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Original Article

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Antifungal activity of polymeric micelles of silver nanoparticles prepared from *Psidium guajava* aqueous extract

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Summary **In the present study, silver nanoparticles (AgNPs) were synthesized by green synthesis using *Psidium guajava* aqueous extract (PE) as a reducing agent and silver nitrate ($AgNO_3$) as a precursor. The obtained AgNPs showed maximum absorbance at 455 nm. The results from energy-dispersive X-ray spectroscopy demonstrate Ag signal at 88.33% weight. The particle image under scanning electron microscopy is spherical shape. The average size of the freshly prepared AgNPs is 96 ± 4 nm but is dramatically increases during storage due to particle aggregation. Coating AgNPs with polymeric micelles of poloxamer 407 (F127) at the suitable ratio can decrease the size of the freshly prepared AgNPs to 70.4 ± 0.8 nm and significantly prevent AgNPs from aggregation. The obtained coated AgNPs showed high effective on inhibition of *Candida albicans*. Isotonic solutions of 0.9% NaCl and phosphate buffer solution pH 7.4 can cause some extend of aggregation and increase the particle size of the coated AgNPs but the increased size is in the colloidal range that no precipitation occurs during 90 days at room temperature. From our results, it is suggested that the 1:1 ratio of AgNPs/F127 is the most suitable ratio to obtain the AgNPs loaded polymeric micelles with high stability, small particle size, and high inhibitory activity against *C. albicans*. These AgNPs are the promising antifungal nanomaterials for further study in animal model.**

Keywords: Silver nanoparticles, green synthesis, polymeric micelles, *Psidium guajava* antifungal activity

1. Introduction

Metal nanoparticles have been recently applied in various fields. Silver nanoparticle (AgNPs) are being successfully used in medical and pharmaceutical fields because of their potential on antimicrobial activity. Moreover, AgNPs also pronounce anticancer and antioxidant activities (*1*). Synthesis of AgNPs can be performed in small scale as in laboratory works or larger scale as in industries by redox reaction between silver salt such as silver nitrate ($AgNO_3$) as a precursor and reducing agent to reduced Ag^+ to Ag^o (*2*). However, some chemical reducing agents can cause harmful to human and environment. Green synthesis using reagents from eco-friendly resources such as bacteria (*3*), fungi (*4*), algae (*5*), and plants (*6*) are received increasing interest nowadays. The extracts from certain plants such as *Plectranthus amboinicus* (*7*), *Lycium barbarum* (*8*), *Alternanthera dentata* (*9*), *Coriandrum sativum* (*10*), *Emblica officinalis* (*11*), and *Sargassum incisifolium* (*12*) have been reported to act as good reducing agents in the green synthesis of AgNPs. The reducing activity is expected to obtain from some phytochemicals in plant extracts (*13*).

Psidium guajava is a plant in family Myrtaceae and is an original native plant of Mexico that extends throughout the South America, European, Africa and Asia (*14*). Many reports indicate that *P. guajava* leaves possess many biological effects that can support the good health of human being such as hepatoprotective

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effect (*15*), antioxidant activity (*16*), anti-inflammatory activity (*17*), antimicrobial activity (*18*), and anti-hyperglycemic as well as anti-hyperlipidemic effects (*19*). The main constituents of *P. guajava* are in the groups of vitamins, tannins, phenolic compounds, flavonoids, sesquiterpene alcohols and triterpenoid acids (*20*). The important phytochemicals of *P. guajava* leaves are gallic acid, catechin, epicatechin, rutin, naringenin, and kaempferol (*14,21,22*). *P. guajava* was previously used as a reducing agent in AgNPs synthesis (*23,24*), however the size of the obtained AgNPs were quite large according to the particle aggregation. Therefore, searching for suitable stabilizing agent is necessary in order to protect AgNPs from aggregation and to maintain the obtained particles with stable small size.

Polymeric micelles have spherical and nanosize (10-100 nm) supramolecular core/shell structures formed by self-assembly of amphiphilic copolymers in aqueous solution. Poloxamers are triblock copolymers composed of poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) (PEO-PPO-PEO). Polymeric micelles of poloxamers have been reported to stabilize AgNPs obtained from chemical and natural reducing agents (*25,26*), however, they have not yet been used with AgNPs obtained from *P. guajava* aqueous extract (PE).

In the present study, Poloxamer 407 (F127) micelles were investigated for possible potential to stabilize AgNPs prepared by using PE as a reducing agent. The effects of F127 micelles on physicochemical characteristics of the obtained AgNPs were evaluated using dynamic light scattering (DLS), scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX). Moreover, the physical stability and antifungal activity of the obtained AgNPs against *Candida albicans* were also studied.

2. Material and Methods

2.1. *Materials*

2,4,6-Tripyridyl-s-triazine (TPTZ) and 2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were from Sigma-Aldrich, Inc (St. Louis, MO, USA). $AgNO_3$ and sodium hydroxide (NaOH) 97% were from RCI Lab-scan Co., Ltd. (Bangkok, Thailand). Hydrochloric acid (HCl) 37% was from Carlo Erba reagents (Rodano, Metropolitan City of Milan, Italy). Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) was from RFCL limited (New Delhi, India). Iron (III) chloride hexahydrate ($FeCl_3$) was from Honeywell Riedel-de-Haën™ (Seelze GmbH Manufacturing Facility, Seelze, Hanover, Germany). Poloxamer 407 (F127) were from O-BASF Co. (Ludwigshafen, Germany). Sabouraud dextrose agar (SDA) and broth (SDB) were purchased from BBL™ (Baltimore, MD, USA). All other chemicals and solvents were of analytical reagent grade or the highest grade available. They were used without further purification.

2.2. *Preparation of PE*

Exact amount of 2 g of dried powder of *P. guajava* leaves was mixed with 100 mL deionized water and stirrer at room temperature for 24 h. The mixture was filtered through a Whatman No.1 filter paper and the filtrate was evaporated using rotary evaporator until the solvent was completely removed. PE obtained was kept in the refrigerator at 4ºC for further study.

2.3. *Synthesis of AgNPs*

A solution containing 0.1 mg/mL of PE in deionized water was firstly prepared and heated to 70ºC. Exact amount of 1 mL $AgNO_3$ 10 mM solution was added drop wise to the heated solution with continuous stirring at 100 rpm for 60 min. The obtained mixture was diluted with deionized water and subjected to centrifugation (Heraeus™ Megafuge™ 40 Centrifuge Series, Thermo Fisher Scientific, Waltham, MA, USA) at 8,000 rpm for 15 min to remove any trace un-utilized phytochemicals. This washing process was done in triplicate. The aqueous colloidal AgNPs were lyophilized using Freeze Dryer (Virtis®, Warminster, PA, USA) to obtained AgNPs powder.

2.4. *AgNPs loaded polymeric micelles*

Certain amount of AgNPs powder was dispersed in ultrapure water to obtain AgNPs at a concentration of 0.1 mg/mL. Aqueous solution containing 5% F127 was prepared and heated to 70°C with continuous stirring at 100 rpm. The AgNPs solution was dropped wise into F127 solution to obtain mixtures with different volume ratios of AgNPs solution to F127 solution (AgNPs/F127) at 1:1, 1:3 and 1:5 in order to load AgNPs in F127 micelles. The obtained AgNPs loaded micelles were called coated AgNPs. The mixtures were continuously stirred for 30 min and washed with ultrapure water at 10,000 rpm for 15 min 3 times by using centrifugation. The uncoated AgNPs were prepared in the same manner as the coated AgNPs but ultrapure water was used instead of F127 solution. The obtained AgNPs were kept at 4ºC for further use.

2.5. *Characterization of AgNPs*

The obtained AgNPs were characterized using UV-visible spectrophotometer (UV-vis). The size, size distribution, and zeta potential of AgNPs particles were investigated using DLS (Malvern, Nano series, UK). The surface morphology was investigated by using

SEM (IT-JSM-300) and the Ag signal was detected by using EDX, a high resolution SEM, in order to confirm Ag elemental from the AgNPs. For this experiment, the AgNPs sample was placed on a stub with graphite tape. The element standard used were C ($CaCO_3$), O (SiO_2), Cl (KCl), K (MAD 10 Feldspar), Cu (Cu) and Ag (Ag).

2.6. *Antifungal activity*

Antifungal activity of the obtained AgNPs was tested by using a method Kirby-Bauer (*27*) with some modification. Fungal strain of *C. albicans* ATCC 10231 was used as a test microorganism. The fungal strain was cultured in SDA at 37ºC for 36-48 h. The fungal suspension was adjusted to a final density of 0.5 McFarland constant by observing the optical density at 600 nm under UV-vis to obtain microbial concentration of 1-2 × 10^5 CFU/mL in SDB. Then the fungal suspension was swabbed on the surface of SDA. The wells were made on the agar plates using a sterile cork borer having a diameter of 6 mm. Each 40 µL sample was filled in the well whereas PE and F127 solutions were used as negative controls. The plates were incubated at 37 ± 2ºC for 48 h. The antimicrobial activity of AgNPs was evaluated by determining the diameter of clear zone of inhibition expressed in millimeter (mm). All samples were done in triplicate.

2.7. *Stability study*

The obtained AgNPs were kept at room temperature for 90 days to study their physical characteristics and antifungal activity. The change in physical characteristics such as particle size, size distribution, and zeta potential was investigated using DLS. Antifungal activity of the samples after keeping was compared to the freshly prepared using the antifungal test method mentioned above.

2.8. *Effect of diluting media*

The coated AgNPs containing 1:1 ratio of AgNPs/F127 was diluted with deionized water, 0.9% sodium chloride (NaCl) and phosphate buffer solution (PBS) pH 7.4 to investigate the effect of diluting media on particle size of AgNPs. The obtained suspensions were kept at room temperature for 90 days. The particles size and antifungal activity of the obtained AgNPs after keeping were investigated using the same methods as mentioned above. The results were compared with those of freshly prepared.

2.9. *Statistical analysis*

Data were reported as a mean ± standard deviation. Statistic analysis was done by means of a one-way analysis of variance (ANOVA) and Duncan's multiple range test ($p < 0.05$) using Statistic a software version 17.

3. Results

3.1. *Biosynthesis of AgNPs*

The original color of PE solution is green-brown whereas that of $AgNO_3$ solution is colorless. After mixing these two solutions, the color of the obtained mixture was light green-brown. During synthesis, the color of the mixture turned to brown color at 30 min. After completely reaction at 60 min, the mixture was dark brown color. The obtained AgNPs powder after dispersing in deionized water showed maximum absorbance at 455 nm as shown in Figure 1A. Morphology of AgNPs observed under SEM was spherical shape as shown in Figure 1B. Confirming Ag element using EDX, the spectrum indicated that the obtained AgNPs were composed of Ag 88.33% weight as seen in Figure 1C.

3.2. *AgNPs loaded polymeric micelles*

Synthesizing AgNPs using several ratios of AgNPs/F127 yielded AgNPs loaded polymeric micelles that so called coated AgNPs and those without F127 yielded

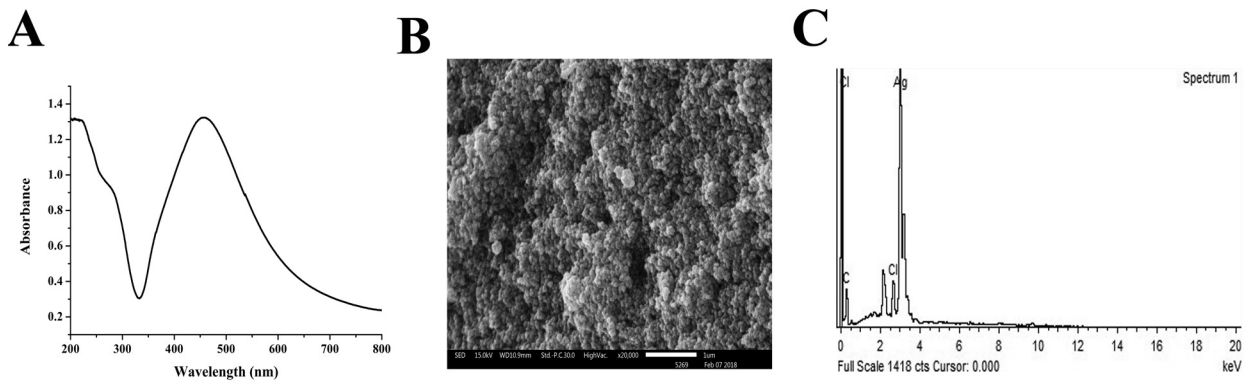

Figure 1. The UV-vis spectrum (A), surface morphology (B), EDX spectrum of Ag signal (C) of AgNPs.

Table 1. Particles size, size distribution, and zeta potential of uncoated AgNPs and AgNPs coated with polymeric micelles

Ratio of AgNPs/F127	Classification of AgNPs	Particles size (nm)	PdI	Zeta potential (mV)
1:0	uncoated	96.4 ± 3.8	0.24 ± 0.1	-18.4 ± 1.6
1:1	coated	70.4 ± 0.8	0.18 ± 0.1	-31.9 ± 0.5
1:3	coated	171.2 ± 2.4	0.22 ± 0.1	-25.5 ± 0.7
1:5	coated	258.6 ± 11.4	0.31 ± 0.1	-22.4 ± 1.2

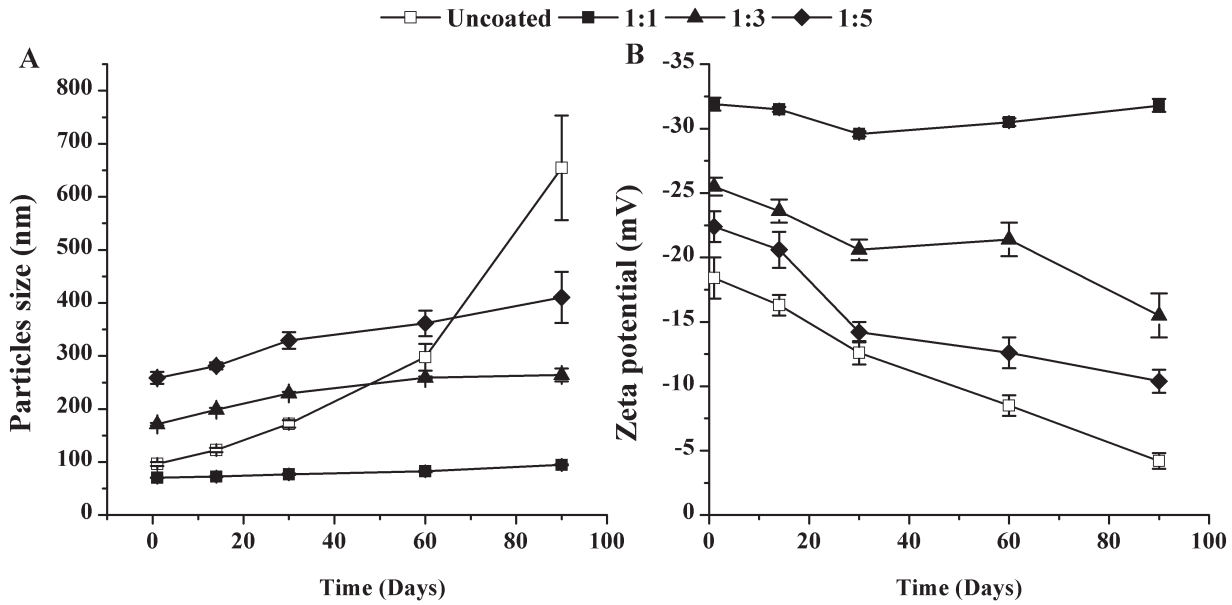

Figure 2. Changes of particles size (A) and zeta potential (B) of the uncoated AgNPs and AgNPs coated F127 with AgNPs/F127 ratios of 1:1, 1:3, and 1:5 after keeping for 90 days.

uncoated AgNPs. The color of the coated AgNPs colloid was lighter than the uncoated AgNPs. The particle size, size distribution, and zeta potential of the coated and uncoated AgNPs were shown in Table 1. It was found that the particle size of the coated AgNPs obtained from the 1:1 ratio of AgNPs/F127 was significantly smaller than the uncoated AgNPs. Increasing this ratio to 1:3 and 1:5 increased the size of the particles. Size distribution of the AgNPs obtained from all ratios was in the range of narrow and acceptable. The negative zeta potential value of the coated AgNPs at 1:1 ratio was higher than those from 1:3 and 1:5 ratios. It is noted that the negative zeta potential value of the coated AgNPs from all ratios was significantly higher than that of the uncoated AgNPs.

3.3. *Stability of AgNPs*

The particles size and zeta potential of the coated and uncoated AgNPs keeping at room temperature for 90 days are shown in Figure 2. It was found that the particle size of the uncoated AgNPs increased promisingly with storage time whereas the size of the coated AgNPs increased at very low rate, particularly those obtained from the ratio of 1:1. The zeta potential of the coated AgNPs obtained from the ratio of 1:1 was not significantly different between the freshly prepared and those kept for 90 days. However, the zeta potential of the uncoated AgNPs was significantly decreased dramatically along the storage time. After 90 days of keeping, the zeta potential of the uncoated AgNPs was nearly zero.

3.4. *Effect of diluting media*

In this study, the coated AgNPs obtained from 1:1 ratio of AgNPs/F127 was used and compared with the uncoated AgNPs. The particles size of AgNPs at day 1, 3, 7, 14, 30, 60, and 90 after contacting with three different diluting media was determined using DLS. The results are shown in Figure 3. It was found that the freshly prepared particles after diluting with 0.9% NaCl and PBS were bigger than those diluted with deionized water. Keeping at room temperature, the relatively high rate of size increase was seen within 15 days after contacting with 0.9% NaCl and PBS but after that the size was not much influenced. The size increase in the uncoated AgNPs occurred during 90 days could be seen more obviously than the coated AgNPs.

3.5. *Antifungal activity of AgNPs*

Antifungal activity of the obtained AgNPs against *C. albicans* was investigated using the diffusion method.

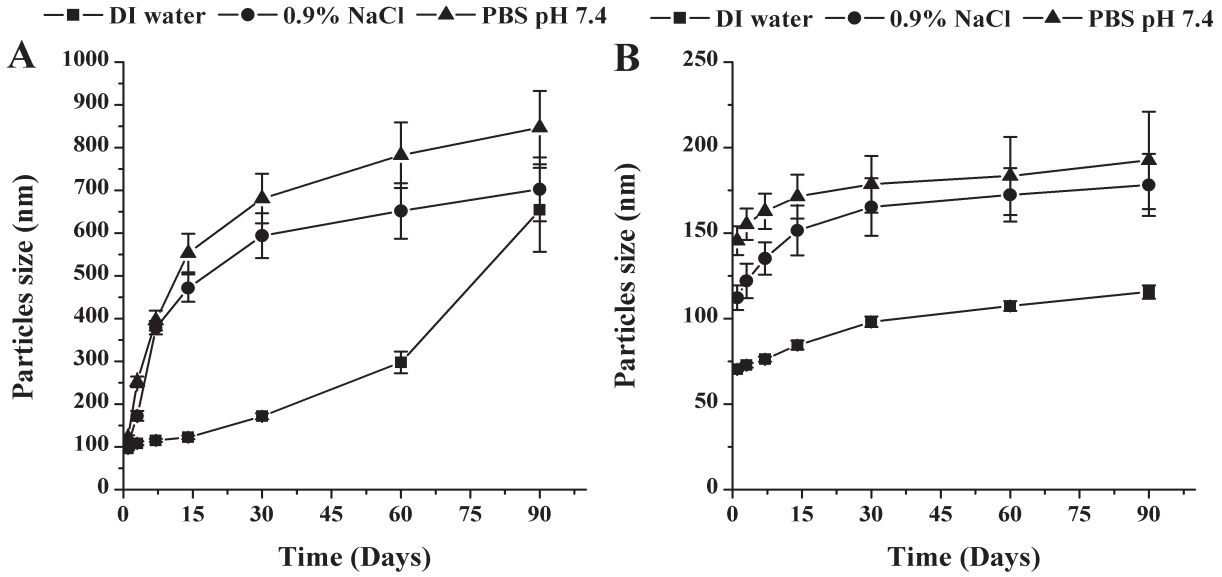

Figure 3. Changes of particle size of the uncoated AgNPs (A) and coated AgNPs (B) after contacting with different media.

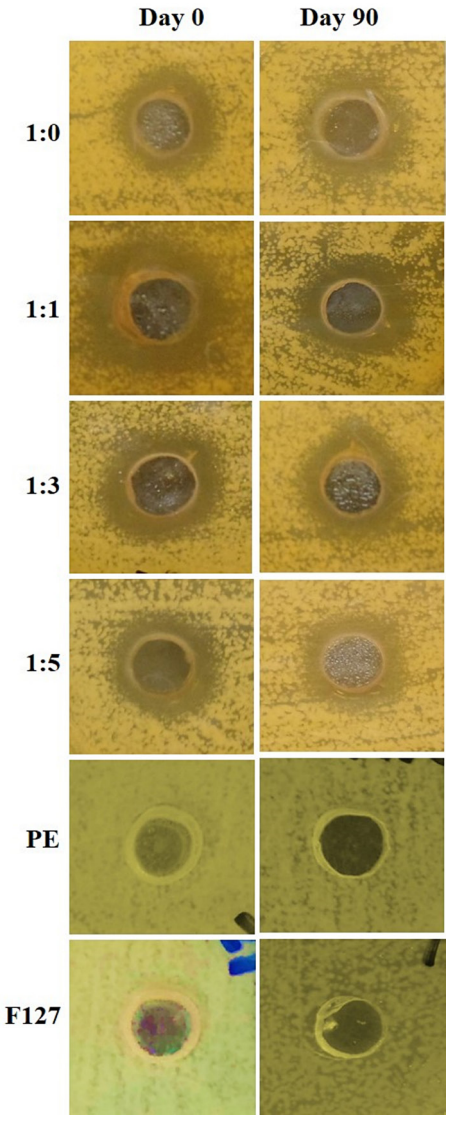

Figure 4. Inhibitory activity of the freshly prepared AgNPs (A) and the AgNPs after keeping for 90 days in comparison with the negative controls.

Table 2. Inhibition zones of the freshly prepared AgNPs *C. albicans* in comparison with AgNPs kept at room temperature for 90 days

Samples	Classification of sample	Day 0	Day 90
PE	negative control	NZ	NZ
F127	negative control	NZ	NZ
1:0 AgNPs/F127	uncoated AgNPs	13.8 ± 0.3	7.6 ± 0.7
1:1 AgNPs/F127	coated AgNPs	14.2 ± 0.7	13.8 ± 0.7
1:3 AgNPs/F127	coated AgNPs	11.4 ± 0.9	9.7 ± 1.2
1:5 AgNPs/F127	coated AgNPs	9.6 ± 0.7	8.4 ± 0.7

NZ: no inhibition zone.

The antifungal activity of the freshly prepared AgNPs was higher than that kept for 90 days as seen in Figure 4. The diameters of the inhibition zones are shown in Table 2. It is noted that the significant decrease in diameter of inhibition zone after keeping for 90 days could be found in both uncoated and coated AgNPs synthesized with 1:3 and 1:5 ratios. The coated AgNPs synthesized with 1:1 ratio showed significantly stably high antifungal activity. PE and F127 as negative controls showed no inhibition zone.

4. Discussions

P. guajava has been reported to have high reducing and antioxidant activities (*21,28,29*). Many active compounds for these activities such as myricetin, apigenin, cathechic, gallic acid, ferutic acid, saponins, oleanolic acid were found in PE (*14,16,20*). Previously, our group reported that quercetin, morin, and quercetin-3-*O*-glucopyranoside isolated from PE possess high reducing power (*21*). Thus, in the present study, PE was used as a reducing agent to synthesized AgNPs from $AgNO_3$. Adding PE solution to an aqueous $AgNO_3$ solution, resulting in color change to dark brown color

within 60 min due to excitation of surface plasmon vibration in metal nanoparticles (*30*). UV-vis absorption is widely used for characterization of AgNPs. Previous report revealed that the UV-vis spectra of AgNPs generally appear in the range of approximately 400-450 nm, depending on the type of plant extract and duration time of synthesis. For example, AgNPs obtained from the extract of *Hibiscus sabdariffa* showed the maximum absorbance at 400 and 410 nm at the reaction time of 10 and 90 min, respectively (*31*). In the current study, the spectra of the mixtures obtained after PE was completely reacted with $AgNO_3$ showed a strong broad peak at 455 nm indicating the presence of AgNPs (*32*). The obtained AgNPs showed spherical shape in the nanosize range corresponding to the size measured by DLS. Moreover, the results presented strong signal from the silver atoms confirming the development of silver nanostructures and the signals of C and O indicating the presence of the metabolites in PE. From these results, it is obviously shown that AgNPs can be obtained from the reduction of $AgNO_3$ by PE.

Stability study indicates that the uncoated AgNPs are not stable. Keeping at room temperature for 90 days, their particle size increases rapidly indicating that particle aggregation occurs. Entrapment of AgNPs inside the polymeric micelles of F127 yields a so-called coated AgNPs with different characteristics and particle stability depending on the ratios of AgNPs/F127 used. The particles size of the uncoated freshly prepared AgNPs was as small as the coated AgNPs obtained from the system of 1:1 ratio and significantly smaller than those from 1:3 and 1:5 ratios. The size and zeta potential of the coated AgNPs particularly of 1:1 ratio is not significantly changed, whereas the size of the uncoated AgNPs significantly increased and their zeta potential value dramatically decreased until almost zero at the end of the studied period. It is considered that negatively charged surfaces of AgNPs can help in preventing the aggregation. F127 as a poloxamer block copolymer has an ability to form micelles in solution and/or on the surface of particles (*33*). It is, therefore, considered that the negative charges in the relative hydrophilic part (PEO) of F127 surrounded at AgNPs surfaces influence the high negative value of the particles and prevent the particles from aggregation. It is also considered that besides enhancing the stability of AgNPs, F127 micelles also play an important role on controlling shape and size of the particles. From our results, it is also suggested that the ratio of AgNPs/F127 is important. Only the 1:1 ratio is found to be suitable for synthesizing AgNPs that the highest stable AgNPs can be achieved.

Green synthesis of AgNPs has been reported using many plant extracts (*7-12*). However, from those reported, it has been shown that AgNPs obtained from different reducing plant extracts possess different characteristics and activity. AgNPs have been reported on activity against *C. albicans* that only the small size of AgNPs showed the effective antifungal activity (*34,35*). The mechanism of action of AgNPs on *C. albicans* has been explored that AgNPs can disrupt fungal cell membrane structure and inhibit normal budding process due to the destruction of the membrane integrity (*36*). AgNPs obtained from the reduction of $AgNO_3$ by PE were previously reported by other groups but the particle size obtained was quite large due to particle aggregation (*23,24*). In the present study, our results show that the aggregation of AgNPs can be prevented by coating AgNPs with polymeric micelles of F127 at suitable ratio of AgNPs/F127. The antifungal activity of the coated AgNPs is higher than that of the uncoated. The activity of the uncoated and the coated AgNPs at ratios of 1:3 and 1:5 decreases dramatically after storage for 90 days. The antifungal activity of the 1:1 ratio AgNPs kept at room temperature is as high as that of freshly prepared, indicating that only the suitable ratio of 1:1 is the best formulation for producing AgNPs with the smallest size, the most stable, and the most effective antifungal activity.

As mentioned above, our results suggest that AgNPs with only small particle size is essential for antifungal activity, if AgNPs are aggregated and the particle size becomes large in the diluting vehicle, it is not possible for AgNPs to have this activity potential. Investigation of effects of diluting media on the particle size is therefore necessary. As the isotonic solutions of 0.9% NaCl and PBS pH 7.4 are often used as diluting vehicle for intravenous injection. The effects of these two solutions are therefore investigated. The results demonstrate that these two solutions cause the increase in AgNPs size compared with those diluted with deionized water. It is considered that the electrolytes in both solutions play an important role on this effect. Our results are in line with the other groups who found that the freshly prepared AgNPs were aggregated and precipitated in a few minutes after contacting with 0.9% NaCl and PBS pH 7.4 (*37*). However, our AgNPs showed no precipitation. The increased size of the AgNPs in the current study are still in the colloidal range that all particles can be suspended in the systems. This is considered to be due to F127 micelles that play an important role on prevention of precipitation. Keeping at room temperature for 90 days, the size of AgNPs in both isotonic and deionized water systems gradually increased but not dramatically. Deionized water is not a good vehicle for intravenous injection as it is not isotonic solution. Comparing between 0.9% NaCl and PBS pH 7.4, the size of AgNPs in 0.9% NaCl is smaller than that in PBS pH 7.4. From these results, it is considered that both isotonic diluting solutions influence the size of AgNPs coated with F127 micelles rapidly at the fresh synthesis but not much effect along the period of storage. It is concluded that entrapment of AgNPs prepared from PE with F127 micelles yield

the promising antifungal AgNPs suitable for further investigation in animal model.

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Original Article

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Silver nanoparticles fabricated by reducing property of cellulose derivatives

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Summary **The aim of this study was to synthesize silver nanoparticles (AgNPs) by using cellulose derivatives as a reducing agent. Methyl cellulose (MC), hydroxy ethylcellulose (HEC), and hydroxypropyl methylcellulose (HPMC) were compared for their reducing property. HPMC presented the highest reducing power, with equilibrium concentration (EC) of 84.6 ± 4.5 µmol Fe^{2+}/g, followed by MC and HEC, with the EC of 62.3 ± 1.4, and 38.1 ± 3.2 µmol Fe^{2+}/g, respectively. Using these cellulose derivatives as a reducing agent and silver nitrate as a precursor in fabrication of silver nanoparticles (AgNPs), three cellulose-AgNPs, HEC-AgNPs, MC-AgNPs, and HPMC-AgNPs, were obtained. The cellulose-AgNPs showed different maximum absorptions confirming AgNPs spectra at 415, 425, and 418 nm, respectively. Reaction parameters such as pH, temperature, and period of reaction affected intensity of the maximum absorptions and size of AgNPs. Using 0.3% cellulose solution at pH 9 and reaction at 70°C for 90 min, the particle size of MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs was 97.7 ± 2.4, 165.6 ± 10.6, and 51.8 ± 1.6 nm, respectively. AgNPs obtained from different cellulose derivatives and various preparation parameters possess different inhibition potential against *Escherichia coli* and *Staphylococcus aureus*. The cellulose-AgNPs have higher effective against *E. coli* than *S. aureus*. HPMC-AgNPs showed significantly higher antibacterial activity than MC- AgNPs and HEC-AgNPs, respectively. These results suggest that the type of cellulose derivatives and the reaction parameters of the synthesis such as pH, temperature, and reaction period play an important role to the yield and physicochemical property of the obtained AgNPs.**

Keywords: Cellulose derivatives, HPMC, green synthesis, AgNPs, antibacterial activity

1. Introduction

Silver nanoparticles (AgNPs) receive increase interest in application for many fields (*1*) due to their efficiency in antibacterial (*2*), antifungal (*3*), antiviral (*4*), anticancer (*5*), and antioxidant activities (*6*). Synthesis of AgNPs can be generally performed by reacting silver salts with certain reducing agents from natural such as plant extracts (*7*) or chemically synthesized agents (*8*). The reducing agents from natural resources have gain increasing interest since there are less hazardous waste than those from chemical synthesis. Polysaccharides are one of the natural interesting groups that some of them, *e.g*. starch, dextran, and cellulose were used as a reducing agent for synthesis of metal nanoparticles. For example, our group previously reported the use of starch derivatives from rice for synthesis of AgNPs (*9*). Bankura *et al*. reported the use of dextran to synthesize gold nanoparticles (*10*). For cellulose, there are some reports on using cellulose derivatives such as methylcellulose and carboxymethyl cellulose to synthesize AgNPs (*11-13*). However, there is still less report on factors affecting the obtained AgNPs synthesized by using cellulose derivatives as a reducing agent as well as the comparison of reducing efficiency among many types of cellulose derivatives.

