindeki protoskolekslerin etrafını sararak lazer ışını altında, ışınların enerjisini absorbe edip ısı enerjisi dönüştüren bir etkileşime sahip edildikleri. Çalışmada, biyo-uyumlu fototermal altın nanopartikülleri (AuNPs) karaciğer hidatik kistlerini tedavi etmek için toksik olmayan bir skolosidal ajan olarak uygulandığında etkisi saptanmıştır.

Gereç ve Yöntem: Protoskoleksler infekte koyun karaciğerlerinden elde edildi. Her bir çalışma grubu için 1,5 mL kist sıvısı içeren tüpler oluşturuldu. Tüpler 5 gruba ayrılır. Kontrol grubu, sadece AuNPs eklenen grup, sadece lazer uygulanan grup, yüksek doz AuNPs eklenip lazer uygulanan grup, düşük doz AuNPs eklenip lazer uygulanan gruplar idi. İki farklı konsantrasyonda (0,4 ve 0,8 mL), üç farklı güçte lazer (30, 50, 150 mW), 30, 60, 120 dakika süresiyle gruplara uygulandı. Daha sonra kist sıvıları ışık mikroskobu altında incelendi ve protoskolekslerin canlılık kriterleri değerlendirildi.

Bulgular: Yüksek doz AuNPs eklenen ve 150 mW lazerin 120 dakika uygulandığı grupta %89.30 protoskoleks ölümü sağlandı. Fakat sadece AuNPs eklenen ve sadece lazer uygulanan gruplarda anlamlı ölüm oranları ile karşılaştırımdı. AuNPs dozunun, lazer gücünün veya sürenin arttırmaları ile protoskoleks ölümünün de arttığını tespit edildi.

Sonuç: Çalışmada, AuNPs’nin lazer ışını altında, hidatik kist tedavisinde etkili bir skolosidal ajan olabildiği başarılı sonuçlar arzetti ve etkisi bir şekilde gösterildi.

Anahtar Kelimeler: Altın, nanoparçacık, lazer, hidatik kist

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