Cellulose is an organic polysaccharide consisting

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of a linear chain of D-glucose units. It is insoluble in organic solvents and water, however, it can be modified to yield cellulose derivatives in order to increase its water solubility (*14*). Cellulose derivatives can be categorized according to their chemical structure into ether and ester groups. Examples of cellulose ether derivatives are methyl cellulose (MC), hydroxyethyl cellulose (HEC), and hydroxypropyl methylcellulose (HPMC) whereas that of cellulose ester derivatives are cellulose acetate, cellulose acetate trimellitate, and cellulose acetate phthalate. Cellulose ester derivatives are less water soluble, therefore, they are commonly used as drug delivery systems in the form of membrane (*15*), nanocrystal (*16*), and fiber (*17*). Comparison between these two groups of cellulose derivatives, those in the ether form are of higher interest as they are more water soluble than those in the ester group. In the present study, three types of cellulose ether derivatives, MC, HEC, and HPMC were used. They are odorless and tasteless, white to slightly off-white, fibrous or granular, free-flowing powder. They were firstly compared for the reducing power by using ferric reducing antioxidant power (FRAP) assay. Synthesis of AgNPs then was prepared from these three cellulose derivatives using silver nitrate ($AgNO_3$) as a precursor. Effects of preparation parameters such as pH, temperature, and duration of reaction were investigated. Three kinds of cellulose-AgNPs, MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs, were obtained from the respective cellulose derivatives. Physicochemical properties of the obtained cellulose-AgNPs were characterized using UV-visible spectrophotometer (UV-vis) and photon correlation spectrophotometer (PCS). The inhibitory activity of AgNPs from the most suitable reacting condition were selected to test against Gram-positive and Gram-negative bacteria. The antibacterial activity was evaluated by measuring the inhibition zones, minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC).

2. Materials and Methods

2.1. *Materials*

MC, HEC, and HPMC were purchased from S. Tong Chemicals Co., Ltd (Bangkok, Thailand). $AgNO_3$ was supplied by RCI Lab-scan Co., Ltd. (Bangkok, Thailand). Ferrous sulfate ($FeSO_4$) and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). Tryptic soy agar (TSA) and tryptic soy broth (TSB) were supplied by Difco™ (Baltimore, MD, USA). All other chemicals and solvents were of AR grade or the highest grade available.

2.2. *Microbial strain*

The aerobic bacterial strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 represented for Gram-positive and Gram-negative bacteria, respectively, were used.

2.3. *Reducing property of the cellulose derivatives*

The reducing power of MC, HEC, and HPMC was determined by using FRAP assay as described previously (*18*) with some modification. Briefly, the FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6. Cellulose derivative solutions having cellulose concentrations of 0.05-0.4% (w/v) were prepared. An amount of 20 µL of each cellulose derivative solution was mixed with 180 µL of FRAP reagent in 96 well plate. Blank samples were prepared by mixing acetate buffer and different concentration of MC, HEC, and HPMC. The test samples and blank were incubated for 10 min at room temperature, then the absorbance was determined at 595 nm using microplate reader (Bio-Rad, Model 680, Hercules, CA, USA). The reducing power of the samples was evaluated by calculating the amount of Fe^{2+} produced by cellulose derivatives samples using the calibration curve of $FeSO_4$. All experiments were run in triplicate.

2.4. *Synthesis of cellulose-AgNPs*

2.4.1. *Effect of pH*

Aqueous solutions containing 0.3% (w/v) of MC, HEC, and HPMC were prepared and the pH was adjusted to 3, 5, 7, 9, and 12 by using 1 M NaOH and 1 M HCl. Then, 10 mM $AgNO_3$ solution was added dropwise to the cellulose solutions at 50ºC with continuous stirring until the volume ratio of the cellulose solution and $AgNO_3$ solution was 100:1. The reaction was kept at this temperature under continuous stirring for 60 min. The obtained cellulose-AgNPs were cooled down to room temperature for further studies.

2.4.2. *Effect of temperature*

Aqueous solutions containing 0.3% (w/v) of MC, HEC, and HPMC were prepared and adjusted to pH 9. Then, 10 mM $AgNO_3$ solution was added dropwise to the cellulose solution with continuous stirring until the volume ratio of the cellulose solution and $AgNO_3$ solution was 100:1. The reaction temperature was studied at 28, 50, 70, and 90°C under continuous stirring for 60 min. The obtained cellulose-AgNPs were cooled down to room temperature for further studies.

2.4.3. *Effect of reaction period*

An aqueous solution containing 0.3% (w/v) of MC, HEC, and HPMC was prepared. Then, 10 mM $AgNO_3$

solution was added dropwise to the cellulose solution at 70ºC with continuous stirring until the volume ratio of the rice solution and $AgNO_3$ solution was 100:1. The reaction was kept at this temperature under continuous stirring for 15, 30, 60, 90, and 120 min. The obtained cellulose-AgNPs from each reaction period were cooled down to room temperature for further studies.

2.5. *Characterization*

2.5.1. *UV-vis*

The dispersion of cellulose-AgNPs obtained from each preparation condition was diluted to 100 fold with deionized water. Outer color appearance of cellulose-AgNPs was observed by visualization. Optical property of the cellulose-AgNPs solution was observed by using UV-vis (Shimadzu-2450, Kyoto, Japan) in the wavelength range of 200-700 nm.

2.5.2. *PCS*

The size, size distribution, and zeta potential of cellulose-AgNPs were investigated using PCS (Malvern Zetasizer Nano ZS, Malvern instrument, Worcestershire, UK) at 25ºC. Each sample was diluted to 100 fold with deionized water before measuring.

2.6. *Evaluation of antimicrobial activity*

2.6.1. *Well diffusion method*

A well diffusion used for investigating antibacterial activity was based on Kirby-Bauer method (*19*). The active test strains of *S. aureus* and *E. coli* grown in TSA at 37ºC for 24 h were diluted in TSB to a final concentration of 1.5×10^6 colony-forming units (CFU)/mL. Bacterial density was adjusted to 0.5 McFarland constant observed at 600 nm using UV-vis. The agar surface was spread with bacterial suspension by using sterile cotton swab. Aqueous solutions (40 µL) of HEC-AgNPs, MC-AgNPs, and HPMC-AgNPs were added into the 6-mm wells of the agar plates. The plates were incubated at 37ºC for 24 h. The antimicrobial activity was investigated by determining the diameter of the clear inhibition zone around the well expressed in millimeter (mm). All samples were done in triplicate.

2.6.2. *Broth dilution method*

In this experiment, the test bacterial suspensions were prepared as the same manner as the well diffusion method. Aqueous solutions containing 0.2 mg/mL of lyophilized cellulose-AgNPs were prepared in deionized water. These solutions were further diluted with TSB to obtained the 2-fold dilution series having cellulose-AgNPs concentration of 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/mL. Subsequently, 50 µL of these 2-fold dilutions were added into the 96-well plates containing 50 µL of TSB and 50 µL of the test bacterial suspension. The plates were incubated at 37ºC. After 24 h of incubation, the minimum cellulose-AgNPs concentration giving clear solution was recorded and this concentration was denoted as MIC. For MBC determination, the clear samples resulted from the MIC series were swabbed on the agar plates. The minimum cellulose-AgNPs concentration showing no bacterial growth in the agar plate was recorded and it was denoted as MBC (*20*). All samples were done in triplicate.

2.7. *Statistical analysis*

Data were analyzed using a One-way analysis of variance (ANOVA) and Duncan's multiple range test Statistic a software version 17 (SPSS Inc., Chicago, USA). The values were presented as means ± standard deviation which a *p*-value less than 0.05 was considered as a significant difference.

3. Results

3.1. *Reducing property of the cellulose derivatives*

The results showed that all three cellulose derivatives exhibited reducing power as a dose dependent manner as seen in Figure 1. Among them, HPMC showed the highest reducing activity of 84.6 ± 4.5 µmol Fe^{2+}/g sample whereas that of MC and HEC were 38.1 ± 3.2 and 62.3 ± 1.4 µmol Fe^{2+}/g sample, respectively. However, high concentration of cellulose derivative, such as 0.3 and 0.4 % (w/v), showed similar activity.

3.2. *Effect of pH*

The effect of pH on cellulose-AgNPs systems was clearly observed by color change. Increasing pH from

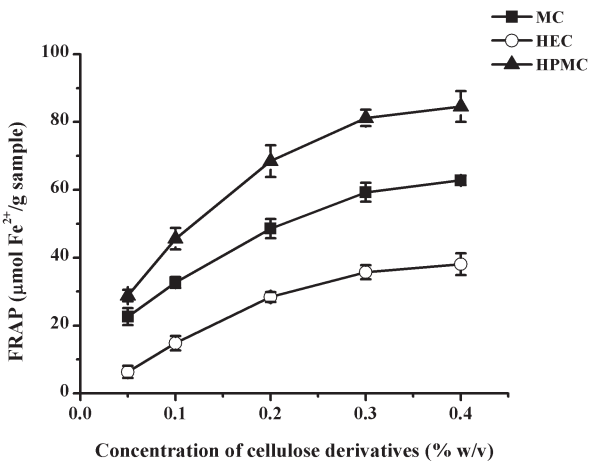

Figure 1. Reducing power of MC, HEC, and HPMC.

3 to 12, *e.g.* at pH 3, 5, 7, 9, and 12, the color of MC-AgNPs, after complete reaction period of 60 min, were light yellow, intense yellow, yellow-brown, brown, and dark brown, respectively. HEC-AgNPs systems were different. At pH 3 and 5, the systems of HEC-AgNPs appeared as mixtures of colorless solution with dark large particles precipitation. Whereas at pH 7, 9, and 12, HEC-AgNPs showed light yellow, yellow, and brown color, respectively. Interestingly, the systems of HPMC-AgNPs at pH 3, 5, 7, 9, and 12 showed light yellow, yellow, orange, red wine, and dark brown, respectively.

Measuring the effect of pH on the absorbance of MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs, the results revealed that the maximum absorption of each system was at 415, 425, and 418 nm, respectively. The quantity of absorbance for each system at its respective maximum absorption was measured and the results are shown in Figure 2A. It was found that the absorbance of cellulose-AgNPs rapidly increased when pH was increased up to around pH 9. After pH 9, such as pH 9 and 12, the absorbance was not significantly different, indicating the systems reached the maximum absorption at about pH 9. Moreover, at pH 12, large precipitation was observed indicating that this pH causes instability of cellulose-AgNPs.

The size of the obtained cellulose-AgNPs was also affected by pH as shown in Figure 2B. The higher pH gave the smaller size until pH 9 that the systems showed the smallest size of the particles. The average particle size of cellulose-AgNPs obtained from the following preparing conditions; 0.3% (w/v) cellulose derivatives, period time at 60 min and 10 mM $AgNO_3$ was 117 ± 6, 183 ± 12, and 53 ± 2 nm for MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs, respectively. The size distribution expressed by polydispersity index (PdI) of each system was not significantly different, *i.e.*, 0.18 ± 0.08, 0.21 ± 0.08, and 0.15 ± 0.04 for MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs, respectively. These PdI values are acceptable as narrow size distribution. It was concluded that pH and type of cellulose derivatives showed the influence on absorption and size of cellulose-AgNPs. Among the obtained cellulose-AgNPs, HPMC-AgNPs showed the highest absorption and the smallest size. pH 9 is considered as the most suitable pH for preparing cellulose-AgNPs.

3.3. *Effect of temperature*

Keeping constant synthesized conditions such as cellulose and $AgNO_3$ concentration, pH of reacting medium, and reaction period, the effects of reaction temperature (at 28, 50, 70, and 90°C) was studied. The results showed that different temperature caused different manner of color changing. After complete fabrication at 90°C, the resulted systems of MC-AgNPs and HEC-AgNPs showed dark brown color whereas that of HPMC-AgNPs was red brown. Synthesizing of MC-AgNPs and HPMC-AgNPs at 28, 50, and 70°C, the color of the obtained systems was light yellow, intense yellow, and red wine, respectively. Whereas that of HEC-AgNPs was light brown, brown, and dark brown, respectively. After standing at room temperature for 24 h, aggregation and precipitation were observed in the cellulose-AgNPs systems obtained from reacting temperature of 28 and 50°C.

The intensity of absorbance of cellulose-AgNPs at their respective maximum absorption was recorded and the results are shown in Figure 3A. It was found that increasing reacting temperature caused the increase of the absorbance intensity of the obtained cellulose-AgNPs. The results revealed that at 90°C, all cellulose-

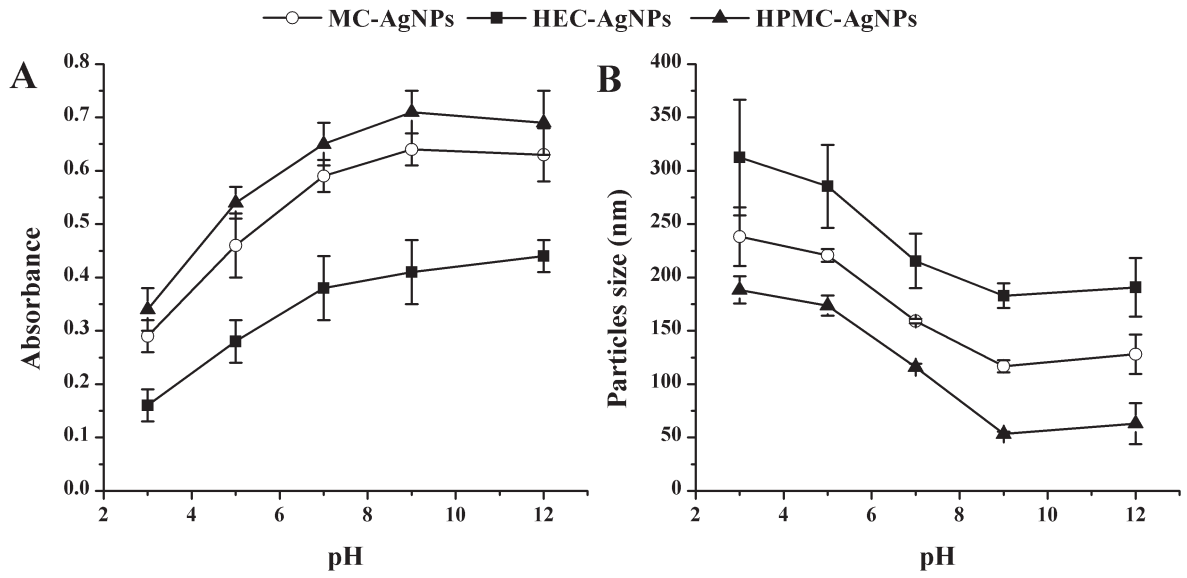

Figure 2. Effects of pH on intensity of maximum absorbance (A) and particle size (B) of cellulose-AgNPs.

AgNPs systems showed the highest intensity of absorption.

The effect of temperature on size and size distribution of the synthesized cellulose-AgNPs was investigated. The results explored that increasing synthesized temperature decreased the particles size of the obtained cellulose-AgNPs as shown in Figure 3B. The temperature at 90°C displayed the smallest size of cellulose-AgNPs. The average size of MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs prepared at this temperature was 168 ± 2, 108 ± 3, and 45 ± 2 nm, respectively with PdI values of 0.19 ± 0.02, 0.21 ± 0.04, and 0.17 ± 0.06, respectively. From these results, the most suitable temperature for synthesizing cellulose-AgNPs was considered to be 90°C.

3.4. *Effects of reaction period*

The effect of reaction period on the synthesized cellulose-AgNPs was investigated using the most suitable pH (pH 9) and reacting temperature (90°C) obtained from the above results. The reaction period of 15, 30, 60, 90, and 120 min was studied. The obtained systems exhibited different physical properties including absorption intensity at different reaction period. The color of MC-AgNPs and MC-AgNPs was slightly brown whereas that of HPMC-AgNPs was slightly red. Standing at room temperature for 24 h, the systems of MC-AgNPs and HEC-AgNPs fabricated with reacting period of 15 and 30 min showed aggregation and large dark brown precipitates were found. Whereas the

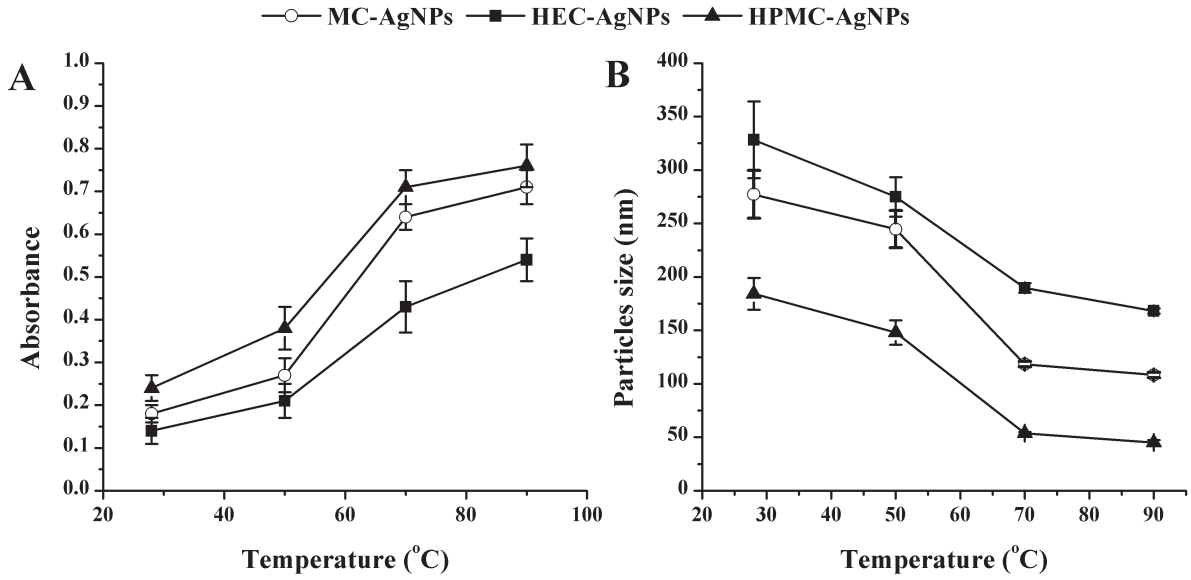

Figure 3. Effects of reaction temperature on intensity of maximum absorbance (A) and particle size (B) of cellulose-AgNPs.

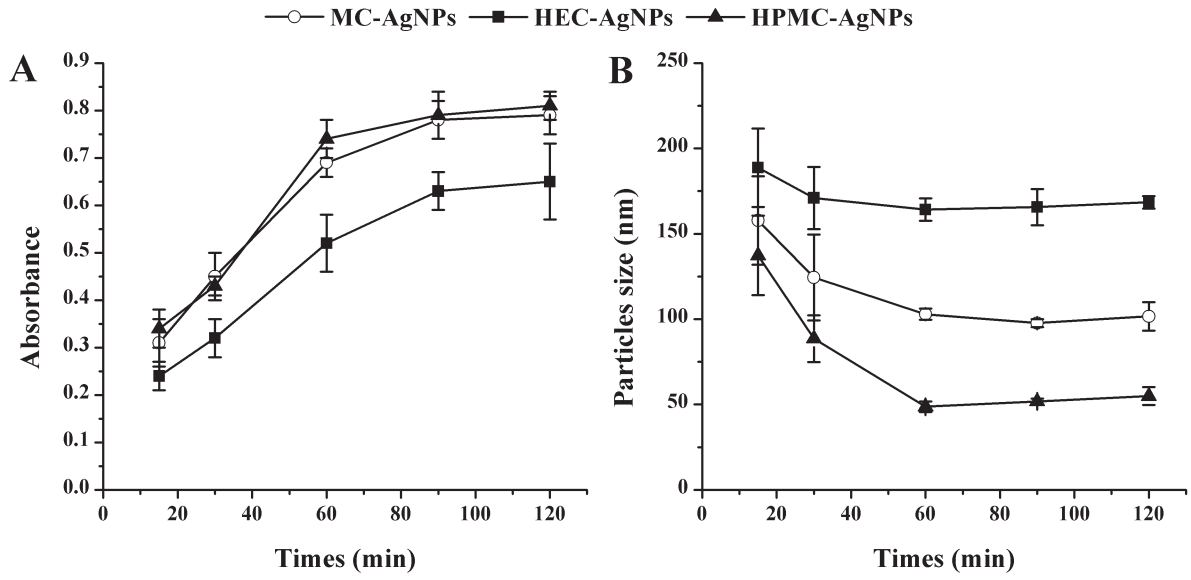

Figure 4. Effects of reaction period on intensity of maximum absorbance (A) and particle size (B) of cellulose-AgNPs.

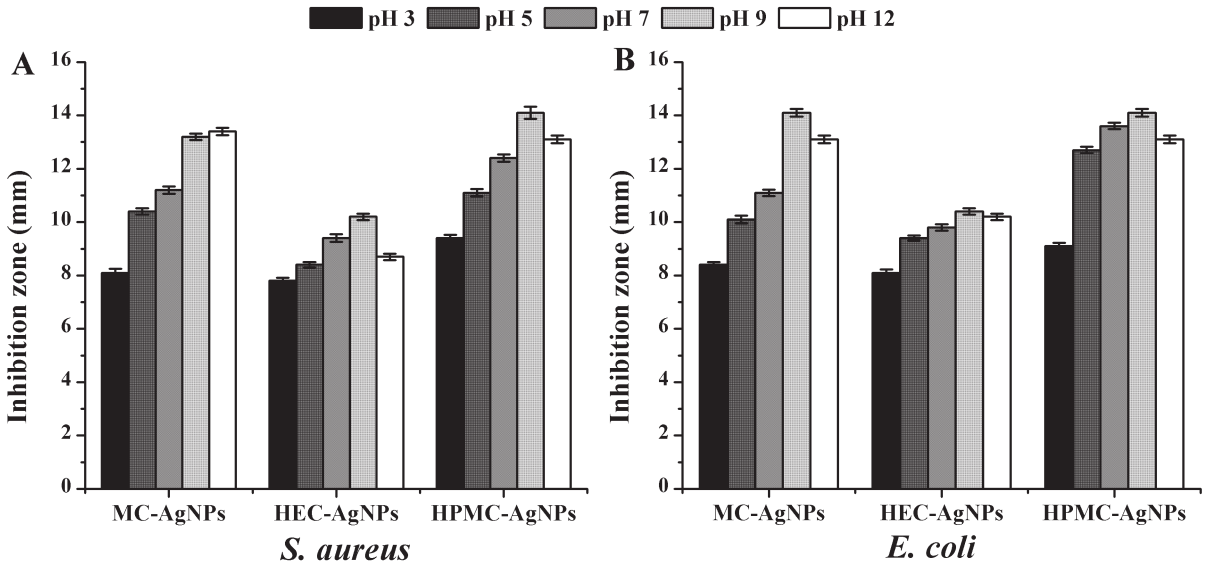

Figure 5. Antibacterial activity of cellulose-AgNPs obtained from different pH of reaction media.

aggregation and dark green precipitates were observed from HPMC-AgNPs system using reacting period of 15 min. Increased reaction period from 15 to 60 min caused significant increase of absorption intensity of the obtained cellulose-AgNPs systems as shown in Figure 4A. Reaction periods of 90 and 120 min gave the high absorbance with no significant different intensity.

The average size of MC-AgNPs and HPMC-AgNPs was significantly decreased when the reaction period was increased whereas that of HEC-AgNPs was slightly insignificantly decreased as shown in Figure 4B. The size change was clearly seen when short reaction period was used. It was found that reaction period of 60-120 min caused no effect on size of cellulose-AgNPs. The average particle size of MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs obtained from the reaction periods of 60-120 min was 98 ± 2, 166 ± 11, 52 ± 2 nm, respectively with PdI values of 0.18 ± 0.08, 0.22 ± 0.06, 0.17 ± 0.04, respectively. Therefore, the suitable reaction period of cellulose-AgNPs was considered to be about 60-120 min.

3.5. *Antibacterial test*

The obtained cellulose-AgNPs showed antibacterial effect against *S. aureus* and *E. coli* whereas the pure cellulose solution could not inhibit the tested strains. The results suggested that the antibacterial activity of cellulose-AgNPs were depended on cellulose type and conditions of AgNPs synthesis. HPMC-AgNPs explored the highest inhibitory activity against *S. aureus* and *E. coli* followed by MC-AgNPs and HEC-AgNPs, respectively. The antibacterial activity of cellulose-AgNPs obtained from different pH condition against *S. aureus* and *E. coli* is shown in Figure 5A and 5B, respectively. The width of inhibition zone indicates the bacterial inhibitory potential. The results showed that the width of inhibition zone of cellulose-AgNPs was depended on pH of the synthesized reaction. Cellulose-AgNPs obtained from reaction at high pH, particularly at pH 9 showed the great inhibition zone for both strains. For reacting temperature condition, cellulose-AgNPs obtained from high temperature displayed the high antibacterial activity, which was clearly seen in the systems of MC-AgNPs and HPMC-AgNPs obtained from the reaction temperature at 50 and 70°C as shown in Figure 6A and 6B for inhibition of *S. aureus* and *E. coli*, respectively. The cellulose-AgNPs obtained from different reaction period showed different antibacterial activity as shown in Figure 7A and 7B for inhibition of *S. aureus* and *E. coli*, respectively. The results showed that increased reaction period yielded the cellulose-AgNPs with high antibacterial activity, except those obtained from 120-min reaction period that the activity was decreased. This might be due to the occurrence of particle aggregation and precipitation in the agar plates. From this experiment, it was noted that the inhibition zones of HPMC-AgNPs against both strains were relatively wider than those of MC-AgNPs and HEC-AgNPs, respectively, indicating that HPMC-AgNPs was the strongest antibacterial activity, followed by MC-AgNPs and HEC-AgNPs, respectively. To confirm these results, MIC and MBC values of the obtained cellulose-AgNPs were investigated. The results showed that the MIC values of MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs against *S. aureus* were 0.05, 0.05, and 0.025 mg/mL, respectively and against *E. coli* were 0.05, 0.05, and 0.025 mg/mL, respectively. The MBC value of all cellulose-AgNPs against *S. aureus* was 0.1 mg/mL whereas that values of MC-AgNPs, HEC-AgNPs, and HPMC-AgNPs against *E. coli* were 0.1, 0.1, and 0.05 mg/mL, respectively. From these results, it was obviously seen that HPMC-AgNPs showed the highest antibacterial activity against both Gram-positive and Gram-negative bacteria whereas MC-AgNPs and HEC-AgNPs had similar potential.

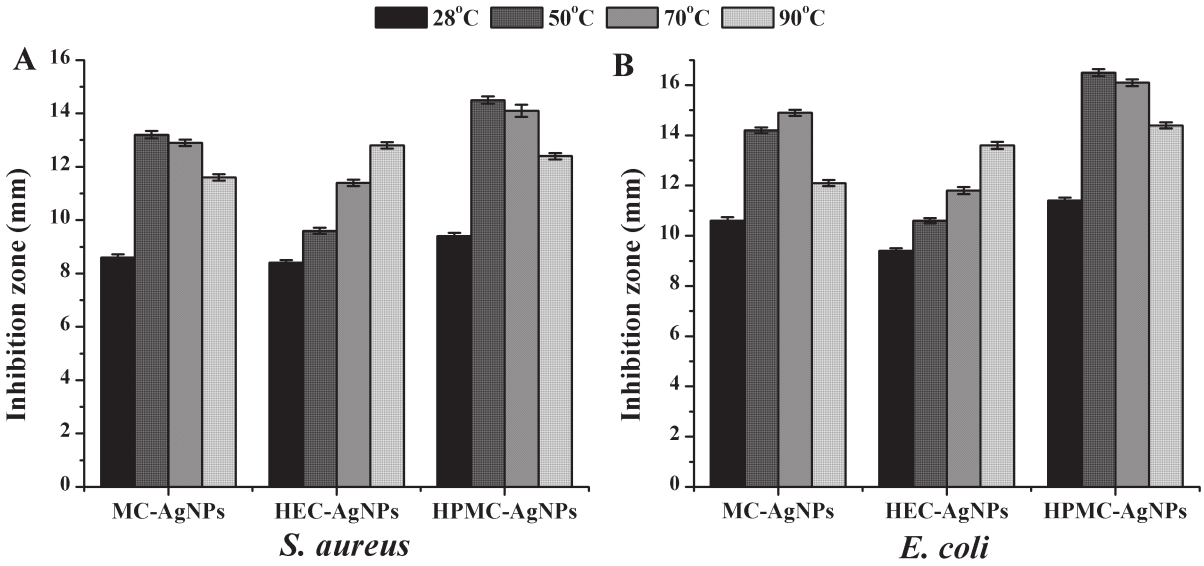

Figure 6. Antibacterial activity of cellulose-AgNPs obtained from different reaction temperature.

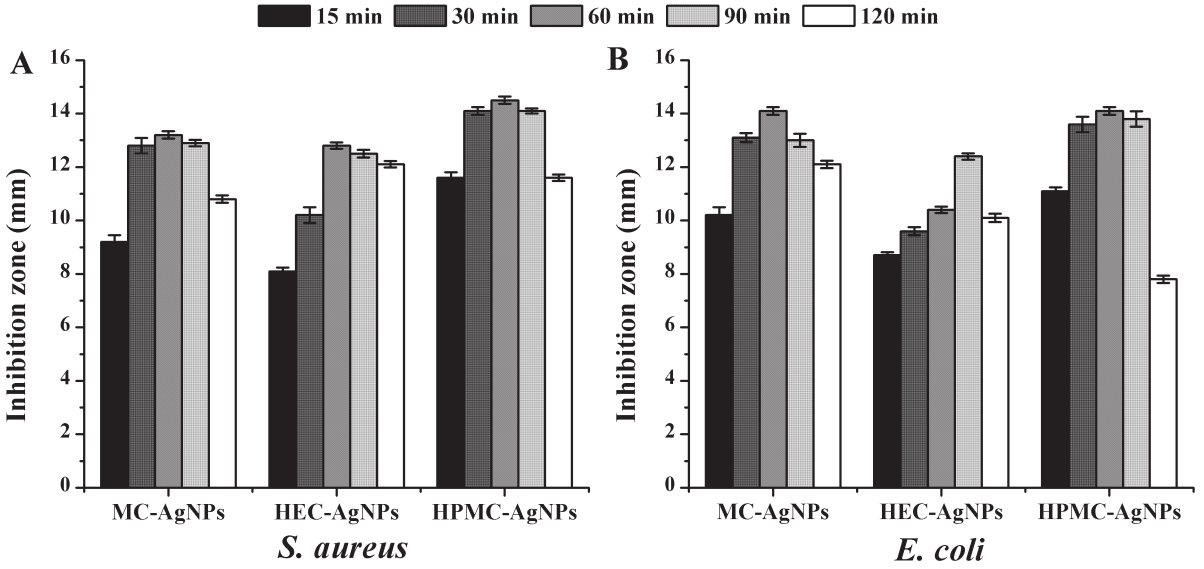

Figure 7. Antibacterial activity of cellulose-AgNPs obtained from different reaction period.

4. Discussion

AgNPs have received significantly increase attention because of their unique physical, chemical, and biological properties (*21,22*). Generally, AgNPs are synthesized by chemical reduction of metal ions from its salt, such as $AgNO_3$, by mostly chemical reducing agents (*23*). However, some chemical reducing agents are toxic to human beings and also to the environment. Therefore, many researchers attempt to synthesize AgNPs using reducing agents from natural such as microorganisms, fungi and plant (*24,25*). Cellulose derivatives are the most abundant resource in nature. They have long been used as a good candidate for fabricating hydrogels in pharmaceutical application owing to their excellent properties in hydrophilicity, biodegradability, and safety (*26*). Moreover, some cellulose derivatives have been demonstrated that they can stabilize the nanoparticles from aggregation (*12,27*). Therefore, at least there are two main actions of cellulose derivatives in the synthesis of AgNPs, one is as a reducing agent and the other is as a stabilizing agent. Three cellulose derivatives, MC, HEC, and HPMC that are chosen for AgNPs synthesis in the present study are non ionic substances and possess positive adsorption at the liquid/air and the other interfaces. To be able to synthesize AgNPs from $AgNO_3$ precursor, a substance should have reducing property, which can be used as an antioxidant. From the best of our knowledge, there is very less report on antioxidant or reducing property of cellulose derivatives. Previously, HPMC was reported to have antioxidant activity but the mechanism of action

was lipid peroxidation (*28*), which might not be related directly to the reducing property. Zimoch-Korzycka *et al*. reported free radical scavenging property of HPMC using 2,2-diphenyl-1-picrylhydrazyl as free radicals (DPPH) and reducing property using FRAP assay (*29*). In the present study, we are the first who demonstrate that the three selected cellulose derivatives; MC, HEC, and HPMC possess reducing activity and in relatively different levels. The reducing activity of the HPMC was found to be the highest among them.

All reactive agents for AgNPs synthesis need to be water soluble because the synthetic medium is aqueous. Therefore, MC, HEC, and HPMC have been selected as representatives of cellulose derivatives for AgNPs synthesis in the present study because they are well water soluble. Our results confirm that MC, HEC, and HPMC can be used as reducing agent in the synthesis of AgNPs. MC has been reported to form a protective colloid against droplets or particles agglomeration (*6*). The cellulose-AgNPs systems of MC, HEC, and HPMC obtained from the suitable conditions showed no aggregation or precipitation, indicating that these cellulose derivatives can act not only as a reducing agent but also a stabilizing or capping agent to protect the resulted AgNPs from aggregation. To obtain AgNPs, a reducing agent should have negative active charge to react with Ag^+ of $AgNO_3$ precursor. Considering the chemical structure of MC, HEC, and HPMC, it is shown that there are linear chains with β-(1→4) linkage but these three types of cellulose derivatives are different to each other in the substitution groups and carbon position (*27*). For being reacted with Ag^+ of $AgNO_3$ precursor, there is a negative charge group ($-COO^-$) in the structure of these cellulose derivatives (*12*).

AgNPs possess optical properties that are depended on size, shape, concentration, and agglomeration state as well as refractive index near the particle surface. These properties can cause UV-vis spectroscopy a valuable tool for identifying, characterizing and studying the obtained AgNPs (*30*). Therefore, the color change of the system after complete reaction can indicate the formation of AgNPs (*31*). Three process parameters of AgNPs synthesis, including pH of reacting media, reacting temperature, and duration of reaction or reaction period are investigated in the present study. The results show that these parameters significantly affected the obtained AgNPs.

Effect of pH on the particle size of the obtained AgNPs is clearly seen. The higher pH causes the smaller particle size. It was reported that slightly acid (pH 6) of Millipore water can retard the reduction of Ag^+ to Ag^o (*32*). Meanwhile, in alkaline or higher pH media, OH^- ions can maintain AgNPs stability by adsorbing these OH^- ions on the particles and providing high repulsive force between the particles. So that it can prevent aggregation of the obtained AgNPs, resulting in maintenance the small size of the AgNPs. Our results are in good agreement with the other groups that in alkaline pH, AgNPs are stable and aggregates formed at lower pH (*33*). Moreover, previous studies reported that alkaline solution increased solubility (*34*) of cellulose derivatives leading to a high viscosity (*35*). The increased viscosity can protect cellulose-AgNPs from aggregation and precipitation. Therefore, it is suggested that synthesis of AgNPs should be performed at alkaline pH.

Studying effect of temperature on the obtained cellulose-AgNPs, it was found that as the temperature increases the absorbance is also increased. Among the studied temperature, synthesis at room temperature at 28ºC gave the least yield of cellulose-AgNPs as the lowest absorbance at the identical wavelength was shown. It is considered that at low temperature, the reduction reaction could not finish completely within the limited time used. The particle size of cellulose-AgNPs decreases with increasing temperature. The smallest size of the particles is obtained when the synthesis was performed at 70ºC or above. Sarkar *et al*. (*36*) reported that increasing temperature, the kinetic energy of the AgNPs in the solution also increases; as a result, the collision frequency between the particles also rises, and this leads to the higher rate of reaction.

For the influence of reaction period, it was observed that within 60 min of reaction period, the absorbance intensity at the fixed wavelength steadily increases as a function of time, indicating that the continued reduction of silver ions. After 60 min, the increasing rate of the absorbance is decreased until 90 min that there is no further increase in the absorbance, indicating the complete reduction of the silver ions. The obtained particles size of cellulose-AgNPs is depended on duration of reaction which decreases with increased time. Among the three cellulose derivatives used, it is found that the particle size of HPMC-AgNPs is the smallest, followed by that of MC-AgNPs and HEC-AgNPs, respectively.

The antibacterial activity of HPMC-AgNPs is significantly higher than MC-AgNPs and HEC-AgNPs, respectively. It has been reported that AgNPs are well adsorbed onto the surface of bacterial cell membrane and can damage the cell by modifying the intracellular structures and inducing cellular toxicity (*37*). The results in the present study indicate that the obtained cellulose-AgNPs are slightly higher effective to Gram-negative bacteria than Gram-positive strains. It is considered that this is due to certain difference between Gram-positive and Gram-negative bacteria, which markedly differ in their cell walls. The cell wall of Gram-positive cells is much thicker with higher amount of peptidoglycan than Gram-negative. The thicker peptidoglycan layer might be therefore extensive practical importance in protecting the cell from penetration of silver ions into the cytoplasm. It is noted that pH, temperature, and period of reaction play an important role on the

antibacterial activity of AgNPs according to the particle size obtained. The smaller particle size gives the higher antibacterial activity. Among the three cellulose-AgNPs, HPMC-AgNPs are the smallest size and possess the most effective activity.

From our study, it can be concluded that HPMC is the most effective reducing agent for synthesizing AgNPs, followed by MC and HEC, respectively. Synthesized parameters such as pH, temperature, and period of reaction play an important role to the color, absorption intensity, particles size, and antibacterial activity of the obtained AgNPs.

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Original Article

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Paraoxonase 1 gene (Q192R) polymorphism confers susceptibility to coronary artery disease in type 2 diabetes patients: Evidence from case-control studies

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Summary **Numerous published studies have investigated the relationship between the paraoxonase 1 (*PON1*) gene Q192R (rs662) polymorphism and the risk of coronary artery disease (CAD) in type 2 diabetes mellitus (T2DM) patients. However, the results are still conflicting and inconclusive. Potentially eligible articles were searched for in related databases. Odds ratios (OR) with 95% confidence intervals (CI) were used to estimate the associations. Subgroup analysis was performed based on ethnicity. Ten case-control studies were included. A significant increase in the susceptibility for CAD in T2DM patients was found in the allelic model (OR = 1.49, $p < 0.001$), homozygote model (OR = 2.47, $p < 0.001$), heterozygote model (OR = 1.47, $p < 0.001$), dominant model (OR = 1.64, $p < 0.001$), and recessive model (OR = 1.74, $p = 0.001$). In subgroup analysis by ethnicity, a significant increase susceptibility was found in Asian populations in the allelic model (OR = 1.39, $p = 0.001$), homozygote model (OR = 2.15, $p = 0.002$), heterozygote model (OR = 1.37, $p = 0.006$), recessive model (OR = 1.65, $p = 0.012$), and dominant model (OR = 1.54, $p < 0.001$). A similar significant increase in susceptibility was found in Caucasian populations in the allelic model (OR = 1.75, $p = 0.002$), homozygote model (OR = 3.39, $p = 0.002$), recessive model (OR = 1.98, $p = 0.030$), heterozygote model (OR = 1.64, $p = 0.001$), and dominant model (OR = 1.83, $p < 0.001$). The results suggest that the *PON1* Q192R polymorphism is associated with a significantly increased risk of CAD in T2DM patients in both Asian and Caucasian populations.**

Keywords: *PON1*, coronary artery disease, type 2 diabetes mellitus, polymorphism, meta-analysis

1. Introduction

Diabetes mellitus along with insulin resistance, hypertension, and hyperlipidemia are closely associated with the development of coronary artery disease (CAD), which is reported to be one of the major causes of morbidity and mortality in certain developed countries (*1,2*). In addition to these traditional cardiovascular risk factors, a genetic predisposition is considered to have an important role in the development of CAD (*3*). Multiple genes are likely to be involved in the pathogenesis of CAD, including those with role in lipoprotein metabolism (*4*). Despite the fact that low- and high-density lipoprotein (LDL and HDL, respectively) cholesterol levels may be normal in patients with T2DM, lipoprotein glycation could be the reason for this abnormality (*5*). Therefore, genetic markers involved in lipoprotein metabolism and modification may be remarkably important in the development process of CAD in patients with T2DM.

Human PON1/arylesterase is an HDL-associated Ca^{2+} dependent glycoprotein (lactonase), which

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presents antioxidant and anti-atherogenic features through paraoxon hydrolysis (*6*). PON1 is an HDL-associated esterase that hydrolyses lipoperoxides. It acts as a protective factor against oxidative modifications of LDL, indicating that it may play a vital role in the prevention of the atherosclerotic process (*7*). The *PON1* gene, localized on chromosome 7q21.3, is 26,857 bp long containing 9 exons, and codes for a glycoprotein that is situated on the surface of HDLs and plays a significant role in preventing LDL oxidation (*8*). Human serum paraoxonase activity towards paraoxon presents varying interindividual mutations and underlies strict genetic control. The molecular basis of this mutation and declined PON1 activity is a polymorphism in the coding region of the gene, which leads to a glutamine (Gln)/arginine (Arg) amino acid substitution in position 192 (*9,10*). Recently, the presence of the Q192R (rs662) polymorphism in the *PON1* gene was announced to be an independent risk factor for CAD in patients with T2DM in various populations (*10-12*). However, studies on the relationship between the *PON1* rs662 polymorphism and CAD in T2DM patients have been inconsistent. A recent study performed in Egypt reported that the Arg allele of the *PON1* Q192R gene polymorphism is an independent risk factor for CAD in T2DM (*6*). A study from China reported that the R allele of the Q192R polymorphism is associated with coronary heart disease in Chinese Han T2DM patients (*11*). It was revealed that the most frequent genotype and allele of the 192 Gln/Arg polymorphism in the *PON1* gene in the Chinese Han population were QR and allele R, respectively. Conversely, genotype QQ and allele Q were the most frequent in the Caucasian populations (*12*). However, they also failed to show evidence of a positive association under the allele, homozygote, recessive, heterozygote, and dominant models (*7,12*). The present study was designed to explore the potential relationship between the *PON1* gene polymorphism and the risk of CAD in T2DM. Therefore, a meta-analysis was performed to conclude a more comprehensive estimation of the relationship between the *PON1* Q192R polymorphism and CAD susceptibility in T2DM.

2. Materials and Methods

2.1. *Search strategies*

The meta-analysis conducted adhered to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (*13*). Four online electronic databases were used to identify relevant studies to include in the present meta-analysis (EMBASE, Web of Science, PubMed, and CBM) for studies published up to October 2, 2018. The following terms were used: (type 2 diabetics or type 2 diabetes mellitus or noninsulin-dependent diabetes mellitus or NIDDM or T2DM) and (coronary artery disease or coronary heart disease or CAD or CHD), (paraoxonase 1 or paraoxonase or PONA or 192 Gln/Arg or Gln^{192}-Arg or PON1 or Q192R or rs662), (polymorphism or SNP or single nucleotide polymorphism or alleles or mutation or variation or genotype). Publications listed in the references cited were also reviewed carefully to identify any potentially relevant studies. There was no limitation placed on languages in the searching process. Divergence regarding the inclusion of a unique paper was also resolved by consensus.

2.2. *Inclusion and exclusion criteria*

All studies included were required to meet the following criteria: (1) studies focused on the role of the *PON1* Q192R polymorphism and the risk of CAD in T2DM; (2) studies with a case-control design; (3) control groups matched with type 2 diabetes patients without CAD; and (4) studies providing detailed genotype frequencies and the total number of cases and controls, especially the number of Q/Q, Q/R, and R/R genotypes in cases and controls groups. The exclusion criteria were: (1) the article was unrelated to the *PON1* rs662 polymorphism; (2) case reports, review article, comments, editorials, or meta-analysis; (3) basic experimental studies or animal studies; and (4) studies without available genotyping data.

2.3. *Data extraction*

Available data were collected and extracted from the included studies by 2 investigators independently, according to a preset data extraction table. Any divergences were resolved by consensus through discussion between all authors. The following detailed information was extracted from each study: surname of the first author, country where the study was carried out, population ethnicity of each study, publication year, source of control (hospital-based or population-based), and the genotype frequencies of the *PON1* rs662 polymorphism. Moreover, if the included paper failed to provide detailed information regarding the Hardy-Weinberg equilibrium (HWE), we further calculated the HWE and provided the related information.

2.4. *Quality assessment*

The quality of all eligible studies was assessed by 2 investigators independently according to a 9-star rating system, the Newcastle-Ottawa Scale (NOS) (*14*), which involve 3 broad aspects as follows: case and control group selection, exposure, and comparability of the groups. High, moderate, and low-quality studies were classified as a study with $\geq$ 7, 4-6, and $<$ 4 scores, respectively.

2.5. *Statistical analysis*

The association strength between the *PON1* Q192R (rs662) polymorphism and VAD risk in T2DM was calculated by odds ratios (OR) with a 95% CI. The pooled ORs were calculated for the allele model, dominant model, recessive model, heterozygote model, and homozygote model. The pooled ORs were measured using the *Z*-test. The HWE for a single study was measured using the χ^2 test, with $p < 0.05$ indicating a significant deviation. The ORs were summarized using either the random-effects model or the fixed effects model according to the heterogeneity assumption. The random effect model was used in the presence of substantial heterogeneity with $I^2 > 50\%$; otherwise, a fixed effect model was used (*15*). To further investigate the individual effect of a single study on the pooled results, sensitivity analysis was also conducted to confirm the stability of the results under all genetic models. We also omitted studies in which the genotype frequencies in the control groups deviated from the HWE. The presence of publication bias was assessed with Egger's funnel plot test, with $p < 0.05$ being considered to indicate statistical significance. All statistical analyses were performed with STATA 12.0 software (Stata Statistical software, College Station, TX, USA).

3. Results

3.1. *Characteristics of the included studies*

The initial search of related electronic databases yielded 98 studies according to the above comprehensive search strategy. A detailed flow chart of the study selection process is shown in Figure 1. After removing duplicate studies, 66 articles remained. A total of 45 obviously irrelevant studies were excluded after careful reading of the titles and abstracts. After the assessment of eligibility using full-text was conducted, 11 articles were excluded. After this procedure, we finally identified 10 relevant case-control studies (*6,7,9-12,16-*

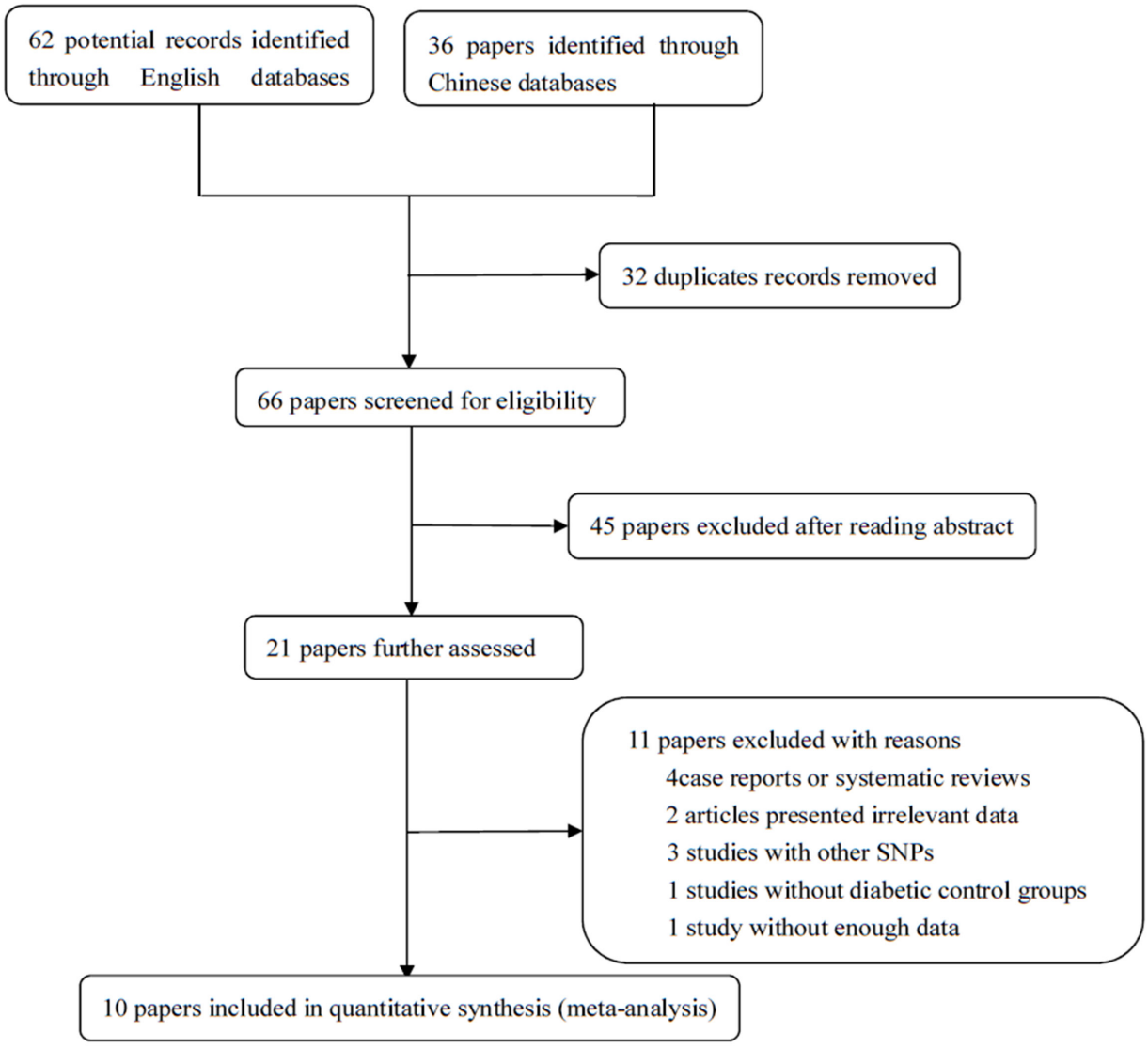

Figure 1. The detailed procedures for the literature search.

19). The control groups of 3 studies were recruited from population-based individuals, and the remaining 7 studies from hospital-based subjects. Four studies were conducted in Caucasian populations, and 6 among Asian populations. In 2 studies, the genotype frequency distribution in the control groups did not deviate from the HWE (*7,16*). The NOS results indicated that the 10 included studies were categorized as 'high quality'. Table 1 shows the detailed characteristics of the included studies.

Table 1. Characteristics of the studies included and genotype frequencies of the Q192R polymorphism in the *PON1* gene and coronary artery disease susceptibility in type 2 diabetes patients

Author	Year	Country	Ethnicity	Genotyping method	Source of control	HWE	NOS	Case			Control		
								QQ	QR	RR	QQ	QR	RR
Bhaskar	2011	India	Asian	PCR	HB	0.043	6	35	97	28	36	87	27
Shao	2014	China	Asian	DHPLC	HB	0.2971	6	19	95	88	31	78	68
Osei-Hyiaman	2001	China	Asian	PCR-SSCP	PB	0.1075	8	136	43	22	181	44	6
James	2000	France	Caucasian	Allele-specific hybridiza-tion	HB	0.1213	7	58	67	12	140	118	15
Ma	2003	China	Asian	PCR-RFLP	HB	0.2242	8	8	42	46	8	42	30
Gupta	2012	India	Asian	PCR and restriction diges-tion	PB	0.6147	7	78	159	63	81	126	43
Qian	2003	China	Asian	PCR	HB	< 0.001	7	9	75	41	20	85	16
Pfohl	1999	Germany	Caucasian	PCR	HB	0.8563	7	73	77	20	66	44	8
Elnoamany	2012	Egypt	Caucasian	PCR	PB	0.2899	7	15	14	19	27	14	4
Odawara	1997	UK	Caucasian	PCR	HB	0.2263	7	1	24	17	25	53	44

PCR, Polymerase Chain Reaction; HWE, Hardy-Weinberg Equilibrium; NOS, Newcastle-Ottawa Scale; RFLP, Restriction Fragment Length Polymorphism; HB, Hospital-Based; PB, Population-Based; PON1, paraoxonase 1; SSCP, Single strand conformational polymorphism; DHPLC, denaturing high performance liquid chromatography.

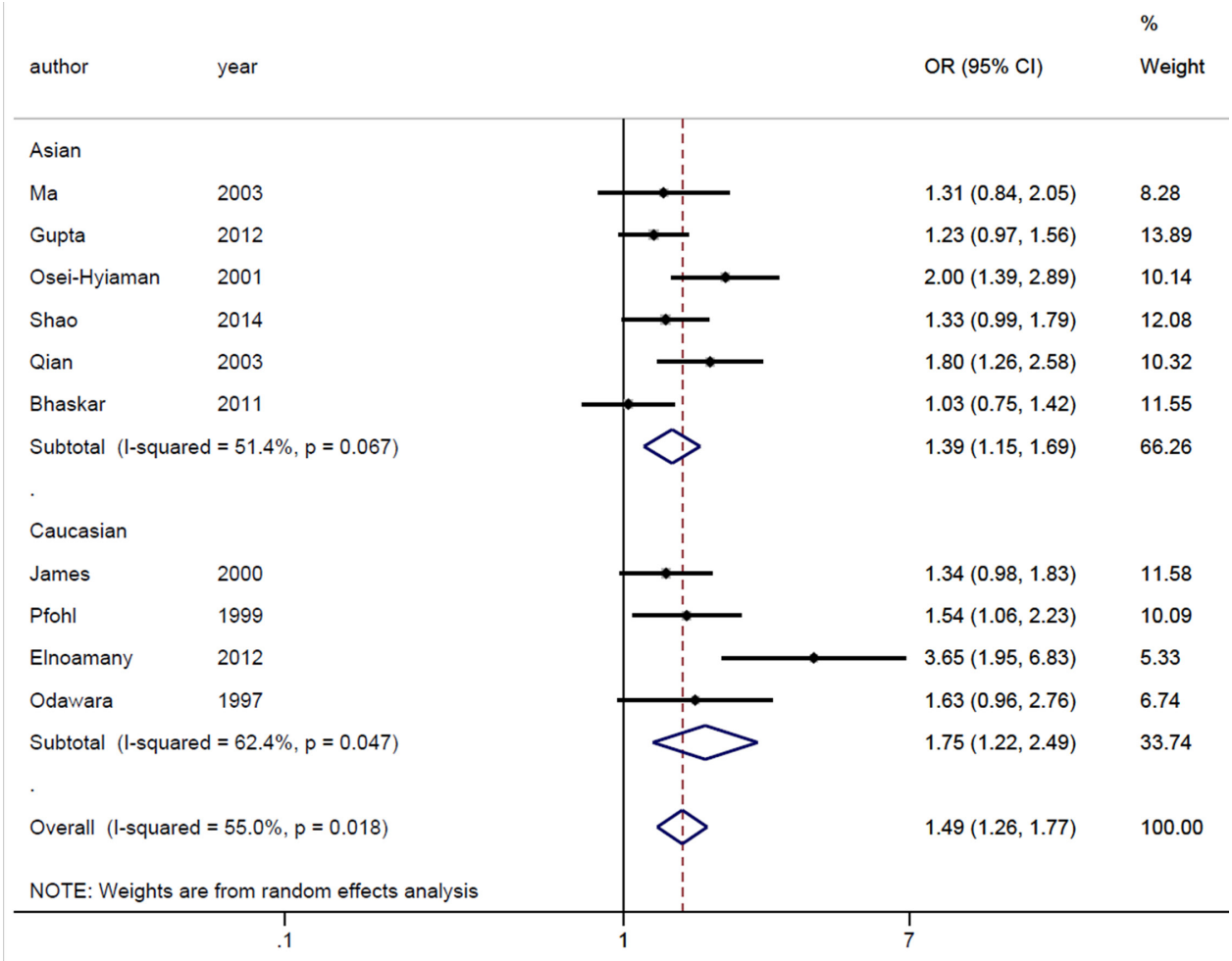

Figure 2. Forest plot for the association between the *PON1* Q192R polymorphism and coronary artery disease susceptibility in type 2 diabetes patients by ethnicity under the allelic model.

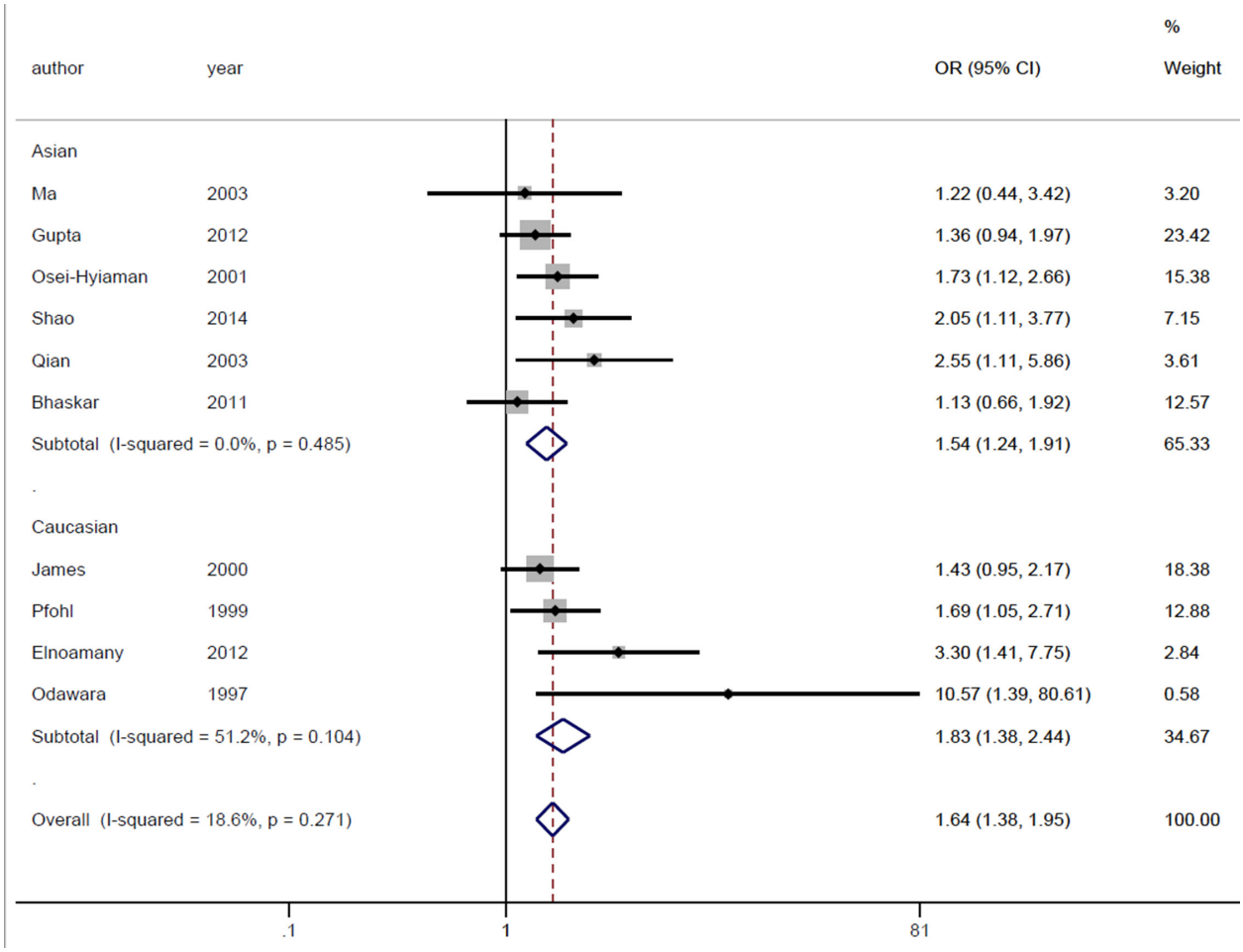

Figure 3. Forest plot for the association between the *PON1* Q192R polymorphism and coronary artery disease susceptibility in type 2 diabetes patients by ethnicity under the dominant model.

3.2. *Main meta-analysis results*

Ten case-control studies were included, reporting a total of 1,481 cases of CAD in T2DM patients and 1,567 T2DM patients without CAD. A significant increase in the susceptibility for CAD in T2DM patients was found in the allele model (R *vs.* Q: OR = 1.49, 95% CI: 1.26-1.77, $p < 0.001$), heterozygote model (QR *vs.* QQ: OR = 1.47, 95% CI: 1.22-1.76, $p < 0.001$), homozygote model (RR *vs.* QQ: OR = 2.47, 95% CI: 1.64-3.71, $p < 0.001$), dominant model (RR + RQ *vs.* QQ: OR = 1.64, 95% CI: 1.38-1.95, $p < 0.001$), and recessive model (RR *vs.* RQ + QQ: OR = 1.74, 95% CI: 1.26-2.39, $p = 0.001$). In the subgroup analysis by ethnicity, a significant increase in the susceptibility CAD in T2DM was found in Asian populations in the allele model (OR = 1.39, 95% CI: 1.15-1.69, $p = 0.001$), homozygote model (OR = 2.15, 95% CI: 1.31-3.51, $p = 0.002$), heterozygote model (OR = 1.37, 95% CI: 1.09-1.73, $p = 0.006$), recessive model (OR = 1.65, 95% CI: 1.11-2.45, $p = 0.012$), and dominant model (OR = 1.54, 95% CI: 1.24-1.91, $p < 0.001$) (Figures 2-6). A similar significant increase in susceptibility was found in Caucasian populations in the allele model (OR = 1.75, 95% CI: 1.22-2.49, $p = 0.002$), homozygote model (OR = 3.39, 95% CI: 1.58-7.27, $p = 0.002$), recessive model (OR = 1.98, 95% CI: 1.07-3.65, $p = 0.030$), heterozygote model (OR = 1.64, 95% CI: 1.21-2.21, $p = 0.001$), and dominant model (OR = 1.83, 95% CI: 1.38-1.95, $p < 0.001$). Subgroup analysis by source of control also yielded positive results in hospital-based population (R *vs.* Q: OR = 1.37, 95% CI: 1.20-1.57, $p < 0.001$; RR *vs.* QQ: OR = 2.16, 95% CI: 1.38-3.36, $p = 0.001$; QR *vs.* QQ: OR = 1.55, 95% CI: 1.23-1.96, $p < 0.001$; RR + RQ *vs.* QQ: OR = 1.65, 95% CI: 1.31-2.06, $p < 0.001$; RR *vs.* RQ + QQ: OR = 1.49, 95% CI: 1.12-2.00, $p = 0.007$) and population-based individuals (R *vs.* Q: OR = 1.96, 95% CI: 1.12-3.42, $p = 0.018$; RR *vs.* QQ: OR = 3.59, 95% CI: 1.21-10.61, $p = 0.021$; QR *vs.* QQ: OR = 1.34, 95% CI: 1.01-1.79, $p = 0.044$; RR + RQ *vs.* QQ: OR = 1.63, 95% CI: 1.25-2.13, $p < 0.001$; RR *vs.* RQ + QQ: OR = 3.11, 95% CI: 1.01-9.57, $p = 0.048$) (Table 2).

3.3. *Sensitivity analysis*

Sensitivity analysis was conducted to investigate the potential influence of a single individual study on the combined OR by eliminating studies one by one in all models. The sensitivity analysis results revealed that no individual study significantly affected the combined

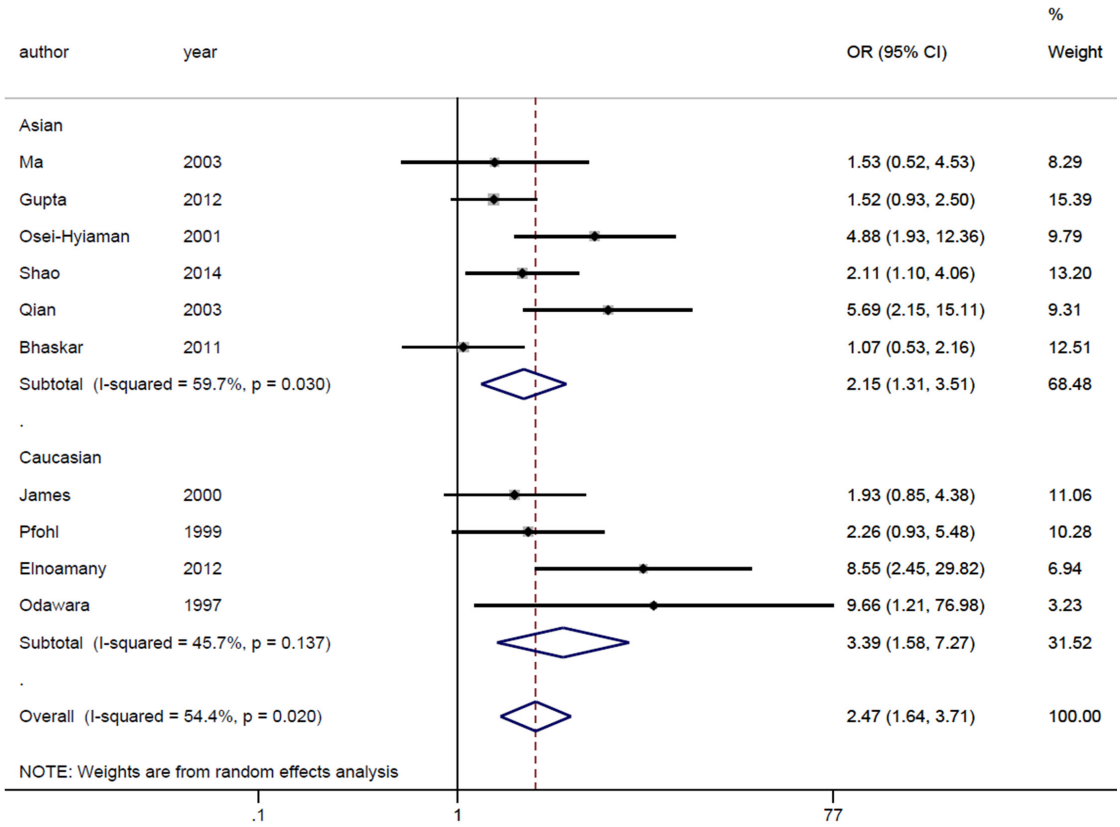

Figure 4. Forest plot for the association between the *PON1* Q192R polymorphism and coronary artery disease susceptibility in type 2 diabetes patients by ethnicity under the recessive model.

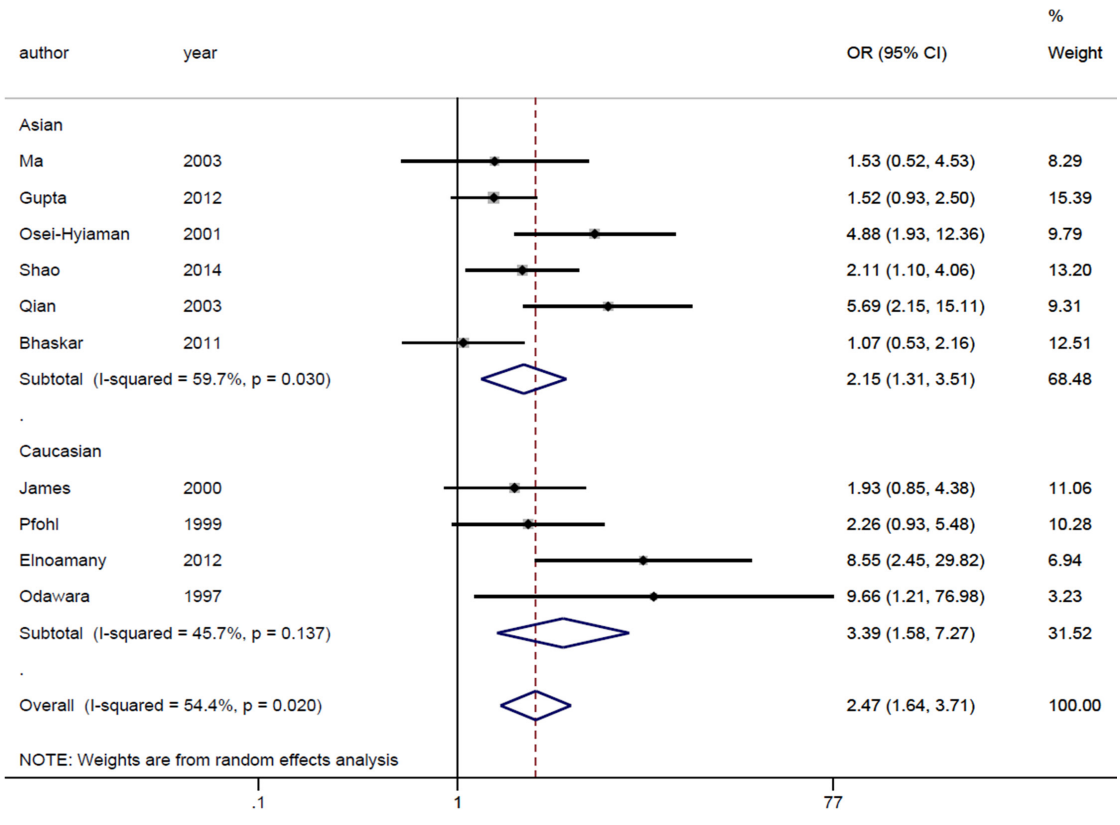

Figure 5. Forest plot for the association between the *PON1* Q192R polymorphism and coronary artery disease susceptibility in type 2 diabetes patients by ethnicity under the homozygous model.

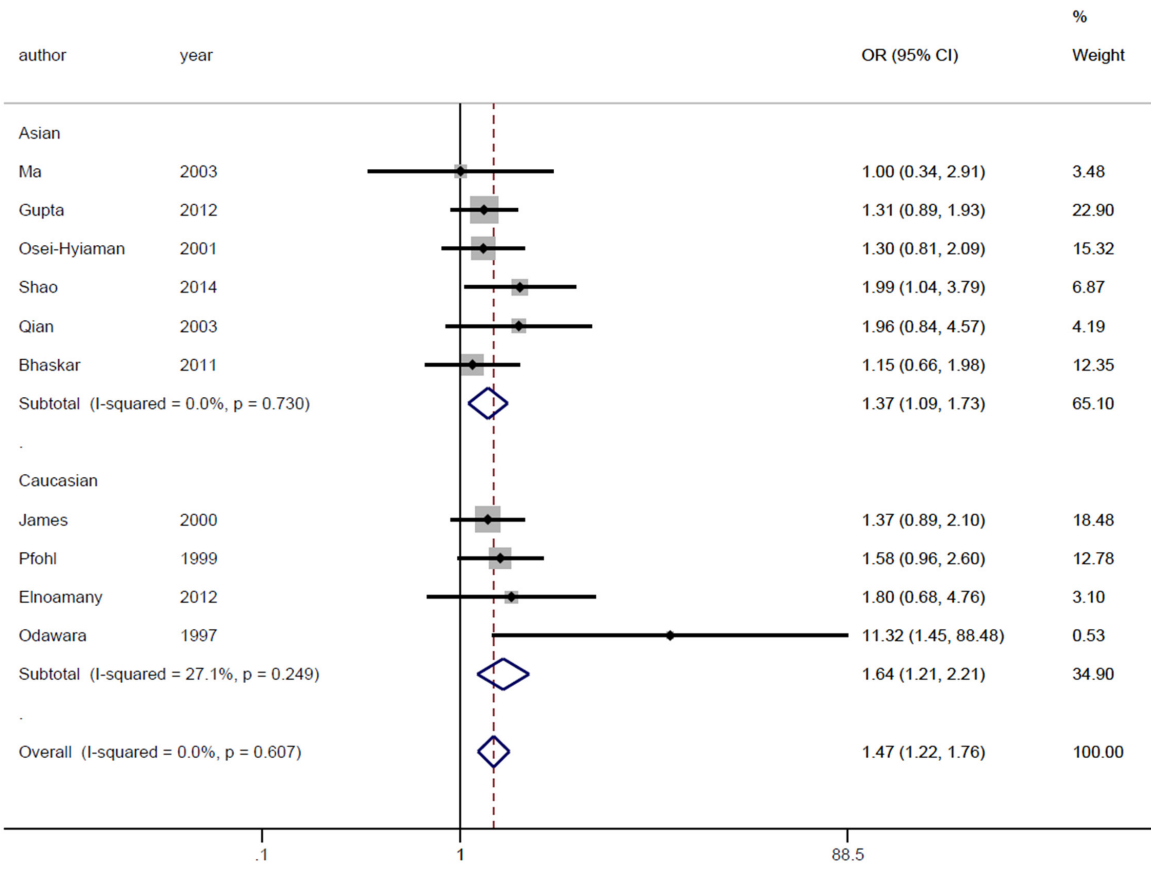

Figure 6. Forest plot for the association between *PON1* the Q192R polymorphism and coronary artery disease susceptibility in type 2 diabetes patients by ethnicity under the heterozygote model.

Table 2. Meta-analysis of the association between the *PON1* Q192R polymorphism and susceptibility of coronary artery disease in type 2 diabetes patients

Subgroup analysis	Study number	OR	95% CI	$P_{(Z-t)}$	I^2 (%)	$P_{(Q-t)}$	Model
R *vs*. Q	10	1.49	1.26-1.77	< 0.001	55	0.018	Random
Asian	6	1.39	1.15-1.69	0.001	51.4	0.067	Random
Caucasian	4	1.75	1.22-2.49	0.002	62.4	0.047	Random
HB	7	1.37	1.20-1.57	< 0.001	3.3	0.4	Fixed
PB	3	1.96	1.12-3.42	0.018	84.2	0.002	Random
RR *vs*. QQ	10	2.47	1.64-3.71	< 0.001	54.4	0.002	Random
Asian	6	2.15	1.31-3.51	0.002	59.7	0.003	Random
Caucasian	4	3.39	1.58-7.27	0.002	45.7	0.137	Fixed
HB	7	2.16	1.38-3.36	0.001	40.4	0.122	Fixed
PB	3	3.59	1.21-10.61	0.021	78.9	0.009	Random
QR *vs*. QQ	10	1.47	1.22-1.76	< 0.001	0	0.607	Fixed
Asian	6	1.37	1.09-1.73	0.006	0	0.73	Fixed
Caucasian	4	1.64	1.21-2.21	0.001	27.1	0.249	Fixed
HB	7	1.55	1.23-1.96	< 0.001	9	0.36	Fixed
PB	3	1.34	1.01-1.79	0.044	0	0.827	Fixed
RR+RQ *vs*. QQ	10	1.64	1.38-1.95	< 0.001	18.6	0.271	Fixed
Asian	6	1.54	1.24-1.91	< 0.001	0	0.485	Fixed
Caucasian	4	1.83	1.38-1.95	< 0.001	51.2	0.104	Random
HB	7	1.65	1.31-2.06	< 0.001	19.9	0.278	Fixed
PB	3	1.63	1.25-2.13	< 0.001	44.3	0.166	Fixed
RR *vs*. RQ+QQ	10	1.74	1.26-2.39	0.001	57.8	0.011	Random
Asian	6	1.65	1.11-2.45	0.012	65.7	0.012	Random
Caucasian	4	1.98	1.07-3.65	0.03	50.6	0.108	Random
HB	7	1.49	1.12-2.00	0.007	32.7	0.178	Fixed
PB	3	3.11	1.01-9.57	0.048	82.2	0.004	Random

OR, odds ratios; CI, confidence intervals; $P_{(Z-t)}$ value for association test; $P_{(Q-t)}$ value for heterogeneity test; HB, Hospital-Based; PB, Population-based.

ORs under any genetic *PON1* rs662 polymorphism model (data not shown), which indicated the robustness and reliability of the results. Sensitivity analyses were further performed by sequentially leaving out the studies that were not consistent with the balance of HWE. The recalculated results remained statistically significant for the *PON1* rs662 polymorphism and CAD risk in T2DM under any of the genetic models (data not shown).

3.4. *Publication bias*

Substantial asymmetry was observed in 4 genetic models by visual inspection of the Begg's funnel plots. In quantitative analysis, no evidence of publication bias was observed in the heterozygote model ($p = 0.051$); however, substantial asymmetry was noted in the other models ($p = 0.032$ for the dominant model, $p = 0.026$ for the allelic model, $p = 0.023$ for the recessive model, and $p = 0.024$ for the homozygous model) (figures not shown).

4. Discussion

Peroxidation of LDL plays a key role in the process of atherogenesis (*20*). Enzymes associated with HDL particles, including PON1, can cleave oxidized lipids from LDL. HDL diminishes the accumulation of lipid peroxides in LDL generally owing to PONA activity (*21*). It was reported that PON1 activity is reduced in patients with CAD and in patients with T2DM (*19,22-24*). The reason for the decreased PON1 activity in these studies could be owed to the increased glycation of HDL cholesterol particles. The predisposition to CAD is determined by a combination of environmental and genetic factors. A single study lacks enough statistical power to confirm the relationship between *PON1* rs662 and the risk of CAD in T2DM owing to the limited sample size. In order to clarify the relationship between these two entities, a comprehensive meta-analysis with a large sample size was performed to identify the associations. To our knowledge, this is the first meta-analysis to reveal that the *PON1* Q192R polymorphism is associated with a significantly increased risk of CAD in T2DM in both Asian and Caucasian populations.

For T2DM patients, the functional role of PONA is more profoundly essential than in non-diabetes individuals. Chronic persistent hyperglycemia results in considerable modification in the protein structure and function, primarily caused by the non-enzymatic glycation of amino acid residues (*5*). Furthermore, LDL cholesterol along with glycated apolipoprotein-B100 interacts with the vascular endothelium and platelets, resulting in elevated production of thromboxanes hence reducing thrombolytic prostaglandins (*25,26*). On the other hand, glycated LDL cholesterol is more readily oxidized, leading to enhanced macrophage uptake by the scavenger receptor pathway (*27*).

It has been revealed that diabetes mellitus patients are at a rising risk for oxidative stress (*28*). The reason may partly be due to features of the diabetic state, markedly hyperglycemia and increased level of glycosylation end products. In accordance with diabetes mellitus, smoking is a well-established risk factor for CAD in which oxidative mechanisms play a significant role. An elevated number of free radicals and their by-products is observed in smokers from lipid peroxidation of oxidized LDL particles compared with non-smokers (*29*).

However, some limitations should be considered when interpreting the results. First, some confounding factors addressed across the various studies, such as age, gender, smoking, family history, and eating habits may have influenced the reliability of the results. Second, 2 studies were not in agreement with HWE; the pooled ORs were not materially altered in the sensitivity analysis. Furthermore, even though we performed a comprehensive literature search, publication bias still remains a problem. Last, owing to the limited studies in certain subgroups, some conclusions should be interpreted with caution in the subgroup analysis. Further prospective studies in a larger population of various races are still required to confirm these findings.

5. Conclusion

In summary, the present meta-analysis results provide strong evidence that the *PON1* Q192R polymorphism is associated with a significantly increased risk of CAD in T2DM patients in both Asian and Caucasian populations.

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Original Article

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A study on factors determining dose of topical lignocaine during broncho-alveolar lavage by spray-as-you-go technique: A single centre observational study

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Summary **Basic bronchoscopic diagnostic procedures like Broncho-alveolar lavage (BAL) are often performed without sedation, using lignocaine administered *via* the working channel of bronchoscope (spray-as-you-go technique) and other routes. Our aim was to evaluate the factors responsible for variation in the total dose of lignocaine administered in individual subjects. We prospectively included consecutive subjects undergoing BAL in an outpatient setting from August 2016 to November 2017 at our centre. The subjects were administered lignocaine *via* nebulization, nasal gel, oropharyngeal spray before and during bronchoscopy ("spray-as-you-go") as per a predefined protocol. The demographic details, high resolution computerized tomography (HRCT) characteristics, procedural details, doses of lignocaine administered and a visual analogue scale (VAS) for satisfaction with the procedure were recorded. Using lignocaine dose as outcome, variables were assessed for effect by univariate and multivariate regression analysis. 96 subjects were included with a mean age of 40 years and male predominance (60.4%). Cough was the most common presenting symptom (64.6%). Predisposing factors included tuberculosis (47.9%) and smoking (23.2%). Maximum variation in lignocaine dose occurred prior to intubating vocal cords using "spray-as-you-go", which was significantly related to history of past tuberculosis ($p = 0.031$), obstructive airway disease ($p = 0.009$), fibrotic sequelae ($p = 0.011$) and bronchiectasis ($p = 0.049$). Obstructive airway disease and fibrotic sequelae were also significant on multivariate analysis ($p = 0.01$ and 0.005 respectively). Obstructive airway disease and architectural distortion due to fibrotic sequelae leads to higher dose requirement for lignocaine during BAL by fibre-optic bronchoscopy. Caution must be maintained during bronchoscopic procedures to avoid exceeding recommended maximum doses in such patients.**

Keywords: Bronchoscopy, broncho-alveolar lavage, lignocaine dose

1. Introduction

Flexible fibre-opticbronchoscopy (FFB) is one of the most commonly used tools in the diagnosis and treatment of broncho-pulmonary diseases throughout the world. It is generally believed to be an extremely safe procedure with low rates of major complications and extremely low mortality (0-0.04% in 68,000 procedures) (*1*). Minor procedure related complications are common, of which cough is often reported by most patients to be particularly distressing (*2*). A number of regimens have been suggested for optimal patient comfort during the procedure, ranging from conscious sedation combined with anticholinergic agents and topical lignocaine to topical lignocaine alone (*3*). While sedatives are often recommended in patients in whom there are no contraindications, actual practice scan vary based on the settings (office, intensive care units, or operating room), complexity and duration of the procedure (advanced diagnostic or therapeutic bronchoscopy) (*4*). In most cases, basic bronchoscopic diagnostic procedures like broncho-alveolar lavage (BAL) can be performed without sedatives while minimizing patient discomfort if adequate topical lignocaine is provided (*5*).

Lignocaine is the most commonly used topical

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anaesthetic for flexible bronchoscopy because of its efficacy in suppressing cough, short half-life, wide safety margin and minimal tissue toxicity (*6*). It can be administered as soaked cotton pledgets, dropper instillation, aerosol spray, nebulization, trans-cricoid or trans-tracheal injection, local nerve block, or "spray-as-you-go" (through the working channel of the bronchoscope) (*4*). Although believed to be safe, systemic absorption of a fraction of administered dose is known, and dose related cardiac (arrhythmias) and neurologic (circum-oral paraesthesia, seizures) side effects can occur if the total topical dose exceeds 8.2 mg/kg or serum lidocaine level exceeds 5 mg/L (*3*). The total dose of lignocaine administered, therefore, needs to be carefully tracked and meticulously recorded throughout the procedure. Studies on lignocaine kinetics have identified subjects with advanced age, impaired liver function, or congestive heart failure at particular risk of toxicity (*7*). However, clinical experience suggests that the dose of lignocaine administered is highly variable among patients irrespective of the presence of aforementioned factors. The objective of this study was to identify patients in whom such high doses could be anticipated and thus could be pre-emptively supplemented with sedatives prior to the procedure to limit total lignocaine used.

2. Materials and Methods

2.1. *Study setting*

The current study was conducted in the bronchoscopy suite of the department of Medicine, All India Institute of Medical Sciences, New Delhi, a tertiary care academic centre in India. We included consecutive subjects undergoing FFB for BAL from August 2016 to November 2017 through a prospectively acquired database. The study protocol was approved by the Ethics Review Committee (Ref. No. IECPG/499/29.8.2016), and written informed consent was obtained from all the subjects.

2.2. *Patient characterization*

The demographic details of the subjects were recorded, including age, sex, education, weight, height, body mass index (BMI), smoking status, and clinical features of underlying broncho-pulmonary disease (shortness of breath, haemoptysis, cough, fever, history of past or present anti-tubercular therapy (ATT) and inhaler use). All the subjects in the study had a recent High-Resolution Computed Tomography (HRCT) chest done, and pertinent findings of the scan were also recorded.

2.3. *Study protocol*

As mentioned previously, the subjects undergoing BAL were not routinely sedated, and bronchoscopy was performed under topical anaesthesia with lignocaine. All subjects were kept fasting overnight prior to the procedure. BAL was done by a single operator (A.R) who has more than 5 years of experience using Pentax EB-1970K with a 6.2 mm distal tube diameter, 2.8 mm working channel and 120° angle of view. BAL was done using standard protocol and 80-100 mL of lignocaine was instilled in a "wedged" segment followed by application of suction through a wall-mounted apparatus keeping the pressure below 100 mm of Hg at all times. The subjects received lignocaine through 4 routes according to a predefined protocol: nebulization, nasal gel, oropharyngeal spray before and "spray-as-you-go" during bronchoscopy. Nebulization: 2.5 mL of 4% lignocaine for 15 minutes. Oropharyngeal spray: Lignocaine spray (10%) was sprayed twice (10 mg/puff) over the oropharynx. Nasal Gel: Approximately 3 mL of lignocaine gel (2%), equivalent to 60 mg of lignocaine, was administered in the nasal cavity prior to the introduction of the bronchoscope. "Spray-as-you-go": Subjects thereafter received 1-mL aliquots of 2% lignocaine solution delivered through the bronchoscope as a rescue therapy to suppress cough, at the discretion of the operator. In general, four aliquots of 1 mL of lignocaine were administered: one each at the vocal cord, tracheal carina, and in the right and left main bronchus.

The sum of the administered dose (2.5 mL of 4% nebulized lignocaine [100 mg] plus 3 mL of 2% lignocaine gel [60 mg] plus two puff s of 10% lignocaine spray [20 mg] plus 20 mg for every additional aliquot of 2% lignocaine during "spray-as-you-go") made up the total dose of lignocaine used. For ease of calculations, the dose administered was recorded as milligrams of lignocaine. The amount of lignocaine administered before and after passing through the vocal cords and the total dose of lignocaine administered per kg body weight were also recorded. The subjects in whom technical difficulty prevented completion of procedure received additional intravenous sedation and were excluded from this study.

Vital parameters, namely pulse rate, respiratory rate, blood pressure (BP), and oxygen saturation (by pulse oximetry) were monitored throughout the procedure. Subjects were also monitored for any adverse effects related to lignocaine use (like arrhythmia, involuntary movements, anaphylaxis, and bronchospasm).

Along with the dose of lignocaine, the amount of normal saline (NS) administered, number of BAL sites and time taken for the procedure was also recorded. A Visual Analogue Scale (VAS) was used to objectively assess satisfaction with the procedure as reported by the patient and bronchoscopist independently. The scale consisted of a 100 mm line, wherein the rating was given by marking a point along this line, where 0 represented complete satisfaction with the procedure and 100 represented no satisfaction at all.

The satisfaction rating thus obtained was assessed for agreement between bronchoscopist and the patient. The dose of lignocaine used across different quartiles of patient satisfaction groups was also analysed.

2.4. *Sample size estimation*

The primary objective of this study was to identify independent factors that affect dosing of lignocaine among patients. For the purpose of sample size estimation, it was assumed that analysing up to 5 variables for independent effect by multivariate analysis would be sufficient. Assuming an alpha of 0.05, power of 0.80, number of predictors to be 5 with moderate effect size ($f^2 = 0.15$), the number of subjects required for analysis was estimated to be 92 (*8*). An additional 8 cases were added to account for requirement of IV sedation and withdrawal of consent.

2.5. *Statistical analysis*

The continuous variables are presented as means with standard deviations or medians with interquartile range depending upon underlying distribution. The categorical variables are presented as percentages for each category. The statistical association of each of the variables with lignocaine dose administered throughout the procedure, prior to passing through vocal cords and thereafter, along with lignocaine dose by body weight was assessed by Student's *t* tests, Mann Whitney *U* tests and chi square tests as appropriate. One-way ANOVA was used for comparing intergroup differences in patient and bronchoscopist reported satisfaction as there were more than 2 groups. Appropriate subsets of the determinant variables were assessed for independent effect on outcome by multivariate linear regression analysis. All analyses were performed using IBM SPSS Statistics Version 20 and Microsoft Excel 2011.

3. Results

A total of 99 consecutive subjects who underwent BAL over the 1-year period gave consent for inclusion in this study. In 3 subjects the procedure couldn't be completed on topical lignocaine alone and required intravenous sedation. They were therefore excluded and the remaining 96 subjects were included in the study. The study population had a mean age of 40 years with male predominance (59.4%) and a relatively high proportion of present or past tuberculosis (8.3% and 39.6%) (Table 1). Cough was the most common presenting symptom (64.6%), although shortness of breath, fever and haemoptysis were fairly common as well (34.4%, 35.8% and 22.9% respectively). The most common computerized tomography (CT) findings included bronchiectasis, centri-lobular nodules, consolidation, cavitation and fibrotic sequelae (26.0%, 28.1%, 27.1%, 21.9% and 28.1% respectively). However almost all CTs had some demonstrable abnormality, and only 2 subjects had near normal CT scans (Table 2).

The mean doses of lignocaine administered to various subgroups of categorical clinical findings were compared by Student's *t* test (Table 3). The maximum variation in lignocaine administered occurred during "spray-as-you-go" prior to intubating vocal cords (VC), with same doses used for nebulization, nasal gel, oropharyngeal spray and nearly similar doses after intubating vocal cords. The time needed for reaching vocal cords during the procedure was also dependent only on the dose of lignocaine needed prior to reaching VC (Spearman's rho: 0.725, $p < 0.001$) (Figure 1).

The total dose of lignocaine was similar irrespective of sex, however, females received significantly lesser lignocaine dose when compared based on dose administered per kg body weight (6.5 mg/kg *vs*. 7.76 mg/kg for males, $p < 0.01$) (Figure 2). No association was noted for history of smoking, inhaler use, shortness of breath, haemoptysis or cough, although there was a trend towards significance for cough when compared for lignocaine needed prior to intubating vocal cords. (66.9 mg *vs*. 55.8 mg, $p = 0.062$). Subjects with history of past ATT (anti-tubercular therapy) intake also required significantly more lignocaine doses prior to vocal cords (70.5 mg *vs*. 58.1 mg, $p = 0.031$). In addition, subjects with fever (53.8 mg *vs*. 68.1 mg, $p = 0.015$) and present ATT use (42.5 mg *vs*. 64.8 mg, $p = 0.028$) appeared to require lesser lignocaine doses than others. The effect of history of fever was also evident on total lignocaine dose

Table 1. Demographic and clinical characteristics of study population

Age (yrs) mean ± SD	40.0 ± 14.8
Females, *n* (%)	39 (40.6%)
Weight (kg) mean ± SD	47.7 ± 11.5
Height (m) mean ± SD	1.61 ± 0.10
BMI (kg/m^2) mean ± SD	18.23 ± 3.83
Smokers, *n* (%)	22 (23.2%)
History of shortness of breath, *n* (%)	33 (34.4%)
History of fever, *n* (%)	34 (35.8%)
History of hemoptysis, n (%)	22 (22.9%)
History of cough, *n* (%)	62 (64.6%)
Presently taking ATT, *n* (%)	8 (8.3%)
Past history of ATT use, *n* (%)	38 (39.6%)
History of inhaler use, *n* (%)	21 (21.9%)

Table 2. High Resolution Computerized Tomography characteristics of study population

Bronchiectasis, *n* (%)	25 (26.0%)
Mass lesion, *n* (%)	9 (9.4%)
Miliary nodules, *n* (%)	3 (3.1%)
Centrilobular nodules, *n* (%)	27 (28.1%)
Consolidation, *n* (%)	26 (27.1%)
Cavitation, *n* (%)	21 (21.9%)
Fibrotic sequelae, *n* (%)	27 (28.1%)
Collapse, *n* (%)	7 (7.3%)
Air trapping, *n* (%)	11 (11.5%)
Normal CT scan	2 (2.1%)

Table 3. Mean lignocaine dose administered across clinical subgroups[$]

Items		Total lignocaine administered*	Sig.[#]	Lignocaine administered prior to VC*	Sig.[#]	Lignocaine administered after VC*	Sig.[#]	Total lignocaine/ Body weight*	Sig.[#]	Total "spray-as-you-go" lignocaine	Sig.[#]
Females	Y	320.7 ± 39.8	NS	64 ± 27.5	NS	76.8 ± 22.2	NS	6.5 ± 1.58	< 0.01	133.2 ± 34	NS
	N	313.3 ± 34.7		61.5 ± 28.4		71.7 ± 18.7		7.76 ± 1.71		140.8 ± 35.3	
Smokers	Y	319 ± 32.9	NS	63.6 ± 25.9	NS	75.4 ± 19.4	NS	6.81 ± 2.06	NS	139.1 ± 32.9	NS
	N	316.9 ± 39.5		62.3 ± 28.3		74.7 ± 21.6		7.04 ± 1.64		137.1 ± 39.4	
History of shortness of breath	Y	326 ± 40.1	NS	69.3 ± 31.4	NS	76.9 ± 22.4	NS	7.28 ± 1.74	NS	146.4 ± 39.7	NS
	N	313.3 ± 36.1		59.6 ± 25.2		73.6 ± 20.2		6.87 ± 1.74		133.3 ± 36.1	
History of fever	Y	307 ± 32.6	0.041	53.8 ± 20.0	0.015	73.5 ± 20.7	NS	6.48 ± 1.78	0.021	127.4 ± 32.2	0.034
	N	323.6 ± 39.7		68.1 ± 30.4		75.4 ± 21.4		7.34 ± 1.66		143.6 ± 39.8	
History of hemoptysis	Y	320 ± 39	NS	63.6 ± 32.4	NS	76.3 ± 19.1	NS	6.79 ± 1.6	NS	140 ± 39.04	NS
	N	317 ± 37.6		62.8 ± 26.4		74.3 ± 21.5		7.08 ± 1.78		137.2 ± 37.5	
History of cough	Y	322.2 ± 38.1	NS	66.9 ± 28.8	0.062	75.4 ± 21.2	NS	7.17 ± 1.76	NS	142.4 ± 37.9	NS
	N	309.4 ± 36.2		55.8 ± 24.5		73.5 ± 20.7		6.74 ± 1.68		129.4 ± 36.3	
Presently taking ATT	Y	295 ± 39.6	0.076	42.5 ± 16.6	0.028	72.5 ± 26	NS	6.04 ± 1.12	NS	115 ± 39.6	NS
	N	319.7 ± 37.2		64.8 ± 27.8		75 ± 20.6		7.09 ± 1.76		139.9 ± 37	
Past history of ATT use	Y	326.8 ± 40.3	0.055	70.5 ± 29.6	0.031	76.3 ± 20.7	NS	7.39 ± 1.75	0.089	146.8 ± 40.3	0.066
	N	311.7 ± 35.1		58.1 ± 25.5		73.7 ± 21.2		6.76 ± 1.7		131.9 ± 34.9	
History of inhaler use	Y	327.6 ± 43.5	NS	70.9 ± 33.4	NS	77.1 ± 23	NS	7.22 ± 1.64	NS	148.1 ± 42.9	NS
	N	314.9 ± 35.8		60.8 ± 25.7		74.1 ± 20.4		6.95 ± 1.77		134.9 ± 35.9	

*: Lignocaine doses are reported as equivalents of milligrams of lignocaine. [#]: Significance is reported for Student's *t* test as *p* values. Only values < 0.10 are reported. Values < 0.05 are accepted as statistically significant. [$]: All Values are reported as means ± Standard deviation in mg, unless stated otherwise.

Table 4. Mean lignocaine dose administered across computerized tomography subgroups[$]

Items		Total lignocaine administered*	Sig.[#]	Lignocaine administered prior to VC*	Sig.[#]	Lignocaine administered after VC*	Sig.[#]	Total lignocaine/ Body weight*	Sig.[#]	Total "spray-as-you-go" lignocaine	Sig.[#]
Bronchiectasis	Y	320.8 ± 37.6	NS	72.4 ± 30.7	0.049	68.8 ± 16.4	NS	7.15 ± 1.42	NS	141.2 ± 37	NS
	N	316.6 ± 38		59.7 ± 26		76.9 ± 22		6.96 ± 1.85		136.6 ± 38.1	
Mass lesion	Y	302.2 ± 25.3	NS	53.3 ± 14.1	NS	68.8 ± 22.6	NS	6.87 ± 2.57	NS	122.2 ± 25.4	NS
	N	319.3 ± 38.6		64 ± 28.6		75.4 ± 20.8		7.03 ± 1.65		139.4 ± 38.5	
Miliary nodules	Y	280 ± 40	0.079	33.3 ± 11.5	0.059	66.6 ± 30.5	NS	6.42 ± 2.13	NS	100 ± 40	0.079
	N	318.9 ± 37.3		63.9 ± 27.6		75 ± 20.7		7.03 ± 1.74		139 ± 37.2	
Centrilobular nodules	Y	322.9 ± 41.7	NS	62.2 ± 27.3	NS	80.7 ± 22.5	0.082	7.04 ± 1.97	NS	143 ± 41.8	NS
	N	315.6 ± 36.2		63.3 ± 28.1		72.4 ± 20		7 ± 1.66		135.8 ± 36.1	
Consolidation	Y	315.3 ± 35.4	NS	58.4 ± 25.2	NS	76.9 ± 21.6	NS	6.75 ± 1.47	NS	135.4 ± 35.5	NS
	N	318.5 ± 38.8		64.7 ± 28.6		74 ± 20.8		7.11 ± 1.83		138.7 ± 38.7	
Cavitation	Y	318 ± 43.7	NS	63.8 ± 33.8	NS	74.2 ± 22.9	NS	7.04 ± 1.76	NS	138.1 ± 43.8	NS
	N	317.6 ± 36.3		62.8 ± 26		74.9 ± 20.5		7 ± 1.74		137.7 ± 36.1	
Fibrotic sequelae	Y	337.7 ± 44.8	0.005	77.7 ± 36.5	0.011	80 ± 19.2	NS	7.41 ± 1.85	NS	157.8 ± 44.8	0.005
	N	309.8 ± 31.7		57.2 ± 21.1		72.7 ± 21.4		6.86 ± 1.68		130 ± 31.5	
Collapse	Y	314.2 ± 37.7	NS	57.1 ± 24.2	NS	77.1 ± 21.3	NS	6.64 ± 2.39	NS	134.3 ± 37.8	NS
	N	317.9 ± 38		63.4 ± 28		74.6 ± 21		7.04 ± 1.69		138.1 ± 37.9	
Air trapping	Y	329 ± 32.6	NS	67.2 ± 16.1	NS	81.8 ± 24.4	NS	7.41 ± 1.77	NS	149.1 ± 32.7	NS
	N	316.2 ± 38.3		62.4 ± 28.9		73.8 ± 20.4		6.96 ± 1.74		136.4 ± 38.2	
Obstructive Airway Disease**	Y	327.3 ± 39.6	0.031	71.4 ± 30.1	0.009	76 ± 21	NS	7.14 ± 1.55	NS	147.6 ± 39.3	0.031
	N	310.5 ± 35		56.7 ± 24.2		73.8 ± 21		6.91 ± 1.88		130.5 ± 35	

*: Lignocaine doses are reported as equivalents of milligrams of lignocaine. [#]: Significance is reported for Student's *t* test as *p* values. Only values < 0.10 are reported. Values < 0.05 are accepted as statistically significant. [$]: All Values are reported as means ± Standard deviation in mg, unless stated otherwise. **: Obstructive airway disease is a composite of bronchiectasis, air trapping and history of inhaler use.

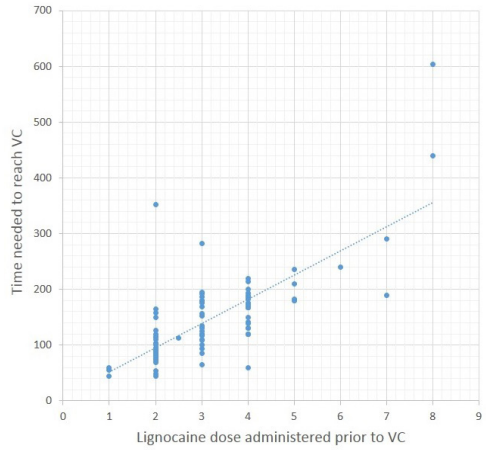

Figure 1. Subjects requiring higher lignocaine dose prior to VC intubation also required longer time for intubating VC.

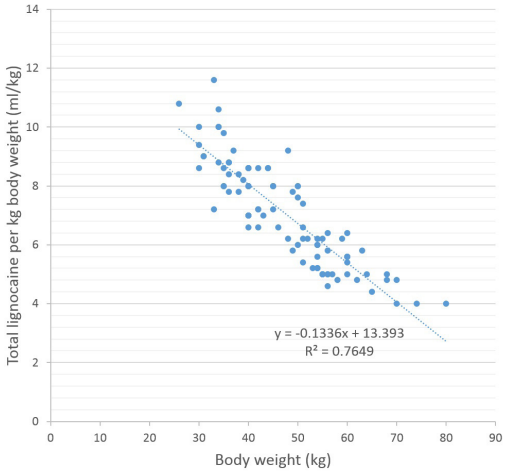

Figure 2. Subjects with higher body weights received significantly lower doses of total lignocaine when assessed based on per kg body weight.

Table 5. Procedural characteristics of subjects undergoing Broncho-alveolar lavage

Total lignocaine dose (mg) mean ± SD	317.71 ± 37.82
Lignocaine administered prior to vocal cords(VC) (mg), mean ± SD	63.02 ± 27.76
Lignocaine administered after vocal cords(VC) (mg), mean ± SD	74.79 ± 20.98
Total lignocaine dose/kg body weight (mg/kg), mean ± SD	7.02 ± 1.74
Time (seconds) needed to reach VC, median(IQR)	130 (93-180)
Time (seconds) needed after passing VC, median(IQR)	245 (195-285)
Lignocaine 4-6 mg/kg	35 (36.5%)
Lignocaine between 6 and 8.2 mg/kg	35 (36.5%)
Lignocaine > 8.2mg/kg	26 (27.1%)
Satisfaction (as assessed by the patient)	50 (20-80)
Satisfaction (as assessed by the bronchoscopist)	30 (20-60)

Table 6. Multivariate linear regression analysis to identify independent predictors of lignocaine dose administered prior to reaching VC

Variable	B (95% Confidence Interval)	Beta	Sig.
(Constant)	46.72 (35.37-58.08)		0.000
History of cough	9.96 (–0.99-20.92)	0.172	0.074
Present ATT use	– 12.79 (–31.77-6.18)	– 0.128	0.184
Past ATT use	– 0.521 (–12.85-11.81)	– 0.009	0.933
Miliary nodules	– 9.128 (–40.11- 21.85)	– 0.058	0.560
Fibrotic sequelae	19.03 (5.83-32.23)	0.310	0.005
Obstructive airway disease	14.21 (3.48-24.93)	0.254	0.010

and lignocaine per kg body weight.

The doses required for subjects with different CT findings were similarly compared (Table 4). Subjects with fibrotic sequelae (77.7 mg *vs*. 57.2 mg, $p = 0.011$) and bronchiectasis (72.4 mg *vs*. 59.7 mg, $p = 0.049$) on CT required significantly higher dose of lignocaine prior to intubating VC. None of the other findings on CT were associated with lignocaine dose.

The continuous variables were assessed across various lignocaine doses by calculating Pearson's correlation coefficients. Statistically significant but overall poor correlation was noted between subjects' age and lignocaine administered after passing VC ($r = 0.202$, $p = 0.048$) (Table 5).

To better explain the variability in lignocaine administered prior to reaching VC, multivariate linear regression analysis was conducted, using lignocaine dose administered prior to reaching VC as dependent variable. To control the degrees of freedom for small sample size, a composite variable of obstructive airway disease was calculated, using past inhaler use for obstructive airway disease, CT features of bronchiectasis or air trapping. The predictors assessed therefore included history of cough, present or past ATT use, miliary nodules on CT, fibrotic sequelae on CT and obstructive airway disease. Multivariate analysis identified presence of obstructive airway disease and fibrotic sequelae as the only independent predictors of lignocaine dose required (Table 6). Together, the presence of these 2 variables explained nearly one

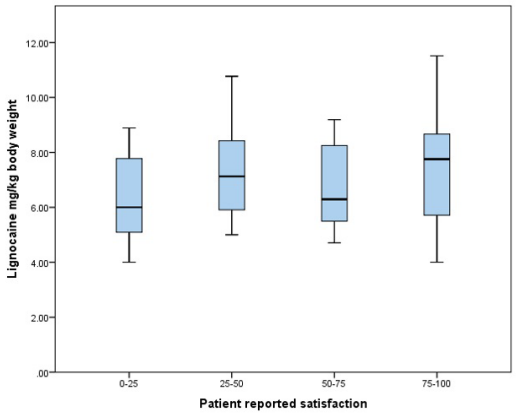

Figure 3. Boxplots of dose of lignocaine used across quartiles of patient reported outcomes.

fourth of variations in lignocaine dose needed (adjusted $R^2 = 0.241$).

Most of the subjects were only partly satisfied with the procedure, with a median self-rated satisfaction of 50% (IQR 20-80). The self-rated index of dissatisfaction was consistently higher when assessed by the subjects compared with that of bronchoscopist (median of 30) (Table 4). There was a significant but only moderate correlation between patient and bronchoscopist assessed outcomes for satisfaction (Spearman's rho = 0.464). While bronchoscopist reported satisfaction was related to the dose of lignocaine ($p = 0.002$, One-way ANOVA), no similar association could be identified for patient reported satisfaction. No definite trend was identified in dose of lignocaine across quartiles of satisfaction (Figure 3).

4. Discussion

To the best of our knowledge this the first study on the possible factors governing the dose of lignocaine during FBB. It is known that different patients require different doses of topical lignocaine during bronchoscopy – but little is known about the factors dictating increased doses of lignocaine.

Our study demonstrated that the presence of obstructive airway disease (history of prior inhaler use for physician diagnosed obstructive airway disease, features of airway trapping or bronchiectasis on CT scan) and fibrosis on CT scan were the only two independent predictors of lignocaine dose. It is known that obstructive airway disease, in the form of both asthma and non-asthmatic conditions can be associated with a heightened cough response which can explain the increased cough and need for increased dose of topical lignocaine in the group of presumed obstructive airway disease as seen in our study (*9*).

Tuberculosis is often associated with architectural compromise of the airways (*10*) and lung parenchyma leading to a gamut of restrictive and obstructive defects in pulmonary function (*11,12*). Badivuku *et al*. have demonstrated increased bronchial reactivity in patients with treated tuberculosis (*13*). The architectural distortion coupled with ongoing inflammation as a result of inter-current infections may predispose these individuals to increased cough reactivity – leading to increased requirement of topical lignocaine.

Also, the maximum variation in lignocaine dose occurred before intubation of vocal cord and the doses were not much different after intubation. The possible reason for the same was that the lignocaine used above the vocal cord also trickled down to the tracheo-bronchial tree producing topical anaesthesia, thus reducing further dose requirement. This is further demonstrated by the fact that the total doses of lignocaine used as "spray-as-you-go" (total lignocaine above and below the vocal cord) showed similar predictors as those for above the vocal cord.

The weight of the patient was not a potential factor dictating the requirement of lignocaine dose. A fixed amount of lignocaine (nebulisation and nasal) was given to the subjects and the "spray-as-you-go" were given according to the cough response of the patient - which did not vary according to weight – signifying that other factors and not the weight determine the dose of lignocaine. Thus, patients with lesser weight are more likely to be administered higher doses when compared on a per kg basis, and appropriate caution must be exercised in such patients.

Morice, *et al*. (*14*) had reported increased cough in females in response to inhalation cough challenge (with 10% citric acid) which suggested that there may be a sex-related difference in cough reflex sensitivity. No such difference was noted on our study. It may however be reasoned, that the smaller diameter of trachea-bronchial tree in females (*15*) may lead to greater deposition of topical lignocaine, protecting against the tussive effect of the bronchoscope in the airway.

There is no standard validated technique for assessing adequacy of anaesthetic dose administered during the procedure, although a number of tools have been used in the past with inconsistent results. The VAS is a simple and easily administered tool that can be used to gain insight to both the doctor's and patients' opinion of the procedure on a common scale. In our study, the bronchoscopist assessed outcomes were similar to that of others (*16*) for non-sedated patients, for satisfaction (Table 4). As reported previously, however, the patient assessed outcomes were only moderately correlated with that of bronchoscopist and were usually higher and more variable. The patient related outcomes also did not show a statistical association with the dose of lignocaine administered, indicating that there would be additional factors responsible for dissatisfaction, which cannot be accounted by the dose of lignocaine used alone. More studies are needed in this particular field for providing better patient comfort and procedure

results.

The limitations of this study are that it was conducted in a single centre (making it less generalizable), cross-sectional design and lack of blinding of the operator to patients' past medical history as well as dose of lignocaine given raising the possibility of bias. As the study was conducted in a tuberculosis endemic region around 40% patients had past history of tuberculosis. The strengths of the study, apart from its novelty, is the uniformity of the study protocol applied to all patients because of the fact that it was done by a single bronchoscopist following a pre-determined protocol.

In conclusion, the topical dose of lignocaine in patients undergoing FFB in a tuberculosis-endemic region is linked to patient factors like existence of obstructive airway disease and fibrotic sequelae. Such patients may require a combination of intravenous sedation and topical anaesthesia rather than topical lignocaine alone.

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Original Article

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Infectious versus non-infectious causes of oligoarticular inflammatory arthritis: A prospective study from a tertiary care hospital in north India

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Summary **Oligoarticular arthritis (inflammation of upto 4 joints) has a wide range of infectious and non-infectious etiologies. The aim of our study was to identify the features which could help in the differentiation of infectious from non-infectious arthritis. The study was prospective and observational, and included 100 patients with oligoarticular inflammatory arthritis. The final diagnosis was made using standard diagnostic criteria and the patients were categorized into infectious and non-infectious groups. Among the 100 patients who were recruited, the following final diagnosis were made: peripheral spondyloarthritis (*n* = 37), axial spondyloarthritis (*n* = 11), tuberculosis (*n* = 19), brucellosis (*n* = 6), septic arthritis (*n* = 6), gouty arthritis (*n* = 5), early rheumatoid arthritis (*n* = 5), non-tubercular mycobacteria (*n* = 2), SLE (*n* = 2), post-chikungunya arthritis (*n* = 2), acute lymphocytic leukaemia (*n* = 1), pachydermoperiostosis (n = 1), sarcoidosis (n = 1) and juvenile idiopathoic arthritis (*n* = 1). The patients were categorized into two groups: infectious (33) and non-infectious (60). The presence of monoarthritis, clinically-significant weight loss, hepatomegaly, splenomegaly and erosive arthritis were significantly more common in the infectious group as compared to the non-infectious group.**

Keywords: Axial spondyloarthritis, peripheral spondyloarthritis, tuberculosis, brucellosis

1. Introduction

Musculoskeletal pain is among the commonest symptoms with which patients present in outpatient clinics. A fraction of these patients are diagnosed with arthritis. Although polyarthritic diseases like rheumatoid arthritis have been widely studied, the epidemiology of oligoarticular arthritis remains less explored. Oligoarticular arthritis, defined as the involvement of upto 4 joints, is routinely encountered in clinical practice. It has a wide range of infectious (*e.g.*: tuberculosis, brucellosis and septic arthritis) as well as non-infectious (*e.g.*: crystal-induced arthritis, peripheral and axial spondyloarthropathies, early rheumatoid arthritis and sarcoidosis) causes. In the studies from developed countries, non-infective causes have been found to be more common than infectious causes. Although not many studies on the clinical and demographic profile of oligoarthritis in Indian patients are available, it is possible that the clinical profile of Indian patients would be different from those of the developed countries due to the higher prevalence of tuberculosis and other infectious diseases.

The clinical presentations of infectious and non-infectious oligoarthritis overlap significantly, which makes a clinical distinction extremely difficult.

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However, it is important to identify the infectious causes of arthritis early as prompt initiation of antibiotics can reduce the extent of the damage to the joint, thereby reducing future disability and morbidity. Therefore, the purpose of the study was to describe the epidemiology of oligoarticular arthritis and identify the demographical, clinical, laboratorical and radiological findings which could help in the differentiation of infectious from non-infectious arthritis.

2. Materials and Methods

The study was designed as a prospective, observational study and prior approval was obtained from the institutional ethics committee. Subsequently, 100 patients who presented to the outpatient and inpatient services of our hospital between June 2017 and November 2018 with oligoarticular arthritis were recruited into the study. An informed consent was taken from all the patients. Patients with clinically evident osteoarthritis, neuropathic joint disease and those with history of significant prior trauma to the bones or joints were excluded from the study. The history and physical examination findings were recorded using a predefined questionnaire. Routine laboratory investigations were performed in all patients, which included routine haemogram, liver and renal function tests, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Apart from these, appropriate specific immunological and microbiological tests like IgM and IgG Enzyme linked immunosorbent assay (ELISA) for brucellosis, Human Leukocyte Antigen-B27 (HLA B-27), rheumatoid factor, anti-cyclic citrullinated peptide (anti-CCP), angiotensin-converting enzyme (ACE) levels, urine for Chlamydial polymerase chain reaction assay (PCR), stool culture for *Shigella* spp. and *Salmonella* spp., stool for *C. difficile* toxin ELISA, synovial fluid for Gene Xpert, Ziehl-Neelsen stain and Mycobacterial culture were performed according to the physician's discretion depending on the clinical presentation of the patient. All patients also underwent a chest radiograph as well as one radiographic projection of the affected joints to look for erosions. Depending on the clinical scenario, other radiographs and magnetic resonance imaging (MRI) were performed on a case to case basis. The patients were then assigned a final diagnosis based on the standard, disease-specific criteria. Axial and peripheral spondyloarthritis were diagnosed according to the Assessment of Spondyloarthritis International Society (ASAS) classification criteria (*1,2*). Crystal-induced arthritis was diagnosed based on the presence of compatible clinical presentation and hyperuricemia with or without demonstration of crystals in the synovial fluid aspirate (*3*). Sarcoid arthropathy was diagnosed on the basis of the clinical, radiological and histopathological findings (*4*). Early Rheumatoid arthritis was diagnosed as per the American College of Rheumatology/European League against Rheumatism Criteria (ACR/EULAR, 2010) (*5*). Systemic lupus erythematosus (SLE) was diagnosed according to the ACR classification criteria (*6*). Arthritis from tubercular and non-tubercular mycobacteria as well as septic arthritis was diagnosed based on the clinical and radiological features with or without microbiological confirmation (*7*). Brucella arthritis was diagnosed based on a positive IgM/ IgG ELISA or standard agglutination test. After assignment of the final diagnosis, all the recruited patients were categorized into the two groups of infectious and non-infectious arthritis. These groups were then compared for the distribution of clinical parameters like age, fever, weight loss, lymphadenopathy, skin rash, hepatomegaly and splenomegaly. The radiological findings which were compared included the presence of lung manifestations on the chest radiograph, the number of joints involved, axial skeleton involvement and presence of erosive arthritis. Among the lab parameters, ESR and CRP were compared between the two groups. The patients who could not be categorized unambiguously into either of the two groups were excluded from the analysis.

For statistical analysis, all the data were presented as mean ± standard deviation (SD) or median with interquartile range where appropriate. The analysis was performed using the STATA software, version 11 (StataCorp, TX, USA). Any differences in the distribution of the evaluated attributes between the two groups (infectious and non-infectious) were evaluated using the chi square or Fisher's exact test. A p-value < 0.05 was considered as statistically significant.

3. Results

The study had a total of 100 patients. The age of the study population ranged from 14 to 60 years, the mean age being 30.5 ± 13 years. A male dominance was observed (67% of the patients). The most common age group was 21-30 years (35%, n = 35).

The most common symptoms were fever and weight loss, which were present in 59% and 31% of the patients respectively. The median duration of fever was 30 days (range: 10-120 days). History of cattle exposure and travel to outside of India were present in 17% and 6% respectively. The most common physical examination finding was skin rash (16%), followed by splenomegaly (12%), lymphadenopathy (12%), hepatomegaly (11%), enthesitis (7%), dactylitis (3%) and orchitis (2%). Serology for the human immunodeficiency virus and hepatitis C virus was positive in 5% and 1% of the patients respectively.

A total of 23% of the patients had acute arthritis (duration < 6 weeks) at presentation, whereas 77% had chronic arthritis (duration above 6 weeks). The median duration of arthritis was 110 days (range: 30-660 days). The average number of joints involved was

2.1, with 33 patients having monoarticular involvement and 39, 12, 16 patients having two, three and four joints involvement respectively. Concomitant sacroiliitis was present in 17%, among whom 12 had unilateral involvement and 5 had bilateral disease. Majority of the patients showed only large joint involvement (95%), with 72% having lower limb involvement, 8% having upper limb involvement and the remaining 15% having involvement of both the upper and lower limbs. On the other hand, exclusive small joint involvement was seen only in 1% of the patients. Both large and small joints were involved in 4%. Knee joint was the most commonly affected joint, followed by the ankle, hip, wrist, elbow, shoulder and metacarpophalangeal joints. Axial skeleton involvement (excluding degenerative changes) was seen in 20 patients, whereas erosive arthritis was present in 23 patients (Figure 1).

The final diagnosis of all patients is compiled in Figure 2. Prodromal diarrhea (n = 2) or concomitant urinary tract infection (n = 5) was present in 7 patients who had axial and peripheral spondyloarthritis. Polymerase chain reaction (PCR) assay for *Chlamydia trachomatis* was positive in the urine sample in 3 patients. Among the 19 patients who were diagnosed with tuberculosis, 15 had tubercular arthritis and 4 had osteomyelitis with secondary involvement of the adjacent joint. Tubercular involvement at other sites was seen in 7 patients. The individual patterns of joint involvement in peripheral and axial spondyloarthritis, tubercular arthritis, brucellosis and septic arthritis are summarized in Table 1.

The patients were categorized into two groups: infectious (n = 33) and non-infectious (n = 60) (Table 2). Six patients were excluded from the analysis since these patients had a mixed infective and inflammatory clinical picture and exact cause of the arthritis could not be established. These included cases of peripheral and axial spondyloarthrithis in patients who had concomitant hepatitis-C (HCV) and human retroviral (HIV) infection as well as those who had post-chikungunya arthritis.

When the infectious and non-infectious groups were compared, the presence of weight loss ($p < 0.001$), hepatomegaly ($p = 0.04$), splenomegaly ($p = 0.001$) and erosive arthritis ($p = 0.001$) were significantly more prevalent in the infectious group. Mono-arthritis was also more common in the infectious group ($p < 0.001$).

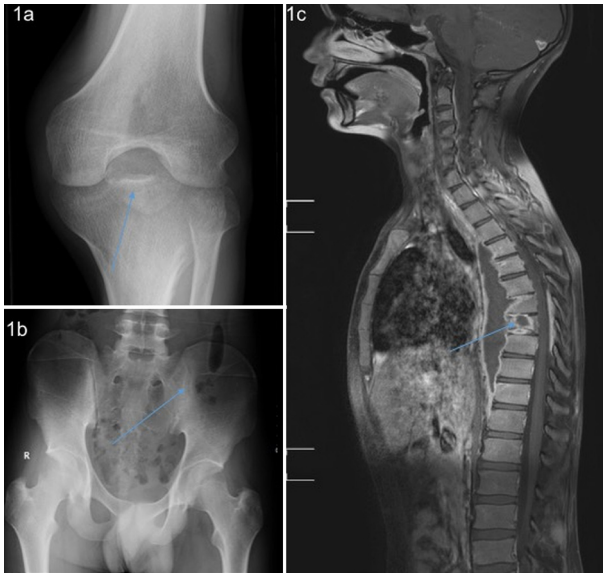

Figure 1. Three patients with different osteoarticular manifestations of tuberculosis. (a) Anteroposterior radiograph of the left knee showing widened intercondylar notch of femur along with small erosion in the tibia (arrow). **(b)** Anteroposterior radiograph of the pelvis showing reduction of the left sacroiliac joint space with sclerosis involving both iliac and sacral sides, suggestive of sacroiliitis. **(c)** Post-contrast T1-weighted sagittal images of the spine showing a large paravertebral collection from D4 toD10 levels along with spondylodiscitis at the D8-D9 level and abscess formation within the D8 vertebra.

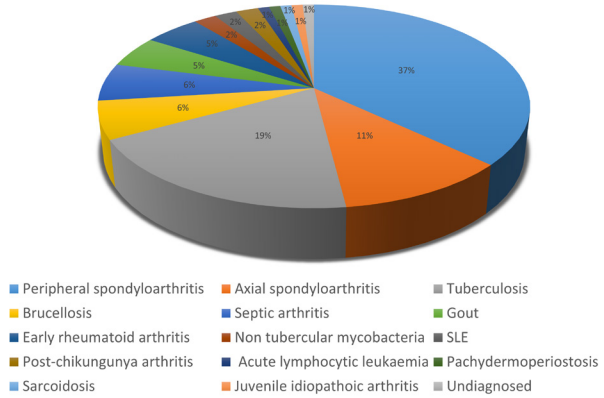

Figure 2. Pie diagram showing the final diagnosis in our patients with oligoarthritis (n = 100).

Table 1. Features of joint involvement in the commonest causes of oligoarthritis in our study

Characteristics	Peripheral spondyloarthritis (37%)	Axial spondyloarthritis (11%)	Tubercular arthritis (19%)	Brucella arthritis (6%)	Septic arthritis (6%)
Number of joints					
1	7	2	14	2	4
> 1	30	9	5	4	2
Most common joint	Knee	Knee	Knee	Knee	Hip
Duration (Acute: Chronic)	13:24	0: 11	2: 17	0: 6	3:3
Axial skeleton involvement	0	11	4	2	2
Erosive arthritis	3	3	10	1	2

Table 2. Comparison of the demographic, clinical, laboratorical and radiological findings in patients with infection and non-infectious causes of oligoarthritis

Characteristics	Infectious (n = 33)	Non-infectious (n = 60)	p-value
Age in years (mean)	29	30.6	0.59
Fever	22 (66.7%)	33 (55%)	0.27
Weight loss	22 (66.7%)	9 (15%)	< 0.001
Lymphadenopathy	7 (21.2%)	5 (8.3%)	0.08
Skin Rash	3 (9.1%)	10 (16.7%)	0.31
Hepatomegaly	7 (21.2%)	4 (6.7%)	0.04
Splenomegaly	8 (24.2%)	4 (6.7%)	0.01
Number of joints			
1	21 (63.6%)	12 (20%)	< 0.001
>1	12 (36.4%)	48 (80%)	
Axial skeleton involvement	7 (21.2%)	11 (18.3%)	0.74
Erosive arthritis	15 (45.4%)	8 (13.3%)	0.01
Erythrocyte sedimentation rate (median)	44	38	0.47
C- reactive protein (median)	59	18.9	0.69

4. Discussion

Axial and peripheral spondyloarthritis was the most common diagnosis in our patients. Similar to previous studies, our study also showed a male dominance in patients with axial and peripheral spondyloarthritis (*8-12*). Lower limb asymmetric oligoarthritis was the most common pattern observed in the above studies, with knee being the commonest joint involved. In one study of axial spondyloarthritis, 65.7% of the patients also had concurrent peripheral joint involvement (*9*). Prodromal diarrhea or urinary tract infection was present in 7 patients with axial and peripheral spondyloarthritis. PCR assay for *Chlamydia trachomatis* was positive in the urine sample in 3 patients. This subset of patients could be classified as reactive arthritis, which usually results from inflammatory response to the microbial invasion of the joints. In a study from India, Chlamydia PCR was positive in the synovial fluid in 23.6% of the patients with reactive arthritis and undifferentiated spondyloarthropathies (*13*). In cases of post-Chlamydial reactive arthritis, treatment with anti-Chlamydial agents have shown no benefits in most studies except for a small randomized trial where prolonged course of combination antibiotics (doxycycline or azithromycin and rifampicin) was associated with better outcome (*14*). We treated all patients who showed urine PCR positivity with anti-inflammatory agents and a single dose of azithromycin.

All the five patients who were diagnosed with gouty arthritis were males and the mean age was 50.6 ± 5.9 years. The most common joint involved was ankle, followed by the metatarsophalangeal joints. Previous studies also have shown a male preponderance with metatarsophalangeal joints, knee and ankle being the most common sites of involvement (*15*). Tubercular arthritis is among the commonest causes of infectious arthritis in endemic areas. It was observed to be the third most common cause of extra-pulmonary tuberculosis in one series (*16*). In a retrospective study of 99 patients who had osteoarticular tuberculosis, arthritis and osteomyelitis was seen in 28% and 10% respectively (*17*). As with many of the previous studies, monoarthritis was the commonest presentation (74%) (*18*). Brucella arthritis was the other major cause of infectious arthritis in our study. Considering the high prevalence of exposure to cattle and unpasteurized milk in the Indian population (17% in our study), this finding is not surprising. Reports of brucellosis have been on a steady rise in India over the past few years. In a review of 792 cases of brucellosis from India, joint involvement was seen in 23.1% (*19*). Hip and knee were the commonest joints involved (*20*). In comparison, in our study, knee was the most common joint involved. Septic arthritis was another important cause of infectious arthritis in our study. Previous studies have shown monoarthritis to be the commonest presentation of septic arthritis, except in 10-20% who may have polyarticular involvement (*21*). 67% of our patients with septic arthritis had monoarthritis. Similar to the previous studies, hip and knee were the commonest joints involved (*21*).

Oligoarthritis from both infectious and non-infectious causes are associated with significant morbidity. However, the infectious arthritis can be treated effectively with anti-microbials. Prompt identification and treatment of infections may prevent further joint damage and reduce future disability and morbidity. Due to similarities in the clinical presentation of infectious and non-infectious causes, it is pertinent to identify the factors which could help in the early identification of infectious arthritis. We found that those patients with clinically-significant weight loss, hepatosplenomegaly, erosive arthritis and monoarthritis were more likely to have an infectious cause for their arthritis than non-infectious causes.

Our study had some limitations. The sample size was too small to draw definite conclusions. However, this study points towards a potential area for future research having significant translational

value and clinical impact. Larger studies would be needed to validate our findings. We also had to exclude patients in whom the cause for the arthritis could not be definitively established as infectious or non-infectious. These included patients with known HIV and HCV infection who were newly diagnosed with spondyloarthropathies since it could not be definitely disproven whether these infections were directly responsible for the joint manifestations or not. Similarly, patients with post-chikungunya arthritis were also excluded as several studies have previously shown that the joint manifestations in chikungunya were secondary to inflammatory response towards the virus from the synovial macrophages.

In conclusion, the presence of clinically-significant weight loss, hepatosplenomegaly, mono-arthritis and erosive arthritis could potentially direct the primary care physician to suspect an infectious cause of arthritis as against a non-infectious cause and start appropriate anti-microbial agents promptly so as to obtain better clinical outcome and limit or prevent future morbidity.

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Original Article

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Clinical spectrum and outcome of critically ill hospitalized patients with acute febrile illness and new-onset organ dysfunction presenting during monsoon season

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Summary **Acute febrile illness (AFI) is one of the commonest indications for hospitalization and can present with varying severity including single or multiple organ dysfunction syndrome (MODS). During monsoon season, there is a spurt of AFI often caused by vector borne diseases leading to substantial morbidity and mortality. Our aim was to determine distribution of etiological causes, differential organ involvement and predictors of mortality in critically ill patients with AFI. It was a hospital based observational study which included patients with AFI with dysfunction of at least one organ system. The study was conducted over 4 months during monsoon season. Admitted patients were included who had been subjected to a standard battery of tests and managed with standard hospital based management protocol. 145 patients were included and etiology of fever was ascertained in 81.4% of patients with the most common single infection being chikungunya (20.7%) followed by dengue (20%) fever. Thrombocytopenia and deranged liver biochemistry each were seen in nearly 75% of the patients. Renal (50.3%) and nervous system (46.2%) dysfunction were the predominant organ failures. 49 patients died (33.8%) which correlated with predicted mortality by APACHE (acute physiological assessment and chronic health evaluation) II score. Independent predictors for mortality were older age (> 55 years) (p = 0.01), acidemia (p = 0.01), altered sensorium (p = 0.02) and coagulopathy (p = 0.048). Sub-group analysis revealed that amongst patients with MODS, hypotension could help differentiate between bacterial and non-bacterial causes (p = 0.01). Critically ill patients with AFI suffer from significant morbidity and mortality. Features like the presence of hypotension in MODS may differentiate between a bacterial cause vis-à-vis viral or protozoal etiology.**

Keywords: Acute febrile illness, MODS, chikungunya, hypotension

1. Introduction

Fever is one of the most common indications for seeking medical attention. The severity of an acute febrile illness (AFI) may vary from a simple self-limiting viral infection to a life threatening multi-organ dysfunction syndrome. The causes of AFI are diverse and frequently the associated symptoms are non-specific. In a resource-limited setting or at the time of an outbreak or epidemic, a severe shortage of local data and epidemiology is often perceived. Investigations need to be rationalized and often, empirical therapy has to be initiated based on clinical findings and assumptions.

The causes of acute febrile illness have been studied in different backdrops. In 2013, a systematic review revealed that only 17.9% cases had been diagnosed with a specific cause. Blood stream infection was the most common diagnosis with bacterial and fungal cultures being positive in 10.3% of patients (*1*). The above mentioned review included two studies from India, the largest of which was from South India – which may not be representative of the entire country because of its unique landscape and characteristic endemicity of pathogens (*2*). Other studies on acute febrile illness have been done where only about 40% cases could

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be given a definitive diagnosis (*3,4*). A study from Dehradun, India showed that dengue followed by enteric fever was the most common etiology (*5*). There are some studies which have focused on individual disease and their pattern but limited data is available about the epidemiology of acute febrile illnesses during monsoon season in the northern parts of India. The mortality of patients with acute febrile illness when complicated by organ dysfunction can be as high as 20% (*6-8*). Febrile illness severe enough to cause dysfunction of an organ or multiple organs leads to significant morbidity and mortality in tropical countries (*8*). Most studies on AFI from India have focused only on etiology with little information on the other important aspects like predictors of poor prognosis and mortality. In 2016, we initiated a hospital based prospective observational study of severe acute febrile illness with organ dysfunction with focus on these hitherto unexplored aspects. We describe the demographic, clinical, and laboratory features, etiology, predictors of mortality, pattern of organ involvement in various diseases as also certain pointers helpful in making a provisional syndromic diagnosis.

2. Materials and Methods

2.1. *Study design*

The study was conducted in a tertiary care centre catering to the national capital as well as the adjoining National Capital region (NCR). It is an apex tertiary care centre which serves as a referral centre for a large part of Northern India. Geographically New Delhi is an inland and surrounded by land on all sides and the population is largely urban.

This is a prospective hospital-based observational study conducted over 4 months (from July 2016 to October 2016 which covered the entire monsoon season). Ethics clearance was obtained from the Institute Ethics Committee prior to start of the study. All patients with history of fever were screened and were included in the study after applying the inclusion and exclusion criteria. We included only admitted patients who had presented with fever of ≤ 7 days at time of presentation, presence of at least one organ dysfunction (according to criteria mentioned below) and age > 13 years. Patients with history of hospitalization in last 3 months or refusal to give written informed consent were excluded from the study.

Organ dysfunction was defined as below (any of the following): 1) Acute kidney injury (AKI): according to acute kidney injury network (AKIN) definition (absolute increase in creatinine ≥ 0.3 mg/dL over 24 hours or a 50% increase in creatinine from baseline or urine output ≤ 0.5 mL/kg/hour for at least 6 hours) (*9*). 2) Acute respiratory distress syndrome (ARDS): as per the Berlin definition, PaO_2/FiO_2 ratio of ≤ 300 with a CPAP of 5 cm water (*10*). 3) Central nervous system: as per Glasgow coma scale when score is less than 15. 4) Coagulopathy: prothrombin time (PT) more than 16 seconds or INR > 1.5. 5) Liver: a serum bilirubin of > 2 mg/dL or patients having encephalopathy with deranged INR. 6) Thrombocytopenia: platelets < 150,000/cu. mm with abnormal bleeding not explained by other causes. 7) Cardiovascular: systolic blood pressure < 90 mm Hg or a drop in SBP > 40 mm Hg from baseline or mean arterial pressure < 65 mm Hg.

After inclusion into the study, demographic and clinical details were recorded at admission in a pre-designed pro forma. The patients were followed up till discharge or death; details of stay in hospital as well as ICU stay (if present) were recorded along with results of laboratory investigations.

2.2. *Laboratory evaluation*

All patients underwent hematological investigations including complete blood count (CBC), liver function tests (LFT), kidney function tests (KFT), arterial/venous blood gas analysis (as required), prothrombin time/INR and blood culture. A urine routine microscopy, urine culture and chest-X ray were done for all patients. Basic work-up for etiology included rapid test (a point of care test based on lactate dehydrogenase and histidine rich protein 2) and peripheral smear for malaria, NS-1 antigen (Panbio Dengue Early ELISA, Standard diagnostics Inc., Republic of Korea) and IgM antibody detection for dengue virus (NIV DEN Immunoglobulin (IgM) Capture ELISA, National Institute of Virology, Pune, India) and IgM antibody against chikungunya virus (NIV Chikungunya IgM ELISA, National Institute of Virology, Pune, India). For dengue, NS-1 antigen was tested if the patient presented in the first 5 days of illness and the IgM test if later than that. When clinically suspected, tests were done for Leptospira (Panbio Leptospira IgM ELISA, Standard Diagnostics, Republic of Korea) and scrub typhus (InBios International scrub typhus IgM indirect ELISA, Seattle, WA). Other investigations were done as required on a case to case basis. The study team collected data independent from the clinical team in-charge and no changes were made to the management of the patient by the study group though relevant investigations reports were timely conveyed to the treating team. The patients with multi organ dysfunction i.e. two or more organ failures, were further divided into sub-groups for analysis. Those with dengue fever, chikungunya, malaria, scrub typhus and confirmed viral illness were put in the sub group of "viral and viral like illness". Those with confirmed or likely bacterial infection and enteric fever were included in "bacterial and presumed bacterial". "Likely bacterial infection" was kept as the diagnosis in patients who had a raised total leukocyte count with polymorphonuclear leukocyte predominance

and any of the following: an area of consolidation on chest x ray or urine routine microscopy showing more than 10 WBC/high power field (hpf) (only valid for newly catheterized or uncatheterized patients) or meningitis with cerebrospinal fluid showing low sugar, high protein with neutrophils is CSF. "Confirmed bacterial infection" was diagnosed when culture grew pathogenic bacteria species.

2.3. *Statistical analysis*

Data was recorded in a predesigned pro forma and subsequently on an excel spreadsheet. Categorical variables were summarized as frequency (percentage) and analyzed using χ^2 (Chi squared) or Fischer's exact test. Continuous variables were summarized as mean and standard deviation (SD) or median and range (when SD was > 50% of mean) and analyzed using parametric tests (*t*-test or one way analysis of variance). When analyzing for predictors of mortality, those with *p* value of < 0.10 in univariable analysis were included for multivariable analysis. A *p*-value of < 0.05 was considered as significant. Statistical analysis was performed using the Stata 12 software (StataCorp [2011], College station, TX).

Table 1. Demographic profile, co morbidities and clinical spectrum of patients with acute febrile illness (*n* = 145)*

Parameter	Overall (n = 145)
Gender	
Males	92 (63.4%)
Females	53 (36.6%)
Age (years)	30 (24.75)
Co morbidities	
Diabetes	21 (14.5%)
Hypertension	27 (18.6%)
Chronic kidney disease	8 (5.5%)
Coronary artery disease	6 (4.2%)
Cerebrovascular disease	7 (4.8%)
Chronic liver disease	6 (4.1%)
Clinical features	
Body ache	65 (44.8%)
Altered sensorium	62 (42.7%)
SOB	52 (35.9%)
Joint pain	48 (33.1%)
Bleeding	41 (28.3%)
Decreased urine output	35 (24.1%)
Cough	24 (16.5%)
Jaundice	15 (10.3%)
Seizure	19 (13.2%)
Tachycardia	89 (61.3%)
Tachypnea	73 (50.3%)
Hypotension	48 (33.0%)
Hepatomegaly	26 (17.9%)
Splenomegaly	20 (13.8%)
Rash	27 (18.6%)
Organ failures	
Renal	73 (50.3%)
Altered sensorium	67 (46.2%)
Pulmonary	49 (33.8%)
Liver	48 (33.1%)
Cardiovascular	42 (29%)
Thrombocytopenia with bleeding	38 (26.2%)
Coagulopathy	33 (22.7%)
MODS	93 (64.1%)
Hospital stay (median)(Days)	6 (6)
ICU stay	29 (20%)
Mortality	49 (33.7%)

*Categorical variables are summarized as *n* (%). Continuous variables are represented as mean ± SD or median (Interquartile range) if SD > 50% of mean.

3. Results

A total of 145 patients were included in the study. They were all followed up for the entire duration of the hospital stay. The median age of participants was 30 years and 63.4% were male. The mean duration of illness at time of presentation was 5.1 ± 2.3 days. Median duration of hospital stay was 6 days. Etiology for fever could be ascertained in 118 patients (81.4%) while 27 remained undifferentiated. Most common specific diagnosis was found to be chikungunya in 30 patients (20.7%) closely followed by dengue (*n* = 29) (20%). Of the group comprising bacterial infection (*n* = 30), 12 (40%) had pneumonia related sepsis, 8 (26.7%) had urosepsis, 4 (13.3%) had tuberculosis, 3 (10%) had leptospirosis, 2 (6.7%) had enteric fever and 1 (3.3%) patient had pyogenic meningitis. Ten patients (6.9%) had a mixed infection with most common co-infection being chikungunya and dengue fever (*n* = 5).

There was significant variation in symptomatology of patients (Table 1). Myalgia was the most common symptom followed by altered mentation. Tachycardia and tachypnea were the most frequently elicited signs. Hypotension requiring inotropic support was seen in around one third of the patients. Baseline laboratory parameters of the participants are listed in the Table 2. Thrombocytopenia was the most common lab finding followed by elevated liver enzymes both of which were seen in three quarters of the patients.

The incidence of different organ system dysfunction in the participants is shown in Table 3. Few diseases

Table 2. Laboratory parameters of patients with acute febrile illness (*n* = 145)*

Laboratory Parameters	Overall (*n* = 145)
Hemoglobin(g/dL)	11.0 ± 2.7
Anemia	86 (59.3%)
TLC (/mm^3)	9,100 (11,600)
Leucopenia (< 4,000/mm^3)	24 (16.5%)
Leucocytosis (>11,000/mm^3)	59 (40.6%)
Platelets(/mm^3)	53,000 (105,250)
Thrombocytopenia	114 (78.6%)
Urea(mg/dL)	56.5 (70.5)
Creatinine(mg/dL)	1.2 (2.5)
Bilirubin(mg/dL)	0.9 (2.6)
AST(IU/L)	114 (155.5)
ALT(IU/L)	80 (152.5)
Elevated transaminases	104 (71.7%)

*Categorical variables are summarized as *n* (%). Continuous variables are represented as mean ± SD or median (Interquartile range) if SD > 50% of mean.

Table 3. Pattern of organ dysfunction with different diagnoses

Organ Dysfunction	Total *n* (%)	Liver (%)	Lung (ARDS) (%)	Renal (AKI) (%)	Cardiovascular (Hypotension) (%)	Central nervous system (%)	Thrombocytopenia with Bleeding (%)
Chikungunya	23 (15.9)	26.1	17.3	39.1	34.8	34.7	30.4
Dengue	21 (14.5)	38.1	0	28.6	14.3	14.3	52.4
Malaria	21 (14.5)	52.4	23.8	38.1	28.6	28.6	28.6
Scrub typhus	10 (6.9)	40	40	60	20	20	0
Leptospira	3 (2.1)	33.3	33.3	100	100	100	33.3
Other bacterial	25 (17.2)	32	44	80	48	72	28
Other viral infections	5 (3.5)	40	60	40	20	100	0
Undifferentiated	27 (18.6)	25.6	48.1	62.3	44.4	25.6	3.7
Co infection Chikungunya & Dengue	5 (3.4)	60	40	20	0	40	60
Other co infections*	5 (3.4)	20	0	20	20	40	40
Total *n* (%)	145 (100)	48 (33)	49 (33.8)	73 (50.3)	42 (29)	67 (46.2)	38 (26.2)

*Other co infections include co infection of dengue with scrub typhus, enteric fever and malaria in one case each; and co infection of chikungunya with Japanese encephalitis, enteric fever in one case each.

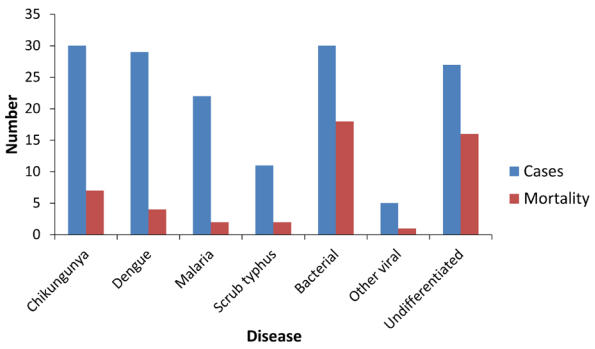

Figure 1. Proportional prevalence of disease and associated mortality.

had predilection for particular systems. Renal dysfunction was more common in leptospirosis, confirmed bacterial infection and scrub typhus as compared to the others. ARDS was most common in the group which remained undifferentiated, followed by bacterial infections and scrub typhus. Hypotension was common in leptospirosis, bacterial and undifferentiated groups. Altered sensorium and acute kidney injury were the most common organ dysfunction seen and occurred in around half of the patients. Dengue, leptospirosis, malaria and chikungunya had a predilection for thrombocytopenia and bleeding. These pointers are not exclusive but may provide a clue to diagnosis in the setting of monsoon season in a tropical country when all these ailments are at their peak incidence. Individual diseases have different patterns of organ dysfunction and this has been depicted in Table 3. Mean APACHE (acute physiological assessment and chronic health evaluation) II score for participants at admission was 21.5 and SOFA (sequential organ failure assessment) score at day 0 was 7.4.

The overall mortality rate was 33.8% (n = 49). This was close to the adjusted death rate (39%) calculated as per the mean APACHE score (21.5) at the time of presentation. Disease wise mortality is depicted in Figure 1. Predictors of mortality were identified by stepwise logistic regression. Previously known diabetes, higher age (> 55 years), smoking, tachycardia, tachypnea, abnormal Glasgow coma score (GCS) (< 15), lower spO_2/FiO_2 ratio (< 315), leucocytosis (TLC > 11,000/cu. mm), acidosis on ABG (pH < 7.35), urea > 40 mg/dL, serum creatinine > 1.2 mg/dL, coagulopathy (INR > 1.5) were identified as significant predictors of mortality while diagnosis of dengue and malaria were associated with relatively lower mortality on univariate analysis. Amongst these, higher age, abnormal GCS, acidosis and coagulopathy were found to be independent predictors (Table 4). Lower SpO_2/FiO_2 ratio as an independent predictor of mortality showed a trend towards statistical significance. APACHE II and SOFA score were also good predictors of mortality. APACHE II score in the range of 10 to 35 was having an odds ratio (OR) of 20 while > 35 had an odds ratio of 117.3 and a p value of < 0.01. SOFA score of 5-10 had an OR of 7.25 and SOFA > 11 had an OR of 49.3 with an overall p value < 0.01.

The sub-groups of patients with "bacterial, presumed bacterial and likely bacterial" was compared with group of patients with "viral or viral like" for possible clinical predictors which could differentiate the patients at the time of presentation. Patients with multi-organ dysfunction (MODS) were selected and compared. It was found that if a patient was having MODS with hypotension, then the diagnosis was more likely to be bacterial than viral or viral like. This difference was statistically significant (p = 0.01) (Table 5).

4. Discussion

Every monsoon season there is a spate of vector borne diseases in India. Many of the patients are critically ill and require hospital admission with significant morbidity and mortality. While some diseases like malaria have point-of-care tests, similar tests for others

Table 4. Predictors of mortality, Univariable and Multivariable Logistic regression analysis

Variable	Outcome		Unadjusted OR (95% CI); *p* value	Adjusted OR (95% CI); *p* value
	Alive (*n* = 96), *n* (%)	Dead (*n* = 49), *n* (%)		
Age > 55	12 (12.5)	14 (28.6)	4 (1.4-11.3); < 0.01	8.1 (1.6-40.5); 0.01
Diabetes	10 (10.4)	11 (22.4)	2.49 (0.9-6.35); 0.06	
Smoking	5 (5.2)	10 (20.4)	4.6 (1.5-14.3); 0.01	
Tachycardia	52 (54.2)	37 (75.5)	2.6 (1.2-5.6); 0.01	
Tachypnea	40 (41.7)	33 (67.3)	2.9 (1.4-5.9); < 0.01	
Abnormal GCS	32 (33.3)	35 (71.3)	5 (2.3-10.6); < 0.01	3.1 (1.2-4.4); 0.02
spO_2/FiO_2 < 315	20 (20.8)	29 (59.2)	5.5 (2.6-11.7); < 0.01	2.94 (0.9-9.2); 0.06
Leucocytosis	28 (29.2)	31 (63.2)	2.8 (1.4-5.7); < 0.01	
Acidosis (pH < 7.35)	18 (18.8)	31 (63.2)	5.7 (2.4-13.3); < 0.01	4.3 (1.3-14.1); 0.01
Urea (> 40 mg/dL)	47 (49)	38 (77.5)	4.6 (1.9-10.8); < 0.01	
Creatinine (> 1.2 mg/dL)	32 (33.3)	35 (71.4)	5 (2.4-10.6); < 0.01	
INR (> 1.5)	30 (31.2)	25 (51)	2.4 (1.1-4.6); 0.02	3.7 (1.0-13.4); 0.048
Dengue	25 (26)	4 (8.2)	0.2 (0.1-0.7); 0.02	
Malaria	22 (22.9)	2 (4.1)	0.1 (0.03-0.64); 0.01	

Table 5. Association of hypotension with multi-organ dysfunction (MODS) with diagnosis

Number of patients	MODS with hypotension	MODS without hypotension	
Bacterial and presumed bacterial	11	4	*p* = 0.01
Viral and viral like illness	18	33	Odds ratio – 4.89, (1.35-17.61)

like scrub typhus are not readily available. Also, in resource limited settings the availability of these tests may be limited. In such a backdrop it is important to suspect these diseases and to timely treat them with appropriate agents.

In our study population, the most common diagnosis was chikungunya followed by presumed bacterial infection. The combination of non-bacterial diseases far outnumbered the bacterial cases. It is known that vector borne diseases like dengue peak during the monsoon season (*11*). The incidence of Chikungunya had increased in recent times and numerous outbreaks have been declared in the recent past in various parts of India (*12-14*). One study had described the etiologies of acute febrile illnesses in northern India during the monsoon season of 2013 and had found dengue to be the commonest cause of fever (*15*). It was a retrospective study designed to include cases positive for dengue, malaria, typhoid and scrub typhus. However, the clinical presentations and organ involvement were not recorded by the authors. A paper from south India had reported on the etiology of fever and the associated case fatality admitted in a period of one month in 2007 (*16*). In the hundred patients studied, 66% had fever of more than 7 days. In such a group of patients, tuberculosis (19%) was reported as the most common cause and the incidence of malaria, scrub typhus and typhoid were 5%, 5%, and 4%, respectively. The deaths observed in the above group were 7%, with bacterial causes responsible for the majority. However, in our study all the patients had acute febrile illnesses with duration of fever ≤ 7 days presenting during the monsoon season (July to October).

In our study, amongst organ involvement neurological and renal involvements were the most common manifestations. Amongst the organ dysfunction no statistically significant predilection was noted for any specific etiology. However acute kidney injury, bleeding and hepatic dysfunction were most common in chikungunya, dengue and malaria respectively.

The most important predictors of mortality were higher age, low GCS, acidemia and coagulopathy. Age and low GCS are a part of simplified acute physiology score II (SAPS II), while coagulopathy is a part of sequential organ failure score (SOFA) in intensive care unit patients (*15,17*). These findings reiterate the importance of these parameters in critically ill patients with acute febrile illness. Amongst the patients with chikungunya, there were 7 deaths. Six (86%) of them had another associated co morbid illness and 5 (71%) were above the age of 55 years.

One of the most important highlights of this study was that in the presence of MODS, hypotension suggested the diagnosis of bacterial cause vis-à-vis non-bacterial cause. This is important as it suggests that MODS without the presence of shock is more common in patients without bacterial etiology. The exact cause of this can be hypothesized in the following way. Bacterial sepsis from a focal infection occurs when the release of proinflammatory mediators breaches the boundaries of the local environment leading to malignant intravascular inflammation (*18*). The reason why localized responses sometimes spread beyond the local boundaries causing sepsis is likely to be multifactorial and may include characteristics of invading microbes, massive release of cytokines,

complement activation or genetic susceptibility of the individuals (*19*). The overflowing cytokines reach distant organs causing cellular injury and resultant multiple organ dysfunction. On the other hand, for non-bacterial infections, the cellular injury is thought to be brought about by the agency of tissue ischaemia, cytopathic injury and apoptosis (*20-22*). Malaria might have a different pathophysiological basis than cytokine overflow for the development of multiple organ dysfunction. Brain dysfunction in malaria occurs due to parasitized erythrocytes in the cerebral vasculature (*23*). In malaria renal dysfunction may result from immune-complex deposition and infected RBC adhesion to renal vasculature (*24*). Rickettsial infections may not lead to exotoxin production but bring about damage to small blood vessels (*25*). Rickettsial organisms may induce rearrangement of cellular actin and engulfment by the cell *via* endocytosis. There is subsequent passage of the organism into neighbouring cells *via* filopodia resulting in spread throughout the body *via* bloodstream or lymphatics (*26*). In non-bacterial infections, organ dysfunction may occur without concomitant shock due to possibly different pathophysiological characteristics as outlined above unlike cytokine overflow in bacterial infections which leads to circulatory shock along with organ dysfunction. Though, cytokine release and amplification may very well be a part of non-bacterial as well as bacterial infection, however the difference in composition and quantum of the same as well as their effects on the host have not been studied. The results from such studies may further throw light on the pathophysiology of organ dysfunctions in the settings discussed above.

This is the first study from India where critically ill patients with acute febrile illness during the monsoon season have been studied extensively with details about the clinical profile, organ dysfunction, morbidity and mortality. This study also highlights the importance of presence/absence of hypotension in such group of patients with multi-organ dysfunction in suggesting a probable cause. A limitation of the study was the small sample size. Also the differentiation between bacterial and non-bacterial causes was at times empirical in the absence of culture positivity for bacteria.

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Original Article

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Impact of non-selective beta blockers on portal hypertension and hepatic elasticity in hepatitis C virus-related liver cirrhosis

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Summary **Portal hypertension and its complications are the leading causes of morbidity and mortality in patients with liver cirrhosis. Noninvasive assessment of liver stiffness had been an effective tool for assessment of fibrosis progression in chronic liver disease. It was intended to assess liver stiffness measurement (LSM), portal vein diameter (PVD), splenic bipolar diameter (SD), and the platelet count/spleen diameter (PC/SD) ratio in patients who test positive for the hepatitis C virus (HCV) and to study the impact of non-selective beta blockers (NSBB) on the grade of esophageal varices (EVs) and liver elasticity. Subjects were 80 patients with Child-Pugh grade A or B compensated cirrhosis who tested positive for HCV. All of the patients underwent a laboratory workup including AFP, HCV antibodies, HCV RNA, HBsAg, LSM according to real-time elastography, upper gastrointestinal endoscopy (UGIE) to detect and grade EVs, calculation of the PC/SD ratio, and measurement of the PVD and SD according to real-time abdominal ultrasonography. All patients were given the maximum tolerated dose of NSBB for three months, and UGIE, LSM, PC/SD, PVD, and SD were subsequently reassessed and reported. LSM and the PC/SD ratio were exceptional noninvasive tools for prediction of significant EVs (grade ≥ II, $p < 0.001$) with a sensitivity 82.4% and a specificity 82.6% at a cutoff point 18 kPa for LSM, and a sensitivity 94.1% and specificity 69.6% at a cutoff point 880 for the PC/SD ratio. LSM is highly correlated with PVD, the PC/SD ratio, SD, and the Child-Pugh score. NSBB significantly decreased PVD. The percent change in PVD significantly correlated with LSM, the grade of EVs, and SD. Findings indicated that LSM is a noninvasive, rapid, and reproducible tool with which to detect portal hypertension and EVs. NSBB therapy can effectively decrease PVD and may consequently improve the EV grade with no significant impact on LSM in patients with liver cirrhosis.**

Keywords: Liver stiffness measurement, portal hypertension, esophageal varices, non-selective beta blockers

1. Introduction

Portal hypertension is believed to be the main trigger for most complications in patients with liver cirrhosis. A hepatic venous pressure gradient (HVPG) ≥ 10 mm Hg is necessary for the development of ascites, esophageal varices (EVs), and all other complications of liver cirrhosis (*1*). Clinically significant portal hypertension (CSPH) is invariably found in patients with decompensated liver disease (*2*); its presence is an independent predictor of clinical decompensation in 50-70% of patients (*3*). The presence of EVs, as a complication of CSPH, is an independent predictor of significant morbidity, so all patients with compensated cirrhosis should be screened for the presence of EVs (*4*).

Patients with liver cirrhosis must sometimes undergo invasive procedures to diagnose EVs and CSPH, such as liver biopsy and hepatic vein catheterization. These procedures require specific experience and carry some

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risk, so simple, noninvasive, accurate, and objective diagnostic tools need to be developed for high-risk patients. A reproducible estimation of liver stiffness (LS) according to transient hepatic elastography has been developed as a noninvasive method to diagnose CSPH and EVs in patients with compensated cirrhosis (*5*).

Non-selective beta-blockers (NSBB) have been used since 1981 as a therapeutic option for portal hypertension in patients with liver cirrhosis. Patients with refractory ascites experience a diminished sensitivity to the NSBB due to increased levels of splanchnic pro-inflammatory cytokines; the beneficial effects of NSBBs may decrease, and NSBBs may even be harmful (*6*).

One aim of the current study was to assess hepatic elasticity, portal vein diameter (PVD), the platelet count/spleen diameter (PC/SD) ratio, and spleen bipolar diameter (SD) in patients with compensated liver cirrhosis who were also infected with HCV. A second aim of this study was to examine the impact of NSBB on the grade of EVs and liver stiffness measurement (LSM).

2. Patients and Methods

2.1. *Study design and settings*

A case control, prospective observational study was conducted in the Hepatogastroenterology and Endoscopy units of the Department of Internal Medicine in cooperation with the Advanced Center for Liver Disease of Zagazig University Hospital, Egypt over a six-month period from November 2017 to April 2018.

2.2. *Subject population*

Potential subjects were 300 patients with liver cirrhosis and who were infected with HCV who were seen at an outpatient clinic and who were scheduled for diagnostic upper GI endoscopy. Subjects were 80 patients with compensated cirrhosis who met the inclusion criteria while not meeting the exclusion criteria (Figure 1).

2.3. *Inclusion and exclusion criteria*

Patients had Child-Pugh A or B grade compensated liver cirrhosis and tested positive for HCV infection. Patients infected with the hepatitis B virus or who had hepatocellular carcinoma, portal vein thrombosis, who had undergone sclerotherapy or band ligation to treat EVs, patients who had previously received or who were ineligible to receive NSBBs (obstructive airway disease, peripheral arterial disease, or brittle diabetes), patients who had Child-Pugh grade C cirrhosis, patients

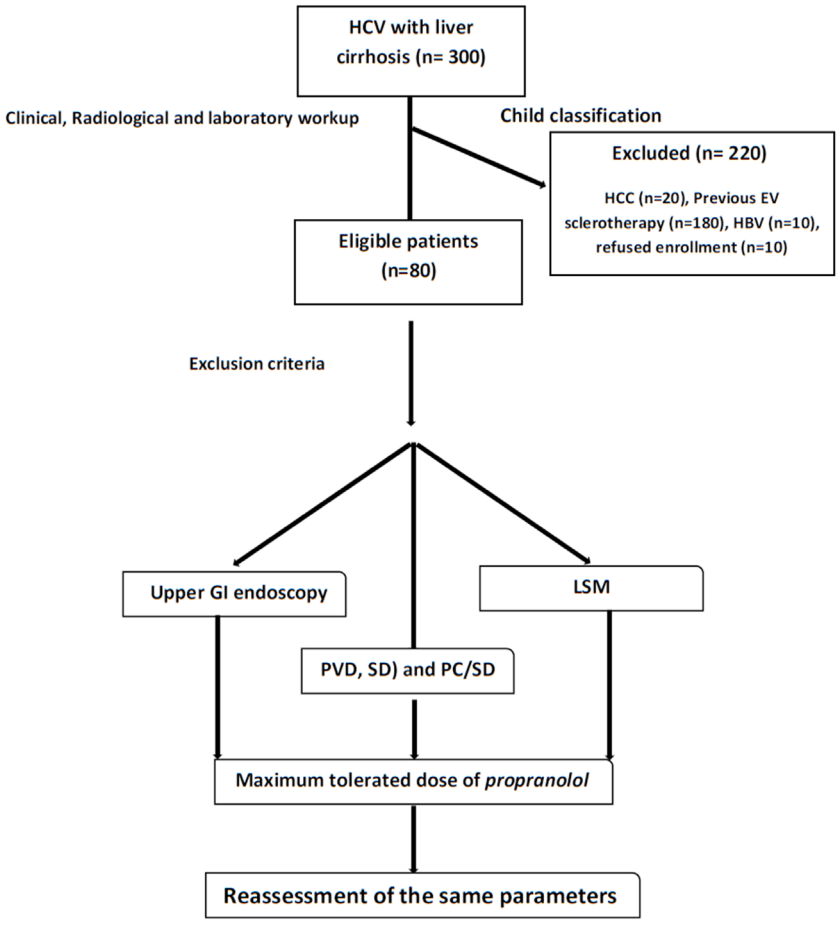

Figure 1. Flowchart for patients in this study.

who did not have EVs, and patients who missed follow-up or who declined to participate in this study were excluded. All of the enrolled patients gave informed consent *via* a form developed by the research team. This study was approved by the ethical committee of Zagazig University. All information gathered from patients was kept confidential.

2.4. *Methods*

All patients had their history taken and underwent a physical examination and laboratory testing that included a complete blood count (CBC), platelet count, liver and kidney function tests, the international normalized ratio (INR), measurement of alpha fetoprotein (AFP), and serum markers for HCV and HBV. PVD and SD had been assessed using trans-abdominal ultrasound (Famio 5 ultrasound Machine, Abex Medical System, Toshiba, Japan). Variations in PVD during respiratory phases were addressed by measuring PVD during inspiration, expiration, and at rest. The normal PVD was less than 13 mm and SD was less than 130 mm. The platelet count (PC) was divided by SD to yield the PC/SD ratio. Upper gastrointestinal endoscopy (UGIE) served as a standard diagnostic modality for EVs. All patients underwent UGIE using GIF- XP160 video endoscopy (Exera 160 series, Olympus Endoscopy System, Japan). EVs were graded into the four grades of I, II, III, and IV according to the modified Paquet classification.

Estimation of liver stiffness using real-time elastography was accomplished by measuring the velocity of elastic shear waves in the liver parenchyma generated by the mechanical push (using a Philips IU22 ultrasound machine). The medium reading of the tissue elasticity was calculated and expressed in kPa. The success rate of the examination was calculated as the ratio of the number of validated measurements made by the machine and the total number of attempted measurements during the same examination. The median value for validated measurements was used to represent liver stiffness (*7*).

All patients had been scheduled to receive the maximum tolerated dose of propranolol that decreases the basal heart rate by 25% but not below 60 beats per minute (*5*). The same patients were re-evaluated after 3 months by measuring UGIE, LSM, PVD, SD, and the PC/SD ratio again.

2.5. *Data processing and analysis*

All calculations were performed using the computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA version 18.0). Data were statistically described in terms of mean ± SD, median and range, or frequencies and percentages when available. The Mann Whitney (U) and Kruskal Wallis tests were used when appropriate. Sensitivity and specificity were used to represent the accuracy of the tests. Receiver operator characteristic (ROC) analysis was used to determine the optimum cutoff value for the diagnostic variables studied. To determine the significant independent predictors for the occurrence and the grade of significant EVs, univariate and multivariate regression models were constructed. *P* values < 0.05 was considered statistically significant.

3. Results

The mean daily dose of propranolol used in the study was 66.95 ± 17 mg (range: 30-80 mg), which caused a significant decrease in the mean heart rate from 79.05 ± 9.02 (range: 60-95 b/m) pre- treatment to 61.15 ± 4.73 (range: 55-71 b/m) post-treatment ($p < 0.001$).

According to endoscopic evaluation pre-treatment, 46 patients (57%) had grade I EVs while 32 (40%) had grade II and only 2 patients had grade III EVs (2.5%). Post-treatment endoscopic examination revealed that 4 patients had no EVs (5%), 42 patients had grade I EVs (52.5%), 32 had grade II EVs (40%) and only 2 patients had grade III EVs (2.5%).

There were no significant changes between pre-treatment and post-treatment values for SD, LSM, and PC/SD ($p = 0.5$, 0.77, and 0.08), but PVD decreased significantly ($p < 0.001$) (Table 1). The percent change in PVD was correlated with the percent change in EV grade, LSM, and SD ($p = 0.05$, 0.001, and 0.05) (Table 2).

Pre- and post-treatment, the grade of EVs was

Table 1. Mean and median values for variables pre-treatment in the patients studied

Variable	Pre-treatment, ($n = 80$)	Post-treatment, ($n = 80$)	Test of sig.	p
PVD: (mm)			t	< 0.001
Mean ± SD	12.8 ± 1.34	11.44 ± 1.93	5.87	
Range	10 – 15	7 - 15		
Splenic diameter: (cm)			t	0.50
Mean ± SD	14.77 ± 2.17	14.8 ± 2.18	0.84	
Range	7.5 – 18.3	7.5 - 18		
LSM: (kpa)			W	0.77
Median (Range)	18.15 (8 – 44.1)	17.6 (7.8 – 43)	0.29	
PC/SD ratio			W	
Median (Range)	800 (188-4667)	720 (226-4440)	1.47	0.08

significantly correlated with PVD, SD, and LSM and inversely correlated with the PC/SD ratio (Table 3). In the pre-treatment evaluation, LSM at a cutoff value of 18 kPa, a PC/SD ratio of 808, and PVD of 12.5 mm predicted EVs (≥ grade II), while LSM at a cutoff value of 16.8 kPa, a PC/SD ratio of 720, and PVD of 11.5 mm post-treatment predicted EVs ≥ grade II, thus indicating the impact of adding NSBB (Table 4). LSM was significantly correlated with both BMI and age ($p < 0.05$ and < 0.001).

The pre- and post-treatment mean values for PVD (12.8 ± 1.34 *vs.*11.44 ± 1.93 mm) differed significantly ($p < 0.001$). PVD at a cutoff value of 12.5 mm had a sensitivity 82.4% and a specificity of 47.8% at predicting significant EVs. PVD was significantly correlated with age and BMI ($p = 0.02$ and 0.001). SD did not change significantly with NSBB (14.77 ± 2.17 cm *vs*. 14.8 ± 2.18, $p = 0.5$) post-treatment. There was no significant correlation between SD and the NSBB dose ($p = 0.88$). The PC/SD ratio was highly inversely correlated with the grade of EVs, PVD, and SD during pre- and post-treatment assessments ($p < 0.001$). The PC/SD ratio was significantly inversely correlated with age and BMI ($p < 0.001$). LSM was highly correlated with the grade of EVs pre- and post-treatment ($p < 0.001$). LSM at a cutoff value 18 kPa had a sensitivity 82.4% and a specificity of 82.6 % at predicting EVs (≥ grade II). The NSBB dose was not significantly correlated with LSM, SD, or the PC/SD ratio but was significantly correlated with the percent change in PVD ($p < 0.001$).

Table 2. Correlation between the percent change in the portal vein diameter (PVD) and the percent change in the grade of esophageal varices, liver stiffness measurement, and splenic diameter in the patients studied

Variable	% change in PVD	
	r	*P*
% change in EV grade	0.33	< 0.05
% change in LSM	0.63	< 0.001
% change in splenic diameter	0.34	< 0.05

4. Discussion

Portal hypertension and its complications are the leading causes of morbidity and mortality in patients with liver cirrhosis. The most important consequences are those that constitute decompensation of cirrhosis, such as ascites, variceal hemorrhage, and encephalopathy. The median survival of a patient without complications of portal hypertension is longer than 12 years, whereas it is shorter than 2 years for a decompensated patient (*1*).

NSBBs are commonly used to decrease portal

Table 3. Correlation between liver stiffness measurements, portal vein diameter, beta blocker dose, grade of esophageal varices, platelet/spleen ratio, and spleen diameter pre- and post-treatment among the patients studied

Variable	PVD ($n = 80$)		BB dose ($n = 80$)		EV grade ($n = 80$)		LSM ($n = 80$)		SD ($n = 80$)	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Pre-treatment										
BB dose	- 0.33	0.02	---	----	---	----	---	----	---	----
EV grade	0.54	< 0.001	- 0.17		---	-----	---	----	---	----
LSM	0.63	< 0.001	0.04	0.83	0.53	< 0.001	---	----	---	----
SD	0.31	0.04	- 0.05	0.77	0.62	< 0.001	0.55	< 0.001	---	----
PC/SD ratio	- 0.49	< 0.001	- 0.11	0.17	- 0.71	< 0.001	- 0.62	< 0.001	- 0.79	< 0.001
Post-treatment										
BB dose	- 0.02	0.90								
EV grade	0.42	0.007	- 0.19	0.25	----	----				
LSM	0.67	< 0.001	- 0.05	0.75	0.49	< 0.001				
SD	0.25	0.12	- 0.03	0.88	0.28	0.08	0.61	< 0.001	----	----
PC/SD ratio	- 0.51	< 0.001	- 0.19	0.23	- 0.53	< 0.001	- 0.63	< 0.001	- 0.79	< 0.001

Table 4. Validity of cutoff values for liver stiffness measurement, the platelet/spleen ratio, and portal vein diameter in diagnosis of esophageal varices pre- and post- treatment

Test	Sensitivity	Specificity	PPV	NPV	Kappa	*p*
Pre-treatment						
LSM > 18 kPa	82.4	82.6	77.8	86.4	0.64	< 0.001
PC/SD ratio ≤ 880	94.1	69.6	69.6	94.1	0.61	< 0.001
PV > 12.5 mm	82.4	47.8	53.8	78.6	0.28	0.04
Post-treatment						
LSM > 16.8 kPa	90.9	62.5	52.6	93.8	0.45	< 0.003
PC/ SD ratio ≤ 720	90.9	62.5	52.6	93.8	0.45	< 0.003
PV > 11.5mm	72.7	62.5	47.1	83.3	0.31	< 0.05

hypertension in patients with compensated cirrhosis in order to facilitate primary and secondary prevention of first variceal bleeding. Propranolol is the most commonly used NSBB that causes a significant reduction in portal pressure. The current study used propranolol at a dose that achieved a 25% reduction in heart rate. In patients with high-risk varices, an NSBB significantly decrease the incidence of first variceal hemorrhage.

In patients who have already bled from varices, an NSBB prevented the recurrence of variceal hemorrhage when used in combination with endoscopic variceal therapy. Recent data suggested that in patients with compensated cirrhosis (*i.e.*, patients who have CSPH with no or small varices), NSBB provides protection from clinical decompensation (*8*).

In the current study, females tended to have a lower PC/SD ratio and PVD but higher LSM. These findings agreed with the results of Castera *et al*. (*9*) regarding a non-significant difference between genders. In the study by Castera *et al*., SD and PVD were significantly correlated with age, and the PC/SD ratio was significantly inversely correlated with age.

The current study found that age was correlated with LSM, but Castera *et al*. (*9*) reported that the two were not significantly correlated. The current study found that LSM was correlated with BMI, and this finding agrees with the results of Castera *et al*. (*9*) and Das *et al*. (*10*); in the latter study, the mean value for LSM was higher in obese individuals compared to that in individuals with a normal BMI.

Few studies have discussed the impact of NSBBs on LSM, but Razavi *et al*. (*2*) studied the correlation between HVPG and LSM before and after NSBB administration, and they concluded that the two were more strongly and directly correlated post-treatment than pre-treatment.

The correlation between LSM and HVPG improved in hemodynamic responders to NSBBs (R = 0.864) but not in non-responders (R = 0.535), whereas changes in LSM, heart rate, and mean arterial pressure were similar in both groups (*11*).

Pre-treatment values for LSM at a cutoff point of 18 kPa had a sensitivity of 82.4% and a specificity of 82.6% at detecting significant EVs (≥ grade II). Accordingly, LSM is an exceptional test with which to predict the presence of significant EVs. However, other studies used different cutoff values for large EVs. A study by Sporea *et al*. (*12*) reported that a cutoff value of 24.8 kPa had a sensitivity of 81% and a specificity of 80.7%, but that study was a retrospective study, and it did not record LSM in real time or whether patients were given NSBBs or not.

Kazmi *et al*. (*13*) conducted a study using cutoff value less than 19 kPa and found that this value was highly predictive for the absence of grade II EVs (sensitivity of 84%, specificity of 78%, PPV of 47%, and NPV of 93%). Francesco *et al*. (*14*) found that LSM at a cutoff value of 17.6 kPa had a sensitivity of 90%, a specificity of 86%, and an NPV of 66% at predicting EVs. Results of both of those studies agreed with the current results. These variations in cutoff values for LSM to detect or rule out EVs may be related to many factors that include the type of patient, sample size, and different etiologies of cirrhosis.

The current study found that LSM was significantly correlated with the Child-Pugh grade during both pre- and post-treatment assessments. This finding agreed with the results of Razavi *et al*. (*2*), Castera *et al*. (*9*), and Foucher *et al*. (*15*), who all reported that LSM was correlated with the Child-Pugh score as well as with the stage of fibrosis and EV grade.

The mean PVD decreased significantly after 3 months of therapy with propranolol. With PVD as a noninvasive predictor for EVs, PVD of 12.5 mm pre-treatment can be used as a cutoff point to predict significant EV with a sensitivity of 82% and a specificity of 47.8%. Schepis *et al*. (*16*) found that PVD of 13 mm is a cutoff value for the presence of EVs.

The PC/SD ratio is considered to be another noninvasive marker for the presence of EVs. In the current study, a PC/SD ratio at a cutoff of 880 had a sensitivity of 94.1% and a specificity of 69.6% at predicting significant EVs. Baig *et al*., (*17*) found that a PC/SD ratio at a cutoff point of 890 was a predictor of EVs with a PPV of 95.5% and NPV of 95.1%, while Giannini *et al*. (*18*) found that a PC/SD ratio at a cutoff point of 909 predicted EVs with a sensitivity of 100% and a specificity of 71%. The mean value for SD did not change significantly after treatment with NSBBs.

One limitation of the current study was that it did not measure HVPG since doing so would be invasive. Instead, this study evaluated the grade of EVs, which are a common complication of a significant increase in HVPG.

In conclusion, LSM is a noninvasive, reproducible, and rapid method with which to evaluate liver cirrhosis and the stage of fibrosis and with which to evaluate the grade of either small or significant EVs. NSBB therapy can effectively decrease PVD and may consequently improve the EV grade with no significant impact on LSM in patients with liver cirrhosis.

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Brief Report

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Carbon dots have antitumor action as monotherapy or combination therapy

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Summary **Carbon dots prepared from different sources have been widely studied in various medical applications. Those dots have been reported to be able to inhibit the proliferation of cancer cells, such as prostate cancer cells. The current study used carbon dots prepared from red beans to determine their effect on 16 cell lines including liver cancer cells, pancreatic cancer cells, intrahepatic cholangiocarcinoma cells, and colorectal adenocarcinoma cells. In a cellular viability experiment, carbon dots were found suppress cancer cell viability in a time- and dose-dependent manner. In a cell migration experiment, cancer cells treated with carbon dots had less ability to heal, suggesting that carbon dots inhibit cell migration. In another cellular viability experiment, a combination of carbon dots and doxorubicin resulted in greater inhibition than cells treated with either therapy alone. These findings suggest that carbon dots could be an alternative and complementary medicine for the treatment of cancers.**

Keywords: Carbon dots, liver cancer, pancreatic cancer, intrahepatic cholangiocarcinoma, colorectal adenocarcinoma, combination therapy

1. Introduction

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body (*1*). In 2018, 18.1 million new cases of cancer and 9.6 million deaths occurred globally. About 20% of males and 17% of females will develop cancer at some point in time, while 13% of males and 9% of females will die from it (*2*). There are many options for cancer treatment; the primary ones include surgery, chemotherapy, radiation therapy, and targeted therapy (*3-7*). However, current therapeutic methods have several drawbacks, such as the inability to kill microscopic disease, damage to nearby normal tissues, toxicities and adverse reactions, and resistance. Based on the limitations of those options, increasing attention is being devoted to the development of novel drugs and medical materials (*8*).

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Due to their unique physicochemical features and their ability to enter cells, carbon nanomaterials possess great potential for biomedical applications (*9-11*). The utilization of carbon nanomaterials as innovative drug delivery systems for chemotherapeutics and other bioactive compounds has extensively been studied over the past few years (*12-14*). Studies have suggested that carbon-based materials, such as carbon fibers, carbon nanotubes, and carbon dots, are able to enhance apoptosis and inhibit proliferation of cancer cells through a series of signaling pathways (*15,16*).

Carbon dots prepared from different sources have been widely studied in imaging and drug delivery but less so in terms of intracellular activity and mechanisms of potential anticancer action (*17*). As an example, carbon dots prepared from a spice have been found to be able to inhibit tumor growth in a xenograft model (*17*). Another study found that carbon dots prepared from tea can bind to certain amino acids on ARF and colocalize with ARF in the nucleus, thus sensitizing cancer cells to rapamycin (*18,19*). The current study used an atomic carbon material (Australian patent number: 2005230336, Figure 1). Carbon dots were prepared from red beans to determine if they could inhibit the proliferation and migration of several cancer

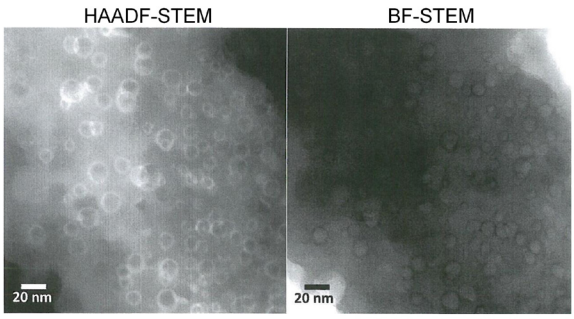

Figure 1. STEM model. HAADF-STEM: High-angle annular dark-field scanning transmission electron microscopy. BF-STEM: Bright-field scanning transmission electron microscopy.

cell lines and if they could promote the inhibitory effect of a conventional chemotherapy drug when administered in combination.

2. Materials and Methods

2.1. *Reagents*

High-glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). Penicillin and streptomycin were also purchased from Gibco (Gaithersburg, MD, USA). Cell Proliferation Kit I was purchased from Roche Applied Science (Penzberg, Upper Bavaria, Germany). Doxorubicin was purchased from Sigma (St. Louis, MO, USA). Carbon dots were donated by the New Japan Medical Institute (Bunkyo, Tokyo, Japan).

2.2. *Cell lines and maintenance*

Liver cancer cell lines (HepG2, HepG2.2.15, PLC/PRF/5, Huh-7, Huh-1, HLE, HLF, SK-Hep-1, Hep3B, and BEL-7402), pancreatic cancer cell lines (Panc-1, CAPAN-1, MIA-Paca-2, and SUIT-2), an intrahepatic cholangiocarcinoma cell line (RBE), and a colorectal adenocarcinoma cell line (DLD-1) were used in this study. These cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

2.3. *Dispersion of carbon dots*

Dispersions of carbon dots were prepared using human serum albumin (HSA; Baxter, Unterschleissheim, Germany). HSA was diluted with phosphate-buffered saline (PBS) and added to the carbon dots at a ratio of 1:1 by weight, followed by sonication with an ultrasonic tip (Sonoplus HD 2070, Bandelin; Heinrichstra, Berlin, Germany) for 5 min at 30% power. Different dilutions were then prepared in cell culture medium.

2.4. *Cell viability assay*

Cells were seeded in 96-well plates at a density of 2×10^3 per well and treated with reagents for 24, 48, and 72 h. The cytotoxicity of CQD was analyzed with an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay using Cell Proliferation Kit I according to the manufacturer's instructions. Each experiment was performed in triplicate. Cell viability was expressed as a percentage of the control.

2.5. *Wound healing assay*

Cells were seeded in six-well plates and cultured until confluent. A sterile 200 μL pipette tip was used to make a straight scratch, simulating a wound. Cells were then rinsed with PBS (very gently as sheets of cells may detach if care is not exercised) and replaced with 1.5 mL of medium containing reagents. Cells were photographed using phase contrast and a magnification of 10×, and cells were photographed again 24, 48 and 72 h later. After each measurement, old medium was replaced with fresh medium containing additives.

2.6. *Statistical analysis*

All experiments were performed in triplicate, and results were analyzed via one-way analysis of variance (ANOVA) using GraphPad Prism 4, followed by the Student's *t*-test performed using the Microsoft Office software Excel. $p < 0.05$ was considered to indicate a significant difference.

3. Results and Discussion

3.1. *Carbon dots inhibited the proliferation of cancer cells*

An MTT assay was used to estimate the extent to which carbon dots inhibited the proliferation of liver cancer cell lines (HepG2, HepG2.2.15, PLC/PRF/5, Huh-7, Huh-1, HLE, HLF, SK-Hep-1, Hep3B, and BEL-7402), pancreatic cancer cell lines (Panc-1, CAPAN-1, MIA-Paca-2, and SUIT-2), an intrahepatic cholangiocarcinoma cell line (RBE), and a colorectal adenocarcinoma cell line (DLD-1). As shown in Figure 2, carbon dots significantly inhibited those cell lines. Cancer cells were treated with various concentrations of carbon dots (5 mg/mL, 0.5 mg/mL, 0.05 mg/mL), and proliferation was measured at 24, 48, and 72 h. Results indicated that the inhibitory effect of carbon dots was dose- and time-dependent and that carbon dots most markedly inhibited cell proliferation at a concentration of 5 mg/mL. These findings suggest that carbon dots inhibit the proliferation of liver cancer cells, pancreatic

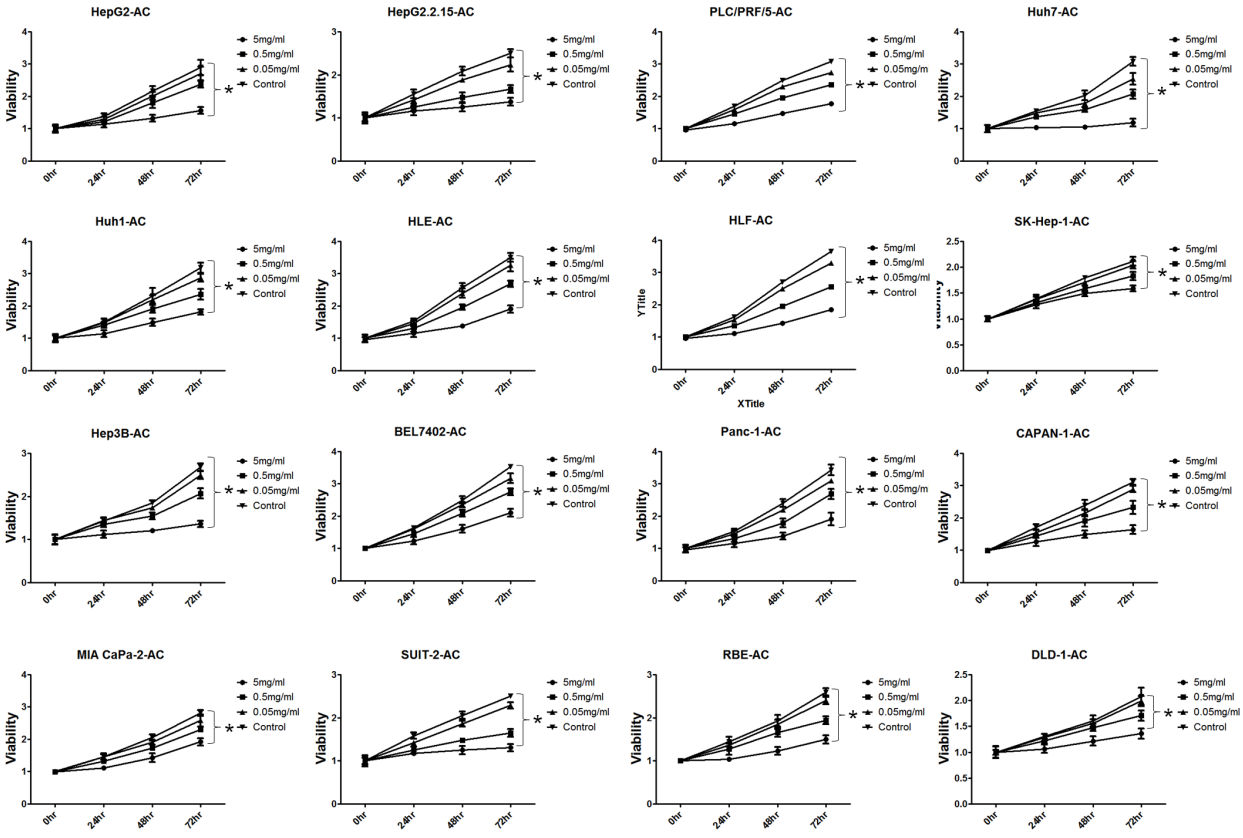

Figure 2. Inhibition of cancer cell growth by carbon dots.

cancer cells, intrahepatic cholangiocarcinoma cells, and colorectal adenocarcinoma cells. They may substantially inhibit other types of cancer cells as well. Further study is needed to determine the molecular mechanism of that inhibitory effect.

3.2. *Carbon dots inhibited the migration of cancer cells*

A wound healing assay was used to investigate the effects of carbon dots on the migratory ability of cancer cells. Six liver cancer cell lines (HLF, SK-Hep-1, Huh-7, HLE, HepG2, and Hep3B) were used in this experiment. After treatment with different reagents for 72 h, marked changes in healing were evident in cancer cells (Figure 3). Changes in the area of the scratch were analyzed in photographs to quantify cell migration. As shown in Figure 3, cells treated with carbon dots had less healing than the control group ($p < 0.05$). These results suggested that carbon dots can decrease the migration of liver cancer cells. Further study is planned to determine if carbon dots act similarly on other types of cancer cells and the molecular mechanism of that action.

3.3. *Combination therapy inhibited cancer cells to a greater extent*

An MTT assay was used to determine the extent to

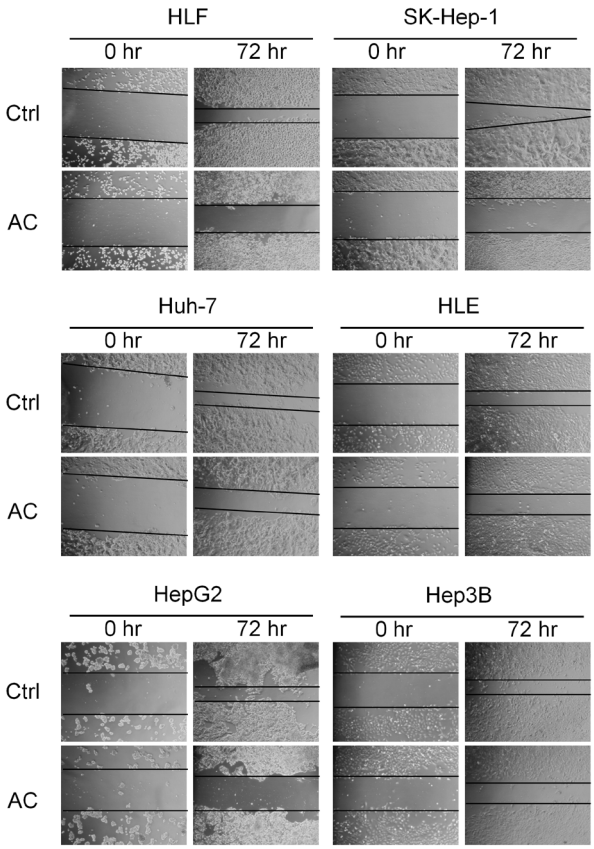

Figure 3. Detection of the extent to which carbon dots inhibited the migration of cancer cells.

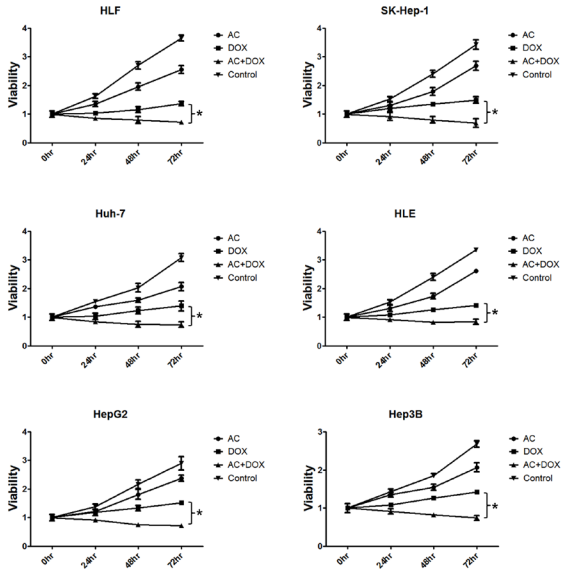

Figure 4. Detection of the extent to which the combined therapies inhibited cancer cells.

which a combination of carbon dots and doxorubicin inhibited cell proliferation. Six liver cancer cell lines (HLF, SK-Hep-1, Huh-7, HLE, HepG2, and Hep3B) were used in this experiment. Four experimental groups were established: a carbon dot group (0.5 mg/mL), a doxorubicin group (800 nM), a combined therapy group (0.5 mg/mL carbon dots + 800 nM doxorubicin), and a control group. As shown in Figure 4, cell inhibition was greater in the combined therapy group than in either the carbon dot group or the doxorubicin group ($p < 0.05$). This may indicate that combined administration could increase the therapeutic efficacy of conventional chemotherapy drugs.

4. Conclusion

The current study found that carbon dots prepared from red beans were able to inhibit the proliferation and migration of various types of cancer cells. A combination of carbon dots and a conventional chemotherapy drug inhibited cancer cells more than either therapy alone. Taken together, these findings suggest that carbon dots might be an effective alternative and complementary medicine for the treatment of cancers.

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Case Report

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Acute mesenteric vein thrombosis after endoscopic injection sclerotherapy for esophageal varices in a patient with liver cirrhosis

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Summary **Portal vein thrombosis (PVT) is a common complication of liver cirrhosis. The association between endoscopic injection sclerotherapy (EIS) and PVT is unclear. In this paper, we reported that a male cirrhotic patient developed acute mesenteric vein thrombosis after EIS for secondary prophylaxis of esophageal variceal bleeding. Immediate anticoagulation therapy was effective and safe in this patient.**

Keywords: Portal vein thrombosis, endoscopic therapy, anticoagulation therapy

1. Introduction

Portal vein thrombosis (PVT) is a common complication of liver cirrhosis with a prevalence of 0.6-26% (*1*). Acute PVT refers to recent (< 30 days) formation of thrombosis in the main portal vein or its branches; by comparison, chronic PVT often has multiple collateral vessels around the thrombosed portal vein (*2*). Risk factors for the development of PVT in cirrhotic patients include reduced portal vein flow velocity, liver dysfunction, portosystemic collateral vessels, splenectomy, and thrombophilia, *etc*. (*3*).

Endoscopic variceal ligation (EVL) is the preferred treatment of choice for both controlling esophageal variceal bleeding and secondary prophylaxis, and endoscopic injection sclerotherapy (EIS) may be performed if EVL is technically difficult (*4*). During EIS procedure, sclerosing agents are injected into varices, thereby achieving variceal occlusion. Notably, this procedure may increase the portal vein blood flow, thereby resulting in a sudden increase of portal vein pressure and turbulent blood flow pooling in the portal venous system, which causes PVT (*5*).

In this paper, we reported a case of acute mesenteric vein thrombosis after EIS for esophageal varices in a patient with liver cirrhosis, in whom anticoagulation with low molecular weight heparin (LMWH) achieved partial mesenteric vein recanalization without any bleeding episode.

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2. Case report

On May 29, 2017, a 53-year-old male with a 23-year history of liver cirrhosis secondary to hepatitis B virus infection was admitted to our department due to nausea and diarrhea for 5 days. He was accompanied by abdominal distension and intermittent fever with body temperature up to 38.5℃ which was normalized after oral antipyretic. There was neither abdominal pain nor bloody purulent stool. During the past 18 years, he was repeatedly admitted to our department due to the development of ascites, encephalopathy, and/or gastroesophageal variceal bleeding. He underwent splenectomy with devascularization in 2007, EIS in 2012 and 2013, and EVL in 2014 and 2016. Laboratory tests demonstrated that red blood cell (RBC) was 3.6×10^{12}/L (reference range: $4.3\text{-}5.8 \times 10^{12}$/L), hemoglobin (Hb) was 118 g/L (reference range: 130-175 g/L), hematocrit (HCT) was 36.2% (reference range: 40-50%), white blood cell (WBC) was 9.5×10^{9}/L (reference range: $3.5\text{-}9.5 \times 10^{9}$/L), percentage of granulocyte (GR%) was 61.1% (reference range: 40-75%), erythrocyte sedimentation rate (ESR) was 17 mm/h (reference range: 0-15 mm/h), C-reactive protein (CRP) was 25.5 mg/L (reference range: 0-8 mg/L), total bilirubin (TBIL) was

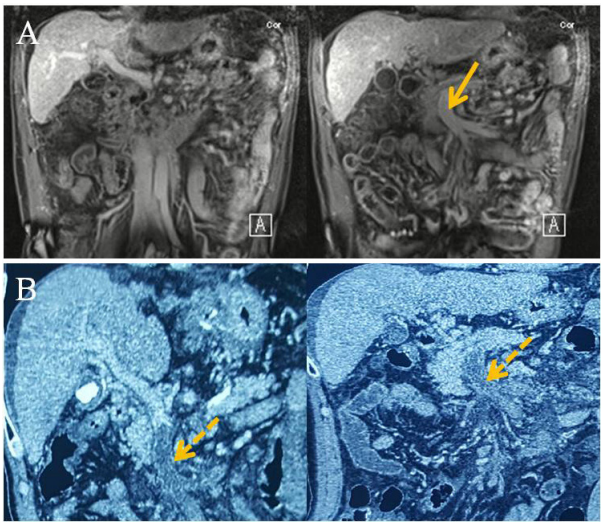

Figure 1. Coronal views. (A) contrast-enhanced magnetic resonance imaging performed on May 29, 2017 showing patent mesenteric vein (solid arrow); **(B)** computed tomography angiography performed on June 13, 2017 showing complete thrombosis in the mesenteric vein (dotted arrow).

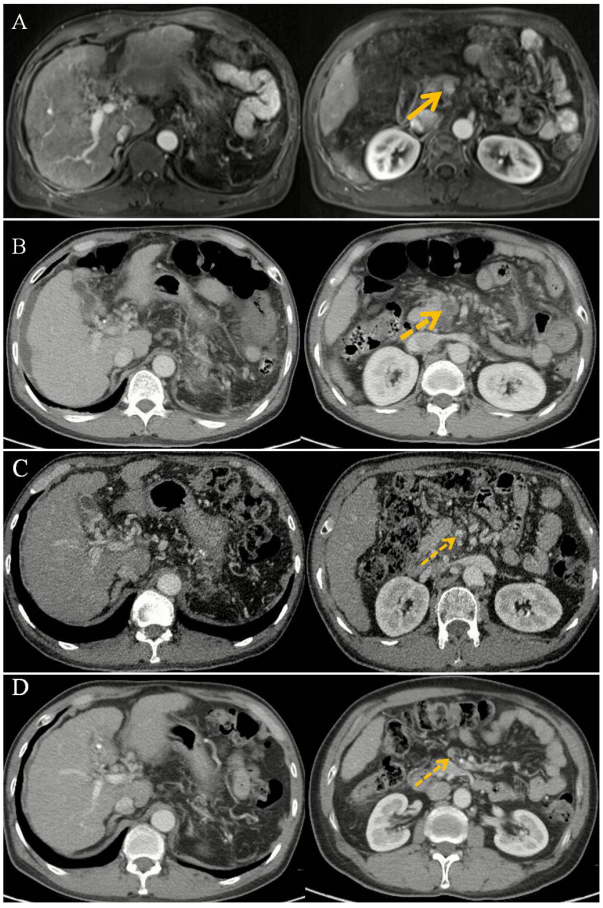

Figure 2. Axial views. (A) contrast-enhanced magnetic resonance imaging performed on May 29, 2017 showing patent mesenteric vein (solid arrow); **(B)** computed tomography angiography performed on June 13, 2017 showing complete thrombosis in the mesenteric vein (dotted arrow); **(C)** computed tomography angiography performed on July 19, 2017 showing partial recanalization of mesenteric vein thrombosis (thin dotted arrow); **(D)** computed tomography angiography performed on June 5, 2018 showing partial recanalization of mesenteric vein (thin dotted arrow) with collateral circulation around the mesenteric vein.

29.4 μmol/L (reference range: 5.1-22.2 μmol/L), direct bilirubin (DBIL) was 10.0 μmol/L (reference range: 0-8.6 μmol/L), alanine amino-transaminase (ALT) was 14.93 U/L (reference range: 9-50 U/L), aspartate amino-transaminase (AST) was 31.55 U/L (reference range: 15-40 U/L), alkaline phosphatase (AKP) was 127.26 U/L (reference range: 45-125 U/L), γ-glutamyl transpeptidase (GGT) was 40.85 U/L (reference range:10-60 U/L), serum albumin (ALB) was 36.2 g/L (reference range: 40-55 g/L), prothrombin time (PT) was 13.9 seconds (reference range: 11.5-14.5 seconds), international normalized ratio (INR) was 1.06, and D-dimer was 2.52 mg/L (reference range: 0.01-0.55 mg/L). Contrast-enhanced axial (Figure 1A) and coronal (Figure 2A) magnetic resonance imaging revealed liver cirrhosis, ascites, and multiple collaterals around the intrahepatic portal vein branch and portal trunk, but patent mesenteric vein. His Child-Pugh score was 6.

On May 30, 2017, the patient developed fever with a body temperature up to 38.8°C and abdominal distension again. Laboratory tests demonstrated that WBC was 13.5 × 10^9/L, GR% was 83.3%, ESR was 33 mm/h, CRP was 42.9 mg/L, and procalcitonin (PCT) was 0.11 ng/mL (reference range: 0-0.05 ng/mL). Blood culture demonstrated the absence of bacteria within 5 days. Cefoperazone sulbactam sodium was given intravenously with a dosage of 1.5 g twice a day. The patient's condition was stable and his body temperature regressed after a 6-day duration of antibiotics.

On June 6, 2017, upper gastrointestinal endoscopy revealed post-ligation scar and mild varices without red color sign at the lower esophagus. Prophylactic EIS was performed.

On June 13, 2017, he developed persistent abdominal pain accompanied by fever. Physical examination showed abdominal softness, mild tenderness in the left upper abdomen, neither rebound tenderness nor muscle tension, negative shifting dullness, and weak borborygmus. Laboratory tests demonstrated that PT was 16.0 seconds, INR was 1.26, fibrinogen degradation product (FDP) was 51.96 mg/L (reference range: 0.01-5.00 mg/L), antithrombin III (ATIII) was 48% (reference range: 80-120 U/L), and D-dimer was 16.05 mg/L. Abdominal X-ray showed that abdominal intestine had gas accumulation and expansion, but no air-fluid level was observed. Computed tomography angiography (CTA) revealed complete thrombosis in the mesenteric vein as well as edematous and thickened small intestine wall (Figures 1B and 2B). Immediately, anticoagulation with LMWH was given subcutaneously with a dosage of 4,250 iu twice a day. Abdominal pain improved gradually.

On June 16, 2017, his upper abdominal pain significantly relieved in the absence of abdominal distension, nausea, or vomiting. The body temperature was gradually normalized. Physical examination

showed soft abdomen, slight tenderness, neither rebound tenderness nor muscle tension, negative shifting dullness, and normal borborygmus.

On June 19, 2017, his body temperature was normal and abdominal pain disappeared. Laboratory tests demonstrated that PT was 15.8 seconds, INR was 1.24, FDP was 18.47 mg/L, ATIII was 52%, and D-dimer was 7.79 mg/L.

On June 22, 2017, the patient was discharged without any complaints. LMWH was recommended after discharge.

On July 19, 2017, CTA revealed partial recanalization of mesenteric vein (Figure 2C).

On January 14, 2018, upper gastrointestinal endoscopy revealed esophageal and gastric varices. Endoscopic treatment was not performed because this patient was receiving anticoagulants at that time.

On April 1, 2018, the patient stopped anticoagulant therapy before prophylactic endoscopic treatment. He did not develop any bleeding episode during the entire 10-month period of anticoagulation therapy.

On June 5, 2018, the CTA revealed partial recanalization of mesenteric vein with collateral circulation around the mesenteric vein (Figure 2D).

On June 8, 2018, the patient underwent EVL for secondary prophylaxis of variceal bleeding. The last follow-up was performed on January 10, 2019 when his general condition was stable without any complaints.

3. Discussion

This patient developed complete thrombosis in the mesenteric vein after EIS for the prophylaxis of esophageal variceal bleeding. His clinical manifestations were abdominal pain and fever. CTA confirmed the diagnosis of PVT. Anticoagulation therapy with LMWH achieved partial recanalization without any bleeding episode.

At present, the first-line choice for secondary prophylaxis of variceal bleeding is a combination of EVL and non-selective beta-blockers (*6*). However, EIS may be performed if EVL was technically difficult or infeasible (*4*). Our endoscopist considered EIS, because EVL was technically difficult in this patient due to post-EVL scars.

Association between EIS and PVT. Incidence of PVT after EIS was heterogeneous among studies. Kawasaki *et al*. observed that none of 22 cirrhotic patients had splenic or portal vein thrombosis during a post-EIS follow-up duration of 26 ± 17 months (*7*). Barsoum *et al*. observed that only 1 of 122 patients had a recent thrombus in the portal vein after EIS, which was confirmed by autopsy (*8*). By comparison, Hou *et al*. observed that 16 of 91 cirrhotic patients had a PVT after cyanoacrylate injection for gastric variceal bleeding (*9*). Amitrano *et al*. observed that 10 of 61 cirrhotic patients developed PVT after EIS for esophageal variceal bleeding (*10*). The difference in the incidence of PVT among studies may be related to the heterogeneity of patient characteristics, date when the imaging was performed, and diagnostic approaches.

Association between EIS and PVT was controversial among comparative studies. Hunter *et al*. found that PVT occurred in 4 of 11 patients who had EIS and only 1 of 10 patients who did not have EIS (4/11 *vs*. 1/10) (*11*). Leach *et al*. retrospectively identified 27 patients who underwent portosystemic shunting after an episode of variceal bleeding. Patients with a history of EIS had a higher incidence of splanchnic vein thrombosis than those without (6/11 *vs*. 2/16) (*12*). By comparison, one prospective controlled study found no significant difference in the incidence of PVT between cirrhotic patients who received EIS for esophageal variceal bleeding and those who did not (8/72 *vs*. 5/52) (*13*).

In our patient, a complete thrombosis in the mesenteric vein was observed on the 7th day after EIS. Therefore, their association should not be ignored. In addition, infection, especially abdominal inflammation, is one of the most frequently acquired prothrombotic states (*2,14*). Our patient might have an intra-abdominal infection manifested as digestive symptoms, fever, and elevated inflammatory indexes at admission. Unfortunately, we did not identify the origin of infection. But antibiotic was potentially effective in our patient.

The cases of portal venous system thrombosis caused by EIS might be extrapolated to other endoscopic variceal therapy, because all types of endoscopic variceal therapy may lead to an increased portal vein pressure and then cause turbulent blood flow pooling in the portal venous system. On the other hand, gastroesophageal varices and variceal rebleeding can be aggravated by PVT (*15*). Thus, endoscopic variceal treatment, PVT, and variceal rebleeding may produce a vicious circle in cirrhotic patients.

Anticoagulation for PVT. Anticoagulation is the major treatment option of PVT (*5*). Baveno VI consensus indicates that systemic anticoagulation with LMWH should be started as soon as a diagnosis of PVT is made (*16*). Guidance for the management of venous thrombosis in unusual sites also indicates that cirrhotic patients with splanchnic vein thrombosis who do not have active bleeding should initiate early anticoagulation with LMWH (*17*). In our patient, anticoagulation with LMWH was started immediately after his diagnosis of PVT.

A systematic review and meta-analysis concluded that anticoagulant therapy increased the rate of portal vein recanalization from 42% in patients who did not receive anticoagulants to 71% in patients who received anticoagulants and reduced the rate of thrombotic progression from 33% to 9%, but did not significantly increase the risk of bleeding, such as variceal bleeding (*18*). Our meta-analysis also supported that anticoagulant therapy increased portal vein recanalization in cirrhotic patients (*19*).

Two recent studies also suggested that anticoagulation therapy could increase the rate of portal recanalization, but not increase the risk of bleeding. A prospective cohort study demonstrated that the recanalization rate was significantly higher in patients treated with anticoagulants than untreated patients (56.8% *vs*. 27.7%). Of the 46 patients who achieved portal recanalization, 67.4% had complete recanalization, but 36% suffered from recurrent thrombosis after stopping anticoagulants. Risk of bleeding complications was similar between the two groups (*20*). Another prospective study also demonstrated that 57.5% of patients treated with anticoagulants achieved complete recanalization, and 25.0% achieved partial recanalization. Of the 40 patients treated with anticoagulants, 37.5% developed bleeding, but none died from bleeding. Notably, 70% of patients had a recurrence or extension of PVT after stopping anticoagulants (*21*). Taken together, both studies indicated that anticoagulation should be maintained in order to avoid recurrence.

In our patient, anticoagulants were started immediately after a diagnosis of PVT. A partial recanalization of mesenteric vein was achieved after anticoagulant therapy for 1 month. Then, anticoagulant therapy was maintained for additional 9 months in order to avoid recurrence. He did not develop any bleeding episode during the entire period of anticoagulation therapy.

In conclusion, EIS may be a risk factor of thrombosis within portal vein system. Once acute thrombus within portal vein system was confirmed, an immediate and continuous treatment with anticoagulants should be effective and safe.

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Case Report

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Two different scenarios of advanced basal cell carcinomas during the use of vismodegib: Cases of oral administration and administration directly to the stomach

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Summary **No effective therapy exists for locally advanced or metastatic basal cell carcinoma (BCC). Vismodegib is a small molecule that is an inhibitor of the hedgehog pathway. An oral treatment to inactivate Smoothened would be a new therapeutic approach to treat advanced BCC. We studied two patients with advanced BCC and analysed variables, including age and sex of the patient, tumour location and size, time of evolution and nature of the tumour (primary or recurrent), type of treatment, route of administration, treatment duration, and treatment response. The most important side effects were determined. The patients received oral vismodegib (150 mg) daily. The male patient experienced difficulty in swallowing, which necessitated administration of the drug using a percutaneous endoscopic gastrostomy tube. In the first few months of treatment, both patients displayed significant improvement with almost complete disappearance of the skin lesions in one case and more than 50% in the other case. The median duration of response was 7.6 months. The side effects observed were of slight relevance; alopecia, dysgeusia, asthenia, and fatigue were easily resolved with the appropriate treatments. Vismodegib appears to be well tolerated and effective in treating advanced and metastatic BCC. No serious adverse events were reported.**

Keywords: Advanced localised basal cell carcinoma (BCC), metastatic BCC, hedgehog pathway inhibitors, vismodegib, route of administration

1. Introduction

Basal cell carcinoma (BCC) has a global incidence of 70 to over 800 new cases per 100,000 persons per year. It is the most common skin tumour, accounting for 80% of the nonmelanoma skin cancer cases. Rarely, BCC progresses to locally advanced or metastatic BCC (*1,2*). However, some cases of BCC involve a more aggressive phenotype and the prognosis is poorer (*3*). Tumours with a diameter greater than 2 cm are associated with a higher risk of recurrence and metastasis. Location of lesions in the centre of the face, especially around the eyes, nose, lips, and ears, is also associated with a higher risk of recurrence (4). Patients with recurrent lesions have a higher risk of metastasis. Mofeiform, micronodular, infiltrative and basosquamous histology subtypes pose an increased risk of recurrence and metastasis. Finally, perineural and/or perivascular involvement confers a greater risk of recurrence.

There is currently no established definition for locally advanced BCCs. It has been proposed to use this term for American Joint Committee on Cancer stage II BCCs. These tumours are > 2 cm in diameter with at least two high risk factors, which include invasion depth > 2 mm, Clark IV level, perineural invasion, facial H location and poor tumour differentiation. Other relevant added characteristics include the presence of large tumours or several tumours, presence of genodermatosis (Gorlin syndrome) and comorbidities.

All these aspects should be considered when selecting a treatment option for patients with BCC, since the

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surgery can be debilitating and sometimes disfiguring. Additionally, surgery and radiotherapy are inappropriate in some cases. In these cases, non-surgical treatments may be required (*5*). Until the availability of approved systemic therapy there has been no effective treatment for this type of patients.

Vismodegib is the first and, so far, only Food and Drug Administration approved oral therapy for advanced and metastatic BCC (*6,7*). Vismodegib is an orally administered compound. It is a selective first-in-class hedgehog pathway inhibitor. The Sonic Hedgehog (SHH) pathway plays a key role in the regulation of cell differentiation and organ formation during embryonic development. In adults the SHH pathway remains inactive in most tissues (*8,9*). Vismodegib specifically binds and inactivates the seven transmembrane helical fold Smoothened receptor (SMO), which slows the activation of the family of transcription factors of the glioma-associated oncogene (GLI) and suppresses proliferation and tumour growth (*10*).

In this manuscript, we review the results of vismodegib treatment of BCC in two patients. One patient had metastatic BCC and the other had advanced localised BCC. We focused on the mechanism of action, clinical efficacy, and safety of vismodegib.

2. Case report

2.1. *Case 1*

A 76-year-old woman presented with a large (8 × 14 cm) ulcerated lesion in the retro-auricular zone, with an extension to the right parieto-occipital region (Figure 1).

The lesion had progressed over a period of 7 years with invasion of the bones and cerebellum. The patient was not a candidate for surgery because the tumour was inoperable. Radiotherapy was contraindicated due to the extension of the tumour, which included invasion of the central nervous system. Skin findings showed BCC with keratotic differentiation. Biopsy of the right parieto-occipital bone revealed BCC infiltration of bone tissue and acute osteomyelitis with gram-positive bacteria. Immunohistochemistry analysis confirmed the absence of CD56 and Cd117 (C-Kitt). The patient had no previous family history of skin cancer, and did not present with other suspicious lesions during clinical examination. Total body computed tomography scans and positron emission tomography imaging revealed the involvement of the underlying bones and cerebellum, with no evidence of distant spread (Figure 2).

The findings from magnetic resonance imaging suggested extensive tumour involvement in the subcutaneous tissue from the right temporal area to the deep right suboccipital area and neck, with the involvement of the skull base, right portion of C1, and extension to the right epidural area at the C1 level of the spinal canal. There was no evidence of marrow involvement. Complete staging including the thorax, abdomen and pelvis did not reveal BCC metastasis. The patient was started on vismodegib with a standard oral dose of 150 mg/day. No other medications were used. The patient was evaluated for the effect of treatment 4, 16 and 36 weeks after the initiation of treatment. Appreciable improves were evident and included decreased tumour size and signs of healing in the periphery (Figure 3).

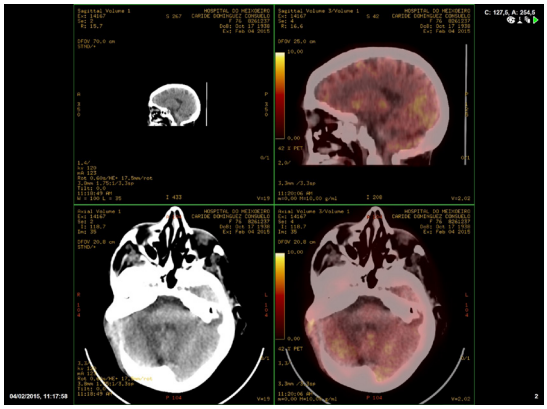

Figure 2. Patient's total-body computed tomography and positron emission tomography imaging at the beginning of treatment. Right skull base shows intensive pathological hypercaptation associated with soft tissue involvement extending into the right paravertebral region of C2.

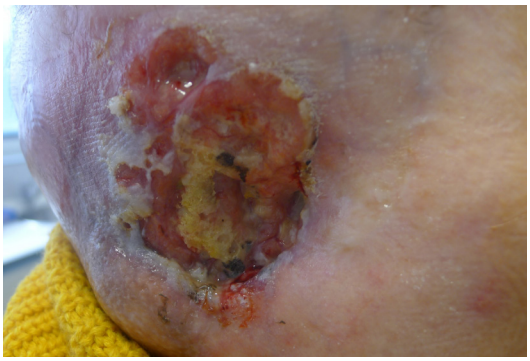

Figure 1. Macroscopic findings at the beginning of treatment with vismodegib. Representative image of the tumour with a size of 10.5 × 13 cm. No auricular palate is observed due to previous surgeries.

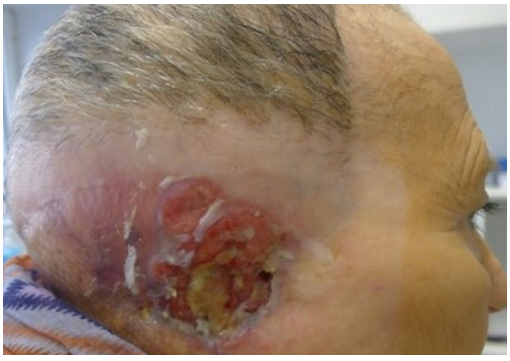

Figure 3. Macroscopic findings after four months of treatment. Marked reduction of the tumour size by 50% and abundant tumour areas of tissue repair.

Table 1. Demographic and clinical characteristics of the patients and follow-up data

BCC type	MBCC (Case 1)	BCC-la (Case 2)
Age, years	76	80
Sex		
Male		1
Female	1	
Contraindications to surgery or radiation therapy	1	1
Inoperable tumour	1	
Surgery inappropriate	1	1
Multiple recurrences	1	1
Substantial morbidity or deformity anticipated	1	
Radiation therapy previously administered	1	1
Treatment	Vismodegib 150 mg/oral/24 h	Vismodegib 150 mg dissolved in 50 cc of hot water per gastrostomy tube/24 h
Radiation therapy inappropriate or contraindicated	1	1
Response Evaluation Criteria (RECIST 1.1)	Decrease of more than 50% of the sum of the diameter of the target lesion	Virtually complete disappearance of target skin lesions (90%)
Clinical benefit rate (partial or complete response at any time (before or after progression)) + stable disease for 4 or more weeks	1	1
Average time to maximum tumour reduction	4 months	3.5 months
Deaths	1	1
Aspiration pneumonia		1
Multi-organ failure	1	

mBCC: Metastatic basal-cell carcinoma. laBCC: localised advanced basal-cell carcinoma.

Table 2. Adverse events

Symptomatology	Patients	Grade	Treatment	Response to treatment
Alopecia	1	1	Minoxidil 5% twice daily	Partial response
Dysgeusia	1	2	Zinc supplements, continuous nutritional support	Regression of symptoms
Asthenia/fatigue	2	1	Physical activity, including yoga, Fluoxetine 20 mg/oral/24 h	Regression of symptoms

The side effects were minimal and included constipation and fatigue. They were manageable. The patient ultimately died of multiple organ failure but in not relationship with the vimodegib treatment (Tables 1 and 2).

2.2. *Case 2*

A 76-year-old man was originally referred to the neurology department for recurrent apnoea and asymmetric parkinsonian onset. He was then referred to our clinic for recurrence of lesions on the scalp and face. He presented with multiple BCC tumours in the left parietal and occipital region, which met the criteria for BCC that was inoperable with multiple recurrences. This is associated with important morbidities and deformities that can include the forehead, temple and external auditory canal. Clinical history-taking revealed more than 20 recurrences of BCC. During the previous 5 years he had been operated on several times, with grafts applied to virtually the entire scalp and 40% of the facial area (Figure 4).

The graft tissue had been obtained from the abdomen, which had produced scarring. Multiple biopsies had been performed. The results were consistent with BCC. Prior magnetic resonance imaging had revealed infiltration of the bone marrow of the left parietal bone in the parasagittal region and diffuse corticosubcortical atrophy

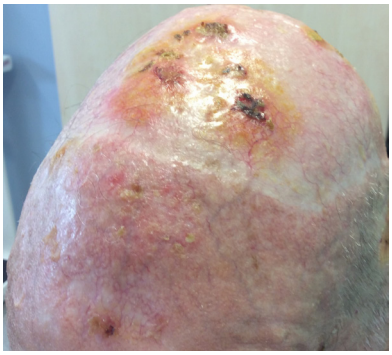

Figure 4. Macroscopic findings before treatment with vismodegib. Crusted ulcerative lesions are observed on the scalp and grafts, after multiple previous surgeries.

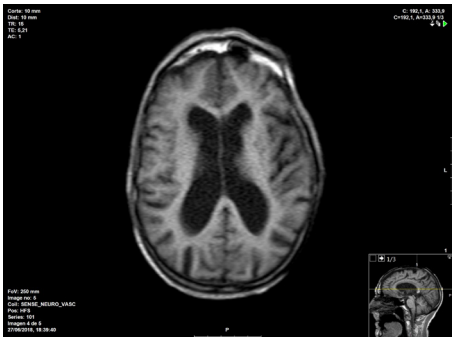

Figure 5. Magnetic resonance image of the tumour. Early phase of the disease showing the important invasion of the tumour towards the left parietal bone.

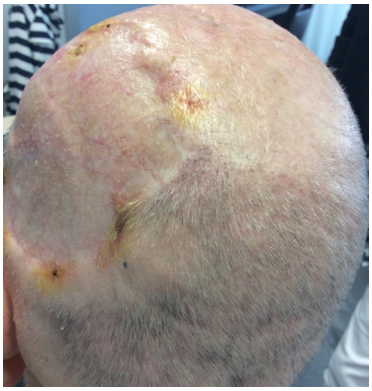

Figure 6. Macroscopic findings. Disappearance of skin lesions after 6 months of treatment with vismodegib.

was observed (Figure 5).

Following admission, swallowing was affected by a concentric stenosis at the level of the pharyngoesophageal junction. Therefore, percutaneous endoscopic gastrostomy (PEG) was done and a 14F retention balloon catheter was installed. There were no immediate or longer-term complications. The patient was treated solely with oral vismodegib 150 mg initially. The same dose was subsequently diluted in 50 cc of warm water and administered *via* the catheter. The treatment was well tolerated with no side effects associated with vismodegib or the other drugs that continued to be used. The patient was evaluated at 4, 16 and 36 weeks after the initiation of treatment. Nearly total remission of the skin lesions was observed (Figure 6).

Ultimately, the patient died. Death was not related to the vismodegib, but rather to aspiration pneumonia (Tables 1 and 2).

3. Discussion

BCC is usually treated by surgery alone or in combination with radiotherapy. However, treatment options are limited for the minority of patients who present with locally advanced or metastatic BCC. Overall survival estimates for patients with metastatic disease are poor, ranging from 8 months to 3.6 years. In one study, the incidence of advanced or complicated BCCs in a tertiary referral centre was 6.6% (640 of 9,652) moderate cases and 0.6% (58 of 9,652) severe cases (*11*).

Vismodegib is a targeted inhibitor of SMO, which decreases the activity of the hedgehog-signalling pathway and subsequently reduces basal cell proliferation. A durable reduction in the size of unresectable, metastatic, and potentially disfiguring or invasive BCC has been a direct clinical benefit of treatment with vismodegib (*12*).

A number of clinical trials have supported the approved use of vismodegib for BCC. In one trial 63 patients with locally advanced BCC and 33 patients with metastatic BCC, the rates of the response of the patients

Table 3. Summary of efficacy findings of the largest clinical trials investigating vismodegib in laBCC and mBCC

No. of patients enrolled (laBBC)	No. of patients enrolled (mBCC)	Study type	Treatment	Mean treatment duration (months)	Response rate (*laBCC)	Response rate (*mBCC)	Reference
62	57	Open-label, multicenter study	Vismodegib 150 mg orally	5.5	46.4%	30.8%	Chang *et al.* 2014 (*19*)
113 (included patients with Gorlin syndrome		Phase II, randomized, controlled, double-blind trial	Vismodegib 150 mg orally	18.5	**58.4%		Dreno *et al.* 2015 (*17*)
71	33	Phase II, two-cohort, multicenter study	Vismodegib 150 mg orally	39	60.3%	48.5%	Sekulic *et al.* 2017 (*15*)
1,119	96	Phase II, single-arm, multicenter, open-label study	Vismodegib 150 mg orally	8.6	***68.5%	***36.9%	Basset-Seguin *et al.* 2017 (*16*)
55	-	Phase II trial multicenter, open-label study	Vismodegib 150 mg orally		80%	-	Mortier *et al.* 2018 (*18*)
-	2	Clinical practice	Vismodegib 150 orally + concurrent radiotherapy (66 Gy in 33 fractions) for case 1 and (12.5 and 22.3 Gy) for case 2	10.5	-	Stable disease apparent on imaging	Pollom *et al.* 2015 (*20*)
-	4	Clinical practice	150 mg orally+ 20% 5-aminolevulinic acid in 1-3 sessions	1	-	100%	Rizzo *et al.* 2017 (*21*)
1	1	Clinical practice	Vismodegib 150 mg orally and through a PEG****	7.5	50%	100% and stable disease apparent on imaging	(Current cases)

* laBCC: locally advanced basal cell carcinoma; mBCC: metastatic basal cell carcinoma. ** 54% for group A, 62.8% for group B. *** laBCC (total 34%, partial in 33%, and stable in 26%); mBCC (2 total, 9 partial and 10% stable). **** PEG: Percutaneous endoscopic gastrostomy.

were 43% and 30% respectively. The median duration of response was 7.6 months in both cohorts. Adverse events occurred in more than 30% of the patients (*13*). In the pivotal nonrandomised ERIVANCE study (NCT00833417) published in 2015 by the same authors, 104 patients (33 metastatic BCC, 71 locally advanced BCC) were enrolled. During a 12-month period the response rate increased with time, with overall response rates of 30.3% for the metastatic BCC patients and 47% for the locally advanced BCC patients. The median duration of response in the latter patients was increased 2.1 months compared to a study published in 2012 (*14*). The final results of ERIVANCE were published in 2017 with a cut-off at 39 months after completion of accrual (*15*). The response rate was 60.3% for locally advanced BCC patients and 48.5% for the metastatic BCC patients (Table 3).

The open-label STEVIE study [NCT01367665 (*15*)] enrolled a total of 1,232 patients. These included 499 patients (468 with locally advanced BCC and 31 with metastatic BCC) who received vismodegib and were followed-up for a minimum of 12 months. The primary objective was drug safety. For the patients with locally advanced BCC, the rate of complete response and partial response was 34% and 33%, respectively, and a stable response was observed in 26% of patients. For the patients with metastatic BCC, the respective response rates were 2%, 9% and 10% respectively (*16*).

The randomised, double-blind, phase II MIKIE study NCT01815840 (*17*) included patients with multiple BCCs, including those with basal cell nevus (Gorlin) syndrome, who required extended treatment. A total of 229 patients were randomised to two treatments (116 in treatment group A and 113 in treatment group B). Both groups received intermittent treatment with vismodegib 150 mg daily and placebo. The decrease in the mean number of tumours at 73 weeks of follow-up was 62.7% in group A and 54.0% in group B (*17*).

The objective of the open-label, phase II VISMONEO study NCT02667574 (*18*) was to evaluate the reduction of the size of locally advanced BCC tumours located on the face after neoadjuvant treatment involving vismodegib, to explore the strategy as a means of reducing the need for surgery. The study enrolled 55 patients who were contraindicated for surgery; four were inoperable, 15 had a risk of major functional damage and 36 had a risk of minor functional risk or major aesthetic risk. Eighty percent of the patients displayed a treatment response and the severity of the surgery was reduced. Chang *et al*. (*19*) described 119 patients treated with vismodegib for over 5 months. Response to treatment was observed in 46.4% of patients with locally advanced BCC and 30.8% of patients with metastatic BCC. The most important side effects were muscle spasms (70.6%), dysgeusia (70.6%), alopecia (58.0) and diarrhoea (25.2%).

Concerning the integration of vismodegib with existing therapies, prior publications have documented similar experiences regarding vismodegib in combination with radiotherapy. The data indicate the excellent response and good tolerance of patients. Pollom *et al*. (*20*) reported on two patients with metastatic BCC treated with vismodegib 150 mg concurrent with radiotherapy. At a mean follow-up of 10.5 months, imaging analyses revealed apparent stable disease in both patients, with reduced facial weakness and absence of pain. Rizzo *et al*. (*21*) obtained excellent results with photodynamic therapy and vismodegib in patients with metastatic BCC, involving three applications of 20% 5-aminolevulinic acid. The photodynamic therapy was applied 7 ± 4 days after starting vismodegib and was repeated at 45 ± 5 days and 90 ± 10 after treatment began. The combination of photodynamic therapy and vismodegib was judged to be a potential safe and effective therapy for the treatment of multiple BCCs.

The present results of our patients corresponded to the usual clinical practice, in which the long-term use of SHH inhibitors are not sustainable, in some cases due to the presence of comorbidities, especially in very elderly patients. Our response rates were similar or better than those reported in the previous tests (*14-17*). It is noteworthy that we did not have to suspend the treatment in any case, irrespective of whether vismodegib was administered orally or *via* catheter. Table 3 summarises studies that involved the treatment of locally advanced or metastatic BCC and metastatic, including the present patients.

The available data, including the present data, indicate that vismodegib is a safe and effective drug for the treatment of locally advanced and metastatic BCC when surgery and radiotherapy are not options. In our patients, the percentage of response to treatment in the cutaneous target lesions varied between 50% and 90%. Adverse effects should be taken into account since they can be frequent. In our patients the adverse events were manageable and resolved without major problem and without having to interrupt the treatment. To our knowledge, this is the first case study to date of treatment with vismodegib in advanced BCC involving administration orally or directly to the stomach *via* a catheter. No loss of efficacy or added adverse side effects were evident.

Our contribution to literature demonstrates the importance of vismodegib treatment in advanced and metastatic BCC as well as a new administration route for patients with difficulties in swallowing.

Availability of data and materials The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate The authors declare that the procedures followed were in accordance

with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Patient consent for publication Written informed consent was obtained from the patient regarding the publication of the case details and any accompanying images.

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